IMMUNO-RELATED GENE EXPRESSION OF ANTI-LIPOPOLYSACCHARIDE FACTORS (ALF) AFTER USING IMMUNOSTIMULANT IN KURUMA SHRIMP (*Marsupenaeus japonicus*)

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ABSTRACT

Supplementation of immuno-stimulant can act as a potent immune modulator and have the effect on inducion of immune- related genes which involved in host defense. In order to clarify the anti-lipopolysaccharide factors (ALF) expression of Kuruma shrimp (*Marsupenaeus japonicus*) after feeding immuno-stimulant, the supplement containing microalgae and Euglena (Ex400) was selected for the trials. Total RNA was extracted by RNA Iso Plus from the lymphoid organ, intestine and blood of shrimp that was fed with Ex400 diet. Tissues were collected at 0, 3, 7, and 10 days after feeding. ALF transcripts were significantly increased by Ex400 compared to control-diet-fed shrimp (with $P<0.01$) at 3 days post-feeding in the intestine and lymphoid organ. This suggested Ex test diet might stimulate ALF of Kuruma shrimp immune defence in the intestine and lymphoid organ.

Keywords: Anti-lipopolysaccharide factor (ALF), gene expression, immunostimulants, Kuruma shrimp, quantitative real-time PCR.

Biểu hiện của gen ALF liên quan đến miễn dịch sau khi sử dụng chất kích thích miễn dịch trong tôm he Nhật Bản

TÓM TẮT

Chất kích thích miễn dịch là chất giúp tăng cường hoạt động của hàng rào miễn dịch và tác động đến các gen liên quan miễn dịch để bảo vệ của vật chủ. Ex400 được sản xuất từ vi tảo và Euglena là chất kích thích miễn dịch trên tôm được sử dụng để xác định biểu hiện của gen ALF trong tôm he Nhật Bản. ARN tổng số được chiết xuất từ các cơ quan lympho, ruột và máu của tôm tại các thời điểm 0, 3, 7 và 10 ngày sau khi cho tôm ăn Ex400. Trong ruột và cơ quan lympho của tôm, biểu hiện của gen ALF cao hơn so với lô đối chứng tại các thời điểm 3 ngày sau khi cho tôm ăn Ex400 ($P< 0.01$). Kết quả mức độ biểu hiện của gen ALF cho thấy chất kích thích miễn dịch có thể kích hoạt ALF trong hàng rào miễn dịch trong ruột và cơ quan lympho của tôm he Nhật Bản..

Từ khóa: Anti-lipopolysaccharide factor (ALF), biểu hiện gen, chất kích thích miễn dịch, định lượng real-time PCR, tôm Kuruma.

1. INTRODUCTION

Shrimp is considered one of the most internationally important traded fishery commodities in terms of value. The production of cultivated penaeid shrimp species increased exponentially since the early 1970s. However, there is rapid increasing problem with serious disease outbreaks. As shrimps lack an adaptive immune system, they rely on innate immune responses against microbial invasion (Tanekhy & Fall, 2015). A better understanding of the innate immune system of shrimp will undoubtedly help develop strategies in disease control and sustainable shrimp farming.

Anti-microbial proteins (AMPs), the cationic and amphipathic proteins of low molecular weight (< 10kDa), play a major role in innate immunity in shrimp lacking adaptive immunity, and studying their functions enriches basic knowledge on immunity and provides possible avenues in formulating disease management strategies in aquaculture (Bachère *et al.*, 2004). AMPs engage mainly to offer an early and first localized line of defense against pathogens (Selsted & Ouellette, 2005). Several AMP families such as penaeidins, crustins, anti-lipopolysaccharide factors (ALFs), histones, and fragments of hemocyanin have so far been described in penaeid shrimps. ALFs are antimicrobial peptides having broad spectra of antimicrobial activity to neutralize gram-negative and gram-positive bacteria, fungi, parasites and viruses (Mekata *et al.*, 2010). ALF, initially isolated and characterized from hemocytes of the horseshoe crab, *Limulus polyphemus (*Miyata *et al.*, 1987), has the endotoxin or lipopolysaccharide (LPS) - mediated coagulation system.

