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N-terminal domain of EcC1INH in *Epinephelus coioides* can antagonize the LPS-stimulated inflammatory response

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ABSTRACT

Complement 1 inhibitor (C1INH) serving as a multifunctional factor can participate in the regulation of complement cascades and attenuate the activation of various proteases. In this study, we obtained EcC1INH cDNA and the tissue-specific analysis indicate that the highest expression level of EcC1INH mRNA was detected in liver. Moreover, *Vibrio alginolyticus* challenge can significantly increase EcC1INH mRNA expression in liver and kidney. N-terminal domain of EcC1INH could decrease LPS binding activity to cell surface, while loss of positively charged residues (PCRs) Arg21, His22, Lys50, Arg61 in N-terminal domain of EcC1INH can significantly reduce its interaction with LPS. Furthermore, LPS injection experiment indicated that the binding of EcC1INH N-terminal domain to LPS can antagonize LPS-induced inflammatory signaling pathway and attenuate the production of proinflammatory cytokines *in vivo*, indicating that EcC1INH was involved in negative regulation of inflammatory response.

1. Introduction

In mammal, cell-killing mechanisms are the most important components of host immune defense against invading microorganisms, infectious tissues and malignant cells via a cytotoxic manner by activating specific proteins such as complement proteins [1]. Complement cascade is one of the central constitutes in mammalian macrophage-activating immune system [2] and serves as professional effectors capable of increasing the phagocytosis [3], generating significant amounts of superoxide production [4] as well as synchronizing nature immune system with acquired immunity [5]. Additionally, classic pathway, alternative pathway and Mannose binding lectin (MBL) pathway can produce active opsonins or recruiters of other proteins and form membrane attack complexes (MAC), thus defending against the invasion of microorganism [6]. Recent studies indicate that mammalian complement system activated by bacterial lipopolysaccharide (LPS) stimulation can effectively confer protection against the subsequent bacterial infection by enhancing phagocytic activity, whereas a destroyed complement system can significantly reduce the *in vivo* clearance of virulent strains, implying that complement activation participates in the host killing mechanism of microorganism [7]. In addition, complement deficiency may relate to a reduced resistance to the microorganism invasion, leading to an increasing prevalence of pyogenic infections and immune complex diseases [8].

Complement 1 inhibitor (C1INH) plays an indispensable role in modulating complement pathways as a negative modulator that inhibits the classical pathway by depressing the activities of complement 1r (C1r) and complement 1s (C1s) [9], exhibits a downregulatory effect in the lectin complement pathway by inhibiting mannose binding lectin associated serum protease (MASP) [10], as well as blocks the alternative complement cascade to decrease complement 3 (C3) cleavage by directly inhibiting the binding activity [11]. Recent findings indicate that C1INH may also inactivate surface-mediated pathway [12,13], which may appear to be associated with the regulation of inflammatory response [14]. In addition, C1INH, a key inhibitor of plasma serine proteases, harboring the characteristic structure of serpin superfamily, may serve as a major downregulator of inflammatory processes in mammal [15].

Mounting evidences indicate that the innate immune defense system in invertebrates harbors pathogen recognition units, but it is only a teleostean basic defense mechanism [16]. In contrast, bony fish contain developed complement cascades [17], and the structures of teleostean

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complements are homologous to those of mammals [18]. Despite previous researches pay more attention to complement cascades in mammals, only a few reports study on the teleostean complement system. In recent years, teleostean C1INHs have been identified in several fish species, including *Oreochromis niloticus* [19,20], *Pseudosciaena croceav* [21], *Oplegnathus fasciatus* [22] and *Sebastes schlegelii* [23]. Most of these studies focus on the gene structure and gene analysis of teleostean C1INHs, but the possible function of teleostean C1INHs in LPS-mediated inflammatory response is still unclear.

