ผลของการเสริมสาหร่ายทะเล *Ascophyllum nodosum* ในอาหาร ต่อความต้านทานโรคของกุ้งกุลาดำ *Penaeus monodon*

นางสาว ศิริกัญญา จึงธนวงศ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต

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EFFECTS OF *Ascophyllum nodosum* SUPPLEMENT IN DIET ON RESISTANCE TO DISEASE IN BLACK TIGER SHRIMP *Penaeus monodon*

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ศิริกัญญา จึงธนวงศ์: ผลของการเสริมสาหร่ายทะเล *Ascophyllum nodosum* ในอาหารต่อความต้านทานโรคของกุ้งกุลาดำ *Penaeus monodon.* (EFFECTS OF KELP *Ascophyllum nodosum* SUPPLEMENT IN DIET ON DISEASE RESISTANCE IN BLACK TIGER SHRIMP *Penaeus monodon*) อ.ที่ปรึกษา: รศ.ดร.สมเกียรติ ปิยะธีรธิติวรกุล 83 หน้า. ISBN 974-53-1147-2

งานวิจัยนี้ศึกษาผลการเสริมสาหร่ายทะเล *Ascophyllum nodosum* ในอาหารต่อการเติบโตและอัตรารอดของกุ้งกุลาดำ *Penaeus monodon* โดยเลี้ยงกุ้งกุลาดำด้วยอาหารผสมสาหร่ายทะเลในอัตรา 0, 2.5, 5, 7.5 และ10% การทดลองแบ่งเป็นสองการทดลอง ได้แก่ การทดลองในกุ้งกุลาดำอายุ 15 วันและกุ้งกุลาดำหลังอนุบาลอายุ 1.5 เดือน การทดลองที่หนึ่งเลี้ยงกุ้งกุลาดำอายุ 15 วัน เป็นระยะเวลา 28 วันในกระชังซึ่งอยู่ภายในบ่อระบบน้ำหมุนเวียนแบบปิด เมื่อสิ้นสุดการทดลองกุ้งมีความยาวเฉลี่ย 2.53±0.61, 2.58±0.54, 2.55±0.51, 2.51±0.50, และ 2.51±0.51 ตามลำดับ ซึ่งไม่แตกต่างกันในเชิงสถิติ อัตรารอดของกุ้งที่กินอาหารเสริมสาหร่าย 2.5% มีอัตรารอดสูงสุด (76.50± 5.44) และแตกต่างจากอัตรารอดของกุ้งที่กินอาหารสูตรอื่นอย่างมีนัยสำคัญ

การทดลองที่สอง เลี้ยงกุ้งกุลาดำหลังอนุบาลอายุ 1.5 เดือน ในกระชังภายในบ่อดิน ด้วยอาหารสูตรที่กำหนดเป็นระยะเวลา 56 วัน เมื่อสิ้นสุดการทดลองพบว่ากุ้งมีน้ำหนักเฉลี่ย 7.62±2.74, 7.53±2.60, 8.00±2.32, 7.23±2.40 และ 7.40±2.43 กรัมตามลำดับ เมื่อทดสอบทางสถิติพบว่าน้ำหนักของกุ้งที่กินอาหารผสมสาหร่าย 5% มีค่าสูงกว่ากุ้งที่กินอาหารผสมสาหร่าย 2.5, 7.5 และ10% อย่างมีนัยสำคัญ ความยาวและผลผลิตรวมเมื่อเลี้ยงครบ 56 วันไม่มีความแตกต่างกันทางสถิติในอาหารทุกสูตร อัตรารอดเมื่อสิ้นสุดการทดลองพบว่ากุ้งที่กินอาหารผสมสาหร่าย 2.5% มีอัตรารอดสูงกว่ากุ้งกินอาหารสูตรควบคุมอย่างมีนัยสำคัญ (86.33±4.16 และ 76.00±8.41) การศึกษาภูมิคุ้มกันในกุ้งกุลาดำที่ได้รับอาหารที่มีสาหร่ายผสมต่างกัน ด้วยการทำให้กุ้งติดเชื้อ *Vibrio harveyi* พบว่ากุ้งกินอาหารเสริมสาหร่าย 2.5% มีอัตรารอดและค่า LD_{50} สูงกว่ากุ้งกินอาหารสูตรอื่นๆ ทั้งในการทดลองระยะวัยรุ่นและกุ้งหลังอายุ 1.5 เดือน การเปรียบเทียบปริมาณเม็ดเลือดและฟีนอลออกซิเดสของกุ้งที่ให้อาหารแต่ละสูตรไม่พบความแตกต่างกัน

ภาควิชา วิทยาศาสตร์ทางทะเล ลายมือชื่อนิสิต.....

สาขาวิชา วิทยาศาสตร์ทางทะเล ลายมือชื่ออาจารย์ที่ปรึกษา.....

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SIRIKANYA CHUNGTHANAWONG: EFFECTS OF KELP *Ascophyllum nodosum* SUPPLEMENT IN DIET ON DISEASE RESISTANCE IN BLACK TIGER SHRIMP *Penaeus monodon*. THESIS ADVISOR: ASST.PROF. SOMKIAT PIYATIRATITIVORAKUL, Ph.D. 83 pp. ISBN 974-53-1147-2.

Effect of kelp *Ascophyllum nodosum* supplemented diets on *Penaeus monodon* was tested in two experiments. All experiments were used diets consisted of 40% protein and 10% fat. *A. nodosum* meal was mixed to shrimp basal diet at concentration of 0, 2.5, 5, 7.5 and 10%. In experimental 1, a 28 days feeding trail was conducted on postlarvae-15. The experiment was tested in cages installed in a closed recirculting water system. At the end of the experiment, shrimp's growth data showed that shrimp fed 2.5% kelp added diet had the highest average length, but was not significantly different from the other diets. Survival rate of shrimp fed 2.5% kelp diet was significant higher than those shrimp fed other diets. In experimental 2, shrimp at the age of 1.5 month-old was tested with the same previous diets for 56 days in cages that were installed in a earthen pond. The resulted showed that the length of shrimp fed different diets were no significant difference. However, when the weight was considered shrimp fed 5% of kelp supplement had significant higher weight (8.00±2.32 g) than those shrimp fed 2.5, 7.5 and 10% kelp added, but not the control one (7.53±2.60, 7.23±2.40, 7.40±2.43 and 7.62±2.74 g, respectively). Shrimp production per cage showed no significant difference amount the diets. Survival rate of shrimp fed 2.5% was significantly higher than those of shrimp fed other diets. In disease resistant test with *Vibrio harveyi*, shrimp fed all the diets did not show any significance in total hemocyte count and phenoloxidase activity. The survival rate and LD_{50} of the shrimp after challenging test indicated that shrimp fed 2.5% kelp added diet was significantly higher than those shrimp fed other diets.

Department MARINE SCIENCE Student's signature……...……

Field of study MARINE SCIENCE Advisor's signature……

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CHAPTER I

INTRODUCTION

Recently, most Thai shrimp farms use an intensive culture which intends to produce high shrimp production. Therefore, high density of shrimp, use of artificial feed and appropriate technologies are applied to this intensive system. Apart from this, some antibiotics such as choramfinicals and nitrofurans are employed by the farmers for disease prevention or treatment. These antibiotics and their residues are contaminated shrimp flesh making the products unacceptable to international consumers. The demands and value of shrimp were declined from 63, 826 (in 2002) to 32,211 million baths (in 2004). From this reason, the use of non-antibiotic products such as natural herbal products, probiotics and prebiotics becomes alternatively importance and need more intensive study to overcome health shrimp products. For example, the uses of astaxantin, HUFA, vitamin C, chitin and chitosan have been studied and showed some positive responses to shrimp health.

Kelp or seaweed is a natural product that provides animals with natural chelated minerals and vitamins which satisfy their need for trace elements lacking or low in mixed feeds, pastures and other forages. The uses of kelp meal as a feed supplement are showed in many livestock, for increasing of milk yield, better fat content and iodine of dairy cow or the increasing of weight gains and reducing of susceptibility to disease in shrimp (http://oldheirloomroses.com/kelp-feed.htm, and http://www.showpoultry.com/bpf/products/kelp.html). Although, there is a small number of the studies of the effect of kelp meal on black tiger shrimp, seaweed could be one of potential biological products used for reducing disease infections in shrimp since Takahashi *et al*., (1998) reported that the product of brown seaweed called “fucoidan”, a sulphate polysaccharide substance, can protect Kurama shrimp from white spot syndrome. Chotigeat *et al*. (2004) also showed that oral administration of fucoidan extracted from *Sargassum polycystum* can reduce the impact of white spot syndrome virus infection in *P. monodon*.

The study of seaweed products on bacterial resistance is usually deal with fucoidan extracts from kelp. In the present study, we would like to test whether crude kelp supplement to diets has any potential on growth promotion or resistant to bacteria, *Vibrio harveyi*, in both postlaval and grow-out stage of black tiger shrimp.

CHAPTER II

LITERATURE REVIEW

2.1 Crustacean immune system

Crustacean immune system is innate immunity which can be aware the different of self and non-self (Ratcliffe *et.al.,* 1985). The immune response system can be divided into 2 groups: cellular immune response and humoral immune response (Lackie, 1980; Ratcliffe *et.al.,* 1985)

2.1.1 Cellular immune response

I. Hemocyte classification

It is active after stimulated by non-self suspicious and the effect is non-specific defense. The response consists of hemolymph clotting, phagocytosis, nodule formation, encapsulation and prophenoloxidase system. Hemocyte is the important cellular component of the immune response and their activities. In crustacean, circulating hemocytes is an open system. Hemopoietic (hematopoietic) tissue is the hemocytes producing organ. It is nearly anterior aorta, under rostrum. Crustacean blood contains hemocyanin pigment which has only gas exchange function while hemocyte is the immune defense. Hemocyte can be divided into 3 basic cell types; one type of hyaline cell and two subgroups of granulocytes, small and large granule hemocytes, (Hose *et al*., 1990).

A. Hyaline cells

Hyaline cells were the most morphologically diverse type of hemocyte. They were generally ovoid in shape, smaller than granulocytes and with a higher nucleocytoplasmic ratio, and either contained few large granules or numerous smaller granules (less than 50 nanometers diameter) when examined by phase contrast microscopy. This means that it has a bigger nucleus and fewer cytoplasms than other hemocyte. (Hose *et al*., 1990)

B. Granular cells

Granulocytes could be divided into 2 groups using phase contrast microscopy as follow:

a. Small granular cell

This granulocytes contained few to many, round, dark, small (usually≤1.0 micrometers diameter) granules and a relatively small, centrally located nucleus. (Hose *et al.*, 1990)

b. Large granular cell

Cytoplasm of large granular cell was packed with larger granule (1.3-2.0 micrometers diameter), refractile granules that obscured the eccentrically placed nucleus. (Hose et al., 1990)

However, both granulocytes usually difficult to differentiate because the sectioned granules are similar in sizes. To distinguish between small and large granule hemocytes the location of the nucleus (centrally or eccentrically placed) and the presence of only large granules (>1.2 micrometer diameter) can be utilized. The granular cell consists of large granules only while the semigranular cell contains both large and small granules. (Hose et al., 1990)

II Cellular defenses

A. Hemolymph clotting

This is the first mechanism which is active after crustacean injure. Hemolymph clotting prevents them from blood loss and the confinement of invasive organisms (Johansson and Soderhall, 1989; Hose *et al*., 1990). This system is functioned by the cooperation between hyaline cell and coagulogen in humeral.