Immuno-stimulants are substances that activate the immune system of animals to make them more resistant to microbial infections (Raa, 1996). The definition has been expanded somewhat to include live organisms or their products that have an impact on the immune system. The use of immuno-stimulants does not generate a specific response to a certain antigen, but causes an overall response that hastens recognition and elimination of a broad range of infectious agents and foreign substances (Sordillo *et al.*, 1997). The present study was carried out to examine the expression of ALF after using immuno-stimulant containing microalgae and Euglena (Ex400) in Kuruma shrimp (*Marsupenaeus japonicus*).

2. MATERIALS AND METHODS

2.1. Animals

Specific pathogen free (SPF) Kuruma shrimps, *Marsupenaeus japonicus,* of 10 ± 0.3 g body weight were obtained from Matsumoto Fisheries, Miyazaki, Japan.

2.2. Methods

2.2.1. Experimental design

Prior to feeding experiment, shrimps were acclimatized, reared in aerated seawater tank at 23°C and 30 ppt salinity, and fed with control diet. 3 days. After that, one group of 12 shrimp was fed with Ex400. The second group, used as the control group (fed with control diet fed shrimp, without Ex400). Three shrimps were collected at 0, 3, 7, and 10 days for experiment. The experiment was conducted triplicate. Shrimp body surfaces were washed and disinfected with 70% ethanol, and then the blood, intestine, lymphoid organ were dissected out. One side of lymphoid organ, 200 µL of blood, and 1/10 of gut were collected.

2.2.2. RNA extraction

Total RNA was extracted from the sample using RNA Iso plus (TAKARA, Japan). The quantity and quality of all RNA samples were checked using a NanoDrop spectrophotometer ND-1000 (Thermo Scientific, Wilmington, DE, USA) at 260 nm and 280 nm.

2.2.3. Synthesis of cDNA

cDNA was synthesized according to the protocol (TOYOBO, Japan) of ReverTra Ace qPCR RT Master Mix with gDNA Remover, using RNA solution resulted from RNA extraction protocol, Nuclease-free water was added to RNA template. Thermal cycler condition was 65°C for 5 minutes, and after rapid cooling on ice, 2 µl of 4×DN Master Mix (gDNA Remover) were added. The thermal cycler profile was 37°C for 10 minutes, then 2 µl of 5×RT Master Mix were added to the mix. The thermal cycler condition for the 3rd step of PCR (reverse transcription reaction) was 37°C for 15 minutes, 98°C for 5 minutes. cDNA was used as template for real-time PCR analysis.

2.2.4. Quantitative RT-PCR for determining gene expression

A qRT-PCR on cDNA specimens was performed using SYBR Green Master Mix (Applied Biosystems). Elongation factor EF-1α gene was used as an internal control. The EF-

1α and their respective primers are presented in Table 1. All PCR reactions were performed in a reaction mixture containing 10.4 µL of SYBR Green Master Mix, 4 µL of 10 pM primer set (ALF/EF-1α), 2 µL of template DNA (10 ng), and 3.6 µL of nuclease-free water.