Stressors such as various pathogenic diseases may exert a harmful effect on the immune system in grouper [24,25]. The recent emergence of global climate anomaly arising a wide public concern may be one of the abnormal phenomena, which can aggravate the expansion of vibrio infection via the long-distance geographical transportation [26]. *Vibrio alginolyticus* is one of vibrio strains can produce a extracellular toxin [27]. In addition, fecal population may facilitate the increasing level of vibrio population [28]. Vibrio strains also contain a TonB/ExbB/ExbD complex that can regulate the iron uptake processes, thus rendering the invading bacteria less susceptible to a microenvironmental condition of limited iron availability [29]. Recent findings indicate that *in vivo* injection of *V. alginolyticus* or its extracellular products can significantly induce toxicological effect in grouper, leading to a slight exophthalmia with corneal opaqueness in moribund/dead fish [27]. Thus, a better understanding of the immunity-related mechanism underlying the change of EcC1INH expression to vibrio stimulation is of great importance and may contribute to the sustainable aquaculture. In this research, we mainly investigated the binding activity of N-terminal domain of EcC1INH to LPS and its protective effect on LPS-induced inflammatory responses.

2. Materials and methods

2.1. Fish preparation

Healthy groupers (approximately 12.50 g) were obtained from a fish farm (Guangdong, China). Groupers were fed twice and acclimatized in aerated seawater before vibrio challenge.

2.2. Cloning

Cloning methods and protocols of full-length EcC1INH cDNA was based on our previous studies using a rapid amplification of cDNA ends (Clontech, China) [30] and the primers for gene cloning were shown in Table 1.

2.3. Bioinformatics analysis

Based on our pervious studies, the domain structures of predicted EcC1INH amino acid sequences were analyzed by using NCBI blast, ExPASy tools, SignalP 4.1, ClustalW and Genedoc and MEGA 6.0, respectively [31,32].

2.4. Vibrio challenge

Vibrio alginolyticus preparation and immune challenge experiment were based on previous research [30]. In brief, 100 μL suspension of 1×10^7 CFU ml^{-1} vibrio/PBS solution was injected intraperitoneally. PBS injection group was used as the control. In addition, 6 fish in each group were collected at various infection times and the liquid nitrogen freezing samples were preserved in −80 °C.

2.5. Tissue distribution and vibrio-induced expression of EcC1INH mRNA

The methods of RNA isolation, cDNA synthesis and qRT-PCR assay were based on our previous studies [31,33,34]. In brief, tissue specific expression and vibrio-induced patterns of EcC1INH (KR071124) were examined in triplicate by qRT-PCR. 18S rRNA was used as internal control. Data were analyzed by $2^{-\triangle\triangle Ct}$ methods and SPSS software.

2.6. Gene mutagenesis and plasmid preparation

N-terminal sequence of EcC1INH (EcC1INH-nonserpin, 248 amino acids) was cloned and used as a template. The EcC1INH-nonserpin mutants (248 amino acids) was conducted by using site-directed mutagenesis with in-fusion HD cloning system (TAKARA, Dalian, China). Positively charged (PCRs) mutant at R21H22 residues (PM1, R21→A21 and H22→A22, the 21st Arg was replaced by Ala and the 22nd His was replaced by Ala, 248 amino acids) and PCRs mutant at R21H22K50R61 residues (PM2, R21→A21, H22→A22, K50→A50 and R61→A61, the 21st Arg was replaced by Ala, the 22nd His was replaced by Ala, the 50th Lys was replaced by Ala and the 61st Arg was replaced by Ala, 248 amino acids) were obtained. Following the double digestion reaction (BamHI and *Hin*dIII), pET32a ligation and sequencing were performed.

2.7. Production, purification and validation of EcC1INH-nonserpins

Production of purified EcC1INH-nonserpin fusion proteins was performed [35]. Briefly, the above constructed PET32a expression plasmids were transformed into competent *E. coil* BL21 (DE3), respectively. Following the sequencing, BL21 clone inserted with a corrected plasmid was incubated in LB medium with 100 μg/ml ampicillin at 37 °C until OD_{600} value reached about 0.7, then IPTG was added to a final concentration of 1 mM for another 4 h induction. After IPTG induction and sonication, pellets were harvested, dissolved in the buffer containing 8 M urea and centrifuged, then the soluble recombinant proteins were obtained and purified by using Ni–NTA resins (Novagen, China). According to purification protocols of His-tag proteins, the Ni-NTA His bind resin was loaded with 1 × binding buffer, Ni ion solution, 1 × binding buffer, soluble recombinant protein samples, 1 × binding buffer, 1 × wash buffer and 1 × elution buffer, respectively. Finally, following proper dialysis, the protein concentration was determined, then mass spectrum analysis (see Table 2) and western blotting were performed in accordance with previous research [36].