B. Phagocytosis

Phagocytosis is the most important process of blood cells to destroy and defend foreign object both living and non-living forms (McKay and Jenkin, 1970; Reade, 1968). Invertebrate phagocytosis mechanism is similar to vertebrate animals. Blood cells attach to foreign object and their cytoplasm enfold it for respiratory burst (Ratcliffe *et al*., 1985). Hemocyte doing this process are found in different types depending on crustacean species. For example, hyaline cells are the phagocytic cell in *Parachaeraps bicarinatus* and *Astacus astacus* but in Penaeid species, granular cells are the phagocytic cell (Mckay and Jenkin, 1970; Smith and Soderhall, 1983; Bachere *et al*, 1995; Hose *et al*, 1990; Itami *et al*, 1998).

C. Nodule formation

In the case that foreign objects are much more than the capability of to phagocytosis, a lot of nodule will form and fix with foreign object to prevent the spread of it. Nodule formation usually forms at gill and hepatopancreas (Ratcliffe *et al*., 1985).

D. Encapsulation

This mechanism functions when the foreign object is bigger than 10 μm (Lackie, 1980) which only one blood cell cannot destroy. Foreign object are namely parasites, fungus and huge unicellular animals. Hose *et al*. (1990) showed the encapsulation of fungal hyphae on spiny lobsters (*Panulirus interruptus*), sheep crabs (*Loxrhynchus grandis*) and maine lobsters (*Homarus americanus*). The observation of initial hemocyte contacting with fungal hyphae showed that approximately two-thirds of adherent cells were large granule hemocytes, between 20% and 30% were small granule hemocytes. For all three species, percentages of adherent large granule cells were enriched 7 to 15 times over those found in hemolymph.

E. Prophenoloxidase system (proPO)

Prophenoloxidase system is the most important defensive mechanism of invertebrate especially crustacean. More than 90% of PO activity was found in heamocytes and the rest are in the serum. Semigranule and granule hemocytes are produce and keep proPO relative enzyme. Proteins of the proPO system occupy a very prominent position in non-self recognition, haemocyte communication and the production of melanin. The inactive proPO is converted to the active phenoloxidase (PO) by prophenoloxidase activating enzyme (ppA) when activation and degranulation of the haemocytes. PO enzyme catalyses oxidation of phenol to quinones that lead to the formation of melanin, a dark brown pigment that sequesters the pathogens, and thus preventing their contact with the host (Braak, 2002). Johansson and Soderhall (1989) reported 76 kDa protein contains in shrimp blood and encourage the function of proPO system. Degranulation and proPO are increasing. Baseline of PO activity of *P. monodon* under farm and laboratory conditions were 335.18±106.29 and 217.84±161.99 unit/min/mg protein respectively (Supamattaya *et al*., 2000).

2.1.2 Humoral immune response

Immunoglobulin is not found in invertebrate serum but the serum contains other component in which function as immune response namely agglutinin, antibacterial activity, cytokine –like factor, modulators and clotting-like factor (Lackie, 1980., Ratcliffe *et al*., 1985 McKay *et al*., 1969).

I. Agglutinins

Agglutinins are substance inducing the forming of foreign object agglutination and phagocytosis. In shrimp plasma, agglutinating activity has been detected in *Penaeus monodon, P. stylirostris, P. carliforniensis. P. japonicus* and *P. indicus.* A 420-kDa glycoprotein of *P. monodon* plasma, formed by 27-kDa identical subunits, was isolated by affinity chromatography. This lactin, named monodin is specific for NANA (N-acetyl neuraminic acid) but its specificity is not absolute and is shared with other N-acetyl amino sugas. Monodin induces the agglutination of Vibrio vulnificus, a major infective bacterium for prawn and this agglutination can be specifically inhibited by NANA (Albores and Plascencia, 1998.)

II. Antibacterial activity

Antibacterial activity is found in plasma, serum and haemocyte lysate supernatant (Adam, 1991; Destoumieux *et al*, 1997; Noga et al, 1996). This substance called bactericidin. The study of bactericidin in lobster, *Panulinus argus*, reported the substance induced by gram negative rod bacteria isolated from lobster intestine (Evans *et al*, 1968). In *P. monodon*, Bactericidin is found in plasma and can resists to *V. anguillarum*, *V.alginolyticus* and *E.coli*(Adams, 1991; Sung *et al*, 1996). Antibacterial activity of *P. monodon* present in plasma which can induced by *Vibrio alginolyticus* (Adam, 1991)

III. Cytokine –like factor

Cytokine –like factor of shrimp is 76 kDa proteins. It is encourage phagocytosis activity and strengthen the attach of foreign object and hemocyte when encapsulated (Smith and Chisholm, 1992). Not only that, it enhances prophenoloxidase activity by stimulated granular cell to increase degranulation activity and releasing of prophenoloxidase (Johansson and Soderhall, 1989).

IV. Modulators

Modulators are controller of crustacean immune system namely proteinase inhibitor and α-macroglobulin, which control the balance of serine proteinase in prophenoloxidase system (Smith and Chisholm, 1992).

V. Clotting-like factor

Coagulogen is plasma protein which prevent blood leaching and diseases infection (Smith and Chisholm, 1992).

2.1.3. Luminous disease

The causes of shrimp diseases can be classified in 4 categories; bacteria, viruses, fungi and parasites.

I. Bacteria

The diseases caused by bacteria are the main cause of shrimp death and the bacteria can develop themselves to resist to antibiotics. Vibrio is the most common bacteria found in shrimp farms such as *V. harveyi, V. parahaemolyticus, V. splendidus, V. fisheri, V. cholerar, and V. vulnificus*. However, *Aeromonas sp*. is also found, but it is trifling effect.

II. Viruses

The diseases caused by viruses make a major negative impact on shrimp production. The virus types found so far are as the following:

- Penaeus monodon baculovirus (MVB)
- Lymphoid organ virus
- Infectious hematopoietic and hypodermal necrosis virus (IHHNV)
- Hepatopancreatic parvo-like virus (HPV)
- Yellow head virus (YHV)
- Systemic ectodermal and mesodermal baculovirus (SEMBV)

III. Fungi

Fungus diseases cause a minor damage on shrimp farming. The common fungi found in shrimp farm are *Lagenidium sp. and Fusarium sp.*

IV. Parasites

They can be divided into external parasite such as *Zoothannium sp*., *Epistylis sp*., *Vorticella sp*., and *Acineta sp*. and internal parasite such as *Agmasoma penaei* (macrosporidian)

In Thailand, luminous disease was reported about its widespread in 1987 which started in the center of Thailand. It has caused the high mortality rate in shrimp hatchery from nauplius until zoea stages of *P. merguiensis* (but not found the mortality at mysis stage). In *P. monodon*, 80-100% of shrimp mortalities (nauplius to mysis state) were caused by this disease. The contaminated tank or pond with this disease can be noticed by the luminescence of water and shrimp. Moreover, *V. harveyi* can also be found on shrimp having tea-brown gill syndrome (TBGS) and red disease syndrome (Tansutapanit and Ruangpan, 1998.).

Vibrio species are part of the autochthonous flora of marine organisms and one of the most important groups in marine environments. In surface water of the western Pacific Ocean got nearly 80% of them (Tsukamoto et al, 1993). From the numerical taxonomic analysis, *Vibrio* species collected from infected shrimp in Thai hatchery are 180 species (Luminous and aluminous species) which 70% of luminous species is *V. harveyi* and 7.7% is *V. fishery*. In 1996, the sample of 210 luminous bacteria in Thai nearshore was reported and showed that 57% is *V. harveyi, V. cholerae* biotype albensis and *photobacterium leiogathi*. This report also found *V. harveyi* showing green or yellow green colonies on TCBS agar. Characteristics of *V. harveyi* from shrimp luminous disease is showen in Table 1. The main pathogenic *Vibrio species* affecting *Penaeid* shrimp are *V. vulnificus, V. alginolyticus, V. campbellii, V. splendidus, V. damsela, V. parahaemolyticus* and *V. harveyi* (Lightner, 1996).

Luminous disease from bacteria is usually widespread in summer because of high temperature. They grow better in 10-40 ppt salinity, temperature between 20-30°C and pH at 8-9. Optimum condition for *Vibrio* species is high organic contents and low oxygen concentration, which mostly occur after shrimp was domesticated for 3 or 4 months in earthen pond (Bhaskar and Setty, 1994).

Table1 Characteristics of *V. harveyi* from shrimp luminous disease (Nihimathachoke *et al*.,1995)

Test	Characteristics
Gram staining	Gram negative rod
Growth on TCBS	Green
Luminescence	+
Mortality	+
Swarming	-
Pigment production	-
Oxidative-fermentative test	Fermentative
Oxidase	+
Catalase	+
Gas from glucose	-
Nitrate reduction	+
Methyl red	+
Voges-Proskauer	-
Indole production	+
Citrate utilization	-
Decarboxylase of:	
Arginine	-
Lysine	+
Ornithine	-
Gelatin liquefaction	+
Growth at temperature	
4°C	+
30°C	+
40°C	-
Growth in % NaCl	
0	-
1	+
3	+
6	+
8	-

Test	Characteristics
100	-
Utilization of	
Glucose	+
Lactose	-
Sucrose	-
Arabinose	-
Mannitol	+
Sensitivity to 0/129	
10 mg	+
150 mg	+
Antibiotic Sensitivity (S)/Resistance (R)	
Chloramphenicol	S
Sulfamenthoxazole-trimethoprim	S
Oxytetracycline	R
Nitrofurantion	Intermediate S
Oxolinic acid	S

2.1.4 The use of kelps

I. *Ascophyllum nodosum*

Ascophyllum nodosum is one species of kelps which is very common wrack of the middle shore of Europe and the Atlantic coast of North America but not in a wide range of habitats. Adult seaweeds requires substrate, such as stones, piers and other solid objects, for their attachment in estuaries and semi-sheltered bays. This species of kelp has many common names, namely knotted wrack, asco, rockweed, yellow weed, sea whishes, and yellow tang.

A. nodosum is most abundant in sheltered areas. When submerged, the plant floats buoyed by its air bladders. At high tide, these beds provide shelter and food for many small fishes and other marine organisms. Because of their association with sheltered area, they can growth in small niches on surprisingly exposed sections of coast such as gently sloping rock platforms which is cut down on wave exposure.

