The Immune Response and Passive Protective Abilities of the Outer Membrane Lipoprotein Slp of *Aeromonas hydrophila* against the Major Pathogenic Bacteria of Freshwater Aquaculture in Fish

Sijie Jian1,2,3,4, Wei Sun1,3, Jia Chao1,2, Na Rong1,4, Xiang Liu1,2,3,4,* and Chen Chen1,2,3,4,*

1Chinese-German Joint Institute for Natural Product Research, College of Biological Science and Engineering, Shaanxi University of Technology, Hanzhong 723000, China
2Shaanxi Province Key Laboratory of Bio-Resources, Hanzhong 723000, China
3QinLing-Bashan Mountains Bioresources Comprehensive Development C.I.C., Hanzhong 723000, China
4Qinba State Key Laboratory of Biological Resources and Ecological Environment, Hanzhong 723000, China

**ABSTRACT**

*Aeromonas hydrophila* and *Pseudomonas fluorescens* are major pathogenic bacteria in freshwater aquaculture and cause huge economic losses. The outer membrane lipoprotein Slp of *A. hydrophila* has potential applications in fish vaccines. Slp bioinformatics analysis showed that anti-Slp serum might provide cross-protection to resist bacterial infection in fish. Slp was obtained by molecular cloning, expression and purification, and the expression conditions were optimized. In mice immunized with Slp, the immune-related factors of LZM and AKP were enhanced (*p* < 0.05), and a specific antiserum titer (1: 3200) was obtained that had immune recognition effects for *A. hydrophila* and *P. fluorescens*. Passive immunization of *Carassius auratus* with Slp mouse serum and challenging with bacteria showed a passive protection rate of Slp serum against *A. hydrophila* infection of 42.5% (*p* < 0.05) and a passive cross-protection rate against *P. fluorescens* of 18.6%; the immune-related factors of LZM, AKP and ACP and the leukocyte phagocytosis of phagocytic percentage (PP) and phagocytic index (PI) increased (*p* < 0.05); the inflammation-related genes expression of IL-6, IL-8, IL-1β and TNF-α decreased (*p* < 0.05) in kidneys and spleens after challenging with *A. hydrophila* and *P. fluorescens*; and reduced injury to the kidneys, spleens and intestines; there was no significant change in the antioxidant-related factors of MDA, SOD, CAT and GSH-PX. Therefore, Slp has passive protection (*A. hydrophila*) and passive cross-protection (*P. fluorescens*) abilities and it can boost resistance to infection using major freshwater aquaculture etiologic agent of fish.

**INTRODUCTION**

*Aeromonas hydrophila* is a gram-negative bacillus with a single flagellum that belongs to a species of g-proteobacteria in the family Aeromonadaceae. It is widely distributed in nature, especially in water, and is a conditional pathogen of a variety of aquatic animals (Dong et al., 2020; Nayak, 2020). Cold-blooded animals, such as fish and frogs, are the natural hosts of *A. hydrophila*, which can produce highly toxic exotoxins, such as hemolysin, tissue toxin, necrosis toxin, enterotoxin, and protease, and cause skin ulcers and sepsis in fish (Barger et al., 2020). *Pseudomonas fluorescens* is a gram-negative bacteria that exists widely in soil and aquatic environments (Marvin et al., 2019) and is a primary pathogen affecting a wide range of farmed fish, such as carp, grass carp, and crucian, causing ascites, visceral organ swelling, bleeding, and necrosis (Wang et al., 2020). These two bacteria cause high mortality, resulting in huge economic losses in...
freshwater aquaculture. They are also zoonotic pathogens and can infect animals and humans (Nayak, 2020; Wang et al., 2020).

Antibiotics are the primary drugs used to prevent and treat A. hydrophila and P. fluorescens infections in freshwater aquaculture fish (Bilen and Ellebshi, 2019; González-Renteria et al., 2020), but the abuse of antibiotics will inevitably lead to bacterial resistance, drug residues, and environmental pollution (Zhang et al., 2020). Many preventative treatments have been researched for fish bacterial diseases, including whole-cell inactivated vaccines, live attenuated vaccines, nucleic acid vaccines, and genetic engineering, but these are still at the experimental stages, and the need to develop new vaccines to prevent and treat aquatic pathogenic bacterial infections in fish.

