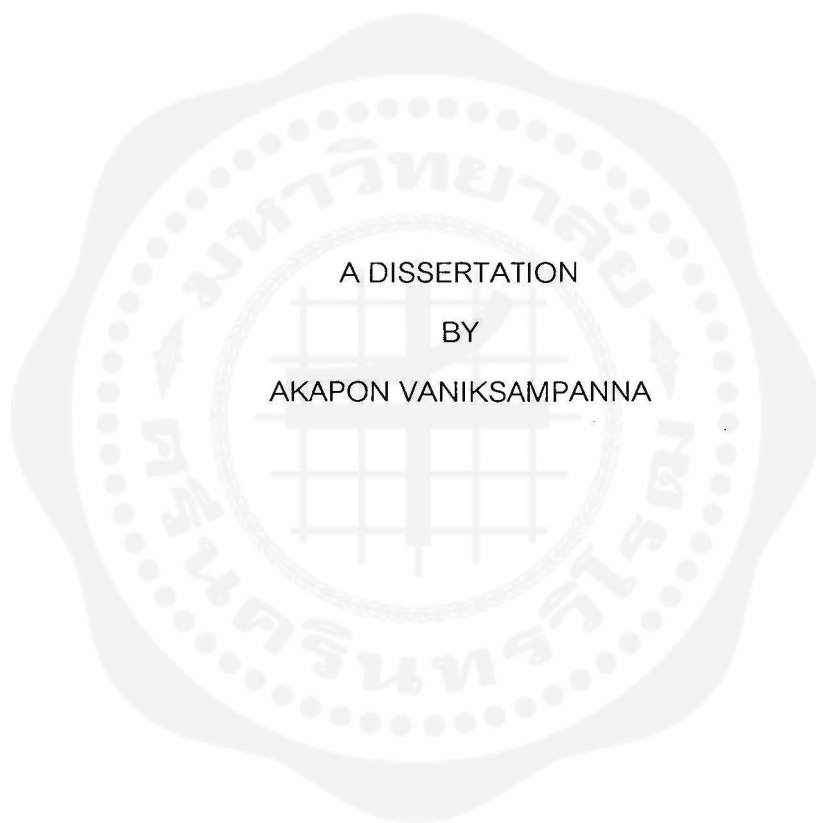


ISOLATION AND MOLECULAR CHARACTERIZATION OF A *SPÄTZLE* GENE FROM
FRESHWATER PRAWN (*MACROBRACHIUM ROSENBERGII*)

A DISSERTATION
BY
AKAPON VANIKSAMPANNA



Presented in Partial Fulfillment of the Requirements for the
Doctor of Philosophy Degree in Biotechnology
at Srinakharinwirot University

July 2018

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ISOLATION AND MOLECULAR CHARACTERIZATION OF A *SPÄTZLE* GENE FROM
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AN ABSTRACT
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AKAPON VANIKSAMPANNA

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Akapon Vaniksampanna. (2018). *Isolation and Molecular Characterization of a Spätzle Gene from Freshwater Prawn (Macrobrachium rosenbergii)*. Dissertation, Ph.D. (Biotechnology). Bangkok: Graduate School, Srinakharinwirot University. Advisor Committee: Assoc. Prof. Dr.Parin Chaivisutthangkura, Dr.Walaiporn Charoensapsri, Assoc. Prof. Dr.Siwaporn Longyant.

Spätzle protein is an extracellular ligand of Toll receptor in Toll signaling pathway involved in the embryonic dorso-ventral patterning and innate immunity. In terms of the innate immunity, pathogen invasion induces the activation of Spätzle processing enzyme to cleave the inactive Spätzle protein. After binding of the cleaved Spätzle protein to Toll receptor, antimicrobial peptides gene expressions were induced. In this study, 2,253 base pairs of *Spätzle* gene from freshwater prawn, *Macrobrachium rosenbergii* (*MrSpz*) was isolated and characterized. The open reading frame of *MrSpz* consisted of 747 nucleotides encoding 248 amino acid residues containing a signal peptide and C-terminal Spätzle activated domain. *MrSpz* protein shared high similarity to Spätzle of *Fenneropenaeus chinensis* (*FcSpz*) at 92% identity, *Marsupenaeus japonicus* (*MjSpz*) at 83% identity and *Penaeus monodon* (*PmSpz*) at 78% identity. The phylogenetic analysis revealed that *MrSpz* was a member of the clade containing *LvSpz3* of *Litopenaeus vannamei*, *FcSpz* and *PmSpz*. The expression distribution at transcriptional level in various tissues of normal prawn revealed that the *MrSpz* was detected in gills, heart and hepatopancreas while undetectable in hemocyte, muscle and stomach. In the *Aeromonas caviae* challenged prawn, the expression level of *MrSpz* in hemocyte was increased gradually at 6, 12 and 24 h post-injection. Furthermore, in *MrSpz* knocked down prawn injected with *A. caviae*, the mortality rate were higher than that of a non-related dsRNA group and a control group. These results suggested that *MrSpz* protein may play a key role in the innate immunity of *M. rosenbergii*, especially in response to Gram-negative bacteria *A. caviae* invasion.

การแยกและศึกษาคุณลักษณะของยีน *spätzle* จากกุ้งก้ามกราม (*Macrobrachium rosenbergii*)



เสนอต่อบัณฑิตวิทยาลัย มหาวิทยาลัยศรีนครินทรวิโรฒ เพื่อเป็นส่วนหนึ่งของการศึกษา
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โปรตีน *spätzle* คือโปรตีนที่ทำหน้าที่เป็น extracellular ligand ของ Toll receptor ใน Toll signaling pathway ซึ่งเป็น pathway ที่เกี่ยวข้องกับกระบวนการ dorso-ventral patterning ของตัวอ่อนและระบบภูมิคุ้มกันที่มาจากกำเนิด ในกรณีของระบบภูมิคุ้มกัน การบุกรุกของเชื้อจุลินทรีย์กระตุ้นให้มีการทำงานของ *spätzle* processing enzyme ที่ทำหน้าที่ตัดโปรตีน *spätzle* ในรูปแบบ inactive form ให้เป็น active form ซึ่งเมื่อโปรตีน *spätzle* เข้าจับกับ Toll receptor ทำให้เกิดการกระตุ้นการแสดงออกของ antimicrobial peptide gene ในการศึกษาครั้งนี้ได้ทำการแยกและศึกษายีน *spätzle* ของกุ้งก้ามกราม *Macrobrachium rosenbergii* (MrSpz) ซึ่งพบว่ามีความยาว 2253 คู่เบส และเป็นส่วนของกรอบการอ่าน 747 คู่เบส เป็นรหัสของกรดอะมิโนจำนวน 248 หน่วย โดยประกอบด้วยส่วนของ signal peptide และ C-terminal *spätzle* domain โปรตีน MrSpz มีความคล้ายคลึงกับโปรตีน FcSpz ของกุ้งขาวจีน *Fenneropenaeus chinensis* 92 เปอร์เซ็นต์ MjSpz ของกุ้งขาวญี่ปุ่น *Marsupenaeus japonicus* 83 เปอร์เซ็นต์ และ PmSpz ของกุ้งกุลาดำ *Penaeus monodon* 78% จากการวิเคราะห์แผนภูมิต้นไม้วิวัฒนาการพบว่าโปรตีน MrSpz ถูกจัดให้อยู่ในกลุ่มของโปรตีน LvSpz3 ของกุ้งขาวแวนนาไม *Penaeus vannamei* โปรตีน FcSpz ของกุ้งขาวจีน และโปรตีน PmSpz ของกุ้งกุลาดำ ในการศึกษาการแสดงออกของยีน MrSpz ในระดับการถอดรหัสพบได้ในเหงือก หัวใจ และตับ-ตับอ่อน และไม่พบการแสดงออกในเม็ดเลือด กล้ามเนื้อ และกระเพาะอาหาร ในกุ้งที่ฉีดด้วยเชื้อ *Aeromonas caviae* พบว่ายีน MrSpz ได้แสดงออกเพิ่มขึ้นเป็นลำดับในช่วงเวลาที่ 6 12 และ 24 ชั่วโมงหลังฉีดเชื้อ นอกจากนี้ยังพบว่ากุ้งก้ามกรามที่ถูกยับยั้งไม่ให้เกิดการแสดงออกของยีน MrSpz และถูกฉีดด้วยเชื้อ *A. caviae* มีอัตราการตายที่สูงกว่ากลุ่มที่ฉีดด้วยอาร์เอ็นเอสายคู่ที่ไม่เกี่ยวข้องและกลุ่มควบคุม ผลการทดลองเหล่านี้ชี้ให้เห็นว่าโปรตีน MrSpz อาจมีบทบาทสำคัญในระบบภูมิคุ้มกันของกุ้ง โดยเฉพาะอย่างยิ่งในการตอบสนองต่อการบุกรุกของเชื้อ *A. caviae* ซึ่งเป็นแบคทีเรียแกรมลบชนิดหนึ่ง

The dissertation titled
"Isolation and Molecular Characterization of a *Spätzle* Gene
from Freshwater Prawn (*Macrobrachium rosenbergii*)"

by

Akapon Vaniksampanna

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Doctor of Philosophy degree in Biotechnology of Srinakharinwirot University.

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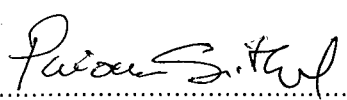
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CHAPTER 1

INTRODUCTION

Macrobrachium rosenbergii, known as giant freshwater prawn is currently one of the most important inland aquacultured crustacean species. *M. rosenbergii* is native to South Asia such as India and Bangladesh, Southeast Asia such as Thailand, Myanmar, Vietnam, Cambodia, Malaysia, Indonesia and Philippines and some parts of the Oceania and the Pacific Islands (Rayong inland fisheries research and development center. 2017, New 1982, 2002). Giant freshwater prawn farming has expanded worldwide and the global production increased gradually from 195,858 tonnes in 2005 to 216,856 tonnes in 2014 (FAO, 2017). In Thailand, freshwater prawn cultured areas are spread in central part of Thailand and extended to some provinces in the northern parts such as Chiang Rai and Phichit (Whangchai; et al. 2007: 89-96). The production of *M. rosenbergii* in Thailand continuously increased from about 4,764 tonnes in 1998 to 27,650 tonnes in 2007 (FAO, 2009).

However, the success of *M. rosenbergii* production is limited by disease outbreaks, especially viral diseases such as white tail disease (WTD) caused by *Macrobrachium rosenbergii* nodavirus (MrNV) and extra small virus (XSV), which result in high mortality rate in post larvae (Bonami; & Lightner. 1991: 597-622, Qian; et al. 2003: 521-527, Hameed; et al. 2004: 127-133, Yoganandhan; et al. 2006: 255-258). However WTD has no effect on the mortality and clinical signs of WTD in adult prawn (Sahul Hameed; et al. 2004: 191-196). In addition, diseases in freshwater prawn are also caused by bacteria or protozoa such as protozoal infection among *Epistylis* sp., *Zoothamnium* sp., *Vorticella* sp. (Mandal; et al. 2015: 115-119), muscular necrosis and mortality by *Enterococcus* like bacterial infection (Cheng; & Chen. 1998: 93-101) and chitinolytic bacterial disease (also called as black spot, brown spot, shell disease or bacterial necrosis) caused by the invasion of chitinolytic bacteria. In 2002, the study on farmed freshwater prawn in Vietnam revealed several bacterial strains including *Aeromonas caviae*, *A. hydrophila* *A. sobria* were found in adult freshwater prawn with sign of bacterial necrosis (Hao; Oanh; & Phuong. 2014: online).

In order to defend pathogen infection, freshwater prawn rely on several innate immune system including cellular immunity and humoral immunity. Toll signaling pathway is an important pathway of humoral immunity, which plays a key role in antimicrobial peptides (AMPs) production, the main effectors for invertebrates to eliminate bacteria and fungi (Lemaitre; & Hoffmann. 2007: 697-743, Rowley; & Powell. 2007: 7209-7214, Zhong; et al. 2012: 514-524, Meng; et al. 2016: 792-797). Toll receptor and its ligand, Spätzle protein have been identified from various species, such as *Caenorhabditis elegans* (Pujol; et al. 2001: 809-821), *Drosophila melanogaster* (Lemaitre; et al. 1996: 973–983), *Aedes aegypti* (Shin; Bian; & Raikhel. 2006: 39388-39395), *Penaeus monodon* (Arts; et al. 2007: 504-513.) and *P. vannamei* (also known as *Litopenaeus vannamei*) (Yang; et al. 2007: 1999-2008). The studies of Toll signalling pathway including Spätzle protein in many species demonstrated their roles in response to bacteria or fungi invasion and some evidence for virus. For the studies in shrimp, in 2009, Shi and colleagues isolated a *Fenneropenaeus chinensis* Spätzle-like protein (*FcSpz*) and the results from challenged shrimp revealed that the expression level of *FcSpz* was significantly up-regulated in all tissues when shrimp were injected with *Vibrio anguillarum* and white spot syndrome virus (WSSV) (Shi; et al. 2009: 610–617). Furthermore, in 2012, *LvToll* and three Spätzle-like proteins were isolated from the whiteleg shrimp, *P. vannamei* and the expression level of *LvSpz1* and *LvSpz3* were found to be strongly upregulated after challenging with *V. alginolyticus* and white spot syndrome virus, while the expression of *LvToll1*, *LvToll2* and *LvToll3* were significantly upregulated after WSSV challenge (Wang; et al. 2012: 359-371).

For *M. rosenbergii*, in 2014, Srisuk and colleagues successfully isolated the full-length cDNA sequence of Toll receptor and named as *MrToll*. In *Aeromonas caviae* challenged experiment, a reverse transcriptase PCR assay showed that the expression level of *MrToll* was significantly increased at 3–12 h post-injection. Furthermore, in *MrToll*-silenced prawn, the mortality and bacterial CFU counts were significantly increased when challenged with *A. caviae*. The researchers proposed that *MrToll* might be involved in host innate defense, especially against the bacteria *A. caviae* infection (Srisuk; et al. 2014: 552-562). However, there is no report about Spätzle protein, a Toll-receptor ligand in *M. rosenbergii*. Therefore, this study aims to isolate and characterize a full-length *Spätzle* cDNA sequence of *M. rosenbergii* (*MrSpz*) and investigate the role of *MrSpz* against *A. caviae* invasion by RNAi assay. The knowledge on *MrSpz* may lead to further understanding of immune response against bacterial infection in *M. rosenbergii*.

Objectives

1. To isolate and characterize a *Spätzle* gene from *M. rosenbergii*
2. To study *Spätzle* gene expression in uninfected and *A. caviae* challenged *M. rosenbergii*
3. To characterize the role of *Spätzle* gene in response to *A. caviae* infection by RNA interference (RNAi) assay

CHAPTER 2

REVIEW LITERATURE

2.1 Giant freshwater prawn (*Macrobrachium rosenbergii*)

The giant freshwater prawn or giant river prawn, *Macrobrachium rosenbergii*, is a commercially important species of inland cultured freshwater prawn industry in many countries such as Bangladesh, India and Thailand. They live in tropical freshwater environments with extremely turbid conditions that connect to brackishwater areas, necessary to initiate the first stage of their life cycle. For general morphology of *M. rosenbergii*, the body of both post larvae and adult prawns consists of cephalothorax (head and thorax fused together) and abdomen which can be divided into 6 segments. Male adult prawns have a large second walking leg, which is blue in color, longer and thicker than that of female (New, 2002).

2.1.1 Taxonomy of giant freshwater prawn (*M. rosenbergii*)



2.1.2 Life cycle of *M. rosenbergii*

M. rosenbergii can reproduce throughout the year. After female sloughed off their shell, in mating, they deposit a spermatophore (gelatinous mass of semen) at the underside of the thorax which located between the walking legs. A few hours after breeding, eggs are ejected via gonopores and fertilized with the semen during this process and then the fertilized egg are attached at the brood chamber. The fertilized eggs take average of 20 days at 28°C to hatch and complete their planktonic larval life (zoea I-XI) by moulting 11 times in brackish water. This process take about 16-30 days, depending on water temperature and feeding conditions. At this stage, their diet is zooplankton or the larvae of other species and they move by swimming with their dorsal side uppermost. Afterward, they metamorphose into post larvae (PL). At this stage, the appearance of post larvae are similar to juvenile and adult prawns and their moving become crawling rather than swimming. Next, the post larvae develop into juveniles, in nature, the juveniles remain in brackish water for 1-2 weeks and then gradually migrate upstream into less saline water and later to freshwater in rainy season. They will grow and sexually mature in about 9 months (Fig. 1) (New. 2002).

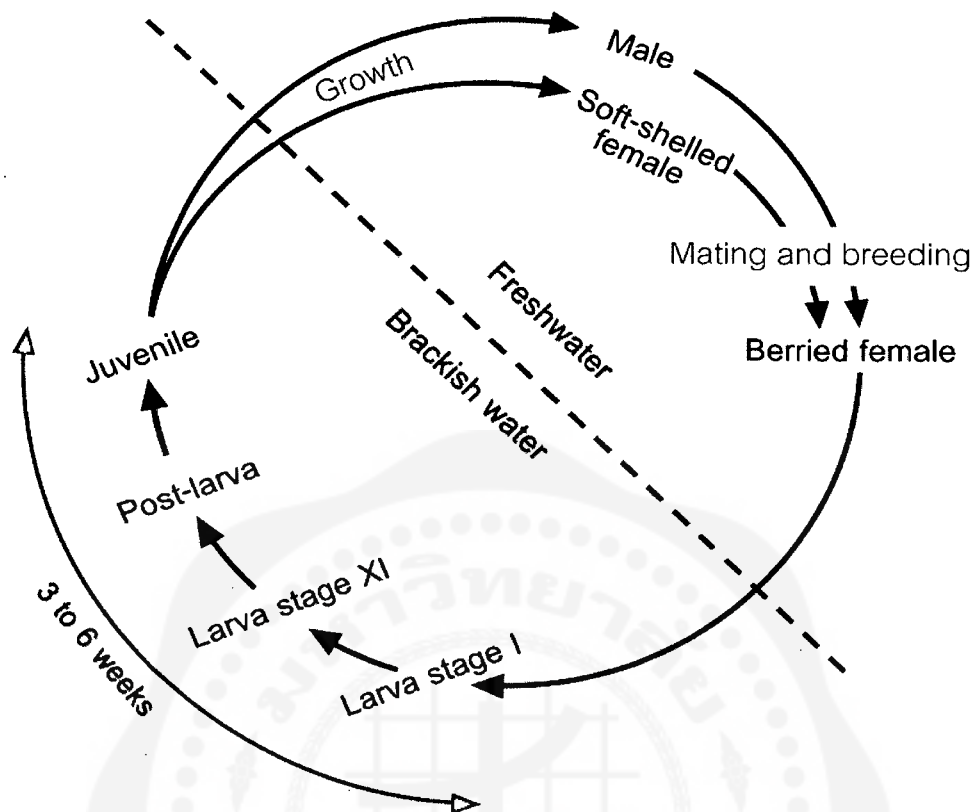


Figure 1 Life cycle of giant freshwater prawn (*M. rosenbergii*). They initiate the first stage of life in brackish water and migrate into freshwater for mating and breeding.

Source: Modified from <http://www.notesonzoology.com/india/prawns-and-shrimp-culture/giant-freshwater-prawn-distribution-biology-and-life-history/1055>

2.2 Crustacean immunity

Crustaceans such as shrimp, crab, mantis shrimp, krill and lobster rely on innate immunity to defend themselves from potential pathogen infection because of lacking an adaptive immune system (Tassanakajon; et al. 2013: 954-967). The innate immunity can be divided into two systems: cellular immunity system include encapsulation, phagocytosis, apoptosis and nodule formation and humoral immunity system, involving humoral defenses such as antimicrobial peptides (AMPs), clotting cascade, agglutinin, cytokine-like, prophenoloxidase system (proPO) and antiviral peptides (Little; Hulmark; & Read. 2005: 651-654, Tassanakajon; et al. 2013: 954-967).

2.2.1 Cellular immunity system

The hemocytes in crustaceans play an important role in a cellular immunity system, and can be generally classified into three categories according to their size and granularity: hyaline (agranulocyte), semigranular and granular cells. They are involved in different reactions depending on different cell types (Bachère. 2000: 3-11).

The hyaline are the smallest hemocytes ($11.1 \times 5.9 \mu\text{m}$) that are generally flat and round in shape, although some cells are fusiform-like shape. They have a high nucleocytoplasmic ratio with a few small granules ($0.2 \mu\text{m}$ in diameter) or no granules in cytoplasm (Fig. 2A). The hyaline cells are involved in phagocytosis (Kondo; et al. 1998: 421-427) and coagulation (Omori; et al. 1989: 117-123). The semigranular cell has small granules ($0.2 \mu\text{m}$ in diameter). These cells are ovoid or fusiform in shape with cell size of $14.6 \times 8.7 \mu\text{m}$ and has a low nucleocytoplasmic ratio (Fig. 2B). For the granular cell, it is the largest cell with size of $19.1 \times 9.8 \mu\text{m}$. Their shape and nucleocytoplasmic ratio similar to that of semigranular cell (Fig. 2C). Both semigranular and granular cells play role in phagocytosis (Kondo; et al. 1998: 421-427) and proPO system (Johansson; & Söderhall. 1985: 175-181). In addition, the semigranular cells are also involved with nodule formation (Söderhäll; & Cerenius. 1998: 23-28) and encapsulation (response to larger microorganisms) (Persson; Vay; & Söderhäll. 1987: 409-415).

Phagocytosis is a conserved cellular response in many organisms, which is the first response of hemocytes that have the ability to recognize and ingest non-self and small particles such as bacteria and spores in the hemocoel or self-senescent cells (Vazquez; et al. 2009: 179-188, Jiravanichpaisal; Lee; & Söderhäll. 2006: 213-236). For the mechanism of phagocytosis, it is initiated by target recognition and binding of the target particle to the hemocyte (Phagocytic cell) followed by uptaking the target particle by intracellular vesicular transport to phagosomes and then the entered vesicle is destroyed (Pearson; et al. 2003: 815-824).

Nodulation or Encapsulation is a restricted cellular immunity to invertebrates, which is response to foreign bodies too large for phagocytosis by individual hemocytes. Nodulation is a process carried out by multicellular hemocytic aggregates, which wrap the invader and finally, the invader or pathogen is lined by a multilayered sheath of hemocytes (encapsulation) (Jiravanichpaisal; Lee; & Söderhäll. 2006: 213-236).

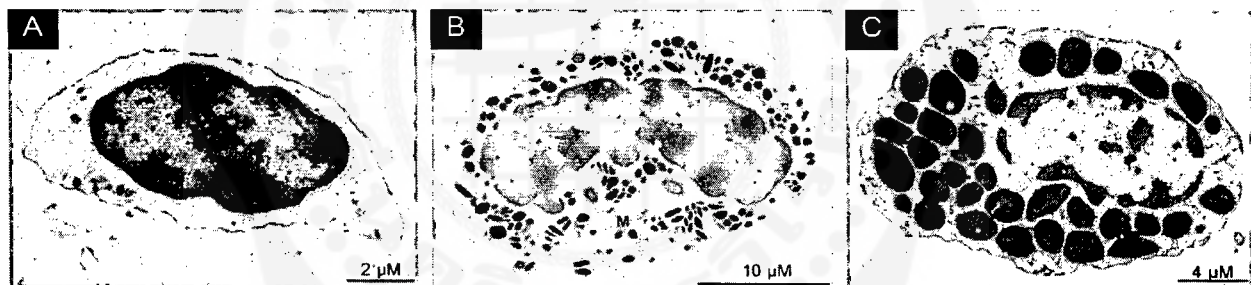


Figure 2 Transmission electron microscopy of *Astacus leptodactylus* hemocytes. Hyaline hemocyte with a high nucleocytoplasmic ratio and a few small granules (A). Semigranular hemocyte (B) and Granular hemocyte (C) filled with the granules and showing a low nucleocytoplasmic ratio

Source: Modified from Giulianini; et al. (2007). Ultrastructural and functional characterization of circulating hemocytes from the freshwater crayfish *Astacus leptodactylus*: Cell types and their role after *in vivo* artificial non-self challenge. P. 49-57.