2.8. Elisa assay

96-well plates were coated with 100 μg/ml LPS (purified from *Escherichia coli* O111:B4, Sigma, USA) at 4 °C overnight. Then, the plate was blocked with 1% BSA/PBS at 37 °C for 2 h. After washing with 0.5% Tween-20/PBS, the plate coated with LPS was incubated with the purified recombinant EcC1INH-nonserpin WT protein or mutant proteins (0, 4, 20, 100 and 500 μg/ml) at room temperature for 2 h, then incubated with His-tag antibody (Tiangen, China) and HRP secondary antibody (Tiangen, China) followed by washing with 0.5% Tween-20/PBS. 100 μl of TMB diluted in substrate buffer was incubated in dark, until the color was developed. Then, 2 M H_2SO_4 was added to stop the reaction. The absorbance at 450 nm was determined by a microplate reader. In this study, The absorbance of OD_{450} value in PBS was served as the negative control and the binding index was calculated as follows: OD_{450} nm absorption in test group/OD_{450} nm absorption in negative control group (n = 3).

2.9. Fluorescence microscopy

Hela cells were plated on the glass coverslips in 24-well plates and incubated at 37 °C for 18 h. Cells were treated with 100 ng/ml FITC-conjugated LPS (Sigma, USA) with or without purified Trx or EcC1INH-nonserpin WT/mutant proteins (150 μg/ml) for 1 h. Then, cells were washed with PBS, fixed with 4% PFA and observed using fluorescence microscopy [37].

Table 1
The primer sequences used in this study.

Primer names	Sequence direction (5' → 3')	Use
C1INH-F	TGTAACCGCTCAGTCGCC	Clone
C1INH-R	GCATTATCCCTCGCACCTA	Clone
C1INH-5gsp	GCACCCCGCTGATACTGATAGGAG	RACE
C1INH-5nest	GGGTTCCCCTGTGCGACTGTTC	RACE
C1INH-3gsp	AACACCCGTAGAGCCATTGAGACAG	RACE
C1INH-3nest	GTTTTATGAAGCAAAGCCCACCAGG	RACE
C1INH-nons-F	ATGAAACTACAGGCCGTACT	Clone
C1INH-nons-R	CTAGAGATTTGAAAACTCCG	Clone
C1INH-nonsNM-F1	ATGAAACTACAGGCCGTACTCT	Mutant
C1INH-nonsNM-R1	TGTCCAGCTGTGTCCTTGTGCCAGCATTGTCCTCT	Mutant
C1INH-nonsNM-F2	CACAAGGACACAGCTGGACACTGAAGACGAGTCAA	Mutant
C1INH-nonsNM-R2	CTAGAGATTTGAAAACTCCGTG	Mutant
C1INH-nonsPM1-F	TTCTTCATGCGCAGCTTTCGAGGTGA	Mutant
C1INH-nonsPM1-R	AGCTCGAAAACGAGCAGCAGCAAGAG	Mutant
C1INH-nonsPM2-F	GGGCATTCAATGGTGAAGATCTAAGTCTCAGCGCTGCGT	Mutant
C1INH-nonsPM2-R	AGGTGATGGTCCCTCCAGTGTAGACATCTT	Mutant
pET-C1INHnons-F	GCGGGATCCATGAAACTACAGGCCGTACT	Vector
pET-C1INHnons-R	CCCAAGCTTTTAGAGATTTGAAAACTCCGTGA	Vector
18S-F	CAGCGAGCGATGGAGACC	qRT-PCR
18S-R	CCAGTTTAGCATAGCGATACCTT	qRT-PCR
RT-C1INH -F	CCACAAACGAGGTCAAATG	qRT-PCR
RT-C1INH-R	CAGTCAAAATCCAGCGGTC	qRT-PCR
RT-Myd88-F	TATGCCTTCATCTGCTACTGCC	qRT-PCR
RT-Myd88-R	ACCATCCGCTTACACCTCTTC	qRT-PCR
RT-TRAF6-F	GCGGAGCCAGGAAGCA	qRT-PCR
RT-TRAF6-R	CAGCAGCCCGTCAAAAGC	qRT-PCR
RT-IRAK4-F	CCGTGTTTGGTGTATGAC	qRT-PCR
RT-IRAK4-R	CTCTGTGGACATGATGGT	qRT-PCR
RT-IL1b-F	GAGACGATTACCAAAGACAGC	qRT-PCR
RT-IL1b-R	GGGGTGAGCGACAGACAT	qRT-PCR
RT-TNF1-F	GCCAGACATCACGACGCA	qRT-PCR
RT-TNF1-R	CCGCCCTGAGCAAACG	qRT-PCR
RT-TNF2-F	ACACTCTGCCTCGCCTCTG	qRT-PCR
RT-TNF2-R	CCTGACTCCTCCATTCCACTG	qRT-PCR

