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Molecular characterization, phylogenetic analysis and adjuvant effect of channel catfish interleukin-1βs against *Streptococcus iniae*

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ABSTRACT

Channel catfish is one of the most extensively cultured species worldwide, which is widely used as a classical model for comparative immunology. Interleukin-1β (IL1β) is an immunoregulatory cytokine with the potential to enhance the immune response induced by vaccines in many animals. To characterize the molecular characterization and identify the immunoadjuvant role of channel catfish IL1β, molecular cloning, phylogenetic analysis, and expression of two IL1β genes were performed, the bioactivity of their recombinant proteins (rIL1β1 and rIL1β2) were detected in vitro and their adjuvant effects on a subunit vaccine encoding C5a peptidase (pSCPI) of *Streptococcus iniae* were evaluated. The results indicated that two IL1βs remained highly conserved possessing five conserved motifs compared with other fish IL1βs, although there were 28 nucleotide differences and 16 amino acid differences between channel catfish IL1β1 and IL1β2. Analysis of the ratios of nonsynonymous (dN) and synonymous (dS) substitutions revealed that fish IL1β genes were subjected to negative/purifying selection with global dN/dS ratios value 0.425. The results of adjuvant effect showed that compared with injection of pSCPI alone, co-injecting pSCPI with both rIL1β1 and rIL1β2 significantly enhanced antibody levels, serum bactericidal activity, lysozyme activity, alternative complement hemolytic activity, and the expression of endogenous IL1β and TNF-α in head kidney and spleen. Although vaccination with rIL1β1 or rIL1β2 failed to offer immunoprotection against *S. iniae* infection, the RPS (relative percent survival) of pSCPI + rIL1β1 and pSCPI + rIL1β2 groups were both higher than pSCPI alone (RPS, 50%), with 64.26% and 60.71%, respectively. Moreover, pSCPI + rIL1β1 + rIL1β2 offered significantly higher ($P < 0.05$) immunoprotection (RPS, 75%) against *S. iniae* infection than pSCPI alone. Our present results not only enrich the molecular structure study of fish IL1βs but also signify that two recombinant channel catfish IL1βs can be used as potential adjuvants in a subunit vaccine model against bacterial infection, which are of profound importance to prevent and control bacterial disease in channel catfish.

1. Introduction

With the increasing development of intensive aquaculture, the problems of fish health are becoming more serious. Outbreaks of fish diseases, caused by microbial pathogens, have become a key issue limiting the vigorous development of aquaculture. Currently, the main methods to control fish disease include chemicals, antibiotics, and vaccines. Chemicals, approved for aquaculture, according to the USA Food and Drug Administration include chloramine-T, formalin, and hydrogen peroxide. Antibiotic drugs include florfenicol, oxytetracyclin, and sulfadimethoxine/ormetoprim, according to the Food and Agriculture Organization. The use of chemical and antibiotics in aquaculture has brought several problems, including the resistance to antibiotics and the presence of antibiotic residues. By contrast, vaccines are regarded as an effective, environmentally-friendly, prophylactic method to prevent bacterial infections [1]. Genetically-engineered vaccine, as one kind of fish vaccines, consists of protective immunogens or antigenic genes and has become a better choice in light of efficiency, stability, safety, and environmental protection [2]. However, in many cases, genetically-engineered vaccines pose the problem of weak

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Table 1
Primers used in this paper.

Primers	Sequences (5'→3')[a]	Target gene	Accession Number	Product Size (bp)
IL1β1-F	GGATCCATGGCTGACAAAGATTT (BamHI)	IL1β1	DQ160229.1	843
IL1β1-R	CTCGAGTCACATCTCATTCTG (XhoI)			
IL1β2-F	GGATCCATGGATGACAAAGATTT (BamHI)	IL1β2	DQ160230.1	843
IL1β2-R	CTCGAGTCAGATCTCATTCTGAG (XhoI)			
18S-F	GGACACGGAAAGGATTGACAGA	18S rRNA	AF021880.1	121
18S-R	GAGGAGTCTCGTTCGTTATCGG			
IL1β-F	GCCATGTTGCTAATGTTGTAATCG	endogenous IL1β	DQ160229.1	174
IL1β-R	TGTCTTGCAGGCTGTAACTCTTG			
TNF-α-F	CGCACAACAAACCAGACGAGAC	TNF-α	AJ417565.2	129
TNF-α-R	ACCACTGCATAGATACGCTCGAA			

[a] Underlined nucleotides are restriction sites of the enzymes indicated in the brackets at the ends.

immunogenicity. Thus, immunoadjuvants or immunestimulants are essential for eliciting an immune response with maximal protection against bacterial diseases [3].

Cytokines have been used as novel adjuvants for vaccination against various pathogens in mammals [4–7]. However, not many studies have focused on the possible use of cytokine genes as immunoadjuvants in fish [3]. Although some attempts have been made to explore the potential of cytokines as adjuvants in some fish species [8–10], details concerning their immunoadjuvant mechanisms are still lacking. Interleukin-1β (IL1β) is a pluripotent cytokine, involved in the activation of the proliferation of T cells and B cells, the activation of the cytotoxic activity of macrophages and natural killer cells, the induction of immunoglobulin secretion, and the expression of immune-related genes [11,12]. Recombinant IL1β has been used as an adjuvant for vaccines in many animals including pigs [13,14], cattle [15,16], and sheep [17,18]. To date, some IL1β genes had been cloned and characterized in fish species, including carp [19], rainbow trout [20–24], sea bass [25,26], Atlantic cod [27], yellowfin sea bream [28], Japanese flounder [29], Nile tilapia [30] and channel catfish [31]. Only a few studies have shown that recombinant IL1β can be used as a possible immunoadjuvant in fish, for example in carp [32], barramundi [32], rainbow trout [34], sea bass [25] and Japanese flounder [35]. It is a valuable research to explore more fish IL1βs to serve as immunoadjuvant in fish vaccination.

Channel catfish (*Ictalurus punctatus*), is the most extensively cultured food-fish species worldwide; it is also widely used as a classical model for comparative immunology [36]. Although two distinct cDNAs encoding channel catfish IL1β have been reported with differential expression profiles after bacterial infection by Wang et al. [31], the potential function and viable application (like immunoadjuvant) of channel catfish IL1βs were still unclear and deserved to study. Compared with the study of Wang, in this study, we used simpler methods (RT-PCR and PCR) to obtain the coding sequences (CDS) of these two IL1β genes and easy-operation methods (prokaryotic expression with *E. coli* BL21) and plasmids (pMD19-T and pET32a) to obtain the recombinant IL1βs. To characterize the molecular characterization and identify the immunoadjuvant role of channel catfish IL1β, sequence analysis, phylogenetic analysis, and expression of two IL1β genes were performed, the bioactivity of their recombinant proteins (rIL1β1 and rIL1β2) were tested in vitro and their adjuvant effects on a subunit vaccine encoding C5a peptidase (pSCPI, a part of the C5a peptidase encoded by the *Scp I* gene) of *Streptococcus iniae* (Gram-positive channel catfish pathogenic bacteria) were evaluated from several aspects of immunity, including antibody levels, serum bactericidal activity, lysozyme activity, alternative haemolytic complement activity, the expression of immune-related genes, and immunoprotection.

2. Materials and methods

2.1. Ethics statement

All animal experiments were approved by the Committee of Ethics on Animal Care and Use of Sichuan Agricultural University (No. XF201418). All experimental procedures were performed in accordance with the guidelines for care and use of experimental animals of the Chinese Ministry of Science and Technology.