The outer membrane proteins (OMPs) of Gram-negative bacteria are located in the outermost layer of the bacteria and are important virulence factors (Xu et al., 2020), helping to maintain cellular integrity, signal transduction, transport nutrients, metabolize energy, promote the formation of biofilms (Egan, 2018; Silva et al., 2020), and contribute to the adhesion and colonization of bacteria in the host (Bonsor and Sundberg, 2019; Vaca et al., 2020). OMPs are also relatively easily recognized by the host’s immune system, and stimulate an immune response for locating on the surface of the bacteria (Cole et al., 2021). Thus, many OMPs have high immunogenicity for potential candidate vaccine proteins, including A, C, F, and H bacterial OMPs (Pore and Chakrabarti, 2013; Liko et al., 2018; Diao et al., 2020; Nie et al., 2020), which can improve the resistance of animals to bacterial infection.

The outer membrane lipoprotein Slp of A. hydrophila is located in the outermost layer of the bacteria and has an eight-stranded beta-barrel domain (Hooda et al., 2017), which contributes to adhesion in the host (Fedorchuk et al., 2019), bacterial antibiotic resistance, and bacterial virulence (Hooda et al., 2016; Price and St-John, 2000). Slp may also be a prime candidate for vaccine development (Hooda et al., 2017), but its immune responses and passive protective abilities are unclear.

In the current study, A. hydrophila Slp protein was obtained by molecular cloning, expression, and purification, and Slp mouse serum was prepared and its immune function analyzed. The passive protective activity was evaluated by immunizing Carassius auratus with Slp mouse serum, challenging with A. hydrophila and P. fluorescens, and analyzing the protection rate, immune-related factors, inflammation-related genes, antioxidant-related factors, and pathological sections. This research is expected to provide the theoretical basis for Slp vaccine development in fish.

MATERIALS AND METHODS

Animals and bacterial strains

C. auratus were purchased from Shanghai Original Ecological Aquarium Co. Ltd. (Shanghai, China), and Kunming mice were purchased from Chongqing Tengxin Biotechnology Co. Ltd. (Chongqing, China). All animal procedures were performed in accordance with the guidelines in the Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Ethics Committee, Shaanxi University of Technology, Hanzhong, China (No. 2020-08).

A. hydrophila ATCC7966, P. fluorescens ATCC13525, Escherichia coli BL21, E. coli DH5a and pET32a plasmid were all preserved in the biochemistry and molecular laboratory of Shaanxi University of Technology.

Homologous and phylogenetic analysis of Slp

The Slp protein sequences of different bacteria were obtained from the NCBI database: A. hydrophila (ABK38004.1), P. fluorescens (SUD28948.1), Escherichia coli (QYG81998.1), Enterobacter hormaechei QLW08570.1), Shigella boydii (QQT76177.1) and Pseudomonas aeruginosa (WP_216825990.1). Multiple sequence alignment maps and phylogenetic trees were constructed using DNAMAN and MEGA software, respectively.

Construction, expression and purification of Slp

The slp gene recombinant strain was constructed by molecular cloning. Briefly, according to the slp gene sequence (GenBank: ABK38004.1) from the NCBI database, primers were designed to amplify the slp gene using the PCR method: F-slp: AGGGAATTCTATGGTGAAGCGTGCAATG; R-slp: T A A C T C G A G T C A T G C G G C G C T G C AGCGGCCGT (the underlined position is the restriction site of EcoR I and Xho I, respectively). The genome of A. hydrophila was extracted according to the instructions of the bacteria genomic DNA extraction kit (Takara, Dalian, China), which was used as a template to amplify the slp gene by PCR following the instructions of the rTaq polymerase kit (Takara, Dalian, China). The PCR product consisted of 30 cycles of pre-denaturing for 3 min at 94 °C, denaturing for 30 s at 94 °C, annealing for 45 s at 55 °C and extension for 90 s at 72 °C, followed by abundant extension for 10 min at 72 °C. The PCR samples were digested with EcoR I and Xho I and connected to pET-32a plasmid by T4-DNA ligase (Takara, Dalian, China). Then, they were transformed to E. coli DH5a to amplify slp recombinant plasmid, which was further identified by restriction enzyme digestion and gene sequencing identify
An orthogonal experimental design \( L_9(3^4) \) was used to optimize the expression conditions of Slp. Briefly, the recombinant plasmid was transformed into \( E. coli \) BL21 to construct a slp gene prokaryotic expression strain (Liu et al., 2018).