2.10. Protective effect of EcC1INH-nonserpin on the immune challenge with LPS

To investigate the protective effect of EcC1INH-nonserpin against LPS stimuli, LPS challenge was performed *in vivo*. 30 μg purified EcC1INH-nonserpin protein was mixed with 100 μg/ml LPS at room temperature for 2 h. In brief, the grouper injected with 30 μg EcC1INH-nonserpin WT/fish was used as EcC1INH-nonserpin WT + PBS group. Besides, the grouper injected with the above mixture/fish was used as EcC1INH-nonserpin WT + LPS group, while the grouper injected with 100 μg/ml LPS was used as LPS + PBS group. 6 fish in each group were collected after vibrio challenge and the liquid nitrogen freezing samples were preserved in −80 °C. As shown above, RNA isolation and the determination of its quality was carried out. In addition, qRT-PCR assay was performed according to the above methods. The primers of Myeloid differentiation primary response 88 (MyD88, JF271883.1), Interleukin-1 receptor-associated kinase 4 (IRAK4, JX856139.1), TNF receptor associated factor 6 (TRAF6, KF137656.1), Interleukin-1β (IL-1β, EF582837.1), Tumor necrosis factor 1 (TNF1, HQ011925.1), Tumor necrosis factor 2 (TNF2, HQ011926.1) and 18S rRNA were shown in Table 1.

2.11. Statistical analyses

SPSS 18 was used to analyze the obtained data and the differences in each samples were subjected to one-way ANOVA ($P < 0.05$) [38].

3. Results

3.1. Bioinformatics analysis of EcC1INH cDNA

In Supplementary Fig. 1A, EcC1INH cDNA of 2219 bp comprised an ORF of 1797 bp with a poly(A) tail. In addition, the molecular mass of the deduced EcC1INH polypeptide (598 amino acids) was about 66.46 KDa.

In Supplementary Fig. 1B, EcC1INH was a member of serpin superfamily and the deduced amino acid sequence of EcC1INH contained two potential functional domains: Ig domain (amino acids 28–106 and amino acids 116–208, respectively) and serpin domain (amino acids 234–598). GenBank analysis indicated that the deduced EcC1INH polypeptide was homologous to those of *Oplegnathus fasciatus* (71% identity, AFO64913.1), L*arimichthys crocea* (64% identity, NP_001290301.1) and Oryz*ias melastigma* (62% identity, AEA30130.1), indicating that EcC1INH contained similar structural motifs as those found in teleostean counterparts [22]. In addition, a signal peptide

Table 2
Peptide information of EcC1INH-nonserpin by LC-MS/MS (Q-TOF) analysis.