2.2. Bacterial strains, plasmids, and growth conditions

Streptococcus iniae DGX07, a fish pathogen, was isolated from sick channel catfish in China; it was cultured in Brain-Heart Infusion (BHI) medium at 37 °C and stored at our laboratory [37]. *Escherichia coli* strains DH5α and BL21 (DE3) (Takara; Dalian, China) were used for cloning and protein expression, respectively. Both strains were grown in Luria-Bertani medium containing 100 μg/ml of ampicillin at 37 °C. Plasmids pMD19-T (Takara) and pET32a (+) (Merck, Germany) were used for T-A cloning and protein expression, respectively. Recombinant protein pSCPI containing the truncated part of C5a peptidase was constructed, expressed, and stored at our laboratory [38,39].

2.3. Molecular cloning and sequences analysis of IL1βs

Total RNA was extracted from the head kidney of channel catfish using TRIzol reagent (Invitrogen, USA). First strand cDNA was synthesized using PrimeScript™ RT reagent kit with gDNA Eraser (Perfect Real Time) (TaKaRa), according to the manufacturer's instructions. The channel catfish genes IL1β1 and IL1β2 were amplified by PCR from the cDNA template with specific primers (Table 1) designed using Primer Premier 5.0 software, according to the sequences (DQ160229 and DQ160230) published in GenBank by Wang et al. [31]. The PCR products were purified using the Agarose Gel DNA Extraction kit (TaKaRa), cloned into the pMD19-T vector, and transformed into *E. coli* DH5α. The recombinant plasmids were identified by PCR, digested with restriction enzymes *BamH* I and *Xho* I, and fractionated on 1% agarose gels. DNA sequencing was performed by TaKaRa Bio Inc and the sequences were deposited on NCBI Genbank to obtain accession numbers. The correct recombinant cloning plasmids were named by T-IL1β1 and T-IL1β2.

The amino acid sequences of channel catfish IL1βs were derived from the results of sequencing, following by alignment with other 14 fish species IL1βs using MUSCLE [40]. To delineate the evolutionary dynamics of fish IL1βs, the conserved domains and conserved motifs were identified using the Conserved Domain Database (CDD) in NCBI [41] and MEME software [42], respectively, the amino acid sequences identity between IL1βs and other 14 fish species IL1βs was calculated using the MegAlign program (DNASTAR, Madison, WI), the phylogenetic tree was constructed using the neighbor-joining method in MEGA 5 [43] with bootstrap test of 1000 replicates. To measure the selection pressures imposed on fish IL1βs, the natural selection analysis was conducted based on the dN/dS ratios (the relative rates of non-synonymous (dN) and synonymous (dS) substitutions) which was calculated using Datamonkey [44].

2.4. Expression and purification of recombinant IL1βs

Expression and purification of recombinant proteins were conducted as described in our previous study [8,45]. Briefly, the plasmids T-IL1β1 and T-IL1β2 were digested with *BamH* I and *Xho* I, and the resulting products inserted into the *BamH* I, Xho I sites of the pET32a (+) vector to construct the plasmids for recombinant expression, which were named P-IL1β1 and P-IL1β2, respectively. The plasmids were transformed into *E. coli* BL21 and induced with 1.0 mM IPTG at 37 °C for 4 h. The cells were centrifuged at 8000 × *g* for 10 min and resuspended in sterile phosphate buffer saline (PBS), followed by ultrasonication, and detection by 12.5% SDS-PAGE. The recombinant proteins expressed in form of inclusion bodies in the sediment were purified using a Ni-NTA-Sefinose Column (Sangon Biotech, Shanghai, China), and refolded by gradient dialysis. To rule out the potential bystander effects of LPS/or other impurities, endotoxin in the recombinant protein was removed using ToxinEraser™ Endotoxin Removal kit (GenScript Corp, Nanjing, China), and the remaining endotoxin levels were measured using the Chromogenic End-point Endotoxin Assay kit (Limulus reagent biotechnology, Xiamen, China). Less than 0.1 EU/ml was detected in the final protein preparations. The quantity was determined using a NanoDrop spectrophotometer (Thermo Scientific) according to the manufacturer's instructions. Purified recombinant proteins, named rIL1β1 and rIL1β2, were stored at −20 °C.

2.5. Western blotting

The western blotting analysis of recombinant proteins was performed as previously described, with minor modifications [8,38]. Briefly, the purified proteins were separated by 12.5% SDS-PAGE and transferred to a PVDF membrane at 150 V for 2 h. After pre-blocking with TBST (containing 3% BSA) for 1 h at 37 °C, the membrane was incubated with rabbit anti-6-His antibody (Sangon Biotech, Shanghai, China) diluted 1:100 in TBST (containing 0.5% BSA) for 12 h at 4 °C. After washing three times with TBST, the membrane was incubated with goat-anti-rabbit IgG (H + L)-HRP (Sigma, Beijing, China), diluted 1:5000 in TBST (containing 0.5% BSA), at 37 °C for 1 h. The reaction was visualized using DAB (Sigma) for 5–15 min, and terminated by rinsing with distilled water.

2.6. Bioactivity test of recombinant rIL1βs

The biological activity of the recombinant rIL1βs was detected through its effect on endogenous IL1β expression in head kidney leukocytes according to the previously described methods [24,25,28] with slight modifications. Briefly, the head kidney leukocytes were obtained and purified as described previously [46], then adjusted to $\sim 1.0 \times 10^{6}$ cells/ml (in 5 ml of L15 medium, (Gibco, USA)) and incubated at 25 °C for 4 h before total RNA extraction using 1, 10, 50 or 100 ng/ml of the recombinant rIL1βs as test groups; no stimulation as negative control, and 5 μg/ml LPS (Sigma) was used as a positive control, which was one of the typical inflammatory stimuli [26,47] and could un-regulate the expression of inflammation and immune related genes such as IL1β [20,22,24]. Each treatment was performed in triplicate. After stimulation, total RNA extraction and cDNA synthesis were conducted as described above. Quantitative real time PCR (qRT-PCR) was performed to investigate the expression of the endogenous IL1β gene using the SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) (TaKaRa) in an ABI StepOnePlus™ System (Applied Biosystems, USA) as described previously [8]. The housekeeping gene 18S rRNA was used as an internal control. The primers used to amplify 18S rRNA and endogenous IL1β are shown in Table 1. All data are presented as relative expression level.

2.7. Preparation of fish and of recombinant vaccines

Channel catfish (50.0 ± 5.0 g) were purchased from a fish farm in Chengdu (Sichuan, China) and acclimatized in the laboratory for 2 weeks, before any experimental manipulation. Fish were fed a commercial diet daily; water was partially replaced every day, maintaining the temperature at 28 ± 1 °C. Before the experiments, blood, liver, kidney, and spleen of fish were sampled. Examination of bacterial recovery indicated the absence of bacteria; agglutination tests showed no reaction between the serum and *S. iniae* DGX07. Fish were anesthetized with MS-222 (Sigma) before experimental manipulation. rIL1β1, rIL1β2, and the recombinant antigen pSCPI and were diluted in PBS to 0.5 mg/ml, 0.5 mg/ml, and 1.0 mg/ml, respectively. To obtain rIL1β1 + PBS, rIL1β2 + PBS and pSCPI + PBS, the recombinant proteins were mixed respectively with equal volumes of PBS. To obtain pSCPI + rIL1β1 and pSCPI + rIL1β2, the recombinant antigen pSCPI was mixed respectively with equal volumes of rIL1β1 and rIL1β2. To obtain pSCPI + rIL1β1 + rIL1β2, the recombinant antigen pSCPI was mixed with rIL1β1 and rIL1β2 at a ratio of 1.0:0.5:0.5 using an ultrasonic disruptor (JY92-IIDN, Ningbo Scientz, China).

