The Effect of Dietary Chitosan and Chitin Supplementation on the Survival and Immune Reactivity of Crayfish, *Procambarus clarkii*

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Abstract. – White spot syndrome virus (WSSV) is an important viral pathogen responsible for severe economic loss to shrimp aquaculture. The effect of oral administration of chitosan and chitin against WSSV in crayfish *Procambarus clarkii* was investigated. Six groups of 20 crayfish, *P. clarkii*, in triplicate were diet supplemented with chitosan or chitin at 5, 10, and 15 mg/g for 4 wks, and orally challenged with WSSV. The cumulative mortalities in the groups fed with chitosan at 10 mg/g was significantly lower (*P* < 0.05) than the control but the other groups were not. The relative percent survival (RPS) showed that chitosan provided better protection against WSSV than chitin (*P* < 0.05). Polymerase chain reaction (PCR) analysis showed that the surviving crayfish were WSSV negative. The immunological parameters analyzed revealed that the crayfish fed with chitosan and chitin showed significantly higher level of total hemocyte count (THC), prophenoloxidase (proPO), and superoxide dismutase (SOD) when compared to the control groups.

White spot syndrome (WSS) is an important shrimp viral disease, which affects many species of commercially cultivated shrimp, not just in Asia but globally. The causative organism, white spot syndrome virus (WSSV), is responsible for huge economic loss to shrimp farmers. WSSV is a rod-shaped, nonoccluded, enveloped and double-stranded DNA virus of a new genus Whispovirus in a new family Nimaviridae (Wongteerasupaya et al. 1995; Lightner 1996; Mayo 2002; Escobedo-Bonilla et al. 2008). Sequencing of three different WSSV isolates revealed that the dsDNA genome is about 300 kb in size (Tsai et al. 2000; Van Hulten et al. 2001; Yang et al. 2001).

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The nucleotide sequence analysis revealed that the WSSV genome encodes approximately 184 open reading frames (ORFs) of 50 amino acids or more. WSSV has been found to be highly pathogenic not only to penaeid shrimp, but also to many species of crustacean including crayfish (Lo et al. 1996; Wang et al. 1998; Jiranvanichpaisal et al. 2001). It causes up to 100% mortality within 7–10 d in commercial shrimp farms, and resulting in large economic losses (Chou et al. 1995).

The immune stimulatory effects of immunostimulants such as glucan, chitosan, chitin, and other polysaccharides have been widely studied in crustaceans (Smith et al. 1984; Itami et al. 1994; Song and Huang 1999; Chang et al. 2000; Wang and Chen 2005; Powell et al. 2007). Polysaccharides also have been successfully used to enhance resistance of crustaceans against bacterial and viral infections (Sung et al. 1994; Itami et al. 1998; Chang et al. 1999; Chang et al. 2003; Chotigeat et al. 2004). Oral administration of β-1,3-glucan extracted from the fungus, *Schizophyllum commune*, has been reported to increase the resistance of *Penaeus monodon* against WSSV infection (Chang et al. 2003).

Chitin is a β-1,4-linked polymer of *N*-acetyl-d-glucosamine, one of the most abundant polysaccharides in nature, and a common constituent of insect, exoskeleton, crustacean shells and fungal cell walls. Chitosan is a linear homopolymer of β-(1,4)-2-amino-2-deoxy-d-glucose, and is prepared by the alkaline deacetylation of chitin obtained from crab shells (Rinaudo 2006). As immunostimulants,
chitosan and chitin have been proved that they can enhance the nonspecific immunity and prevent resistance against disease (Esteban et al. 2000; Esteban et al. 2001; Wang and Chen 2005; Powell and Rowley 2007). *Litopenaeus vannamei* received chitin at 6 μg/g or chitosan at 4 μg/g may less increased its immune ability and resistance to *Vibrio alginolyticus* infection (Wang and Chen 2005). Chitosan could inhibit viroid infection in tomato plants when added to the inoculum and when sprayed into the leaves prior to viroid inoculation (Pospieszny 1997).

This study was conducted in an attempt to optimize the dietary use of chitosan and chitin to protect *Procambarus clarkii* against WSSV. The mortality was measured during the course of challenging to assess its resistance to WSSV when the crayfish were fed with chitosan or chitin for 4 wks.

**Materials and Methods**

**Crayfish and Virus**

Crayfish, *P. clarkii*, approximately 20 g, were reared at 25 ± 1 C. They were kept in tanks with sand-filtered, ozone-treated, flow-through freshwater and fed with commercial crayfish pellet feed (Dajiang Feed Company, Huaian, China) at 5% of body weight per day. The first walking legs from randomly selected individuals were subjected to polymerase chain reaction (PCR) assays to ensure that the crayfish were WSSV-free before experimental challenge. WSSV-infected shrimp, *Fenneropenaeus chinensis*, were collected from shrimp farms located near Ningbo, China. The tail muscle of shrimp was removed and used to infect crayfish for the challenge test.

