Aquaculture International (2020) 28:1787–1796
https://doi.org/10.1007/s10499-020-00557-6

Effects of astragalus polysaccharides on hemocytes phagocytosis and gene expression of immune-related factors in *Eriocheir sinensis*

Qingman Cui[1] · Ziyue Zhao[1] · Chunying Yuan[1]

Received: 23 March 2020 / Accepted: 28 April 2020 / Published online: 13 May 2020

Abstract
The 4-week feeding experiments were conducted to investigate the effects of astragalus polysaccharides (APS) on hemocytes phagocytosis and gene expression of immune-related factors in *Eriocheir sinensis*—with initial weights of 6.11 ± 2.25 g. The phagocytic activity of hemocytes in *E. sinensis* was determined by fluorescence labeling, and the expression of immune-related factor genes in different tissues of *E. sinensis* was analyzed by quantitative real-time PCR (qPCR). Results showed that APS significantly improved the phagocytic activity of hemocytes in *E. sinensis* ($p < 0.05$), and the phagocytic activity of hemocytes in the 0.1% APS group was the highest (50.84%). Moreover, 0.05% APS significantly increased the expression of glutathione peroxidase (GSH-Px), anti-lipopolysaccharide (ALF), phenol oxidase (PO) and heat shock protein (HSP) genes in the hemocytes, and HSP in the hepatopancreas, and masquerade-like protein (MasL) in the gill ($p < 0.05$). Furthermore, 0.1% APS significantly increased the expression levels of PO, GSH-Px, ALF, MasL, and HSP genes in the hemocytes and gill ($p < 0.05$) and significantly increased the expression levels of PO, Crusl, and HSP genes in crab hepatopancreas ($p < 0.05$). In addition, 0.2% APS significantly increased the expression levels of all immune-related factor genes in the hemocytes and gill ($p < 0.05$) and significantly increased the expression levels of PO, ALF, Crusl, and HSP genes in the crab hepatopancreas ($p < 0.05$). It is estimated that APS could enhance the immune function of *E. sinensis* by increasing the phagocytic activity of hemocytes and the expression levels of the PO, GSH-Px, ALF, Crus1, MasL, and HSP genes.

Keywords *Eriocheir sinensis* · Astragalus polysaccharides · Hemocytes phagocytosis · Gene expression · Immune-related factors

✉ Qingman Cui
cqm80@163.com

[1] College of Marine & Environment, Tianjin University of Science and Technology, Tianjin, China

Introduction

Eriocheir sinensis, which is commonly known as river crab, is a special aquatic product with delicious meat and unique flavor and important economic value (Wang et al. 2013). With the continuous expansion of crab culture, improvement in the intensive degree, and deterioration of the culture environment, their diseases are becoming increasingly severe, such as "water turtle," trembling, and gill rot diseases, which have become major obstacles in the steady development of the breeding industry of *E. sinensis* (Shen et al. 2000; Song et al. 2007). Antibiotics and other chemical drugs are abused in aquaculture, to prevent and control these diseases; misuse can increase the drug resistance of pathogenic bacteria, lead to a decline in crab immunity, and exacerbate the risk of drug residues (Xu et al. 2009). Therefore, pollution-free substances must be urgently identified for the disease control and immune enhancement of crab. Dietary glutathione supplementation enhances antioxidant activity and protects against lipopolysaccharide-induced acute hepatopancreatic injury and cell apoptosis in *Eriocheir sinensis* (Liu et al. 2020).

Astragalus polysaccharides (APS) is a heteropolysaccharide extracted from the root of *Astragalus membranaceus*; it is mainly composed of glucose, galactose, and arabinose. APS has an immunomodulatory effect and has been used as a feed additive in *Procambarus clarkii*, *Amyda sincnsis*, hybrid snakehead, and livestock (Hong et al. 2013; Zhang 2003; Yang et al. 2018; Wang et al. 2018; Wu et al. 2018). Fu et al. (2017) pointed out that astragalus polysaccharide 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. In this study, APS was applied to a culture of *E. sinensis* to investigate its effect on the phagocytosis of hemocytes and the expression of immune-related factors.

