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Effects of *Astragalus* polysaccharides on antioxidant abilities and non-specific immune responses of Chinese mitten crab, *Eriocheir sinensis*

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Abstract This study assessed the effects of *Astragalus* polysaccharides (APS) on antioxidant abilities, non-specific immune responses, and immune protective efficacy (attacked by *Aeromonas hydrophila*) of *Eriocheir sinensis*, the most important Chinese freshwater crabs. A total of 720 crabs (initial mean weight 10.27 ± 1.58 g) were fed 60 days with six kinds of experimental diets containing graded dosages of APS (0, 300, 600, 900, 1200, 1500 mg/kg diets) in 18 outdoor cement tanks. The results showed that superoxide dismutase (SOD), catalase (CAT), total antioxidant capacity (T-AOC), lysozyme (LZM), and phenoloxidase (PO) activities of serum significantly increased ($P < 0.05$) with increasing APS dosages (0–900 mg/kg diets), but alkaline phosphatase (AKP) and acid phosphatase (ACP) activities of serum did not significantly changed ($P > 0.05$); SOD, CAT, T-AOC, LZM, AKP, and ACP activities of hepatopancreas significantly increased ($P < 0.05$) with increasing APS dosages (0–1200 mg/kg diets); the increased maximal multiples of LZM and PO activities were higher than SOD, CAT, and T-AOC which increased. The results of *A. hydrophila* attack test showed that mortality rates significantly decreased ($P < 0.05$) with increasing APS dosages (0–600 mg/kg diets), and the highest immune protective rate was 49.4%. In short, APS could help *E. sinensis* to improve immune responses and may reduce the risk of disease attacks as one kind of effective immunopotentiator in diets, and the best additive dosage was 1200 mg/kg.

Keywords *Eriocheir sinensis* · *Astragalus* polysaccharides · Antioxidant ability · Non-specific immune response · Immune protective rate

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Introduction

Eriocheir sinensis is the most important freshwater crabs with tremendous economic value in China. It belongs to the phylum Arthropoda, class Crustacea, order Decapoda, family Grapsidae, and genus *Eriocheir*. The annual culture area and output of *E. sinensis* in China are more than 1 million hm^2 and 600,000 t, respectively. However, there are many kinds of diseases that have attacked *E. sinensis* so that fishermen suffered losses with extensive farming in recent 20 years. Even though no any disease had happened, the survival rates were low (<50%) in the process of *E. sinensis* farming with high water temperature, low dissolved oxygen, river pollution, abuse of medical drugs in aquaculture farming and other stresses, and the low immunity and poor stress responses of *E. sinensis* may be the primary causes. In recent years, many kinds of immunopotentiators (such as Chinese herbal polysaccharides, vitamins, probiotics, and so on)were used to help aquatic animals to reduce the risk of bacterium and virus infections, since the significance of disease prevention is much larger than treatments in aquatic animals. Intensive researches had been conducted on the efficacy of multitudinous immunopotentiators on the antioxidant ability and non-specific immune responses of crabs and shrimps all over the world (Ai et al. 2008; Bidhan et al. 2014; Chen et al. 2014; Hou et al. 2015; Li et al. 2014; Li et al. 2015a, b; Ma and Chen 2013; Montero-Rocha et al. 2006; Song et al. 2005; Shen et al. 2004; Smith et al. 2003; Wei et al. 2015; Xu et al. 2005; Yuan et al. 2014; Yang et al. 2005; Zokaeifar et al. 2012; Zhao et al. 2016).

Astragalus polysaccharides (APS) are the most effective components of *Astragalus* which is a traditional herbal medicine in China. APS have many clinical efficacies, such as anti-tumor, antibacterium, anti-virus, regulating the body's humoral immune, activating the immune cytokines, and so on(Chen and Huang 2008). In recent years, using the APS to carry on the antibacterium and anti-virus and regulating the body's humoral immune treatments becomes one of focuses of increasing production in animal husbandry (Yao et al. 2009) and fishery (Liu et al. 2014) owing to significant effects and advantages (no side effects, rich resources, and low cost). However, to date, no investigations about APS had been conducted on crabs all over the world. This study investigated the use of APS to feed *E. sinensis* as a feed additive and assessed the effects on antioxidant abilities, non-specific immune responses and immune protective efficacy (attacked by *Aeromonas hydrophila*).

