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Changes in the immune-related gene expression of kuruma shrimp *Marsupenaeus japonicus* in response to dietary inclusion of Macrophage Activating Chinese Herbs (MACH).

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ABSTRACT

Immunostimulants are a category of the natural substances, which proved effective in controlling the disease in wide range of terrestrial animals and in the field of aquaculture. Plant mixed feed named MACH is a preparation made from four plants; namely, pumpkin (C*ucurbita moschata*) seeds, plantain (*Plantago asiatica*) seeds, honeysuckle (*Lonicera japonica*) flowers, and safflower (*Carthamus tinctorius*) flowers was added to shrimp feed at concentration of 0.2%; shrimp were fed on this mixture for seven days. Immune-related gene expressions were measured on zero, one, three and seven days after start feeding. Prophenol oxidase, antilipopolysaccharide factor and lysozyme genes were studied. The results showed that the antilipopolysacchride factor gene (ALF) was highly expressed on the 3rd day from feeding and returned to its normal levels on the 7th day. Lysozyme gene expression reached its peak on the 3rd day after feeding and returned to its normal values on the 7th day. The Prophenol oxidase (proPO) gene expression showed gradient increase in its expression with the sharp increase on the 7th day after MACH feeding.

Key words: kuruma shrimp, immune related genes, expression, Real time PCR

INTRODUCTION

The global production of farmed shrimp reached 3, 275,728 metric tons yearly (FAO, 2009). Shrimp possess neither immunoglobulins nor memory following the first encounter with a pathogen, and it obvious that they must rely on an efficient system of defenses (Roch, 1999). They depend mainly on the innate immune system, which involve haemocytes (for encapsulation, nodule formation and phagocytosis), several plasma components (antimicrobial peptides, histones, lysosomal enzymes, lipopolysaccharide, β-1,3-glucan binding proteins, and recognition molecules), and multimeric systems (clotting protein cascade, prophenoloxidase system). When these defense mechanisms fail to protect the shrimp against bacteria, viruses, fungi, protozoa and their products, disease develops and a

negative impact takes place in the shrimp culture system (Guzman *et al*., 2009).

Immunostimulants include bacteria and bacterial products, complex carbohydrates, nutritional factors, animal extracts, cytokines, lectins, synthetic drugs and plant extracts (Galeotti, 1998; Sakai, 1999). The immunostimulatory effects of plant extracts have been widely studied in fish and crustaceans (Kim *et al*., 1998; Logambal *et al*., 2000; Dügenci *et al*., 2003., Citarasu *et al*., 2006; Balasubramanian *et al.*, 2007; Balasubramanian *et al*., 2008 a, b). MACH (Macrophage Activating Chinaese Herbs) that have been used in the current study is a preparation made from four plants [pumpkin (*Cucurbita moschata*) seeds, plantain (*Plantago asiatica*) seeds, honeysuckle (*Lonicera japonica*) flowers, and safflower (*Carthamus tinctorius*) flowers]. It has been reported that MACH is effective in a wide range of animals (Yoshida *et al*., 2000; Yoshida *et al*., 2006 a, b) humans (Ushiroyama *et al*., 2004 a, b; Kaji, *et al.*, 2004) fish (Chansue *et al*., 2000; Ponpornpisit *et al*., 2001), and shrimp (Amel *et al*., 2010). Reverse Transcription-Polymerase Chain Reaction (RT-PCR) is a highly sensitive and specific method useful for the detection and analysis of samples available in limited amounts (Carding *et al*., 1992). Also, it is one of the accurate methods to calculate the relative changes in gene expression (Livak and Schmittgen., 2001).

MATERIALS AND METHODS

Test shrimp

Batches of juvenile kuruma shrimp, *Marsupenaeus japonicus (M. Japonicus)*, with average body weights ranging from 10 to14g were used. They were transported from the farm to the wet lab belonging to (Department of Biological Production and Environmental Science, Faculty of agriculture, University of Miyazaki), in well-sealed aerated foam boxes. Acclimation of shrimp were done by keeping the shrimp in their water at the room temperature, with gradual exchanging to their water with that of the already prepared tanks till the temperature of the both water become almost similar, where we can transfer shrimp to the well prepared fiber glass aquaria (60 × 30 × 35 cm) their bottoms were covered with approximately 3 cm thick washed plastic sand. An aeration system, heater, and surface-filtering device were installed, and approximately 40 litre of artificial seawater (Sealife, MarineTech, Japan) was added with 30 ppt salinity, which was measured using salinometer. The water temperature was set at 21 to 22°C during the study.