The seaweed is an olive green to yellow, turning greenish black when it is dried. They usually grow up to 2 meters in length (after 3-5 years) and have long,

tough, leathery, elastic, slender, strap-like, irregularly-branched fronds (with no mid rib) growing from a crowded, disc-shaped holdfast. The basal disc supports numerous basal fronds, often numbering > 50. Spaced at intervals along the middle of the main fronds and sometimes occupying their full widths are the characteristic long, egg-shaped bladders, which contain gas. The number of bladders present gives an indication of the age of the plant after allowing 2 years of initial growth. One bladder is generally formed each year. When submerge by the tide, these bladders float the fronds up towards the light maximizing the photosynthetic potential of the seaweed.

The harvesting of the seaweed is start when this kelp is 3-5 years old, depend on the area. Sustainable harvesting requires that between 3-5 years is allowed to pass before the next cut of weed is taken from the same area. The regular harvesting gives greater production.

This seaweed is valued for their proven record of beneficial effects on plant growth and health attributed to their concentrations of important trace elements. The regular application of seaweed manure or spray is said to have the following benefits:

- the promotion of seed germination and growth
- increases resistance to frost damage and drought
- increases resistance to fungal and insect pest
- fruit trees produce larger and more abundant crops of apples, oranges, bananas, tomatoes, pineapples, grapes, strawberries, peaches, etc.
- crops of tomatoes, carrots, Brussels sprouts, beetroot also show increased yields.

A. nodosum contain high level of nutrients and vitamins and trace elements which showed in Table 2. (Morrissey, Kraan and Guiry. 2001)

Table2 Nutritional analysis of *A. nodosum* (Morrissey, Kraan and Guiry. 2001)

Protein	5-12%
Fat	2-4%
Carbohydrate	42-64%
Mannitol	4.20%
Alginic acid	26%
Laminaran	10%
Vitamin C	500-1650ppm
Beta-carotene	35-80ppm
Vitamin B1	1-5ppm
Vitamin B2	5-10ppm
Vitamin B3	10-30ppm
Vitamin B6	0.1-0.5ppm
Vitamin B12	0.8-3ppm
Vitamin E	260-450ppm
Vitamin H	0.1-0.4ppm
Vitamin K3	10ppm
Calcium	1-3%
Iodine	700-120ppm
Iron	101-176ppm
Magnesium	0.5-0.9%
Manganese	10-15ppm
Sodium	3-4%
Zinc	70-240ppm
Fucoidan	4-10%

The uses of kelp, *A. nodosum*, as a feed supplement were showed in many livestock. This studies have proven the benefit of this kelp for animal consumption. Many studies are the battle against kinds of cancers. (http://oldheirloomroses.com/kelp-feed.htm.; http://www.noamkelp.com/slfeed.html.)

Benefits of kelp for livestock

- Dairy and beef cattle:
  1. Increased milk production and butterfat content. More efficient feed utilization thus requiring less feed
  2. Longer lactation periods
  3. Dramatic reduction of pink eye
  4. Increased iodine and vitamin A content of milk in cows feed kelp
  5. Improved conception rate and increase in birth of healthy cows
  6. Increased hemoglobin levels in blood
  7. Less problems caused by dietary deficiencies such as mastitis, retained placenta, milk fever, abortions and infertility. Milk fever can usually be averted by feeding mothers for 3-4 months before calving.
  8. In beef cattle- increased weight of 5 lb/day on less feed. Increased loin size and reduction of black foot.
- Swine:
  1. Better feed conversion, rapid gains make for marketing weeks earlier
  2. Increased litters
  3. More meat and less fat per carcass and reduction in black fat thickness
  4. Helps drastically reduce the occurrence of liver parasites thus salable livers
- Horses:
  1. Brood mares especially benefit by the addition of kelp to their diet and increased fertility
  2. Helps prevent cracked hooves
  3. Reduces feed bills due to increased utilization of feed
  4. Stable vices and nervous habits are often minimized or eliminated
  5. Skin texture and elasticity are improved along with coat gloss
  6. Higher resistance to infections and faster healing time from injuries
- Sheep:
  1. Increases of up to 20% in wool productions due to the ability of kelp to prevent molting
  2. Better quality of wool as it is longer, stronger fibers
  3. Reduction of lamb losses caused by white muscle disease

4. Increased lambs born per ewe. Easier drops during lambing
5. Reduced levels of internal parasites and better growth rates

- Poultry:
  1. Brighter plumage, increased weight and general alertness
  2. Marked increase in iodine content of eggs produced
  3. Yolks are deeper colored with better pigmentation
  4. Improved hatchability of eggs
  5. Reduction of blood spots in eggs
  6. Reduced coccidiosis
  7. Harder shells thus less egg breakage
  8. Improved health resulting in less diseases and usage of antibiotics

II. Fucoidan

Fucoidan is a complex polysaccharide composed largely of fucopyranoside and natural sulphate. Fucoidan also has trace elements of galactose, xylose and glucolonic acid- three different types of sugar molecules. The polymers can be broke down by hydrolysis. (Marais and Joseleau, 2001.). The core region of the fucan is composed primarily of a polymer of alpha 1-3-linked fucose with sulphate groups substituted at the 4 position on some of the fucose residues. Fucose is also attached to this polymer to form branch points, one for every 2-3 fucose residues within the chain (http://www.ncbi.nih.gov/entrez/query.fcgi?cmd). Chevolot et al. (2001) report that fucoidan from *A. nodosum*, prepared by acid hydrolysis and centrifucal partition chromatography (LMWF), is the repeating unit of monosaccharide and sulphate group.

$$[\rightarrow 3)\text{-}\alpha\text{-L-Fuc}(2SO_3^-)\text{-}(1\rightarrow 4)\text{-}\alpha\text{-L-Fuc}(2,3diSO_3^-)\text{-}(1]_n$$

There are three types of Kombu fucoidan which have different chemical structures: U-Fucoidan, F-Fucoidan, and G-Fucoidan. Each different structure is different activities and the structure of them showed in Figure 1

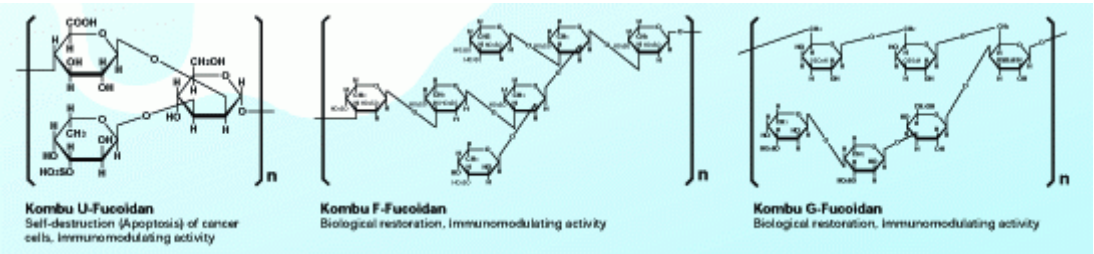

Figure 1 Kombu fucoidan types: U, F, and G-fucoidan**.**
(http://takara-bio.co.jp/english/products/pdfs/fuco.pdf).

- U-fucoidan causes cancer cells to self-destruct, induce apoptosis in cancer cells. This mechanism can be distinguished from necrosis, which is the death of cells directly brought about physical and chemical damages. Thus, U-fucoidan has an anticancer effect, which demonstrates no adverse effect (no damage to normal cells). In animal studies, it was demonstrated to reduce tumor size and to have a life-extending effect in nude mice implanted with human colon cancer cells when the mice were allowed to consume Kombu fucoidan and libitum. In rat and mice with chemically induced-cancer, a marked life-extending effect was confirmed when these animals were allowed to consume it.

- F-fucoidan and G-fucoidan were determined, which then led to the discovery that these fucoidans induce the production of Hepatocyte Growth Factor (HGF) in cultured cells. Subsequent studies have demonstrated that HGF prosesses growth promoting and activating effects in various cells.

All types of fucoidan have immunomodulating activity. Fucoidan have been found to induce the production of interferon-γ(IFN- γ) and interleukin-12(IL-12) in immunocytes. IFN- γ and IL-12 enhance the immunological ability to attack foreign invasion (antigen), while they inhibit the overproduction of IgE, which cause the immune system to overreact such as pollen hypersensitivity (http://takara-bio.co.jp/english/products/pdfs/fuco.pdf).

The effect of fucoidan on anticoagulant was reported by Tholacius *et al.*, 2000 (*site in* Alejandro, Mayer and Hamann, 2000). Fucoidan was affected on P- and L-selectin, two members of the selectin family of adhesion molecules. It inhibited thrombus formation in arterioles and venules in vivo with no effect on P and L-seletin function suggested that the anticoagulant effect of fucoidan was mainly responsible

for its powerful antithrombotic property in vivo. Albuquerque *et al*.(2004) reported that fucoidan isolated from different algaes has different anticoagulant activity; the activities depend on their sulphate content and molecular weight.

The studies of fucoidan on aquatic domestication are not abundant. In shrimp domestication, a purified preparation fucoidan (>70%) extracting from *Chladosiphon okamuranus* supplemented in Kurama shrimp diet for two dosages, 60 and 100 mg/kg/body weight per day and subsequently exposed to the white spot syndrome virus (WSSV). Shrimp were fed for 15 days with the challenge beginning by water borne exposure to carriers at day 4. Shrimp which fed by supplementary diet have more than 80% survival. This values were significantly higher than the value in the control group (Takahashi *et al*.,1998)

Velasco *et al*. (2002) were study the resistant of WSSV on pacific white shrimp (*Litopenaeus vannamei*) by kelp meal. 3.5% of kelp was mixed with the diet and fed for 90 days. Their result reported that shrimp growth and survival were not different from shrimp fed by control diet.

The study of Chotigeat *et al*. (2004) indicated that oral administration of fucoidan extracted from *Sargassum polycystum* could reduce the impact of white spot syndrome virus infection on *P. monodon* in two span weight 5-8 and 12-15 g. Survival rate after 10 days infection are 46% and 93% respectively. And the minimum fucoidan concentration for inhibiting the disease are 12.0 and 6.0 mg/ml.

CHAPTER III

MATERIALS AND METHODS

3.1 Experimental design

This study consisted of 2 experiments: the effect of kelp meal on postlarva-15 (P-15) black tiger shrimp and the effect of kelp meal on grow-out black tiger shrimp (1.5 month-old). A completely randomized design (CRD) was used in the study. Five concentrations of kelp *A. nodosum* supplemented diets (0%, 2.5%, 5%, 7.5%, and 10 %) were utilized and four replications were done in each treatment. The first experiment was carried out for 28 days and the second one for 56 days.

3.2 Experimental diets

A. nodosum meal was added to a basal diet, composed of 40% protein and 10% fat, at a proportion of 0, 2.5, 5, 7.5 and 10% of the diet. The basal diet ingredients are shown in **Table 3.** All ingredients were grounded into 200 mesh-size powder and mixed by a twin blade-rolling mixer for 30 minutes. After the mixtures became homogenized, each mash was pelleted using California Pelleting Machine (CPM) at a size of 2.2 mm in diameters and 5.0 cm in length, steamed at 95 ^{o}C for 5 minutes and dried by hot air oven at 60 ^{o}C for 2 hours. For postlarval experiment, the diets were crushed into a size of nearly 1-2 mm. All diets were kept in dry-dark container and kept at -20°C until use. The stability and the proximate analysis of experimental diets (crude protein, lipid, ash, fiber and moisture contents) was determined using AOAC (1990).