Material and methods

Materials

In March 2016, a total of 720 *E. sinensis* (initial mean weight 10.27 ± 1.58 g) were picked out at Chinese Mitten Crab Farm of Freshwater Fisheries Research Institute of Jiangsu Province, China.

APS was purchased from Nuoweikang Biology Science and Technology Co., Ltd., Anhui Province, China.

Fish meal, soybean meal, corn meal, wheat bran, $Ca(H_2PO_4)_2$, NaH_2PO_4, and binder were used to make the basic diets. Five different dosages of APS were added to the basic diets to make the experimental diets: 300 mg/kg (group A1), 600 mg/kg (group A2), 900 mg/kg (group A3), 1200 mg/kg (group A4), and 1500 mg/kg (group A5). The control group (group A0) was treated with the basic diets. All the diets were made by the Haipurui Feed Co., Ltd., Jiangsu Province, China, and were stored in the refrigerator at −20 °C before using (Table 1).

Table 1 Compositions and nutritional levels of the basic diets

Items	Content (%)
Compositions	
Fish meal	35
Soybean meal	25
Corn meal	22
Wheat bran	15
$Ca(H_2PO_4)_2$	2
NaH_2PO_4	0.5
Binder	0.5
Total	100
Nutritional levels	
Crude protein	42.3
Crude fat	8.6
Crude ash	13.5
Dry matter	90.2

Methods

A total of 720 *E. sinensis* were averagely divided into three parts at random, i.e., three repeats. Eath part was divided into six groups at random. Five different experimental diets and the basic diets were used to feed them. In eath group, 40 *E. sinensis* were placed in an outdoor cement tank (2.0 × 2.0 × 1.0 m) for farming, and four tiles were placed at the bottom of each tank for shelters. During the experiment, feeding amount (3% of the total crabs weight in a cement tank in accordance with normal farming methods in the ourdoor pond) and feeding time (7 pm each day; *E. sinensis* is a nocturnal aquatic animal.) of each group were the same, and the water was changed weekly in each pond. Continuous feeding was terminated after 60 days under the natural conditions: water temperature 15.8–27.2 °C, dissolved oxygen 4.3–7.8 mg/L, and pH 7.2–8.2.

Sample preparations and parameter determinations

A total of six *E. sinensis* were picked out at each group randomly, and their hemolymphs were respectively extracted from the root of the third paraeiopod which were broken off. After 24 h in the Eppendorf centrifugal tubes under the 4 °C condition, all the hemolymphs were centrifuged 10 mins under the conditions: 4 °C and 5000 r/min, then supernatant serum were sucked up for parameter determinations. At the same time, every crab was dissected to get the hepatopancreas which then were stored in a −80 °C refrigerator after quick-freezing of liquid nitrogen. When the parameters would be determinated, stroke-physiological saline solution (0.65%) was added in the hepatopancreas according to 1:9 volume. After being broken by the ultrasonic, all the hepatopancreas were centrifuged 10 mins under the conditions: 4 °C and 5000 r/min, then supernatant serum were sucked up for parameter determinations.

All the parameters, including superoxide dismutase (SOD), catalase (CAT), total antioxidant capacity (T-AOC), lysozyme (LZM), phenoloxidase (PO), alkaline phosphatase (AKP), and acid phosphatase (ACP) activities, were determinated with test kits which were produced by the Nanjing Jiancheng Bioengineering Institute, Jiangsu Province, China. The determination methods were carried out in accordance with all the test kit instructions (SOD: WST-1 method, reaction temperature 37 °C, wave length 450 nm; CAT: visible light method, reaction

temperature 37 °C, wave length 405 nm; T-AOC: colorimetry method, reaction temperature 37 °C, wave length 520 nm; LZM: turbidimetry method, reaction temperature 37 °C, wave length 530 nm; PO: ELISA method, reaction temperature 37 °C, wave length 450 nm; AKP: visible light colorimetry method, reaction temperature 37 °C, wave length 520 nm; ACP: spectrophotometry method, reaction temperature 37 °C, wave length 520 nm).

Bacteria attack test

The strains of *A. hydrophila* were purchased from Shanghai Baili Biology Science and Technology Co., Ltd., China.