Materials and methods

Experimental materials

E. sinensis with a body weight of 6.11 ± 2.25 g, a length of 2.19 ± 0.26 cm, and a width of 2.39 ± 0.29 cm were obtained from Tianjin Xieyuan Aquaculture Co., Ltd.

Vibrio anguillarum was donated by the Tianjin Fisheries Research Institute.

Astragalus polysaccharides was purchased Beijing Bailingwei Technology Co., Ltd.

The basic feed formula was shown in Table 1. Feed with a diameter of 2.5 mm was made following the given formula.

Vertical steam sterilizer was from Shanghai Boxun Industrial Co. Ltd., Bioprep-6 biological sample homogeneous instrument was from Hangzhou Aoxeng Instrument Co. Ltd., polymerase chain reaction (PCR) amplifier and Gel Doc EZ gel imaging system were from Bio-Rad, USA, MIKRO 200R and UNIVERSAL 320R desktop refrigerated centrifuges were from Hettich, Germany, Nikon-90i microscope was from Nikon, Japan, and StepOnePlus™ Real-Time PCR System was from Applied Biosystems, USA.

TRIzol™ reagent kit, M-MLV, random primer, and oligo dT were obtained from Invitrogen Corporation; RNase inhibitor, dNTPs, and dNTPs were acquired from Shanghai biotechnology; Chloroform, isopropanol, and ethanol were obtained from Beijing Solabo Technology Co., Ltd.

Table 1 Basic feed formula of *Eriocheir sinensis*

Feed components	%
Foreign fishmeal	15
Potato	6
Fermented soybean meal	10
Expanded soybean	10
Peanut meal	8
Rapeseed meal	4
Shrimp shell powder	5
Shrimp meal	5
Wheat bran	15
Flour	15
Fish oil	1
Phospholipid	1
Calcium dihydrogen phosphate	4
Compound vitamin	0.3
Composite minerals	0.4
Vc	0.3

Experimental methods

Experimental design and feeding management

The experiment was divided into four groups including control group, 0.05% APS, 0.1% APS, and 0.2% APS. Each group had three replications, and each replicate had 40 crabs cultivated in tank (75 × 55 × 45 cm). Several arched tiles were placed at the bottom of the aquarium for crabs to inhabit.

The feeding amount was 3–5% of the crabs' body weight. The crabs were fed three times a day (8:00, 14:00, and 20:00). The averages of water temperature, pH, and dissolved oxygen values were 25.2 °C, 8.0, and 6.3 mg/L, respectively. The entire feeding experiment lasted 4 weeks.

Phagocytosis

Hemolymph was collected from the cheliped using a syringe with an equal volume of anticoagulant (100 mM glucose, 26 mM citric acid, 415 mM NaCl, 30 mM sodium citrate, 30 mM EDTA, pH 4.6) (Soderhall and Smith 1983) and immediately centrifuged at 1000×*g* for 10 min at 4 °C to harvest the hemocytes.

About 20 μL of FITC (1 mg/mL) was added into 1 mL of *Vibrio parahaemolyticus* suspension (10^6 CFU/mL) for fluorescence labeling for 16 h. Subsequently, 20 μL of FITC labeled bacteria was added into the hemocytes suspension and incubated at 25 °C for 2 h. We then added 20 μL of Hoechast cell staining solution to dye the cells for 20 min. After staining, the slides were washed with PBS three times and observed with a microscope. Each field counted 200 cells, and the phagocytic activity was expressed by the phagocytic percentage:

Phagocytic percentage (%) = (number of phagocytic cells/total number of cells) × 100%

Total RNA extraction

Total RNA of the hepatopancreas, gill, and hemocytes was extracted using TRIzol reagent in accordance with the manufacture's protocol (Invitrogen).

Detection of immune-related factor gene expression in crabs by real-time quantitative polymerase chain reaction (qPCR)

Six immune-related factors were selected: phenol oxidase (PO), glutathione peroxidase (GSH-Px), heat shock protein (HSP), Crus1 (a kind of chitin antibacterial peptide), masquerade like protein (MasL), and anti-lipopolysaccharide factor (ALF). The primer sequences were shown in Table 2.