**PCR Analysis and Microscopy for WSSV**

Total DNA was extracted from walking legs of crayfish with an animal tissue genomic DNA mini-prep kit (Sangon, Shanghai, China). The samples were tested with the primer set VP28-FW (5′-CTTTCACTCTTTCCGTCGTG-3′) and VP28-RV (5′-TTCTGCCCCACAGTCACCTC-3′) (AF502435), amplifying part of WSSV VP28 gene, was used to screen for WSSV-positive animals. PCR was performed with the VP28 primer pair using the following protocol: 5 min at 94 C followed by 35 cycles at 94 C for 1 min, 55 C for 1 min and 72 C for 1 min (Jha et al. 2006). The PCR products were analyzed by electrophoresis on 1% agarose gels stained with ethidium bromide and visualized by ultraviolet transillumination. Then collected hemolymph of crayfish that PCR analysis showed positive or negative were negatively stained with 2% sodium phosphotungstate (PTA, pH 7.0) on collodion-carbon coated grids and made observations with TEM immediately to detect WSSV.

**Experimental Procedure**

Chitosan (Sigma C-3646, St. Louis, MO, USA) and chitin (Sigma C-9752) used in this study were blended with commercial crayfish feed powder (Dajiang Feed Company, Huaian, China) at 5, 10, or 15 mg/g to formulate two experimental diets. Crayfish, *P. clarkii*, three test groups of 20 crayfish in triplicate were fed with 5, 10, and 15 mg/g chitosan, and another three test groups of 20 crayfish in triplicate were fed with 5, 10, and 15 mg/g chitin for 4 wks. The positive control and the negative control of 20 crayfish were fed with common crayfish feed. For the study of resistance of crayfish to WSSV, six test groups and the positive control were orally challenged with tail muscle from crayfish infected with WSSV at the dosage of 1 g per crayfish. And the negative control was fed with tail muscle from healthy crayfish. The animals were observed twice a day for clinical signs of disease and mortality; the number of deaths was recorded and the cumulative percentage of mortality was calculated.

**Total Hemocyte Count (THC) Assay**

Hemolymph (100 μL) was withdrawn from the ventral sinus of each crayfish into a 1 mL sterile syringe (25 gauge) containing 0.9 mL anticoagulant solution (trisodium citrate 30 mM, sodium chloride 0.34 M, ethylenediaminetetraacetic acid (EDTA) 10 mM, pH 7.55, osmolality adjusted with glucose to 780 mOsm/
A drop of the anticoagulant-hemolymph mixture (100 μL) was placed on a hemocytometer, and a THC was made under an inverted phase-contrast microscope (Leica DMIL, Germany).

**Prophenoloxidase (proPO) Assay**

ProPO activity was measured spectrophotometrically by recording the formation of dopachrome produced from L-dihydroxyphenyl alanine (L-DOPA) according to the method of Hernandez-Lopez et al. (1996). Briefly, the diluted hemolymph was centrifuged at 800 \( \times \) g at 4 \( ^\circ \)C for 20 min to collect the pellet which was resuspended gently in cacodylate buffer (0.01 M sodium cacodylate, 0.45 M sodium chloride, 0.10 M trisodium citrate, pH 7.0). The suspended pellet was centrifuged again and the pellet was resuspended with 100 μL of cacodylate buffer. The resuspended pellet was incubated with 50 μL trypsin (T-0303, Sigma, 1mg/mL) at 25°C for 10 min, which served as an activator; 50 μL L-DOPA was then added followed by 800 μL of cacodylate buffer 5 min later. The optical density at 490 nm was measured using a UV-VIS spectrophotometer-117 (Systronics, Shanghai, China).

**Superoxide Dismutase (SOD) Assay**

SOD activity was determined according to the method of Beauchamp and Fridovich (1971) using nitro blue tetrazolium (NBT) chloride in the presence of riboflavin. Briefly, 100 μL of hemolymph was homogenized in a mechanical homogenizer containing 0.5 mL of phosphate buffer (50 mM, pH 7.8). The homogenate was centrifuged for 5 min at 5724 \( \times \) g at 4°C and the supernatant recovered was heated for 5 min at 65°C to obtain a new supernatant after centrifugation (crude extract), which was stored at −20°C until use. Samples were maintained on ice at all times to avoid protein denaturation. A mixture of NBT, 20 mM of reaction mixture (0.1 mM EDTA, 13 mM methionine, 0.75 mM NBT, and 20 mM riboflavin in phosphate buffer, 50 mM, pH 7.8) and 0–100 μL of the crude extract were placed under fluorescent light for 2 min or until A560 in the control tubes reached 0.2–0.25 OD. The results were expressed as relative enzyme activity.