2.2.2 Humoral immunity system

The humoral immunity is mediated by the molecules which activated and released from hemocytes and other cells, such as anticoagulant proteins, agglutinins, phenoloxidase enzyme, antimicrobial peptides, protease inhibitors, etc. (Jiravanichpaisal; et al. 2006: 213-236, Holmblad; & Söderhäll. 1999: 111-123).

One of the important system in humoral immunity is the proPO system, which consists of an enzyme cascade that activates the proPO or other proteins that are involved in melanization, encapsulation and phagocytosis. The proPO system is activated by the presence of bacterial components such as lipopolysaccharide (LPS), peptidoglycan (PG) and β -1,3-glucans (β G) to the semigranular or granular cells and initially triggered by the interaction of pattern-recognition proteins (PRPs) on the cell membrane of hemocyte, which comprise of β -1,3-glucan binding protein (β GBP), LPS and β -1,3-glucan binding protein (LGBP) and peptidoglycan binding protein (PGBP) (Jiravanichpaisal; Lee; & Söderhäll. 2006: 213-236). These interaction induced the activation of serine protease named the prophenoloxidase activating enzyme (ppA) to cleave and convert an inactive proPO to active PO. The activated PO oxidize phenols to quinones and finally the synthesis of melanin (Sritunyalucksana; & Söderhäll. 2000: 53-69, Tassanakajon; et al. 2013: 954-967, Holmblad; & Söderhäll. 1999: 111-123, Jiravanichpaisal; Lee; & Söderhäll. 2006: 213-236).

In the case of antimicrobial peptides (AMPs), they play a key role in innate immunity, conserved in evolution, and present in all organisms include plants, vertebrates and invertebrate (Gueguen; et al. 2006: 283-288). These AMPs are main effectors for bacteria or fungi elimination and destruction in invertebrates. The AMPs are generally small molecules about 150-200 amino acid residues and have an amphipathic structure with anionic or cationic properties (Tassanakajon; et al. 2013: 954-967). The studies in *Drosophila* revealed that, AMPs of *Drosophila* are drosomycins, defensins, attacins, dipterocins, cecropins, metchnikowins and drosocins, while AMPs of shrimp are crustins, penaeidins and hemocyanins (Rowley; & Powell. 2007: 7209-7214).

2.3 Toll signaling pathway

In invertebrates including *M. rosenbergii* rely largely on their cellular and humoral innate immune responses to resist the pathogen infection that are involved with plenty of signaling pathway. Toll signaling pathway is one of the key pathway in the humoral innate immunity that are evolutionarily conserved in all animals including mammals.

In 1980, the Toll receptor was initially identified and described as a receptor that controls the dorso-ventral patterning in early embryonic development of *Drosophila* (Volhard; & Wieschaus. 1980: 795-801). Subsequently, the Toll pathway was also identified as an activator of the immune response in a *Drosophila* cell line in 1995 by Rosetto and colleagues (Rosetto; et al. 1995: 111-116). In 1996, Lemaitre and colleagues demonstrated that the dorso-ventral regulatory gene was also involved in the innate immune response of insects to combat invading Gram-positive bacteria and fungi (Lemaitre; et al. 1996: 973–983). At the same time, Taguchi and colleagues identified a human homolog of Toll and mapped this gene to chromosome 4p14. In 2006, Akira and co-worker identified vertebrate homolog of Toll and named Toll-like receptors (TLRs) (Akira; Uematsu; & Takeuchi. 2006: 783-801).

To activate the Toll signaling pathway of invertebrates in both dorso-ventral patterning and Toll-dependent antimicrobial response (innate immunity), Toll receptor require binding of the Spätzle, whereas the Toll-like receptor of vertebrates can bind directly to cell membrane components of invading microbes known as pathogen-associated molecular patterns (PAMP) (Imler; & Hoffmann. 2001: 304-311, Valanne; Wang; & Rämét. 2011: 649-656). Spätzle protein was synthesized and secreted from cell as an inactive form that contained a prodomain and a C-terminal Spätzle activated domain. The prodomain is required for Spätzle protein secretion and also functions to prevent the C-terminal Spätzle activated domain from binding to Toll receptor in non-signaling conditions (Shi; et al. 2009: 610-617, Valanne; Wang; & Rämét. 2011: 649-656).

In *Drosophila melanogaster*, after invasion of the pathogens to the host, cell wall components are recognized by extracellular recognition factors including gram negative binding protein (GNPB3) and peptidoglycan (PGN) recognition protein (PGRP). Then the signals from recognition factor converge a modular serine protease (ModSP) and activate the Gram-positive-specific serine protease (Grass), respectively. Activation of Grass leads to

activation of downstream serine protease including spirit, spheroid, sphinx1 and sphinx2 which induce the activation of Spätzle processing enzyme (SPE) (Buchon; et al. 2009: 12442-12447). Thereafter, activated SPE cleave the full-length Spätzle protein and then the proteolytically cleaved Spätzle binds to the Toll receptor. There are two models for the binding of cleaved Spätzle to Toll receptor. The first model is one Spätzle dimer interacts with two Toll receptors (Weber; et al. 2005: 22793–22799) and the second model is two Spätzle dimers binding to each ectodomain of two Toll receptors (Gangloff; et al. 2008: 14629–14635). The binding of Spätzle dimer to Toll receptor induces a conformational change of Toll receptor and initiates intracellular signaling result in activation of downstream components of Toll signaling pathway (Fig. 3). To transmit the signals from Toll receptors to cytosolic molecules, the activated Toll receptor interacts with their adaptor protein MyD88 by homophilic TIR-TIR interaction between Toll/interleukin-1 receptor (TIR) domain of MyD88 and TIR domain of Toll receptors (Janssens; & Beyaert. 2002: 474-482). For next signal transduction, the heterotrimeric formation of the MyD88, Pelle (which is the protein kinase) and Tube are required. The evidence from coimmunoprecipitation studies by Sun and colleague in 2002 suggested that the MyD88's death domain binds to death domain of Tube. In addition, by using crystallographic studies, Pelle formed the heterotrimer by two distinct binding surfaces (i.e. death domain and catalytic domain) to Tube death domain (Fig. 4) (Sun; Bristow; & Wasserman. 2002: 12871–12876). The heterotrimer acts as an upstream signal which induces the phosphorylation and degradation of I κ B factor Cactus result in the nuclear translocation of Dif and Dorsal (released from Dorsal/Dif-Cactus complex) and activate transcription of antimicrobial peptides (AMPs) gene (Wu; & Anderson. 1998: 93–97).

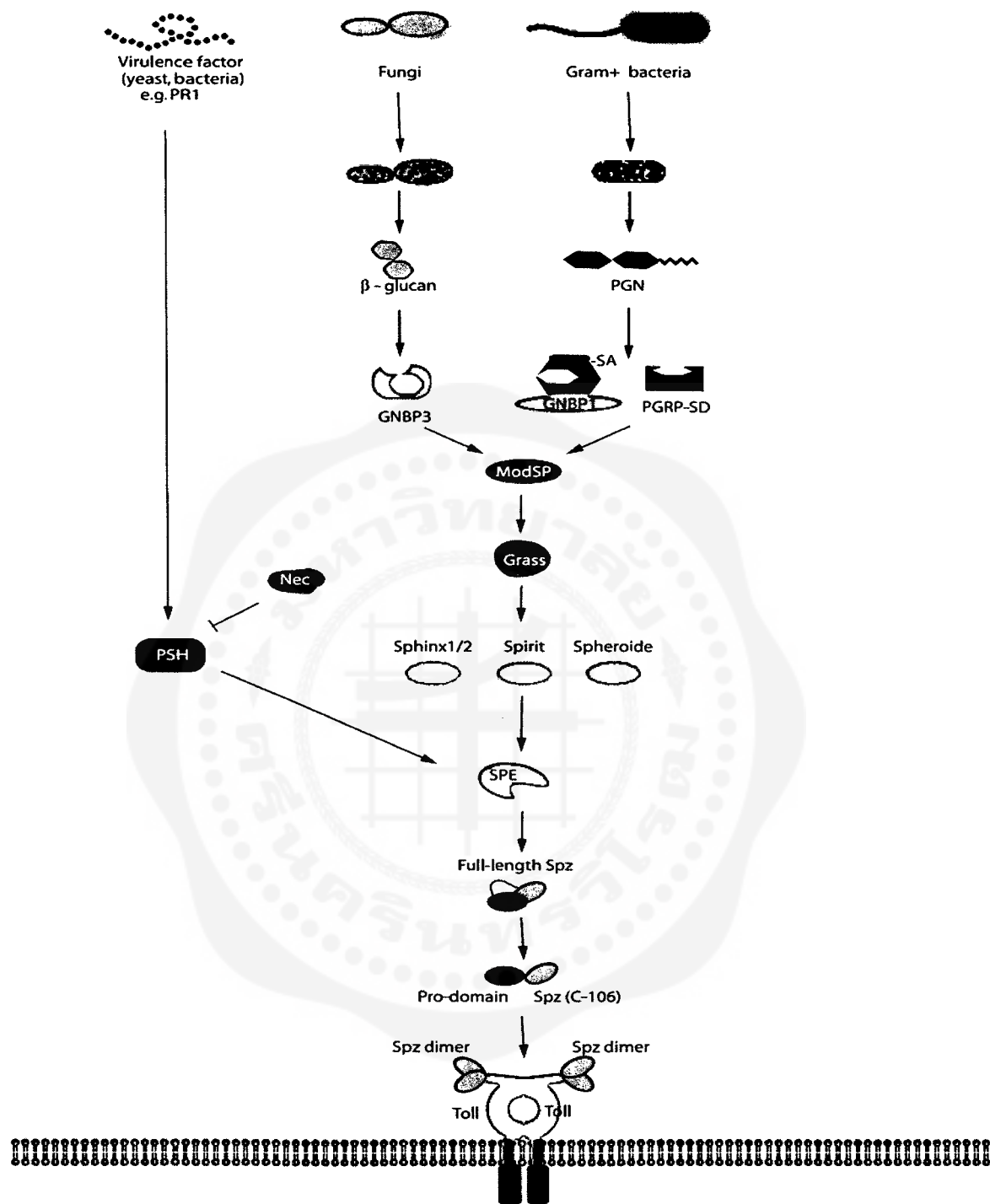


Figure 3 The activation of Toll pathway by proteolytic cleavage of Spätzle protein in a *Drosophila melanogaster* model

Source: Valanne; Wang; & Rämét. (2011). The *Drosophila* Toll signaling pathway. P. 649-656.

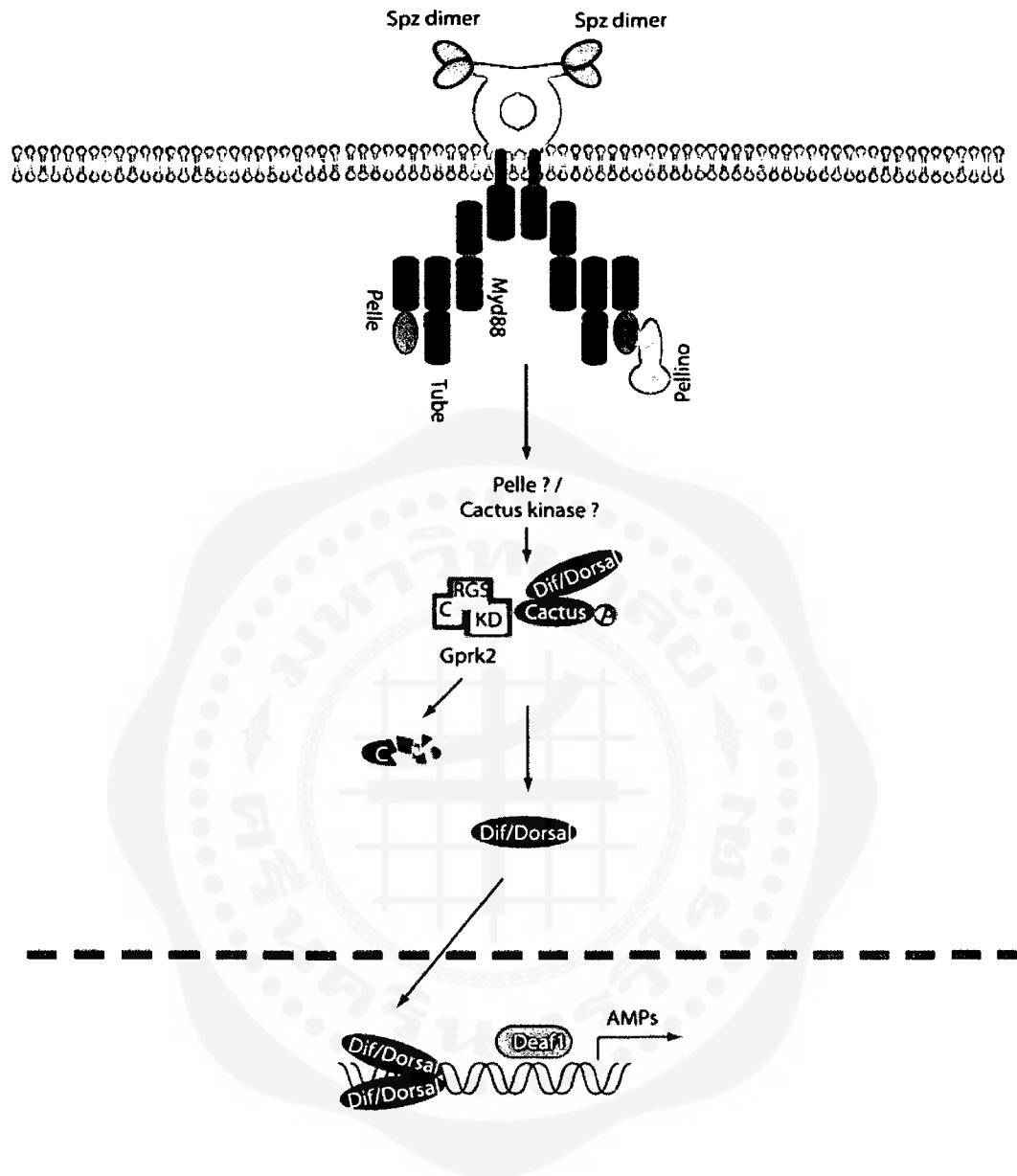


Figure 4 Downstream signaling of Toll pathway. Spz dimers bind to N-terminus of Toll receptor to form 4Spz:2Toll complex and trigger a phosphorylation of cytosolic molecules result in Cactus degradation which required for Dif/Dorsal nuclear translocation. The transcription of antimicrobial peptide genes are induced by binding to transcription control element.

Source: Valanne; Wang; & R  met. (2011). The *Drosophila* Toll signaling pathway.

P. 649-656.

2.4 Spätzle protein

Spätzle protein is a protein that acts as a ligand of Toll receptor. Spätzle protein is synthesized and secreted as an inactive form and requires the enzymatic cleavage by Spätzle processing enzyme (SPE) for its activation (Jang; et al. 2006: 45-55). Spätzle protein is a member of cysteine-knot superfamily (contains cysteine residues) as well as several growth factors such as platelet-derived growth factor BB (PDGF-BB), nerve growth factor (NGF) and transforming growth factor β (TGF- β) (Mizuguchi; et al. 1998: 239-242).

The pro-Spätzle protein (inactive form) consists of Spätzle pro-domain and C-terminal Spätzle activated domain. The Spätzle pro-domain is required for secretion of the synthesized protein from cells. According to Weber and colleagues, removal of the pro-domain from cell line 648-1B6 (derived from S2 cells) resulted in drastically reduced C106 (C-terminal Spätzle activated domain) secreted into the cell culture medium while the synthesized protein is present in the cellular fraction (Weber; et al. 2007: 13522-13531). Furthermore, the pro-domain also acts to prevent the pro-Spätzle protein from binding to Toll receptor in non-signaling conditions (Weber; et al. 2003: 794-800). However, after pro-Spätzle protein cleavage by Spätzle processing enzyme, the pro-domain is still associated with C-terminal Spätzle activated domain by non-covalent interaction and replaced by N-terminal ectodomain of Toll receptor (Weber; et al. 2007: 13522-13531).

There are many researches indicating the importance of Spätzle in invertebrate immune system. In 2012, Zhong and colleagues investigated the interaction between Toll receptor and Spätzle protein of tobacco hornworm, *Manduca sexta* by co-immunoprecipitation assays, the results showed that the ecto-domain of Toll receptor could bind with the C-terminal of Spätzle protein (MsSpz-C108). No interaction was observed in the experiment of Toll receptor with full-length MsSpz, suggesting that Toll receptor could bind to C-terminal Spätzle activated domain only. To confirm a Toll-Spz pathway in *M. sexta*, researchers used an antibody blocking assay to test the expression of AMP genes in *M. sexta* larvae, including *attacin*, *cecropin*, *lebocin* and *moricin* induced by purified recombinant MsSpz-C108 injection. The results showed that these AMP genes could be blocked by pre-injection of antibody against MsToll (Zhong; et al. 2012: 514-524). In the same year, Zheng and colleagues isolated the full-length *Spätzle* gene from *Artemia sinica* by RACE technique and studied the expression

pattern of Spätzle protein during embryonic development and bacteria challenge. The *A. sinica Spätzle* gene was 2,400 nucleotide with, a 2,010 bp of ORF encoding a 669 amino acid residues. The protein analysis revealed that the molecular weight of *A. sinica* Spätzle protein was 76.9 kDa with a signal peptide at N-terminus which indicated that this protein could be a secreted protein. A specific serine endoprotease cleavage site was found at the C-terminus. In the bacteria challenged experiment, mRNA expression was gradually increased after immersed *A. sinica* cysts with Gram-positive bacteria *Micrococcus lysodeikticus* (Zheng; et al. 2012: 6035-6042).

The study of shrimp Spätzle proteins are rather few. In Chinese shrimp, *Fenneropenaeus chinensis*, Shi and colleagues identified a *F. chinensis* Spätzle-like protein (*FcSpz*) by using a 726 bp DNA sequence of expressed sequence tag (EST) from random sequencing of a hemocytes cDNA library to design specific primers used in 5' cDNA end amplification. The *FcSpz* cDNA was 997 bp containing an open reading frame of 768 bp encoding 255 amino acids with 19 residues of signal peptide and had molecular mass of 28.89 kDa. BLASTp analysis showed that *FcSpz* had 54% identity with Spätzle-2-like protein of salmon louse, *Lepeophtheirus salmonis*, 52% identity with Spätzle-2 of *Aedes aegypti* and 54% identity with Spätzle-like protein of *Anopheles gambiae*. Researcher used semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) to analyze tissue distribution and expression profiles of *FcSpz* gene in normal shrimp. The results revealed that *FcSpz* mRNA was expressed in gills, heart, hemocytes, hepatopancreas, intestine and stomach. When shrimp were injected with *Vibrio anguillarum* and white spot syndrome virus (WSSV), the expression level was significantly up-regulated in all tissues excepted in stomach. Furthermore, crayfish injected with recombinant protein *FcSpz* C114 (C-terminal activated Spätzle domain) the transcription level of *crustin 2* analyzed by semi-quantitative RT-PCR was significantly up-regulated (Shi; et al. 2009: 610–617).

In 2012, Wang and colleagues isolated two novel Tolls (*LvToll2* and *LvToll3*) and Spätzle-like proteins from whiteleg shrimp, *Litopenaeus vannamei*. For Spätzle proteins, gene specific primers were designed based on DNA sequences of expressed sequence tag (EST), which shared similarity to the *Drosophila Spätzle* gene. They obtained three full-length Spätzle cDNA sequences of *LvSpz1*, *LvSpz2* and *LvSpz3*. The *LvSpz1* was 1,082 bp, with an ORF of

714 bp. It shared 38.4% identity with *FcSpz* and 20.7% identity with *DmSpz1*. The *LvSpz2* was 1,733 bp, with an ORF of 750 bp. It had 26.5% identity with *LvSpz1*, 24.5% identity with *FcSpz* and 19.4% identity with *DmSpz1*, while the *LvSpz3* had an ORF of 768 bp and it had 96.5% identity with *FcSpz*, 38.9% identity with *LvSpz1* and 19.7% identity with *DmSpz1*. *LvToll1-3* were expressed in several tissues, while the *LvSpz1-3* displayed tissue-specific expressions (epithelium, eyestalk and intestine for *LvSpz1* and 3 or gill, eyestalk, epithelium and muscle for *LvSpz2*). After shrimp were injected with *V. alginolyticus* and white spot syndrome virus, expression level of *LvSpz1* and *LvSpz3* were strongly up-regulated at all time points, while *LvSpz2* was slightly downregulated (Wang; et al. 2012: 359-371). Recently, in 2017, a 2,642 bp of *LvSpz4* was isolated. The ORF was 918 bp, which encoded 305 amino acids that contained signal peptide and C-terminal Spätzle activated domain. The *LvSpz4* shared 55% identity to *Zootermopsis nevadensis* Spz and 33% identity to *Penaeus monodon* Spz. However, it shared lower identities to *LvSpz1-3*. The expression of *LvSpz4* was found in various tissues and mainly expressed in gill and hemocyte. In immune challenge shrimp, *LvSpz4* expression was increased after stimulated with LPS, *Staphylococcus aureus* and *V. alginolyticus*. Furthermore, in *V. alginolyticus* injected shrimp, the cumulative mortality of *LvSpz4* knocked down was significantly increased when compared to PBS and double-stranded eGFP injected shrimp (Yuan; et al. 2017: 46-57).

In 2017, Boonrawd and colleagues identified three isoforms of Spätzle proteins of black tiger shrimp, *P. monodon* (*PmSpz1*, 2 and 3) from expressed sequence tag (EST) data. They chose *PmSpz1* to study the active domain, expression profile of healthy shrimp and expression of *PmSpz1* gene in response to WSSV infection. The result revealed that the protein sequence of *PmSpz1* is highly homologous to those of *L. vannamei*, and consisted of an open reading frame of 714 bp containing 25 amino acid residues of N-terminal signal peptide and C-terminal Spätzle activated domain at 116 - 208 amino acid residues. The expression of *PmSpz1* gene analyzed by reverse transcriptase PCR was found in hemocytes, stomach, hepatopancreas, gill, lymphoid organ, eyestalk, heart, intestine and heart. The expression of *PmSpz1* gene in hemocytes was up-regulated after injected with WSSV compared to the control group injected with normal saline. Furthermore, expression levels of AMP genes in hemocytes including *ALFPm3*, *crustinPm1*, *crustinPm7*, *penaeidin3* and

penaeidin5 were up-regulated after shrimp injected with 3 nmol of recombinant active domain of PmSpz1 protein (racPmSpz1) (Boonrawd; et al. 2017: 88-95).

2.5 Rapid amplification of cDNA ends (RACE)

Rapid amplification of cDNA ends (RACE) is a technique used for PCR amplification of either the 5' or 3' end of a specific cDNA preparing from a total mRNA molecules to obtain the full length mRNA sequence. RACE allows the amplification of unknown sequence regions and only requires partial or a small known sequence within the mRNA of interest to generate the gene specific primers (GSPs). Partial sequence of mRNA are mostly obtained from PCR using degenerate primers designed based on conserved regions of the interest gene.