2.8. Fish vaccination and bacterial challenge

Vaccination experiments were performed in duplicate. Healthy channel catfish were divided randomly into seven groups (60 fish per group, two duplicate tanks per group, 30 fish per tank) and injected intraperitoneally with 2 μg/g of fish of recombinant antigen pSCPI in 0.2 ml. Details of the vaccination schemes are shown in Table 2. Secondary vaccinations, with the same method and doses, were performed to obtain an optimal immune response 2 weeks after the first vaccination. Four weeks after the secondary vaccination (psv, post-secondary vaccination), 30 fish of each group from duplicate tanks (15 fish per) mixed together and were challenged by intraperitoneal injection with 0.2 ml of 6×10^{7} CFU/ml of *S. iniae* DGX07 resuspended in PBS [8],

Table 2
Details of the vaccination schemes.

Groups	Fish No.	First vaccination		Second vaccination (2 weeks after first vaccination)	
		Antigen/adjuvant amount (μg/g fish)	Injected dosage (ml)	Antigen/adjuvant amount (μg/g fish)	Injected dosage (ml)
PBS (Control)	60	–	0.2	–	0.2
rIL1β1 + PBS	60	1	0.2 (0.1 + 0.1)	1	0.2 (0.1 + 0.1)
rIL1β1 + PBS	60	1	0.2 (0.1 + 0.1)	1	0.2 (0.1 + 0.1)
pSCPI + PBS	60	2	0.2 (0.1 + 0.1)	2	0.2 (0.1 + 0.1)
pSCPI + rIL1β1	60	2 + 1	0.2 (0.1 + 0.1)	2 + 1	0.2 (0.1 + 0.1)
pSCPI + rIL1β2	60	2 + 1	0.2 (0.1 + 0.1)	2 + 1	0.2 (0.1 + 0.1)
pSCPI + rIL1β1 + rIL1β2	60	2 + 0.5 + 0.5	0.2 (0.1 + 0.05 + 0.05)	2 + 0.5 + 0.5	0.2 (0.1 + 0.05 + 0.05)

then were distributed randomly into 2 tanks. Mortality was monitored for 14 days after the bacterial challenge. Dying fish were randomly selected for examination of bacterial recovery from liver, kidney, and spleen. Relative percent of survival (RPS) was calculated according to the following formula: RPS = [1 − (% mortality of vaccinated fish/% mortality of control fish)] × 100 [48]. Serum samples of five fish from each group were collected for the assessment of immune-related indexes from 3rd to 7th -week psv. Head kidney and spleen tissues of five fish were taken for qRT-PCR analysis at 24 h post challenge.

2.9. Preparation of rabbit anti-channel catfish IgM antiserum

Rabbit anti-channel catfish IgM antisera was prepared using serum obtained from healthy channel catfish according to the method described in our previous study [38]. Rabbit anti-channel catfish IgM antiserum (Immunoglobulin G, IgG) was collected from New Zealand white rabbits, and purified by the ammonium sulfate precipitation method [49]; it was stored at −20 °C until required.

2.10. Enzyme-linked immunosorbent assay (ELISA)

Sera were collected from the caudal vein of vaccinated fish from 3rd to 7th -week psv. Antibody levels against pSCPI were measured by ELISA as described before [8,38] with minor modifications. Briefly, pSCPI was diluted to 50 μg/ml in a carbonate buffer (pH = 9.6). Each well of 96-well plates was covered with 100 μL diluted pSCPI overnight at 4 °C, followed by washing with PBST (0.1% Tween-20 in PBS), and then blocking with 3% BSA in PBST for 2 h at 37 °C. Serial 2-fold dilutions of sera were added into the wells in triplicate, and the plates incubated for 2 h at 37 °C. Rabbit anti-channel catfish IgM antiserum (1:200) and goat-anti-rabbit IgG (H + L)-HRP (1:2000, Sangon Biotech, Shanghai, China) were used as first and secondary antibodies, respectively. Color development was performed using the TMB kit (Tiangen, Beijing, China). The reaction was terminated with 2 M H_2SO_4. The plates were read at 450 nm with a microplate reader (Bio-Rad, Hercules, USA).

2.11. Serum bactericidal activity

Serum bactericidal activity was measured by the method of Sun et al. [50], with minor modifications. Briefly, 25 μl *S. iniae* DGX07 bacterial suspensions containing 1.0×10^6 CFU/ml were mixed with 75 μl untreated serum, or heat-treated serum (44 °C for 30 min), in a total volume of 100 μl. Besides, in order to examine the role of antibodies, 25 μl of the bacterial suspensions as above were mixed with 75 μl of serum from vaccinated fish, which was first heated at 100 °C for 10 min. The mixture was incubated at 30 °C for 3 h, and after diluting in BHI, plated on BHI agar plates. The survival rate was calculated based on the viable counts of bacteria as follows: 100 × (the number of bacteria survived with untreated or treated serum/the number of bacteria survived with control serum).

2.12. Serum lysozyme activity

Serum lysozyme activity was measured by the turbidimetric method as described previously with minor modifications [51]. Briefly, 150 μl *Micrococcus lysodeikticus* at 0.2 mg/ml (in 0.02 M PBS, pH = 5.5) was added to 20 μl of serum in a 96-well U-bottom microtiter plate, and quickly mixed by vortexing. The initial optical density (OD) was detected at 450 nm immediately after adding *M. lysodeikticus*; the final OD was measured after incubation for 1 h at 37 °C. Lyophilized hen egg-white lysozyme (Sigma) was used to create a standard curve. Each test was performed in triplicate. One activity unit of lysozyme (U) was defined as the amount of serum lysozyme that caused a decrease of 0.001 in absorbance per min at 450 nm.

2.13. Alternative hemolytic complement activity

Alternative hemolytic complement activity was detected according to the method described by Sunyer and Tort [52], which is based on the hemolysis of sheep red blood cells. The volume of serum yielding 50% hemolysis (ACH50) was determined and used to calculate the complement activity of the samples (value of ACH50 is in units per ml).

2.14. qRT-PCR analysis of the expression of IL1β and TNF-α

Head kidney and spleen tissues were taken at 24 h post-challenge. Total RNA extraction and cDNA synthesis were carried out as described above. qRT-PCR was performed using SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) (TaKaRa) in an ABI StepOnePlus™ System (Applied Biosystems, USA) as described previously [8]. Each assay was performed in triplicate, using the housekeeping gene 18S rRNA as an internal control. The primers used to amplify the genes of the cytokines IL1β and TNF-α are shown in Table 1. The relative expression levels of these two genes were analyzed by the $2^{-\Delta\Delta CT}$ method. All data are presented as relative mRNA expression.

2.15. Statistical analysis

Statistical analysis was performed using SPSS 19.0 software (SPSS Inc., USA). Mortality data from the bacterial challenge experiments were analyzed by the Kaplan–Meier methods, and differences among groups were tested using log-rank tests. The differences between data were determined using a one-way analysis of variance (ANOVA). In all cases, the significance level was defined as $P < 0.05$ and the results were presented as mean ± SD (standard deviation).