The Slp protein was expressed and purified as previously described (Chen et al., 2019). Briefly, the recombinant Slp strain was cultured overnight at 37°C and transferred to 600 mL fresh LB medium until bacterial concentration reached 0.6 (OD\(_{600}\) nm). Then, isopropyl-\(\beta\)-D-thiogalactoside (IPTG) (Sigma, St. Louis, USA) was added and induced at 20 °C for 24 h to express Slp protein. After centrifugation, bacteria were disrupted with ultrasonic crushing, and Slp was purified with Ni-NTA flow resin (Sigma, St. Louis, USA).

**The optimal expression conditions of Slp**

The expression conditions of Slp was optimized by an orthogonal experimental design \( L_9(3^4) \) as previously described (Chen et al., 2019). Briefly, the four-factor parameters are the strain-inducing concentration of OD\(_{600}\) value (A), IPTG inducing concentration (B), inducing time (C) and inducing temperature (D); the three-level parameters are shown in Table II. According to the \( L_9(3^4) \) model, once bacteria were cultured to the designed OD\(_{600}\) value, different concentrations of IPTG were added to induce Slp expression at settings temperature and time. Next, 2×SDS protein loading buffer (300 μL) was added after the bacterial solution (1 mL) was harvested by centrifugation; it was then heated for 5 min in boiling water. Next, 10 μL samples were used for SDS-PAGE gel electrophoresis, and the Slp expression was visualised by G-250 staining. The density of Slp bands was analysed using Phoretix 1D software to assess the density dataset. Range analysis and SPSS software were employed to analyse optimal expression conditions.

**Preparation, specificity, titre and immune-related factors analysis of Slp polyclonal antibody**

Mice were immunized with Slp (50 μg per mouse) to prepare the polyclonal antibody. Freund’s complete adjuvant (Sigma, St. Louis, USA) was used in the first immunization, and the second and third immunizations consisted of Freund’s incomplete adjuvant (Sigma, St. Louis, USA). The second immunization occurred at 14-day intervals, and the third immunization occurred at 7-days intervals. Seven days after the third immunization, mice plasma was collected from the eyeballs under anaesthesia. The serum was harvested overnight culture for SDS-PAGE gel electrophoresis, and the proteins were transferred from SDS-PAGE gel to the NC membrane at 80 V for 1 h. Skim milk (5%) was used to block the NC membrane for 2 h at room temperature. The serum of different dilutions (1: 800, 1: 1600, 1: 3200) was added to the NC membrane and incubated at 37 °C for 1 h, and received blank serum (NC) as a control. Then, the NC membrane was incubated with secondary goat anti-mouse antibodies (Sigma, St. Louis, USA), and a dimethylaminobenzene (DAB) substrate system (Sigma, St. Louis, USA) was employed to visualise bands.

The immune-related factors of lysozyme (LZM), acid phosphatase (ACP), and alkaline phosphatase (AKP) were assessed in serum according to the manufacturer’s instructions (Jiancheng Institute of Biotechnology Co. Ltd., Nanjing, China).

**Analysis of immune recognition between antiserum and bacteria**

The recognition between the antiserum and bacteria was identified using ELISA (Liu et al., 2021). Briefly, bacteria were harvested after being cultured overnight and were inactivated with oxymethylene (1%, W/V), and the bacterial concentration was adjusted to 6×10\(^8\) CFU/mL with normal saline. The bacteria and antiserum at the various dilutions were mixed and added to 1.5 mL tubes to incubate at 37 °C for 1 h. After the second antibody was added, the samples were suspended with PBS solution (20 μL) and transfer to a 96-well enzyme-linked plate. After the addition of colouration liquid (50 μL TMB and 50 μL H\(_2\)O\(_2\)) and 50 μL stop solution (2 M H\(_2\)SO\(_4\)) to the wells, the absorbance values were analysed using a microplate reader at OD\(_{450}\) nm (Bio-Rad, Hercules, USA).

