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EFFECTS OF POLYSACCHARIDES INJECTION ON THE NON-SPECIFIC IMMUNE RESPONSES OF AMUR STURGEON *ACIPENSER SCHRENKI*

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Abstract This study was conducted to investigate the effect of different polysaccharides on non-specific immune responses in Amur sturgeons (*Acipenser schrenki*). Four polysaccharides (chitosan, stachyose, yeast polysaccharide and lipopolysaccharide) in rice bran extract (LPS_R) were injected intra-peritoneally into Amur sturgeons. After 9 days, α-naphthyl acetate esterase (ANAE) positivity in peripheral blood, bacteriolytic and hemolytic activities were measured. The results showed that among four polysaccharides, chitosan was the most effective immunostimulator, with significant increase in all three immune parameters compared to the control. The ANAE positivity and lysozyme activity were higher than those in other treatment groups. Stachyose, yeast polysaccharide and LPS_R showed no significant improvement in ANAE activities and bacteriolytic activities, but all displayed higher hemolytic activity than the control.

Key words *Acipenser schrenki*; Polysaccharides; ANAE positivity; Bacteriolytic activity; Alternative complement pathway hemolytic activity

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Since the discovery that adjuvants used in the application of vaccines confer considerable resistance in themselves[1], a wide range of products have been tested for immunostimulatory effects on diverse fish species[2]. The use of immunostimulants is being introduced into fish farming routine procedures for prophylactic purpose. Many fish species showed enhanced non-specific responses with immunostimulants[3—6]. Another advantage of immunostimulants is their use as adjuvants for fish vaccination[7, 8]. As few negative side-effects have been found, immunostimulants are presented as an attractive alternative to control bacterial infections[9, 10].

The effects of immunostimulants are affected by the timing, dosage and method of administration. The most effective method of administration of immunostimulants to fish is injection[2]. Respiratory burst and phagocytic activities of turbot (*Scophthalmus maximus*) leucocytes were enhanced after an in vitro incubation of head kidneys with different doses of yeast glucan, and the optimum dose was 50 μg/mL[11]. The resistance against *Edwardsiella tarda* was enhanced after tilapia (*Oreochromis niloticus*) intraperitoneal injected with 0. 1 mg PS-K (a protein-bound polysaccharide preparetion)/g body weight, and the maximal resistance was developed in fish 1 week after injection[12]. Turbot (*Sophthalmus maximus* L.) intraperitoneal injecting with β-1, 3 glucan, the production of O^{-2} in head kidney leucocytes and lysozyme concentration were higher at day 7 when compared with days 14 and 21[13].

Among polysaccharides from various biological origins (yeasts, algae, bacteria, higher plants and especially fungi), β-glucans are the most intensively studied one in fish[14—17]. Relatively few researches conducted

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on other polysaccharides, such as chitosan and lipopolysaccharide (LPS) etc.[2]. Amur sturgeon (*Acipenser schrenki*), an original species of Osteichithyes, is an important aquaculture species, famous for its caviar. So far, few studies have evaluated the effect of immunostimulants on the immune responses of this fish.

The present study aimed to evaluate the effects of chitosan, stachyose, yeast polysaccharide, and lipopolysaccharide (LPS_R) on the non-specific immune responses of Amur sturgeon, and to compare the immunostimulatory effects of the four polysaccharides.

1 Materials and methods

1.1 Experimental fish Amur sturgeons weighing (35.9±5.7) g (mean±S.D.) were acquired from the sturgeon development center of Beijing Fisheries Research Institute, China, and allocated in 50L opaque plastic tanks with a flow through system at a density of eight fish per tank. The depth of the water is always 50cm. Fish were acclimated to laboratory conditions for 14 days before the experiment. The water temperature was (18±1)℃ and dissolved oxygen was saturated. Fish were fed twice daily with commercial pellets.

1.2 Polysaccharide treatment Tab.1 lists the polysaccharides tested in the experiment. All polysaccharides were provided by Beijing Research Institute for Nutritional Resources except chitosan, which was provided by Institute of Zoology, Chinese Academy of Sciences.

Each polysaccharide powder was suspended at 5 mg/mL in sterile phosphate buffered saline (PBS, 7.3 mmol/L monosodium phosphate, 18.0 mmol/L disodium phosphate, 0.15 mol/L sodium chloride, pH 7.2). Every experimental fish was injected intraperitoneally with 100 μL of the polysaccharide suspension.

Eight fish were injected with polysaccharide in each treatment group. In addition to the treatments in Tab.1, eight fish were injected with sterile PBS in the control group.