3. Results

3.1. Molecular characterization and phylogenetic analysis of IL1β1 and IL1β2

The full-length of the coding sequences of IL1β1 and IL1β2 obtained by PCR in this study were both 843 bp, which were identical to those obtained by Wang et al. [31]. The nucleotide sequences of IL1β1 and IL1β2 were submitted to the GenBank of NCBI, and assigned the accession numbers MF504016 and MF504017, respectively. These two IL1β genes contained a complete open reading frame and both encoded 280 amino acid (a.a.) with a predicted molecular weight of 31.97 kDa and 31.95 kDa, and their theoretical isoelectric points were 4.95 and 4.72, respectively. There were 28 nucleotide differences, corresponding to 16 a.a. differences between IL1β1 and IL1β2 although they both contained an IL1 superfamily conserved domain and 17 IL-1 receptor binding sites at the N-terminus at positions 121–256. Compared with other fish IL1βs, channel catfish IL1β1 and IL1β2 in this study were highly conserved and both possessed five conserved motifs, which comprised about 56.8% of total length of IL1β.

The results of amino acid sequences identity showed that IL1β1 and IL1β2 in this study shared 100% identity respectively with those obtained by Wang et al. [31]. Besides, IL1β1 shared high identity (78.9%–93.9%) with other Siluriformes species IL1βs (i.e. *Tachysurus vachellii* and *Hemibagrus macropterus*), but low identity (31.5%–56.4%) with other fish IL1βs. IL1β2 shared 79.3%–93.9% identity with other Siluriformes species IL1βs, but low identity (32.3%–57.1%) with other fish IL1βs. The phylogenetic analysis showed that IL1β1 and IL1β2 clustered with channel catfish IL1β subtype 1 and IL1β subtype 2, respectively, displaying the closest relationship between them, and a more distant relationship with other fish IL1βs. In addition, the result of natural selection pressure analysis indicated that the global dN/dS ratios of fish IL1βs was 0.425 with 0 positive/diversifying selection sites and 38 negative/purifying selection sites, which was well below 1.0, a theoretical boundary for positive and negative selection.

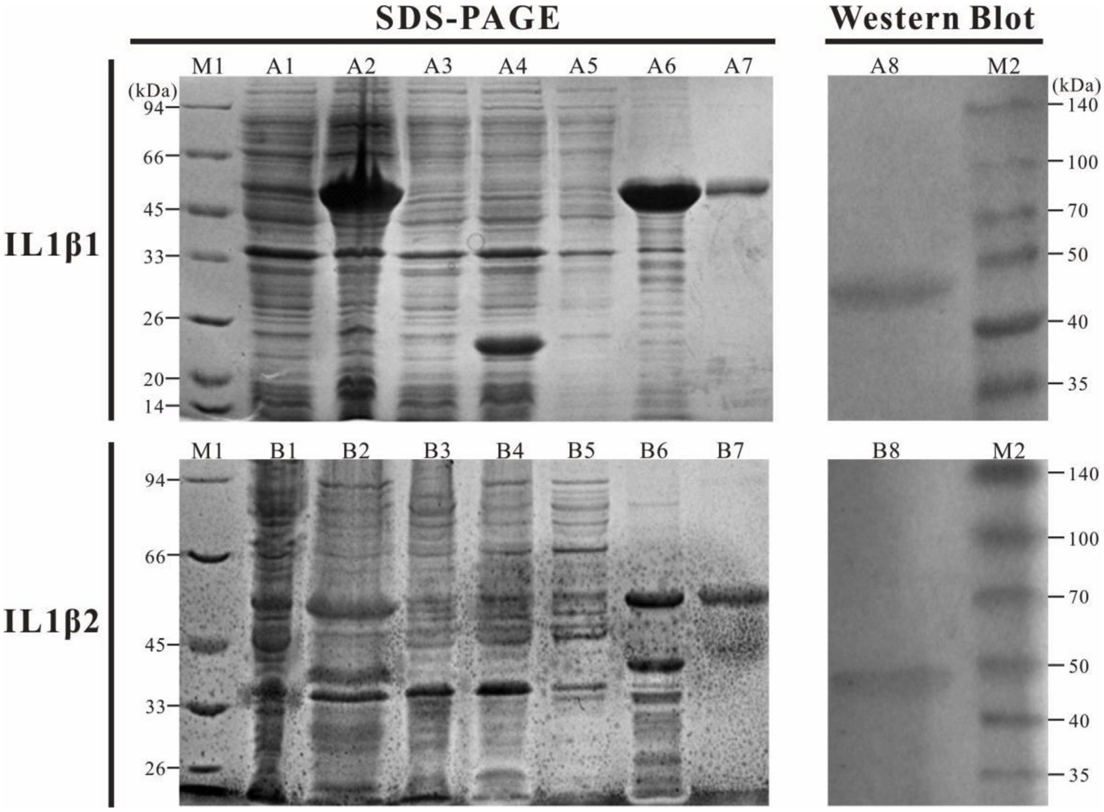


Fig. 1. Expression, purification and western blotting analysis of recombinant proteins. M1 and M2: Protein markers. A1 ~ A8: uninduced BL21 (P-IL1β1) whole bacteria, induced BL21 (P-IL1β1) whole bacteria, uninduced BL21 (pET32a) whole bacteria, induced BL21 (pET32a) whole bacteria, induced BL21 (P-IL1β1) supernatant, induced BL21 (P-IL1β1) sediment, purification of recombinant rIL1β1, recombinant protein rIL1β1. B1 ~ B8: uninduced BL21 (P-IL1β2) whole bacteria, induced BL21 (P-IL1β2) whole bacteria, uninduced BL21 (pET32a) whole bacteria, induced BL21 (pET32a) whole bacteria, induced BL21 (P-IL1β2) supernatant, induced BL21 (P-IL1β2) sediment, purification of recombinant rIL1β2, recombinant protein rIL1β2.

3.2. Expression, purification and western blotting analysis of recombinant IL1β1 and IL1β2

Recombinant expression plasmids were constructed by inserting the digested IL1β1 and IL1β2 products into digested pET32a (+) vector. The expression vector contained a 6 × His-tag, which was the basis for the subsequent detection and purification of the recombinant proteins. The recombinant IL1β1 and IL1β2 proteins were expressed in *E. coli* BL21 cells, then the expression products were purified by His-tag Ni affinity chromatography, and analyzed by SDS-PAGE. The results showed that as the fusion protein including His-tag, recombinant IL1β1 and IL1β2 were both approximately 48 kDa, consisting of the sequence of IL1β1 (~ 31.97 kDa)/IL1β2 (~ 31.95 kDa), the His-tag sequences and some sequences of expression plasmid pET32a. They were both expressed in the form of inclusion bodies in the sediment (Fig. 1). The expression correctness of recombinant IL1β1 and IL1β2 was determined by western blotting analysis using rabbit anti-6-His antibody. Rabbit anti-6-His antibody specifically detected rIL1β1 and rIL1β2 as single bands of approximately 48 kDa, which suggested that the recombinant IL1β1 and IL1β2 were expressed correctly and could be used for the subsequent experiments.