Number	Mr (expt)	Mr (Calc)	Peptides	Location
1	3294.652	3294.6271	HFEVIPGSTLELPCLSFQDVYTGGTITWK	22–50
2	1508.645	1508.6227	TQLDTEDESSDNR	150–162
3	1858.0763	1858.0452	VNLLYPLDLDQTITIK	163–178
4	1776.8989	1776.8781	TGEPILQESITEFSNL	233–248

(amino acid 1–20) was found in EcC1INH (Human C1INH, amino acid 1–22; Chicken C1INH, amino acid 1–16; Zebrafish C1INH, amino acid 1–17). Similarly, a signal peptide is also found at the N-terminal of C1INH (amino acid 1–20) in rock bream, suggesting that the mature C1INH may be an secreted protein harboring two major domain architecture of serpin superfamily [22]. Additionally, glycosylation in mammalian C1INH is playing a vital role in the plasma clearance [39] and the maintenance of the metastable serpin conformation, similar to the effects of heparin [40], and the affinity of C1INH to endotoxin LPS may depend on the glycosylated N-terminal residue in the non-serpin domain [41]. Evidences are emerging that mammalian C1INH contains a large number of potential N- glycosylation sites (NSS) and O-glycosylation sites [9,42], while teleostean C1INHs only harbor two or three glycosylation sites [43]. In this study, we found that EcC1INH polypeptide contained 3 potential NSS (146–149 “NGTR”, 330–333 “NLSE” and 337–340“NQSI”, respectively).

According to the obtained C1INH polypeptide, phylogenetic analysis was constructed (Supplementary Fig. 1C). EcC1INH polypeptide showed a high similarity to C1INHs in *Oplegnathus fasciatus* and *Larimichthys crocea*.

3.2. Expressions of tissue-specific EcC1INH mRNA and V. alginolyticus-stimulated EcC1INH mRNA

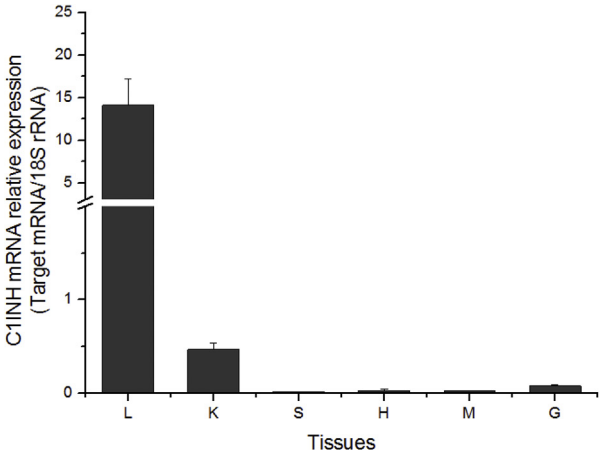
In Fig. 1A, tissue-specific expression measured by qRT-PCR revealed that liver and kidney EcC1INH expression were stronger. Similarly, a high-level of liver C1INH mRNA was observed in Nile tilapia [20]. In mammal, liver is able to produce approximately 80% of PRRs, which can enable the activation of nature immunity to defense against bacterial or viral invasion [44]. Additionally, liver serves as a predominant site in host immunity and is able to produce leukocyte lineages and enhance complement signalings [45]. In previous research, a high-level of EcC3 and EcC8 beta expression is observed in grouper [18,46]. Taken together, liver may be the major organ for C1INH synthesis.

In Fig. 1B, a fluctuation of liver EcC1INH mRNA was detected from 6 h to 24 h following vibrio challenge. EcC1INH mRNA increased to the peak at 36 h, but it decreased at 48 h post-injection.

In Fig. 1C, kidney EcC1INH mRNA decreased gradually from 6 h to 24 h after vibrio challenge, but it reached a peaked level at 36 h.

