The effect of sodium alginate on the immune response of tiger shrimp via dietary administration: Activity and gene transcription

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Abstract

The total haemocyte count (THC), phenoloxidase (PO) activity, respiratory bursts (release of superoxide anions), and superoxide dismutase (SOD) activity, as well as expressions of β-1,3-glucan-binding protein (βGBP), prophenoloxidase (proPO), peroxinectin (PE), cytosolic SOD (cyt-SOD), penaeidin-5 (PA-5), and a single whey acidic protein (WAP) domain protein (SWDP) gene were determined in the tiger shrimp Penaeus monodon (15.6–19.5 g) which had individually been fed diets containing sodium alginate at 0, 1.0, or 2.0 g kg⁻¹ for 5 months. Results showed that shrimp fed a diet containing 1.0 and 2.0 g kg⁻¹ sodium alginate had significantly increased SOD activity but decreased respiratory bursts. The expressions of βGBP, PE, cyt-SOD, PA-5, and SWDP were significantly elevated in shrimp fed the ≤2.0 g kg⁻¹ sodium alginate-containing diet for 5 months. However, no significant differences in THC, PO activity, or proPO mRNA transcription in shrimp were observed among the three treatments. It was concluded that sodium alginate can be used as an immunomodulator for shrimp through dietary administration to modify immune genes expression of shrimp.

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1. Introduction

Shrimp are one of the most important aquatic organisms in aquaculture. During the past few years, commercial shrimp farming has been severely hit due to epidemics associated with viruses and vibriosis, which have caused serious economic losses. Disease outbreaks are resulting from interactions of the three factors of a deteriorating

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environment, weakened hosts, and aggressive pathogens. Crustaceans do not have acquired immunity; instead they have an innate immune system, which includes melanisation by activation of the prophenoloxidase-activating system (proPO system), a clotting process, phagocytosis, encapsulation of foreign material, antimicrobial action, and cell agglutination [1]. Several components or associated factors of the proPO system have been found in crustaceans including the pattern recognition protein, proPO zymogen, a serine protease of the prophenoloxidase-activating enzyme (ppAE), pacifastin, a serine protease inhibitor, and a membrane receptor of the β-1,3-glucan-binding protein, cell adhesive protein; these are reviewed by Sritunyalucksana and Söderhäll [2].

In addition, several oxygen species are produced during phagocytosis, which play important roles in microbicidal activity [3], and are regulated by antioxidant molecules and antioxidant enzymes. A single WAP domain-containing protein which is probably related to serine proteinase inhibitors has been found in Litopenaeus vannamei and Penaeus monodon, and the expression of this molecule is modified by the inoculation of Vibrio alginolyticus showing its possible participation in the shrimp immune response [4]. The immune parameters described above could be used as indicators to evaluate the immune ability of shrimp under different situations.

The immune stimulatory effects of immunostimulants like glucan, chitosan, and other polysaccharides have been widely studied in fish and crustaceans and were reviewed by Sakai [5]. Administration of hot-water extracts of Porphyra yezoensis and Gloiopeltis furcata increased the resistance of common carp Cyprinus carpio, against Edwardsiella tarda, and yellowtail Seriola quinqueradiata against Streptococcus sp. infection [6]. Sodium alginate has been reported to enhance the resistance of C. carpio against E. tarda infection [7], and increase the non-specific defence system of C. carpio [8]. Immunostimulants increase the immune responses in several shrimp species by promoting phagocytosis, bactericidal activity, proPO activity, and respiratory bursts, and enhancing resistance against pathogens [9,10]. Administration of sodium alginate extracted from the brown algae, Macrocystis pyrifera and Lessonia nigrescens, has been reported to enhance the immune ability of L. vannamei, and increase its resistance against V. alginolyticus [11,12]. L. vannamei fed a diet containing 2.0 g kg⁻¹ sodium alginate had increased phenoloxidase activity, respiratory burst and superoxide dismutase (SOD) activity, and phagocytic activity to V. alginolyticus. The shrimp fed a diet containing sodium alginate at 0.5, 1.0 or 2.0 g kg⁻¹ had increased clearance efficiency to V. alginolyticus [12]. However, the immune genes transcription of shrimp following immunostimulators administration is yet to be clarified.