Table 3 Feed ingredients of experimental diet

Ingredients	Dry weight (g 100^{-1} g of diet)				
	0%kelp	2.5%kelp	5.0%kelp	7.5%kelp	10.0%kelp
Fish meal	47.0	47.0	47.0	47.0	47.0
Wheat flour	25.0	22.5	20.0	17.5	15.0
Soybean meal	5.0	5.0	5.0	5.0	5.0
Shrimp head meal	7.0	7.0	7.0	7.0	7.0
Wheat gluten	6.0	6.0	6.0	6.0	6.0
Refined tuna fish oil	2.0	2.0	2.0	2.0	2.0
Mineral mixture[a]	2.0	2.0	2.0	2.0	2.0
Vitamin mixture[b]	1.5	1.5	1.5	1.5	1.5
Cholesterol[c]	1.5	1.5	1.5	1.5	1.5
Lecithin[d]	2.0	2.0	2.0	2.0	2.0
Vitamin C[e]	0.5	0.5	0.5	0.5	0.5
Cellulose	0.5	0.5	0.5	0.5	0.5
Kelp	0.0	2.5	5.0	7.5	10.0

[a]Mineral mixture 100 g contains: K_2HPO_4 2.0 g, $Ca_3(PO_4)_2$ 2.720 g, $MgSO_47H_2O$ 3.041 g, $NaH_2PO_42H_2O$ 0.790 g.

[b]Vitamin mixture 100 g contains: ρ-aminobenzoic acid 10.0 mg, biotin 0.40 mg, inositol 400.0 mg, nicotinic acid 40.0 mg, Ca-pantothenate 60.0 mg, pyridoxine-HCl 12.0 mg, riboflavin 8.0 mg, thiamin-HCl 4.0 mg, menadione 4.0 mg, cyanocobalamine 0.08 mg, calciferol 1.20 mg, folic acid 0.80 mg, choline chloride 120.0 mg.

[c]Ninety five percent cholesterol, laboratory grade, Sigma.

[d]Soy lecithin, feed grade.

[e]Stay C 35 %, Roch®.

3.3 The effect of kelp meal on postlarva-15 (P-15) black tiger shrimp

Experimental pond

In this experiment an in-house closed recirculating water system with a volume of 25 m^3 described by Menasveta *et al*. (1991) was used. The system consisted of a rounded cement pond with 7.0 m in diameter and 1.0 m in depth. The system has an inner pond with 4.0 m in diameter worked as a biological filter unit. This pond was filled with layers of sea sand and oyster shell for sediment filtration and biological treatment. The culture section (ring) is 1.5 m in breadth with water depth of 80 cm. Air lift system was used to operate filtering system and kept dissolved oxygen above 6 mg/l.

For postlarval culture, a set of cultured cages was installed in the culture section of pond. The cage size is 1x1x1 m^3. All experiments were done in these cages (Figure 2). Water salinity was controlled at 20 ppt for the whole period of experiment.

Figure 2 Rearing system of black tiger shrimp for postlarva-15 experiment

Rearing animals and feeding regime

Postlarva-10 of black tiger shrimp from the same female broodstock were used for this experiment. The shrimp were initially acclimated for 5 days before the experiment. One hundred of P-15 black tiger shrimp were selected and transferred to each cage. Feeding regime was 4 times daily; 6.00, 12.00, 18.00 and 22.00 hour as a feeding rate of approximately 10% the body weight per day. The experiment was performed for 28 days. Growth rate of the shrimp was monitored their length every

two weeks from 20% of total shrimp in each treatment randomly. Survival of shrimp in each cage was determined at the end of the experiment. A number of shrimp were randomly in each treatment and carried for testing the disease resistance and immune response.

Disease resistant test

Shrimp with the same size were acclimated for 5 days at the laboratory condition (salinity 20 ppt, tempterature 28±2°C) before the challenging experiment. Feeding regime of the shrimp in each experimental diet was carried out in the same way as above. Ten shrimp were randomly selected and released to each rearing unit containing 10 liters of 20 ppt water before 10^6 CFU/ml of *V. harveyi* were added into the water. The mortality rate was measured everyday for a week.

3.4 The effect of kelp meal on grow out black tiger shrimp

One hundred black tiger shrimp (1.5 month-old) at the average size of 3.8 g were selected from a nursery pond and transferred to 2x2x1.5 m^3 cage floating in an 30x30x2 m^3 earthen pond at 3 ppt water salinity. The shrimp were acclimated under experimental condition for at least 7 days before starting the experiment. Aeration was provided for the whole period of the experiment. Each diet treatment was run in 4 replicates. The experiment was carried out for 56 days. The shrimp was fed 3 times daily; 6.00, 13.00 and 19.00 hour. Feeding rate was approximately 5% body weight per day. Every two weeks, 20% of shrimp in each cage were randomly caught for growth rate determination. Survival rate was done at the end of the experiment and shrimp was randomly chosen to test the resistant to *V. harveyi.*

Figure 3 Rearing system of black tiger shrimp for grow out experiment

Disease resistant test

Shrimp with the same size were collected and acclimated for 5 days before the experiment started. Feeding regime was the same as 3.3. Ten shrimp were randomly put into each rearing unit which contains 100 liters of water with salinity of 20 ppt. Then 10^8 CFU/ml of bacteria *V. harveyi* were added into the water. The mortality rate was determined everyday until day 7. Each dead shrimp was tested to confirm the death of Vibriosis. Hepatopancreas and rearing water were cultured to find the bacteria type.

3.5 The resistant to luminous disease

3.5.1. Tested bacteria preparation

V. harveyi type 1526 was cultured in marine broth for 18-20 hours on shaker at room temperature and used immediately. Bacterial concentration was measured by spectrophotometer (GENESIS®; model 10 UV scanning) at 600 nm.

Bacterial concentration = $(10^9 x) - (2 \times 10^7)$

x = The absorbance of the solution was measured by spectrophotometer

3.5.2. Haemolymph analysis

3.5.2.1 Shrimp haemolymph preparation

Approximately 200 µL blood was collected from the last pair of walking legs (Supamattaya *et al*, 2000a) by small syringe (1 mL #26). The blood was mixed with 200 µL anticoagulant (10% sodium citrate, pH 7.2) and kept in 1.5 ml microcentrifuge tube in an ice box for future analysis.

Figure 4 Haemolymph sampling from the last pair of walking leg of challenging Shrimp

3.5.2.2 Total haemocyte counts (THCs)

This method was modified from Supamattaya *et al*, 2000a. Shrimp blood approximately 50 µL was mixed with anticoagulant 50 µL and stained with 100 µL trypan blue. The mixture was stored in an ice box. THCs were done by haemacytometer under compound microscope.

Total haemocyte counts (cell/ml) = total of cell count x 5 x 10^4 x dilution factor

3.5.2.3 Phenoloxidase activity and haemolymph protein analysis

3.5.2.3.1 Haemocyte lysate supernatant; HLS preparation

Shrimp blood approximately 100 µL was mixed with anticoagulant 100 µL and centrifuged at 8,000 rpm for 5 minutes (at 4°C). Haemocyte pellet was washed with 500 µL CAC buffer (10 mM Ca-codylic acid with 100 mM $CaCl_2$ in deionised water; pH 6.8) after that resuspended in 500 µL CAC buffer and the cell were broken by sonicator for 2 minutes. Finally, the sample was centrifuged at 10,000 rpm for 10 minutes (at 4°C). Haemocyte lysate supernatant was collected for the study.

3.5.2.3.2 Phenoloxidase activity

Hundred microliter of HLS was mixed with 100 µL trypsin solution (0.1% trypsin in CAC buffer) and incubated for 10 minutes at room temperature. Then it was mixed with 100 µL L-dihydroxyphenylalanine (L-DOPA 4 mg/ml in CAC buffer) and measured the kinetic activity of phenoloxidase enzyme by microplate reader (BIO-RAD® model 550) at 490 nm for 10 minutes.

Phenoloxidase activity was calculated as the following formula:

Phenoloxidase activity = unit at $0.001(\Delta OD_{490})$/minute/mg.protein in solution

3.5.2.3.3 Haemolymph protein

HLS was diluted into the optimum concentration (compared with the standard protein). Two hundreds microliters of diluted HLS was mixed with 1 mL of protein reagent and incubated for 10 minutes at room temperature. After that it was mixed with 100 µL of solution D (Folin-ciocalteau reagent 1:1 (v/v) deionized water) and incubated for 30 minutes at room temperature. The absorbance of the solution was measured by spectrophotometer (GENESIS®; model 10 UV scanning) at 660 nm. Haemolymph protein was calculated by compared with standard protein.

3.6 Water quality

Temperature, salinity and pH were determined by YSI model 57. Alkalinity, ammonia, dissolved oxygen and nitrite were determined weekly by test kids developed by Faculty of Veterinarian Science, Chulalongkorn University, Thailand. All parameters were contained in the range which the shrimp can survive.

3.7 Statistical analysis

Effects of the experimental diet on length, weight gain and survival rate were analyzed using analysis of variance and Duncan's New Multiple Range Test.

CHAPTER IV

RESULTS

4.1 Experimental diets

Results of proximate analysis of experimental diet is described in Table 4 and diet stability is described in Table 5. Average protein of the diets was 42.09-42.64% and the lipid was 9.14-9.73%, respectively. Average moisture and fiber of all diet formulae were similar, but average ash was different. Stability of the experiment diets are approximately 2.5 hours.

Table 4 The proximate analysis of experimental diet

Diet formulas (%Kelp)	Protein (%)	Lipid (%)	Ash (%)	Moisture (%)	Fiber (%)
0	42.64±0.27	9.73±0.09	12.66±0.04	6.90±0.47	2.57±0.39
2.5	42.12±0.40	9.14±0.44	12.49±0.16	6.49±0.70	3.00±0.45
5.0	42.09±0.72	9.30±0.14	13.13±0.14	6.68±0.78	2.77±0.25
7.5	42.35±0.26	9.61±0.20	14.04±0.18	6.58±0.29	3.04±0.32
10.0	42.55±0.29	9.30±0.46	15.02±0.22	6.30±0.29	2.94±0.40

Table 5 The stability of experimental diet

Diet formulas (%Kelp)	Stability (Hours)
0	2.5
2.5	2.5
5.0	2.5
7.5	2.5
10.0	2.5

4.2 The effect of kelp meal on postlarva-15 (P-15) black tiger shrimp

4.2.1 Water quality of postlarva-15 ponds

Water quality of the experiment is described in Table 6. The quality was within recommended level for shrimp (Appendix B). Ammonia and nitrite were not detected throughout the domestic period and nitrate was detected at low level which does not affect shrimp. Dissolved oxygen was rather stable because of aerating system.