The strains were inoculated on agar culture medium and then rejuvenated for 48 h in a 30 °C biochemical incubator. The solution concentration of rejuvenated strains which were then put in stroke-physiological saline solution (0.65%) was 1.0×10^7 cfu/mL. The solution concentration, which was confirmed by a preliminary test, was the median lethal dose.

According to the original design of experiment, 30 *E. sinensis* were picked out at each group randomly and then placed in 18 plastic cases (0.8 × 0.5 × 0.5 m), respectively. Each crab was injected with *A. hydrophila* solution 0.1 mL at the root of the third paraeiopod and cultured tentatively in corresponding plastic cases. The numbers of dead crabs were recorded in each plastic case every 24 h, and the mortality rates and immune protective rates were calculated respectively.

$$\text{Immune protective rate}(\%) = (\text{Mortality rate of the control group} - \text{Mortality rate of the test group})/\text{Mortality rate of the control group} \times 100\%$$

Data processing and statistical analysis

SPSS19.0 was used to construct the data processing for a comparison of means and variance analysis. Two multiple comparison methods, least significant difference and least significant ranges, were used to compare the results ($P < 0.05$).

Results

Effects of APS on SOD, CAT, T-AOC, LZM, PO, AKP, ACP activities of serum and hepatopancreas of *E. sinensis*

SOD, CAT, T-AOC, LZM, PO, AKP, and ACP activities of serum and hepatopancreas of *E. sinensis* of different groups after APS feeding were shown in Figs. 1, 2, 3, 4, 5, 6, and 7.

Figure 1 showed that SOD activities of serum and hepatopancreas gradually increased after APS feeding with increasing dosages. SOD activities of serum of groups A0, A1, A2, A3, A4, and A5 were 86.36 ± 4.13, 87.72 ± 4.51, 115.64 ± 7.57, 138.22 ± 6.93, 145.28 ± 10.97, and 141.28 ± 9.37 U/mL, respectively; SOD activities of hepatopancreas of the abovementioned groups were 116.78 ± 6.21, 120.40 ± 8.57, 133.56 ± 10.57, 168.97 ± 11.86, 175.76 ± 8.94, and 169.37 ± 10.56 U/mL, respectively.

Figure 2 showed that CAT activities of serum and hepatopancreas gradually increased after APS feeding with increasing dosages. CAT activities of serum of the abovementioned groups

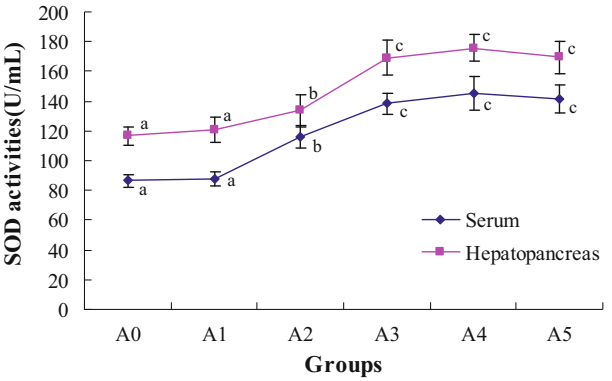

Fig. 1 Effects of APS on SOD activities of serum and hepatopancreas of *E. sinensis*. *Values in each column not sharing the same letter* are significantly different ($P < 0.05$). Values are given as means ± S.E

were 1.46 ± 0.12, 1.52 ± 0.15, 1.84 ± 0.17, 2.32 ± 0.29, 2.27 ± 0.27, and 2.41 ± 0.37 U/mL, respectively; CAT activities of hepatopancreas of the abovementioned groups were 1.86 ± 0.09, 1.92 ± 0.19, 2.34 ± 0.31, 2.78 ± 0.40, 2.71 ± 0.42, and 2.86 ± 0.57 U/mL, respectively.

Figure 3 showed that T-AOC activities of serum and hepatopancreas gradually increased after APS feeding with increasing dosages. T-AOC activities of serum of the above-mentioned groups were 8.16 ± 0.82, 8.02 ± 0.55, 9.76 ± 0.97, 11.58 ± 1.09, 11.34 ± 0.87, and 11.48 ± 0.69 U/mL, respectively; T-AOC activities of hepatopancreas of the abovementioned groups were 8.56 ± 0.76, 8.72 ± 0.59, 10.54 ± 0.71, 12.82 ± 1.02, 13.08 ± 0.86, and 12.92 ± 1.17 U/mL, respectively.