The purpose of this study was to examine the immune parameters, and to evaluate the immune gene expression of the tiger shrimp Penaeus monodon, after feeding diets containing sodium alginate from L. nigrescens. Several immune parameters such as THC, PO activity, respiratory bursts, and SOD activity, as well as the immune gene transcripts of β-1,3-glucan binding protein (βGBP), prophenoloxidase (proPO), peroxinectin (PE), cytosolic superoxide dismutase (cyt-SOD), penaeidin-5 (PA-5), and a single WAP domain protein (SWDP) were determined when shrimp were fed diets containing different concentrations of sodium alginate for 5 months.

2. Materials and methods

2.1. Diet preparations

Diets containing three different levels of sodium alginate were prepared by the Shinta Feed Company (Pingtung, Taiwan). The basal diet contained 0.2% cellulose. Sodium alginate (Kimitsu Algina I-1, Kimitsu Chemical Industries, Chiba, Japan) was added to the test diets at levels of 1.0 and 2.0 g (kg diet)⁻¹ with a corresponding decrease in the amount of cellulose. The finished pellets were stored at 4 °C until use.

2.2. Experimental design

Postlarvae of P. monodon obtained from a private farm (Linyuan, Kaohsiung, Taiwan) were shipped to the Department of Aquaculture, and were acclimated in indoor cement ponds (12 × 12 × 1 m), and fed the control diet for 1 week. At the beginning of the trial, six ponds (6 × 6 × 1 m) were each stocked with 5000 juveniles (equivalent to a density of 139 shrimp m⁻³). Each diet was fed to two groups of shrimp. Ponds received continuous aeration, and 50% of the water was exchanged weekly to maintain the water quality. During the grow-out periods, water temperature ranged from 18.5 to 28 °C, pH from 6.8 to 8.4, salinity from 15 to 25‰, and the dissolved oxygen concentration from 6.5 to 7.7 mg L⁻¹. Shrimp were fed their respective diets at rate of 2–5% body weight at 08:00, 15:00,
and 22:00 h. After 5 months, the shrimp were sampled and acclimated in the laboratory for 1 week for subsequent tests. During the acclimation period, the shrimp continued to be fed the test diets twice daily. Only shrimp in the intermolt stage were used for the subsequent tests. The molt stage was determined by examining the uropoda in which partial retraction of the epidermis could be distinguished [13].

Two studies were conducted. For the studies of immune parameters, tests were carried out in ten-replicate test and control groups consisting of one shrimp for each treatment. For the studies of immune gene expression, tests were carried out in ten-replicate test and control groups consisting of one shrimp for each treatment. The shrimp ranged from 15.6 to 19.5 g, averaging 16.6 ± 1.2 g (mean ± SD) with no significant size differences among treatments.

2.3. Immune parameters of *P. monodon*

Haemolymph (100 μl) was withdrawn from the ventral sinus of each shrimp into a 1-ml sterile syringe (with 25-gauge needle) containing 0.9 ml of an anticoagulant solution (30 mM sodium citrate, 0.34 M sodium chloride, 10 mM EDTA (pH 7.55), with the osmolality adjusted with glucose to 780 mOsm kg⁻¹). A drop of the anticoagulant-haemolymph mixture was placed on a haemocytometer to measure THC using an inverted phase-contrast microscope (Leica DMIL, Leica Microsystems, Wetzlar GmbH, Germany). The remainder of the haemolymph mixture was used for subsequent tests.

Phenoloxidase activity was measured spectrophotometrically at 490 nm by recording the formation of dopachrome following the procedures of Mason [14] and Hernández-López et al. [15]. The optical density of the background phenoloxidase activity was in the range of 0.02–0.06. The optical density of the shrimp’s phenoloxidase activity was expressed as dopachrome formation in 50 μl of haemolymph.

Respiratory bursts of haemocytes were quantified using the reduction of NBT (nitroblue tetrazolium) to formasan as a measure of superoxide anions (O₂⁻) as described previously [16,17]. The optical density at 630 nm was measured using a microplate reader (Model VERSAmax, Molecular Devices, Sunnyvale, CA, USA). Respiratory bursts were expressed as NBT-reduction in 10 μl of haemolymph.

