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Immune stimulant effects of a nucleotide-rich baker's yeast extract in the kuruma shrimp, *Marsupenaeus japonicus*

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ABSTRACT

Immune stimulant effects of a nucleotide-rich baker's yeast extract (Vertex IG20) were investigated in the kuruma shrimp, *Marsupenaeus japonicus* by examining expression of anti-microbial peptides/proteins (AMPs) such as penaeidin (MjPen), crustin (MjCrus) and lysozyme (MjLyz) genes. Furthermore, to confirm that the baker's yeast extract-induced AMPs were functional, we also assessed the effect of its oral administration on resistance to *Vibrio nigripulchritudo* infection in the kuruma shrimp. Our results demonstrate that baker's yeast extract-injected and fed shrimps displayed a significant up-regulation of MjPen, MjCrus and MjLyz gene expression in the lymphoid organ. Moreover, significantly increased ($P<0.01$) resistance to the bacterial pathogen in term of better post infection survival (66.6%) was observed in the shrimp fed with the yeast extract-incorporated diet compared with the control diet fed group (8.3%). The present study indicates the immunostimulatory effects of the nucleotide-rich baker's yeast extract on the kuruma shrimp immune system and supports its potential use in aquaculture.

1. Introduction

Aquaculture represents the fastest-growing animal based primary food producing sector with 63.6 million tons (MT) production and 8.8% annual growth rate in 2011 (FAO, 2012). The contribution from crustacean aquaculture is 5.7 MT. Kuruma shrimp, *Marsupenaeus japonicus* is the highest priced shrimp species among the farmed crustaceans and widely cultured in Japan, China, Australia and Southeast Asian countries (Rosenberry, 2001). However, disease occurrences have led to considerable economic loss in shrimp farming industry. Treatment of diseases using chemotherapeutics and antibiotics at farm level is either infeasible or prohibited. Therefore, enhancement of immune status using bio-products could render resistance to diseases and thus prevention of these disease occurrences is possible.

Shrimps, like other invertebrates, depend on innate immune system rather than on non-existent acquired immunity for protection against invading pathogens (Lee and Söderhäll, 2002; Loker et al., 2004). Cationic anti-microbial peptides/proteins (AMPs) play a major role in innate immunity in shrimp. AMPs are amphipathic proteins of low molecular weight (<10 kDa) and mainly offer an early and localized first line of defense against pathogens (Selsted and Ouellette, 2005; Zasloff, 2002). To date, several AMP families including penaeidins (Destoumieux et al., 1997), crustins (Bartlett et al., 2002), anti-lipopolysaccharide factors (Somboonwiwat et al., 2005), histones (Patat et al., 2004), and fragments of hemocyanin (Destoumieux-Garzón et al., 2001) have been described in penaeid shrimps. Penaeidins with chitin-binding properties (Destoumieux et al., 2000a) are ubiquitous in penaeid shrimps (Gueguen et al., 2006) and act against Gram-positive bacteria, filamentous fungi (Destoumieux et al., 1997), viruses and protozoans (Bachère, 2003). Crustins, first identified in shore crab *Carcinus maenas* (Relf et al., 1999) and later also described in *M. japonicus* (Rattanachai et al., 2004), have antimicrobial activity against Gram-positive bacteria. Lysozyme acts against Gram-negative bacteria by degrading the cell wall mucopolysaccharides, allowing their recognition by phagocytic cells (Aguirre-Guzmán et al., 2009). Kuruma shrimp lysozyme also displayed glycolytic activities to the pathogenic *Vibrio* species (Hikima et al., 2003). Therefore, studying the functions of AMPs enriches basic knowledge on shrimp immunity and provides possible avenues in formulating disease management strategies in aquaculture (Bachère et al., 2004).

Nucleotides are low molecular weight biological compounds that play important role in essential physiological and biochemical functions (Carver and Walker, 1995; Cosgrove, 1998). Nucleotides are synthesized *de novo* in most of the tissues, but some immune and intestinal cells lack this process and depend on exogenous supply (Quan, 1992). Hence, administration of pure nucleotides guarantees

Abbreviations: RT, reverse transcription; PCR, polymerase chain reaction.

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increased availability to the body at the time of high demand for various physiological activities (Whitehead et al., 2006). The immunomodulatory effects and genetic expression due to dietary nucleotides supplementation have been reported in higher animals (Gil, 2002; Singhal et al., 2008). Since last three decades, in several studies involving aquatic species, dietary nucleotides have elevated cellular and humoral immune responses in Atlantic salmon (Burrells et al., 2001), catla (Jha et al., 2007), common carp (Sakai et al., 2001), grouper (Lin et al., 2009), hybrid striped bass (Li et al., 2004), rainbow trout (Leonardi et al., 2003; Tahmasebi-Kohyani et al., 2011) and red drum (Cheng et al., 2011). However, few nucleotide nutrition researches in Pacific white shrimp have shown beneficial results on growth, survival (Li et al., 2007) and cellular immunity (Murthy et al., 2009), lacking elucidation of molecular immune functions. There is no information regarding the expression of innate immune genes, especially AMPs in kuruma shrimp administered with a baker's yeast extract preparation. Therefore, we tested the efficacy of a nucleotide-rich baker's yeast extract (Vertex IG20) administration in regulation of AMP genes, such as penaeidin (MjPen), crustin (MjCrus) and lysozyme (MjLyz) in the lymphoid organ (LO) of kuruma shrimp. Additionally, we assessed resistance to *Vibrio nigripulchritudo* infection in the Vertex IG20 fed kuruma shrimps to confirm functionality of the elevated innate immune system.

