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Immunostimulation and yellow head virus (YHV) disease resistance induced by a lignin-based pulping by-product in black tiger shrimp (*Penaeus monodon* Linn.)

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ABSTRACT

Yellow head virus (YHV) is classified as one of the most serious pathogens causing a harmful disease in many penaeids, especially black tiger shrimp (*Penaeus monodon*), with high economic loss. To determine a potent and practical prophylactic strategy for controlling this disease, the toxicity of the by-product kraft lignin and its ability to control severe YHV infection were investigated in juvenile black tiger shrimp (15.9 ± 1.2 g body weight). The median lethal dosage at 96 h (96-hrs LD_{50}) of lignin in shrimp was 297 mg/L. Lignin was further added to shrimp diets via top-dressing to assess its ability to elicit immune stimulation activity. At 14 days after feeding, shrimp fed 1, 3, 5 and 10 g of lignin/kg of diet exhibited significantly higher levels of phagocytic activity (PA) than the control group ($P < 0.05$). However, differences in total hemocyte count among treatments were not significant during the experimental period ($P > 0.05$). Additionally, lignin supplementation at 1-10 g/kg for 14 days failed to protect experimental shrimp against YHV infection. The antiviral activity of lignin against YHV in black tiger shrimp was notable *in vitro* because compared to control shrimp (96.7 ± 5.8%; $P < 0.05$), shrimp injected with a pre-incubated solution of YHV and lignin at 1, 5, 10 and 20 mg/L exhibited significantly lower mortality rates, 23.3 ± 5.8, 16.7 ± 5.8, 23.3 ± 5.8, and 20.0 ± 0.0%, respectively, after a lethal dose of YHV at 14–20 days after injection. These potent effects were clearly supported and confirmed by histopathological and RT-PCR analyses. Based on these results, the pulping by-product kraft lignin efficiently inhibits YHV infection in black tiger shrimp. This information will facilitate the development of practical methods to control yellow head disease in the marine shrimp culture industry.

1. Introduction

Until the 2000s, the black tiger shrimp (*Penaeus monodon*) was among the most highly valued shrimp species, providing significant income to Thailand and other Asian countries. However, serious disease outbreaks caused by deadly viruses, such as white spot syndrome virus (WSSV) and yellow head virus (YHV), have seriously hampered black tiger shrimp culture, resulting in heavy economic losses [1]. Until recently, the culture of black tiger shrimp was almost completely replaced by that of exotic Pacific white shrimp (*Litopenaeus vannamei*). Black tiger shrimp culture represents 1-5% of the total annual shrimp production in Thailand, and WSSV and YHV remain the primary causative agents of severe diseases destroying the Thai shrimp industry. Several attempts have been made to mitigate the impact of viral diseases, such as the application of chemicals, drugs, medicinal herbs and several probiotics [2]. However, no effective and safe agents have been reported to control viral diseases during growth. Additionally, the use of certain antimicrobial agents for disease prevention and therapy has serious negative impacts, such as the emergence of drug-resistant microorganisms and the accumulation of antibiotic residue in shrimp and their environment [3,4].

YHV is a serious pathogen that causes a harmful disease called “yellow head disease”. Additionally, YHV rapidly kills black tiger shrimp and causes mass mortality in several other types of crustaceans, including *Metapenaeus ensis* [5], *P. vannamei* and *P. stylrostris* [6], *P. setiferus*, *P. aztecus* and *P. duorarum* [7]. Additionally, the red claw crayfish (Cherax quadricarinatus) was found to be susceptible without mortality but may be a perfect carrier of YHV for transmitting this harmful virus to black tiger shrimp [8]. YHV type-1 has been identified as a severe agent causing rapid and harmful morbidity, while YHV type-

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2 (gill-associated virus; GAV) is a less virulent or non-virulent virus [9]. Lethal type-1 YHV causes marked economic losses for both the *P. monodon* and *P. vannamei* farming industries in Thailand. Recently, there have been several attempts to generate a powerful tool to prevent or treat yellow head disease, including molecular-based strategies and gene-silencing procedures [10–12]. Reports have preliminarily described the efficacy of both prophylactic and therapeutic strategies for controlling yellow head disease, but no strategies have been introduced to shrimp farms due to costs and the requirement for complicated techniques.

Lignins are naturally found in the middle lamella of higher plants, such as Angiosperms, Gymnosperms and Monocotyledons. Lignins coat the cellulose and hemi-cellulose layers [13] and comprise 10-35% of plant components. The molecular weights of lignins range from 10 to 10,000 kDa or higher. Structurally, lignins are polymerized products derived from 3 phenylpropanoid subunits: *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol [14]. Lignins are nontoxic and extremely versatile in terms of performance; thus, these compounds are increasingly important in many industrial applications as emulsifiers, sequestrants, dispersants and binders. Commercial lignin, which is available in the forms of lignosulfonate and kraft lignins, is currently produced as the primary waste by-product of the paper pulping process [15]. Overall, kraft lignin possesses fewer sulfur groups and anionic charges and is less water soluble than lignosulfonates. Kraft lignin contains low sulfur and sulfonate contents and charge density, at 0.23–3.0% (by weight), 0 mM/g, 0.01 meq/g and 1500–25,000 g/M, respectively, whereas the values for lignosulfonates are 3.5–8.0% (by weight), 0.7–2.5 mM/g, 0.9 meq/g and 1000–150,000 g/M, respectively [15].