RACE ready cDNA is required for both 5' and 3' RACE PCR reactions. The first-strand cDNA was synthesized by reverse transcriptase for 5' and 3' RACE. In the 3' RACE ready cDNA synthesis, the first-strand of cDNA is synthesized by using a traditional reverse transcription with a modified oligo(dT) primer and after elimination of mRNA, the second-strand of cDNA is synthesized by using gene specific primer (GSP) (Fig. 5). In the case of 5' RACE ready cDNA synthesis, gene specific primer anneal to a known sequence in the middle of the RNA transcript and the first-strand cDNA is synthesized by reverse transcriptase. When the reverse transcriptase reaching the end of an RNA template and the complementary RNA is eliminated, homopolymeric tail is added to the 3' end of the first-strand cDNA by terminal transferase. The oligo(dT) anneals to these homopolymeric tail and subsequently the second-strand of cDNA is synthesized from 3' end of the oligo(dT) to generate a complete double-stranded cDNA (Fig. 6). To amplify the cDNA fragment either 5' and 3' end, the RACE PCR can be performed by using different gene-specific primers for different gene of interest with the primer specific to oligo(dT) adaptor (Yeku; & Frohman. 2011: 107-122, Chaivisuthangkura. 2007: 612 pp.).

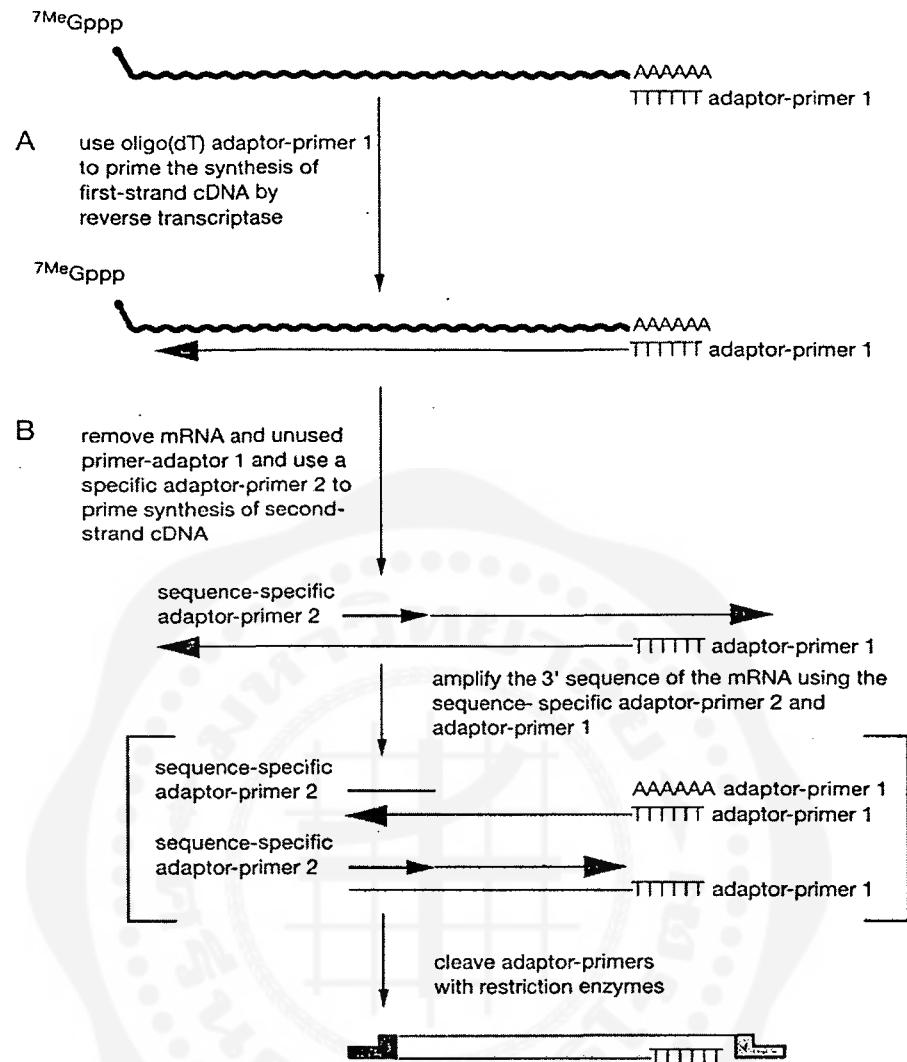


Figure 5 3' RACE ready cDNA synthesis. The synthesis of first-strand cDNA by using oligo(dT) with reverse transcriptase (A). The synthesis of second-strand cDNA by using gene specific primer (B).

Source: Sambrook, J.; & Russell, D.W. (2001). *Molecular cloning: Cold Spring Harbor Laboratory Press (Third Edition)*.

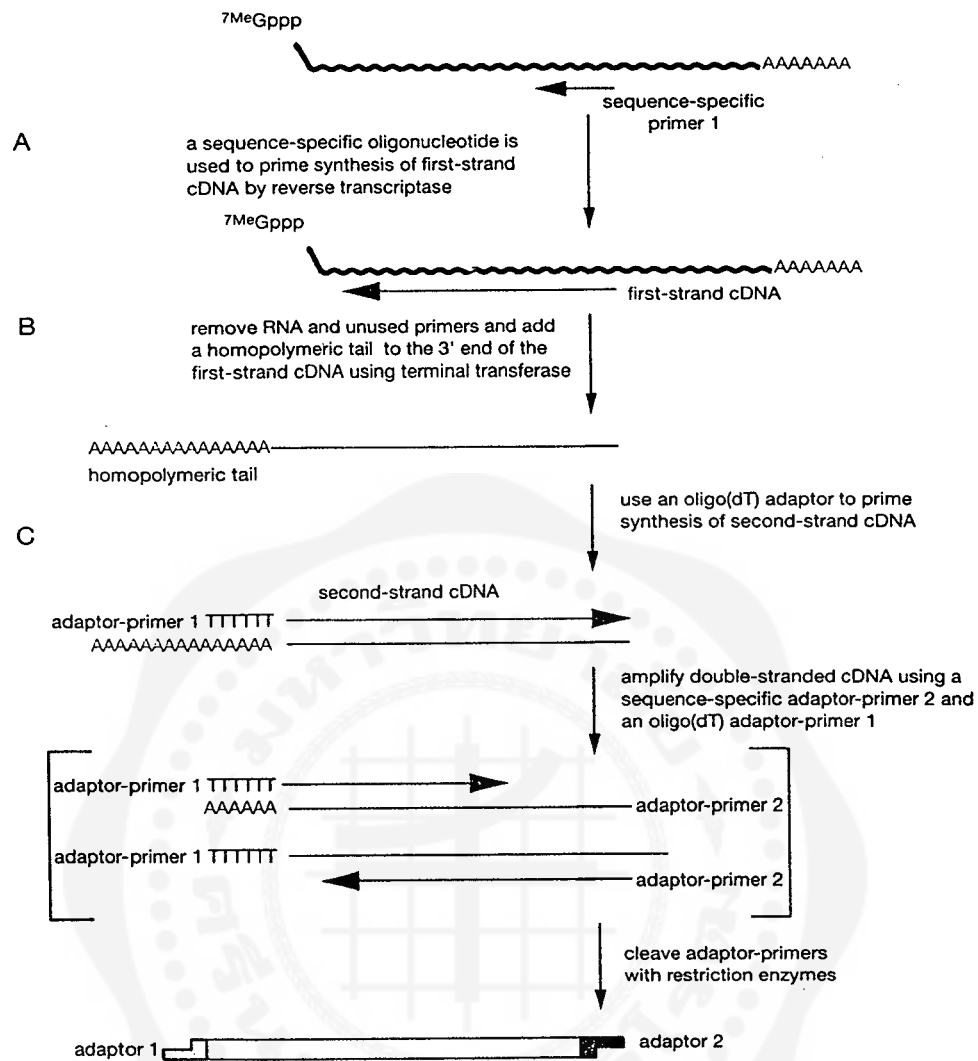


Figure 6 5' RACE ready cDNA synthesis. Gene specific primer is used to synthesize the first-strand cDNA (A). Homopolymeric tail adding by terminal transferase (B). Amplification of double-stranded cDNA by using an oligo(dT) specific to homopolymeric tail (C).

Source: Sambrook, J.; & Russell, D.W. (2001). *Molecular cloning: Cold Spring Harbor Laboratory Press (Third Edition)*.

2.6 RNA interference (RNAi)

In 1990, Napoli, Lemieux and Jorgensen attempted to induce the overexpression of chalcone synthase (CHS) (involved with flavonoid biosynthesis that are responsible for plant coloration) in violet petunia by introducing a chimeric petunia CHS gene. However, the experiments gave the opposite result, the petunia injected with double-stranded RNA of CHS produced white flowers and/or pale sectors on a wild-type pigmented background (Napoli; Lemieux; & Jorgensen. 1990: 279-289). At that time, the understanding about RNAi by dsRNA molecules are still deficient. The researchers have proposed the hypothesis of this phenomenon, which is caused by overexpression of CHS mRNA as post-transcriptional gene silencing (PTGS) process.

In 1998, Fire and colleagues injected RNA populations with different RNA structure consists of sense or antisense single-stranded RNA and double-stranded RNA of *unc-22* gene (encode myofilament protein) into *Caenorhabditis elegans*. They discovered that the double-stranded RNAs caused potent and specific gene silencing (increased the twitching phenotype), while the experiment using single-stranded RNA either sense or antisense strand did not affect the phenotype of *C. elegans* (wild type). They called the gene suppression induced by double-stranded RNAs as RNA interference (RNAi). RNAi technique is a powerful tool for gene knock-down and is widely used to study gene function, prevent viral infection and screen for candidates to treat cancer and infectious diseases (Fire; et al. 1998: 806-811). RNAi is a cellular process that uses dsRNA for suppression of gene expression based on sequence similarity of dsRNA to its target gene (Li; Li; & Ding. 2002: 1319-1321, Robalino; et al. 2005: 13561-13571).

There are two major proteins involved in the RNAi pathway, Dicer and Argonaute (Bohmert; et al 1998: 170-180, Höck; & Meister. 2008: 210.2-210.8, Saksmerprome; et al. 2009: 213-217). Dicer has been identified in various organisms from plants to human (Su; et al. 2008: 223-233). This protein is a member of RNase III endonuclease that cleaves long dsRNA into short interfering RNAs (siRNAs) to initiate the silencing process (Su; et al. 2008: 223-233, Escobedo-Bonilla. 2011: 1-12). In the case of Argonaute proteins (Ago), they are key players in gene-silencing pathway. They bind to siRNAs or microRNAs (miRNAs) to repress specific target RNAs either by RNA degrading in the case of short interfering RNA

(siRNA) or by inhibiting translation in the case of microRNA (miRNA) (Höck; & Meister. 2008: 210.2-210.8, Bernstein; et al. 2001: 363-366)

The mechanism of RNAi involves the cleavage of long dsRNA into short dsRNA around 21-28 bp with 5' phosphate and 3' dinucleotide overhangs by the performing of ribonuclease III-like enzyme called Dicer. For dsRNAs delivery, they can be introduced into cells by injection, electroporation or chemical mediated transfection (Ongvarrasopone; et al. 2007: 35-39). When siRNA present within cells, it is incorporated with many factors including DNA/RNA helicase, PAZ proteins (Piwi/Argonaute/Zwille/Sting), RDE-1 and MUT-7 into RNA induced silencing complexes (RISC) (Hammond; et al. 2000: 293-296), unwound the dsRNA into single-stranded RNA (ssRNA) and the single stranded antisense siRNA is targeted to a specific region of their complementary RNA molecule (Fire; et al. 1998: 806-811). After that, endonuclease digest the complementary target mRNAs at the central nucleotide of mRNA-siRNA complementary region and then mRNA was degraded by the exonuclease. These processes resulted in suppression of target gene expression (Fig. 7) (Martinez; et al. 2002: 563-574). In nature, the initiator of RNAi starts with the appearance of dsRNA, aberrant single-stranded RNA (required RNA dependent RNA polymerase for dsRNA synthesis), transposons or transgenes in cell (Akashi; et al. 2001: 359-367).

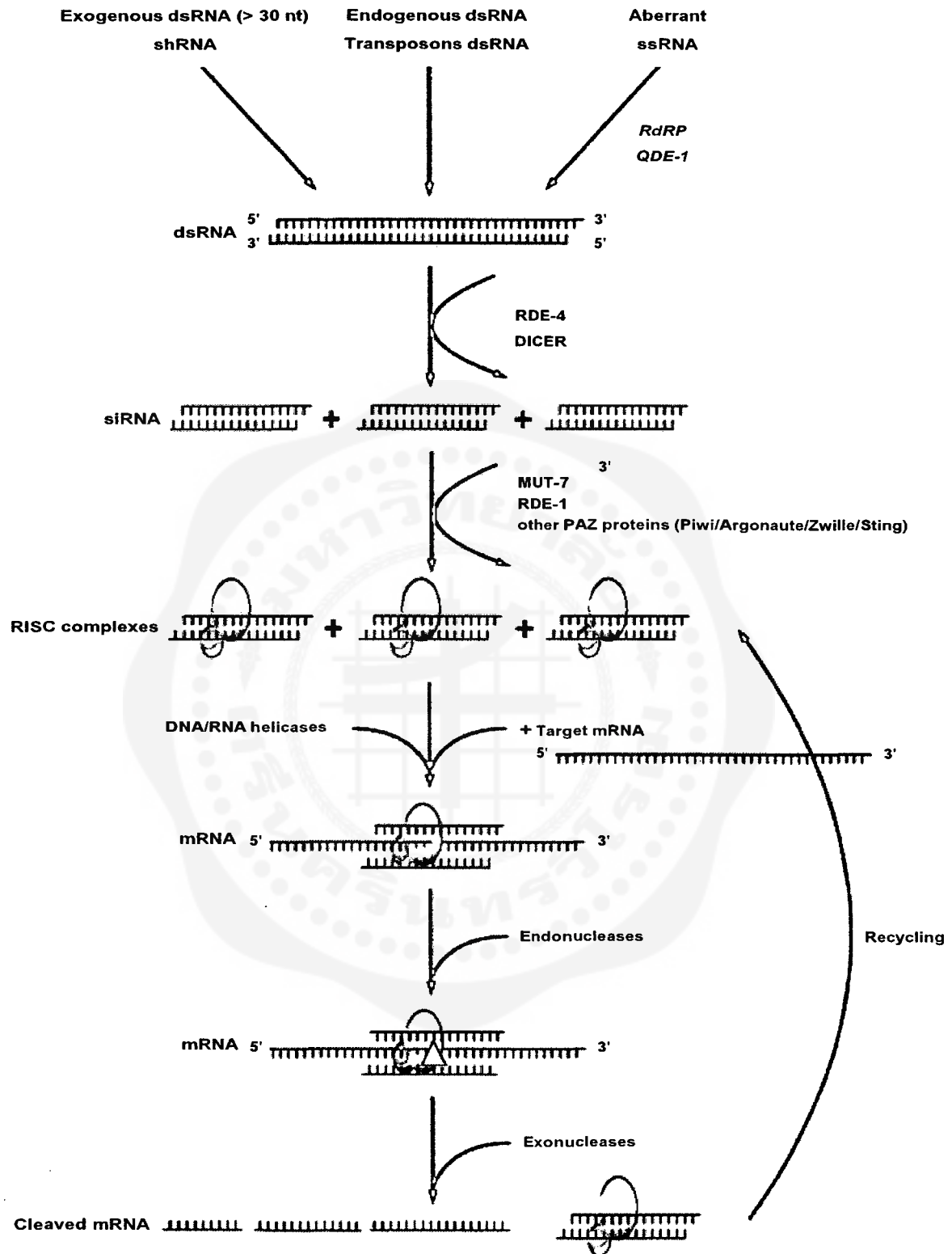


Figure 7 Mechanism of RNAi pathway

CHAPTER 3

MATERIALS AND METHODS

3.1 Research outline

This research consists of 4 major steps. Firstly, isolation of a partial *Macrobrachium rosenbergii* Spätzle gene (*MrSpz*) by PCR using degenerate primers. Secondly, isolation of the full-length cDNA of *MrSpz* by rapid amplification of cDNA ends (RACE) technique. Thirdly, *MrSpz* expression analysis in *Aeromonas caviae* challenged prawn at the transcriptional level by RT-PCR. Finally, characterization of the role of *MrSpz* in immune defense mechanism by suppression of *MrSpz* expression by RNAi technique (Fig. 8).

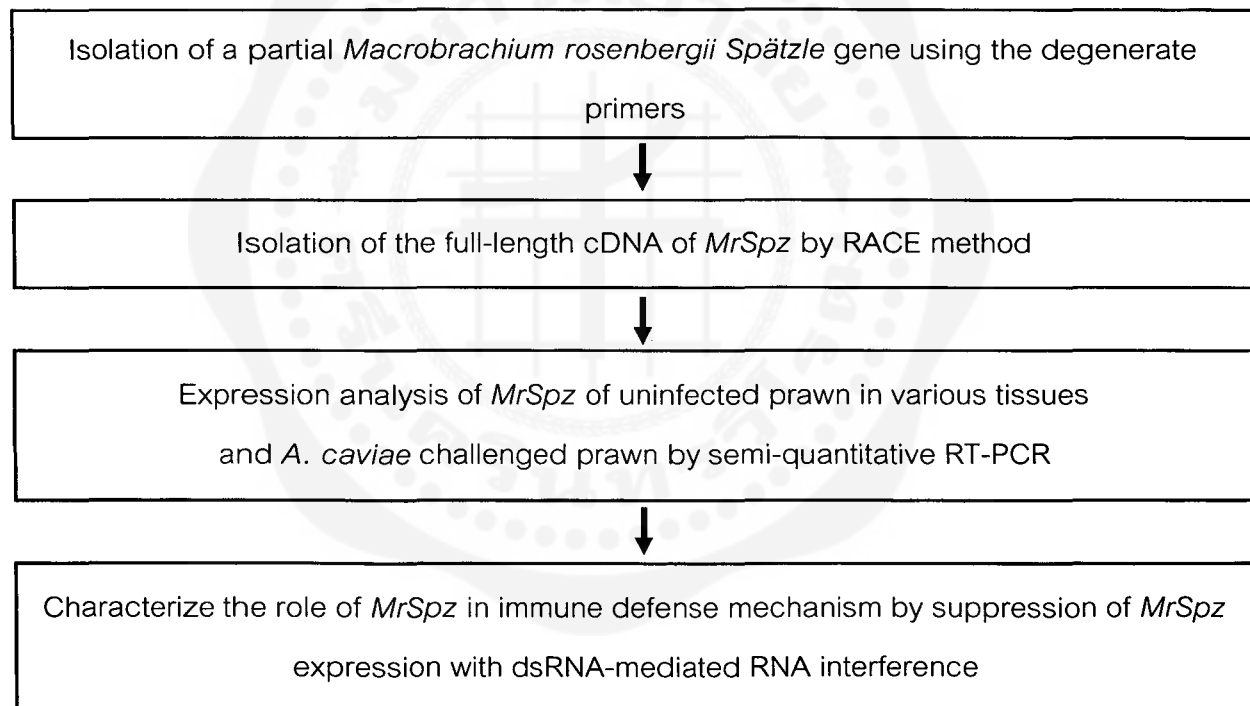


Figure 8 Flowchart demonstrated the overview of research outline

3.2 Isolation of a partial *M. rosenbergii* Spätzle (*MrSpz*) gene

3.2.1 Extraction of total RNA from *M. rosenbergii* and cDNA synthesis

Total RNA was extracted from gill using NucleoSpin® RNA XS isolation Kit following the manufacturer's manual (MACHEREY-NAGEL, GmbH & Co. KG, Düren, Germany). Gill sample of 5 mg was homogenized in 100 µl of lysis buffer RA1 with 2 µl of reducing agent TCEP. Then 5 µl of carrier RNA working solution was added and mixed twice by vortexing for 5 sec. The lysate was filtrated using NucleoSpin® Filter by centrifugation at 11,000 x *g* for 30 sec. To adjust RNA binding condition, the flow-through was mixed with 200 µl of 70% ethanol by pipetting up and down for 5 times and placed the mixed solution into NucleoSpin® RNA XS column and then centrifuged at 11,000 x *g* for 30 sec. The NucleoSpin® RNA XS column was added with 100 µl of membrane desalting buffer (MDB) and centrifuged at 11,000 x *g* for 30 sec. After that, the column was incubated with 25 µl of rDNase reaction mixture to eliminate the contaminating DNA. For washing the silica membrane, the column was incubated with 100 µl of buffer RA2 at room temperature for 2 min and centrifuged at 11,000 x *g* for 30 sec and washed twice with 400 µl and 200 µl of buffer RA3, respectively, then centrifuged at 11,000 x *g* for 2 min to dry the silica membrane. The total RNA was eluted with 10 µl of RNase-free water and stored at -70°C until use. The concentration of total RNA was measured by using NANODROP LITE Spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Total RNA was reverse transcribed to cDNA using the SuperScript® III First-Strand Synthesis following the manufacturer's manual (Invitrogen, Co., Carlsbad, CA, USA). The reaction mixture (5 µg RNA, 50 µM oligo(DT)₂₀ and 10 mM dNTP mix) as shown in Table 1 was heated at 65°C for 5 min and placed on ice for at least 1 min then the reaction mixture was mixed with cDNA synthesis mixture (10X RT buffer, 25 mM MgCl₂, 0.1 M DTT, RNase OUT™ and SuperScript® III RT) as indicated in Table 2 and incubated at 50°C for 50 min. The reaction was terminated at 85°C for 5 min. After that, 1 µl of *E. coli* RNase H was added and incubated at 37°C for 20 min to remove the RNA complementary from cDNA.

Table 1 Reaction mixtures of the SuperScript® III First-Strand Synthesis

Components	Volume (μl)
Total RNA (1 μg/μl)	5
50 μM Oligo(dT) ₂₀	1
10 mM dNTP mix	1
DEPC-treated water	3
Total volume	10

Table 2 cDNA synthesis mixtures

Components	Volume (μl)
10X RT buffer	2
25 mM MgCl ₂	4
0.1 M DTT	2
RNaseOUT™ (40 U/ μl)	1
SuperScript® III RT (200 U/ μl)	1
Total volume	10

3.2.2 Cloning of partial cDNA fragment of *M. rosenbergii* Spätzle (*MrSpz*)

To isolate a partial cDNA fragment of *MrSpz*, three degenerate primers were designed based on the conserved regions of amino acid sequences of Spätzle protein from *F. chinensis* (ACD36030.1), Spätzle 1 (AEK86522.1) and Spätzle 3 (AEK86524.1) from *P. vannamei* which generated by using the Clustal Omega program (Fig. 9). The sequence of all primers were demonstrated in Table 3.

The primary PCR amplification was initially performed using cDNA as a template with a pair of primers Spzl-UPF1 and Spzl-R followed by nested PCR using 50 fold diluted primary PCR products in Tricine-EDTA as a PCR template with a second set of primers namely Spzl-UPF2 and Spzl-NGSP1. The PCR amplification conditions were 1 cycle of 94°C for 2 min, 35 cycles of 94°C for 30 sec, 42°C for 1 min and 72°C for 1 min and 1 cycle of 72°C for 10 min. The reaction mixture of both primary and nested PCR were shown in Table 4. PCR products were analyzed by 2% agarose gel electrophoresis and the expected band was gel purified by using NucleoSpin® Gel and PCR Clean-up Kit (MACHEREY-NAGEL, GmbH & Co. KG, Düren, Germany). The purified PCR products were cloned into pGEM®-T Easy Vector Systems (Promega, Co., Madison, WI, USA) (Fig. 10). The ligation mixture was demonstrated in Table 5 and the reaction was incubated for overnight at 16°C and transformed into XL1-Blue competent cells for sequencing.