Superoxide dismutase (SOD) activity was measured by its ability to inhibit superoxide radical-dependent reactions using the Ransod Kit (Randox, Crumlin, UK) based on previously described methods [18,19]. One unit of SOD was defined as the amount required for inhibiting the rate of xanthine reduction by 50%. Specific activity was expressed as SOD units (g protein)⁻¹. The protein was quantified by the method described by Bradford [20] using a Bio-Rad protein assay kit (no. 500-0006, Bio-Rad Laboratories, USA) with bovine serum albumin as the standard.

2.4. Immune gene expression of *P. monodon*

2.4.1. Haemocyte collection

Haemolymph (0.50 ml) was individually withdrawn from the ventral sinus cavity of each shrimp into a 1-ml sterile syringe (with a 25-gauge needle) containing 0.5 ml of precooled (4 °C) anticoagulant solution (0.45 M NaCl, 0.1 M glucose, 30 mM sodium citrate, 26 mM citric acid, 10 mM EDTA, at pH 7.5 and with an osmolality of 780 mOsm kg⁻¹). The diluted haemolymph was centrifuged at 500 × g and 4 °C for 20 min, and the haemocyte pellet was washed once with cacodylate buffer (10 mM sodium cacodylate, 0.45 M sodium chloride, 20 mM calcium chloride; pH 7.0). The resulting haemocyte pellet was then used for the total RNA isolation.

2.4.2. Total RNA isolation and reverse transcription (RT)

Total RNA was extracted and purified using the guanidinium thiocyanate method described by Chomczynski and Sacchi [21]. First-strand cDNA synthesis by reverse transcription (RT) was as described previously [22].

2.4.3. Primer design

The specific primer pairs were designed for each experimental gene’s mRNA sequence using primer express software (Applied Biosystems, Foster City, CA, USA) or according to previous descriptions as shown below.

- βGBP forward primer, 5′-CATGTCGAACCTTCGTTTCAGA-3′ and reverse primer, 5′-CACCCGCAGTGGCATCTTG-3′ (accession no.: AF368168);
- proPO forward primer, 5′-CGACTCCTGGATGCCACATCAT-3′ and reverse primer, 5′-CATCCGGAAGAGGAACCTTGGT-3′ (AF521948);
- PE forward primer, 5′-GGACATGGTCTAATG-3′ and reverse primer, 5′-GTTCGCGGGCGACATCT-3′ (AF188840);
- cyt-SOD forward primer,
5'-GGCTGGTACAGTCAGTCCTCAGA-3' and reverse primer, 5'-CCTCACCCAATTCAGCATTGA-3' (BI784454); PA-5 forward primer, 5'-TCCCCAGACCACCCTATGG-3' and reverse primer, 5'-GGCTAAGCCTGTGGCATGA-3' (AY326471); SWDP forward primer, 5'-GCCAGACGGATTGGGATTG-3' and reverse primer, 5'-AGCCATTTTACGGCAGCATATG-3' (BI784457); and β-actin forward primer, 5'-CACCACCGCTGAACGAGAA-3' and reverse primer, 5'-AAGGGCGACATAGCAAAGTTTC-3' (AF100986).

2.4.4. Quantification of immune gene expression by real-time RT-PCR

The mRNA expressions of immune genes of shrimp, which had been individually fed diets containing sodium alginate at 0, 1.0, or 2.0 g kg\(^{-1}\) for 5 months, were measured by real-time RT-PCR. The cDNA was used for the assay of real-time RT-PCR. The SYBR green I real-time RT-PCR assay was carried out in an ABI PRISM 7900 Sequence Detection System (Applied Biosystems). The amplifications were performed in a 96-well plate in a 25-μl reaction volume containing 12.5 μl of 2 × SYBR Green Master Mix (Perkin-Elmer Applied Biosystems), 2.5 μl each of the forward and reverse primers (10 μM), 1 μl of template (1 μg cDNA), and 9 μl of DEPC-water. The thermal profile for the SYBR green real-time RT-PCR was 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. In a 96-well plate, each sample was run in duplicate. DEPC-water replaced the template as the negative control.