**Passive protective ability of Slp mouse serum in C. auratus**

The passive protective ability of Slp mouse serum was evaluated using \( C. auratus \) immunized with serum and challenged with \( A. hydrophila \) and \( P. fluorescens \) (Liu et al., 2018). Briefly, \( C. auratus \) were divided into four groups with 15 fish per group. Groups 1 and 2 were intraperitoneally immunized with 30 μL Slp serum, and groups 3 and 4 received blank serum (NC) as a control. After 2 h, fish of groups 1 and 3 were challenged with \( A. hydrophila \) (3 × 10\(^6\) CFU), and groups 2 and 4 with \( P. fluorescens \) (1.5 × 10\(^8\) CFU). Then, fish mortality was counted for 14 days, and the immune protection rate (RPS) of \( C. auratus \) was calculated using the formula RPS (\%) = \left[1 - (\% \text{ vaccinated mortality}/\% \text{ control mortality})\right] × 100. SPSS software was used to analyse the statistical significance.
Table I. Primers applied to qRT-PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession number</th>
<th>Forward primer (5'-3')</th>
<th>Reverse primer (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6</td>
<td>XM_026289280.1</td>
<td>TCTCCTCAGACCTCGAGAG</td>
<td>CGTTTGGTCCCGTGTTTGAC</td>
</tr>
<tr>
<td>IL-8</td>
<td>XM_026267284.1</td>
<td>GGAGTGCAGGCCACTGTTAG</td>
<td>ATCAGAAGCATGAAGCCGA</td>
</tr>
<tr>
<td>IL-1β</td>
<td>AJ249136.1</td>
<td>TCCAGGAAAAGACGGGACAC</td>
<td>GTCAATGGCACCTGTATCA</td>
</tr>
<tr>
<td>TNF-α</td>
<td>EU069817.1</td>
<td>GGGCCACATCGGTATGTA</td>
<td>GCTCCCAAGTGCACGTGTA</td>
</tr>
<tr>
<td>GAPDH</td>
<td>XM_026284269.1</td>
<td>GATTTCAACGGGGATGTGCG</td>
<td>TCACACACACGGTTGCTGA</td>
</tr>
</tbody>
</table>

Table II. The expressing conditions of Slp were optimized by optical density analysis.

<table>
<thead>
<tr>
<th>Factors</th>
<th>A (OD600)</th>
<th>B (mmol/L)</th>
<th>C (h)</th>
<th>D (℃)</th>
<th>Optical density ±SD (×10^-6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1B1C1D1</td>
<td>0.5</td>
<td>0.1</td>
<td>3</td>
<td>28</td>
<td>1.657 ± 0.406</td>
</tr>
<tr>
<td>A1B2C2D2</td>
<td>0.5</td>
<td>0.3</td>
<td>8</td>
<td>32</td>
<td>1.705 ± 0.318</td>
</tr>
<tr>
<td>A1B3C3D3</td>
<td>0.5</td>
<td>0.5</td>
<td>12</td>
<td>37</td>
<td>1.580 ± 0.245</td>
</tr>
<tr>
<td>A2B1C2D3</td>
<td>0.8</td>
<td>0.1</td>
<td>8</td>
<td>37</td>
<td>1.427 ± 0.286</td>
</tr>
<tr>
<td>A2B2C3D1</td>
<td>0.8</td>
<td>0.3</td>
<td>12</td>
<td>28</td>
<td>1.243 ± 0.292</td>
</tr>
<tr>
<td>A2B3C1D2</td>
<td>0.8</td>
<td>0.5</td>
<td>3</td>
<td>32</td>
<td>1.394 ± 0.139</td>
</tr>
<tr>
<td>A3B1C3D2</td>
<td>1.0</td>
<td>0.1</td>
<td>12</td>
<td>32</td>
<td>1.420 ± 0.221</td>
</tr>
<tr>
<td>A3B2C1D3</td>
<td>1.0</td>
<td>0.3</td>
<td>3</td>
<td>37</td>
<td>1.341 ± 0.271</td>
</tr>
<tr>
<td>A3B3C2D1</td>
<td>1.0</td>
<td>0.5</td>
<td>8</td>
<td>28</td>
<td>1.309 ± 0.177</td>
</tr>
</tbody>
</table>

K1(Mean 1) | 1.543 ± 1.398
K2(Mean 2) | 1.320 ± 1.384
K3(Mean 3) | 1.284 ± 1.365
Range | 0.260 ± 0.033

Significance * — — —

Note: *p < 0.05 (compared with control). A, B, C and D are the strain OD600 values, IPTG concentration, induction time, and induction temperature, respectively. K1, K2 and K3 are the mean values. The optimal expressing conditions of Slp were A1, B1, C2 and D2.