DH-LFAKKYADIADQSADDLVDMVTKEQEEAFDYSYYTGASTGDSFYDATHW
 HYEKLLSLYADVADLNTEL-----SVDRPNT
 HYEKLLSLYADVADLNTEL-----SVERPNT
 .: : . ***:** .:: : :
 PSEVVYAMPKRAQNVEGKWRVIVNDVH---YYTQTARLETCLFPEAACRAL
 PSETAYVRPLRAQNTEGKWRVVVNNIDAHYQTLTQTTRIEECLSSADACPLV
 PSETAYVRPLRAQNTEGKWRVIVNNIDAHYQTLTQTTRIEECLTSADACPLV
 . . * *.*****:**:.. ***:* * ** Spz
 TQKSVYHRLLSYDPCDPYKGLFIDIYKLPASCSCHLPA----- 237
 |||||
 LQKSVYHRFLVYDPYDQYFPFAIETFKLPASCACLLGAYTIDH 255
 |||||
 LQKSIYHRFLVYDPYDQYFPFAIETFKLPASCACLLGAYTIDH 255
 Spzl-R
 :**:* *** * * : *: ::*** * *

Table 3 Primers used in this study

Primers	Primer sequence (5'→3')
5'-RACE CDS Primer A	5'-(T)25V N-3' (N = A, C, G, or T; V = A, G, or C)
3'-RACE CDS Primer A	5'-AAG CAG TGG TAT CAA CGC AGA GTA C(T)30 V N-3' (N = A, C, G, or T; V = A, G, or C)
Universal Primer A Mix	Long: 5'-CTA ATA CGA CTC ACT ATA GGG CAA GCA GTG GTA TCA ACG CAG AGT-3' Short: 5'-CTA ATA CGA CTC ACT ATA GGG C-3'
Nested Universal Primer A	5'-AAG CAG TGG TAT CAA CGC AGA GT-3'
SpzI-UPF1	5'-CAY CCI GCI CCI GCI TAY CA-3'
SpzI-UPF2	5'-GAR TGY GCI GCI AAY ACI AC-3'
SpzI-NGSP1	5'-ATT CAT AGC AGT CAG GGA CCT TGG GAC-3'
SpzI-R	5'-GCG GTG GTA SAY KGA CTT CTG-3'
Spz_new3-GSP1	5'-GCC CTC AAG GGC CTG ACG TAC GCG GTC TCG-3'
Spz_new3-GSP2	5'-GCC CTG GTG CCT TGA GGA CCC CGA GTA CCC-3'
Spz_new4-NGSP1	5'-AGG TTG GGT ACT CGG GGT CCT CAA GGC ACC-3'
Spz_new4-NGSP2	5'-CGA GAC CGC GTA CGT CAG GCC CTT GAG GGC-3'
Spz_exp-R1	5'-GTC GTA GGG GTC GTA GAC GAG GAA ACG GTG-3'
<i>β-actin</i> -F	5'-CCC AGA GCA AGA GAG GTA-3'
<i>β-actin</i> -R	5'-GCG TAT CCT TCG TAG ATG GG-3'
ACF	5'-GGC GAG CCG CAG GCA CCC-3'
ACR	5'-CTC GAC GAA GGC CTT GAT GCC C-3'
Spz_RNAi-F	5'-ACC ATC ACA GAG CTC CAT CC-3'
Spz_RNAi-R	5'-ATG GAC TTC TGC AGG CAC TT-3'

Table 4 Primary and nested PCR reaction mixtures of a partial *MrSpz* isolation

PCR reaction mixtures	Volume (μ l)	
	Primary PCR	Nested PCR
cDNA	3	-
[1:50] diluted primary PCR product in Tricine-EDTA	-	5
10X PCR Buffer (Rabbit)	5	5
50 mM MgCl ₂	1.5	1.5
2.5 mM dNTP Mix	4	4
50 μ M SpzI-UPF1	1	-
50 μ M SpzI-UPF2	-	1
50 μ M SpzI-R	1	-
50 μ M N-GSP1	-	1
Hot Start <i>Taq</i> DNA polymerase (Rabbit)	0.5	0.5
PCR-grade water	34.0	32.0
Total volume	50	50

Table 5 Ligation mixtures of a partial *MrSpz* cDNA fragment

Ligation mixtures	Volume (μ l)
pGEM [®] -T easy vector (25 fmole)	1
2X ligation buffer	5
T4 DNA ligase (4U/ μ l)	1
Purified PCR products (250 fmole)	1
Deionized water	2
Total volume	10

3.3 Isolation of full-length *MrSpz* by rapid amplification of cDNA ends (RACE)

3.3.1 The 5' and 3' RACE-Ready cDNA synthesis

The 5' and 3' RACE-Ready cDNA were synthesized from total RNA by SMARTer™ RACE cDNA Amplification Kit with 5'-RACE CDS Primer A and 3'-RACE CDS Primer A, respectively (Table 3) (Clontech Laboratory, Inc., Mountain View, CA, USA) for using as DNA template in the RACE PCR. The 5' and 3' RACE-ready cDNA synthesis reactions were prepared separately. Firstly, the buffer mix (1) for all of 5' and 3' consist of 4.0 µl of 5X First-Strand Buffer, 0.5 µl of 100 mM DTT and 1.0 µl of 20 mM dNTPs were prepared and set aside at room temperatures. Secondly, the reaction mixtures in separate microcentrifuge tubes for 5' and 3' RACE-ready cDNA synthesis were combined with 10 µl of RNA (1 µg) and 1.0 µl of 5'-CDS Primer A for preparation of 5'-RACE-Ready cDNA. For the 3'-RACE-Ready cDNA synthesis, reaction mixtures were mixed with 11 µl of RNA (1 µg) and 1.0 µl of 3'-CDS Primer A (Table 6). After mixing the contents, both microcentrifuge tubes were centrifuged briefly and then incubated at 72°C for 3 min followed by 42°C for 2 min. After cooling, the tubes were briefly centrifuged for 10 sec at 14,000 x g to collect the contents at the bottom. In case of 5' RACE-Ready cDNA, 1 µl of the SMARTer II A Oligonucleotide was added to prepare the 5'-RACE cDNA synthesis reaction.

The master mix for 5' and 3' RACE-Ready cDNA synthesis reactions were prepared by mixing 5.5 µl of the buffer mix (1) from the first step with 0.5 µl of RNase Inhibitor (40 U/µl) and 2.0 µl of SMARTScribe™ Reverse Transcriptase (100 U). These master mix was added to the denatured RNA from the second step for a total volume of 20 µl per cDNA synthesis reaction and then the reaction mixture was mixed by gently pipetting, and centrifuged briefly. The reaction mixture tubes were incubated at 42°C for 90 minutes in a hot-lid thermal cycler (T100™ Thermal Cycler (Bio-Rad Laboratories, Inc., California)) and heated at 70°C for 10 min to stop the reaction. To dilute the 5' and 3' RACE-Ready cDNA reaction, 10 µl of Tricine-EDTA Buffer was added and stored at -20°C for later use.

Table 6 Reaction mixtures for 5' and 3' RACE ready cDNA synthesis

Reaction/Mixtures	Components/Primers	Volume (μl)
Buffer mix (1)	5X First-Strand Buffer	4
	100 mM DTT	0.5
	20 mM dNTPs	1
Reaction mixtures	RNA (1 μg)	10
for 5' RACE-ready	5'-CDS Primer A	1
cDNA	SMARTer II A Oligonucleotide	1
Reaction mixtures	RNA (1 μg)	11
for 3' RACE-ready	3'-CDS Primer A	1
cDNA		
Master mix	Buffer mix (1)	5.5
	RNase Inhibitor (40 U/μl)	0.5
	SMARTScribe™ Reverse Transcriptase (100 U)	2

3.3.2 Rapid amplification of cDNA ends (RACE)

To obtain the full-length cDNA sequence of *MrSpz*, RACE reactions were performed. Gene specific primers for both 5' and 3' RACE PCR reactions were designed based on the partial *MrSpz* cDNA sequence (Table 3). To isolate the 5' end of *MrSpz*, 5' RACE touchdown PCR was firstly performed with gene specific primer Spz_new3-GSP1 and Universal Primer A Mix (UPM) followed by a nested PCR with gene specific primer Spz_new4-NGSP1 and Nested Universal Primer A (NUP). For 3' RACE, touchdown PCR was performed with gene specific primer Spz_new3-GSP2 and UPM followed by a nested PCR with gene specific primer called Spz_new4-NGSP2 and NUP. The reaction mixture for 5' and 3' RACE were shown in Table 7.

The touchdown PCR conditions were 5 cycles of 94°C for 30 sec and 72°C for 3 min, 5 cycles of 94°C for 30 sec, 70°C for 30 sec and 72°C for 3 min and 20 cycles of 94°C for 30 sec, 68°C for 30 sec and 72°C for 3 min. For nested PCR, conditions were 94°C for 2 min followed by 35 cycles of 94°C for 30 sec, 68°C for 30 sec and 72°C for 3 min and then 72°C for 10 min.

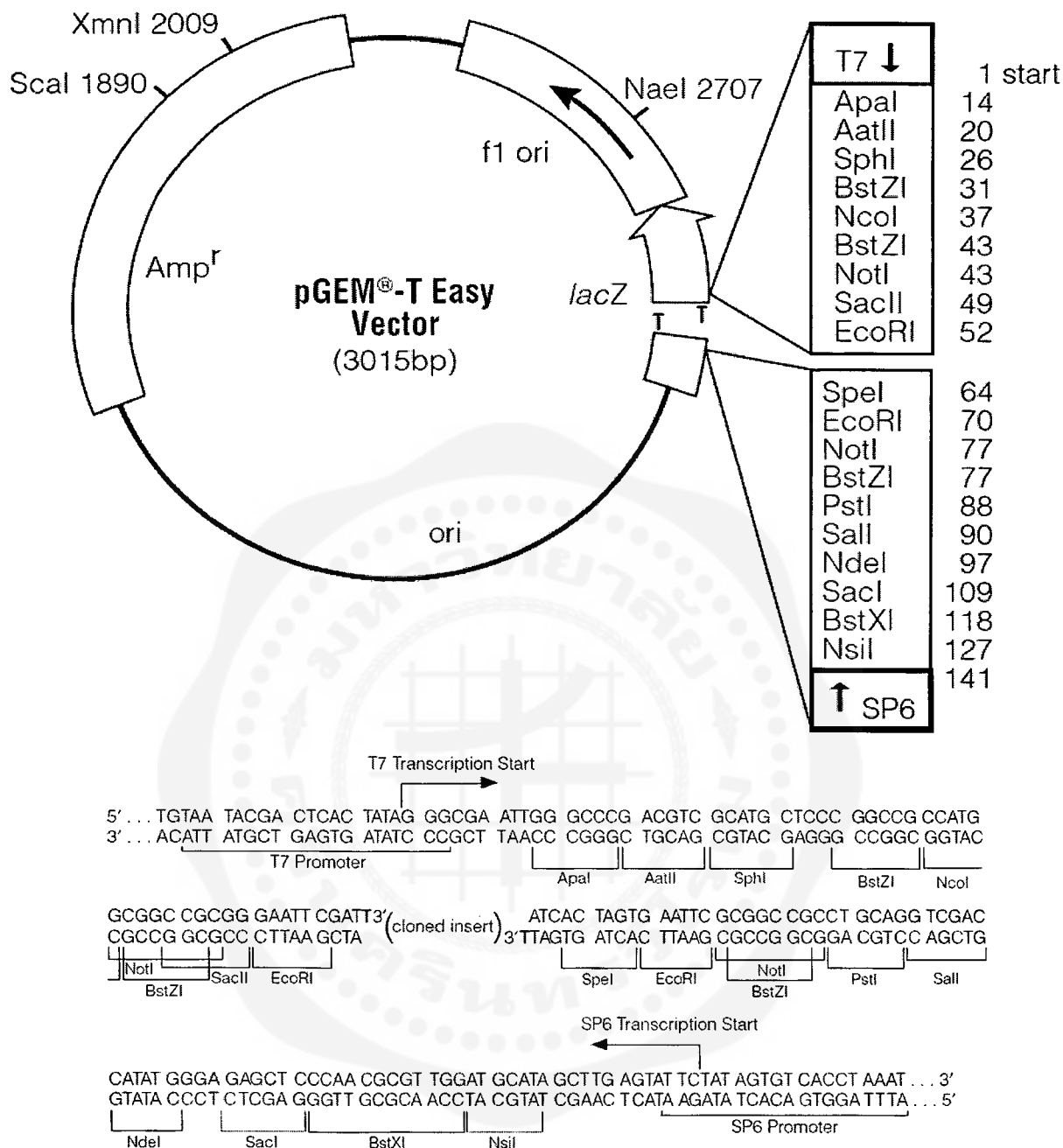


Figure 10 pGEM®-T Easy Vector circle map and their cloning sites

Source: <https://www.promega.com/~media/files/resources/protocols/technical%20manuals/0/pgem-t%20and%20pgemt%20easy%20vector%20systems%20protocol.pdf>

The PCR products were separated on 2% agarose gel and stained with SERVA DNA Stain G (SERVA) and observed under the UV light by using Gel Doc™ XR⁺ (Bio-Rad Laboratories, Inc., California). The expected band was cut from the gel, purified using NucleoSpin® Gel and PCR Clean-up Kit and cloned into pCR™ 2.1-TOPO® TA vector (Invitrogen, Co., Carlsbad, CA, USA) (Fig. 11) and transformed into Top-10 competent cells for sequencing. The ligation mixture was demonstrated in Table 8 and the reaction was incubated at 22°C for 30 min.

Table 7 Reaction mixtures of 5' and 3' RACE

PCR reaction mixtures	Volume (µl)			
	5' RACE		3' RACE	
	Touchdown	Nested	Touchdown	Nested
	PCR	PCR	PCR	PCR
5' or 3' RACE ready cDNA	2.5	-	2.5	-
[1:50] primary 5' or 3' RACE PCR	-	5	-	5
10X UPM	5	-	5	-
10 µM NUP	-	1	-	1
10 µM Spz_new3-GSP1	1	-	-	-
10 µM Spz_new3-GSP2	-	-	1	-
10 µM Spz_new4-NGSP1	-	1	-	-
10 µM Spz_new4-NGSP2	-	-	-	1
10X Advantage 2 PCR Buffer	5	5	5	5
10 mM dNTP Mix	1	1	1	1
50X Advantage 2 Polymerase Mix	1	1	1	1
PCR-grade water	34.5	36	34.5	36
Total volume	50	50	50	50

Table 8 Ligation mixtures of 5' and 3' RACE fragment

Ligation mixtures	Volume (μl)
Purified 5' or 3' RACE PCR products	4
Salt solution	1
pCR®2.1-TOPO®	1
Total volume	6

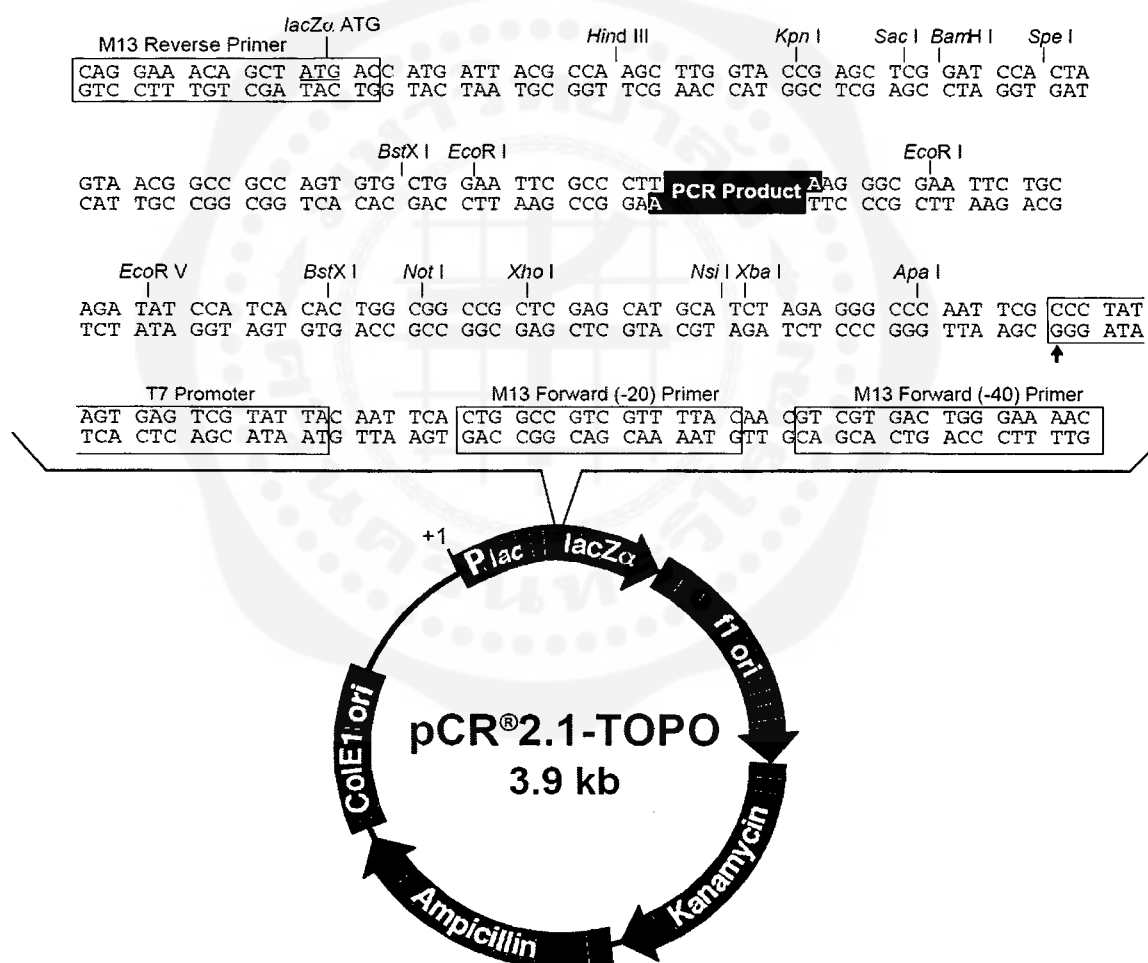


Figure 11 pCR®2.1-TOPO® vector circle map and their cloning sites for TA cloning of PCR products derived from RACE PCR

3.4 Expression analysis of *MrSpz* in different tissues of uninfected and pathogens challenged *M. rosenbergii* by semi-quantitative RT-PCR

3.4.1 Expression analysis of *MrSpz* in uninfected *M. rosenbergii*

In order to determine the expression profile of *MrSpz* in normal prawn at transcriptional level, total RNA was extracted from different tissues including gills, heart, hepatopancreas, intestine, stomach, muscle and hemocyte using NucleoSpin® RNA XS isolation Kit (MACHEREY-NAGEL, GmbH & Co. KG, Düren, Germany). The semi-quantitative RT-PCR was performed using SuperScript™ III One-Step RT-PCR System (Invitrogen, Co., Carlsbad, CA, USA) and total RNA (5 ng) from each tissue was used as a PCR template. The conditions of RT-PCR consist of cDNA synthesis at 50°C for 30 min followed by incubation at 94°C for 5 min and 4°C for 5 min. Then, the PCR amplification was performed with 35 cycles of 94°C for 15 sec, 55°C for 30 sec and 68°C for 30 sec and then the PCR reaction was incubated at 68°C for 5 min. For an internal control, the RT-PCR of *M. rosenbergii* β -actin gene was performed. The RT-PCR reaction mixture was demonstrated in Table 9. The primers for both *MrSpz* and β -actin were shown in Table 3. The PCR products were separated using 2% agarose gel electrophoresis and stained with SERVA DNA Stain G (SERVA, Germany), and then observed under the UV light by using Gel Doc™ XR⁺ (Bio-Rad).

Table 9 RT-PCR reaction mixtures for *MrSpz* expression analysis of uninfected *M. rosenbergii*

RT-PCR reaction mixtures	Volume (μl)
Total RNA (1 ng/μl)	5.0
2X Reaction Mix	12.5
10 μM Spz_new3-GSP2	0.5
10 μM N-GSP1	0.5
SuperScript™ III RT/Platinum™ Taq Mix	0.5
PCR-grade water	6.0
Total volume	25.0

3.4.2 Expression analysis of *MrSpz* in hemocyte of *M. rosenbergii* challenged with *Aeromonas caviae*

To prepare *A. caviae* (AH06103), the bacteria was cultured in tryptic soy agar (TSA; Difco, Sparks, MD, USA) overnight at 37°C and a single colonies from TSA were cultured in 4 ml of alkaline peptone water (APW, HiMedia, Marg, Mumbai, India) overnight at 37°C with shaking at 225 rpm. Then the bacterial cultures were centrifuged at 800 X g for 1 min, the bacteria pellet was resuspended in a 2X PBS (Phosphate buffered saline; 135 mM NaCl, 15 mM sodium phosphate and pH 7.2).

For time course analysis of *MrSpz* expression in hemocyte of *A. caviae* challenged prawn, healthy *M. rosenbergii*, about 25 - 30 g each, were divided into two groups; *A. caviae* challenged group and control group (PBS). Each group will have 21 individuals of prawn. In *A. caviae* challenged group, each prawn was injected with 100 µl of *A. caviae* (5×10^2 CFU/µl) suspended in 2X PBS solution into the ventral sinus. In control group, each prawn was injected with 100 µl of 2X PBS. The hemocyte from each group was randomly collected at 0, 3, 6, 12, 24, 36 and 48 hour post injection (hpi). To collect the hemocytes, hemolymph was collected from the ventral sinus and mixed with equal volume of Alsever's Solution and then centrifuged at 800 X g for 10 min followed by cell pellet washing with 1 ml of Alsever's Solution for three times and preserving at -70°C until use. The total RNA from hemocyte samples were extracted using NucleoSpin® RNA XS isolation Kit (MACHEREY-NAGEL, GmbH & Co. KG, Düren, Germany).

The RT-PCR reaction for expression analysis of *MrSpz* in hemocyte of *M. rosenbergii* challenged with *Aeromonas caviae* was performed as described previously in *MrSpz* expression analysis of normal prawn (section 3.4.1).

To confirm *A. caviae* infection in challenged prawn, 10 µl of hemolymph was collected from ventral sinuses and mixed with 90 µl of sterilized 2X PBS and then diluted to 10^3 and 10^4 fold before spreading each dilution onto TSA for bacterial cultivation. After the TSA plate was incubated at 37°C for 18 h, bacterial colonies were tested by PCR using allele specific primer extension technique with a pair of primers ACF and ACR (Payattikul; et al. 2015: 164-171)

3.5 Median lethal dose (LD₅₀) of the *Aeromonas caviae* to juveniles of *M. rosenbergii*

To determine the bacterial doses using in bioassay of *A. caviae* challenge in *MrSpz* knocked down *M. rosenbergii*, the LD₅₀ of the *A. caviae* to juveniles of *M. rosenbergii* was evaluated by the dose required for 50% mortality within 3 days.