Data analysis of the RT-PCR was performed with SDS software version 2.0 (PE Applied Biosystems). Relative quantitation of gene expression was performed according to the manufacturer’s instructions. Briefly, the threshold PCR cycle (C\(_t\)) is defined as the cycle number at which a statistically significant increase in the fluorescence of SYBR green against the internal passive dye, ROX (ΔR\(_o\)), is first detected. The copy number of the target gene and C\(_t\) values are inversely related; thus, a sample containing a higher number of copies of the target gene has a lower

![Graph A](A) Respiratory bursts vs Sodium alginate (g kg\(^{-1}\))

![Graph B](B) SOD activity vs Sodium alginate (g kg\(^{-1}\))

Fig. 1. Respiratory bursts (A) and superoxide dismutase activity (B) of Penaeus monodon fed the control and 1.0 and 2.0 g kg\(^{-1}\) sodium alginate-containing diets. Each bar represents the mean value from ten determinations with the standard error. Data (mean ± SE) with different letters significantly different (\(p < 0.05\)) among treatments.
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 β-actin gene, called ΔCt, were calculated to normalise 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 ΔCt for the immunostimulated sample was subtracted from the value of ΔCt of the control sample without immunostimulation. The difference was expressed as the ΔΔCt value that allowed measurement of the change in expression of immune genes in the immunostimulated sample relative to the control sample. A 3.3-fold change in the Ct value was considered to be equivalent to a 10-fold change in expression level.

2.5. Statistical analysis

Using SAS computer software (SAS Institute, Cary, NC, USA), a multiple comparison (Tukey) test was conducted to compare the significant differences of immune parameter activities and gene expressions among treatments. Statistically significant differences required that P < 0.05.

3. Results

3.1. Immune parameters of P. monodon

No significant differences in THC or PO activity were observed among the shrimp fed 1.0 and 2.0 g kg\(^{-1}\) sodium alginate-containing diets and the control diet. The mean (±SE) THC values varied from 206.9 ± 25.8 to 244.8 ± 24.3 \(\times\) 10\(^5\) cells ml\(^{-1}\).

![Graph A](image1.png)

**Fig. 2.** Measurement of β-1,3-glucan-binding protein (βGBP) mRNA expression in *Penaeus monodon* fed the control and 1.0 and 2.0 g kg\(^{-1}\) sodium alginate-containing diets by SYBR green RT-PCR. Each bar represents the ΔCt of βGBP (the Ct value of the βGBP gene minus the Ct value of the β-actin gene) as measured by SYBR green RT-PCR. Numbers above the bars indicate the ΔΔCt value (the ΔCt value of βGBP in each treatment shrimp minus the ΔCt value of βGBP in control shrimp) (A). A change in the Ct value (ΔΔCt) of 3.3 is equivalent to a 10-fold difference between treatment and control shrimp (B). Data (mean ± SE) with different letters significantly different (p < 0.05) among treatments.
Respiratory bursts of shrimp that were fed 1.0 and 2.0 g kg\(^{-1}\) sodium alginate containing diets were significantly lower than those of shrimp that were fed the control diet. The relative level of respiratory bursts (compared to the activity of control group) of shrimp fed 1.0 and 2.0 g kg\(^{-1}\) sodium alginate-containing diets decreased to 53.4% and 52.7%, respectively (Fig. 1A).

The SOD activity of shrimp fed the 1.0 and 2.0 g kg\(^{-1}\) sodium alginate-containing diets were significantly higher than that of shrimp fed the control diet and increased to 141.6% and 143.1%, respectively (Fig. 1B).

### 3.2. Immune gene expressions

The mRNA expressions of immune genes of shrimp fed the 0, 1.0, and 2.0 g kg\(^{-1}\) sodium alginate-containing diets for 5 months were measured using real-time RT–PCR. The βGBP gene expression increased with an increase of sodium alginate in the diet and was significantly higher in shrimp fed the 2.0 g kg\(^{-1}\) sodium alginate-containing diet than those fed the control diet (0 g kg\(^{-1}\)). The βGBP gene expression levels (expressed as Δ\(C_t\)) were 9.2 ± 0.4, 8.2 ± 0.2, and 7.2 ± 0.5 in shrimp fed 0, 1.0, and 2.0 g kg\(^{-1}\) sodium alginate-containing diets, respectively. Therefore, the βGBP gene expression difference (ΔΔ\(C_t\)) of shrimp fed the 1.0 and 2.0 g kg\(^{-1}\) sodium alginate-containing diets were −0.97 and −1.97, respectively, relative to shrimp fed the control diet (Fig. 2A). Considering that a ΔΔ\(C_t\) value of 3.3 is equivalent to a 10-fold difference, shrimp fed the 1.0 and 2.0 g kg\(^{-1}\) sodium alginate-containing diets had approximately 2.9- and 6.0-fold higher expressions of βGBP mRNA, respectively, than did shrimp fed the control diet (Fig. 2B).