Table 6 Water quality of postlarva-15 ponds during rearing period

Parameter	Range
Salinity (ppt)	19.7-20.2
Temperature (^{o}C)	26.9-27.2
Dissolved oxygen (mg/l)	3.7-4.2
Alkalinity (mg/l)	136-187
pH	8.04-8.24
Ammonia (mg/l)	0
Nitrate (mg/l)	>0.3-16.5
Nitrite (mg/l)	0

4.2.2 Length, weight and growth rate (28 days) of postlarval black tiger shrimp in a closed recirculation system

Effect of kelp meal on postlarvae-15 is reported in Table 7. Shrimp length was similar in all diets both in the beginning and the end of the experiment. The length was 1.39-1.42 cm and 2.51-2.58 cm, respectively. Survival rate of shrimp fed 2.5% kelp was significantly different from shrimp fed other diet.

Table 7 Length, weight and growth rate (28 days) of postlarval black tiger shrimp

Diet formula (%Kelp)	Length (cm.)		Survival rate (%)
	Day-0	Day-28	
0	1.42±0.19[a]	2.53±0.61[a]	59.25±3.20[b]
2.5	1.39±0.09[a]	2.58±0.54[a]	76.50±5.44[a]
5.0	1.40±0.11[a]	2.55±0.51[a]	61.00±5.16[b]
7.5	1.39±0.09[a]	2.51±0.50[a]	63.25±6.18[b]
10.0	1.40±0.09[a]	2.51±0.51[a]	64.75±6.34[b]

Note: means in the same column with the same superscript indicate non significant difference ($p>0.05$)

4.2.3 Disease resistant test

Survival of shrimp fed 2.5% kelp was higher than those of other treatments and 7.5% kelp feeding treatment showed the lowest survival rate (Figure 5). However, LD50 showed shrimp in the control group had the lowest value and was significantly different from those of the other diets. Shrimp fed 2.5, 5 and 7.5% kelp diets showed non significant difference among them (Figure 6).

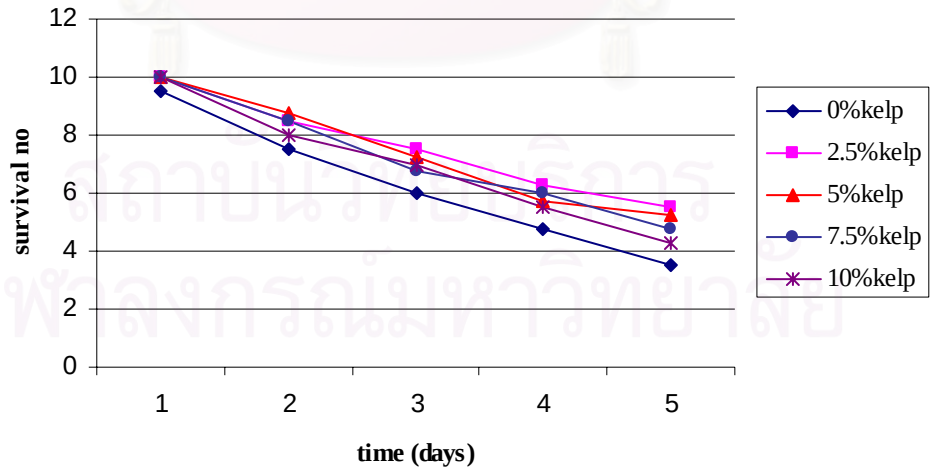

Figure 5 Survival of P15 black tiger shrimp fed different kelp added diets after challenging with *V. harveyi*

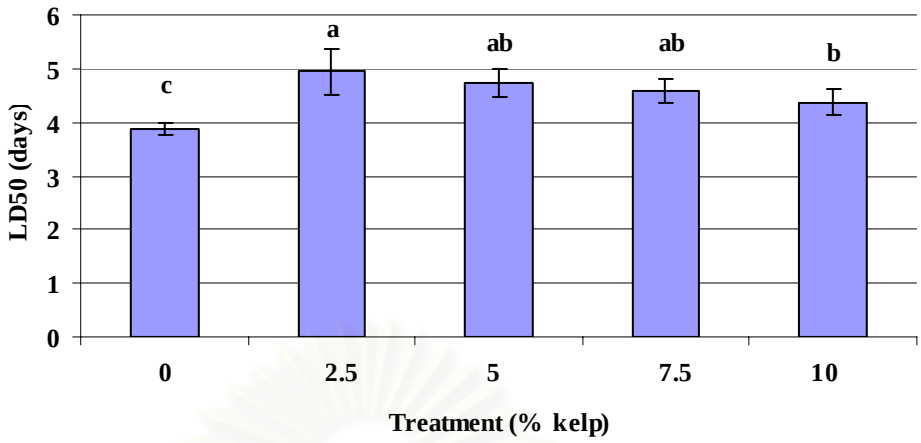

Figure 6 LD50 of P15 black tiger shrimp fed different kelp added diets after challenging with *V. harveyi*.

4.3 Effect of kelp meal on grow-out black tiger shrimp

4.3.1 Water quality of grow out black tiger shrimp ponds

Water quality of the experiment is described in Table 8. The quality was within recommended level for shrimp. Ammonia, nitrite and nitrate were similar to those in postlarval experiment. Dissolved oxygen was rather stable because of aerating system but the range was wider than the postlarval experiment.

Table 8 Water quality of grow out shrimp ponds during rearing period

Parameter	Range
Salinity (ppt)	3.0-4.3
Temperature (^{o}C)	27.9-32.2
Dissolved oxygen (mg/l)	3.2-4.1
Alkalinity (mg/l)	102-119
pH	7.0-7.53
Ammonia (mg/l)	0
Nitrate (mg/l)	0.3-16.5
Nitrite (mg/l)	0

4.3.2 Length, weight and survival rate of black tiger shrimp reared in cage with different kelp added diets

A study of 1.5 month-old shrimp in earth pond is reported in Table 9. Length of shrimp was not significantly different in all diets. Shrimp weight fed 5% kelp (8.00±2.32 g), was higher than those fed 2.5, 7.5 and 10% kelp (7.53±2.60g, 7.23±2.40 g and 7.40±2.43 g, respectively) but not different from the control (0% seaweed, 7.62±2.74 cm). Shrimp biomass showed no significant difference amount the diets. Survival rate of shrimp fed 2.5% kelp (86.33±4.16) was significantly higher than those of the control (76.00±8.41) but did not different with 5, 7.5 and 10% kelp (79.50±2.89, 77.75±4.79 and 76.25±7.41).

4.3.3 Disease resistant test

4.3.3.1 Shrimp mortality

Survival of shrimp fed 2.5% kelp was higher than other treatment (5, 7.5, and 10%kelp and control) which number of shrimp mortality was nearly among them (Figure 7). Not only that, statistical analysis, LD50, indicated that shrimp fed 2.5% kelp was significant higher than those of shrimp fed other diets. However, shrimp fed 5, 7.5 and 10% kelp and control did not showed any significant difference among them (Figure 8).

Table 9 Length, weight and survival rate of black tiger shrimp reared in cage with different kelp added diets

Diet formula (% Kelp)	Day-0		Day-56		Biomass (g)	Survival rate (%)
	Length (cm.)	Weight (g)	Length (cm.)	Weight (g)		
0	7.81±0.66	3.97±0.64	9.68±1.15^{a}	7.62±2.74^{ab}	580.18±120.64^{a}	76.00±8.41^{b}
2.5	7.81±0.66	3.97±0.64	9.60±1.11^{a}	7.53±2.60^{b}	654.78±57.96^{a}	86.33±4.16^{a}
5.0	7.81±0.66	3.97±0.64	9.77±1.00^{a}	8.00±2.32^{a}	637.97±49.45^{a}	79.50±2.89^{ab}
7.5	7.81±0.66	3.97±0.64	9.52±1.08^{a}	7.23±2.40^{b}	614.73±89.49^{a}	77.75±4.79^{ab}
10.0	7.81±0.66	3.97±0.64	9.61±1.13^{a}	7.40±2.43^{b}	564.84±124.86^{a}	76.25±7.41^{ab}

Note: means in the same column with the same superscript indicate non significant difference ($p>0.05$)

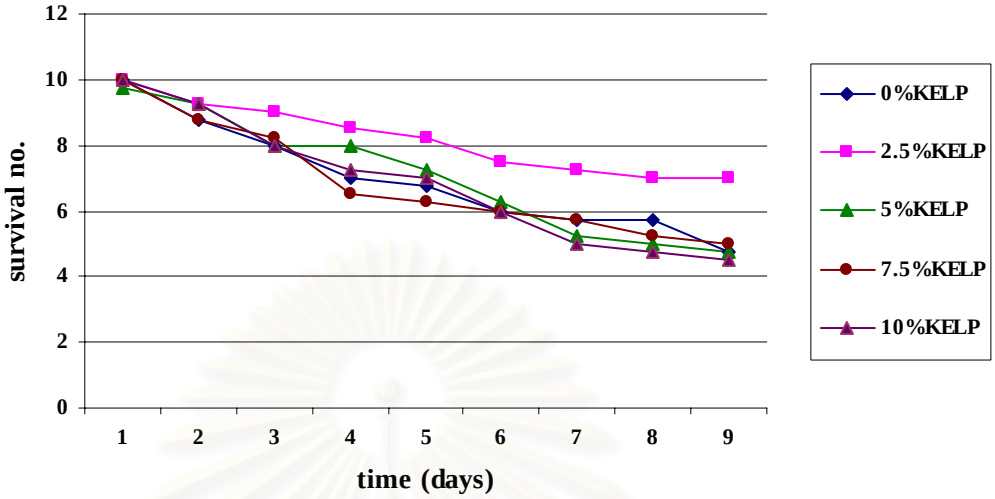

Figure 7 Survival of grow out black tiger shrimp fed different kelp added diets after challenging with *V. harveyi* on different kelp added diets.

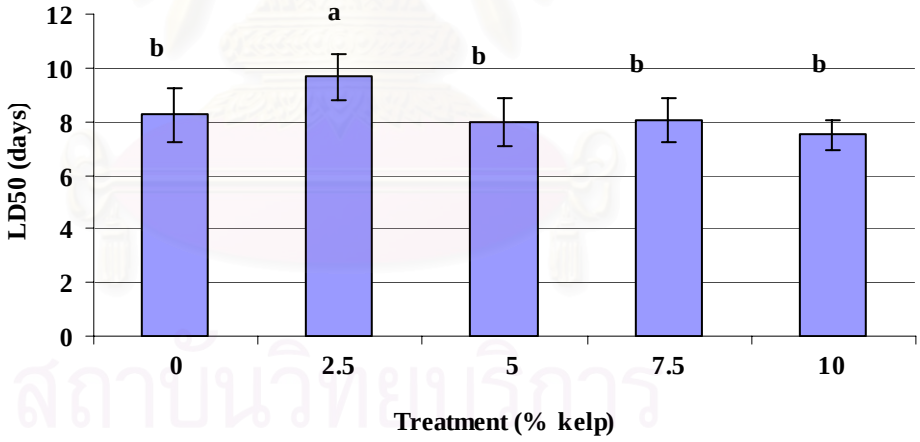

Figure 8 LD50 of grow out black tiger shrimp fed different kelp added diets after challenging with *V. harveyi*.