Bacteria was cultured in Alkaline peptone water (APW) (HiMedia, Marg, Mumbai, India) at 37°C with shaking at 225 rpm for 2 h until OD₆₀₀ reaches 0.5-0.7 and harvested the bacterial cells by centrifuged at 2000x g for 20 min. The bacterial pellet was washed twice and resuspended with 2X PBS. After that the cell suspensions were adjusted to an OD₆₀₀ equal to 1 which the bacterial cells concentration are 1×10^9 CFU/mL. The bacterial cell suspensions were diluted to 4×10^6 CFU/ μ l and tenfold dilutions to 4×10^5 , 4×10^4 , 4×10^3 , and 4×10^2 and 400 CFU/ μ l. The 15 individual of *M. rosenbergii* body weight of 1.5-2 g were injected with 50 μ l of each concentration to ventral sinus and the final concentration were 2×10^7 , 2×10^6 , 2×10^5 , 2×10^4 , 2×10^3 CFU/prawn respectively. In the control group, prawns were injected with 50 μ l of 2X PBS. The mortality rate of each group were recorded at 0, 1, 2, 3, 4, 5, 6 and 7 day post injection.

3.6 RNA interference assay

3.6.1 Construction of recombinant plasmid pCR[®]-Blunt II TOPO[®] *MrSpz* for *in vitro* transcription

For preparation of double-stranded RNA (dsRNA), the recombinant plasmid pCR[®]-Blunt II-TOPO[®] *MrSpz* was used as DNA template for *in vitro* transcription (Fig. 12). A 499 bp DNA fragment and gene specific primers were designed by online E-RNAi3 program (<http://www.dkfz.de/signaling/e-rnai3/>). A 499 bp of *MrSpz* for dsRNA synthesis was generated by the PCR using *Pfx* DNA polymerase. The reaction mixture of PCR was shown in Table 10. The PCR reaction was incubated at 94°C for 5 min followed by 35 cycles of 94°C for 15 sec, 53°C for 30 sec and 68°C for 45 sec. The PCR products were separated on 2% agarose gel then the expected band was purified from agarose gel by using NucleoSpin[®] Gel and PCR Clean-up (MACHEREY-NAGEL, GmbH & Co. KG, Düren, Germany) and cloned into pCR[®]-Blunt II TOPO[®] (Figure 12). The ligation mixture as shown in Table 11 was incubated at 22°C for 30 min and transformed into *E. coli* TOP10 competent cells for blue-white screening. The white colonies were picked and cultured in LB broth medium with 100 µg/ml ampicillin. The inoculated medium was incubated at 37°C for 16 h with shaking at 250 rpm.

To verify insert orientation, the recombinant plasmid was extracted from overnight bacterial culture using NucleoSpin[®] Plasmid (MACHEREY-NAGEL, GmbH & Co. KG, Düren, Germany). The purified recombinant plasmid was digested with *Bam*HI restriction enzyme (New England Biolabs, Inc., Ipswich, MA, England) (Table 11) then separated on 2% agarose gel electrophoresis stained with Serva DNA Stain G and observed under the UV light.

The *E. coli* TOP10 competent cells containing recombinant plasmid pCR[®]-Blunt II TOPO[®] *MrSpz* of both sense and antisense strand were cultured in 50 ml LB broth with 100 µg/ml ampicillin. The sense and antisense strand recombinant plasmid were extracted from *E. coli* using QIAGEN Plasmid Midi Kit (QIAGEN, Inc., Germantown, MD, USA). To harvest bacterial cells, 50 ml of bacterial cultures were centrifuged at 3,000 x *g* for 15 min at room temperature then cell pellet was resuspended in 4 ml of PM1 buffer by vortexing. For cell lysis, 4 ml of PM2 buffer was added to the resuspended bacterial cells then mixed gently by inverting the tube 10 times and incubated at room temperature for 2 min. To neutralize the cell lysate, 4 ml of PM3 buffer was mixed immediately by inverting the tube 10 times.

Afterwards, all of the suspension was transferred to the filter column in collection tube and centrifuged at $3,000 \times g$ for 2 min at room temperature and then transferred the flow-through to the equilibrated plasmid midi column and let the column empty completely by gravity flow. The plasmid midi column was washed by adding 12 ml of PW Buffer and allow the column empty completely by gravity flow. The plasmid DNA was eluted with 8 ml of PEL buffer into a clean 50 ml centrifuge tube by gravity flow. Next, the plasmid DNA was precipitated by adding 6 ml of isopropanol to eluted DNA from previous step and mixed completely by inverting then centrifuged at $15,000 \times g$ for 30 min at 4°C . After centrifugation, the supernatant was removed and DNA pellet was washed with 5 ml of 75% ethanol then centrifuged at $15,000 \times g$ for 10 min at 4°C and air-dried the DNA pellet for 10 min. The dried DNA pellet was dissolved by adding 2 ml of RNase, DNase-free water then incubated at 60°C in water bath for 5-10 min. The concentration of plasmid DNA was measured by using NANODROP LITE Spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

To prepare the linearized recombinant plasmid of both sense and antisense strand, the purified circular recombinant plasmid were digested with *KpnI* restriction enzyme (New England Biolabs, Inc., Ipswich, MA, England) (Table 12) and purified by using NucleoSpin[®] Gel and PCR Clean-up to use as DNA template in *in vitro* transcription for single-stranded RNA synthesis.

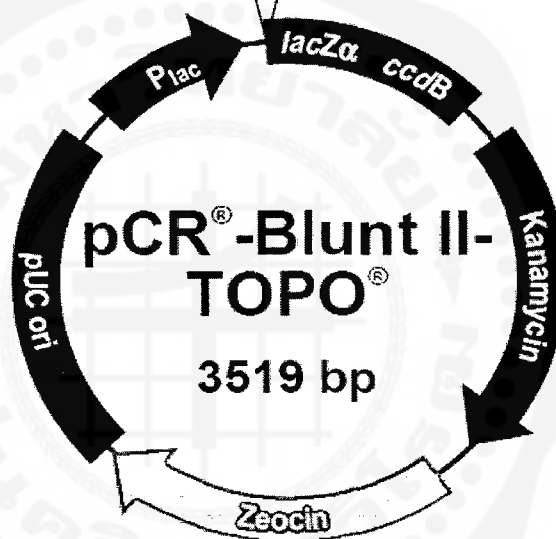
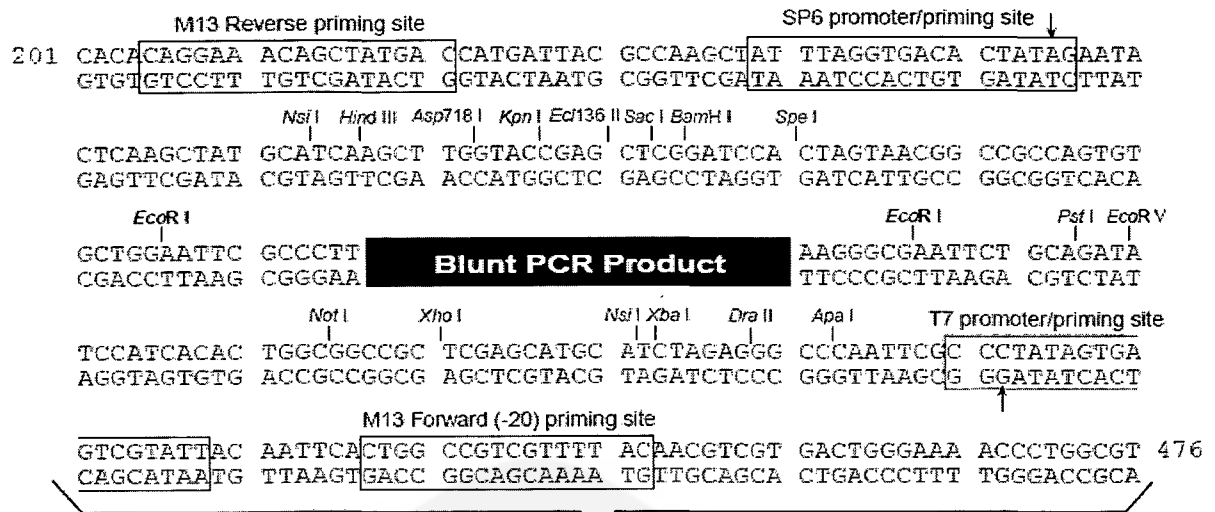


Figure 12 pCR[®]-Blunt II-TOPO[®] vector circle map carries an antibiotic resistant genes and their cloning sites.

Source: https://tools.thermofisher.com/content/sfs/vectors/pcrbluntiiitopo_map.pdf

Table 10 PCR reaction mixtures for recombinant plasmid pCR[®]-Blunt II-TOPO[®] *MrSpz* construction

PCR reaction mixtures	Volume (μl)
cDNA	5
10X <i>Pfx</i> Amplification Buffer	5
50 mM MgSO ₄	1
10 μM Spz_RNAi-F	1.5
10 μM Spz_RNAi-R	1.5
10 mM dNTP mixture	1.5
Platinum™ <i>Pfx</i> DNA Polymerase	1
Distilled water	33.5
Total volume	50

Table 11 Ligation mixtures for recombinant plasmid pCR[®]-Blunt II-TOPO[®] *MrSpz* construction

Ligation mixtures	Volume (μl)
The purified 499 bp DNA fragment (40 fmole)	1
pCR [®] -Blunt II-TOPO [®] (4.3 fmole)	1
Salt Solution	1
Distilled water	3
Total volume	6

Table 12 Digestion mixtures of linear plasmid preparation

Digestion mixtures	Volume (μl)
The purified plasmid pCR [®] -Blunt II-TOPO [®] <i>MrSpz</i> sense/antisense strand (100 μg)	150
<i>KpnI</i> (10 U/ μl)	40
10X NEBuffer 1.1	50
Distilled water	260
Total volume	500

3.6.2 Double-stranded RNA (dsRNA) preparation

To synthesize single-stranded RNA, *in vitro* transcription by T7 RNA polymerase of both sense and antisense strand were performed separately and linearized recombinant plasmid pCR[®]-Blunt II-TOPO[®] *MrSpz* were used as DNA template. The *in vitro* transcription mixtures shown in Table 13 was incubated at 37°C for 5 h to produce dsRNA and then equal volumes of both sense and antisense reaction mixtures were pooled together. The mixture was incubated at 90°C for 6 min followed by 37°C for 1 h to produce dsRNA. Afterwards, the reaction mixture was treated with DNase I, RNase-free (New England Biolabs, Inc., Ipswich, MA, England) at 37°C for 15 min to remove the DNA template. The equal volume of isopropanol and 0.1 volume of 3M sodium acetate (pH 5.2) was added and then chilled at -20°C for 20 min to precipitate dsRNAs. For dsRNA pellets washing, 1 ml of 70% ethanol was added and centrifuge at 12,000 x g at 4°C for 20 min and then removed all of ethanol from dsRNA pellets and air-dried at room temperatures. The dsRNA pellet was dissolved in 100 μl of the RNase-free water and concentrations of dsRNA was measured by using NANODROP LITE spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA) at OD₂₆₀. The dsRNA was stored at -70°C until used.

Table 13 *in vitro* transcription reaction mixtures for single-stranded RNA synthesis of sense and antisense strand

<i>in vitro</i> transcription mixtures	Volume (μ l)	
	Sense strand	Antisense strand
5X buffer	60	60
100 mM DTT	30	30
rNTPs (25 mM each)	25	25
linearized recombinant DNA (50 μ g)	118	147
T7 RNA polymerase (20U/ μ l)	12.5	12.5
DEPC H ₂ O	54.5	25.5
Total volume	300	300

To verify the synthesized dsRNAs, enzymatic testing comprise of DNase I, RNase A and RNase III digestion were performed (Table 14). The digested dsRNA was separated on 2% agarose gel stained with ethidium bromide and observed under the UV light. For a non-related dsRNA used in control group was designed base on the infectious myonecrosis virus (IMNV) genome (a 282 bp fragment of IMNV was obtained by PCR amplification using a pair of primers corresponding to nucleotides 5,789 to 6,070 and viral RNA using as RNA template was extracted from IMNV-infected *P. vannamei*) kindly provided by Dr.Saengchan Senapin (Center of Excellence for Shrimp Molecular Biology and Biotechnology; Centex Shrimp) (Senapin; et al. 2007: 32-38).

Table 14 Enzymatic testing for dsRNA verification based on resistance of dsRNA to degradation by DNase I, RNase A and RNase III

Digestion test	Mixtures	Volume (μ l)	Conditions
DNA digestion	dsRNA (0.2 μ g/ μ l)	5.0	Incubate at 37 °C for 30 min.
	DNase I	0.5	Add 2 μ l of 0.5 M EDTA and
	10X buffer	1.0	incubate at 65 °C for 10 min
	H ₂ O	3.5	to stop reaction.
ssRNA digestion	dsRNA (0.2 μ g/ μ l)	5.0	Incubate at room
	RNase A (0.1 mg/ml)	0.5	temperature for 5 min, then
	20X SSC	1.0	add 2 μ l of 6X loading dye
	H ₂ O	3.5	to stop reaction.
dsRNA digestion	dsRNA (0.2 μ g/ μ l)	5.0	Incubate at 37°C for 30 min.
	RNase III	0.5	Add 1 μ l of 10X EDTA to
	10X buffer	1.0	terminate reaction.
	H ₂ O	3.5	

3.6.3 Knock-down of *MrSpz* gene *in vivo* expression by dsRNA-mediated RNAi

M. rosenbergii body weight ranged from 1.5 to 2 g were obtained from Kanokpon farm at Song Phi Nong district, Suphanburi province, Thailand and acclimated in laboratory for 7 days before dsRNA injections. The prawns were divided into 3 groups. Each group composes of 30 individuals. In dsRNA groups, each prawn was injected with dsRNA-*MrSpz* by intramuscular injection into the third abdominal segment (5 µg/g shrimp) and in the case of non-related dsRNA group, each prawn was injected with dsRNA-IMNV (5 µg/g shrimp). In 2X PBS group, each prawn was injected with 50 µl of 2X PBS. The gill of three individuals from each group were randomly collected at 0, 1, 2, 3, 4, 5, 6 and 7 day post-injection (pi). The total RNA was extracted from gill by using NucleoSpin® RNA XS isolation Kit and analyzed by semiquantitative RT-PCR as indicated in Table 15. The semiquantitative RT-PCR conditions consist of cDNA synthesis at 50 °C for 30 min followed by incubation at 94 °C for 2 min and the PCR amplification conditions were 35 cycles of denaturation at 94 °C for 15 sec, annealing at 55 °C for 30 sec and extension at 68 °C for 30 sec then final extension at 68 °C for 5 min. This experiments were carried out in triplicate.

Table 15 RT-PCR reaction mixtures for gene silencing efficiency

RT-PCR reaction mixtures	Volume (µl)
Total RNA (1 ng/µl)	5.0
2X Reaction Mix	12.5
10 µM Spz_new3-GSP2	0.5
10 µM Spz_exp-R1	0.5
SuperScript™ III RT/Platinum™ Taq Mix	0.5
PCR-grade water	6.0
Total volume	25.0

3.6.4 Bioassay of *A. caviae* and 2X PBS challenge in *MrSpz* knocked down

M. rosenbergii

Total of 120 prawn (1-1.5 g body weight) were divided into 3 groups consisted of dsRNA-*MrSpz* group, dsRNA-IMNV group and 2X PBS group. After acclimatization, 40 prawns were treated with 5 µg of dsRNA-*MrSpz* or dsRNA-IMNV and 2X PBS. After the dsRNA and 2X PBS injection, each group was subdivided into 2 subgroups and challenged with 2X PBS and *A. caviae*, then mortality rate was recorded at 0, 3, 6, 9, 24, 48, 72, 96, 120 and 144 h. This assays were carried out in three replicates. Gill sample from each time point was collected and used for RNA extraction by using NucleoSpin[®] RNA XS isolation Kit. The knock-down efficiency of *MrSpz* in prawn injected with *A. caviae* was determined at the mRNA level by semiquantitative RT-PCR as described previously (3.5.3 Knock-down of *MrSpz* *in vivo* expression by dsRNA-mediated RNAi).

3.7 Bioinformatics tools for *MrSpz* cDNA sequence analysis

The nucleotide sequence of *MrSpz* was analyzed by using the BLAST program (<http://www.ncbi.nlm.nih.gov/BLAST/>) and was translated into the deduced amino acid sequence by Expasy Translate Tool (<http://web.expasy.org/translate/>). Multiple alignments of nucleotide and amino acid sequences were performed by using Clustal Omega (<http://www.ebi.ac.uk/Tools/msa/clustalo/>). The deduced molecular mass and isoelectric point (theoretical pI) of *MrSpz* protein were calculated using Expasy Compute pI/Mw. (http://web.expasy.org/compute_pi/). Identification and analysis of protein domains within *MrSpz* protein sequence were carried out by using Simple Modular Architecture Research Tool SMART (<http://smart.embl-heidelberg.de/>).

3.8 Phylogenetic analysis of MrSpz protein

The multiple alignment of full-length Spätzle amino acid sequence of various species including crustaceans, insects and copepod (Table 16) were analyzed by using clustalw. A Neighbor-joining tree was constructed with 1000 iterations of bootstrap sampling by using MEGA7.0.

Table 16 Spätzle amino acid sequences in phylogenetic analysis

Spätzle protein	Accession no.
<i>Fenneropenaeus chinensis</i> Spätzle	ACD36030.1
<i>Litopenaeus vannamei</i> Spätzle 1	AEK86522.1
<i>Litopenaeus vannamei</i> Spätzle 2	AEK86523.1
<i>Litopenaeus vannamei</i> Spätzle 3	AEK86524.1
<i>Litopenaeus vannamei</i> Spätzle 4	ANJ04742.1
<i>Penaeus monodon</i> Spätzle	AIZ03421.1
<i>Acyrtosiphon pisum</i> Spätzle 1-1 precursor	NP_001153589.1
<i>Artemia sinica</i> Spätzle	ADQ43816.1
<i>Bombyx mori</i> Spätzle 1 precursor	NP_001108066.1
<i>Culex quinquefasciatus</i> Spätzle 1B	XP_001864596.1
<i>Danaus plexippus</i> Spätzle	OWR42582.1
<i>Daphnia magna</i> Spätzle	JAN84466.1
<i>Drosophila melanogaster</i> Spz D	NP_733195.1
<i>Folsomia candida</i> Spätzle	OXA38108.1
<i>Lepeophtheirus salmonis</i> Spätzle 6	CDW20343.1
<i>Plutella xylostella</i> Spätzle	AIW49878.1
<i>Tribolium castaneum</i> Spätzle X2	XP_975083.1

CHAPTER 4

RESULTS

4.1 Isolation of a partial *M. rosenbergii* Spätzle (*MrSpz*) gene

A partial cDNA sequence of *MrSpz* was isolated by using polymerase chain reaction (PCR) with degenerate primers and transcribed cDNA was used as a DNA template. The primary PCR was performed with a pair of primers SpzI-UPF1 and SpzI-R, a DNA fragment of 400 bp and 300 bp were obtained (Fig. 13A). The primary PCR products were diluted and used as a DNA template for nested-PCR, which provided a 350 bp DNA fragment (Fig. 13B). The DNA fragment was purified and cloned into pGEM[®]-T Easy Vector (Promega, Co., Madison, WI, USA) for sequencing.

Analysis of a 350 bp DNA fragment by tblastx revealed that MrSpz protein shared high similarity to those of FcSpz (91% identity), PmSpz (86% similarity), LvSpz3 (87% identity) and MjSpz (81% identity). Based on the obtained partial cDNA sequence, gene specific primers of both 5' and 3' RACE PCR were designed.

4.2 Isolation of full-length *MrSpz* by rapid amplification of cDNA ends (RACE)

4.2.1 The 5' RACE

To isolate the 5' end of *MrSpz* cDNA sequence, touch down PCR was performed by using of gene specific primers, Spz_new3-GSP1 designed based on an obtained partial *MrSpz* cDNA sequence and Universal Primer A Mix (UPM). After agarose gel electrophoresis, a 1,150 bp DNA fragment was obtained (Fig. 14A). The diluted touch down PCR products were used as a DNA template for nested PCR, using Spz_new4-NGSP1 and Nested Universal Primer A (NUP). A DNA fragment of 1,100 bp (Fig. 14B) was obtained and cloned into pCR[®] 2.1-TOPO[®] TA vector (Invitrogen, Co., Carlsbad, CA, USA) for sequencing.

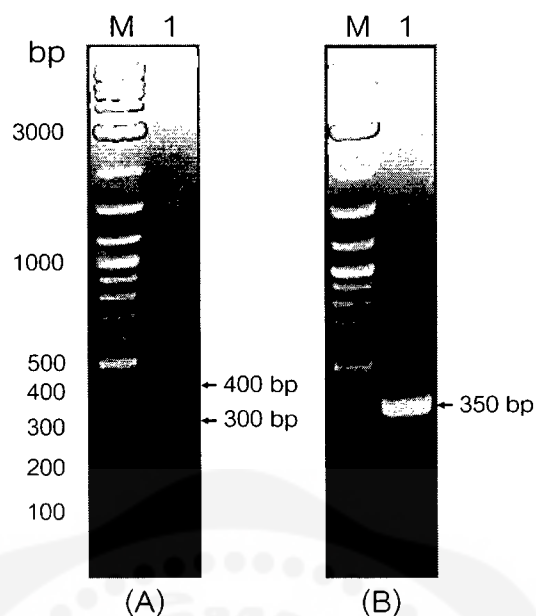


Figure 13 The PCR product of primary PCR (A) and nested PCR (B) by using degenerate primers for a partial *MrSpz* cDNA sequence isolation. Synthesized cDNA was used as template for primary PCR that provided various DNA fragments with the major band of 400 bp and 300 bp (A, lane 1). After the nested PCR, the DNA fragment was 350 bp in length (B, lane 1). Lane M is a 2-Log DNA Ladder (0.1-10 kb).

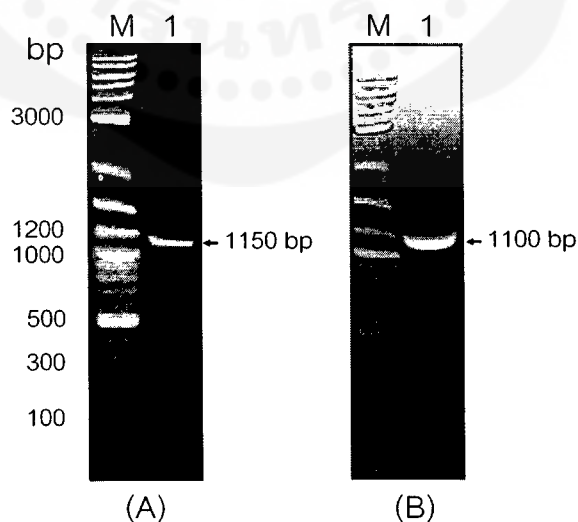


Figure 14 Agarose gel electrophoresis showing 5' RACE touch down PCR products (A, lane 1) and nested PCR products (B, lane 1). Lane M is a 2-Log DNA Ladder (0.1-10 kb).

4.2.1 The 3' RACE

For the 3' end of *MrSpz* cDNA sequence isolation, touch down PCR was performed with using of gene specific primers, Spz_new3-GSP2 and UPM that provided a 950 bp DNA fragment (Fig. 15A) while nested PCR using a pair of primers Spz_new4-NGSP2 and NUP provided a longer PCR products, consisted of 1,400 bp and 1,050 bp (Fig. 15B). However, both of them were cloned and sequenced as well as in 5' RACE.