The β-actin from *E. sinensis* was chosen as reference gene for internal standardization. The 10 μL SYBR Mix, 0.4 μL 50 × ROX, 0.5 μL Primer-F, 0.5 μL Primer-R, 1 μL Template, and 7.6 μL RNase-free water were added into 20 μL reaction system. The reaction conditions were as follows: pre-denaturation at 95 °C for 30 s; PCR amplification at 95 °C for 5 s, 60 °C for 34 s, 50 cycles; and melt curve at 95 °C for 15 s, 60 °C for 60 s, 95 °C for 15 s. All data were given in terms of relative expression.

Statistical analysis

All experimental data were analyzed by one-way ANOVA. The analysis software was SPSS 22.0, and the experimental data were all expressed by mean ± SD.

Results

Effect of APS on the phagocytic activity of hemocytes in *E. sinensis*

The results were shown in Fig. 1 and Table 3. The blue sphere or oval in Fig. 1 was the nucleus of shrimp hemocytes; the green globule was fluorescently labeled *V. anguillarum*, and the green

Table 2 Primer sequence of immune related factors in *Eriocheir sinensis*

Name	Sequence	Length
β-actin-F	5′-GCATCCACGAGACCACTTACA-3′	262.5 bp
β-actin-R	5′-CTCCTGCTTGCTGATCCACATC-3′	
PO-F	5′-CCATCCCTTCCTGCTTACCA-3′	155 bp
PO-R	5′-CTCCATCACAAACCCTAACGACTT-3′	
GSH-Px-F	5′-CAACGGGGCTGATGAAGAC-3′	163 bp
GPX-Px-R	5′-AGGGTGGTAGCGGGTGTAT-3′	
HSP-F	5′-CGGTGGCGGAGAAGTTGTC-3′	162.4 bp
HSP-R	5′-ATCTTGGAGGCGAGGGGA-3′	
Crus1-F	5′-GCTCTATGGCGGAGGATGTCA-3′	125 bp
Crus1-R	5′-CGGGCTTCAGACCCACTTTAC-3′	
MasL-F	5′-TGGGCATCGTCTTTTTCAGG-3′	332.2 bp
MasL-R	5′-TGGCAATGTCGTAGTCCTCGT-3′	
ALF-F	5′-GACCCTTTGCTGAATGCTTGA-3′	108 bp
ALF-R	5′-CTGCTCTACAATGTCGCCTGA-3′	

globule next to the nucleus was the *V. anguillarum* swallowed into the cell. Different contents of APS significantly increased the phagocytic activity of hemocytes in *E. sinensis* ($p < 0.05$), and the phagocytic activity of hemocytes in the 0.1% APS group crabs was the highest (50.84%).

Effect of APS on the gene expression of immune-related factors in hemocytes of *E. sinensis*

qPCR was employed to quantify the mRNA expression of PO, GSH-Px, ALF, Crus1, MasL, and HSP in the hemocytes of crabs (Table 4). The findings showed that 0.05% APS significantly increased the expression levels of PO, GSH-Px, ALF, and HSP genes ($p < 0.05$), and 0.1% APS significantly increased the expression levels of PO, GSH-Px, ALF, MasL, and HSP genes ($p < 0.05$); 0.2% APS significantly improved the expression levels of all genes tested in the hemocytes ($p < 0.05$). However, 0.05% and 0.1% APS had no significant effect on the Crus1 gene expression in the hemocytes of crabs.