Figure 4 showed that LZM activities of serum and hepatopancreas gradually increased after APS feeding with increasing dosages. LZM activities of serum of the above-mentioned Groups were 0.16 ± 0.02, 0.25 ± 0.03, 0.30 ± 0.02, 0.33 ± 0.03, 0.42 ± 0.03, 0.43 ± 0.02 U/mL, respectively; LZM activities of hepatopancreas of the above-mentioned Groups were 0.13 ± 0.01, 0.19 ± 0.02, 0.25 ± 0.03, 0.27 ± 0.02, 0.35 ± 0.02, 0.35 ± 0.02 U/mL, respectively.

Figure 5 showed that PO activities of serum gradually increased after APS feeding with increasing dosages. PO activities of serum of the above-mentioned Groups were 11.13 ± 1.22, 16.23 ± 1.14, 23.30 ± 2.26, 28.61 ± 3.02, 34.63 ± 3.26, 35.50 ± 1.93 U/mL, respectively.

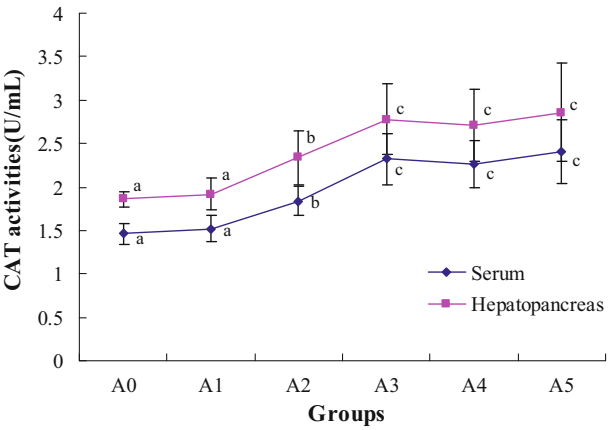

Fig. 2 Effects of APS on CAT activities of serum and hepatopancreas of *E. sinensis*. *Values in each column not sharing the same letter* are significantly different ($P < 0.05$). Values are given as means ± S.E

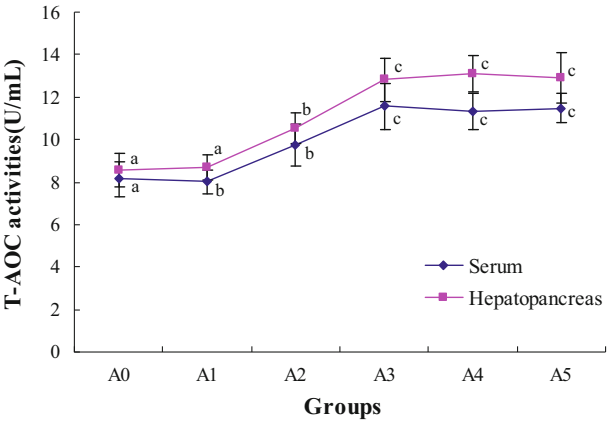

Fig. 3 Effects of APS on T-AOC activities of serum and hepatopancreas of *E. sinensis*. *Values in each column not sharing the same letter* are significantly different ($P < 0.05$). Values are given as means ± S.E

Figure 6 showed that AKP activities of hepatopancreas gradually increased after APS feeding with increasing dosages, but AKP activities of serum had not significantly changed. AKP activities of serum of the above-mentioned Groups were 4.26 ± 0.72, 4.35 ± 0.93, 4.46 ± 1.32, 4.33 ± 1.11, 4.22 ± 0.98, 4.63 ± 1.41 U/mg, respectively; AKP activities of hepatopancreas of the above-mentioned Groups were 9.45 ± 1.75, 10.15 ± 2.02, 14.58 ± 2.93, 16.27 ± 3.33, 24.87 ± 4.21, 25.66 ± 3.47 U/mg, respectively.