3.3. Biological activity of recombinant rIL1βs

The biological activity of rIL1β1 and rIL1β2 in increasing concentrations was tested in head kidney leukocyte using LPS (5 μg/ml) as a positive control. The products obtained using primers for channel catfish endogenous IL1β gene and 18S gene (internal control) were displayed on 1.0% agarose gels (Fig. 2A), the relative expression levels of endogenous IL1β were detected using qRT-PCR. The results showed that weak signals and very low expression level of endogenous IL1β were detected in the negative group (Fig. 2), while in cells treated with 1, 10, 50 and 100 ng/ml of rIL1β1 and rIL1β2, the relative expression levels of endogenous IL1β gradually increased, in a dose-dependent manner (Fig. 2B). The relative expression levels of endogenous IL1β in cells treated with 100 ng/ml rIL1βs were similar or slightly higher than that of cells treated with 5 μg/ml LPS. Moreover, rIL1β1 induced a higher expression of channel catfish IL1β than rIL1β2 at the same concentration.

3.4. Serum antibody production

Serum pSCPI-specific antibody was evaluated continuously by ELISA from 3rd to 7th-week psv. (Fig. 3). The results showed that the vaccines pSCPI + rIL1β1, pSCPI + rIL1β2, and pSCPI + rIL1β1 + rIL1β2 induced significantly higher levels of antibodies than pSCPI + PBS from 3rd to 7th-week psv, with the highest antibody level peaking at fourth week psv. The absorbance values in all of pSCPI groups were significantly higher ($P < 0.05$) than that of the PBS group. Moreover, the antibody level in fish vaccinated with pSCPI + rIL1β1 + rIL1β2 was significantly higher ($P < 0.05$) than that in fish vaccinated with pSCPI + rIL1β1 at third week psv, and than in fish vaccinated with pSCPI + rIL1β2 at 3rd and 4th-week psv. There were no significant differences in antibody levels in fish vaccinated with pSCPI + rIL1β1 or pSCPI + rIL1β2 over the experimental period. Besides, the absorbance values in fish vaccinated with rIL1β1 + PBS and rIL1β2 + PBS showed no significant differences compared with that of fish vaccinated with PBS.

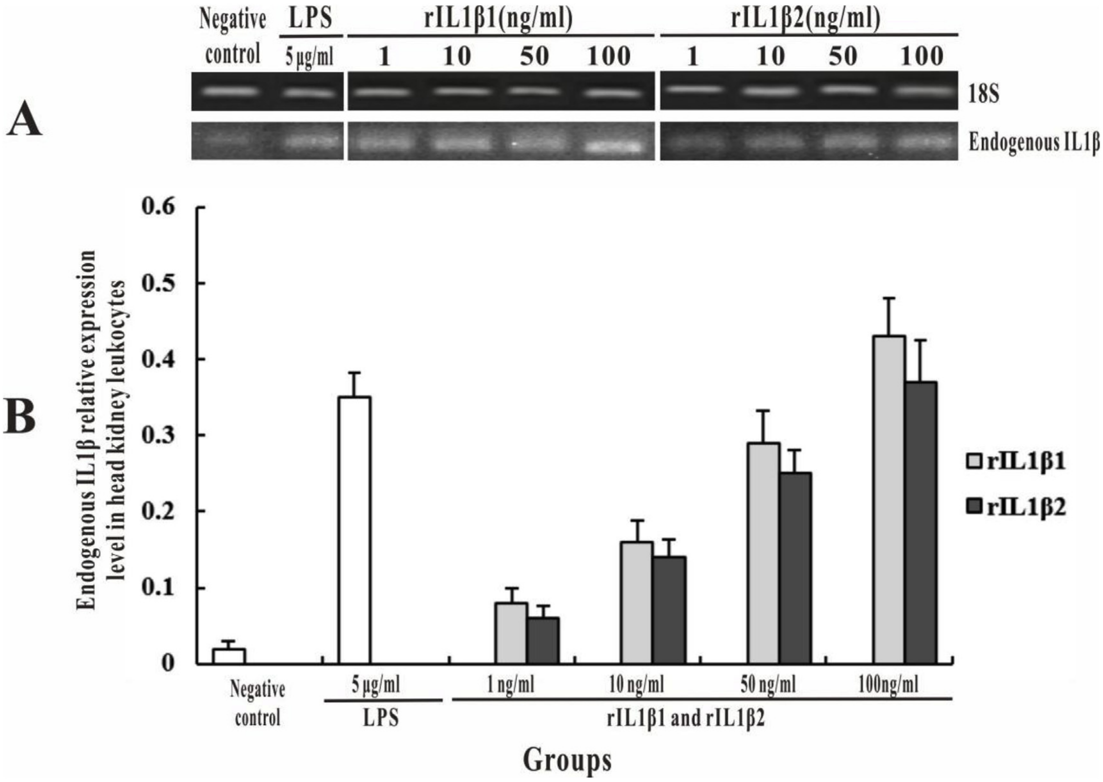


Fig. 2. Channel catfish endogenous IL1β expression in head kidney leukocytes. After isolation, head kidney leukocytes were stimulated without (negative control) or with LPS (positive control, 5 µg/ml), or different concentrations of recombinant rIL1βs (1, 10, 50 and 100 ng/ml) for 4 h before total RNA extraction. (A). The products obtained using primers for channel catfish endogenous IL1β gene and 18S gene (internal control) were displayed on 1.0% agarose gels. (B). The relative expression levels of endogenous IL1β in head kidney leukocytes were detected using qRT-PCR. Three replications of each group were done independently and the data are presented as means ± SD (n = 3).

3.5. Serum bactericidal activity

To examine whether vaccine-induced antibodies had any protective effect on natural immunity (as reflected by serum bactericidal activity), *S. iniae* DGX07 was incubated with the fish serum of PBS group and four pSCPI + PBS groups at 4th-week psv. Then bacterial survival rate was determined by plate counts. The survival rates of *S. iniae* in untreated serum were the lowest, followed by the 44 °C treated serum and the 100 °C treated serum (Fig. 4). Both in untreated and treated serum, the bacteria survival rates in three adjuvant groups (pSCPI + rIL1β1, pSCPI + rIL1β2, and pSCPI + rIL1β1 + rIL1β2) were significantly lower ($P < 0.05$) than that in PBS group and pSCPI + PBS group. While the bacteria survival rates of pSCPI + PBS groups were significantly lower ($P < 0.05$) than that of PBS groups in both untreated and 44 °C treated

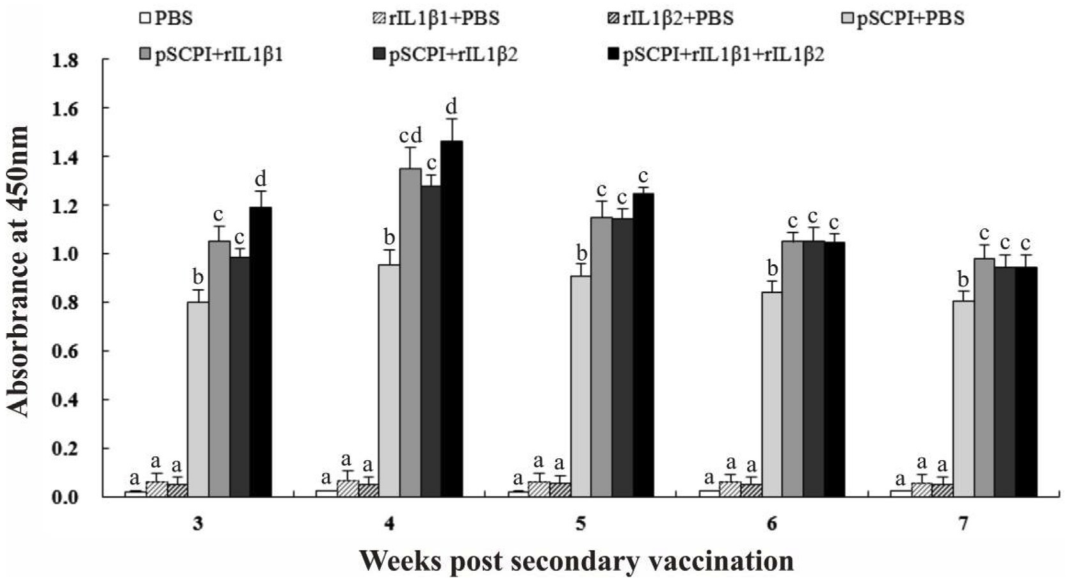


Fig. 3. Serum antibody levels detection in vaccinated fish by ELISA. Channel catfish were vaccinated twice at 2-week intervals. Sera were collected from the fish at 3rd to 7th-week psv. Data are presented as means ± SD (n = 5). Different letters above a bar denote significant difference ($P < 0.05$).