Leukocyte phagocytosis of plasma

Leukocyte phagocytosis was performed as follows: 2 mL of S. aureus (6×10^8 CFU/mL) and C. auratus plasma were incubated at 25℃ for 60 min; 10 µL mixture solution was drawn to glass slide; and methanol was added. After Giemsa dyeing solution performed for 30 min, the slide was washed and air dried for observation with an oil microscope. The phagocytic percentage (PP %) was obtained using the following formula: WBCs involved phagocytosis of 100 WBCs / 100 ×100%, and phagocytic index (PI %): Bacteria phagocytised/ WBCs phagocytising bacteria. The results were analysed the ANOVA method, and the significance was determined using SPSS19.0 software.

Analysis of inflammation-related gene expression using qRT-PCR

After 2 days of challenging with bacteria, C. auratus serum was harvested at 3000 r/min and was used to analyse the antioxidant index of visceral organs using malondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GSH-PX) and catalase (CAT) according to the kit instructions (Jiancheng Institute of Biotechnology Co. Ltd., Nanjing, China).

Histopathological observation of visceral organs

Pathological sections of the kidneys, spleens and intestines of the C. auratus were prepared with H&E staining (Liu et al., 2020). Briefly, the kidneys, spleens and intestines were obtained after 2 days of challenging with bacteria and were dehydrated for 1 h with an alcohol gradient and added to alcohol: xylene mixture (1:1, V/V) for 25 min, xylene for 10 min; xylene: paraffin solution (1:1, V/V) for 30 min and paraffin at 60℃ for 1 h. Next, slices of roughly 5 µm thickness were cut, stained with H & E, observed and photographed with a microscope (Leica, Wetzlar, Germany).

RESULTS

Homologous and phylogenetic analysis of Slp

Based on the Slp amino acid sequences of the different bacteria, homologous analysis showed that the species of bacteria have homology (Fig. 1A); and the phylogenetic trees showed that the relationship among species of Enterobacteria (E. coli, E. hormaechei and S. boydii) was closer than other kinds of bacteria, and the relationship was obtained from the kidney, spleen and gill tissues of C. auratus using an RNA isolation kit (Takara, Tokyo, Japan), and qRT-PCR was performed as previously described (Liu et al., 2021). A commercial kit (Takara, Tokyo, Japan) was utilised to prepare cDNA with mRNA reverse transcription, and qRT-PCR was implemented using a qRT-PCR system (ABI Applied Biosystems, Waltham, USA) with a SYBR® Green Permix Pro Taq HSqPCR kit (Takara, Tokyo, Japan) and synthetic primers (Vosun Biotechnology Co. Ltd.) (Table I). The 2^(-ΔΔCt) method was employed to analyse the mRNA expression with the internal control gene GAPDH.

Analysis of antioxidant index of C. auratus

After 2 days of challenging with bacteria, C. auratus serum was harvested at 3000 r/min and was used to analyse the antioxidant index of visceral organs using malondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GSH-PX) and catalase (CAT) according to the kit instructions (Jiancheng Institute of Biotechnology Co. Ltd., Nanjing, China).
between *A. hydrophila* and *P. fluorescens* was closer than others (Fig. 1B). Therefore, anti-Slp serum may provide cross-protection against *A. hydrophila* and *P. fluorescens* infection in animals.

**Construction, expression and purification of recombinant Slp**

The *slp* gene was amplified from the *A. hydrophila* genome with PCR. DNA electrophoresis showed there was a single band at about 500 bp (Fig. 2A), which was consistent with the theoretical value. The recombinant *slp* plasmid was digested by *EcoR* I and *Xho* I, and a 500 bp band was obtained (Fig. 2B). The results of gene sequencing showed the target gene is consistent with the *slp* sequence of the NCBI database.
After the recombinant strain of Slp was induced with IPTG, the results of SDS-PAGE electrophoresis showed the target protein was about 39 kDa, including 19 kDa of the target protein and 20.4 kDa of the fusion protein, which was consistent with the theoretical value. The Slp was purified with Ni-NTA flow resin (Fig. 2C).

**The optimal expressing conditions of Slp**

According to the L9(3^4) orthogonal design SDS-PAGE electrophoretic maps were obtained (Fig. 3), and the optical density of the bands was analysed using Phoretix 1D software. The mean value analysis showed the optimal Slp expressing conditions were A1, B1, C2 and D2, which means a strain OD_{600} value of 0.5, IPTG concentration of 0.1 mmol/L, inducing time of 8 h and inducing temperature of 32 °C (Table II).

**Immune activity of Slp in mice**

In order to assess the effects of nonspecific immunity, the immune-related factors of LZM, AKP and ACP in Slp mouse serum were determined and the results showed that LZM and AKP increased (p < 0.05) and ACP unchanged (Figure 4), which means Slp could activate nonspecific immunity in mice.