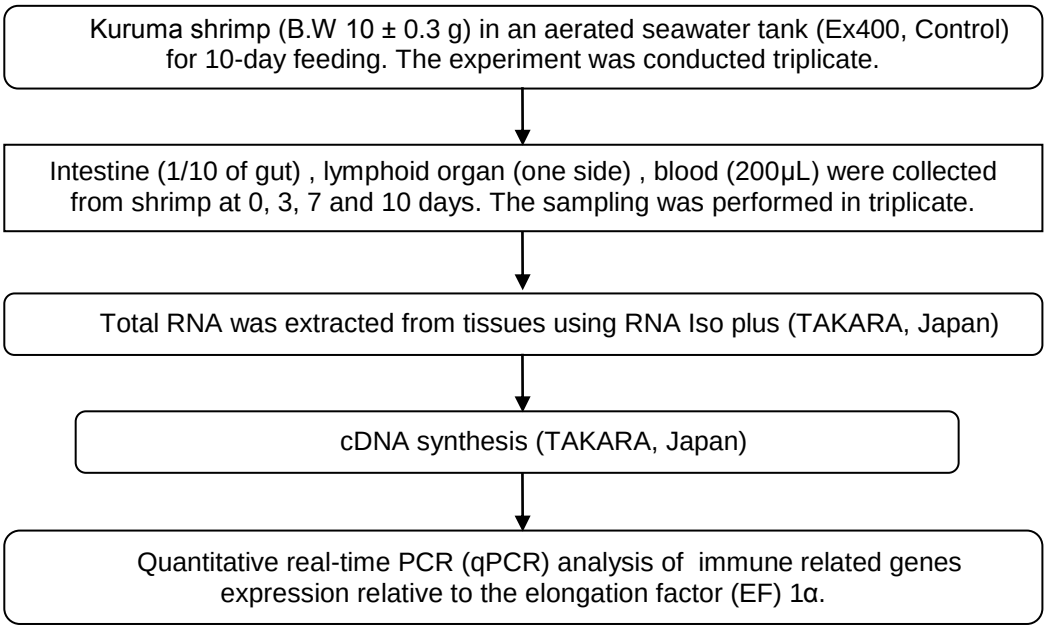

Diagram of experimental design

Table 1. The primers used to amplify EF-1 α and ALF of Kuruma shrimp

Gene	Sequence (5' to 3')	Accession number
[a]EF-1 α- Forward	TTCGCTGAACTGCTGACCAA	AB458256
[a]EF-1 α- Reverse	GCTTGCTGGGAACCATCTTG	
ALF- Forward	CCAACGCCCAACCTTCTACA	AB453738
ALF- Reverse	GGCTGCGGGTCATAGATCTG	

Note: [a] EF-1α specific primer were taken from a previous publication

Source: Maeda et al., 2014

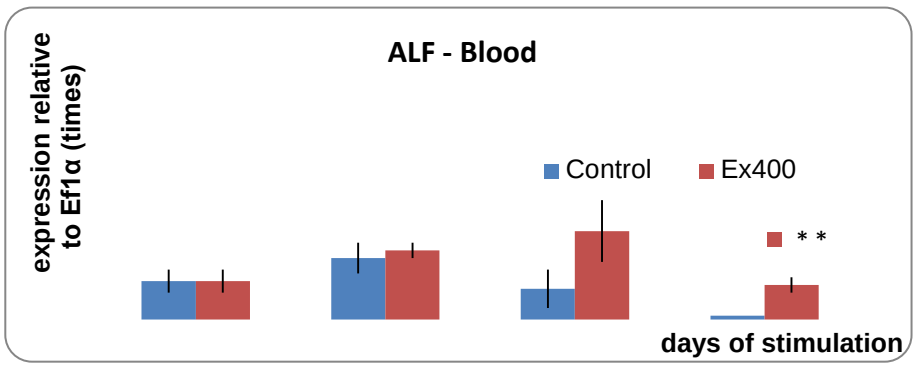

Fig. 1. Quantitative real-time PCR analysis of ALF expression relative to (EF)-1α gene trancript in blood of control and Ex400 diet-fed Kuruma shrimp

*Note: Data are presented as mean ± SD. Differences were considered significant at P<0.05 and P<0.01 as indicated by asterisk * and **, respectively*

Amplification was carried out as follows: 60s at 95°C, 40 cycles of 15s at 95°C, and 40s at 60°C. Thermal cycling and fluorescence detection were conducted using RT-PCR system (Applied Biosystem) with detection run in duplicate. The threshold cycle (C_T) representing the PCR cycle at which an increase in reporter fluorescence above signal was first detected. The comparative C_T method $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001) was used to analyze the expression level of the shrimp genes.

2.3. Statistical analysis

Analysis of variance was carried out using SPSS statistics version 18, to see the significance of expression of the gene at various time points. Independent t-test was performed to see significance in expression between Ex400 fed shrimp and control diet-fed shrimp.

3. RESULTS

3.1. Gene expression of ALF in blood

After 0, 3, 7 and 10 days of feeding, samples from 12 shrimps in each tank (Ex400 and control) were collected for checking expression by real time PCR. The levels of ALF lever in control group and experimental group (added Ex400 of the Ex supplementation) are shown in Fig.1. At 3 and 7 days post-feeding, although higher expression of ALF was observed in the blood of Ex400-fed shrimps, the expression levels was not different (P >0.05) from the expression in control diet-fed shrimp. ALF transcripts were significantly increased by Ex400 supplementation compared to control diet-fed shrimp (P <0.01) at 10 days post feeding. However, the level of expression of ALF in blood of both control diet-fed shrimp and Ex400 diet-fed shrimp at 10 day were lower than that 0 day post-feeding.