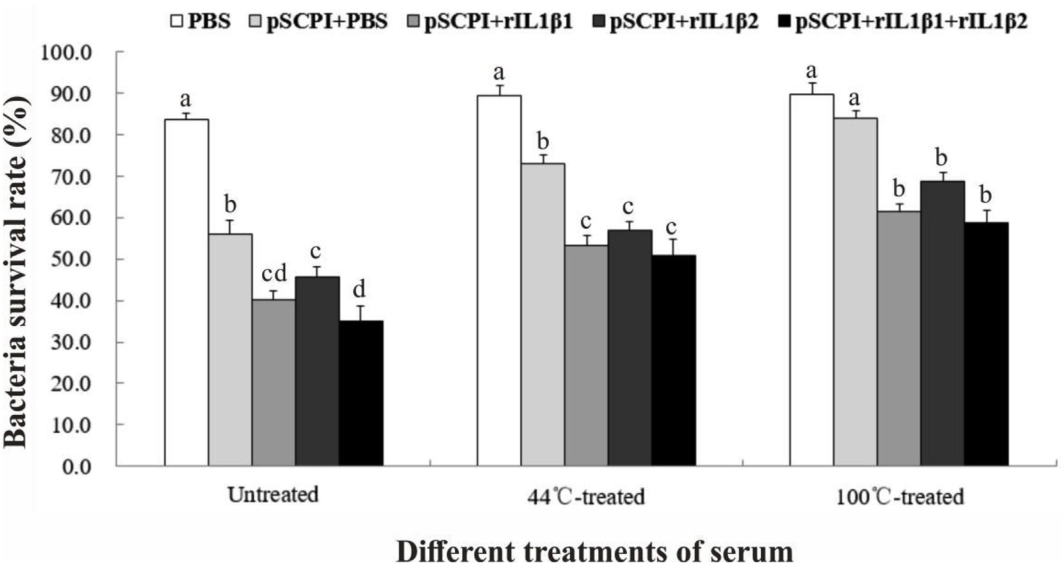


Fig. 4. Serum bactericidal activity of vaccinated fish. *S. iniae* was incubated with serum from channel catfish vaccinated with PBS, pSCPI + PBS, pSCPI + rIL1β1, pSCPI + rIL1β2, and pSCPI + rIL1β1 + rIL1β2 under different treatments. Sera were collected from the fish at 4th-week psv. Bacterial survival rate was determined by plate count. Data are presented as means ± SD (n = 5). Different letters above a bar denote significant difference ($P < 0.05$).

serum, and slightly lower in 100 °C treated serum. Moreover, in untreated serum, the bacterial survival rates of pSCPI + rIL1β1 + rIL1β2 (35.0%) was slightly lower than that of pSCPI + rIL1β1 (40.11%), but significantly lower than that of pSCPI + rIL1β2 (45.56%).

3.6. Serum lysozyme activity

The serum lysozyme activity of fish in PBS group and four pSCPI + PBS groups was detected by a turbidimetric assay at 4th-week psv (Fig. 5A). The three vaccines pSCPI + rIL1β1, pSCPI + rIL1β2, and pSCPI + rIL1β1 + rIL1β2, significantly ($P < 0.05$) enhanced serum lysozyme activity, compared with PBS and pSCPI + PBS. The serum lysozyme activity induced by the pSCPI + PBS vaccine was significantly higher than that of PBS. Moreover, the highest serum lysozyme activity was induced by the vaccine pSCPI + rIL1β1 + rIL1β2, which was slightly higher than that induced by the vaccine pSCPI + rIL1β1, and significantly higher ($P < 0.05$) than that of the vaccine pSCPI + rIL1β2.

3.7. Serum ACH50 activity

The serum ACH50 activity of vaccinated fish was measured by the mean number of ACH50 units/ml serum at 4th-week psv (Fig. 5B). The three vaccines, pSCPI + rIL1β1, pSCPI + rIL1β2, and pSCPI + rIL1β1 + rIL1β2, significantly ($P < 0.05$) increased serum ACH50 activity, compared with PBS and pSCPI + PBS. The serum ACH50 activity induced by pSCPI + PBS was significantly higher ($P < 0.05$) than that induced by PBS. Moreover, the serum ACH50 activity induced by pSCPI + rIL1β1 + rIL1β2 was the highest, followed by that induced by pSCPI + rIL1β2 and pSCPI + rIL1β1.

3.8. Expression of IL1β and TNF-α in head kidney and spleen

Gene expression of endogenous IL1β and TNF-α in head kidney and spleen was evaluated by qRT-PCR at 24 h post challenge as displayed in Fig. 6. The expression of IL1β of fish in three adjuvant groups (pSCPI + rIL1β1, pSCPI + rIL1β2, and pSCPI + rIL1β1 + rIL1β2) was significantly higher ($P < 0.05$) than that in pSCPI + PBS group in head kidney and spleen, except pSCPI + rIL1β2 group in spleen. Moreover, compared with pSCPI + rIL1β1 and pSCPI + rIL1β2, pSCPI + rIL1β1 + rIL1β2 increased significantly the expression of IL1β in head kidney and spleen. The expression of TNF-α of fish in three adjuvant groups was significantly higher than that in pSCPI + PBS group in the

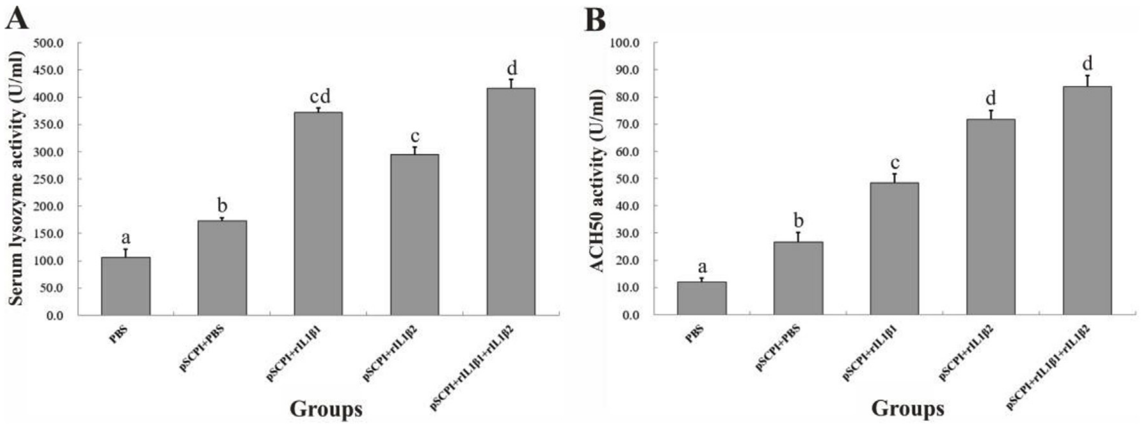


Fig. 5. Serum lysozyme activity (A) and ACH50 activity (B) of vaccinated fish. Channel catfish were vaccinated twice at 2-week intervals, with PBS, pSCPI + PBS, pSCPI + rIL1β1, pSCPI + rIL1β2, and pSCPI + rIL1β1 + rIL1β2 respectively. Sera were collected from the fish at 4th-week psv. Data are presented as means ± SD (n = 5). Different letters above a bar denote significant difference ($P < 0.05$).