Figure 7 showed that ACP activities of hepatopancreas gradually increased after APS feeding with increasing dosages, but ACP activities of serum had not significantly changed. ACP activities of serum of the abovementioned groups were 4.29 ± 0.62, 4.40 ± 0.98, 4.69 ± 1.08, 4.55 ± 1.17, 4.48 ± 1.06, and 4.31 ± 0.89 U/mg; ACP activities of hepatopancreas of the abovementioned groups were 9.52 ± 1.66, 10.21 ± 2.43, 15.98 ± 2.82, 17.48 ± 3.09, 25.55 ± 3.63, and 25.27 ± 3.28 U/mg, respectively.

With regard to SOD, CAT, T-AOC, LZM, PO, AKP, and ACP activities, significant differences of the abovementioned groups were all shown in the figures by sharing the different letter ($P < 0.05$).

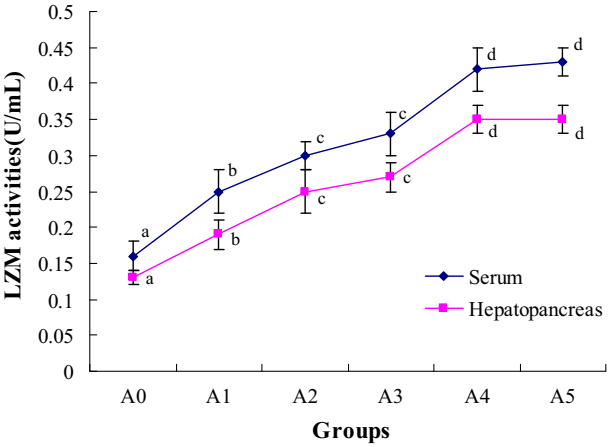

Fig. 4 Effects of APS on LZM activities of serum and hepatopancreas of *E. sinensis*

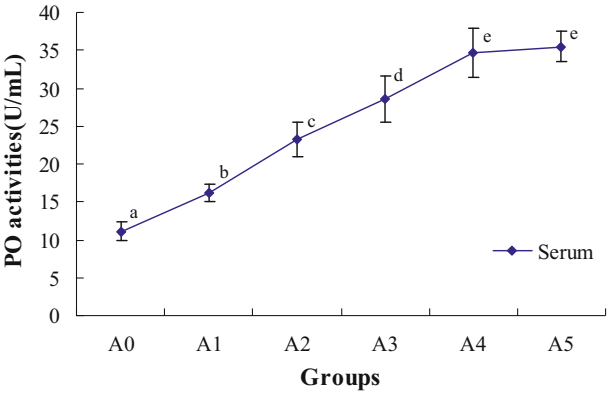

Fig. 5 Effects of APS on PO activities of serum and hepatopancreas of *E. sinensis*. *Values in each column not sharing the same letter* are significantly different ($P < 0.05$). Values are given as means ± S.E

Evaluation of APS on immune protective efficacy *of E. sinensis* which were injected *A. hydrophila*

The mortality rates and immune protective rates of different groups which were injected *A. hydrophila* were shown in Fig. 8. Figure 8 showed that the mortality rates gradually decreased with increasing APS dosages after *A. hydrophila* attack. The mortality rates of groups A0, A1, A2, A3, A4, and A5 were 87.8 ± 3.8, 71.1 ± 3.0, 47.8 ± 2.1, 44.4 ± 3.1, 52.2 ± 2.8, and 47.8 ± 1.9%, respectively; the immune protective rates of groups A0, A1, A2, A3, A4, and A5 were 0, 19.0, 45.6, 49.4, 40.5, and 45.6%, respectively.

Discussion

Effects of APS on antioxidant abilities and non-specific immune responses of *E. sinensis*

The results of this study showed that the antioxidant abilities and non-specific immune responses of *E. sinensis* had been significantly improved after APS feeding. That APS could

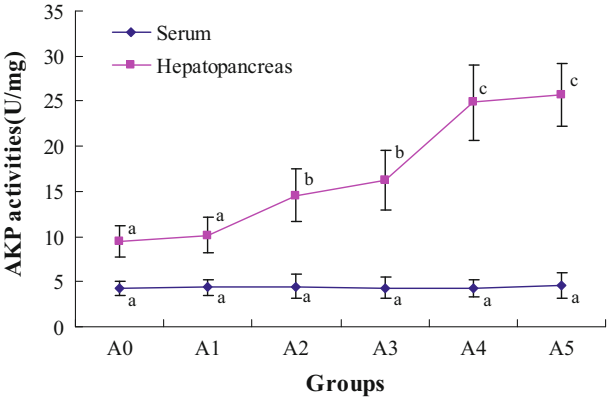

Fig. 6 Effects of APS on AKP activities of serum and hepatopancreas of *E. sinensis*. *Values in each column not sharing the same letter* are significantly different ($P < 0.05$). Values are given as means ± S.E