3.2. ALF expression in intestine

After 0, 3, 7, and 10 days of feeding experiment, intestine from 12 shrimps in each tank (Ex400 and control) were collected. The levels of ALF expression of control (without Ex400) and Ex400 of the Ex supplementation are given in Fig. 2. Transcriptions of ALF were significantly increased by Ex400 in comparision to control diet fed-shrimp (with P <0.01) at 3 days post-feeding in the intestine. At 7 and 10 days post-feeding, although higher expression of ALF was observed in the intestine of Ex400-fed shrimps, the expression levels was not high from the control diet-fed shrimp.

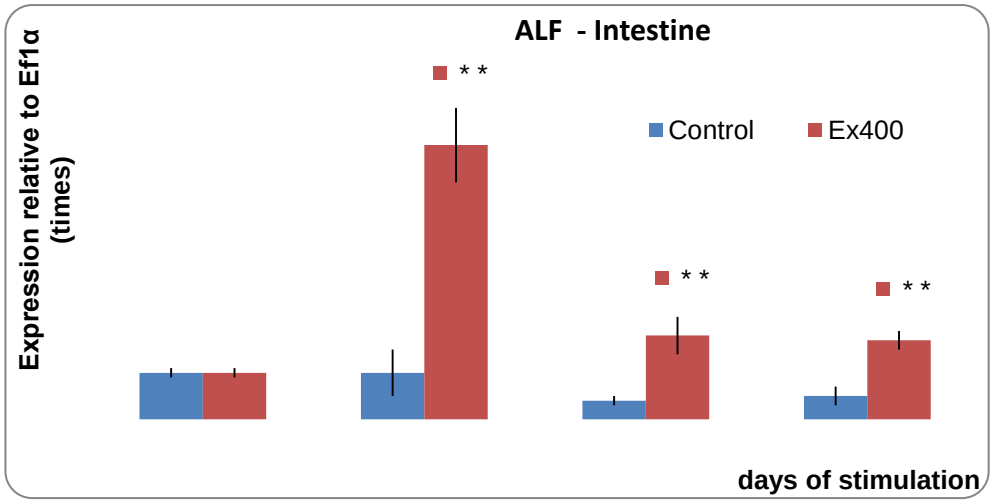

Fig. 2. Quantitative real-time PCR analysis of ALF expression relative to (EF)-1α gene trancript in the intestine of control and Ex400 diet-fed kuruma shrimp

*Note: Data are presented as mean ± SD. Differences were considered significant at P<0.05 and P<0.01 as indicated by asterisk * and **, respectively*

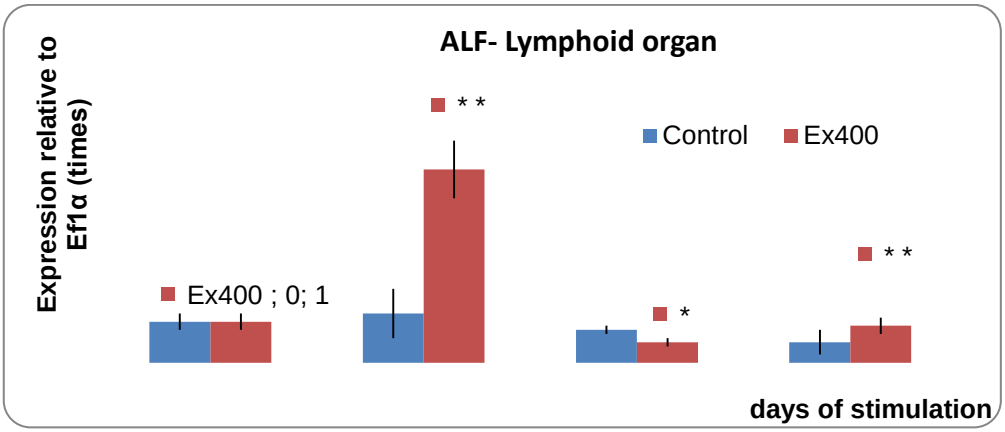

Fig. 3. Quantitative real-time PCR analysis of ALF gene expression relative to (EF)-1α gene trancript in the lymphoid organ of control and Ex400 diet-fed kuruma shrimp

*Note:Data are presented as mean ± SD. Differences were considered significant at P<0.05 and P<0.01 as indicated by asterisk * and **, respectively*

3.3. ALF expression gene in lymphoid organ

After 0, 3, 7 and 10 days of feeding, samples from 12 shrimps in each tank (Ex400 and control) were collected for checking expression by real time PCR. The levels of ALF lever in control group and experimental group (added Ex400 of the Ex supplementation) are shown in Fig.3. In the lymphoid organ, the transcript level of ALF in Ex400-fed shrimp was significantly higher (P <0.01) than control diet-fed shrimp at 3 days post feeding. ALF transcripts were significantly increased by Ex400 supplementation compared to control diet-fed shrimp (P <0.01) at 10 days post feeding, but the level of ALF expression was lower than that of 0 day.