No significant difference in proPO mRNA transcription of shrimp was observed among the three treatments.

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**Fig. 3.** Measurement of peroxinectin (PE) mRNA expression in *Penaeus monodon* fed the control and 1.0 and 2.0 g kg\(^{-1}\) sodium alginate-containing diets by SYBR green RT-PCR. Each bar represents the Δ\(C_t\) of PE (the \(C_t\) value of the PE gene minus the \(C_t\) value of the β-actin gene) as measured by SYBR green RT-PCR. The ΔΔ\(C_t\) (the Δ\(C_t\) value of PE in each treatment shrimp minus the Δ\(C_t\) value of PE in control shrimp) represents the relative expression of PE between two treatments (A). A change in the \(C_t\) value (ΔΔ\(C_t\)) of 3.3 is equivalent to a 10-fold difference between treatment and control shrimp (B). Data (mean ± SE) with different letters is significantly different (\(p < 0.05\)) among treatments.
PE mRNA transcription of shrimp fed the 1.0 and 2.0 g kg\(^{-1}\) sodium alginate-containing diets was significantly higher than those fed the control diets. PE gene expression of shrimp fed the 1.0 and 2.0 g kg\(^{-1}\) sodium alginate-containing diets were −0.74 and −1.0, respectively, relative to shrimp fed the control diet (Fig. 3A). Shrimp fed the 1.0 and 2.0 g kg\(^{-1}\) sodium alginate-containing diets had approximately 2.2- and 3.0-fold higher expressions of PE mRNA, respectively, than did shrimp fed the control diet (Fig. 3B).

cyt-SOD gene expression was significantly higher in shrimp fed the 2.0 g kg\(^{-1}\) sodium alginate-containing diet than those fed the control diet and the 1.0 g kg\(^{-1}\) sodium alginate-containing diet (Fig. 4A). The cyt-SOD gene expression was 1.9-fold (\(\Delta\Delta C_t = -0.63\)) higher in shrimp fed the 2.0 g kg\(^{-1}\) sodium alginate-containing diet than in shrimp fed the control diet (Fig. 4B).

PA-5 mRNA expression of shrimp fed the 1.0 and 2.0 g kg\(^{-1}\) sodium alginate-containing diets were significantly higher than those fed the control diet (Fig. 5A). PA-5 gene expressions were 1.9- (\(\Delta\Delta C_t = -0.62\)) and 1.4-fold (\(\Delta\Delta C_t = -0.47\)) higher in shrimp fed the 1.0 and 2.0 g kg\(^{-1}\) sodium alginate-containing diets, respectively, compared to values of shrimp fed the control diet (Fig. 5B).

SWDP gene expression was significantly higher in shrimp fed the 2.0 g kg\(^{-1}\) sodium alginate-containing diet than those fed the control diet and the 1.0 g kg\(^{-1}\) sodium alginate-containing diet (Fig. 6A). WAP gene expression was 4.6-fold (\(\Delta\Delta C_t = -1.51\)) higher in shrimp fed the 2.0 g kg\(^{-1}\) sodium alginate-containing diet than in shrimp fed the control diet (Fig. 6B).