Effect of APS on the expression of hepatopancreas immune-related factors in *E. sinensis*

The results were shown in Table 5. 0.05% APS significantly increased the expression level of HSP gene in the hepatopancreas ($p < 0.05$), and 0.1% APS significantly increased the

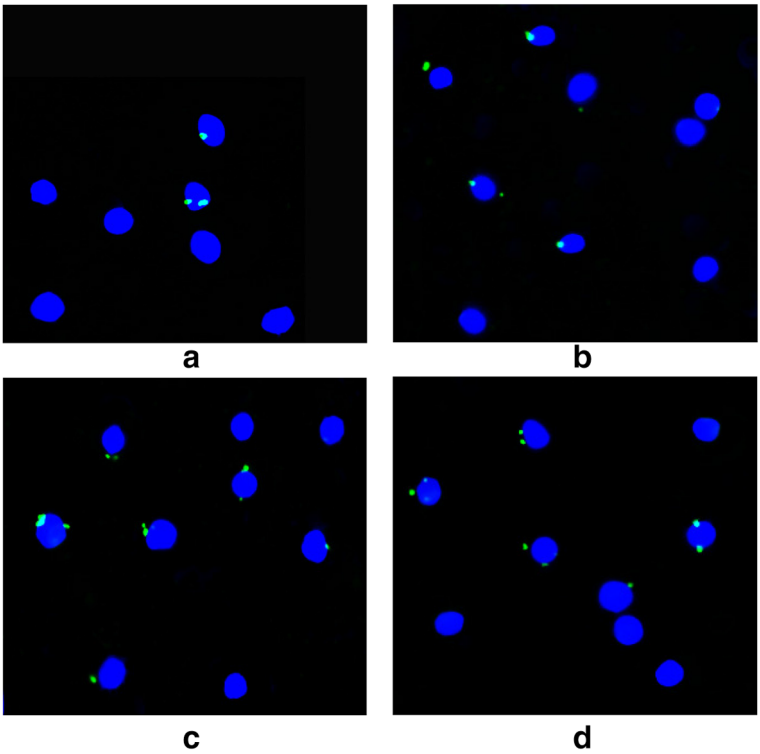

Fig. 1 Fluorescence micrograph of hemophagocytosis in *Eriocheir sinensis*. **a** Control. **b** 0.05%APS. **c** 0.1%APS. **d** 0.2%APS

Table 3 Effect of APS on phagocytic activity of the hemocytes in *Eriocheir sinensis*

Groups	Control	0.05% APS	0.1% APS	0.2% APS
Phagocytosis percentage (%)	19.84 ± 4.81	42.86 ± 12.12*	50.84 ± 16.73*	48.86 ± 12.03*

*$p < 0.05$ vs control group

expression levels of the PO, Crus1, and HSP genes in the hepatopancreas ($p < 0.05$), and 0.2% APS significantly improved the expression levels of the PO, ALF, Crus1, and HSP genes in the hepatopancreas ($p < 0.05$). However, the APS had no significant effect on the GSH-Px and MasL genes expression in the hepatopancreas of crabs.

Effect of APS on the expression of gill immune-related factors in *E. sinensis*

As shown in Table 6, 0.05% APS significantly increased the expression level of MasL gene in the gill ($p < 0.05$), and 0.1% APS significantly improved the expression levels of the PO, GSH-Px, ALF, MasL, and HSP genes in the gill ($p < 0.05$), and 0.2% APS significantly improved the expression levels of all genes tested in the gill ($p < 0.05$).

Discussion

Crustaceans have only a non-specific immune defense mechanism to resist foreign invaders and pathogens. When foreign invaders enter the circulatory system, the hemocytes are affected first (Soderhall and Cerenius 1993; Liang et al. 2012; Roch 1999). Blood lymphocyte is an important defense line in the non-specific immune defense system of crustaceans, and the most important defense function is phagocytosis (Liu and Lu 2007). In this study, APS could significantly improve the phagocytic activity of hemocytes in *E. sinensis*. Li et al. (2014) and Cui et al. (2001) obtained similar results.

In addition to the phagocytosis of hemocytes, crustaceans can resist foreign invaders and pathogens through humoral immunity, such as phenol oxidase system, GSH-Px, Crus1, MasL, ALF, and HSP. The immune function of an animal body is inferred by detecting these genes' expression levels.