The recognition of Slp mouse serum to A. hydrophila and P. fluorescens in vitro was simulated by the ELISA method. With the increases in serum dilution, the ability of SLP serum to recognize A. hydrophila and P. fluorescens decreased gradually, and the recognition between Slp serum and these two kinds of bacteria was evaluated with the dilution of 1:12 800 (Fig. 5A). Western blotting results showed that Slp serum was specifically combined with Slp and that the negative control (blank antiserum) had no band. Slp could stimulate the mice to produce a specific antiserum with a titre of 1: 3200 (Fig. 5B). These results suggest that Slp may have antigenicity.

**Passive protective and passive cross-protective rate of Slp mouse serum in C. auratus**

In order to assess the effects of passive protective and passive cross-protective abilities, C. auratus was immunized with Slp mouse serum and challenged with P. fluorescens and A. hydrophila. C. auratus showed obvious toxic symptoms after being challenged with the bacteria, including less food intake and sluggish activity. The majority of the C. auratus in the control group died within 5 days; the remainder survived and gradually recovered after 6 days (Fig. 6). The passive protective rate of Slp mouse serum against A. hydrophila was 42.5 % (p < 0.05), and the passive cross-protective rate of Slp mouse serum against P. fluorescens infection was 18.6%. Therefore, the Slp mouse serum has passive protective and passive cross-protective abilities.

**Immune-related factors and leukocyte phagocytosis of C. auratus plasma to evaluate passive protective abilities**

The immune-related factors of LZM, AKP and ACP and the leukocyte phagocytosis of C. auratus plasma were determined on 2 days after the C. auratus were passive immunized with Slp mouse serum. The results showed that LZM, AKP and ACP increased (p < 0.01) (Fig. 7), and the leukocyte phagocytosis indexes of PP and PI were higher than those of the NC group (p < 0.01) (Table III). These results suggest that passive immunization with Slp mouse serum can activate a nonspecific immunity in C. auratus.
Immune Response and Passive Protective Abilities of Slp

Fig. 5. The recognition between antibodies and bacteria (A) and specificity and titre of antibody (B). Panel A shows Slp serum can recognize to *A. hydrophila* and *P. fluorescens* with the dilution of 1:12 800 *in vitro*. Panel B shows that Slp serum has specificity and of the titre is 1:3200 times.

Table III. Leukocyte phagocytosis of *C. auratus* plasma after passive immunization with Slp mouse serum.

<table>
<thead>
<tr>
<th>Index</th>
<th>Immunizing blank mouse serum (control)</th>
<th>Immunizing Slp mouse serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phagocytic percentage (PP %)</td>
<td>13.65±0.29</td>
<td>24.87±3.14**</td>
</tr>
<tr>
<td>Phagocytic index (PI %)</td>
<td>6.04±0.57</td>
<td>7.64±0.85*</td>
</tr>
</tbody>
</table>

Note: *p < 0.05, **p < 0.01 (compared with control). The leukocyte phagocytosis effects of Slp serum were higher than the control group (p < 0.05) after passive immunization with Slp mouse serum.

Fig. 6. Percent survival of passive immune protection on *C. auratus*. *p < 0.05 (compared with control). A and B represent to challenge with *A. hydrophila* and *P. fluorescens*, respectively.

Inflammation-related gene expression to evaluate passive protective abilities

In order to assess the passive protective and passive cross-protective abilities of Slp mouse serum, the mRNA expression of inflammation-related genes (IL-6, IL-8, IL-1β and TNF-α) were analysed in the kidneys and spleens on 2 days after the *C. auratus* were immunized with Slp mouse serum and challenged with bacteria. Compared to the NC groups, most of the mRNA expression of IL-6, IL-8, IL-1β and TNF-α decreased (p < 0.05) in kidneys and spleens after being challenged with *A. hydrophila* (Fig. 8A). In addition, after being challenged with *P. fluorescens*, most of the mRNA expression of IL-6, IL-8, IL-1β and TNF-α decreased (p < 0.05) in kidneys and spleens (Fig. 8B). The results suggest that passive immunization with Slp mouse serum could reduce the inflammatory reaction induced by *A. hydrophila* and *P. fluorescens* infection in *C. auratus*.

Fig. 7. The immune-related factors of *C. auratus* plasma after passive immunization with Slp mouse serum. **p < 0.01 (compared with control). LZM, AKP and ACP increased (p < 0.01), meaning that passive immunization with Slp mouse serum may activate a nonspecific immunity in *C. auratus*.

