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Enhancement of non-specific immune response in sea cucumber (*Apostichopus japonicus*) by *Astragalus membranaceus* and its polysaccharides

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ABSTRACT

In this study, the immunostimulatory effect of oral administration of different preparations (conventional fine powder [CP] and superfine powder [SP]) of *Astragalus membranaceus* root or its polysaccharides (APS) in sea cucumber (*Apostichopus japonicus*) was investigated. Sea cucumbers with an average initial weight of 49.3 ± 5.65 g were fed with a diet containing 3% CP or SP or 0.3% APS over a period of 60 days. The non-specific humoral (phenoloxidase, lysozyme and agglutination titer) and cellular (phagocytic capacity and reactive oxygen species) responses were determined and compared with controls (no supplement) after 20, 40 and 60 days of feeding. Variation in the levels of responses was evident among different supplements. SP and APS significantly enhanced most of the immune parameters tested. Among the humoral responses, lysozyme activity significantly increased after feeding with SP-supplemented diet for 20, 40 or 60 days. Furthermore, lectin titer showed significant enhancement after 20 and 60 days of feeding with APS-supplemented diet. Significant increase in the production of reactive oxygen species was evident for all three supplements after 20 days of feeding, but no significant change in serum phenoloxidase activity was observed for any of the three supplements over the three different periods. Overall, significant modulation of the cellular responses was only noticed after 20 days of feeding with SP- or APS-supplemented diet. After 60 days, these two groups also exhibited a decrease in the cumulative symptom rates compared to the controls when challenged with *Vibrio splendidus*. These results indicated that dietary intake containing *A. membranaceus* root or its polysaccharides could enhance the immune responses of *A. japonicus* and improve its resistance to infection by *V. splendidus*.

1. Introduction

Apostichopus japonicus (sea cucumber) is one of the economically important farmed echinoderm species in Northern China [1]. However, infectious diseases are becoming a severe problem with increasing culturing. Disease caused by *Vibrio splendidus* is most widespread in sea cucumber farming [2]. Antibiotics and chemotherapeutics used to control these diseases can result in the development of drug-resistant bacteria, environmental pollution and unwanted residues in aquaculture [3]. One of the most promising methods for controlling sea cucumber diseases in aquaculture is by strengthening their defense mechanisms through prophylactic administration of immunostimulants [4].

Chinese herbs have been used as immunostimulants in human for thousands of years in China. Recently, much attention has been paid to the immune stimulating function of some herbs in aquaculture. Shrimps and fishes fed with diets containing certain Chinese herbs were reported to show improved non-specific immunity, such as bacteriolytic activity and leukocyte function [5–8]. Among the many herbs used in Traditional Chinese Medicine, the root of *Astragalus membranaceus* has been used as an immune booster for nearly 2000 years, and it has been shown to have significant immunostimulatory effects [9–11]. *A. membranaceus* has been reported to significantly improve the non-specific immunity of fish [12,13]. *A. membranaceus* polysaccharide (APS) is one of the major active substances of *A. membranaceus*, and it plays an important role in the specific and non-specific immune responses [14]. It can activate mouse B cells and macrophages [15]. Although *A. membranaceus* and APS have been shown to enhance the non-specific immunity of some animals, their immunostimulatory effects in sea cucumber have not been investigated.

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Our previous work has revealed that APS could significantly promote phagocytosis and superoxide anion (O_2^-) production in *A. japonicus* coelomocytes *in vitro* at 22 and 25 °C [16]. In this study we investigated the effects of *A. membranaceus* root (prepared by conventional and superfine milling) or APS on the non-specific immune responses of *A. japonicus* as well as its resistance to disease caused by *V. splendidus*.

2. Materials and methods

2.1. Collection and maintenance of animals

Sea cucumbers (49.3 ± 5.65 g) were collected from an aquaculture farm in China, held in a recirculation system in Key Laboratory of Marine Culture and Biotechnology of Agriculture Ministry, Dalian Fisheries University (DLFU, Dalian, China), and kept in recirculating aquarium tanks (100 cm × 30 cm × 40 cm) at water temperature of 15–19 °C. During the experiment, the pH of water was maintained at 7.8–8.2, and the salinity at 31–32‰.

2.2. Immunostimulant preparation

A. membranaceus was purchased from the local Nepstar Chain Drugstore (Dalian, China). The roots were removed, washed with water, and then dried at 40 °C. The dry roots were processed in a laboratory pulverizer and passed through a 0.25 mm aperture sieve. This product was designated as CP. CP was further processed with WZJ(BFM)-6J vibrational mill (Billionpower Tech & Engineering Co. Ltd. China) to yield a superfine form, designated as SP. The particle size of SP was about 48 μm. APS (purity: 70%) was commercially obtained from Xian, Tianyuan Biological Preparation Industry and Trade Ltd, China. Basal diet without supplement or supplemented with 3% (w/w) CP or SP or 0.3% (w/w) APS according to the common dose in aquaculture [17–19], and used as feed. The nutritional compositions of each diet supplement are shown in Table 1.