2. Materials and methods

2.1. Experimental shrimp

Healthy kuruma shrimps, *M. japonicus* (mean body weight, 10 ± 1 g) were obtained from Matsumoto Fisheries Farm, Miyazaki, Japan. Shrimps were firstly acclimatized in an aerated seawater tank at 20 °C and fed with a commercial diet (Higashimaru, Japan) once a day for 2 weeks under a natural photoperiod prior to their use in the experiment. The health status of experimental shrimps was checked by culturing hemolymph and hepatopancreas smears from few sampled animals on Marine Agar Broth 2216E (Difco, Detroit, Michigan, USA) for the presence of any bacterial pathogens. The results showed existence of no pathogenic bacteria in shrimps.

2.2. Preparation, injection and feeding of baker's yeast extract to shrimp

In this study, we used a commercial baker's yeast extract, Vertex IG20 (TableMark Co., Ltd., Tokyo, Japan). Vertex IG20 was processed as per the protocol described by Biswas et al. (2012). However, the final product was in the form of fine powder and it contained nucleotides (36.7%) as major components (Table 1). This yeast extract did not contain β-glucan measured using mushroom and yeast β-glucan assay kit (Megazyme, Bray, Ireland). Presence of any microbial contamination in the baker's yeast extract was examined by culturing the yeast extract dissolved in phosphate-buffered saline (PBS) on Marine Agar Broth 2216E (Difco) and growth of no viable bacteria was detected. For injection experiment, shrimps were divided into two groups (n = 20), treatment and control. Shrimps of treatment group were injected with 0.1 mL of Vertex IG20 dissolved in PBS at 5 mg shrimp^{-1}, whereas control group shrimps received an injection of 0.1 mL PBS. Shrimps of both the groups had an intramuscular (i.m.) injection in the second abdominal segment. For feeding experiment, the yeast extract was mixed at 5% (w/w) with the commercial diet mentioned above. Shrimps were divided into two groups (n = 140), *viz.* treatment (Vertex IG20 fed) and control group. Shrimps of the treatment group were fed with the yeast extract mixed diet, whereas control group shrimps were fed with the unmixed commercial diet once a day at 10% body weight for 7 days. Shrimps in both the experiments were maintained in seawater flow-through system at 22 ± 2 °C under a natural photoperiod.

Table 1
Composition of baker's yeast extract (Vertex IG20) used in the study.

Component	%
Nucleotides[a]	36.70
Total amino acids	38.02
Minerals	12.94
Organic acids	2.95
Total vitamins	1.55
Crude fat	0.30
Moisture	3.71
Others	3.83

[a] Nucleotides (36.70%) composed of disodium inosine-5′-monophosphate (IMP)- 10.59%, disodium guanidine-5′-monophosphate (GMP)- 10.02%, disodium cytidine-5′-monophosphate (CMP)- 7.33%, disodium uridine-5′-monophosphate (UMP)- 8.68% and disodium adenosine-5′-monophosphate (AMP) - 0.08%.

2.3. Expression analysis of innate immune-related genes by semi-quantitative RT-PCR

The lymphoid organ (LO) was dissected out from the PBS and Vertex IG20 injected kuruma shrimps (n = 5) at 1, 3 and 5 days post injection, whereas LO from control and Vertex IG20 mixed diet fed shrimps (n = 5) was collected at 0, 1, 3 and 5 days after feeding. Total RNA was extracted from the LO using ISOGEN (Nippon Gene, Osaka, Japan) as per the manufacturer's instructions. Poly (A) RNA was then purified using a quick prep micro mRNA kit (Amersham Pharmacia Biotech, Sweden). To avoid the presence of DNA, RNA samples were treated with recombinant DNase (RNase-free) at 37 °C for 30 min according to the manufacturer's protocol (Takara Bio Inc., Shiga, Japan). Quantity and quality of RNA in all samples were checked using a NanoDrop spectrophotometer, ND-1000 (Thermo Scientific, Wilmington, DE, USA). cDNA synthesis was performed using ReverTra Ace qPCR RT kit (Toboya, Osaka, Japan) and this cDNA served as a template for PCR. All PCR reactions were performed as per the protocol described by Biswas et al. (2012). Amplification of elongation factor (EF)-1α gene was used as an internal control. The immune-related genes, internal control, their respective primers and annealing conditions are presented in Table 2. PCR products were electrophoresed on a 1.5% agarose gel to detect specific bands. To conduct a semi-quantitative approach of gene expression, kuruma shrimp innate-immune related genes and EF-1α gene were amplified using a series of cycle numbers (20–35) under the above mentioned conditions. After determining the optimal cycle number, specific PCR was conducted and the expression ratio of each innate immune

Table 2
Gene specific primers and annealing temperature of kuruma shrimp EF-1α and innate-immune related genes used for PCR analysis in the study.

Gene	Primer sequence (5′→3′)	Annealing temperature (°C)	Accession number
MjEF-1α Fw[a]	GTCTTCCCCTTCAGGACGTA	55	AB458256
MjEF-1α Rv[b]	GAACTTGCAGGCAATGTGAG		
MjPenaeidin Fw	GCTGCACCCACTATAGTCTTT	60	AU175636
MjPenaeidin Rv	CTACCATGGTGATGAAACAAA		
MjCrustin Fw	CACCTTCAGGGACCTTGAA	62	AB121740
MjCrustin Rv	GTAGTCGTTGGAGCAGGTTA		
MjLysozyme Fw	TCCTAATCTAGTCTGCAGGGA	58	AB080238
MjLysozyme Rv	CTAGAATGGGTAGATGGA		

[a] Fw = Forward;
[b] Rv = Reverse.