表 1 各种多糖的有效成分

Tab.1 Active ingredients of polysaccharides

多糖 Polysaccharide	有效成分 Active ingredient
壳聚糖 Chitosan	壳聚糖标准品 Standard sample of chitosan
水苏糖 Stachyose	含 18% 水苏糖 18% stachyose
酵母多糖 Yeast polysaccharide	酵母提取物(含 20% β-1, 3葡聚糖和 β-1, 6葡聚糖, 18% 甘露聚糖) Yeast extract containing 20% β-1, 3 and 1, 6 Glucans, 18% mannan
米糠脂多糖 LPS_R (lipopolysaccharide)	米糠提取物(含 8%－9% 脂多糖) Rice bran extract containing 8%－9% lipopolysaccharide

1.3 Sampling protocol Fish were sacrificed by a sharp blow at the head 9d after injection, and blood samples were collected from the caudal vein by cutting the tails immediately. About 0.5mL blood was laid to clot for 2h at room temperature (25℃) and centrifuged at 10,000 × g for 15 min. Serum was then collected and stored at −80℃ until processing. About 0.5mL blood was dropped into Eppendorff tubes containing heparin sodium. The sodium heparinized (Na-heparin) blood was used immediately in the α-naphthyl acetate esterase (ANAE) positivity assay.

1.4 ANAE positivity assay Fresh leucocytes were collected from the heparinized blood by diluting (1∶1) with Hank's balanced salt solution. Leucocytes were isolated through a lymphocyte separation medium (d=1.077 g/mL, China, TianjinTBD). Cell suspensions were spun for 5 min at 65 × g to remove erythrocytes and debris. The leucocyte-enriched interphase was drawn off and used to make the smears.

Smears were fixed for 30s at room temperature in citrate-acetone-formaldehyde solution and stained with a fresh solution consisting of 3 mL pararosaniline hydrochloride and 3 mL sodium nitrite (4%), 89mL PBS (1/15 mol/L, pH 7.6), 1 mL α-naphthyl acetate (Sigma-Aldrich Chemical, Illkirsh, France) solution (2%) for 12h at 4℃, protected from light. Then the smears were stained again by methyl green (Sigma M-8884).

A total of 200 lymphocytes were counted under a microscope. The percentage of ANAE positive cells

among total lymphocytes were determined by the formula as follows:

Percentage of ANAE positive lymphocyte = ANAE positive lymphocyte amount/200 × 100%

1.5 Bacteriolytic activity assay The bacteriolytic activity of serum was measured using lyophilised *Micrococcus lysodeikticus* as an indicator of serum lysis according to the method described by Hultmark, *et al*[18] and Wang *et al*[19]. Lyophilised *M. lysodeikticus* (Sigma) were suspended in ice-cold 0.1 mol/L potassium phosphate buffer (pH 6.4). The amount of bacteria in the suspension was chosen to give an optical reading of 0.3—0.5 at a wavelength of 570 nm. A 50 μL sample of serum was added to 3 mL bacterial suspension in an ice-bath, and the absorbance of this mixture was measured at 570 nm (A_0). After incubation at 37℃ for 30 min, the mixture was transferred back to the ice-bath for 10 min to stop the reaction. The absorbance of the mixture was again measured at 570 nm (A). Bacteriolytic activity U_L was calculated by the following formula: $U_L = (A_0 - A)/A$.

1.6 Hemolytic activity assay The hemolytic activity of serum was determined by assaying the alternative complement pathway activity (ACP). The hemolytic assay was carried out in Eppendorff tubes following the technique described by Sunyer and Tort (1995)[20]. Briefly, a volume of 25μL of rabbit erythrocytes (RaRBC) suspension was added to 100μL of serially diluted *A. schrenki* serum in Mg^{2+} EGTA-GVB buffer. The tubes were incubated at room temperature for 1h with occasional shaking. The reaction was stopped by adding 1 mL of cold EDTA-GVB. The tubes were centrifuged at 660 × g for 5 min. The extent of hemolysis was estimated by measuring the optical density of the supernatant at 414 nm (OD_{414}). Total hemolysis or 100% hemolysis is given by the optical reading of the supernatant from 25 μL of the same RaRBC suspension added to 1100 μL of distilled water. The alternative complement pathway hemolytic activity was demonstrated by the ACH50 titer, and the reciprocal of the serum dilution causing 50% lysis of RaRBC was designated as the ACH50 titer, the results are presented as ACH50 units/mL. Rabbit blood was used for this assay since it was previously demonstrated that it is better activators of lytic reaction among mammal bloods.

1.7 Statistical analysis One-way ANOVA was utilized to test the significance of means. When significance was observed, LSD test was used for multiple compositions. All statistical analyses were performed using SPSS 11.5 for windows software.

2 Results

2.1 ANAE positivity

Fig. 1 shows the percentage of ANAE positive lymphocyte in heparinized blood of the Amur sturgeons. Fish injected with chitosan showed significantly higher ANAE activity, while no significant difference was found between other groups ($p > 0.05$).