4. Discussion

proPO is one of the proteins of the proPO system that plays a role in an important defence reaction in crustaceans [23,24]. Conversion of proPO to PO occurs by a serine protease named ppAE [25]. In the previous study, *L. vannamei*
fed a diet containing sodium alginate at 1.0 and 2.0 g kg\(^{-1}\) for 5 months had increased PO activity, and at 2.0 g kg\(^{-1}\) had increased respiratory bursts and SOD activity. However, no significant differences in the total haemocyte count (THC), SGCs, or GCs were observed among juvenile *L. vannamei* shrimp fed diets containing 0, 0.5, 1.0, and 2.0 g kg\(^{-1}\) sodium alginate [12]. In the present study, *Penaeus monodon* fed a diet containing sodium alginate at 1.0 and 2.0 g kg\(^{-1}\) for 5 months had significantly increased SOD activity but decreased respiratory bursts. However, no significant differences in the THC and PO activity were observed among the three treatments. The diversity of PO activity and respiratory bursts induced by sodium alginate may be related to the species studied, and the increase of respiratory bursts may also be resulted from the increase of SOD activity.

With injections of laminarin, increased levels of proPO mRNA in haemocytes resulted, whereas levels of actin or PE transcripts remained unchanged in *Astacus astacus* [26]. However, no significant differences in the levels of proPO, actin, or PE mRNA transcripts were observed in *Pacifastacus leniusculus* [26]. The increase in proPO mRNA in *A. astacus* resulted from both an increase in granular haemocytes (GCs) as well as increased expression of this transcript in semi-granular haemocytes (SGCs) [26]. In the present study, no significant differences in proPO mRNA transcription or THC were observed in *P. monodon* fed a diet containing 0, 1.0, and 2.0 g kg\(^{-1}\) sodium alginate. However, PE mRNA transcription significantly increased when the shrimp were fed diets containing sodium alginate at 1.0 and 2.0 g kg\(^{-1}\). These results suggest that the levels of proPO and PE mRNA expression of crustaceans induced by immunostimulants may be related to the species of crustaceans, the type of stimulants, or the methods of administration.

Several pattern recognition proteins (PRPs), such as LPS-binding protein (LBP), \(\beta\)-1,3-glucan-binding protein (\(\beta\)GBP), and lipopolysaccharide- and \(\beta\)-1,3-glucan-binding protein (LGBP), play critical roles in crustaceans-pathogen interactions.

**Fig. 5.** Measurement of penaeidin-5 (PA-5) mRNA expression in *Penaeus monodon* fed the control and 1.0 and 2.0 g kg\(^{-1}\) sodium alginate-containing diets by SYBR green RT-PCR. Each bar represents the \(\Delta C_t\) of PA-5 (the \(C_t\) value of the PA-5 gene minus the \(C_t\) value of the \(\beta\)-actin gene) as measured by SYBR green RT-PCR. The \(\Delta\Delta C_t\) (the \(C_t\) value of PA-5 in each treatment shrimp minus the \(C_t\) value of PA-5 in control shrimp) represents the relative expression of PA-5 between two treatments (A). A change in the \(C_t\) value (\(\Delta\Delta C_t\)) of 3.3 is equivalent to a 10-fold difference between treatment and control shrimp (B). Data (mean ± SE) with different letters significantly different \((p < 0.05)\) among treatments.
interactions. They recognise and respond to intruders and are involved in activation of the proPO system, the coagulation cascade, and expression for antibacterial effector proteins [27], and act as an opsonin to increase the rate of phagocytosis [28]. Dietary administration of sodium alginate extracted from *L. nigrescens* has been reported to increase PO activity, phagocytic activity, and the clearance efficiency of *L. vannamei* [12]. In the present study, GBP and penaeidin gene expressions were significantly increased in shrimp fed a diet containing sodium alginate at 2.0 g kg$^{-1}$ for 5 months. The results suggest that sodium alginate orally administered enhances the activation and/or activity of the proPO system, the clearance efficiency, and the mechanism of phagocytosis which might result from GBP and antibacterial peptide gene transcriptions in shrimp.

PE has multiple biological functions of cell adhesion [29], degranulation [27], encapsulation enhancement [29], opsonisation [30], and peroxidation [31]. These biological activities of PE are generated concomitant with activation of the proPO system [32]. When a foreign particle enters the haemolymph of a host, haemocytes recognise the foreign intruder as a non-self molecule and change from non-adhesive cells to adhesive cells, and strongly adhere to the foreign target. Haemocytes attach and spread across the surface of the foreign intruder, and form a multilayered sheath of cells during encapsulation. Therefore, PE is essential in the crustacean cellular defence reaction for enhancement of encapsulation and phagocytosis [33]. The present study indicated that *P. monodon* fed diets containing 1.0 and 2.0 g kg$^{-1}$ sodium alginate had approximately 2.3- and 13.1-fold higher expressions of PE mRNA, respectively, than did shrimp fed the control diet. These results suggest that sodium alginate enhances the cellular defence reaction by enhancing encapsulation and phagocytosis resulting from activation of the proPO system in *P. monodon*.