TEST IMMUNOSTIMULANT

Macrophage Activating Chinese Herbs (MACH) is a preparation made from four plants; namely, pumpkin (*Cucurbita moschata*) seeds, plantain (*Plantago asiatica*) seeds, honeysuckle (*Lonicera japonica*) flowers, and safflower (*Carthamus tinctorius*) flowers (Matsuura Yakugyo Co., Ltd. Japan).

THE EXPERIMENTAL DESIGN

Shrimp of average body weight 14g (n=15/ treatment in duplicate), were fed with MACH-treated food at concentration of 0.2%. Haemocyte were withdrawn at day zero, one, three and seven from start feeding, with measurement to the immune-related gene expressions (Anti lipopolysacchride factor, Lysozyme and Prophenol oxidase) using SYBR green real -time PCR.

TOTAL RNA ISOLATION AND REVERSE TRANSCRIPTION

RNA extraction was done according to TRIzol protocols (Invitrogen). Before cDNA

synthesis, concentration of the RNA at each sample was quantified using Nano-Drop spectrophotometer (Thermo Scientific USA) at 26 and 280 nm, and only RNAs with absorbance ratios (A260: A280) over 1.80 were used for further cDNA synthesis. The concentrations of RNA were adjusted by using Nuclease free water at 1µg. cDNA synthesis was done by transferring two µl of RNA to new tubes to which [0.63µl DNase, 0.63µl 10X DNase reaction Buffer and 3.04µl DNase free water] (Invetrogen, USA) were added. The samples were stored at room temperature for 15 min and to stop the reaction 0.7µl EDTA were used, and then using thermal cycler for heat shock (65ºC, 10 minutes then 4ºC). A mixture of 5X RT buffer, Enzyme and primer were added for each RNA samples tubes followed by using the thermal cycler for heat shock (37ºC for 20 minutes, 98ºC for 5 minutes then 4ºC). The final cDNA amount in the tube became ten µl

QUANTITATIVE IMMUNE- RELATED GENE EXPRESSION

Real-time RT-PCR was carried out in a 7300 Real-Time PCR System with Dell™ Notebook (Applied Biosystem, Japan). The amplification was performed in a total volume of 20 µl, containing 10 µl of SYBR Green real-time PCR Master Mix (TOYOBO), 2 µl of the diluted cDNA, and 2 µl of each primer. The real-time PCR program was, 95^{o} C for 10 min, followed by 45 cycles of 95^{o} C for 15 s and 60 ^{o} C for 40 s. Dissociation analysis of amplification products was performed at the end of each PCR reaction to confirm that only one PCR product was amplified and detected. Each sample was run in triplicate for each gene using shrimp elongation factor 1α gene as the internal control. Negative control reaction was included for every primer set by omitting template cDNA.

DATA ANALYSIS

Relative quantitation of gene expression was performed according to the manufacturer's instructions. Briefly, the threshold PCR cycle (Ct) is defined as the cycle number at which a statistically significant increase in the fluorescence of SYBR green against the internal passive dye, ROX (Δ Rn), is first detected. The copy number of the target gene and Ct values are inversely related; thus, a sample containing a higher number of copies of the target gene has a lower Ct value than that of a sample with a lower number of copies of the same target. Differences in the Ct values of immune genes and the corresponding internal control EF1α gene, called Δ Ct, were calculated to normalize for any difference in the amount of total RNA added to the cDNA reaction mixture and the efficiency of the reverse-transcription reaction. The value of Δ Ct for the immunostimulated sample was subtracted from the value of Δ Ct of the control sample without immunostimulation. The difference was expressed as the ΔΔ Ct value that allowed measurement of the change in expression of immune genes in the immunostimulated sample relative to the control sample.