**Statistical Analysis**

All data obtained from the experiments were analyzed using one-way analysis of variance (ANOVA) \( (P < 0.05 \) as significant level). Statistical calculations were performed using SPSS 11.0 software. The protection against WSSV after vaccination is given as the relative percent survival (RPS), calculated as (1-vaccinated group mortality/control group mortality) × 100% as described by Amend (1981) (Table 1).

**Results**

**WSSV Challenge Test**

The cumulative mortality and RPS in chitosan or chitin groups are given in Table 1, whereas the resulting time-mortality relationships in the second experiment are shown in Figure 1. The mortality of 100% was recorded in the

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of crayfish</th>
<th>Mortality (%)</th>
<th>RPS (%)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 mg/g chitosan</td>
<td>20 ( \times ) 3</td>
<td>75 ± 5</td>
<td>25 ± 5</td>
<td>0.056</td>
</tr>
<tr>
<td>10 mg/g chitosan</td>
<td>20 ( \times ) 3</td>
<td>60 ± 5</td>
<td>40 ± 5</td>
<td>0.006*</td>
</tr>
<tr>
<td>15 mg/g chitosan</td>
<td>20 ( \times ) 3</td>
<td>75 ± 5</td>
<td>25 ± 5</td>
<td>0.056</td>
</tr>
<tr>
<td>5 mg/g chitin</td>
<td>20 ( \times ) 3</td>
<td>95</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>10 mg/g chitin</td>
<td>20 ( \times ) 3</td>
<td>85 ± 5</td>
<td>15 ± 5</td>
<td>0.23</td>
</tr>
<tr>
<td>15 mg/g chitin</td>
<td>20 ( \times ) 3</td>
<td>85 ± 5</td>
<td>15 ± 5</td>
<td>0.23</td>
</tr>
<tr>
<td>Positive control</td>
<td>20</td>
<td>100</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Negative control</td>
<td>20</td>
<td>0</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

*Significant difference (5% level) compared with the corresponding unvaccinated group is indicated by *.

Table 1. Resistance against experimental WSSV infection in Procambarus clarkii fed with 5, 10, and 15 mg/g chitosan or chitin.
positive control group on the 15th day and no mortality was found in the negative group. Clinical signs were observed after 3 d post-challenge of WSSV and WSSV infections in crayfish caused a rapid reduction in feed intake and lethargy. Then death occurred significantly after 5 d for the challenged crayfish (Fig. 1).

The RPS of crayfish that received chitosan at a dose of 5, 10, and 15 mg/g were higher than that of crayfish that received chitin at a dose of 5, 10, and 15 mg/g and the control crayfish on Day 18 post-challenge. RPS indicated that *P. clarkii* received chitosan at a dose of 10 mg/g increased its resistance against
WSSV significantly compared with the control ($P < 0.05$). All dead crayfish were WSSV positive but all surviving crayfish were WSSV negative by PCR detection and transmission electron microscopy. It is therefore suggested that the virus was not able to multiply in the surviving crayfish.

**Immunological Parameters**

The THC of crayfish fed the diets supplemented with chitosan or chitin was significantly higher ($P < 0.05$) than the control group (Table 2), and crayfish fed diets supplemented with chitosan had significantly higher ($P < 0.05$) THC than in chitin (Table 2). Similarly, proPO activity in plasma was significantly higher ($P < 0.05$) in the crayfish fed the diets supplemented with chitosan or chitin than in the control group (0.21 ± 0.02 Unit), but crayfish fed diets supplemented with 10 and 15 mg/g chitosan had significantly higher ($P < 0.05$) proPO activity than in chitin (Table 2). However, no significant differences were found between the 5 mg/g chitosan and 10 or 15 mg/g chitin. SOD activity in plasma was also significantly higher ($P < 0.05$) in the crayfish fed the diets supplemented with chitosan and chitin than in the control group (0.22 ± 0.02 Unit), and crayfish fed diets supplemented with chitosan had significantly higher ($P < 0.05$) SOD activity than in chitin (Table 2).