Levels of antioxidant-related factors to evaluate passive protective abilities

In order to assess the antioxidant effects of passive immunization with Slp mouse serum, the antioxidant-related factors of MDA, SOD, GSH-PX and CAT in *C. auratus* serum were determined on 2 days after immunizing with Slp serum and challenging with bacteria. The results showed that MDA, SOD, CAT and GSH-PX were no significant change in *C. auratus* serum after challenging with *A. hydrophila* and *P. fluorescens* (Fig. 9). These results suggest that passive immunization with Slp mouse serum has no antioxidative effect against *A. hydrophila* and *fluorescens* infection in *C. auratus*. 
Fig. 8. Analysis of the inflammation-related genes expression after immunizing Slp mouse serum and challenging bacteria in *C. auratus*. *\( p < 0.05 \), **\( p < 0.01 \) (compared with control). Most of the mRNA expression of IL-6, IL-8, IL-1β and TNF-α decreased (\( p < 0.05 \)) in the kidney and spleen after being challenged with *A. hydrophila* (A) and *P. fluorescens* (B).

Fig. 9. The antioxidant effects of passive immunizing Slp mouse serum and challenging *A. hydrophila* (A) and *P. fluorescens* (B) in *C. auratus*. MDA, SOD, CAT and GSH-PX were no significant change in *C. auratus* serum.

**Histopathology of *C. auratus* to evaluate passive protective abilities**

In order to assess the protective abilities of Slp mouse serum for viscera against bacterial infection, sections of the kidneys, spleens and intestines were prepared on 2 days after *C. auratus* were immunized with Slp serum and challenged with *A. hydrophila* and *P. fluorescens* to observe any injury. Compared to the kidneys of *C. auratus* immunized with Slp serum, the kidneys of *C. auratus* that received NC appeared to have glomerular atrophy, nuclear apoptosis and incomplete organisational structure (Fig. 10Aa and 10Ac); the spleens that received NC appeared to less nuclear density and loose organisational structure (Fig. 10Ba and 10Bc); the intestines that received NC appeared to have abscission of intestinal villos epithelial cells and myometrial cell apoptosis and degeneration (Fig. 10Ca and 10Cc). Therefore, Slp serum can reduce the injury to the kidneys, spleens and intestines in *C. auratus* caused by *P. fluorescens* and *A. hydrophila*. 
Fig. 10. Histopathology of kidneys, spleens and intestines of *C. auratus* after immunization and bacteria challenge (H & E ×400 magnification). A, B and C represent the sections of the kidneys, spleens and intestines, respectively. *C. auratus* were passive immunization NC (blank serum) and challenged with *A. hydrophila* (a) and *P. fluorescens* (c). *C. auratus* were passive immunization Slp mouse serum and challenged with *A. hydrophila* (b) and *P. fluorescens* (d). After bacteria challenge, glomerular atrophy, nuclear apoptosis and incomplete organisational structure were observed (Aa and Ac); less nuclear density and loose organisational structure (Ba and Bc); and abscission of intestinal villous epithelial cells, myometrial cells apoptosis, and degeneration were observed (Ca and Cc). Thus, passive immunization with Slp mouse serum can reduce the injury to the kidney, spleen and intestine.

*A. hydrophila* and *P. fluorescens* are major freshwater aquaculture etiologic agent in fish, and protein vaccines are particularly important (Circella et al., 2020; Dong et al., 2020). *A. hydrophila* Slp is located in the outermost layer of the cell membrane and belongs to an OMP protein; it interacts with the host and has immune activity (Hooda et al., 2017). In current research, the recombinant protein of *A. hydrophila* Slp was constructed by molecular cloning and purified with Ni-NTA flow resin. The optimal expressing conditions of Slp were strain OD$_{600}$ of 0.5,
IPTG concentration of 0.1 mmol/L, induction temperature of 32 °C and time of 8 h. We found a low concentration of IPTG is beneficial to the expression of Slp, which may be related to the cytotoxicity of IPTG, and is consistent with our previous study showing that protein expression needs a low concentration of IPTG (Chen et al., 2019). These results lay the foundation for Slp immune function.