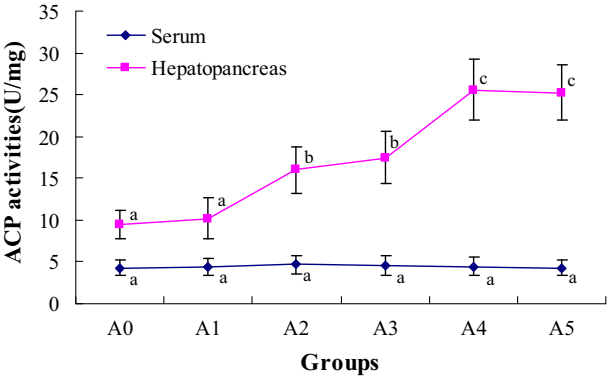

Fig. 7 Effects of APS on ACP activities of serum and hepatopancreas of *E. sinensis*. *Values in each column not sharing the same letter* are significantly different ($P < 0.05$). Values are given as means ± S.E

improve the antioxidant abilities and non-specific immune responses of fishes was in conformity with this study. After being perfused, different concentrations of APS decoction (0.5, 1.0, 2.0%, respectively), SOD activities of serum and livers of *Acipenser schrencki* Brandt had been significantly improved compared to the control group, and the malondialdehyde content gradually decreased(Liu et al. 2006). When the additive dosages of APS in formula feeds were 1000–2000 mg/kg, SOD and CAT activities of the tilapia had been significantly improved (Zhang et al. 2010). That the yellow catfish were fed formula feeds which contained appropriate levels (300–1500 mg/kg) of APS could significantly improve the SOD and CAT activities and decreased the malondialdehyde content, and the highest immune protective rate after Edwardsiella tarda attack was 50.0% (group 1200, 1500 mg/kg) (Bai et al. 2011). In addition, that other Chinese herbal medicines or immunopotentiators could improve the antioxidant abilities and non-specific immune responses of *E. sinensis* were in conformity with this study. The antioxidant abilities and non-specific immune responses were significantly promoted after *E. sinensis* were fed the test feeds which contained six kinds of Chinese herbal medicines (dosage, 2.4%): Folium Isatidis, *Cordate Houttuynia*, *Epimedium brevicornu*, *Rheum officinale*, *Andrographis paniculata* and Radix Scutellariae, and the immune protective rate after *A. hydrophila* attack was 62.5% (Liu et al. 2008). The antioxidant abilities and non-specific immune responses of *E. sinensis* showed the largest increase when the feeds *Asparagus officinalis*, Menthahaplocalyx, *Crataegus pinnatifida*, *Astragalus membranaceus* and

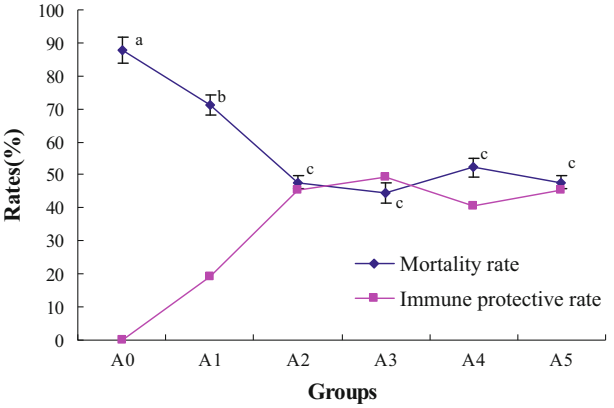

Fig. 8 Mortality rates and immune protective rates of different groups of *E. sinensis* which were injected *A. hydrophila*. *Values in each column not sharing the same letter* are significantly different ($P < 0.05$). Values are given as means ± S.E

Epimedium brevicornu (dosage: 1.0%) were added (Li et al. 2015a, b). Vc could significantly promote non-specific immune function including SOD, LZM, PO, AKP, and ACP activities in *E.sinensis*, and the optimum dosages were 500–1000 mg/100 g diets (Ai et al. 2008). Bamboo shoots polysaccharide could significantly promote non-specific immune function including SOD, LZM, respiratory burst activities, total hemocyte count, phagocytic percentage, and phagocytic index in *E.sinensis*, and the optimum dosage was 10 mg/kg body weight (Li et al. 2014).