**Discussion**

The main finding was that the dietary addition of chitosan and chitin reduced the mortality of crayfish (*P. clarkii*) when orally challenged with WSSV. However, the cumulative RPS of chitosan was higher than chitin but not significantly ($P > 0.05$). Interestingly, 10 mg/g chitosan (RPS, 60%) showed better protective effect than 5 and 15 mg/g chitosan (RPS, 75%). Powell et al. (2007) reported that 5% chitin-fed group had the lowest mortality of 61.5% but in the 10% chitin-fed group the mortality was 85.7%.

Analysis of immunological parameters also confirmed that crayfish fed diets supplemented with chitosan had significantly higher ($P < 0.05$) THC, proPO, and SOD activity than chitin. A wide range of glucans, polysaccharides, and microbial products has been claimed to act as immunostimulants in crustaceans, although the efficacy of these has been questioned (Smith et al. 2003). Dietary administration of chitosan has been reported to increase the resistance of rainbow trout, *Oncorhynchus mykiss*, against *Aeromonas salmonicida* (Siwicki et al. 1994). Although it has been shown that chitin and chitosan have often

<table>
<thead>
<tr>
<th>Treatments</th>
<th>THC ($\times 10^6$ cells/mL)</th>
<th>proPO activity (Unit)</th>
<th>SOD activity (Unit)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>11.97 ± 0.20$^a$</td>
<td>0.21 ± 0.02$^a$</td>
<td>0.22 ± 0.02$^a$</td>
</tr>
<tr>
<td>5 mg/g chitosan</td>
<td>17.10 ± 0.31$^b$</td>
<td>0.29 ± 0.03$^{b,d}$</td>
<td>0.30 ± 0.01$^{b,c}$</td>
</tr>
<tr>
<td>10 mg/g chitosan</td>
<td>17.93 ± 0.30$^c$</td>
<td>0.31 ± 0.02$^b$</td>
<td>0.33 ± 0.01$^c$</td>
</tr>
<tr>
<td>15 mg/g chitosan</td>
<td>17.17 ± 0.28$^b$</td>
<td>0.30 ± 0.02$^b$</td>
<td>0.29 ± 0.02$^{b,c}$</td>
</tr>
<tr>
<td>5 mg/g chitin</td>
<td>15.39 ± 0.43$^d$</td>
<td>0.26 ± 0.02$^e$</td>
<td>0.25 ± 0.01$^d$</td>
</tr>
<tr>
<td>10 mg/g chitin</td>
<td>15.91 ± 0.24$^c$</td>
<td>0.28 ± 0.01$^{c,d}$</td>
<td>0.27 ± 0.01$^e$</td>
</tr>
<tr>
<td>15 mg/g chitin</td>
<td>16.01 ± 0.19$^e$</td>
<td>0.28 ± 0.02$^{c,d}$</td>
<td>0.25 ± 0.02$^d$</td>
</tr>
<tr>
<td>ANOVA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F-value</td>
<td>45.478</td>
<td>30.002</td>
<td>33.069</td>
</tr>
<tr>
<td>P-value</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
</tr>
</tbody>
</table>

ANOVA = analysis of variance; proPO = phenoloxidase; SOD = superoxide dismutase; THC = total hemocyte count.

$^1$Values are expressed as means ± SD ($n = 7$). Means in the same column sharing a same superscript letter are not significantly different and determined by Tukey’s test ($P > 0.05$).
potent immunostimulatory properties in various species of fish, but only a limited number of studies have sought to determine whether these substances have such activity in invertebrates. Chitosan and chitin have been reported to increase the resistance of Litopenaeus vannamei against Vibrio alginolyticus and enhance THC and hemocyte phagocytic activity (Wang and Chen 2005). However, Powell et al. (2007) found no increase in either THC or phagocytic activity of hemocyte in the shore crab, Carcinus maenas maintaining on a fish-based diet supplemented with chitin. Furthermore, challenge of crabs with a pathogenic bacterium (Vibrio alginolyticus) showed that by the end of the experiment the 5% chitin-fed group had the lowest mortality of 61.5% and in the control group the mortality was 77.8%. Shiau and Yu (1998) showed that significantly higher body weight gains were observed in shrimp fed the 10% chitin diet than in those fed the 5% chitin diet. The weight gain of shrimp decreased as dietary chitosan supplementation level increased.

The effect of dietary chitosan and chitin supplementation on the survival and immune reactivity of crustacean was disputed all along. But in this study, crayfish fed diets supplemented with 10 mg/g chitosan showed a significantly higher RPS than the control. And in 10 mg/g chitosan-fed crayfish THC, proPO, and SOD activity was also significantly higher than in chitin and the control. We think chitosan at the concentration of 10 mg/g may be used as a potential effective immunostimulant for resisting pathogen in commercial shrimp.

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Literature Cited