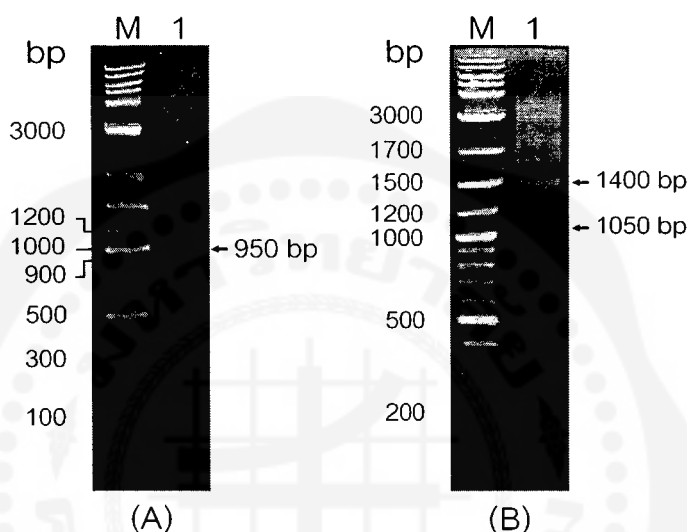


Figure 15 DNA fragment obtained from 3' RACE touch down PCR (A, lane 1) and nested PCR (B, lane 1). Lane M is a 2-Log DNA Ladder (0.1-10 kb).

The obtained cDNA sequence of 5' and 1,050 bp of 3' RACE nested PCR were combined into the full length cDNA sequence by using an overlapping region on a partial *MrSpz* cDNA sequence. A pair of primers Spz-CDS-F and Spz-CDS-R were used to proof the coding sequence of the full length *MrSpz*.

The deduced *MrSpz* was analyzed and the results revealed that *MrSpz* cDNA sequence consisted of 2,253 nucleotides containing a 5' untranslated region (5' UTR) of 825 nucleotides with CAAT signal at positions -312 to -308 (5'-CCAAT-3'). The ATG start codon at position 1 matched 5 out of 7 of the consensus sequence (A/GCCAUGG) initiation codon (Kozak, 1991: 19867-19870). The open reading frame (ORF) consisted of 747 nucleotides encoding 248 amino acid residues. The 3'-UTR consisted of 681 nucleotides with

polyadenylation signal (5'-AATAAA-3') at positions 969 to 974 (Fig. 16). The complete *MrSpz* sequence was deposited into the GenBank with the accession numbers KT809363.

The analysis by using BLASTp revealed that *MrSpz* protein shared 92% identity to *FcSpz* (ACD36030.1); 83% identity to *MjSpz* (AOF79109.1); 79% identity to *LvSpz3* (AEK86524.1); 78% identity to *PmSpz* (AIZ03421.1); 39% identity to *LvSpz1* (AEK86522.1); 28% identity to *LvSpz2* (AEK86523.1) (Fig. 17). Furthermore, *MrSpz* protein also shared slightly similarity to those of spätzle protein of insects and copepod such as *Lepeophtheirus salmonis* Spätzle 2-like protein (ABU41133.1, 40% identity), *Danaus plexippus* (OWR42582.1, 36% identity), *Orchesella cincta* (ODM98902.1, 27% identity), and *Folsomia candida* (OXA38108.1, 26% identity) (Fig. 18) (Table 17).

Table 17 Percent identity between *MrSpz* protein and other Spätzle proteins obtained from BLASTp analysis

Spätzle protein	Accession number	% identity
<i>Fenneropenaeus chinensis</i> Spätzle	ACD36030.1	92
<i>Marsupenaeus japonicus</i> Spätzle	AOF79109.1	83
<i>Litopenaeus vannamei</i> Spätzle 3	AEK86524.1	79
<i>Penaeus monodon</i> Spätzle	AIZ03421.1	78
<i>Litopenaeus vannamei</i> Spätzle 1	AEK86522.1	39
<i>Litopenaeus vannamei</i> Spätzle 2	AEK86523.1	28
<i>Lepeophtheirus salmonis</i> Spätzle 2-like protein	ABU41133.1	40
<i>Danaus plexippus</i> Spätzle	OWR42582.1	36
<i>Orchesella cincta</i> Spätzle	ODM98902.1	27
<i>Folsomia candida</i> Spätzle	OXA38108.1	26

The mature MrSpz protein had a theoretical pI of 5.70 and a molecular mass of 28.3 kDa. Protein domains analysis by InterPro (<http://www.ebi.ac.uk/interpro>) revealed that the mature MrSpz protein contained the C-terminal active Spätzle domain at amino acid positions 143-240 amino acid residues and cysteine-knot domain with nine Cys residues at amino acid positions 85, 94, 145, 187, 194, 200, 205, 237 and 239. The signal peptide located at 1-21 amino acid residues was identified by using SignalP-4.1 analysis (<http://www.cbs.dtu.dk/services/SignalP/>) and the results also showed that it had a cleavage site between positions 21 and 22 with the sequence of Ser Leu Ala – Asp Gln (Fig. 19).

The multiple alignment among shrimp Spätzle protein was also constructed as shown in Figure 20. The result revealed high homology of amino acid residues at C-terminal Spätzle activated domain. Although LvSpz1, LvSpz2 and LvSpz4 are different from other Spätzle, especially LvSpz2 and LvSpz4, seven conserved cysteine residues were observed in all shrimp Spätzle proteins and five conserved cysteine residues were found in LvSpz4 (Fig. 20). In addition, pairwise alignment among the full-length Spätzle protein and C-terminal Spätzle activated domain of shrimps including MrSpz, LvSpz1-4, FcSpz and PmSpz demonstrated that percent identity obtained from C-terminal Spätzle activated domain alignment was higher than that of the full-length Spätzle alignment (Fig. 21).

Figure 16 The cDNA sequence of *MrSpz* and its open reading frame with one-letter codes of the deduced amino acid. The signal peptide was shown in frame. The C-terminal Spätzle activated domain was underlined. The CAAT signal and the polyadenylation signal was double underlined. The cysteine residues of cysteine-knot domain were displayed in circle.

spatzle protein, partial [Fenneropenaeus chinensis]

Sequence ID: [ACD36030.1](#) Length: 166 Number of Matches: 1

A

Range 1: 1 to 166 [GenPept](#) [Graphics](#)[Next Match](#) [Prev. Match](#)

Score	Expect	Method	Identities	Positives	Gaps
324 bits(830)	1e-110	Compositional matrix adjust.	153/166(92%)	159/166(95%)	0/166(0%)
Query 83	PACADNTTKPWCLEDEPEYPSYEIKHAAEYHYEKVLSLYADVADLNTELSVERPNTLEEET	142			
Sbjct 1	PACA NTTKPWCLEDEYP+YEIKHAAEYHYEKVLSLYADV DLNTELSVERPNTLEEET	60			
Query 143	YLCPSETAYVRPLRAQNTEGKWRVIVNNVDVHYETLTQTTRIEECLTGGDACPLVPECYD	202			
Sbjct 61	YLCPSETAYVRPLRAQNTE KWRVIVNN+D HY+TLTQTTRIEECLT GDACPLVP+CY+	120			
Query 203	SKCLQKSIYHRFLVDPYDQYFPFAIETFKLPASCACLLGAFTIDH	248			
Sbjct 121	SKCLQKSIYHRFLV DPYDQYFPFAIETFKLPASCACLLGA+TIDH	166			

spatzle, partial [Marsupenaeus japonicus]

Sequence ID: [AOF79109.1](#) Length: 83 Number of Matches: 1

B

Range 1: 1 to 83 [GenPept](#) [Graphics](#)[Next Match](#) [Prev. Match](#)

Score	Expect	Method	Identities	Positives	Gaps
141 bits(355)	1e-39	Compositional matrix adjust.	69/83(83%)	73/83(87%)	0/83(0%)
Query 82	VPACADNTTKPWCLEDEPEYPSYEIKHAAEYHYEKVLSLYADVADLNTELSVERPNTLEEE	141			
Sbjct 1	VP CA + KPW LED EYPSYEIK AAEYHYEK+LSLYADVADLNTELSVERP L+EE	60			
Query 142	TYLCPSETAYVRPLRAQNTEGKW	164			
Sbjct 61	TYLCPSETAYV PLRAQNT+GKW	83			

Spz3 [Litopenaeus vannamei]

Sequence ID: [AEK86524.1](#) Length: 255 Number of Matches: 1

C

Range 1: 9 to 255 [GenPept](#) [Graphics](#)[Next Match](#) [Prev. Match](#)

Score	Expect	Method	Identities	Positives	Gaps
396 bits(1018)	6e-138	Compositional matrix adjust.	196/248(79%)	221/248(89%)	8/248(3%)
Query 8	VMQVLLAIVGVSLADQSSHVTFSLGGAPAVNNHSS-----SHHHRAPSYQPRPSYHPQP	61			
Sbjct 9	++QV L++ LADQ+SHV+ SLGGAPAV+HHSS S+HH+ P+Y +PSYHPQP	67			
Query 62	SYHPQPSYHPQPKYK-KYEPEVPACADNTTKPWCLEDEPEYPSYEIKHAAEYHYEKVLSLY	120			
Sbjct 68	SYHP P+YHPQP Y+ ++EP VP CA NTK WCLED YP+YEIKHAAEYHYEK+LSLY	127			
Query 121	ADVADLNTELSVERPNTLEEETYLCPSETAYVRPLRAQNTEGKWRVIVNNVDVHYETLTQ	180			
Sbjct 128	ADVADLNTELSV+RPNTLEEETYLCPSETAYVRPLRAQNTEGKWRV+VNN+D HY+TLTQ	187			
Query 181	TTRIEECLTGGDACPLVPECYDSKCLQKSIYHRFLVDPYDQYFPFAIETFKLPASCACL	240			
Sbjct 188	TTRIEECLSSADACPLVPECYESKCLQKSVYHRFLVDPYDQYFPFAIETFKLPASCACL	247			
Query 241	LGAFTIDH	248			
Sbjct 248	LGA+TIDH	255			

Figure 17 The deduced amino acid sequence of MrSpz shared high similarity to Spätzle protein of *F. chinensis* (ACD36030.1) (A), *M. japonicus* (AOF79109.1) (B) and Spätzle 3 of *P. vannamei* (AEK86524.1) (C).

Sptzle 2-like protein, partial [*Lepeophtheirus salmonis*]
Sequence ID: [ABU41133.1](#) Length: 201 Number of Matches: 1

A

Range 1: 20 to 197 [GenPept](#) [Graphics](#)

Score	Expect	Method	Identities	Positives	Gaps
121 bits(303)	2e-30	Compositional matrix adjust.	74/185(40%)	97/185(52%)	35/185(18%)
Query 83	PACADNTTKPWCL	EDPEYPSYEIKHA--AEYHYEKVLSLYADVADLNTELSVERPNTLEE	140		
Sbjct 20	PACA++T +CLED	YP YE+K A A+Y + K Y+D+ D + V+ +E	76		
Query 141	ET-----	-----YLCPSETAYVRPLRAQNTGKWRVIVNNVDVH	174		
Sbjct 77	E	YLCPS+ Y P RA+N G+WRVIVN+V H	134		
Query 175	YETLTQTTRIEECL	TGGDACPLVPECYDSKCLQKSIYHRFLVDPYDQYFPFAIETFKLP	234		
Sbjct 135	Y TQTR E CL	AC L+ CY SKC QK +Y R + YDP D Y I+ +K P	192		
Query 235	ASCAC	239			
Sbjct 193	++C+C	SACSC 197			

spaetzle [*Danaus plexippus plexippus*]

Sequence ID: [OWR42582.1](#) Length: 885 Number of Matches: 1

B

Range 1: 465 to 563 [GenPept](#) [Graphics](#)

Score	Expect	Method	Identities	Positives	Gaps
79.3 bits(194)	3e-13	Compositional matrix adjust.	38/107(36%)	61/107(57%)	8/107(7%)
Query 140	EETYLCPSETAYVRPLRAQNTGKWRVIVNNVDVHYETLTQTTRIEECLTGGDACPLVPE	199			
Sbjct 465	E ++CPS Y RP RA+ T G+W+ IVN +	TQT R+E+CL ++C + +	519		
Query 200	CYDSKCLQKSIYHRFLVDPYDQYFPFAIETFKLPASCACLLGAFTI	246			
Sbjct 520	+ SKC+Q YHR L +DQ ++ FK+P C+C + +++	NFKSKCVQVYNYHRLLT---WDQQNGLHMDIFKVPTCCSCHIEGYSV	563		

Protein spaetzle, partial [*Orchesella cincta*]

Sequence ID: [ODM98902.1](#) Length: 299 Number of Matches: 1

C

Range 1: 134 to 298 [GenPept](#) [Graphics](#)

Score	Expect	Method	Identities	Positives	Gaps
86.7 bits(213)	2e-16	Compositional matrix adjust.	47/174(27%)	88/174(50%)	13/174(7%)
Query 77	KYEPEVPACADNTTKPWCL	EDPEYPSYEIKHAAEYHYEKVLSLYADVADLNTELSVERPN	136		
Sbjct 134	KY+P D+ +CL D EYP ++ + A+++ ++ LY ++ + + N	KYKPTCAGSGDS---YCLYDQEYPMKVSNIQAQHYQNQLQPLYEEMNHYSLSDYIISHDN	189		
Query 137	TLE----EETYLCPSETAYVRPLRAQNTGKWRVIVNNVDVHYETLTQTTRIEECLTGGD	192			
Sbjct 190	+ ++C S+ +YVRP A+N +G+W ++N + Q+ RIE C	ATHRYRHKGHFICESDVSIVRPGWAKNWKGEWVAVLNT-----DVFPQSVRIETCKYPKK	244		
Query 193	ACPLVPECYDSKCLQKSIYHRFLVDPYDQYFPFAIETFKLPASCACLLGAFTI	246			
Sbjct 245	C +P CY S C+Q+ Y + + DPY+ + F++P++C+C + +F I	KCEYMPPCYKSYCVQRYTYVKLICLDPYNPGKRPTTDVFEIPSACSCFVESFPI	298		

Figure 18 MrSpz shared slightly similarity to Spätzle protein of insects and copepod include *L. salmonis* Spätzle 2-like protein (ABU41133.1), *D. plexippus* (OWR42582.1) and *O. cincta* (ODM98902.1).

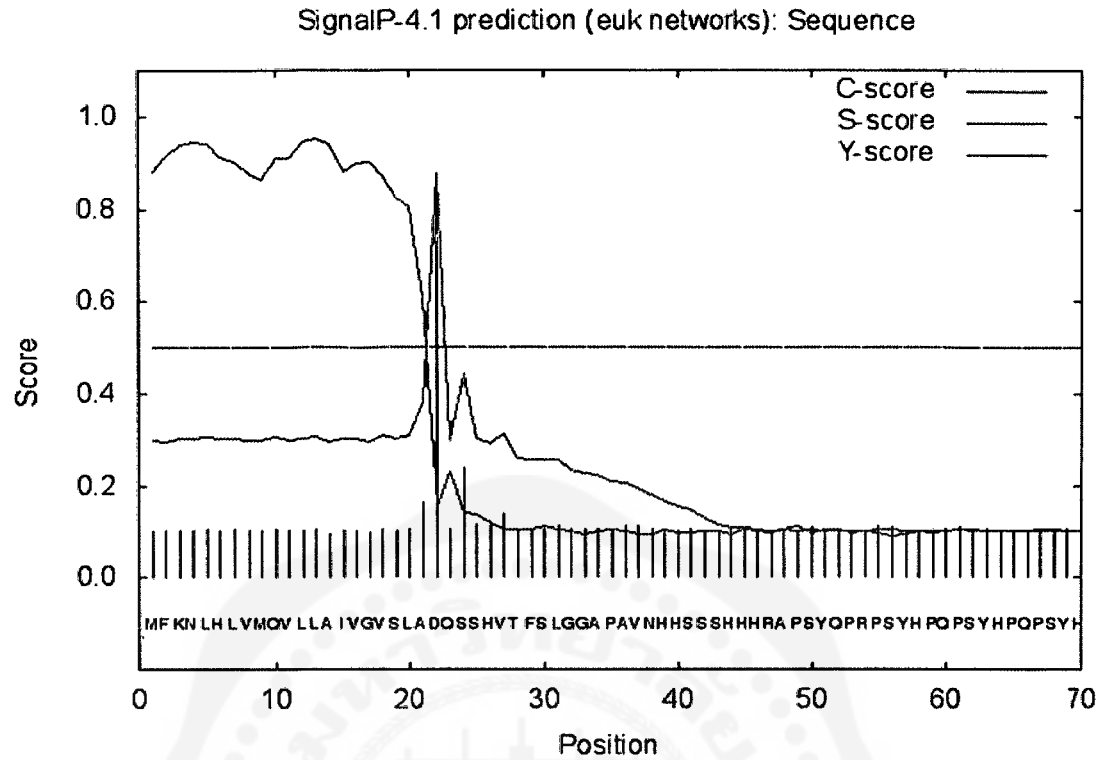


Figure 19 The output from SignalP-4.1 prediction showing a cleavage site of signal peptide on MrSpz protein. Three output scores for each position in MrSpz sequence; C-score (The output from the CS networks), S-score (The output from the SP networks) and Y-score (combined cleavage site of C-score and the slope of the S-score) were produced.

```

PTYEIKHAAEYHYEKL LSLYADVAD---LNTE-----LS-----
PTYEIKHAAEYHYEKL LSLYADVAD---LNTE-----LS-----
*   .:   .       .       *

```

YLPPRQNYSSASKPGYAPPPSPSKYGPPTSNYDHPQTSSYLYDPPMPTYD
-----D-----
-----VTKE-----

Multiple sequence alignment of shrimp Spätzle protein. Amino a

Figure 20 Multiple sequence alignment of shrimp Spätzle protein. Amino acid sequence of LvSpz1 (accession no. AEK86522.1), LvSpz2 (accession no. AEK86523.1), LvSpz3 (accession no. AEK86524.1), LvSpz4 (ANJ04742.1), PmSpz (accession no. AI203421.1), FcSpz (accession no. ACD36030.1) and MrSpz (highlighted with yellow) were analyzed by Clustal Omega (<https://www.ebi.ac.uk/Tools/msa/clustalo/>). The identical residues are indicated by asterisk (*). The highly similar residues are indicated by dot (. :).

LvSpz4	PKSPPGSYLPPRIMRSSLRQRROASSM	DLCPYESEFIMPRVGLNNKGDWMFLVNLNEVS	235
LvSpz2	-----NRTGGRHGGH	FVCESAVQYVRPGWAQNIRGEWVAVINTDK--	179
LvSpz1	-----YTGASTGDSFYDATHWGGPEG	YICPSEVVYAMPKRAQNVEGKWRVIVNDVHY-	171
MrSpz	-----VERPNTLEEET	YLCPSETAYVRPLRAQNTGKWRVIVNNVDVH	174
PmSpz	-----VDRPNTLEEET	YLCPSETAYIRPLRVQNTGKWRVVVNNIDVH	172
LvSpz3	-----VDRPNTLEEET	YLCPSETAYVRPLRAQNTGKWRVVVNNIDAH	181
FcSpz	-----VERPNTLDEET	YLCPSETAYVRPLRAQNTGKWRVIVNNIDAH	181
		: * : * * . * . : : * .	
LvSpz4	-SEYTLVESKKGLTNTCTGACNVPDGFSTVC	QQQYVQKRLIALDASG--MALSTDTFWF	292
LvSpz2	---YQSVRVESCKYKNK-RCEFLPPGYSKCVQRYSYVKLLSVD	PYRPEYKPTVDVFEV	235
LvSpz1	---YTQTARLETGLFPEA-ACRALAPCYQSHCTQKSVYHRLLSYDPCDPYKGLFIDYKL		227
MrSpz	YETLTQTTRIEECLTGGD-ACPLVPECYDSKCLQKSIYHRFLVYDPYDQYFPFAIETFKL		233
PmSpz	YQTLTQSTRIEECLTSGD-ACPLVPDQYESKCLQKSIYHRFLVYDSYDQYFPFAIETFKL		231
LvSpz3	YQTLTQSTRIEECLSSAD-ACPLVPECYESKCLQKSVYHRFLVYDPYDQYFPFAIETFKL		240
FcSpz	YQTLTQSTRIEECLTSAD-ACPLVPECYESKCLQKSIYHRFLVYDPYDQYFPFAIETFKL		240
	* . . : *	. : : * * * : : : *	: : .
LvSpz4	PSCCVCKLNPIGG--		305
LvSpz2	PSACSCFVEDFIYY-		249
LvSpz1	PSACSCHLPA----		237
MrSpz	PASCACLLGAFTIDH		248
PmSpz	PASCACLLGAYTIDH		246
LvSpz3	PASCACLLGAYTIDH		255
FcSpz	PASCACLLGAYTIDH		255
	* : . * * :		

Figure 20 (continued) multiple sequence alignment of shrimp Spätzle protein. The C-terminal Spätzle activated domain was boxed. The conserved cysteine residues were colored grey.

	MrSpz	LvSpz1	LvSpz2	LvSpz3	LvSpz4	FcSpz	PmSpz	
MrSpz		38.62%	27.69%	78.03%	22.92%	78.82%	76.28%	Identity among full-length spätzle protein
LvSpz1	53.77%		26.08%	39.29%	22.40%	37.94%	38.62%	
LvSpz2	38.31%	39.21%		26.61%	20.75%	26.23%	27.09%	
LvSpz3	90.56%	52.83%	37.38%		21.51%	96.47%	90.19%	
LvSpz4	29.35%	31.06%	22.42%	27.52%		21.83%	22.53%	
FcSpz	93.39%	52.83%	37.38%	97.16%	27.52%		89.41%	
PmSpz	89.62%	50.00%	35.51%	91.50%	31.81%	92.45%		
Identity among C-terminal spätzle activated domain								

Figure 21 Amino acid pairwise alignment of the full-length Spätzle protein and C-terminal Spätzle activated domain of shrimp

4.3 Phylogenetic analysis of Spätzle protein

To investigate the relationship among Spätzle protein from various species, phylogenetic tree of Spätzle protein of shrimp, insect and copepod was constructed with 1000 iterations of bootstrap sampling. The result showed that the phylogenetic tree could be divided into two major groups and group 1 could be further divided into 2 subgroups. MrSpz protein belonged to the subgroup 1 that contained Spätzle proteins of shrimp, copepod and insects including FcSpz (accession no. ACD36030.1), LvSpz3 (accession no. AEK86524.1), PmSpz (accession no. AIZ03421.1), LvSpz1 (accession no. AEK86522.1), *Lepeophtheirus salmonis* Spätzle 6 (accession no. CDW20343.1), *Danaus plexippus* Spz (accession no. OWR42582.1), LvSpz2 (accession no. AEK86523.1) and *Folsomia candida* (accession no. OXA38108.1). Subgroup 2 contained Spätzle proteins of crustaceans and insects, the members were LvSpz4 (accession no. ANJ04742.1), *Daphnia magna* Spätzle (accession no. JAN84466.1), *Artemia sinica* spätzle (accession no. ADQ43816.1) and

Plutella xylostella spätzle (accession no. AIW49878.1). Group 2 contained Spätzle proteins of insects including *Tribolium castaneum* Spätzle X2 (accession no. XP_975083.1), *Culex quinquefasciatus* Spätzle 1B (accession no. XP_001864596.1), *D. melanogaster* Spz D (accession no. NP_733195.1), *Acyrtosiphon pisum* Spätzle 1-1 precursor (accession no. NP_001153589.1) and *Bombyx mori* Spätzle 1 precursor (accession no. NP_001108066.1) (Fig. 22).