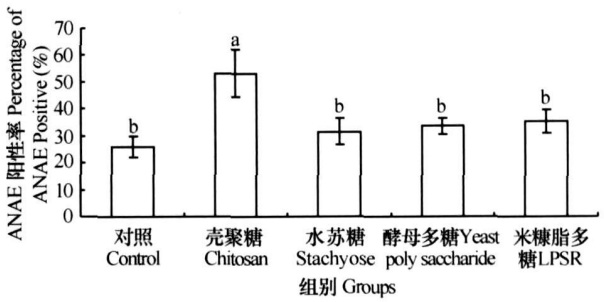

图 1　各组史氏鲟血液中淋巴细胞 ANAE 阳性率

Fig. 1　Percentage of ANAE positive lymphocyte in hepanized blood of Amur sturgeons

纵坐标的数值为各组的平均值 ±标准误, 不同字母代表组间有显著差异 ($p < 0.05$)

Values are means ± S.E. for each group. Different letters denote statistically extreme significant differences ($p < 0.05$) among groups

2.2 Bacteriolytic activity

Chitosan group had significantly higher serum baeteriolytic activity while no significant difference was found between other groups ($p > 0.05$) (Fig. 2).

2.3 Hemolytic activity

All treatment groups showed significantly higher hemolytic activity than the control group (chitosan group, $p = 0.002$; stachyose group, $p = 0.032$; yeast extract group, $p = 0.013$; LPS_R group, $p = 0.008$) (Fig. 3).

There was no difference between each of the groups of fish injected with chitosan, stachyose, yeast polysaccharide, and LPS_R ($p > 0.05$).

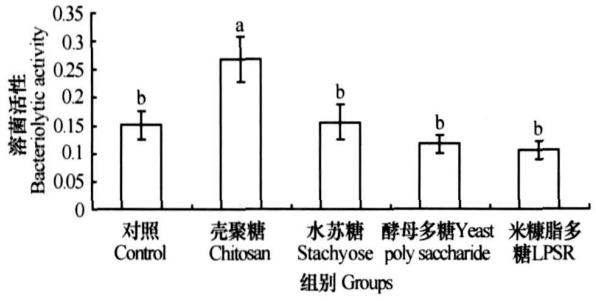

图 2 各组史氏鲟血清溶菌活性

Fig. 2 Serum bacteriolytic activity of the Amur sturgeons

纵坐标的数值为各组的平均值 ± 标准误,不同字母代表组间有显著差异($p < 0.01$)

Values are means ± S. E. for each group; Different letters denote statistically extreme significant differences ($p < 0.01$) among groups

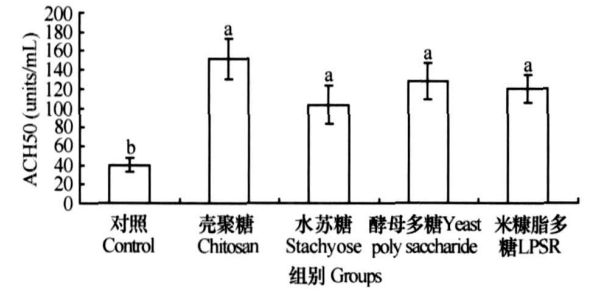

图 3 各组史氏鲟血清旁路补体途径溶血活性(ACH50)

Fig. 3 Serum hemolytic activity of the Amur sturgeons. Values are means ± S. E. for each group

纵坐标的数值为各组的平均值 ± 标准误,不同字母代表组间有显著差异($p < 0.05$)

Different letters denote statistically extreme significant differences ($p < 0.05$) among groups

3 Discussion

In the present study, we evaluated four polysaccharides, chitosan, yeast polysaccharide, stachyose and LPS_R for their ability to stimulate the non-specific immune response of Amur sturgeon, and found that chitosan was the most effective one. Brook trout, *Salvelinus fontinalis*, injected or immersed in chitosan solution showed improved protection against *Aeromonas salmonicida* infection[21]. Similar results were reported in rainbow trout orally administered chitosan [22, 23]. We observed that fish injected with chitosan showed significantly enhanced ANAE positivity and bacteriolytic activity in relation to the control and higher hemolytic activity than the other treatment groups. Although the immune parameters reported varied in different studies, all the results suggested that chitosan has immunostimulatory properties not only in cellular innate immune response, but also in other serum responses. These benefits indicate that chitosan could be a potential alternative and/or supplement to chemotherapy and vaccination for maintaining fish health.