4. DISCUSSION

The intestine was a favorable site for invasion of pathogens carried in the water, food, and sediment (Jayabalan *et al.*, 1982). It was previously demonstrated that an influx of hemocytes entered the intestine of *Penaeus monodon* following exposure to *Vibrio harveyi*. Besides, the hemocytes associated with the basal lamina of *S. ingentis* were reported to fight pathogens entering the body via the midgut (Liuxy *et al.*, 1996). Therefore, intestine which have immune functions in immune system effectively protects against pathogens. The lymphoid organ, first described in *Penaeus orientalis* (Oka, 1969), which consists of folded tubules with a central hemal lumen and a wall, layered with cells, was a site of bacterial uptake and phagocytosis by hemocytes (Van de Braak *et al.*, 2002). In most crustaceans, such as crabs and lobsters, that do not possess the lymphoid organ, phagocytes are involved in the uptake of foreign materials (Johnson, 1987). However, in those that do possess a lymphoid organ, including shrimps, it is the main site of bacteriostasis (Burgents *et al.*, 2005). These results are consistent with our study because we found that ALF was significantly increased after 3 days post feeding in intestine and lymphoid organ. Besides, at 3 and 7 days post-feeding, although higher expression of ALF was observed in the blood of Ex400-fed shrimps, the expression levels were not different (P >0.05) from the control diet-fed shrimp, and the levels of expression of ALF of both control diet-fed shrimp and Ex400 diet-fed shrimp at 10 day were lower than that 0 day post-feeding in blood. This suggestion may be supported by the hypothesis of Beale who attributed the increase in ALF gene expression in tissue to the higher concentration of the haemocytes following bacterial infection (Beale *et al.*, 2008).

Regarding the effect of immuno-stimulant on the gene expression of the immune-related genes. ALF gene expression showed significant increase (eight fold) on the 3rd day, which followed by sharp decrease nearly towards the control on the day seven (El-Asely *et al.*, 2011). The effect of the immuno-stimulants on the ALF gene expression was recorded by Mekata who observed the highest expression of ALF at 48, 8 and 12 h after lipopolysaccharide (LPS) injection at 1, 10 and 100 μg, respectively (Mekata *et al.*, 2010). Other works manipulated the expression of the ALF following bacterial challenge where they showed an increase in its expression short time after challenge (Beale *et al.*, 2008). The sharp increase of ALF gene expression recorded on the day three following administration of MACH may be associated with the fast and significant increase in the THC at the highest dose 0.2% MACH fed shrimp (El-Asely *et al.*, 2011).

In addition, the Ex400 diet test containing Euglena and microalgae produced Polysaccharide and beta-glucan. Polysaccharide is produced by microalgae and applied as anti-virus agent, antioxidant, anti inflammation and as part of immunomodulatory system. Beta-glucan is able to activate phagocytes effectively in invertebrates. According to a previous study, shrimp fed with peptidoglycan-supplemented feed showed better growth and feed conversion rates than those fed a normal diet, and demonstrated that black tiger shrimp grew faster with glucan immersion which could be attributed to the higher activity of glucan delivered by immersion compared to oral administration (Boonyaratpalin *et al.*, 1995).

In this context, we observed the up-regulated ALF transcription in intestine, lymphoid organ of Ex400-fed shrimps at 3 days post-feeding. This result coupled with our findings, therefore, indicated that ALF is elicited by immune-stimulating substances and acts as an integral component of the shrimp antibacterial defense mechanism.

Based on the results obtained, it will be of great interest to determine the gene expression such as Crustins, Penaeidins, Toll receptor in Kuruma shrimp in response to an in *vivo* stimulation, and its resistance against viruses or bacteria.

5. CONCLUSIONS

ALF transcripts were significantly increased by Ex400-fed shrimps compared to control diet-fed shrimps (P <0.01) at 3 post-feeding in lymphoid organ and intestine. The ALF gene expression could activate ALF in the immune system of Kuruma shrimp and suggested that Ex400 has a potential use as immuno-stimulant for shrimps.

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