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A sulfated galactans supplemented diet enhances the expression of immune genes and protects against *Vibrio parahaemolyticus* infection in shrimp

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ABSTRACT

A sulfated galactans (SG) supplemented diet was evaluated for the potential to stimulate immune activity in shrimp *Penaeus vannamei (P. vannamei)*. Shrimp given the SG supplemented diet (0.5, 1 and 2% w/w) for 7 days showed enhanced expression of the downstream signaling mediator of lipopolysaccharide and β-1,3-glucan binding protein (LGBP) and immune related genes including p-NF-κB, IMD, IKKβ and IKKε, antimicrobial peptide PEN-4, proPO-I and II. Following immersion with *Vibrio parahaemolyticus* (*V. parahaemolyticus*) for 14 days, the shrimp given the SG supplemented diet (1 and 2% w/w) showed a decrease in bacterial colonies and bacterial toxin gene expression, compared to shrimp given a normal diet, and they reached 50% mortality at day 14. However, shrimp given the normal diet and challenged with the bacteria reached 100% mortality at day 6. SG-fed shrimp increased expression of immune genes related to LGBP signaling at day 1 after the bacterial immersion compared to control (no immersion), which later decreased to control levels. Shrimp on the normal diet also increased expression of immune related genes at day 1 after immersion which however decreased below control levels by day 3. Taken together, the results indicate the efficacy of the SG supplemented diet to enhance the immune activity in shrimp which could offer protection from *V. parahaemolyticus* infection.

1. Introduction

Disease outbreaks from viral and bacterial infection in shrimp have caused massive mortality and a great loss to the shrimp cultivation industry worldwide [1]. Since 2012, the shrimp production in Thailand has been threatened with early mortality syndrome (EMS) or acute hepatopancreatic necrosis disease (AHPND). AHPND mostly affects post larval and juvenile shrimp and produces up to 100% mortality [2,3].

The AHPND disease is histopathologically characterized by severe atrophy in shrimp hepatopancreas with massive sloughing, collapse of hepatopancreatic epithelial cells and unique vacuolization of embryonic cells [4]. Recently, the causative agent of the disease was reported to be the bacterium *Vibrio parahaemolyticus* (*V. parahaemolyticus*). *V. parahaemolyticus* is a Gram-negative halophilic, non-spore forming, curved rod-shaped bacterium that naturally lives in estuarine and marine environments worldwide [5]. Shrimp infected with *V. parahaemolyticus* showed slightly expanded chromatophores, lethargy, erratic swimming, and empty guts and presented discoloration of hepatopancreas [6]. Recently, ToxA and ToxB released from *V. parahaemolyticus* have been reported to cause AHPND pathology in shrimp. Detection of the pathogenic *V. parahaemolyticus* in shrimp was evaluated by using the AP3 primer specific to the *tox*A gene [7].

Enhancement of the immune system of shrimp and other aquatic animals is one of strategies for protecting against pathogenic infection [8], and is crucial for the sustained and expanded growth in the aquatic culture industry. Shrimp have innate immune responses like other invertebrates, which include cellular and humoral immune responses [9]. When the immune cells are triggered by foreign particles several immune pathways are initiated including Toll and immune deficiency (IMD) immune signaling

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pathways [10]. The activation of lipopolysaccharide and β-1,3-glucan binding protein (LGBP) on the haemocyte membrane of shrimp initiates the downstream signaling pathway of the IMD and proPO cascade, and triggers expression of the proPO system, the antimicrobial peptides (AMPs) and the antiviral proteins of shrimp [10]. Indeed, in recent studies it has been proposed that immunostimulatory natural plant extracts may provide protection against specific diseases in shrimp [11–13]. A number of seaweed compounds show inhibitory activities against aquatic pathogens. For instance, it has been reported that the ethanol extract from *Gracilaria fisheri* (*G. fisheri*) provides potent antibacterial activity against *V. harveyi* in shrimp by increasing the immune response [14]; sulfated polysaccharides from brown and red seaweeds also enhance the immune response and protect against viral infection [11,15]. It has further been shown that shrimp fed with diet containing *Sargassum wightii* (*S. wightii*) [16] and *S. cristaefolium* [17] extracts produced an increased immune response and protected against *Vibrio* infection. However, studies such as these are limited in number.

Our previous work has demonstrated that sulfated galactans (SG) from the red seaweed *G. fisheri* stimulate immune activity in shrimp haemocyte culture, which is mediated, in part, through the LGBP, and IMD-NF-κB pathway [18]. Therefore, the present study was undertaken to investigate the efficacy of SG supplemented diet to enhance immune activity and increase resistance against *V. parahaemolyticus* infection in shrimp.

