SHRIMP EXTRACT IMMUNE STIMULANT HAD UP REGULATED THE EXPRESSION OF ANTI-LIPOPOLYSACCHARIDE FACTOR (ALF) OF PENAEUS VANNAMEI

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ABSTRACT

Pacific white shrimp (*Penaeus vannamei*) is an important aquatic animal culture of Thailand. In 2018, its production had reached more than 345,000 tons which could generate several farming activities and incomes to the farmers and finally increase the export values to Thailand more than 60,000 million baht per year. However, in 2012, it has been reported about an outbreak of infectious disease named as acute hepatopancreatic necrosis disease (AHPND) in Thailand and the causative agent came from Gram negative bacterium, *Vibrio parahaemolyticus* specific isolates (VP_{AHPND} isolates). This pathogen produces two important types of toxin including PirA and PirB which affect hepatopancreas of shrimp and cause sudden mass mortalities.

The present study aimed to solve the problem of VP_{AHPND} infection by using immune stimulant strategies. Shrimp shell obtained from frozen industry was employed as the main source to extract lipid metabolites and used as the immune stimulant. Shrimp fed with lipid metabolites mixed feed for 1 week were subjected to investigate an immune gene expression by RT-PCR analysis. Compared with a control group, shrimp fed with immune stimulant feed formula showed the up regulation of anti-lipopolysaccharide factor (ALF) gene by 1.3-fold. This result indicated that lipid metabolites extracted from shrimp shell can induce the expression of ALF, one of the essential immune genes in shrimp and it had high potential to further apply for protection of VP_{AHPND} infection in shrimp aquaculture.

Keyword: Pacific white shrimp, *Penaeus vannamei*, hepatopancreatic necrosis disease, AHPND, immune stimulant

INTRODUCTION

Pacific white shrimp is an important aquatic animal culture of Thailand. Shrimp exporting to the world market more than 345,000 tons per year can earn about 60,000 million baht per year [1]. However, during 2012-2013 it was reported that shrimp production in Thailand was continuously dropped due to the outbreak of infectious diseases. The acute hepatopancreatic necrosis disease (AHPND) is one of the important diseases which caused by Gram negative bacterium *Vibrio parahaemolyticus* specific subtype infection [2]. The bacterial can produce toxins similar to insecticidal toxins which can affect the hepatopancreatic organ of shrimp and lead to failure of organ function and death [3]. AHPND was previously known as early mortality syndrome (EMS), a newly emerging shrimp disease, which caused serious reductions in shrimp production and financial losses to the global shrimp aquaculture industry [4, 5]. Characterization of this pathogen should help to understand host-pathogen interaction that can help to develop antibacterial strategies. *V. parahaemolyticus* AHPND strain was characterized its ability to produce toxins $PirA^{vp}/PirB^{vp}$ which were determined to be the primary virulence factors destroying shrimp hepatopancreas [3]. The opportunistic marine pathogen *V. parahaemolyticus* becomes virulent by acquiring a plasmid that expresses a deadly toxin [6]. Based on their structure, theses toxins are similar to Cry insecticidal toxin-like proteins that encode a pore-forming activity used to kill host cells [3, 6]. AHPND, it remains unknown whether other virulent factors are commonly present in *V. parahaemolyticus* and might play important roles during shrimp infection.

Shrimp have an immunity system like other invertebrates, which were based on cellular and humoral innate immune responses to defense against invading microorganisms. Major immune reactions take place in haemolymph, which contains three types of haemocytes: the hyaline (or agranular) cells, semi-granular (or small granular) cells, and granular (or large granular) cells [7]. One of an effective immunity response was the superoxide anion that was the first product response process, which is known as a respiratory burst (RB) [8]. Another was anti-lipopolysaccharide factor (ALF), a type of antimicrobial peptide (AMP) with broad-spectrum antimicrobial activities against bacteria, fungi, parasites, and viruses [9]. The ALF, which could bind and neutralize the activity of lipopolysaccharide (LPS), was first isolated from the haemocytes of the horseshoe crab

Limulus polyphemus [10, 11]. A number of ALFs were then identified and characterized in different crustacean species, including crab, shrimp, crayfish, and lobster [12, 13, 14]. ALF proteins exhibited antibacterial and antiviral activities *in vitro* and *in vivo* assays. Both the recombinant ALF proteins and synthetic LBD peptides exhibited antimicrobial activities [13, 15, 16,].