RESULTS AND DISCUSSION

Real-time Polymerase Chain Reaction (PCR) is the ability to monitor the progress of the PCR as it occurs (i.e., in real time). There are many benefits of using real-time PCR over other methods to quantify gene expression. It can produce quantitative data with an accurate dynamic range of seven to eight log orders of magnitude and does not require post-amplification manipulation (Wong and Medrano, 2005). Regarding the effect of MACH on the gene expression of the immune-related genes. Antilipopolyscharide factor (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. The effect of the immunostimulants on

the ALF gene expression was recorded by Mekata *et al*. (2010) who observed the highest expression of ALF at 48, 8 and 12 h after lipopolysaccharide (LPS) injection in 1, 10 and 100 µg respectively.

Other works manipulated the expression of the ALF following bacterial challenge where they showed an increase in its expression short time after challenge (Liu *et al*., 2005; Beale *et al*., 2008; Somboonwiwat *et al*., 2008). The sharp increase of ALF gene expression was recorded on the day three following administration of MACH it may be associated with the fast and significant increase in the THC in the highest dose 0.2% MACH fed shrimp. This suggestion may be supported by the hypothesis of Beale *et al*. (2008) who attributed the increase in ALF gene expression in gill tissue to the higher concentration of the haemocytes following bacterial infection. Lysozyme gene expression reached the peak on the 3rd day. These results came in correlation with Aoki and Hirono (2005), Wang *et al*. (2008) and Ji *et al*. (2009) who observed an increase in the lysozyme gene expression after three day in peptidoglycan-fed kuruma shrimp, 6 h in Schizophyllan-fed white shrimp and three hours post-injecting *L. vannamei* with laminarin, respectively. The results revealed decrease in the lysozyme gene expression reaching its lower level by the 7th day.The results came in the same respect with those works of Wang *et al*. (2008) who found reduction in the lysozyme gene expression which returned to the control level the at 72 h in Schizophyllan-fed white shrimp. Moreover, Ji *et al*. (2009) observed gradual decrease in the expression profiles of lysozyme in haemocytes after injecting *L. vannamei* with laminarin, LPS and poly I: C immunostimulants where it recovered to the pre-injection level at 48 h.

proPO gene expression showed a gradient increase in its expression, with sharp increase on the 7th day (13 fold). These findings came in accordance with that recorded by Lu *et al*., (2006) who found an increase in the proPO gene expression after injection to *M. rosenbergii* with CPG oligodeoxynucleotide (ODN). On the other hand, Aoki and Hirono (2005); Hauton *et al*. (2005); Liu *et al*. (2006) and Okumura (2007) observed that administration of the immunostimulants have no effect on the proPO gene expression, or even it reduced their expression. Moreover, Ji *et al*. (2009) found that the level of proPO transcripts significantly decreased and remained at a lower level in laminarin, LPS and poly I: C immunostimulants injected *L. vannamei*. Chiu *et al*. (2007) found that proPO was highly expressed in probiotic fed groups compared to the control one.

CONCLUSION

The immunostimulants can be used as a good alternative for using chemicals and antibiotic for controlling diseases, which depends mainly on enhancing the immune-status of shrimp with no residual effect. MACH acts as immunostimulant through the activation of both cellular and humoral immunity. Gene expression is a promising tool for tracing the effect of the immunostimulants from the molecular aspect; however, it needs further studies.

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Table 1: Primers used for quantitative real-time PCR study.

Target gene	Forward/reverse sequence	Product (bp)
EF1α	GTCTTCCCCTTCAGGACGTA	377
	GAACTTGCAGGCAATGTGAG	
Lysozyme F	AAACGAGGTATTATCTCTCCAGG	515
Lysozyme R	TACACTTGCTGTTGTAAGCCACC	
Pro Po F	GGGAGTTCGTGGACATCACT	313
Pro PO R	CTGGAACAAGTCATCCACGA	
ALF F	CCAGATCCTTGCATCATCCT	600
ALF R	CCAGATCCTTGCATCATCCT	

