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Dietary chitosan nanoparticles protect crayfish *Procambarus clarkii* against white spot syndrome virus (WSSV) infection

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ABSTRACT

Chitosan nanoparticles have exhibited potential antibacterial activity or anticancer activity as their unique character. In this study, we investigated the effect of chitosan nanoparticles protect crayfish *Procambarus clarkii* against WSSV. Chitosan (from crab shell) nanoparticles were prepared by ultrafine milling. The physicochemical properties of the nanoparticles were determined by particle size measure, zeta potential analysis and scanning electron microscope observation. The total hemocyte count (THC), phenoloxidase (PO) and superoxide dismutase (SOD) activity were measured at days 1, 4, 9 and 12, and the survival rate was also recorded after WSSV challenge. The results showed that chitosan nanoparticles could enhance the survival rate of WSSV-challenged crayfish. And crayfish fed diets supplemented with 10 mg/g chitosan nanoparticles (65% mortality) showed a significantly higher survival rate when compared to the control group (100% mortality). The analysis of immunological parameters revealed that 10 mg/g chitosan nanoparticles showed significantly higher level of prophenoloxidase (proPO), superoxide dismutase (SOD) and total hemocyte count (THC) when compared to the control group. It was found that chitosan nanoparticles could inhibit WSSV replication in crayfish. Our results demonstrated that dietary chitosan nanoparticles effectively improve innate immunity and survival of *P. clarkii* challenged with WSSV.

1. Introduction

Chitosan, derived by the deacetylation of chitin, is a natural polysaccharide composed of randomly distributed chains of β-(1-4) D-glucosamine and *N*-acetyl-D-glucosamine. Chitosan nanoparticles serve a better inhibitory activity against *Candida albicans* and the inhibitory effect was influenced by particle size [1]. Chitosan nanoparticles also could inhibit the growth of *Escherichia coli*, *Salmonella choleraesuis*, *Salmonella typhimurium* and *Staphylococcus aureus* [2]. Chitosan nanoparticles showed strong activity to inhibit the proliferation of human lymphoma cells through inducing apoptosis [3]. Chitosan nanoparticles have showed the antibacterial or anticancer activity in many studies. However, little research show the effect of chitosan nanoparticles on antiviral immunity of animals.

White spot syndrome virus (WSSV), which was first discovered in Taiwan in 1992, has caused mass mortalities and devastating production losses to shrimp farming over many areas [4–6]. The DNA genome of WSSV is about 300 kb, and WSSV may be the sole member of Nimaviridae, genus Whispovirus [7,8]. WSSV is known to infect many crustacean species, including crayfish [9,10]. Both farmed and wild *Procambarus clarkii* in Louisiana (USA) were natural hosts for WSSV [11], and *P. clarkii* has also been used for the study of antiviral immunity in shrimp [12]. In this study, we investigated the effect of chitosan nanoparticles protect *P. clarkii* against WSSV infection. We demonstrated that chitosan nanoparticles could improve the immuno-enzyme activities and enhance the antiviral defenses in crayfish.

2. Materials and methods

2.1. Materials

Chitosan from Alaska snow crab shell was purchased from Qianguang biotechnology company (Tsingtao, China) and the degree of deacetylation is above 90%. The chitosan nanoparticles were prepared according to the previous report [13].

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2.2. Characterizations of the nanoparticles

Particle size was measured using a Zetasizer Nano-ZS-90 (Malvern Instruments, UK). The analysis was performed at a scattering angle of 90° under 25 °C in triplicate.

2.3. Crayfish and WSSV stock

Crayfish *P. clarkii*, approximately 20 g and 8 cm each, were reared at 25 °C. They were kept in tanks with sand-filtered, ozone-treated and flow-through freshwater and fed with commercial pellet feed at 5% of body weight per day. Walking legs from randomly selected individuals were subjected to PCR assays to ensure that the crayfish were WSSV-free before experimental challenge. Then the crayfish was used for the oral challenge test as the previous report [14]. White spot syndrome virus (LD50 = 1.5×10^5 copies/μl) -infected crayfish was used for the challenge test.

2.4. Experimental procedure

Chitosan nanoparticles or chitosan used in this study were blended with commercial crayfish feed powder (Dajiang Feed Company, Huai'an, China) at 1, 10 and 20 mg/g to formulate two experimental diets. Three test groups of 20 crayfish in triplicate were fed with 1, 10 and 20 mg/g chitosan nanoparticles, and another three test groups of 20 crayfish in triplicate were fed with 1, 10 and 20 mg/g chitosan for 3 weeks. And 3 crayfish in triplicate were fed with 1, 10 and 20 mg/g chitosan nanoparticles or common crayfish feed for the analysis of immunological parameters. The positive control and the negative control of 20 crayfish in triplicate 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 WSSV-infected crayfish at the dosage of 1 g per crayfish after 10 feed days. 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.

2.5. 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). 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).

2.6. 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 [15]. Briefly, the diluted hemolymph was centrifuged at 800 × g at 4 °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, 1 mg/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).

2.7. Superoxide dismutase (SOD) assay

SOD activity was determined according to the previous report [16] 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 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.

2.8. WSSV detection and quantitative analysis by PCR

The methods is according to the previous report [17].

2.9. Statistical analysis

The mortalities of the tested and control groups were compared statistically using the chi-square test (c2) at a significance level of 5%. The relative percent survival (RPS) values were calculated according to Amend [17]. Cumulative mortalities, RPS values and P values were determined at the termination (24th day) of challenge test made seven days after the last vaccination. The protection against WSSV after administration was calculated as the relative percent survival (RPS) [(1−oral administrated group mortality/control group mortality) × 100].

3. Results

3.1. The particle size of chitosan nanoparticles

The size is essential characteristic parameters for a kind of nanoparticles. The data showed the size of the chitosan nanoparticles and most chitosan nanoparticles had a diameter between 10 nm and 20 nm (Fig. 1B). And the untreated chitosan particles had a diameter above 1000 nm (Fig. 1A). Using transmission electron microscopy, the morphology of chitosan and chitosan nanoparticles was shown about 1000 and 20 nm in diameter with spherical in shape and found to be agreement (Fig. 2).

3.2. Effects of chitosan nanoparticles on the mortality of WSSV-challenged crayfish

To evaluate the effects of chitosan nanoparticles on the mortality of WSSV-challenged crayfish, chitosan nanoparticles were feed to crayfish for ten days and then challenged with WSSV. In the previous study, chitosan nanoparticles have showed that it had no toxicity to crayfish in 30 days (data not shown). The cumulative mortality and RPS in all groups are given in Table 1. Interestingly, the cumulative mortality of WSSV-infected crayfish was significantly reduced by the chitosan nanoparticles treatment ($P < 0.05$) (Table 1). The negative control showed the cumulative mortality is zero (Fig. 3), and the chitosan nanoparticles showed no toxicity to crayfish (data not shown). By comparing the crayfish mortalities between the chitosan nanoparticles and WSSV treatments, it was demonstrated that chitosan nanoparticles could inhibit WSSV infection in crayfish. The mortality of 100% was recorded in the positive control group on the 11th