According to previous studies, several forms of lignin exhibit antioxidative stress [16,17], phagocytosis-enhancing [18,19], and antiviral properties [20–23]. Among lignin forms, kraft lignin is less utilized for these purposes. However, it is worth studying these important properties of kraft lignin to establish a new strategy to control some harmful viral diseases in black tiger shrimp. Thus, the aim of the present study was to evaluate the efficacy of the pulping by-product kraft lignin in modulating immune responses and antiviral functions against YHV and its toxicity in black tiger shrimp. This information may be valuable for generating new prophylactic methods and environment- or human-friendly products from inexpensive waste products available in large volumes and ready supply that are highly effective at controlling harmful viral diseases in an efficacious and safe manner in the marine shrimp culture industry.

2. Materials and methods

2.1. Lethal dose assay

2.1.1. Experimental animals

One thousand healthy black tiger shrimp (15.9 ± 1.2 g body weight) were purchased from a private farm in Chacherngsao Province, Thailand. The shrimp were acclimatized in four fiberglass tanks containing 300 L of 20 ppt seawater for one week and fed with 5% body weight commercial feed twice per day.

2.1.2. The median lethal dosage at 96 h (96-hrs LD_{50})

After acclimatization, 10 shrimp were randomly selected to rear in each of 21 glass tanks containing 80 L of 20 ppt seawater for two days (7 different groups in triplicate). The 96-hrs LD_{50} of lignin for juvenile shrimp was evaluated according to the method of Reed and Muench (1938) [24]. Briefly, kraft lignin [containing 2% of sulfur (w/w) provided by Nippon Paper Chemicals Co., Ltd., Japan] was prepared in phosphate-buffered saline (PBS, pH 7.4) at 6 different concentrations (100, 150, 200, 250, 300 and 400 mg/L; the final pH for all concentrations ranged 7.28–7.34). Every shrimp in each group was injected with 100 μL of 100, 150, 200, 250, 300 or 400 mg/L kraft lignin. As a negative control, shrimp in the remaining group were injected with 100 μL of PBS. The mortality of the shrimp in each group was recorded at 6, 12, 24, 48, 72 and 96 h after injection. Moribund shrimp were collected and preserved in Davidson's fixative for histopathology examination according to Bell and Lightner (1988) [25].

2.2. Effects of feed supplemented with kraft lignin on immune responses in black tiger shrimp

2.2.1. Experimental animals and disease evaluation

Ten shrimp from each group described in section 2.1.1 were randomly sacrificed, and DNA and RNA were isolated for subsequent PCR analysis of WSSV, infectious hypodermal and hematopoietic necrosis virus (IHHNV), Taura syndrome virus (TSV) and YHV according to the methods described in section 2.5. Two hundred microliters of hemolymph was withdrawn from the vetral sinus of each shrimp, and one hundred microliters was spread onto thiosulfate citrate bile-salt sucrose (TCBS) agar and trypticase soy agar supplemented with 1.5% NaCl. Hepatopancreas samples from the same shrimp were aseptically loop-streaked onto both TCBS and TS agar plates. The agar plates were incubated at 30 °C in an incubator for 24 h to observe bacterial contamination. After the individuals were proven to be free of any pathogens, these shrimp were used for this and other experiments. Twenty out of three hundred shrimp were separately raised in 20 glass tanks, comprising a total of 5 groups with four experimental replicates each. The shrimp were acclimatized for 7 days using a protocol similar to that described in section 2.1.

2.2.2. Experimental design

Two hundred and twenty-five shrimp from the samples prepared as described in section 2.2.1 were randomly collected, and 15 shrimp each were placed into 15 aquarium glass tanks (120-L capacity) containing 80 L of 20 ppt seawater to set three replicates for each treatment. All shrimp groups were acclimatized for 7 days prior to the experiment using to the methods described in section 2.1.1.

2.2.3. Preparation of feed and feeding

Experimental feed was prepared via top-dressing with kraft lignin solution added at 5 different concentrations (0, 1, 3, 5 and 10 g/kg of lignin) to commercial feed. All experimental feeds were finally coated with squid liver oil at a ratio of 20 mL/kg feed. The shrimp in the first group served as a control and were provided feed without lignin. The shrimp in groups 2, 3, 4 and 5 were provided feed containing lignin at 1, 3, 5 and 10 g/kg, respectively. The shrimp were fed at 5% body weight/day per feeding to satiation two times per day for 14 days and until the end after viral injection experiment.

2.2.4. Determination of immune parameters

On days 0, 3, 7 and 14 after the initiation of feeding, three shrimp from each group (one shrimp per tank) were separately sampled. Five hundred microliters of hemolymph was withdrawn from the ventral sinus of each shrimp using a 1-mL syringe containing 0.5 mL of anticoagulant (10% sodium citrate in RPMI medium). Total hemocytes were counted in 15 μL of diluted hemolymph. The remaining hemolymph was further centrifuged at 6500 rpm for 10 min. Phagocytic activity was subsequently analyzed according to the modified method of Weeks-Perkins et al. (1995) [26]. The details of the measurements were previously described [27]. Briefly, 200 μL of PBS-diluted hemocytes (5×10^6 cells/mL) of each shrimp was loaded onto 22×22 mm^2 cover glasses, and the cells were allowed to adhere to the glass surface for 2 h. The cells were washed 3 times with PBS. Then, 200 μL of the latex bead solution (Sigma, USA) containing 10^8 beads/mL was added to the hemocyte monolayer and incubated at room temperature for 1.5 h to allow particle uptake. Subsequently, the attached cells and beads were stained with Diff-Quick staining dye (Fisher Scientific, USA), and 200 cells were counted under a light microscope. Percent phagocytic