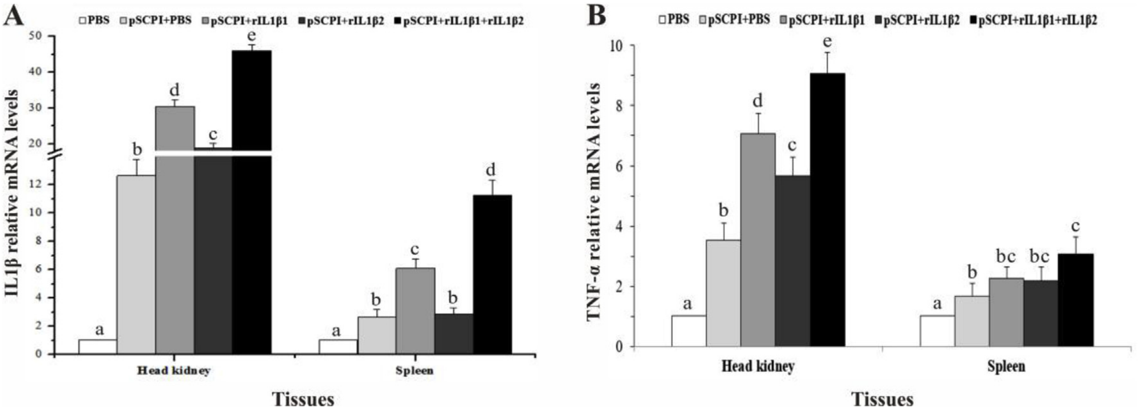


Fig. 6. Expression levels of endogenous IL1β and TNF-α in head kidney and spleen of vaccinated fish determined by qRT-PCR. For each gene, the mRNA level of the PBS-vaccinated fish was set as 1. Data are presented as means ± SD (n = 5). Different letters above a bar denote significant difference ($P < 0.05$).

head kidney. In spleen, the expression of TNF-α in the pSCPI + rIL1β1 + rIL1β2 group was significantly higher ($P < 0.05$) than that in pSCPI + PBS group. In addition, the expression of TNF-α induced by pSCPI + rIL1β1 + rIL1β2 was significantly higher ($P < 0.05$) in head kidney, and slightly higher in spleen, compared with that induced by pSCPI + rIL1β1 and pSCPI + rIL1β2. Interestingly, no matter IL1β or TNF-α, the expression levels of these two genes in all groups were higher in head kidney than that in spleen.

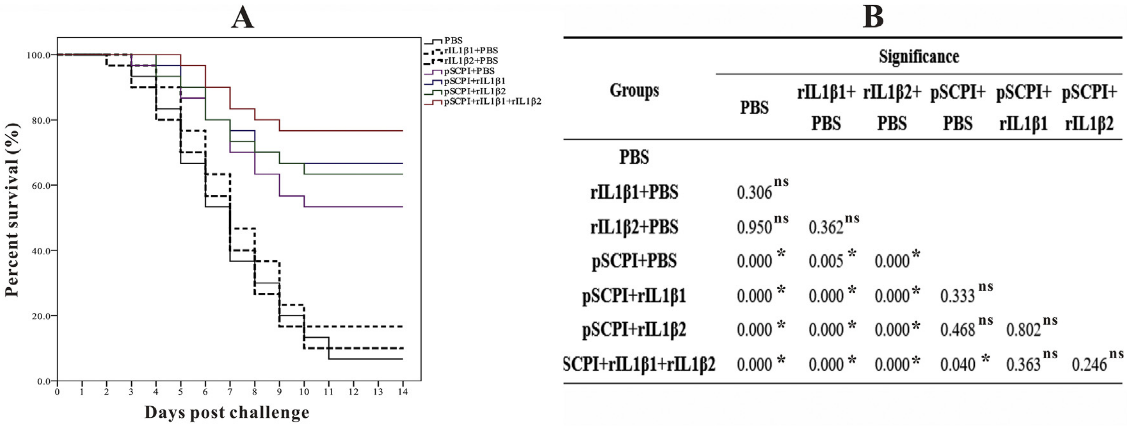
3.9. Immunoprotection efficacy against S. iniae

After challenge with pathogenic *S.iniae* DGX07 at 4th-week psv, the survival of fish was 6.67% for PBS, 16.67% for rIL1β1 + PBS, 10% for rIL1β2 + PBS, 53.33% for pSCPI + PBS, 66.67% for pSCPI + rIL1β1, 63.33% for pSCPI + rIL1β2, and 76.67% for pSCPI + rIL1β1 + rIL1β2 (Fig. 7A). The survival of fish vaccinated with rIL1β1 + PBS and rIL1β2 + PBS was slightly higher than that of fish vaccinated with PBS. The survival of fish in four pSCPI groups was all significantly higher ($P < 0.05$) than in the PBS group. The survival of fish vaccinated with pSCPI + rIL1β1 and pSCPI + rIL1β2 was higher than that of fish vaccinated with pSCPI + PBS, while the survival of fish vaccinated with pSCPI + rIL1β1 + rIL1β2 was significantly higher ($P < 0.05$) than that of fish vaccinated with pSCPI alone by log-rank test (Fig. 7B). In addition, compared with PBS group, the immunoprotection efficacy (in terms of RPS) of rIL1β1 + PBS, rIL1β2 + PBS, pSCPI + PBS, pSCPI + rIL1β1, pSCPI + rIL1β2, and pSCPI + rIL1β1 + rIL1β2 were 10.71%, 3.57%, 50%, 64.26%, 60.71% and 75%, respectively. *S.iniae* DGX07 was the only type of bacterial strain detected in the liver, and kidney of moribund fish, suggesting that mortality was indeed caused by *S.iniae* DGX07 infection.

4. Discussion

Advances in molecular biology have provided many contributions to vaccine research, particularly related to recombinant vaccine development in fish vaccinology [2]. In the past decade, many recombinant production (antigens/vaccines and adjuvants) have been explored and analyzed for their ability to induce immune responses and protective immunity in some fish species including Japanese flounder [53,54], rainbow trout [55–57], Nile tilapia [58–60], and channel catfish [61–64]. Although, the recombinant vaccine is of inferior immunogenicity compared with inactivated or whole cell/virus vaccines, it has advantages for some micro-organisms that are difficult to culture and is safe for use. Although only two recombinant vaccines were reported and approved for use in farmed fish [65,66], it will be established as an economically viable alternative for fish vaccines in the near future. Moreover, an increasing number of adjuvants have been studied to improve the immunogenicity of recombinant vaccines in fish [25,32–35].