Many studies have shown yeast extracts containing β-glucans and mannans have immunostimulatory properties[2]. Yeast glucan enhances the complement-mediated haemolytic activity in Altlantic salmon blood[24]. It is also reported that the effect of β-glucans is dose dependent, low or high doses afford no improvement in immune system, and very high doses may be inhibitory. In our work we found similar enhance effects of yeast extracts (containing 20% β-1, 3 and 1, 6 Glucans, 18% unspecified mannan) on Amur sturgeon serum hemolytic activity. But whether the enhance effect is the largest or not remained unknown, as we did not examine the appropriate dose rate for Amur sturgeons.

There are many reports showed that LPS (lipopolysaccharide), extracted from cell wall of Gram-negative bacteria can enhance macrophage phagocytic activity of various fishes[2]. Jian etc reported that serum antibody titer, lysozyme and antibacterial activities could be improved by the injection of LPS from *Vibrio alginolyticus*[26]. The LPS_R used in our experiment was a rice bran extract. We found Amur sturgeon injected with LPS_R showed increased hemolytic activity. Different LPS sources may induce different results, and the differences need further researches. Few reports have addressed the effect of stachyose on fish nonspecific immune responses, and we found no comparative data.

Serum complement activity has been identified as a powerful non-specific defence mechanism, protecting fish against bacteria, fungi, viruses and parasites[20, 27, 28]. In this study, only the ACP hemolytic activity was enhanced in all polysaccharides injected groups. Therefore, the ACH50 titer for hemolytic aetivity may be a rapid and sensitive indicator of non-specific immune response to polysaccharide immunostimulants. The ACH50 values of Amur sturgeon sera in control group were higher ((40 ± 8) ACH50 U/mL) than the ACH50 titer found in mammal sera such as human (16 ACH50 U/mL), pig (13.6 ACH50 U/mL), sheep (15.4 ACH50 U/mL), dog (14.4

ACH50 U/mL) and guinea pig (11.9 ACH50 U/mL)[27]. The titration of ACP in the carp *Cyprinus carpio*, sea bass *Dicentrarchus labrax* and sea bream *Sparus aurata*, have been reported to be (68±23) ACH50 U/mL, (460±75) ACH50 U/mL and (170±18) ACH50 U/mL respectively. These values are also much high in relation to those of mammals[20, 29, 30]. In fish, the specific response is not well developed. Its antibody response has low affinity, limited heterogeneity, poor anamnestic qualities and the response time is longer than that of mammals. Hence, high ACP hemolytic activity represents a powerful nonspecific defense mechanism which is required to delay as much as possible the establishment of invading organisms, until the specific response is developed.

The result showed that Amur sturgeon serum displayed a bacteriolytic action, because when serum was incubated with Lyophilised *M. lysodeikticus*, the absorbance of the mixture after incubation was lower than the absorbance at 0.00. Bacteriolysis is an event that may occur when normal microbial multiplication is altered due to an uncontrolled activation of a series of autolytic cell-wall breaking enzymes (muramidases). Cell wall breakdown may occur following bacteriolysis induced by a large variety of bacteriolysis-inducing cationic peptides, such as lysozyme and peptides from leucocytes. Sunyer[20] have reported that both bacteriolytic and hemolytic activities were related to the putative third component of sea bream component (C3). We observed the hemolytic activity significantly increased in all treatment groups, while the bacteriolytic activity significantly increased only in chitosan group. Hemolytic activity reflected the character of complement system, while bacteriolytic activity was affected by more complex components of serum.

The enhancement of nonspecific immune response indexes reveals the response manner of Amur sturgeon to the injection with polysaccharides. Though certain polysaccharides enhance the nonspecific immune response of Amur sturgeon, whether they could really protect fish against infection, and how to use the polysaccharides to reach the best effect still need further studies. The mechanisms behind the immunostimulatory effects are also should be considered for applied studies.

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不同多糖对史氏鲟非特异性免疫反应的影响

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摘要: 本文旨在探讨注射不同多糖后史氏鲟非特异性免疫反应的差异。分别将4种不同来源的多糖(壳聚糖、水苏糖、酵母聚糖和米糠脂多糖)腹腔注射到史氏鲟体内, 注射9d后, 观测血液中淋巴细胞α-醋酸萘酯酶(α-naphthyl acetate esterase, ANAE)阳性率、血清溶菌活性(Bacteriolytic activity)和血清旁路补体途径溶血活性(ACP hemolytic activity)。结果显示壳聚糖(Chitosan)在几种多糖中免疫刺激作用最强。壳聚糖组与对照组相比, 所有的免疫指标活性均有显著提高。壳聚糖组ANAE活性和溶菌活性与其他实验组相比也有显著提高。水苏糖(Stachyose)组、酵母多糖(Yeast polysaccharide)组和脂多糖(LPS_R)组与对照相比血清旁路补体途径溶血活性增强, 而对ANAE活性和溶菌活性没有显著影响。

关键词: 史氏鲟; 多糖; ANAE阳性率; 溶菌活性; 血清旁路途径溶血活性