2.3. Experimental design and sampling procedure

Water flow of the recirculating system was maintained at 625 ml/min. One third of the water volume of the recirculating system was replaced by fresh seawater once a day to maintain the water quality. Sea cucumbers were randomly divided into four treatments, with each treatment consisted of three tanks, with 15 animals per tank. The animals were fed with either basal diet only (control group), basal diet supplemented with CP (CP group), SP (SP group) or APS (APS group) at a rate of 3% body weight per day for 60 days.

Coelomic fluid was collected from each individual sea cucumber by tail-cutting method. For serum separation, the collected coelomic fluid was spun down at 3000 × *g* for 10 min at 4 °C. The supernatant was stored in sterile microcentrifuge tubes at −70 °C until use.

Table 1
Nutritional component of different diets for *A. japonicus* (g kg^{-1}).

	Control	CP	SP	APS
Crude protein	152	158	162	151
Crude fat	39	41	40	39
Total carbohydrate	31	31	35	38
Ash	535	530	515	498
Ca/P	7.9	7.5	7.6	8.0
Lys	7.73	7.91	7.90	7.38

Basal diet: soil 38%, seaweed 35%, fishmeal 15%, shrimp powder 5%, shell powder 3%, rice hull 2% and premix 2%.

2.4. Phagocytic activity

Phagocytic activity was evaluated by the method of Barracco et al. [20] and Ordás et al. [21] with slight modifications. Briefly, yeast cells (*Saccharomyces cerevisiae*) were washed in filtered seawater two times and harvested by centrifugation at 3000 × g for 5 min, and then resuspended in filtered seawater. A calibrated concentration of 10.0 ± 2.0 yeast/phagocytic amoebocytes for each animal was prepared in sterilized microcentrifuge tube. For the phagocytic activity assay, 0.1 ml of fresh coelomic fluid was added to 0.l ml of yeast suspension, and after thorough mixing, 0.15 ml aliquot was dispensed onto a glass slide. The slide was placed immediately into an aluminum box with wet filter paper and incubated at 17–20 °C for 1 h. The slide was gently washed twice with filtered seawater, and the adherent phagocytic amoebocytes (PA) were fixed for 3 min with methanol for enumeration. Phagocytic capacity was determined as described by Silva and Peck [22]:

$$\text{Phagocytic capacity} = \frac{\text{No. of PA with yeast inside}}{\text{Total no. of PA}} \times 100\%$$

2.5. Reactive oxygen species (ROS) production

Intracellular ROS production was measured according to Chen et al. [23]. The coelmocytes collected from coelomic fluid by centrifugation were adjusted to 5 × 10^6 cells/ml with culture medium (5% foetal bovine serum, 0.5% penicillin/streptomycin solution in filtered (0.22 μm) seawater). Five hundred microliters of diluted coelmocytes was mixed with 89 μl of culture medium, and nitroblue tetrazolium (NBT) and phorbol 12-myristate 13-acetate (PMA) were then added to a final concentration of 0.1% and 0.01 μM, respectively, and the sample was incubated at room temperature for 1 h. After incubation the supernatant was removed by centrifugation at 540 × g for 10 min, and the cells were fixed with 35% of methanol. After washing twice with 70% methanol, the cells were resuspended in 0.92 M potassium hydroxide and 54% dimethyl sulphoxide in a final volume of 1.3 ml to dissolve the reduced-NBT (in the form of formazan), and the optical density of the sample was measured by absorbance at 625 nm.

2.6. Assay for serum phenoloxidase activity

Phenoloxidase activity in the coelomic fluid was assayed as described by Ashida and Söderhäll [24] using a 96-microtiter plate method. Fifty microliters of the coelomic fluid was mixed with 0.03 M phosphate buffer (pH = 6.0) and 4.6 mM L-dihydroxyphenylalanine in a final volume of 240 μl in a 96-microtiter plate. For control, 50 μl of filtered seawater was used to substitute for the coelomic fluid. The OD value of the sample was measured at 1 min intervals for a total of 10 min at 490 nm. One unit of enzyme activity was defined as the amount of enzyme causing an increase in absorbance of 0.001 per min per ml serum.

2.7. Assay for serum lysozyme activity

Lysozyme activity was measured by a spectrophotometric method based on the lysis of *Micrococcus lysodeikticus* (No.1.0634, Institute of Microbiology, Chinese Academy of Sciences [Beijing, China]) [25]. Briefly, 3 ml of *M lysodeikticus* (OD about 0.3) in phosphate buffer (0.067 M, pH 6.4) was added to 50 μl of serum at 25 ± 1 °C. The reduction in the absorbance of the sample at 540 nm was determined after 0.5 and 4.5 min of incubation. One unit of lysozyme activity was defined as the amount of enzyme causing a reduction in absorbance of 0.001 per min.