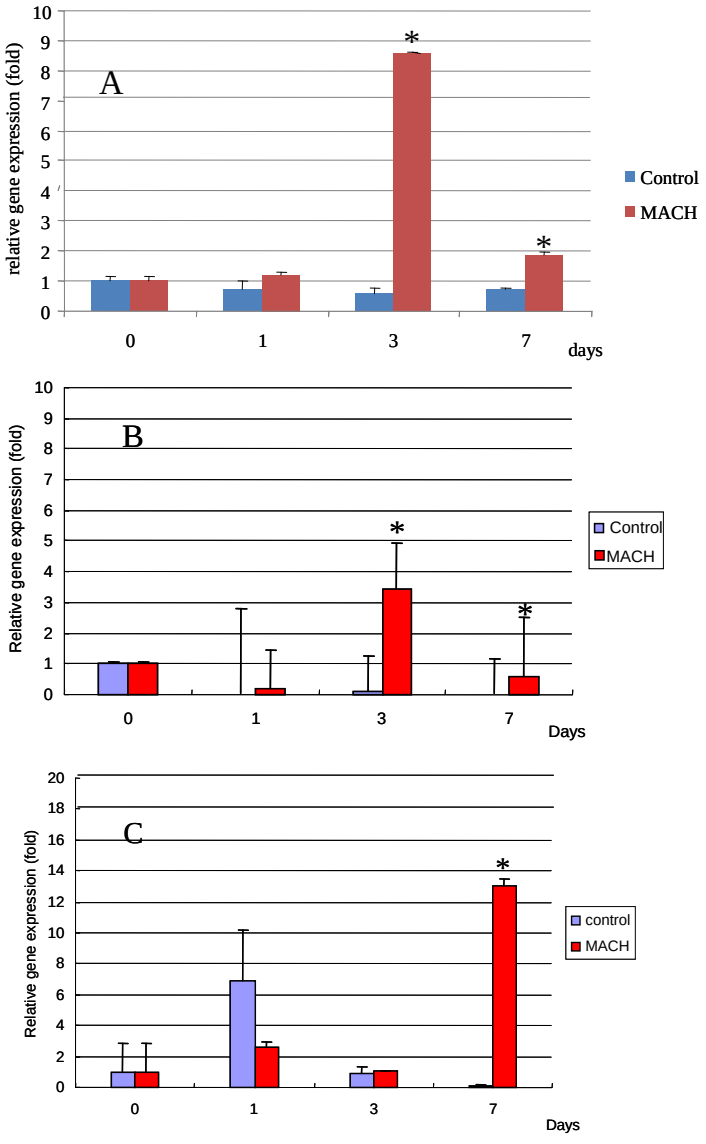
A
relative gene expression (fold)
10
9
8
7
6
5
4
3
2
1
0
0
1
3
7
days
Control
MACH
*
*
B
Relative gene expression (fold)
Days
Control
MACH
C
Relative gene expression (fold)
20
18
16
14
12
10
8
6
4
2
0
Days
control
MACH

Fig. 1: Transcriptional analysis of A) ALF, B) lysozyme and C) pro PO gene in haemocytes of kuruma shrimp fed with 0.2% MACH at different time intervals (zero, one, three, and seven days). Data with * show significant differences $P< 0.05$.

التغيرات فى التعبير الجينى للجينات المرتبطه بالمناعه لجمبرى الكروما عند تغذيته على عليقه بها خليط الاعشاب الصينيه المحفزة للمناعة (الماكة)

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يعد استزراع الجمبرى من المجالات الواعدة فى صناعة الغذاء . كما تعد المستحثات المناعية واحدة من المكونا ت الطبيعية التى لديها القدرة فى السيطرة على بعض الأمراض فى حقل الاستزراع السمكى . فى هذة التجربة تم تجميع دفعات من جمبرى الكروما من مزراع خاص ة بمحافظة ميازاكى باليابان وهذه الدفعات تم استخدامها لمعرفة مدى تاثير الماكه (خليط من اربعة نباتات) على الحالة المناعية لجمبرى الكروما مع تتبع التغيرات فى التعبير الجينى لبعض الجينات المقترنة بالمناعة عند تغذية الجمبرى ٠.٢% ماكه، وقد أوضحت النتائج أن تعبي ر جين معامل أنتى بولى ليبوسكارايد قد ازداد بقوة فى اليوم الثالث ،ثم مالبثت أن رجعت الى مستواها الأصلى فى اليوم السابع . أما عن التعبير الجينى لليسوزيم فقد وصل قمته عند اليوم الثالث وارتدت مرة أخرى فى اليوم السابع . بينما التعبير الجينى للبروفينول أوكسيداز فقد كان ارتفاعه متدرج وكان الارتفاع الحاد له عند اليوم السابع .