4.3.3.2 Physiological response of shrimp exposed to *V. harveyi*

Physiological response of shrimp after challenging with *V. harveyi* was illustrated in Figure 9-11. Immune response of *P. monodon* was indicated by total haemocyte count (THCs), haemolymph protein and phenoloxidase activity.

A. Total haemocyte count (THCs)

THCs, is reported in Figure 9, showing no significant difference among shrimp fed different diets. THCs change with time had similar trend. All of THCs were high in day 3 and 6, then declined in day 9 which the value were lower than day 0.

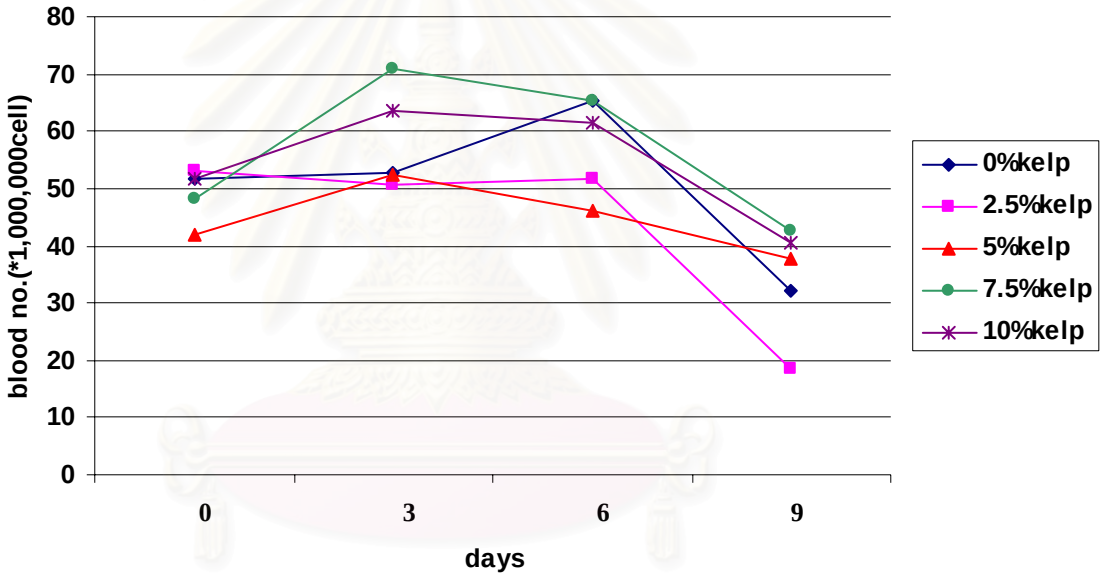

Figure 9 Total blood count of black tiger shrimp after being challenged with *V. harveyi* on different kelp added diets.

B. Phenoloxidase activity

Immune response of *P. monodon*, indicated by phenoloxidase activity, had high fluctuation. This activity showed non significant difference among treatments in time period. At the beginning of trial, phenoloxidase activity value was high and the value increase in day 3 and day 6 and then decline in day 9 (Figure 10), the result was similar trend with THCs. This study contains high standard deviation (not showed in the figure).

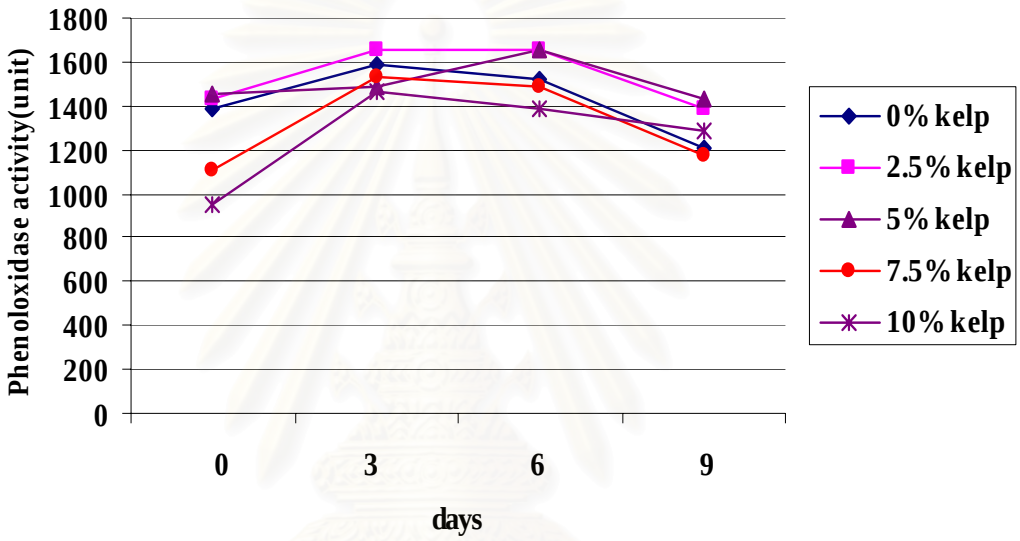

Figure 10 Phenoloxidase activity of black tiger shrimp after challenging with *V. harveyi* on different kelp added diets.

C. Haemolymph protein

Haemolymph protein of shrimp after challenging test showed their result alike THCs and phenoloxidase activity, non significant different. Figure 11 indicated large number of stadard deviation. However, trend of them were similar, these protein were the lowest at beginning then increased on day 3 and 6 and decreased on day 9.

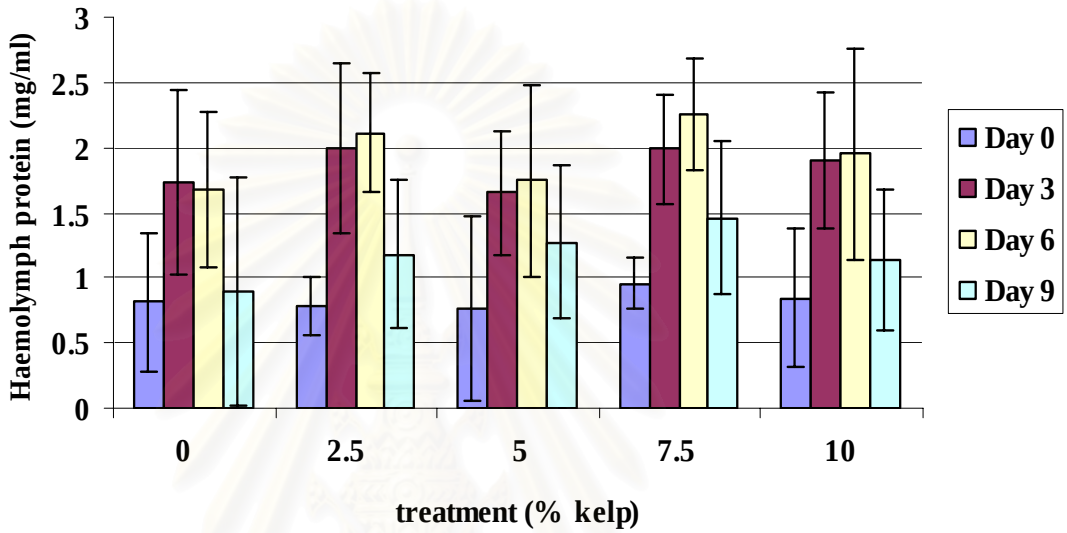

Figure 11 Haemolymph protein of black tiger shrimp after challenging with *V. harveyi* on different kelp added diets.

CHAPTER V

DISCUSSION

5.1 Experimental diets

The proximate analysis of experimental diet showed that average protein and lipid concentrations were within recommended level for *Penaeus monodon*; 35-40% protein and 6 -10% lipid respectively (Shiau, 1998). The experimental diets contained high variation of ash content (12-15% respectively). This may be affected by kelp content which consisted of high minerals (Table 2). Therefore 10% kelp added diet contained the highest ash contend but its nutritional value is still within recommended level (Shiau, 1998). Stability of the experiment diets are approximately 2.5 hours which was enough for feeding period.

5.2 Water quality of black tiger shrimp ponds

The water quality in both experiments was quite similar and within recommended level for shrimp (Appendix B). Ammonia, nitrite and nitrate of both experiments were in the same manner and were non toxic to shrimp. Dissolved oxygen (DO) was rather stable due to a continuous aeration of the rearing system. In the grow-out pond DO was widely fluctuated due to photosynthesis. Water pH of postlarval experiment was higher and more stable than the grow out experiment since the biological filter and bacteria in the system may help to adjust balance of water quality.

5.3 The effect of *A. nodosum* supplemented diet on shrimp growth

A. nodosum contain high level of nutrients, vitamins and metal elements showed in Table 2. It contains 4-10% fucoidan, which reported to have high potential anticabterial, antiviral, anticoagulant activities. However, most study had been done on fucoidan extraction on shrimp diseases resistance to white sport syndrome virus (Albuquerque *et al*., 2004; Chotigeat *et al*.,2004; Takahashi *et al*.,1998.). Our study was to investigate the effects of kelp, *A. nodosum*, meal on growth and resistance to pathogenic bacteria in *P. monodon*. In postlarval experiment, the result indicated no effect of *A. nodosum* on total length of shrimp (Table 7). Survival rate of shrimp fed

2.5% kelp was significantly higher than those of the other diets. With our result, we can recommend that 2.5%kelp meal added to the basal diets may be a good portion for nursery of *P. monodon* postlarvae. In larval stages, zoeal to early postlarval, Yoddee *et al.* (2004) reported that 3-6% kelp added diet could promote growth rate and salinity stress in *P. monodon*

The result of grow out shrimp indicated that kelp supplemental did not effect shrimp length. Whereas in term of weight, shrimp fed 5% kelp and control were significantly higher than those shrimp fed 2.5, 7.5 and 10% kelp. But the survival rate of shrimp fed 2.5% kelp was significantly higher than those shrimp fed only control, but not for those shrimp fed 5, 7.5 and 10% kelp diets. Thus the addition of 2.5-5 % kelp supplemented diets can be recommended for shrimp grow-out. In contract with the result of Velasco *et al*. (2002) which reported that 3% kelp added diet was non significantly different of survival, final weight, FCR and biomass from control diet.

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5.4. Disease resistant test

5.4.1 Shrimp mortality

After 5 days *V. harveyi* challenging test, survival of the postlarva fed 2.5% kelp was higher than those of other diets. The LD_{50} result indicated that shrimp fed 2.5% kelp was significantly different from those shrimp fed control and 10% kelp added diets, but did not different from those shrimp fed 5 and 7.5% kelp added diets. The results indicates that 2.5% kelp added to the diet may be good ratio in *P. monodon* grow-out. This kelp concentration in diet can increase shrimp health and survival. The similar result was found by Chotigeat *et al*. (2004) who reported that the crude fucoidan extracted from *Sargassum polycystum* can inhibit growth of *V. harveyi, Staphylococcus aureus* and *Escherichia coli* at minimal inhibition concentrations of 12.0, 12.0 and 6.0 mg/ml, respectively. Moreover, Araújo et al.(2004) reported that xylofucoglucuronan from *Spatoglossum schröederi* algae could be used for antibiotic immobilization (Gentamicin) to inhibit *Staphylococcus aureus* or *Klebsiella pneumoniae* on *in vivo* study.