IL1β, as one of the earliest secreted cytokines, has a wide range of biological activities mainly involved in the regulation of inflammation and the induction of immune responses. Considering these activities, IL1β has been speculated as a potential immunostimulant used with the vaccine in mammals [13–18] and fish [25,32–35]. Based on the analysis of conserved domains and motifs, multiple sequences alignment and phylogenetic analysis, we speculated that even though the



Groups	Significance					
	PBS	rIL1β1+ PBS	rIL1β2+ PBS	pSCPI+ PBS	pSCPI+ rIL1β1	pSCPI+ rIL1β2
PBS						
rIL1β1+PBS	0.306 ns					
rIL1β2+PBS	0.950 ns	0.362 ns				
pSCPI+PBS	0.000 *	0.005 *	0.000*			
pSCPI+rIL1β1	0.000 *	0.000 *	0.000*	0.333 ns		
pSCPI+rIL1β2	0.000 *	0.000 *	0.000*	0.468 ns	0.802 ns	
SCPI+rIL1β1+rIL1β2	0.000 *	0.000 *	0.000*	0.040 *	0.363 ns	0.246 ns

Fig. 7. Percent survivals of vaccinated fish by Kaplan-Meier method (A) and significant difference analysis among groups by log-rank test (B). "*" denotes significant difference ($P < 0.05$), "ns" means not significant.

sequence divergences existed among fish IL1βs, the components and positions of conserved domains and motifs of fish IL1βs remained highly conserved. Besides, natural selection pressure, as the major force that shapes the genetic variation within populations [67,68], was also analyzed through the dN/dS ratio in this study. The result showed that negative selection ($dN/dS = 0.425, < 1$) played a critical role to remove nonsynonymous substitutions from the IL1βs genes, indicating fish IL1βs were subjected to negative/purifying selection and highly conserved throughout the evolution. However, there were 28 nucleotide differences and 16 amino acid differences between channel catfish IL1β1 and IL1β2, which may be partly responsible for the different expression profiles in the same tissues after bacterial infection [31]. Furthermore, two recombinant rIL1βs possessed biological activity to induce the expression of endogenous IL1β in head kidney leukocytes, and rIL1β1 induced higher expression levels of endogenous IL1β than rIL1β2, which could result in unequal immunoadjuvant role in immune response.

Serum specific antibody induced by protein antigens or vaccines had been proven to be the basic mechanism of immunoprotection against bacteria infection [69–71]. In carp it was reported that injecting killed bacteria plus a recombinant IL1β peptide induced higher agglutinating antibody level compared with killed bacteria alone [32]. In this study, pSCPI-specific antibody levels were continuously detected by ELISA from 3rd to 7th-week, the antibody levels of fish in pSCPI + rIL1β1 and pSCPI + rIL1β2 groups were significantly higher than that of pSCPI alone, and co-injection of rIL1β1 + rIL1β2 induced higher antibody levels compared with rIL1β1 or rIL1β2 alone. Besides, the antibody levels of fish in four pSCPI groups were all maximal at f4th-week psv, which was earlier than the time (fifth week psv.) of pSCPI + FIA (Freund's incomplete adjuvant) group when the highest antibody levels appearing. While the highest antibody levels of fish in three pSCPI plus adjuvant groups in this study were little lower than that of pSCPI + FIA group shown in our previous study [32], mainly because of the difference of types, functions and mechanisms of different adjuvants.

To further examine whether the antibody induced by pSCPI had any protective effects against *S. iniae* infection, we measured the serum bactericidal activity with different treatment. The survival rate of *S. iniae* in untreated serum was the lowest, followed by the 44 °C treated serum and 100 °C treated serum, suggesting that the bactericidal effect was dependent on both antibodies and complement. Moreover, the bacterial survival rates in fish vaccinated with pSCPI + rIL1β1 + rIL1β2 was the lowest, which was in line with the ELISA results, indicating that co-injection of rIL1β1 and rIL1β2 increased antibody levels and complement activity, resulting in higher bactericidal activity. In fish, lysozyme activity and alternative complement haemolytic activity (ACH50) are two important indicators of the innate immune response [72–74]. We found that both rIL1β1 and rIL1β2 stimulated lysozyme activity, and that co-injection of them induced higher lysozyme activity. Similar results were also observed on the ACH50 activity, separate injection or co-injection of rIL1β1 and rIL1β2 with pSCPI all significantly enhanced alternative complement haemolytic activity.

Various studies have reported that recombinant IL1β increased the expression of endogenous IL1β and TNF-α genes in fish [24]. In our study, compared with pSCPI vaccine alone, pSCPI vaccine plus adjuvant (pSCPI + rIL1β1, pSCPI + rIL1β2, and pSCPI + rIL1β1 + rIL1β2) significantly induced the expression of endogenous IL1β and TNF-α genes in head kidney, which was consistent with the results of Hong [34] and Laing [75]. While only pSCPI + rIL1β1 + rIL1β2 significantly enhanced the expression of endogenous IL1β and TNF-α genes in spleen. Besides, the relative expression of endogenous IL1β gene induced by pSCPI + rIL1β1 were higher than that induced by pSCPI + rIL1β2 no matter in head kidney or spleen, which was consistent with the results of their bioactivities in vitro. Interestingly, the expression levels of endogenous IL1β and TNF-α genes in head kidney were higher than in spleen, suggesting that the head kidney may be the primary site where IL1β and TNF-α function during the immune response in channel catfish.

RPS is the most visual index to evaluate vaccine efficacy against bacterial infection. Our results indicated that vaccination with adjuvants rIL1β1 or rIL1β2 alone failed to confer immunoprotection against *S.iniae* infection, only 10.71% and 3.57% respectively, in terms of RPS. While vaccination with pSCPI plus adjuvants rIL1β1 or rIL1β2 enhanced little higher immunoprotection with the RPS of 64.26% and 60.71% respectively. Moreover, vaccination with a combination of rIL1β1, rIL1β2, and pSCPI provided significantly higher protection against infection (RPS was 75%) than vaccination with pSCPI alone (RPS was 50%), which was in line with the results of ELISA, serum bactericidal activity, and lysozyme activity. However, the RPS values of pSCPI + rIL1β1, pSCPI + rIL1β2 and pSCPI + rIL1β1 + rIL1β2 were all lower than that of pSCPI + FIA (RPS was 85.71%, shown in our previous study [38]), mainly due to the different adjuvant types and functional mechanism.

5. Conclusions

In conclusion, channel catfish IL1βs were highly conserved with five conserved motifs compared with other fish IL1βs, there were only 28 nucleotide differences, corresponding to 16 amino acid differences between IL1β1 and IL1β2. Fish IL1β genes were subjected to negative/purifying selection and highly conserved throughout the evolution with global dN/dS ratios value of 0.425. In addition, rIL1β1 and rIL1β2 enhanced the immune response when co-injected with the vaccine pSCPI, by increasing antibody levels, serum bactericidal activity, lysozyme activity, alternative complement haemolytic activity, the expression of immune-related genes, and immunoprotection against *S. iniae* infection. Although rIL1β1 was slightly better than rIL1β2, the co-injection of rIL1β1 and rIL1β2 with pSCPI induced a higher immune response and provided higher immunoprotection than the separate injection. Our present results not only enrich the molecular structure study of fish IL1β, but also indicate that channel catfish IL1βs can be used as potential adjuvants in subunit vaccine models against bacterial infection, which are of profound importance to prevent and control bacterial disease in channel catfish.

Conflicts of interest

The authors declare that there is no conflict of interest.

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