Mice polyclonal antibodies have been widely used in the analysis of immune protein function due to their convenience and shorter preparation time, relatively simple preparation compared to monoclonal antibodies (Beigel, 2018). The research of protein immune function usually needs to be prepared its antibody. In previous studies, we have obtained OMPs antisera of OmpA, OmpC, OmpK, and found they have an immune protective function (Liu et al., 2018; Chen et al., 2019). In current research, a specificity Slp mouse serum was prepared with a titre of 3200 times. Slp serum could recognize A. hydrophila and P. fluorescens to form the compound of Slp antibodies-bacteria in vitro, and the complex may provide an opportunity for antigen presentation and phagocytic removal of bacteria in fish (Nelson et al., 2020). In addition, we found that anti-Slp serum may provide cross-protection against A. hydrophila and P. fluorescens infection in animals by bioinformatics. The immune-related factors of LZM, AKP and ACP can improve the immune ability of animals and promote the elimination of bacteria and viruses (Zhong et al., 2019; Niu et al., 2020). In this research, we found that the immune-related factors increased (p < 0.05) by immunizing mice with Slp. These results suggest that Slp can activate the immune activity of mice.

Mice have a more sophisticated immune system than fish and can activate a higher antibody titre, and passive immunization of fish with mouse antiserum can be used to screen for protective immunogens to assess immune protein ability (Li et al., 2010; Peng et al., 2016, 2018). Peng et al. (2016) have used passive immunization antiserum method to identify 17 OMPs of Vibrio parahaemolyticus, and VP2309, VP0887, VPA0548 and VP1019 have been found to have efficiently protective immunogens. In this research, the passive protection rate of Slp mouse serum against A. hydrophila infection was 42.5 % (p < 0.05), and the passive cross-protective rate of Slp mouse serum against P. fluorescens infection was 18.6 % in C. auratus. In addition, passive immunization with Slp mouse serum enhanced the immune-related factors expressing and the leukocyte phagocytosis of C. auratus, indicating that Slp serum might activate a nonspecific immunity in C. auratus. The results suggest that Slp mouse serum has passive protection abilities (against A. hydrophila) and passive cross-protection abilities (against P. fluorescens) in C. auratus.

Passive protective abilities can also be assessed by inflammation-related genes, antioxidant factors and visceral organ injury after passive immunization with animal antiserum and challenging with pathogenic bacteria (Samblas et al., 2019; Hoseinifar et al., 2020; Solana et al., 2020; Liu et al., 2021). OmpA protein is located in the outermost layer of E. coli, and we found that OmpA nanoparticles can decrease the expressing of inflammation-related genes and decrease visceral organ injury in mice (Liu et al., 2021). In current research, passive immunization with Slp serum to C. auratus was shown to decrease the expression of the inflammation-related genes IL-6, IL-8, IL-1β and TNF-α after being challenged with A. hydrophila and P. fluorescens; and the histopathological sections showed that Slp serum had protective abilities to visceral structure integrity (kidneys, spleens and intestines) against A. hydrophila and P. fluorescens infection. Therefore, Slp serum can reduce the inflammatory reaction induced by A. hydrophila and P. fluorescens infection in C. auratus. These results suggest that Slp mouse serum has passive protective and passive cross-protective abilities.

CONCLUSIONS AND RECOMMENDATIONS

Slp was analysed by bioinformatics and obtained by molecular cloning, expression and purification, and the expressing conditions of Slp were optimized. Slp can activate nonspecific immunity in mice, and a high titre of Slp mouse serum was prepared that recognized to A. hydrophila and P. fluorescens. Slp mouse serum has the passive protective and passive cross-protective abilities against A. hydrophila and P. fluorescens infection, and can activate a nonspecific immunity in C. auratus. Passive immunization with Slp serum can down-regulate the expression of inflammation-related genes, and reduce visceral organ injury caused by A. hydrophila and P. fluorescens in C. auratus. This study contributes to the development of a Slp polyvalent protective immunogen that can be used to boost resistance to infection with major freshwater aquaculture etiologic agents in fish.

Funding
This research was supported by the Natural Science Basic Research Program of Shaanxi Province (2022JM-120) and Project of Shaanxi University of Technology (SLQGD1803), China.

IRB approval and ethical statement
All animal procedures were performed in accordance with the guidelines in the Guide for the Care and Use of
Laboratory Animals and were approved by the Institutional Animal Ethics Committee, Shaanxi University of Technology, Hanzhong, China (No. 2020-08).

Data availability statement
The data that support the findings of this study are available on request from the corresponding author.

Statement of conflict of interest
The authors have declared no conflict of interest.

REFERENCES
Online First Article