However, little is known about the activation of shrimp immunity by some newly discovered immune stimulants. In this study, we tried to extract lipid metabolites from shrimp shell and investigated whether it can be used as immune stimulant for protection of shrimp against infectious diseases. Accordingly, we examined that shrimp shell metabolite can activate shrimp immunity by up regulation of ALF gene expression after feeding the shrimp with metabolite extracts.

MATERIALS AND METHODS

Extraction of metabolites from shrimp shell

Shrimp shell wastes from frozen industry were dried in hot air oven at 60°C for 48 hours and it was ground with mortar and pestle. The sample was subjected to extract twice with semi polar solvent, dichloromethane. After that, solvent was evaporated with rotary evaporation (Buchi Rotavapor® R-300). The obtained crude extract was measured semi-dried weight and purified through silica gel (Merck silica gel 60) column (Fig. 1). The partial purified shrimp shell extracts were kept at -20°C until used for the next experiment.

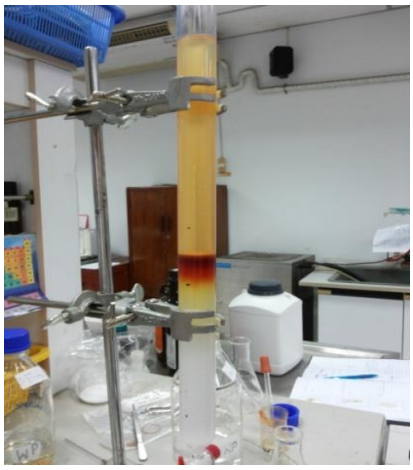


Figure 1. A silica gel column chromatography used for separation of shrimp shell crude extracts.

Preparation of shrimp feed supplemented with metabolite extracts

Shrimp feed were formulated with the metabolite extracts that were prepared as described above. The feed formulation was designed for two recipes as follows.

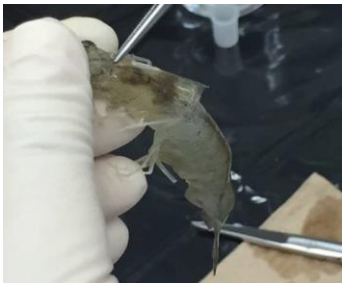
I) Immune stimulant feed was prepared by mixing a 1 kg of commercial shrimp feed (CP 9044 Turbo) with 1.938 g of shrimp shell crude extracts in the solvent solution of ethanol 20 mL and dichloromethane 20 mL. After thoroughly mixing the solution and feed, formulated feed was dried in hot air oven at 50°C for 24 hours. The final concentration of shrimp shell extracts was 500 mg/kg shrimp feed.

II) Control feed was formulated by using 1 kg of commercial shrimp feed (CP 9044 Turbo) mixed with 20 mL of ethanol and 20 mL of dichloromethane. The control feed was also dried in hot air oven at 50°C for 24 hours.

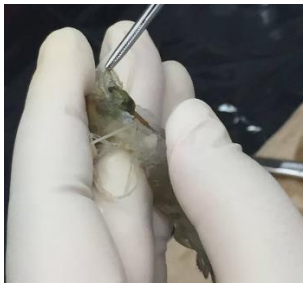
Experimental design and shrimp tissue sampling

Shrimp (5-6 g body weight) were acclimatized in a wet laboratory in 500 L aquaria containing continuously aerated artificial seawater at 20 ppt and 28°C for 3 days before the experiments began. Two treatments were separated in to six groups (60 individuals each) in which group 1, 2, and 3 were fed with Immune stimulant feed whereas group 4, 5, and 6 were fed with the control shrimp feed. Feeding dose rate was at 3% of their body weight, and shrimp were fed two times a day (i.e., 8.00 am and 5.00 pm) [17]. Condition of shrimp cultivation were monitored as followed; the salinity (20 ppt), pH (7.8-8.2), total ammonia (less than 1 mm/L), nitrite (less than 0.1 mg/L) [18], nitrate (less than 20 mg/L) [19]. Artificial seawater was also changed for 30% v/v in weekly. After 7 days feeding program, shrimp samples including haemolymph, stomach, and hepatopancreas (Fig. 2) were collected and preserved in Trizol reagent (Invitrogen) and kept at -80°C until used for RNA extraction.

haemolymph sample



stomach sample



hepatopancreas sample

Figure 2. Shrimp samples were collected using a syringe to withdraw haemolymph, and a knife to dissect stomach and hepatopancreas.