5.4.2. Total haemocyte count (THCs)

THCs is a parameter related with immune defense system in shrimp, and more than 90% phenoloxidase activity is found in haemocyte (Cheng *et al*., 2004 and Galvan *et al*., 1999). THCs of infected shrimp is lower than uninfected shrimp because haemocyte is attach to non-self object and haemocyte will decrease when shrimp have tention or stress. Not only that, *Vibrio* species can produce haemolysin but it also destroy blood cell. Decrease of blood cell can be affected by prophenoloxidase activity, phagocytosis and shrimp immune response (Supamattaya *et al.*,2000; Lee *et al*., 1995).

THCs is non significant different among all treatmented diets in each sampling periods. However, THCs in all treatments were similar. The THCs peaks were high on day 3 and 6, and then declined after that to day 9 (the lowest value).

5.4.3. Phenoloxidase activity

Phenoloxidase activity is related to THCs, so trend of phenoloxidase activity is a similarity of THCs. The present result showed a high fluctuation of phenoloxidase activity which is similar to a study of Supamattaya *et al.*, 2000. This activity had no significantly difference among the dietary treatments and time period. At the beginning of the trial, phenoloxidase activity was high and declined on day 3 until day 9 most of the treatments, except in 7.5 and 10% kelp added diets.

5.4.4. Haemolymph protein

Haemolymph protein of shrimp after challenging test, showed alike result to THCs and phenoloxidase activity with non significant difference and large stadard deviation. However, trends of them were similar, these proteins were the lowest at beginning then increased on day 3 and 6 and then decreased on day 9. Trend of haemolymph protein was similar to THCs, because the protein was measured from heamolymph lysat supernatant.

The physiological response study in all treatments was not significant difference in HTCs, phenoloxidase activity and haemolymph protein. But shrimp mortality indicated that an addition of 2.5% kelp in the diet can promote shrimp survival rate after *V. harveyi* challenging test in both postlarval and grow out experiment.

CHAPTER VI

CONCLUSIONS

2.5% kelp, *A. nodosum*, supplemented diet increased the survival of postlarva 15 but did not effect to shrimp growth (length). A. nodosum supplemented diet at 2.5% also promotes shrimp survival rate after bacterial challenging both postlarval and grow out experiment. The addition of 2.5-5% kelp can raise shrimp production by increased weight gain and decreased mortality of grow out shrimp but was not effected to shrimp length. Kelp supplemented diets did not clearly enhance immune responses in shrimp.

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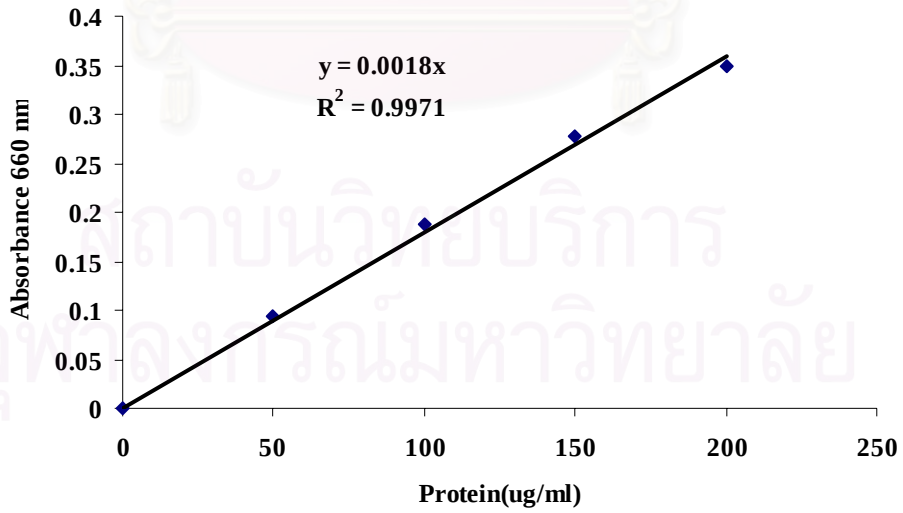
APPENDIX

Appendix A Recommended level of Water quality for shrimp

Water quality	Range	References
Salinity (psu)	<2-40	Howerton(2001)
Temperature (^{o}C)	25-30	Boy and Tucker(1992)
Dissolved oxygen (mg/l)	≥3.5-saturated	Boy and Tucker(1992)
	5-7.5	Department of Fishery(1991)
Alkalinity (mg/l)	75-150	Howerton(2001)
pH	7-9	Boy and Tucker(1992)
	7.5-8.5	Department of Fishery(1991)
Ammonia (mg/l)	0.4-2.0	Spotte(1979)
Nitrate (mg/l)	<20	Spotte(1979)
Nitrite(mg/l)	<0.1	Spotte(1979)

Appendix B Standard graph of *P.monodon* haemolymph protein.



Appendix C Chemical preparation

Marine broth = 5 g Peptone + 1 g Yeast extract + 20 g NaCl + 1000 ml Water

Marine agar = 5 g Peptone + 1 g Yeast extract + 20 g NaCl + 20 g Agar + 1000 ml Water

TCBS media = 8.6 g TCBS + 100 ml Water

CAC Buffer = 10 mM Cacodylic acid + 100 mM $CaCl_2$
adjust pH to 6.8

Anticoaggulant:

10% Sodium citrate = 20g tri-sodium citrate + 200 ml deionized water
adjust pH to 7.2

Phenoloxidase activity chemical:

Trypsin = 0.1%trypsin in CAC Buffer

L-DOPA = 4 mg/ml in CAC Buffer

Protein analysis chemical:

Solution A = 2%sodium carbonate, 0.4%sodium hydroxide and 0.16% sodium potassium tartrate in deionized water

Solution B = 0.04% $CuSO_4.3H_2O$

Solution C = 0.5 mL of solution A + 50 mL of solution B (Protein reagent)

Solution D = Folin-ciocalteau reagent 1:1 (v/v) deionized water

Standard protein = 200 µg/ml bovine serum albumin

Appendix D Statistical test of total haemocyte count to *V. harveyi* on grow out black tiger shrimp.

```
The SAS System       09:32 Wednesday, March 25, 1998   1

---------------------------------------- TIME=0 ----------------------------------------

                            General Linear Models Procedure
                               Class Level Information

                          Class    Levels    Values

                          TRT           5    0 5 10 2.5 7.5


                        Number of observations in by group = 25
```

---------- TIME=0 ----------

General Linear Models Procedure

Dependent Variable: BC

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	400.57414400	100.14353600	0.25	0.9054
Error	20	7965.21552000	398.26077600		
Corrected Total	24	8365.78966400			

R-Square	C.V.	Root MSE	BC Mean
0.047882	40.50332	19.95647203	49.27120000

Source	DF	Type I SS	Mean Square	F Value	Pr > F
TRT	4	400.57414400	100.14353600	0.25	0.9054

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	4	400.57414400	100.14353600	0.25	0.9054

---------- TIME=0 ----------

General Linear Models Procedure

Duncan's Multiple Range Test for variable: BC

NOTE: This test controls the type I comparisonwise error rate, not the experimentwise error rate

Alpha= 0.05 df= 20 MSE= 398.2608

Number of Means	2	3	4	5
Critical Range	26.33	27.64	28.47	29.05

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	TRT
A	53.08	5	2.5
A			
A	51.62	5	0
A			
A	51.58	5	10
A			
A	48.12	5	7.5
A			
A	41.96	5	5

---------- TIME=3 ----------

General Linear Models Procedure
Class Level Information

Class	Levels	Values
TRT	5	0 5 10 2.5 7.5

Number of observations in by group = 25

---------- TIME=3 ----------

General Linear Models Procedure

Dependent Variable: BC

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	1556.94000000	389.23500000	0.77	0.5563
Error	20	10086.34000000	504.31700000		
Corrected Total	24	11643.28000000			

R-Square	C.V.	Root MSE	BC Mean
0.133720	38.75907	22.45700336	57.94000000

Source	DF	Type I SS	Mean Square	F Value	Pr > F
TRT	4	1556.94000000	389.23500000	0.77	0.5563

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	4	1556.94000000	389.23500000	0.77	0.5563

---------- TIME=3 ----------

General Linear Models Procedure

Duncan's Multiple Range Test for variable: BC

NOTE: This test controls the type I comparisonwise error rate, not the experimentwise error rate

Alpha= 0.05 df= 20 MSE= 504.317

Number of Means	2	3	4	5
Critical Range	29.63	31.10	32.03	32.69

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	TRT
A	70.84	5	7.5
A			
A	63.42	5	10
A			
A	52.60	5	0
A			
A	52.26	5	5
A			
A	50.58	5	2.5

---------- TIME=6 ----------

General Linear Models Procedure
Class Level Information

Class	Levels	Values
TRT	5	0 5 10 2.5 7.5

Number of observations in by group = 25

---------- TIME=6 ----------

General Linear Models Procedure

Dependent Variable: BC

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	1490.74800000	372.68700000	0.82	0.5252
Error	20	9045.35200000	452.26760000		
Corrected Total	24	10536.10000000			

R-Square	C.V.	Root MSE	BC Mean
0.141490	36.61602	21.26658412	58.08000000

Source	DF	Type I SS	Mean Square	F Value	Pr > F
TRT	4	1490.74800000	372.68700000	0.82	0.5252

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	4	1490.74800000	372.68700000	0.82	0.5252

---------- TIME=6 ----------

General Linear Models Procedure

Duncan's Multiple Range Test for variable: BC

NOTE: This test controls the type I comparisonwise error rate, not the experimentwise error rate

Alpha= 0.05 df= 20 MSE= 452.2676

Number of Means	2	3	4	5
Critical Range	28.06	29.45	30.34	30.95

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	TRT
A	65.48	5	0
A			
A	65.30	5	7.5
A			
A	61.56	5	10
A			
A	51.80	5	2.5
A			
A	46.26	5	5

---------- TIME=9 ----------

General Linear Models Procedure
Class Level Information

Class	Levels	Values
TRT	5	0 5 10 2.5 7.5

Number of observations in by group = 25

---------- TIME=9 ----------

General Linear Models Procedure

Dependent Variable: BC

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	1859.75110400	464.93777600	1.51	0.2368
Error	20	6152.82032000	307.64101600		
Corrected Total	24	8012.57142400			

R-Square	C.V.	Root MSE	BC Mean
0.232104	51.03972	17.53969829	34.36480000

Source	DF	Type I SS	Mean Square	F Value	Pr > F
TRT	4	1859.75110400	464.93777600	1.51	0.2368

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	4	1859.75110400	464.93777600	1.51	0.2368

---------- TIME=9 ----------

General Linear Models Procedure

Duncan's Multiple Range Test for variable: BC

NOTE: This test controls the type I comparisonwise error rate, not the experimentwise error rate

Alpha= 0.05 df= 20 MSE= 307.641

Number of Means	2	3	4	5
Critical Range	23.14	24.29	25.02	25.53

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	TRT
A	42.78	5	7.5
A			
A	40.60	5	10
A			
A	37.58	5	5
A			
A	32.22	5	0
A			
A	18.64	5	2.5