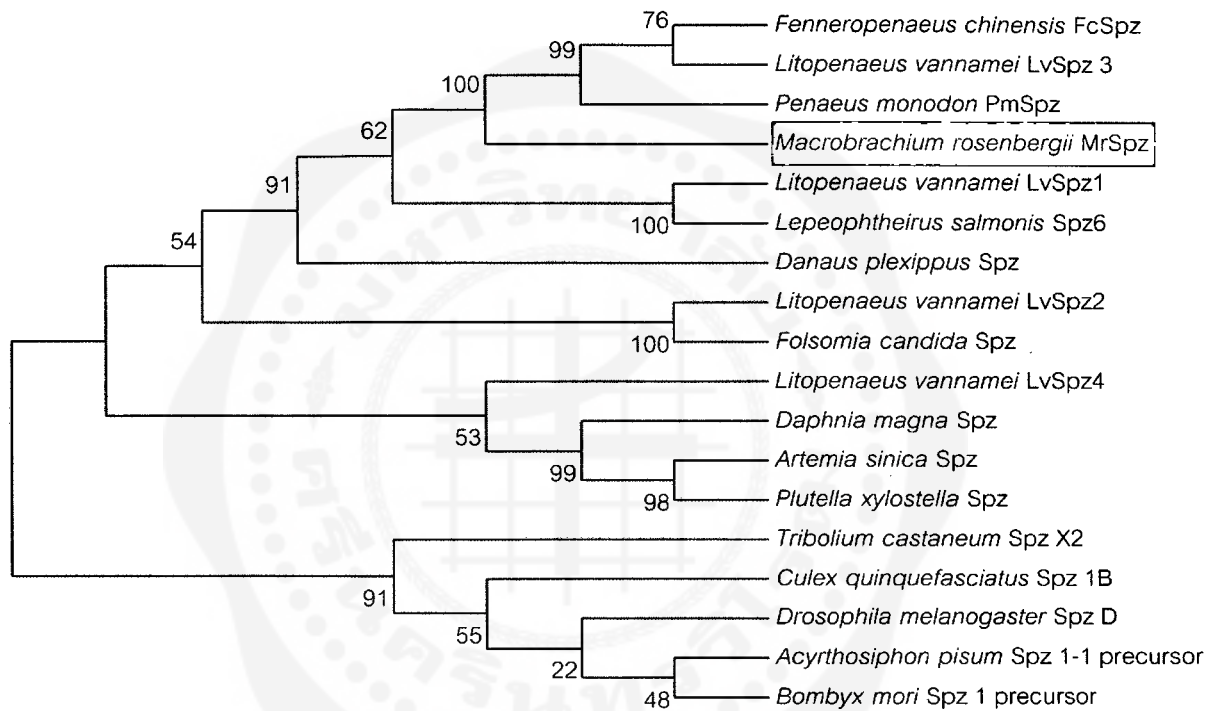


Figure 22 Neighbor-joining tree of the Spätzle amino acid sequences with 1,000 bootstrap iterations generated by MEGA 7.0 software. MrSpz was highlighted in yellow.

4.4 Expression profile analysis of *MrSpz* of uninfected prawn by semi-quantitative RT-PCR

The transcriptional expression of *MrSpz* gene from various tissues of uninfected prawn including gill, heart, hepatopancreas, hemocyte, muscle and stomach were investigated. Total RNAs of 5 ng were used as RNA template in semi-quantitative RT-PCR assay using SuperScript™ III One-Step RT-PCR System (Invitrogen) with the primer Spz-GSP2 and Spz-NGSP1 that generated a 335 bp DNA fragment. For an internal control, the RT-PCR of *M. rosenbergii* β -actin gene was performed with the primers β -actin-F and β -actin-R which provided a 337 bp DNA fragment. The results showed that the expression of *MrSpz* gene in transcriptional level was strongly expressed in gill and weakly expressed in heart and hepatopancreas while there was no expression observed in hemocytes, muscle and stomach (Fig. 23).

4.5 Expression analysis of *MrSpz* in *M. rosenbergii* challenged with *A. caviae*

Time course analysis of *MrSpz* expression in hemocytes of *A. caviae* challenged prawn was performed by semi-quantitative RT-PCR. The result showed that the expression level of *MrSpz* was not observed at 0 and 3 hpi, then significantly increased from 6 to 24 hpi and no expression was observed at 36 and 48 h post injection (Student's t-test, $P < 0.05$) (Fig. 24B). In the control group, there is no *MrSpz* expression at any time point of investigation (Fig. 24A). In addition, bacterial count was performed and *A. caviae* colony was demonstrated at 0, 3, 6, 12 and 24 hpi with the bacterial counts of 0, 2.2×10^3 , 8×10^3 , 2×10^3 and 3×10^4 CFU/ml, respectively while *A. caviae* colony counts at 36 and 48 hpi were only 10 CFU/ml. The *A. caviae* colonies were randomly collected and confirmed by allele specific PCR analysis (Fig. 25).

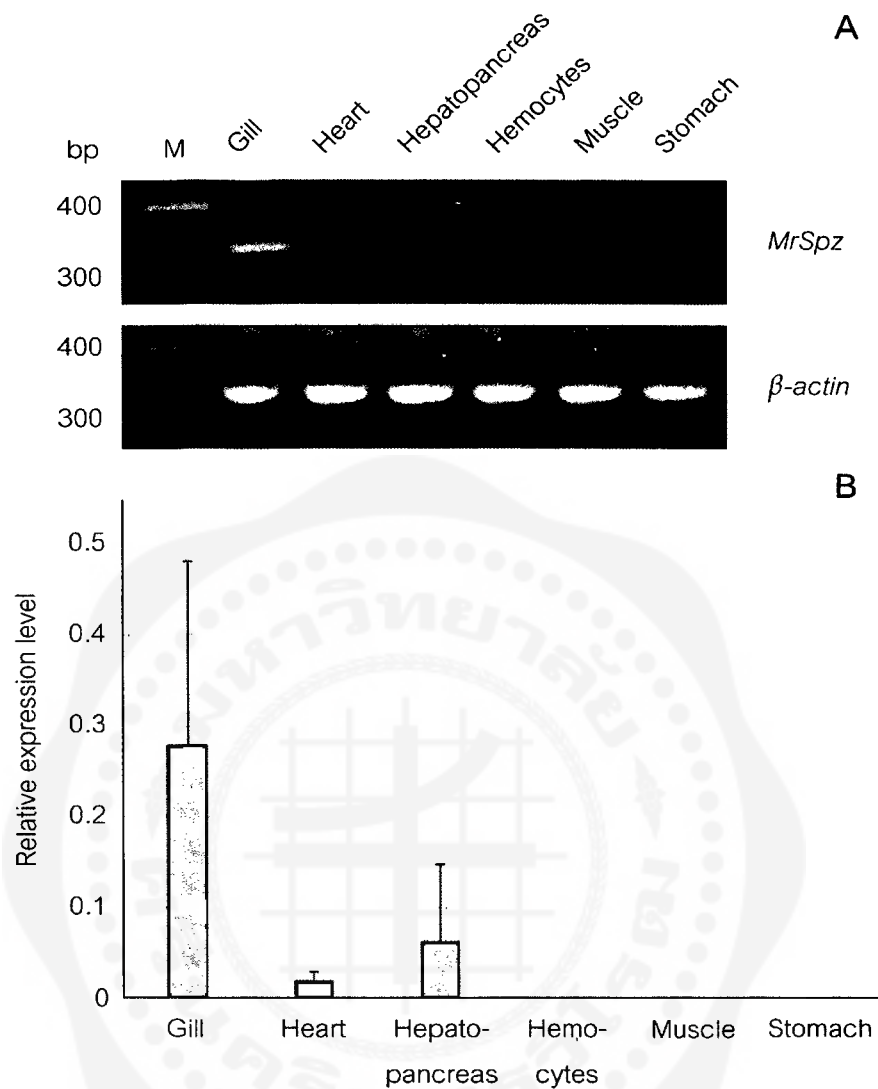


Figure 23 Expression profile of *MrSpz* in different tissues analyzed by semi-quantitative RT-PCR. In uninfected prawn, *MrSpz* was strongly expressed in gill, slightly expressed in heart and hepatopancreas and undetectable in hemocytes, muscle and stomach.

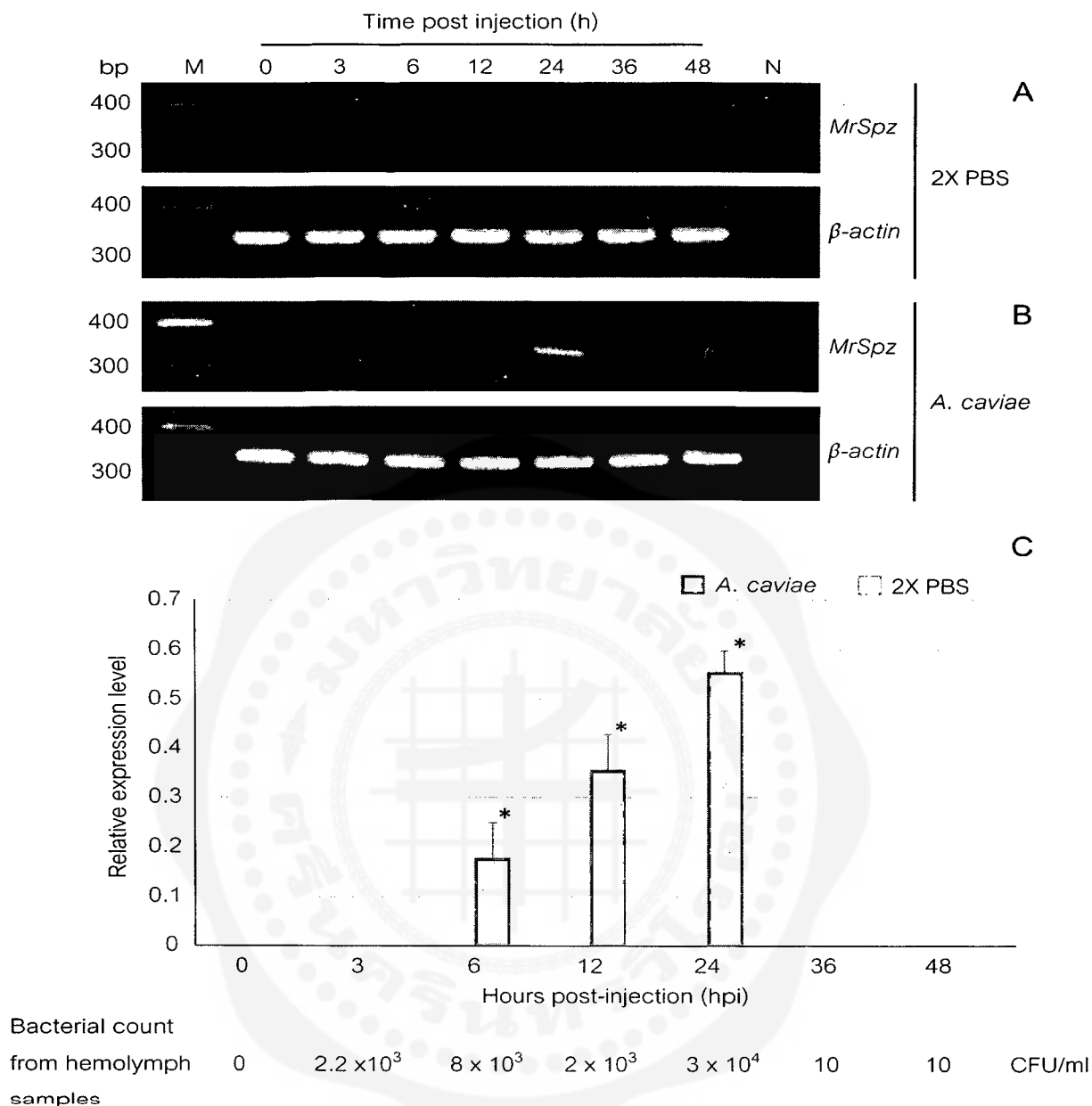


Figure 24 Time course analysis of *MrSpz* expression in hemocytes by using semi-quantitative RT-PCR assay. Prawn were injected with 2X PBS (A) and *A. caviae* (B), then hemolymph samples from each time point were collected for RNA extraction. In *A. caviae* injected prawn, *MrSpz* increased gradually from 6 to 24 hpi while no *MrSpz* expression was detected in a control group. The *MrSpz* relative expression (C) represent the mean $\pm$ S.D. of three independent PCR amplifications and asterisks indicate the significant differences between *MrSpz* expression level of *A. caviae* challenged group and 2X PBS group ($P < 0.05$).

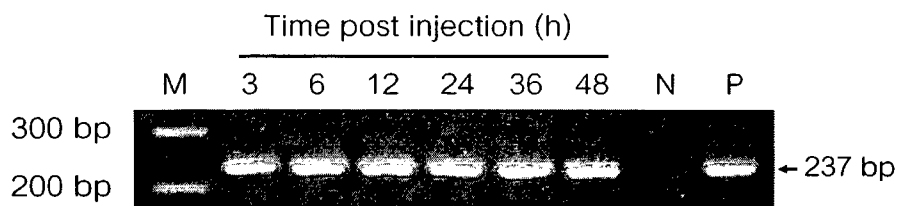


Figure 25 A representative gel of colony PCR for *A. caviae* identification. Various colonies in TSA plate at different time points were randomly collected. A positive result was indicated by DNA fragment of 237 bp. In a positive control, PCR was performed using *A. caviae* genomic DNA as a template.

4.6 Median lethal dose (LD₅₀) of the *A. caviae* to juveniles of *M. rosenbergii*

The LD₅₀ of the *A. caviae* to *M. rosenbergii* juvenile body weight about 1.5-2 g was evaluated. The bacterial dose required for 50% cumulative mortality within 3 days was determined as LD₅₀. After prawns were injected with 50 µl of *A. caviae* inoculum with the concentrates of 2×10^7 , 2×10^6 , 2×10^5 , 2×10^4 and 2×10^3 CFU, the mortality rate of each group were recorded at 0, 1, 2, 3, 4, 5, 6 and 7 day post injection (Table 18). The result demonstrated that 50% cumulative mortality occurred at day 3 in prawn injected with the bacterial dose of 2×10^6 CFU/prawn and no mortality was observed in 2X PBS group.

Table 18 Percent cumulative mortality of prawn injected with *A. caviae* and 2X PBS

Experiments		% Cumulative mortality							
		Days							
		0	1	2	3	4	5	6	7
<i>A. caviae</i> (CFU/prawn)	2×10^3	0	0	0	0	0	6.67	13.33	20.00
	2×10^4	0	6.67	6.67	6.67	6.67	13.33	26.67	26.67
	2×10^5	13.33	20.00	20.00	20.00	20.00	26.67	33.33	33.33
	2×10^6	13.33	20.00	40.00	60.00	60.00	66.67	73.33	73.33
	2×10^7	73.33	100.00	100.00	100.00	100.00	100.00	100.00	100.00
2X PBS		0	0	0	0	0	0	0	0

4.7 Construction of recombinant plasmid pCR[®]-Blunt II TOPO[®] *MrSpz* for *in vitro* transcription

To construct recombinant plasmid *MrSpz*- pCR[®]-Blunt II TOPO[®] for *in vitro* transcription, a 499 bp fragment was designed by using online program E-RNAi3 (<http://www.dkfz.de/signaling/e-rnai3/>). The expected DNA fragment was amplified by using PCR with *Pfx* DNA polymerase, primers Spz-RNAi-F and Spz-RNAi-R. The PCR products were gel purified and cloned into pCR[®] -Blunt II TOPO[®]. Insert orientation was verified by *Bam*HI restriction enzyme that provided a 382 bp and 201 bp DNA fragment for plus and minus insert orientation, respectively. The result showed that plus strand insertion was detected in recombinant plasmid extracted from bacterial culture no. 5, 7 and 12. For minus strand insertion, it was detected in bacterial culture no. 1, 2, 4, 6, 8, 9, 10 and 11. However, there was no DNA insertion observed in bacteria culture no. 3, 13 and 14 (Fig. 26). To prepare linearized recombinant *MrSpz*-pCR[®]-Blunt II TOPO[®] both plus and minus strand, circular recombinant plasmids were digested with *Kpn*I restriction enzyme and purified by using NucleoSpin[®] Gel and PCR Clean-up (MACHEREY-NAGEL, GmbH & Co. KG, Düren, Germany) (Fig. 27).

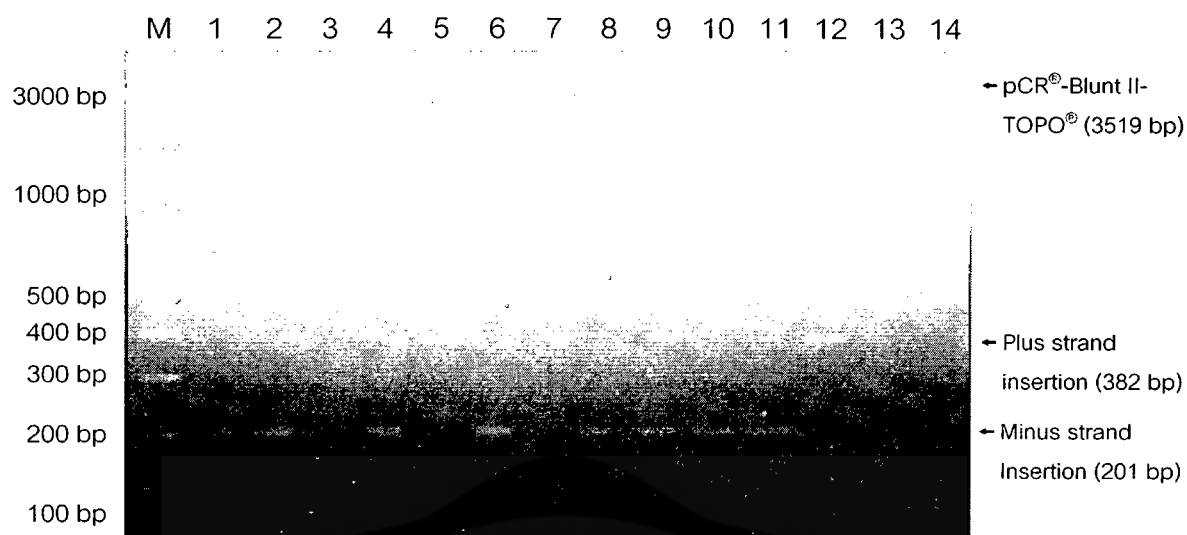


Figure 26 DNA insert orientation analysis by using *Bam*HI restriction enzyme. The presence of a 382 bp DNA fragment indicates the plus strand insertion (lanes 5, 7 and 12) and a 201 bp denotes the minus strand insertion (lanes 1, 2, 4, 6, 8, 9, 10 and 11).

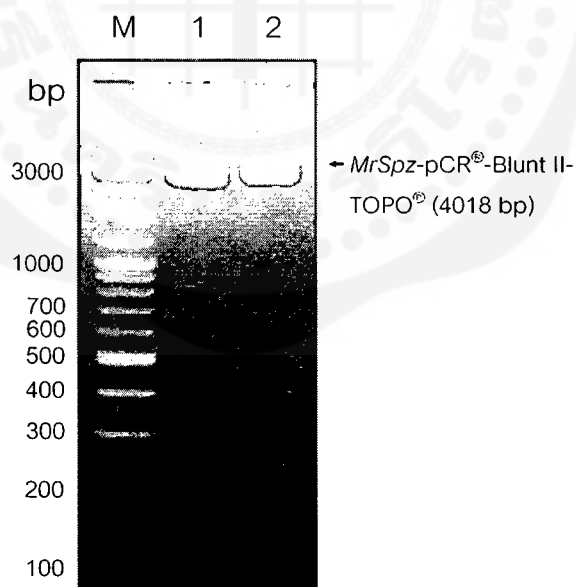


Figure 27 Linearized recombinant *MrSpz*-pCR®-Blunt II TOPO® of plus strand (lane 1) and minus strand (Lane 2) prepared by using *Kpn*I digestion.

4.8 Double-stranded RNA (dsRNA) preparation

To prepare double-stranded RNA, plus and minus linearized recombinant *MrSpz*-pCR®-Blunt II TOPO® both plus and minus strands were transcribed into single-stranded RNA separately by using T7 RNA polymerase (Promega, Co., Madison, WI, USA). After that, equal volumes of each reaction mixture was pooled together to hybridize each single-stranded RNA into double-stranded RNA. The synthesized dsRNA was verified by enzymatic digestion using DNase I, RNase A and RNase III (Fig. 28).

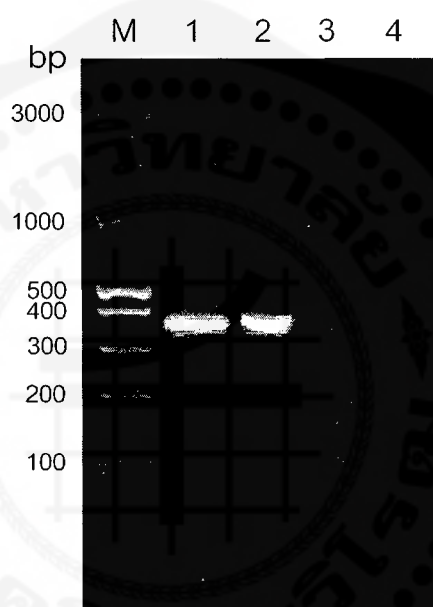


Figure 28 dsRNA-*MrSpz* synthesis and their enzymatic digestion. The synthesized dsRNA-*MrSpz* was demonstrated in lane 1. Lane 2, dsRNA-*MrSpz* digested with DNase I. Lane 3 dsRNA-*MrSpz* digested with RNase A. Lane 4, dsRNA-*MrSpz* digested with RNase III. Lane M, 2 Log DNA Ladder.

4.9 Knock-down *MrSpz* *in vivo* expression by dsRNA-mediated RNA interference

To study the role of *MrSpz* against bacterial invasion, the expression of *MrSpz* in translational level was obstructed by dsRNA-mediated RNA interference. After prawn were injected with dsRNA designed specific to *MrSpz*, the expression of *MrSpz* was analyzed by using RT-PCR. The result showed that *MrSpz* mRNA expression was completely knocked down from days 5 to 7 post-injection (Fig. 29A). In the control group, IMNV-specific dsRNA and 2X PBS injection had no effect to the expression of *MrSpz* (Fig. 29B and 29C). The expression of β -actin was used as the internal control and it was not affected by 2X PBS, dsRNA-*MrSpz* and dsRNA-IMNV injection.

