The effects of 5 aminolevulinic acid supplementation in Pacific white shrimp Litopenaeus vannamei

「課程博士・論文博士共通」

博士学位論文内容要旨
Abstract

専　攻 Major	APPLIED MARINE BIOSCIENCES	氏　名 Name	ヘラスミオ イバネ ペドロサ GERASMIO IVANE PEDROSA
論文題目 Title	The effects of 5-aminolevulinic acid supplementation in Pacific white shrimp *Litopenaeus vannamei* (バナメイエビ *Litopenaeus vannamei* における 5-アミノレブリン酸の経口投与がもたらす効果)		

The Pacific white shrimp *Litopenaeus vannamei* is the most important commodity in shrimp aquaculture, contributing about 80% of the total shrimp production (quantity) worldwide. To increase profits and sustain the increasing demand for shrimp, intensification of shrimp farms has been practiced. However, intensive shrimp farming has led to growing problems with infectious diseases, increasing our reliance to the use of antibiotics for prophylaxis and treatment. The heavy use and abuse of antibiotics has resulted in the spread of antibiotic-resistant bacteria and build-up of antibiotic residues, both in shrimp tissues and the environment. These issues have prompted the search for alternative ways to treat infections in shrimp aquaculture and one method considered to be promising in improving the health status of shrimp is immunostimulation.

An immunostimulant is any substance or action that enhances the immune system of an organism, making it less susceptible to pathogenic infections. One immunostimulant candidate is 5-aminolevulinc acid (5-ALA), an endogenous non-proteinogenic amino acid that plays a rate-limiting role in the synthesis of all tetrapyrroles, including heme. Previous studies conducted in vertebrates have suggested the positive effects of 5-ALA on the immune response of the organism and is mainly attributed to the increased activity of heme-containing proteins (hemoproteins) having various important functions in the body. Some examples of hemoproteins are catalase (antioxidation), respiratory cytochromes (ATP production), nuclear receptor E75 (ecdysone synthesis), cytochrome P450 (detoxification) and nitric oxide synthase (nitric oxide synthesis). Provided the beneficial effects of dietary 5-ALA in vertebrates, the general objective of this dissertation is to investigate the effects of 5-ALA supplementation on the gene expression profile, immune response, ATP level, ecdysis, growth and bacterial community profile in *L. vannamei*.

In the first feeding trial, 5-ALA group was supplemented with 15-ppm of 5-ALA and the control group was fed with basal diet (BD) for two weeks. In this experiment, the following were determined: (1) gene expression profile by microarray analysis; (2) ATP levels; (3) immune response against experimental *Vibrio parahaemolyticus* infection; and, (4) expression of some heme pathway genes and hemoproteins. Results showed that out of 15, 745 *L. vanna*mei putative genes spotted on the microarray, 101 genes were differentially expressed by more than four-fold ($p<0.05$) between 5-ALA-supplemented and control shrimp hepatopancreas. Based on sequence homology of known genes, 41 (41/101) genes were immune- and defense-related. Interestingly, seven of these genes are involved in chitin binding and metabolism and 5 of these 7 genes were highly upregulated, by more than eight-fold in 5-ALA group compared to the control. 5-ALA-supplemented shrimp also had a higher ATP level and survival rate in the challenge test (95%) compared to the control (0%) two weeks post infection. The heme synthesis pathway genes (porphobilinogen synthase and ferrochelatase) and hemoproteins (catalase and nuclear receptor E75) were also relatively higher in 5-ALA group compared to the control group.

In the second feeding trial, three 5-ALA concentrations were used and compared with the control group after 12 weeks of feeding. Dietary groups included the control group fed with BD and 5-ALA groups fed with BD plus 15, 30 and 60 ppm of 5-ALA (ALA15, ALA30 and ALA60, respectively). Since it was previously mentioned that 5-ALA upregulated genes involved in chitin binding and metabolism, we added an investigation

on the effect of 5-ALA on ecdysis (=molting). Chitin, which is the main component of the exoskeleton or cuticle of arthropods including shrimp, is synthesized and cleaved during ecdysis. Here, the effect of 5-ALA on the (1) relative expression of ecdysis-related genes; (2) ecdysis frequency and growth; (3) relative expression of catalase (CAT) and prophenoloxidase (proPO) genes and total hemocyte count (THC) after the feeding trial and after infection; and, (4) survival rate after bacterial challenge test, were investigated. After 12 weeks of feeding, most 5-ALA diets increased the expression of genes associated with ecdysis (nuclear receptor E75, cytochrome P450 Shade and chitinases 3 and 4) compared to the control. Ecdysis frequency and growth were highest in ALA30, although growth difference was not statistically significant compared to the control group. CAT and proPO gene expression levels were also significantly higher in ALA60 after the 12-week feeding trial. Six hours post-*V. parahaemolyticus* infection, THC (ALA60) and CAT gene expression levels were significantly higher in 5-ALA groups (ALA30, ALA60) than the control. ALA15 and ALA60 diets also increased resistance of *L. vannamei* against *V. parahaemolyticus*, with higher survival rate (90%) compared to the control (50%).

The effect of 5-ALA supplementation (15 ppm) on the bacterial community profile of *L. vannamei* was also investigated. Bacterial structure and function of the stomach and intestine after 5-ALA supplementation were determined using MiSeq Illumina sequencing of the V4 region of the 16S rRNA gene. Results revealed that 5-ALA did not cause a significant change in the alpha diversity indices (within sample) in both tissues, but beta diversity (between samples) shown by the principal coordinate analysis plots revealed alteration of the bacterial community structure after 1 week of 5-ALA supplementation. Differentially abundant taxa having potential benefits like *Enhydrobacter* and *Oceaniovalibus* were higher in 5-ALA group while potential pathogens like *Tenacibaculum* and *Mycobacterium* were more abundant in the control group. For the microbiome function prediction, control group have higher counts matching with the KEGG pathway 'human diseases' both in the intestine and stomach, compared to 5-ALA group, after 1 week of supplementation.

5-aminolevulinic acid synthase (ALAS), the first and rate-limiting enzyme in heme synthesis, condenses glycine and succinyl-CoA to form endogenous 5-ALA. To better understand ALAS in *L. vannamei* (LvALAS), cloning, characterization, gene expression analysis, and gene silencing of LvALAS were conducted. Cloning revealed that LvALAS is composed of an open reading frame of 1599 bp, encoding 532 aa and is ubiquitously present in all the *L. vannamei* tissues tested. Relative gene expression of LvALAS decreased with age and significantly increased after bacterial infection in the stomach. Silencing of LvALAS resulted in lower mRNA transcripts after 12- and 24- post-injection, compared to the PBS-injected group. LvALAS-silenced shrimp cannot molt normally even if they were already at the late pre-molt stage (compared to the PBS-injected control group which molted every 8-10 days) and died eventually after 2 weeks post injection.

Taken together, these results suggest that supplementing *L. vannamei* feeds with 5-ALA can enhance gene expression of heme synthesis pathway genes, upregulates several immune- and defense-related genes (including some hemoproteins), increases ATP production and enhances immune response against *V. parahaemolyticus* infection. 5-ALA can also induce ecdysis and expression of ecdysis-related genes, has a positive effect on growth, and may potentially promote the formation of a beneficial bacterial community structure in *L. vannamei*. These results also provide evidence that only the housekeeping form of ALAS exists in *L. vannamei*, LvALAS expression decreases as a function of age and that an ALAS-dependent pathway is indispensable for the proper molting process in *L. vannamei*.