The PorPO system in crustaceans is an enzyme cascade system similar to vertebrates' complement system. The factors in the system exist in the granules of blood cells in an inactive state, and a small amount of microbial polysaccharide can activate the porPO system (Meng et al. 1999). As the terminal enzyme of the system, PO can play a role in melanization when

Table 4 Effect of APS on gene expression of hemocytes immune-related factors in *Eriocheir sinensis*

Factors	Control	0.05% APS	0.1% APS	0.2% APS
PO	1.00 ± 0.00	2.71 ± 0.25*	3.05 ± 0.16*	3.29 ± 0.18*
GSH-Px	1.00 ± 0.00	1.55 ± 0.21*	3.11 ± 0.31*	4.64 ± 0.46*
ALF	1.00 ± 0.00	1.46 ± 0.19*	1.85 ± 0.27*	2.87 ± 0.32*
Crus1	1.00 ± 0.00	1.09 ± 0.11	1.22 ± 0.31	1.69 ± 0.24*
MasL	1.00 ± 0.00	1.06 ± 0.32	1.52 ± 0.22*	1.46 ± 0.17*
HSP	1.00 ± 0.00	1.71 ± 0.15*	1.93 ± 0.13*	4.12 ± 0.37*

*$p < 0.05$ vs control group

Table 5 Effect of APS on gene expression of hepatopancreas immune-related factors in *Eriocheir sinensis*

Factors	Control	0.05% APS	0.1% APS	0.2% APS
PO	1.00 ± 0.00	1.31 ± 0.27	1.48 ± 0.24*	1.76 ± 0.13*
GSH-Px	1.00 ± 0.00	1.02 ± 0.17	1.21 ± 0.24	1.13 ± 0.22
ALF	1.00 ± 0.00	1.08 ± 0.21	1.28 ± 0.26	1.72 ± 0.29*
Crus1	1.00 ± 0.00	1.31 ± 0.38	1.85 ± 0.18*	2.03 ± 0.30*
MasL	1.00 ± 0.00	1.08 ± 0.29	1.19 ± 0.26	1.27 ± 0.24
HSP	1.00 ± 0.00	2.07 ± 0.14*	3.38 ± 0.33*	3.23 ± 0.41*

*$p < 0.05$ vs control group

pathogens invade crustaceans (Soderhall 1993). Gai et al. (2008) stated that the mRNA transcript activities of EsproPO and PO can be detected in all examined tissues (hepatopancreas, gill, gonad, muscle, heart, eye, and hemocytes) with the highest level both in hepatopancreas. This study confirmed that the APS significantly increased the expression level of the PO gene in the hemocytes, and 0.1% APS and 0.2% APS significantly increased the expression level of the PO gene in the hepatopancreas and gill. The expression level of the PO gene in the hemocytes and gill was obviously higher than that in the hepatopancreas.

GSH-Px is an important peroxide-degrading enzyme that is widely distributed in the body. It can catalyze GSH to GSSG, reduce toxic peroxides to non-toxic hydroxyl compounds, and promote the decomposition of hydrogen peroxide, thereby protecting the structure and function of cells from interference and damage by peroxides. We found that APS had different effects on the gene expression of GSH-Px in the different tissues of *E. sinensis*. The APS significantly increased the gene expression level of GSH-Px in hemocytes, and 0.1% and 0.2% APS significantly increased the gene expression level of GSH-Px in the gill. The expression level of GSH-Px gene in the hemocytes and gill was higher than those in the hepatopancreas.

ALF was first found in the amoeba like cells of *Limulus polyphemus*. It binds to lipopolysaccharide and has a strong inhibitory effect on R-type Gram-negative bacteria (Li et al. 2008). Multiplicate ALF isoforms coexist in *E. sinensis*, and the diversity of the immune effector molecules in the innate immune system, such as ALF, helps Chinese mitten crabs deal with various pathogens in the aquatic environment (Wang et al. 2011). In this study, we found that the APS had significant effect on the gene expression level of ALF in the hemocytes, and 0.2% APS significantly increased the gene expression level of ALF in the hepatopancreas, and 0.1% and 0.2% APS significantly increased the gene expression level of ALF in the gill. The expression level of the ALF gene in the hemocytes and gill was obviously higher than that in the hepatopancreas.