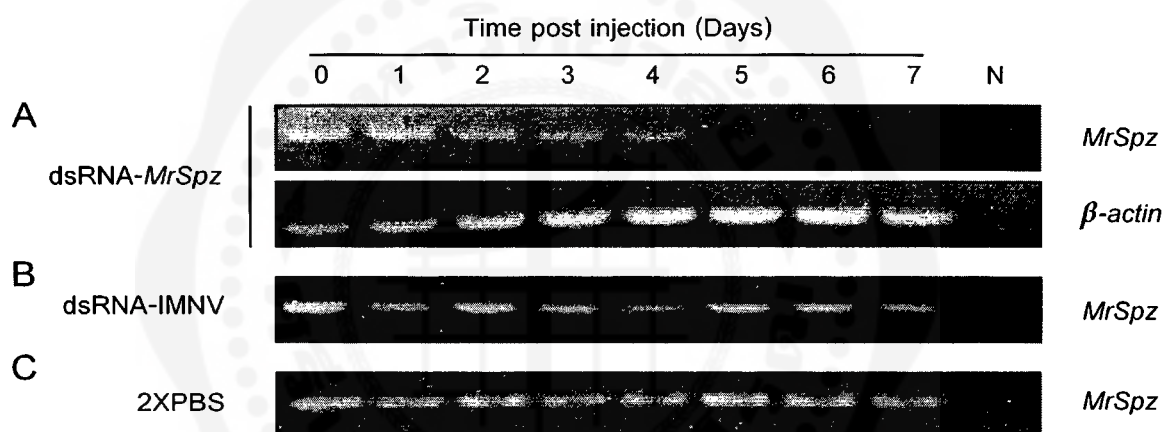


Figure 29 Efficiency of dsRNA-*MrSpz* for *in vivo* knock-down of *MrSpz* expression. Prawn were divided into three groups and injected with 5 μ g/ g shrimp of dsRNA-*MrSpz*, dsRNA-IMNV and 50 μ l of 2X PBS, then the expression of *MrSpz* was monitored in gill for 7 days by using RT-PCR. In the dsRNA-*MrSpz* injection group, the *MrSpz* mRNA expression was knocked down from days 5 to 7 post injection (A) while in the dsRNA-IMNV and 2X PBS groups, the *MrSpz* was expressed normally (B and C).

4.10 Mortality rate of prawn challenged with *A. caviae* increased by *MrSpz* suppression

To assure the role of *MrSpz* of freshwater prawn in innate immunity against certain pathogen invasion, the *in vivo* mRNA expression of *MrSpz* was knocked down by dsRNA mediated RNA interference. At day 5 post dsRNA injection, in which *MrSpz* expression was completely knocked down, prawn were challenged with *A. caviae* with the dose of LD₅₀.

In the control group, three subgroups including dsRNA-IMNV, dsRNA-*MrSpz* and 2X PBS groups were injected with 2X PBS and cumulative mortality data of those groups were referenced as a basal cumulative mortality. The cumulative mortality of the control group at the last time point were 20%, 13.33% and 13.33% for dsRNA-IMNV, dsRNA-*MrSpz* and 2X PBS respectively (Fig. 30A). It was observed that most of the dead prawn were in the molting stage leading to cannibalism among shrimp.

In bacteria challenged group, three subgroups were challenged with *A. caviae*. In dsRNA-*MrSpz* group, cumulative mortality began to increase higher than that of the control groups (dsRNA-IMNV and 2X PBS) at 6 hpi and rapidly increased more than dsRNA-IMNV and 2X PBS at 24 hpi. Final cumulative mortality of dsRNA-*MrSpz*, dsRNA-IMNV and 2X PBS were 96.67%, 50.00% and 46.67% respectively (Fig. 30B). High mortality rates of *MrSpz* knocked down prawn challenged with *A. caviae* indicates that the *MrSpz* play an important role in protection against the invasion of gram-negative bacteria.

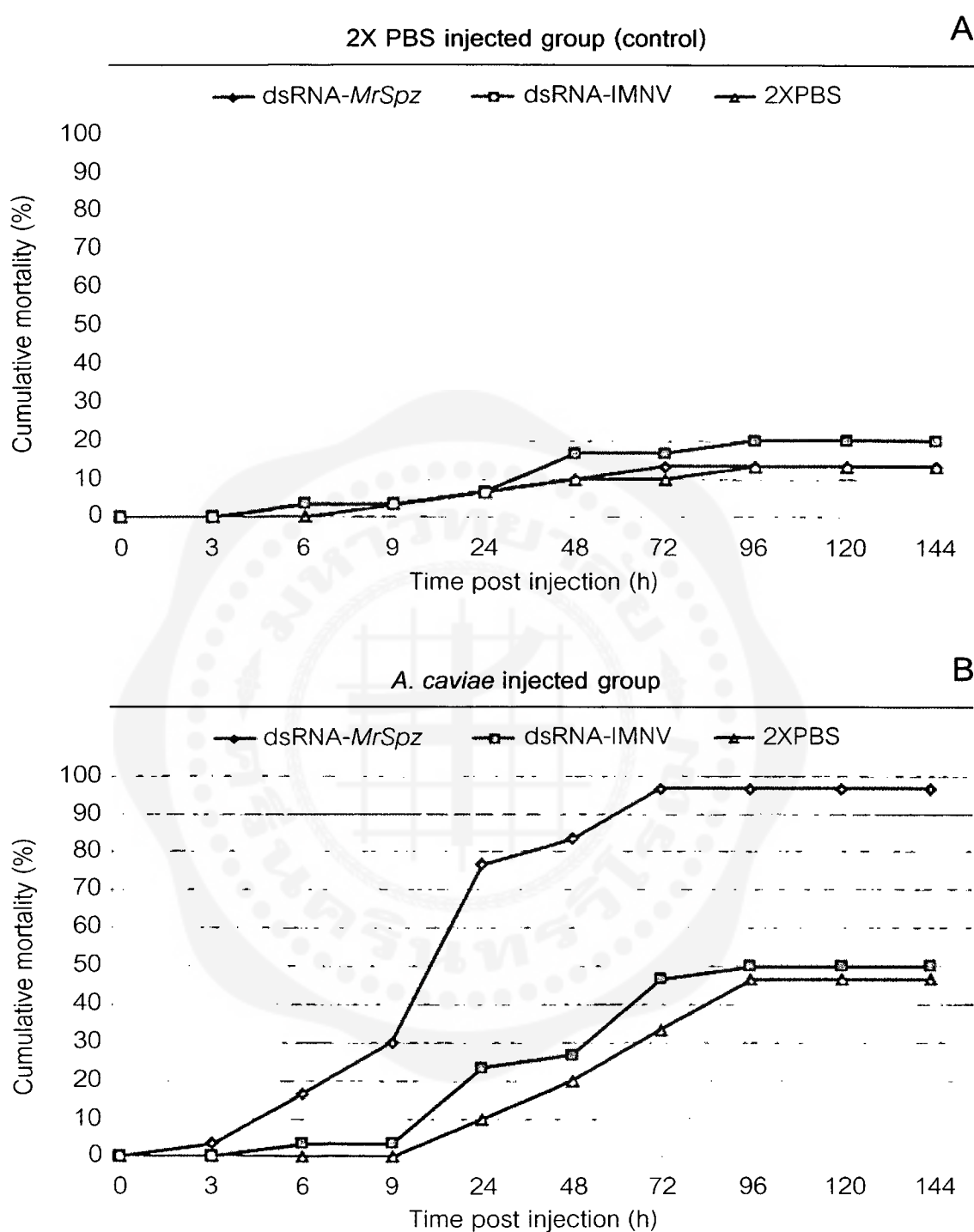


Figure 30 Cumulative mortality of *A. caviae* injected prawn increased after MrSpz gene silencing. At day 5 of the initial injection, the experimental groups were injected with 2X PBS (A) and *A. caviae* (B). The cumulative mortality of each subgroup (dsRNA-MrSpz, dsRNA-IMNV and 2X PBS, $n = 15$) was recorded at 0, 3, 6, 9, 24, 48, 72, 96, 120 and 144 hours post injection.

To identify the bacterial species isolated from the hemolymph of *MrSpz* knocked down prawn challenged with *A. caviae*, the colony PCR was performed. Bacterial colonies in TSA plate were randomly collected and mixed with the PCR reaction mixtures for using as DNA template. PCR products were analysed by gel electrophoresis, the results showed that all sampling colonies were *A. caviae* (Fig. 31).

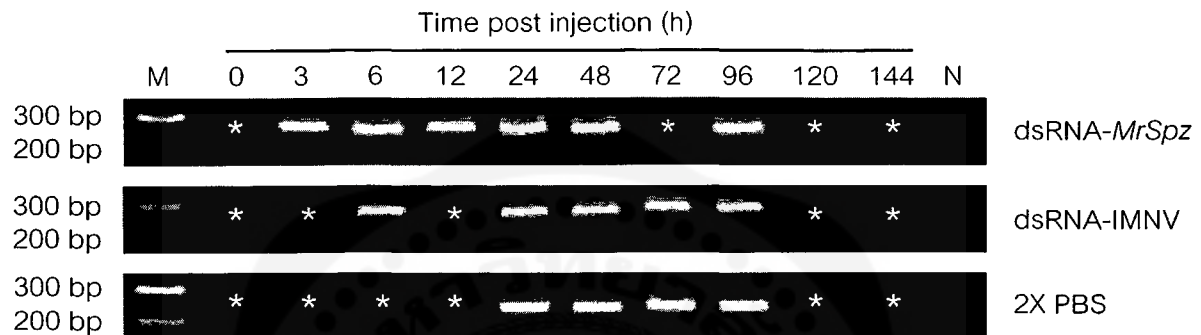


Figure 31 A representative gel of colony PCR for *A. caviae* identification. A single colony in TSA plate of *A. caviae* challenged prawn of dsRNA-*MrSpz*, dsRNA-IMNV and 2X PBS group was collected randomly. A positive result was indicated by DNA fragment of 237 bp. Asterisks (*) indicate no dead prawn sample at that time point.

CHAPTER 5

CONCLUSIONS AND DISCUSSIONS

Study of Toll signaling pathway and the molecules involved in this pathway are mostly investigated in *Drosophila*. In the case of Spätzle protein of shrimp, it has been reported in *F. chinensis* (Shi; et al. 2009: 610-617), *P. vannamei* (Wang; et al. 2012: 359-371, Yuan; et al. 2017: 46-57) and *P. monodon* (Boonrawd; et al. 2017: 88-95). Here, for the first time, a *Spätzle* gene from *M. rosenbergii* was isolated and characterized. The full-length cDNA of *MrSpz* gene was 2,253 bp with an ORF of 747 bp encoding 248 amino acid residues. The signal peptide located at amino acid residues 1-21 and C-terminal Spätzle activated domain located at amino acid residues 143-240 were identified on the mature *MrSpz* protein. The presence of signal peptide at the N-terminus of mature *MrSpz* protein agreed with other Spätzle protein of shrimps as mentioned above, and also with insects such as tobacco hornworm (*Manduca sexta*) (Zhong; et al. 2012: 514-524) and Silkworm (*B. mori*) (Wang; et al. 2007: 1002-1012). It supports the fact that the *MrSpz* protein is an extracellular protein, synthesized in cell and required signal peptide for secretion (Weber; et al. 2007: 13522-13531). Furthermore, nine cysteine residues of cysteine-knot domain were also observed in *MrSpz* and seven of which were dispersed in C-terminal Spätzle activated domain. Such characteristics were consistent with the Spätzle of *D. melanogaster* in which 7 out of 9 cysteine residues were clustered in the C-terminal Spätzle activated domain (C-106) (Morisato; & Anderson. 1994: 677-688). In shrimp, this feature was also found in *FcSpz* (Shi; et al. 2009: 610-617), *LvSpz2*, *LvSpz3* (Wang; et al. 2012: 359-371) and *LvSpz4* (Yuan; et al. 2017: 46-57). It is interesting that although each shrimp Spätzle had different amino acids in various positions, but they shared highly conserved cysteine residues in both numbers and positions except for *LvSpz4* in which 2 out of 7 cysteine residues located at different positions from Spätzle proteins of other shrimp species. Cysteine residues are necessary for dimer formation by disulfide bridges of protein in the cysteine-knot superfamily before binding to specific receptors that includes nerve growth factors (NGF), platelet-derived growth factor BB (PDGF-BB), transforming growth factor β (TGF- β), human chorionic gonadotropin (hCG) and Spätzle protein (Morisato; & Anderson. 1994: 677-688,

Gangloff; et al. 2008: 14629-14635). Based on BLASTp analysis, MjSpz shared high similarity with MrSpz, but the MjSpz amino acid sequence was not analyzed in multiple sequence alignment because there were only 22 amino acid residues of C-terminal Spätzle activated domain reported in the GenBank.

Analysis of deduced amino acid sequence revealed that MrSpz showed high similarity to that of penaeid shrimp including FcSpz, MjSpz, LvSpz3 and PmSpz. For LvSpz1, LvSpz2 and LvSpz4, it is noteworthy that they shared slightly similarity with MrSpz at 39%, 28% and 23% identity, respectively. Therefore, similarity among a full length spätzle and C-terminal Spätzle activated domain were analyzed. The results showed that the percent identity obtained from the C-terminal Spätzle activated domain of all shrimp Spätzle proteins were higher than that of the full-length pairwise analysis, especially in the case of similarity between MrSpz and LvSpz1; MrSpz and LvSpz2; MrSpz and LvSpz4 which demonstrated 54%, 38% and 29% identity, respectively. These data indicate that Spätzle protein of each shrimp species differ in its signal peptide and pro-domain, while the C-terminal Spätzle activated domain is more conserved, which confirms the role of the C-terminal Spätzle activated domain that stimulates the Toll signaling pathway. In addition, MrSpz also shared 26-40% identity to Spätzle protein of insects and copepod.

A neighbor-joining tree was constructed to investigate the phylogenetic relationship. The *M. rosenbergii* Spätzle protein was closely related to the Spätzle proteins of penaeid shrimp species, including *F. chinensis*, *P. monodon* and *L. vannamei* Spz3. In the case of LvSpz1, LvSpz2 and LvSpz4, they were different from other shrimp Spätzle proteins and grouped with insects and copepod. It was interestingly that *L. salmonis* Spz6 and *F. candida* Spz were placed in the cluster of shrimp Spätzle. Our analysis revealed that their C-terminal Spätzle activated domain shared high similarity to Spätzle proteins of shrimps than that of other insect species and they shared 5 conserved cysteine residues to shrimp Spätzle proteins. For LvSpz4, it showed low identity with other shrimp Spätzle and belonged to the group containing *D. magna*, *A. sinica* and *P. xylostella* Spätzle proteins.

In uninfected adult *M. rosenbergii*, MrSpz highly expressed in gill and weakly expressed in hepatopancreas and heart, while undetectable in hemocytes, muscle and stomach. This results suggested that the MrSpz was expressed in a tissue specific manner.

The tissue-specific expression of Spätzle proteins were found in *LvSpz1-3* of *L. vannamei* that mainly expressed in epithelium, gill, intestine and eyestalk. It is interesting that *LvSpz1* and *LvSpz3* had a very low expression level in hemocyte, and almost no expression in hemocyte for the *LvSpz2* (Wang; et al. 2012: 359-371), which corresponded to no expression of *MrSpz* in hemocyte. In contrast, the expression of *FcSpz*, *PmSpz* and *LvSpz4* were found in a various tissues including hemocyte, heart, hepatopancreas, gill, stomach and intestine (Shi; et al. 2009: 610-617, Boonrawd; et al. 2017: 88-95, Yuan; et al. 2017: 46-57). In addition, tissue-specific expression of *Spätzle* was also found in mosquito *Aedes aegypti* in that the *Spätzle 1B* was expressed in ovarian tissue, *Spätzle 1C* was expressed in fat body and *Spätzle 1A* was expressed in both ovary and fat body, while no expression of those *Spätzle* in midgut (Shin; Bian; & Raikhel. 2006: 39388-39395).

Although there was no *MrSpz* gene expression in hemocytes of healthy prawn, after injection of *A. caviae* via ventral sinus, the expression of *MrSpz* was significantly upregulated at 6, 12 and 24 hpi. After *MrSpz* expression level reached the highest level at 24 hpi, bacterial CFU was dramatically decreased to 10 CFU/mL at 36 and 48 hpi, respectively. This result suggested that *MrSpz* may be implicated in defense mechanism against *A. caviae*.

In Chinese shrimp *F. chinensis*, *FcSpz* expression in hemocyte is significantly up-regulated at 6, 12 and 24 h after injection with Gram-negative bacteria *V. anguillarum* and WSSV at 12 and 24 h post injection (Shi; et al. 2009: 610-617). In *L. vannamei*, *LvSpz1* and *LvSpz3* are induced by *V. alginolyticus* and WSSV injection. For *LvSpz1*, its expression is up-regulated at all time points and the highest expression is 155-fold at 12 h post injection and by the WSSV challenge, it also up-regulated about 120 and 160-fold at 3 and 12 h post-injection respectively. In the case of *LvToll3*, after *V. alginolyticus* challenge, it was up-regulated to 340-fold at 12 h post-injection and by WSSV challenge, *LvToll3* is gradually up-regulated at 3, 9 and 12 h post-injection. However, in *V. alginolyticus* challenged shrimp, *LvSpz2* expression has no increased and slightly down-regulated in WSSV challenged shrimp (Wang; et al. 2012: 359-371). Recently, *LvSpz4* is isolated and its expression is upregulated after LPS, *V. alginolyticus* and *S. aureus* challenges (Yuan; et al. 2017: 46-57). In *P. monodon*, with the injection of recombinant protein *PmSpz1* (r*PmSpz1*), the survival

rate of WSSV challenged shrimp is higher than 40 % during the 10 day trial period while WSSV challenged shrimp without *rPmSpz1* injection had a survival rate of 0% within 4 days (Boonrawd; et al. 2017: 88-95).

To further characterize the role of MrSpz protein in the innate immunity by *MrSpz* specific dsRNA injection, in *A. caviae* challenged group, mortality rate of dsRNA-*MrSpz* were increased up to 96.67% within 72 hours post injection while the mortality rate of non-related dsRNA and 2X PBS were 50.00% and 46.67% as a result of the LD₅₀ dose of *A. caviae* injection. In the 2X PBS injected group, the mortality rate of dsRNA-*MrSpz*, dsRNA-IMNV and 2X PBS were only 13.33%, 20% and 13.33% respectively. These results agreed with the study of *M. rosenbergii* Toll receptor (*MrToll*) that the *MrToll* knocked-down prawn challenged with *A. caviae* had a significantly higher mortality rate than that of the dsRNA-IMNV and 2X PBS about 4 and 7-folds, respectively (Srisuk; et al. 2014: 552-562) Therefore MrSpz protein is one of important protein in toll signaling pathway in response to *A. caviae* invasion.

In summary, the first *MrSpz* cDNA was isolated and characterized its role in innate immune system. The full-length MrSpz amino acid sequence was closely related to the Spätzle protein of *F. chinensis*, LvSpz3 of *P. vannamei* and PmSpz of *P. monodon*. The mature MrSpz protein consisted of three protein domains; signal peptide, pro-domain and C-terminal Spätzle activated domain. In the C-terminal Spätzle activated domain, 7 conserved cysteine residues were observed. In uninfected prawn, MrSpz expression was found in gill, heart and hepatopancreas. *MrSpz* expression in hemocytes could be induced by *A. caviae* injection. In *MrSpz* knocked down prawn, mortality rate of *A. caviae* injected group were significantly increased. These results indicated that MrSpz was one of the molecules involved in the defense mechanism of Toll signaling pathway against bacterial infection. These data may provide a better understanding of Toll signaling pathway in prawn innate immunity.



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APPENDIX A
BUFFER AND REAGENT PREPARATION

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BUFFER AND REAGENT PREPARATION

1. 0.15 M Phosphate buffered saline (2X PBS) pH7.2

NaCl	8.00	g
KCl	0.20	g
KH ₂ PO ₄	0.20	g
Na ₂ HPO ₄	1.15	g
Distilled water adjust volume to	500.00	ml

2. IPTG (100 mM IPTG)

IPTG	238.00	mg
Distilled water	10.00	ml
Aliquoted 1 ml to 1.5 ml tube and stored at -20°C		

3. X-gal (40mg/ml)

X-gal	200.00	mg
Diethyl formamide	5.00	ml
Aliquoted 1 ml to 1.5 ml tube and stored at -20°C		

3. Ampicilin (100mg/ml)

Ampicilin	1.00	g
Distilled water	10.00	ml
Aliquoted 1 ml to 1.5 ml tube and stored at -20°C		

4. Kanamycin (50 mg/ml)

Kanamycin	0.50	g
Distilled water	10.00	ml

Aliquoted 1 ml to 1.5 ml tube and stored at -20°C

5. 10X TBE buffer

Tris-HCl (pH 8.3)	108.00	g
Boric acid	55.00	g
EDTA	9.00	g
Distilled water adjust volume to	1000.00	ml

6. 20X SSC buffer, pH 7.2

NaCl	87.65	g
Sodium citrate	44.10	g
Distilled water adjust volume to	500.00	ml



APPENDIX B

MEDIA

APPENDIX B

MEDIA

1. LB broth

Pancreatic Digest of Casein (Tryptone)	5.00	g
Yeast extract	2.50	g
NaCl	5.00	g
Distilled water adjust volume to	500.00	ml
Autoclaved		

2. LB agar

Pancreatic Digest of Casein (Tryptone)	5.00	g
Yeast extract	2.50	g
NaCl	5.00	g
Agar	7.00	g
Distilled water adjust volume to	500.00	ml
Autoclaved and poured 15 ml into plate		

3. LB agar + ampicillin

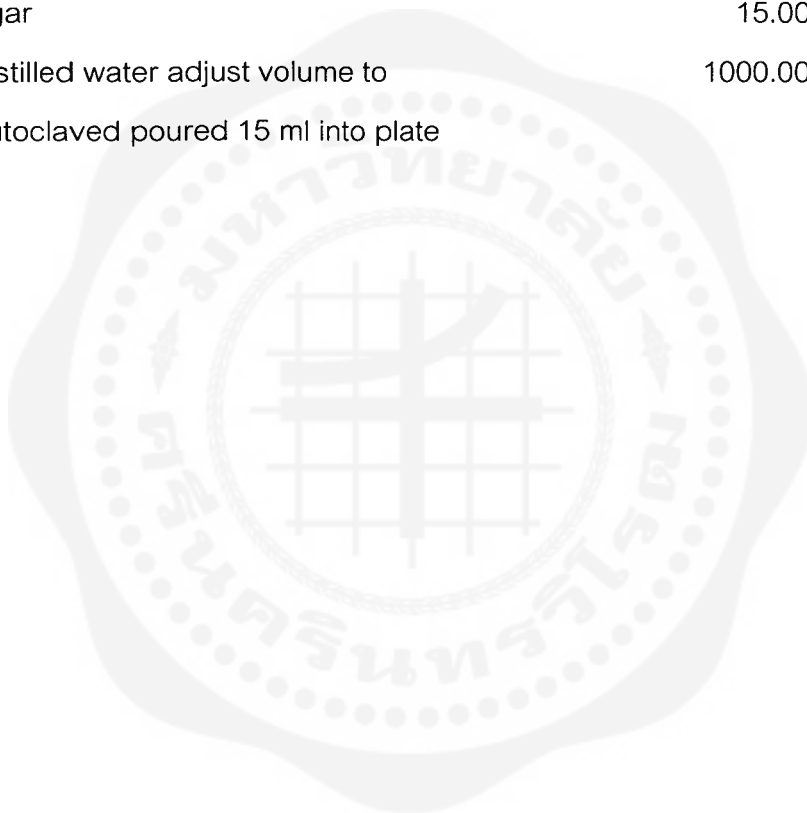
Pancreatic Digest of Casein (Tryptone)	5.00	g
Yeast extract	2.50	g
NaCl	5.00	g
Agar	7.00	g
Distilled water adjust volume to	500.00	ml
Autoclaved, added 100 mg/ml ampicillin 200 µl and poured 15 ml into plate		

4. Alkaline Peptone Water (APW), pH 9.2

Peptone	10.00	g
NaCl	10.00	g
Distilled water adjust volume to	1000.00	ml
Autoclaved		

5. Tryptic soy agar

Trypticase soy broth	30.00	g
Agar	15.00	g
Distilled water adjust volume to	1000.00	ml
Autoclaved poured 15 ml into plate		





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