2. Materials and methods

2.1. Sulfated galactans

Sulfated galactans (SG) was extracted from the red seaweed *G. fisheri* and purified following the method previously described [11,18]. The chemical structure of SG was characterized by nuclear magnetic resonance (NMR) and fourier transformed infrared spectroscopy (FT-IR) analysis. SG is composed of the linear backbone of alternating 3-linked β-D-galactopyranose and 4-linked 3,6-anhydro-α-L-galactopyranose or α-L-galactose 6-sulfate units. Sulfations are on C-4 and C-6 of D-galactopyranose units.

2.2. Preparation of SG supplemented diet

SG was formulated to provide four different concentrations of feed diet as shown in Table 1. Fresh whole minced prawn *(Metupenaeus bennettue),* squid oil and, where applicable, the SG (0, 0.5, 1 and 2% w/w of diet) were added together and mixed thoroughly in a Hobart mixer (Hobart Manufacturing Company, London). Water was then added to the mixture, if required, until the dough was formed. The dough was extruded twice through a mincer attachment with one mm lengths. The resulting strands were then cut into 3 mm lengths and steamed for 10 min. The formulated diet was dried at 60 °C until they contained about 10–12% moisture, and stored at 4 °C until used.

2.3. Administration of SG supplemented diet and determination of immune related gene expression

Post larvae shrimp (150–300 mg) were kept in bio-filter laboratory tanks containing artificial seawater at 26 °C and daily fed with the SG supplemented diet in amounts of 5% of the body weight for 7 days. Shrimp were divided into 4 groups (90 shrimp/group) and each group assayed in triplicate (30 shrimp/assay). Group 1 was composed of shrimp fed with the control diet (control). Group 2, 3 and 4 were composed of shrimp fed with SG supplemented diet at 0.5, 1 and 2% w/w of diet (0.5 SG, 1 SG and 2 SG), respectively. Seven days after SG supplemented diet administration; shrimp (10 shrimp/group) were collected in order to determine expression of the phosphorylated-NF-κB (p-NF-κB) protein, which is a downstream signaling mediator of shrimp immunity, by Western blot analysis. The expression of immune related genes including the signaling mediators of shrimp immunity (IMD, IKKβ, IKKε), antimicrobial peptides (PEN-4 and crustin), and prophenoloxidase system (proPO-I and proPO-II) were determined by reverse transcription polymerase chain reaction (RT-PCR) analysis.

2.3.1. Expression of p-NF-κB in shrimp by western blotting analysis

The whole shrimp (5 shrimp/group) were protein extracted using a protein lysis buffer [20 mM Tris-HCl, 100 mM NaCl, 5 mM phenylmethylsulfonyl fluoride (PMSF), protease inhibitor mix]. The protein was separated on 12.5% SDS-PAGE and transferred to a nitrocellulose membrane (Whatman, UK). The membrane was blocked with 5% (w/v) non-fat dry milk in 1 × Tris-buffered saline at room temperature for 2 h, incubated with anti p-NF-κB antibody (1:1000 dilution, Cell Signaling Technology, USA.) at 4 °C overnight, followed by a secondary antibody, HRP-conjugated goat anti-mouse IgG (1:2000 dilution). The immunoreactive protein was determined using a Chemiluminescence ECL Western blotting detection kit (GE Healthcare, UK). The intensity of the protein band was measured relative to an internal control (β-actin) using a densitometry Scion image software package (a version of the NIH image program developed at the US National Institutes of Health and available at http://rsb.info.nih.gov/nih-image/distributed by the Scion Corporation, Frederick, MD).

2.3.2. Expression of immune related genes in shrimp by RT-PCR analysis

The whole shrimp (5 shrimp/group) were RNA extracted in 200 μl TRI reagent according to the manufacturer's protocol (Sigma

Table 1
Formulation of SG supplemented diet (% on a dry mass: w/w).

Ingredients	Control	0.5 SG	1 SG	2 SG
SG	0	0.5	1.0	2.0
Fish meal	30.0	30.0	30.0	30.0
Prawn mince	5.0	5.0	5.0	5.0
Soybean meal	9.3	9.3	9.3	9.3
Wheat gluten	6.0	6.0	6.0	6.0
Squid oil	4.0	4.0	4.0	4.0
Soybean lecithin	3.0	3.0	3.0	3.0
Cholesterol	0.5	0.5	0.5	0.5
Wheat flour	26.0	26.0	26.0	26.0
Cellulose	13.0	12.5	12.0	11.0
Mineral premix	2.3	2.3	2.3	2.3
Vitamin premix	0.9	0.9	0.9	0.9