RNA extraction

Shrimp specimens were homogenized in Trizol reagent and total RNA were isolated following manufacturer's instructions. Briefly, 0.2 mL of chloroform were used to separate RNA in 1 mL of Trizol by centrifugation at 12000 g, 4°C for 15 min. The aqueous phase was transferred to the new tube and precipitated the RNA with isopropanol. After centrifugation, RNA pellets were washed with 75% ethanol and re-suspended with DEPC water. The RNA concentration was measured by using NanoDrop (ThermoScientific) with the absorbance at 260 nm.

Gene expression analysis by RT-PCR

RT-PCR was employed to evaluate the expression profiles of anti-lipopolysaccharide factor (ALF) gene in stomach tissue. A partial fragment of 231 bp of ALF was amplified using the primer pair LvALF-F (5'-TCGCCAGCAAGATCGTTGGG-3') and LvALF-R (5'-TGAGGCCATCCCTGAAGGC-3') which were designed based on the NCBI database (GenBank accession no. GQ227486). Partial *P. vannamei* beta-actin gene (GenBank accession no. AF300705) amplified using primers Actin-F (5'-AGGCTCCCCTCAACCCCAAGG-3') and Actin-R (5'-GCAGTGATTCTGCATGCG-3') was used as a control. RT-PCR reactions were carried out in a 25 µL reaction solution containing of 100 ng RNA template, 200 nM of each forward and reverse primer and 1x one-step RT-PCR master mix (GeneAll®). The reaction protocol comprised reverse transcription at 50°C for 30 min followed by denaturation at 94°C for 2 min followed by PCR cycling consisting of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 30 s. The PCR cycling was optimized to detect each transcript as follows: 35 cycles for ALF gene and 25 cycles for beta-actin. RT-PCR products were analyzed by agarose gel electrophoresis. The density of PCR products were measured using Quantity One® software (Bio-RAD).

RESULTS AND DISCUSSIONS

The experimental shrimp were fed with immune stimulant feed at 500 mg of the crude extract from shrimp shell mixed with 1 kg of standard shrimp feed and compared with the control feed for 7 days. The survival rates of two treatments were not significant difference in which they showed approximately 95% survival rate. At 7 day feeding program, shrimp tissues were collected including haemolymph, stomach, and heptopancreas. Triplicates of pooled samples were performed with 3 individuals in each pool. The control groups were composed of C-pool A, C-pool B and C-pool C whereas the immune stimulant groups were composed of B-pool A, B-pool B and B-pool C.

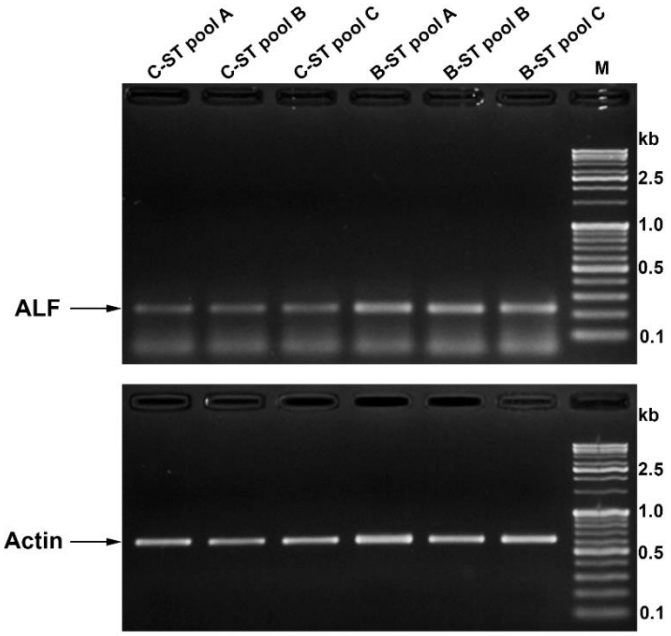


Figure 3. The RT-PCR amplification of ALF gene compared with actin gene in shrimp stomach. Each lane represents the pooled samples from 3 individual shrimp. C is control group whereas B is immune stimulant feeding. M is VC DNA ladder mix (Vivantis).