Differential effects of APS on antioxidant abilities and non-specific immune responses of serum and hepatopancreas of *E. sinensis*

The results of this study showed that SOD, CAT, T-AOC, AKP, and ACP activities of hepatopancreas were higher than serum, but LZM activity was lower than serum. Hepatopancreas, the nutrient regulatory center of *E. sinensis* plays the most important role in the process of metabolism. In this process, some harmful products which contain peroxy radical, superoxide, hydroxyl radical, hydrogen peroxide, singlet oxygen, and so on appear in tissues. That the antioxidant system which was constituted by SOD, CAT, T-AOC, and so on was in charge of clearing them protects some biological membranes from damages (Ortuno et al. 1999).

The results showed that AKP and ACP activities of serum of *E. sinensis* did not change significantly, this was because AKP and ACP were released to serum when the hepatopancreas were damaged or taken some diseases. AKP and ACP, mainly existing in hepatopancreas of *E. sinensis*, catalyze the transfer reactions of all the phosphate monoesters and groups and play an important role in absorbing of calcium, calcium phosphate deposition, chitin secretion, and so on, since crabs molt many times in the process of growth(Chen et al. 1996; Kobayashi et al. 1983). In addition, ACP is one kind of lysosomal enzyme produced by activated macrophages as an important indicator in the assessment of immune state, as it can kill some pathogenic microorganisms (Li et al. 2015a, b).

The results showed that LZM activities of serum and hepatopancreas and PO activities of serum were increased significantly after APS feeding, and the maximums which increased contrasting with the control group were 168.9, 169.2, and 219.0%, respectively. These were higher than SOD, CAT, and T-AOC activities which increased, only 50.5, 53.8, and 52.8%, respectively. The main factor for this phenomenon was likely that APS could stimulate to produce LZM and PO or take part in producing something which was closely related to LZM and PO, and it was consistent with the significant clinical efficacy of APS: antibacterium and anti-virus. LZM, one kind of hydrolytic enzymes which specially act on cell walls of microorganisms, is an important non-specific immune factor in body fluids of crustaceans. Animals can enhance their disease resistances through much stronger LZM activities (Ma et al. 2006). PO which is produced from prPO system in blood cells of crustaceans plays an important role in foreign matter recognitions, opsonin releases, improving phagocytosis of cells, producing agglutinins and LZM and so on, and it has a close connection with immune functions of body (Soderhall 1999; Wang 1993). Since the body fluids of crustaceans have not immunoglobulins, so LZM and PO play the leading roles in humoral immunity.

When the additive dosage of APS in the basic diets was more than 900 mg/kg, SOD, CAT, and T-AOC activities did not change significantly; when the additive dosage of APS in the basic diets was more than 1200 mg/kg, LZM, PO, AKP, and ACP activities did not change significantly. This fact showed that the antibacterium and anti-virus efficacy of APS needed higher additive dosages for *E. sinensis*.

Evaluation of APS on immune protective efficacy of *E. sinensis* which were injected *A. hydrophila*

The results of bacteria attack test showed that the mortality rates gradually decreased with increasing APS dosages. When the additive dosage of APS in the basic diets was more than 600 mg/kg, mortality rates did not change significantly. This phenomenon was not consistent with the change rules of the abovementioned parameters, and this was likely related to experimental errors and precision. In short, APS could help *E.sinensis* to improve immune responses and may reduce the risk of disease attacks as one kind of effective immunopotentiator in diets, and the best additive dosage was 1200 mg/kg.

Acknowledgments This work was supported by grants from the Jiangsu Agriculture Science and Technology Innovation Fund (JASTIF)(CX(15)1011)and the Aquatic Sanxin Project of Jiangsu Province (D2015-5, D2016-1, Y2014-24).

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