Table 6 Effect of APS on gene expression of immune-related factors in gill of *Eriocheir sinensis*

Factors	Control	0.05% APS	0.1% APS	0.2% APS
PO	1.00 ± 0.00	1.37 ± 0.25	2.52 ± 0.19*	2.89 ± 0.68*
GSH-Px	1.00 ± 0.00	1.23 ± 0.25	2.76 ± 0.38*	4.08 ± 0.76*
ALF	1.00 ± 0.00	1.19 ± 0.47	1.69 ± 0.21*	2.29 ± 0.61*
Crus1	1.00 ± 0.00	1.08 ± 0.56	1.17 ± 0.14	1.42 ± 0.21*
MasL	1.00 ± 0.00	1.91 ± 0.34*	3.69 ± 0.43*	4.73 ± 0.61*
HSP	1.00 ± 0.00	1.35 ± 0.22	5.55 ± 0.78*	6.94 ± 0.38*

*$p < 0.05$ vs control group

Crustin is another member of the antimicrobial peptide family. It is rich in cysteine and has a whey acidic protein domain. It has a strong inhibitory effect on Gram-positive bacteria (Mu et al. 2010). The mRNA transcripts of CrusEs2 could be detected in hemocytes and gill; its expression level in hemocytes was upregulated after *Listonella anguillarum* challenge, but it decreased after *Micrococcus luteus* challenge (Mu et al. 2011). In this study, 0.1% and 0.2% APS significantly improved the expression levels of Crus1 in the hepatopancreas, and 0.2% APS significantly improved the expression levels of Crus1 in the hemocytes and gill of *E. sinensis*. The expression level of the Crus1 gene in the hemocytes and hepatopancreas was obviously higher than that in the gill.

MasL is a heterodimer protein similar to serine protease, which has a bacterial binding domain; it also has the functions of opsonin, cell adhesion protein, and pattern recognition protein and participates in the antibacterial activity of river crabs (Kawabata et al. 1996). A multifunctional masquerade-like protein has first been isolated, purified, and characterized from hemocytes of *Pacifastacus leniusculus* (Lee and Soderhall 2001). A kind of MasL in *Penaeus monodon* has a noticeable response to *Vibrio* stimulation (Amparyup et al. 2007). In this study, we found that 0.1% and 0.2% APS significantly increased the expression level of MasL gene in the hemocytes of *E. sinensis*, and the APS significantly increased the expression level of MasL gene in the gill. Moreover, the expression level of the MasL gene in the gill was obviously higher than that in the hemocytes and hepatopancreas.

HSP is a protein that is sensitive to environmental stimulation; it repairs or reduces harmful stimulation to the body (Yuan et al. 2008). Nitrite can increase the relative expression level of HSP in the gill of *E. sinensis* (Hong et al. 2011). Du et al. (2013) concluded that APS can protect immune cells from stress injury by inducing HSP70 gene expression in Gifu tilapia. In this study, the APS significantly increased the expression level of HSP gene in hemocytes and hepatopancreas of *E. sinensis*, and 0.1% and 0.2% APS significantly increased the expression level of HSP gene in gill of *E. sinensis.* The expression level of HSP gene in the hemocytes and hepatopancreas of crabs was lower than those in the gill.

In conclusion

Astragalus polysaccharide significantly increased the hemocytes phagocytic activity of crabs and increased the expression levels of all immune-related factor genes tested in crabs to varying degrees. The recommended level of ASP in feed is 0.1–0.2%.

Acknowledgments We are grateful to the Tianjin Key Laboratory of Marine Resource and Chemistry for providing technical assistances.

Funding information This research was financially supported by the innovation team for Tianjin modern agricultural industry technical system (ITTFRS2017006) and Tianjin Binhai New Area Science and Technology Project (BHXQKJXM-SF-2018-26).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical statement All applicable international, national, and/or institutional guidelines for the care and use of animals were followed by the authors.

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