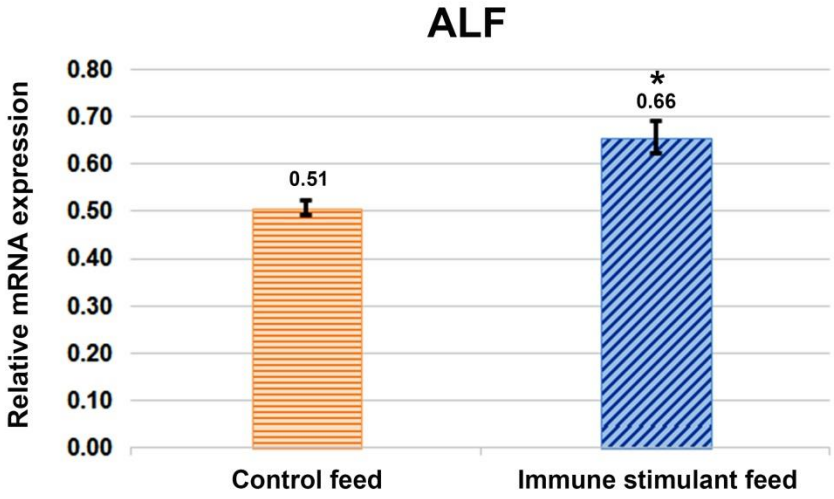


Figure 4. Relative mRNA expression of ALF normalized to actin gene. The average relative expressions are representative of triplicates and displayed as the mean ± 1 SD (error bar). Significant difference compared with control is indicated by an asterisk ($p < 0.05$).

The representative of ALF gene expression was described in the stomach tissue because this organ was the target for VP_{AHPND} colonization [20]. It was shown that up regulation of ALF transcript were clearly observed in shrimp fed with immune stimulant extracts (Fig. 3). As shown in gel electrophoresis, the intensity of RT-PCR products of 231-bp ALF gene in lane B-ST pool A, B-ST pool B, and B-ST pool C were higher than that of control groups in lane C-ST pool A, C-ST pool B, and C-ST pool C, indicating that shrimp shell extracts could enhance ALF gene expression. The observation of ALF gene expression was compared with the internal control beta-actin gene which was constantly expressed in both control and immune stimulant groups. Therefore, the up regulation of ALF gene expression by immune stimulant extracts was genuine.

As shown in Fig. 4, the relative mRNA expression of ALF gene normalized to actin gene was analyzed by measurement of intensity of PCR products in figure 3. Compared with control feed, the immune stimulant feed can increase the ALF gene expression by1.3-fold. The significant up regulation of ALF gene by shrimp shell extracts suggested that it had high potential to be used as immune stimulant in shrimp farming. Since it had been reported that recombinant ALF protein of *P. vannamei* exhibited antibacterial activity against VP_{AHPND} [21], the shrimp shell extracts in this study may represented as one of the important immune stimulants that can be used to control VP_{AHPND} infection in shrimp aquaculture.

CONCLUSION

Shrimp shell extracts in this study revealed immune stimulant property. It was showed that the metabolite extracts could induce the expression of ALF gene, one of the important antimicrobial peptides in shrimp that capable to defense against *V. parahaemolyticus* specific AHPND isolate. The immune stimulant feed formulation described in this study was the proportion of shrimp shell extracts 500 mg per 1 kg of shrimp feed and administration of this feed for 7 days can boost shrimp immune gene expression. Further study is being carried out to investigate whether this shrimp shell extracts can induce the expression of other immune genes in shrimp. This research could lead to development of immune stimulant strategies to control VP_{AHPND} infection in shrimp farms.

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