Fig. 6. Measurement of single whey acidic protein (WAP) domain protein (SWDP) mRNA expression in *Penaeus monodon* fed the control and 1.0 and 2.0 g kg$^{-1}$ sodium alginate-containing diets by SYBR green RT-PCR. Each bar represents the $\Delta C_t$ of SWDP (the $C_t$ value of the SWDP gene minus the $C_t$ value of the $\beta$-actin gene) as measured by SYBR green RT-PCR. The $\Delta \Delta C_t$ (the $C_t$ value of SWDP of treatment shrimp minus the $C_t$ value of SWDP of control shrimp) represents relative expression of SWDP between two treatments (A). A change in the $C_t$ value ($\Delta \Delta C_t$) of 3.3 is equivalent to a 10-fold difference between treatment and control shrimp (B). Data (mean ± SE) with different letters significantly different ($p < 0.05$) among treatments.
Reactive oxygen intermediates (ROIs) are released during respiratory bursts of phagocytosis, which represent a defence mechanism against microbial infection [3]. However, the excessive accumulation of ROIs is extremely toxic to host cells. Under a normal physiological state, harmful effects of ROIs are effectively neutralised by the antioxidative defence system of organisms, which in general comprises enzymes like superoxide dismutases, catalase, and various peroxidases, and small antioxidant molecules like ascorbate, polyunsaturated fatty acids, and sugars. Therefore, the activity of an antioxidant is usually considered to be related to the defence response in living systems [34].

SOD and PE belong to antioxidative enzymes that scavenge superoxide anions (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$), respectively, in crustaceans [34]. In the present study, dietary administration of sodium alginate significantly decreased respiratory bursts, and increased SOD activity, and PE and SOD gene expressions in haemocytes of _P. monodon_. These facts suggest that sodium alginate enhances regulation of the antioxidative mechanism in _P. monodon_ by increasing the expression of antioxidative enzymes. Similar to PO activity and antibiotic peptides, the antioxidative enzymes are important components necessary for a fully functioning crustacean immune system [34]. Therefore, the increased PE and SOD transcripts in _P. monodon_ also suggest that sodium alginate enhances both the immune and antioxidative defence systems in shrimp.

Proteins containing the whey acidic protein (WAP) domain were initially associated with serine proteinase inhibitors [35–37]; however, this domain is present in other proteins as well and with a variety of functions. A single WAP domain protein (SWDP), similar to mouse single WAP motif (SWAM) proteins, was described in haemocytes of penaeid shrimp (_L. vannamei_ and _P. monodon_), and modification of its expression was observed in _L. vannamei_ after inoculation with _V. alginolyticus_, indicating the probable role of this protein in the immune response [4]. SWAMs are antibacterial proteins, which have been identified in the mouse [37]. In the present study, the expression of SWDP in _P. monodon_ was remarkably increased after shrimp were fed a 2.0 g kg$^{-1}$ sodium alginate-containing diet. These facts suggest that sodium alginate enhances serine proteinase inhibitors and/or antibacterial activity to help regulate the immunity of shrimp.

In conclusion, the present investigations clearly indicate that _P. monodon_ fed a diet containing 1.0 or 2.0 g kg$^{-1}$ sodium alginate experienced enhanced SOD activity, and βGBP, PE, SOD, PA, and SWDP mRNA transcriptions, which may be resulting in increases in the immune ability of the shrimp. Sodium alginate can be used as an immunomodulator for shrimp through diet administration to modify immune genes expression of shrimp.

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**References**


Liu CH, Yeh ST, Cheng SY, Chen JC. The immune response of the white shrimp *Litopenaeus vannamei* and its susceptibility to *Vibrio* infection in relation with the moult cycle. Fish and Shellfish Immunology 2004;16:151–61.