Appendix E Statistical test of phenoloxidase activity to *V. harveyi* on grow out black tiger shrimp.

```
The SAS System            02:09 Tuesday, May 6, 1997   1

---------------------------------------- TIME=0 ----------------------------------------

                        General Linear Models Procedure
                           Class Level Information

                  Class    Levels    Values

                  TRT           5    1 2 3 4 5


                Number of observations in by group = 25
```

The SAS System 02:09 Tuesday, May 6, 1997 2

---------- TIME=0 ----------

General Linear Models Procedure

Dependent Variable: PO

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	1036939.33503498	259234.83375874	1.25	0.3238
Error	20	4162516.41601700	208125.82080085		
Corrected Total	24	5199455.75105198			

R-Square	C.V.	Root MSE	PO Mean
0.199432	36.00770	456.20808936	1266.97369313

Source	DF	Type I SS	Mean Square	F Value	Pr > F
TRT	4	1036939.33503497	259234.83375874	1.25	0.3238

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	4	1036939.33503497	259234.83375874	1.25	0.3238

The SAS System 09:50 Wednesday, March 25, 1998 3

---------- TIME=0 ----------

General Linear Models Procedure

Duncan's Multiple Range Test for variable: PO

NOTE: This test controls the type I comparisonwise error rate, not the experimentwise error rate

Alpha= 0.05 df= 20 MSE= 208125.8

Number of Means	2	3	4	5
Critical Range	601.9	631.8	650.8	664.0

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	TRT
A	1457.9	5	3
A			
A	1432.4	5	2
A			
A	1391.0	5	1
A			
A	1106.6	5	4
A			
A	946.9	5	5

---------- TIME=3 ----------

General Linear Models Procedure
Class Level Information

Class	Levels	Values
TRT	5	1 2 3 4 5

Number of observations in by group = 25

---------- TIME=3 ----------

General Linear Models Procedure

Dependent Variable: PO

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	115388.41891871	28847.10472968	0.07	0.9904
Error	20	8263067.84200645	413153.39210032		
Corrected Total	24	8378456.26092515			

R-Square	C.V.	Root MSE	PO Mean
0.013772	41.58999	642.77009272	1545.49232608

Source	DF	Type I SS	Mean Square	F Value	Pr > F
TRT	4	115388.41891870	28847.10472968	0.07	0.9904

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	4	115388.41891870	28847.10472968	0.07	0.9904

---------- TIME=3 ----------

General Linear Models Procedure

Duncan's Multiple Range Test for variable: PO

NOTE: This test controls the type I comparisonwise error rate, not the experimentwise error rate

Alpha= 0.05 df= 20 MSE= 413153.4

Number of Means	2	3	4	5
Critical Range	848.0	890.1	916.9	935.6

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	TRT
A	1650.0	5	2
A			
A	1589.3	5	1
A			
A	1537.2	5	4
A			
A	1488.4	5	3
A			
A	1462.4	5	5

---------- TIME=6 ----------

General Linear Models Procedure
Class Level Information

Class	Levels	Values
TRT	5	1 2 3 4 5

Number of observations in by group = 25

---------- TIME=6 ----------

General Linear Models Procedure

Dependent Variable: PO

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	254952.47648862	63738.11912215	0.42	0.7934
Error	20	3046810.63361152	152340.53168058		
Corrected Total	24	3301763.11010014			

R-Square	C.V.	Root MSE	PO Mean
0.077217	25.33582	390.30825213	1540.53900410

Source	DF	Type I SS	Mean Square	F Value	Pr > F
TRT	4	254952.47648861	63738.11912215	0.42	0.7934

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	4	254952.47648861	63738.11912215	0.42	0.7934

---------- TIME=6 ----------

General Linear Models Procedure

Duncan's Multiple Range Test for variable: PO

NOTE: This test controls the type I comparisonwise error rate, not the experimentwise error rate

Alpha= 0.05 df= 20 MSE= 152340.5

Number of Means	2	3	4	5
Critical Range	514.9	540.5	556.8	568.1

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	TRT
A	1653.7	5	3
A			
A	1651.0	5	2
A			
A	1523.4	5	1
A			
A	1484.1	5	4
A			
A	1390.5	5	5

---------- TIME=9 ----------

General Linear Models Procedure
Class Level Information

Class	Levels	Values
TRT	5	1 2 3 4 5

Number of observations in by group = 25

---------- TIME=9 ----------

General Linear Models Procedure

Dependent Variable: PO

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	239660.85298715	59915.21324679	0.06	0.9919
Error	20	18800366.09992930	940018.30499647		
Corrected Total	24	19040026.95291650			

R-Square	C.V.	Root MSE	PO Mean
0.012587	74.80234	969.54541152	1296.14317061

Source	DF	Type I SS	Mean Square	F Value	Pr > F
TRT	4	239660.85298714	59915.21324679	0.06	0.9919

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	4	239660.85298714	59915.21324679	0.06	0.9919

---------- TIME=9 ----------

General Linear Models Procedure

Duncan's Multiple Range Test for variable: PO

NOTE: This test controls the type I comparisonwise error rate, not the experimentwise error rate

Alpha= 0.05 df= 20 MSE= 940018.3

Number of Means	2	3	4	5
Critical Range	1279	1343	1383	1411

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	TRT
A	1425.7	5	3
A			
A	1385.7	5	2
A			
A	1287.1	5	5
A			
A	1213.0	5	1
A			
A	1169.2	5	4

Appendix F Statistical test of haemolymph protein to *V. harveyi* on grow out black tiger shrimp.

```
The SAS System       10:04 Wednesday, March 25, 1998   1

---------------------------------------- TIME=0 ----------------------------------------

                            General Linear Models Procedure
                               Class Level Information

                         Class    Levels    Values

                         TRT           5    0 5 10 2.5 7.5


                        Number of observations in by group = 25
```

---------- TIME=0 ----------

General Linear Models Procedure

Dependent Variable: PROTEIN

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	0.12306032	0.03076508	0.13	0.9677
Error	20	4.56731973	0.22836599		
Corrected Total	24	4.69038004			

R-Square	C.V.	Root MSE	PROTEIN Mean
0.026237	57.55567	0.47787654	0.83028571

Source	DF	Type I SS	Mean Square	F Value	Pr > F
TRT	4	0.12306032	0.03076508	0.13	0.9677

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	4	0.12306032	0.03076508	0.13	0.9677

---------- TIME=0 ----------

General Linear Models Procedure

Duncan's Multiple Range Test for variable: PROTEIN

NOTE: This test controls the type I comparisonwise error rate, not the experimentwise error rate

Alpha= 0.05 df= 20 MSE= 0.228366

Number of Means	2	3	4	5
Critical Range	.6305	.6618	.6817	.6956

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	TRT
A	0.9590	5	7.5
A			
A	0.8419	5	10
A			
A	0.8124	5	0
A			
A	0.7752	5	2.5
A			
A	0.7629	5	5

---------- TIME=3 ----------

General Linear Models Procedure
Class Level Information

Class	Levels	Values
TRT	5	0 5 10 2.5 7.5

Number of observations in by group = 25

---------- TIME=3 ----------

General Linear Models Procedure

Dependent Variable: PROTEIN

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	0.36395425	0.09098856	0.28	0.8876
Error	20	6.50383270	0.32519164		
Corrected Total	24	6.86778695			

R-Square	C.V.	Root MSE	PROTEIN Mean
0.052994	30.78185	0.57025576	1.85257143

Source	DF	Type I SS	Mean Square	F Value	Pr > F
TRT	4	0.36395425	0.09098856	0.28	0.8876

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	4	0.36395425	0.09098856	0.28	0.8876

---------- TIME=3 ----------

General Linear Models Procedure

Duncan's Multiple Range Test for variable: PROTEIN

NOTE: This test controls the type I comparisonwise error rate, not the experimentwise error rate

Alpha= 0.05 df= 20 MSE= 0.325192

Number of Means	2	3	4	5
Critical Range	.7523	.7897	.8134	.8300

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	TRT
A	2.0483	5	10
A			
A	1.9016	5	2.5
A			
A	1.8610	5	5
A			
A	1.7270	5	0
A			
A	1.7251	5	7.5

---------- TIME=6 ----------

General Linear Models Procedure
Class Level Information

Class	Levels	Values
TRT	5	0 5 10 2.5 7.5

Number of observations in by group = 25

---------- TIME=6 ----------

General Linear Models Procedure

Dependent Variable: PROTEIN

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	0.84100129	0.21025032	0.51	0.7272
Error	20	8.20279890	0.41013994		
Corrected Total	24	9.04380018			

R-Square	C.V.	Root MSE	PROTEIN Mean
0.092992	33.75594	0.64042169	1.89721212

Source	DF	Type I SS	Mean Square	F Value	Pr > F
TRT	4	0.84100129	0.21025032	0.51	0.7272

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	4	0.84100129	0.21025032	0.51	0.7272

---------- TIME=6 ----------

General Linear Models Procedure

Duncan's Multiple Range Test for variable: PROTEIN

NOTE: This test controls the type I comparisonwise error rate, not the experimentwise error rate

Alpha= 0.05 df= 20 MSE= 0.41014

Number of Means	2	3	4	5
Critical Range	.8449	.8869	.9135	.9321

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	TRT
A	2.1358	5	7.5
A			
A	2.0515	5	2.5
A			
A	1.9097	5	10
A			
A	1.7455	5	5
A			
A	1.6436	5	0

---------- TIME=9 ----------

General Linear Models Procedure
Class Level Information

Class	Levels	Values
TRT	5	0 5 10 2.5 7.5

Number of observations in by group = 25

---------- TIME=9 ----------

General Linear Models Procedure

Dependent Variable: PROTEIN

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	0.94262899	0.23565725	0.63	0.6443
Error	20	7.43688766	0.37184438		
Corrected Total	24	8.37951665			

R-Square	C.V.	Root MSE	PROTEIN Mean
0.112492	50.30400	0.60979044	1.21221053

Source	DF	Type I SS	Mean Square	F Value	Pr > F
TRT	4	0.94262899	0.23565725	0.63	0.6443

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRT	4	0.94262899	0.23565725	0.63	0.6443

---------- TIME=9 ----------

General Linear Models Procedure

Duncan's Multiple Range Test for variable: PROTEIN

NOTE: This test controls the type I comparisonwise error rate, not the experimentwise error rate

Alpha= 0.05 df= 20 MSE= 0.371844

Number of Means	2	3	4	5
Critical Range	.8045	.8444	.8698	.8876

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	TRT
A	1.5074	5	7.5
A			
A	1.2723	5	5
A			
A	1.2660	5	2.5
A			
A	1.0828	5	10
A			
A	0.9326	5	0

BIOGRAPHY

Miss Sirikanya Chungthanawong was born on August 29, 1980 in Srisaket. Graduated with the primary education at Orrachan Wittaya School, secondary education at Kanthralak Wittaya School in Srisaket and Bachelor of Science from Department of Biology, Faculty of Science, Khonkean University in 2001.