3.3. Prokaryotic expression and fusion protein validation

The binding ability of mammalian C1INH to LPS may rely on NSS and PCRs located at N-terminal 97 amino acid sequence, while the O-linked glycosylation and C-terminal residue play no role in mediating the LPS binding activity [41,47]. Additionally, the binding to LPS of mammalian C1INH only requires Asn3, while the N-glycosylated Asn47 and Asn59 plays no role in mediating LPS binding [48]. In contrast, N-terminal of teleostean C1INH lacks NSS but contains PCRs. To investigate functionality of EcC1INH N-terminal domain, we performed the cloning assay and performed the gene mutagenesis, thus the expression plasmids of PCRs mutant at R21H22 residues (PM1, R21→A21 and H22→A22, the 21st Arg was replaced by Ala and the 22nd His was replaced by Ala) and PCRs mutant at R21H22K50R61 residues (PM2, R21→A21, H22→A22, K50→A50 and R61→A61, the 21st Arg was replaced by Ala, the 22nd His was replaced by Ala, the 50th Lys was replaced by Ala and the 61st Arg was replaced by Ala) were obtained. The diagrams of the EcC1INH-nonserpin WT/PM1/PM2 were shown in Fig. 2A. Before the induction of recombinant proteins, recombinant plasmids pET32a-EcC1INH-nonserpin WT/PM1/PM2 were subjected to DNA sequencing. In Fig. 2B and C, protein bands at about 55 kDa were strongly visualized. Following sonication, Trx or EcC1INH-nonserpin WT/PM1/PM2 proteins were purified.



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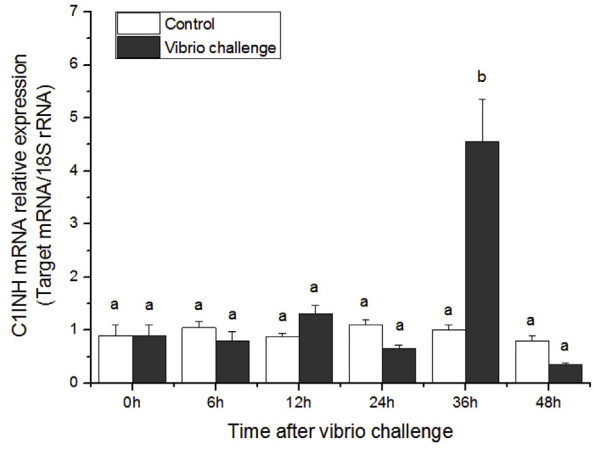

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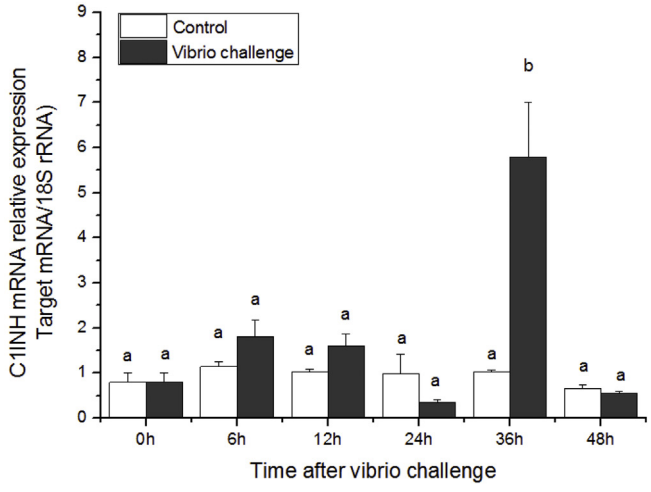

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Fig. 1. qRT-PCR analysis of EcC1INH mRNA expression. (A) qRT-PCR analysis of tissue-specific EcC1INH mRNA expression. The relative mRNA level was compared with muscle expression. (L: liver; G: gill; S: spleen; H: heart; K: kidney; M: muscle). (B) qRT-PCR analysis of EcC1INH mRNA expression in liver and kidney at 0, 6, 12, 24, 36 and 48 h post-injection. The relative EcC1INH transcript expression of each tissue were calculated by the $2^{-\triangle\triangle Ct}$ methods using 18S rRNA as a reference gene. The calculated data (mean ± SD) of six individuals (n = 6) with different letters were significantly different ($P < 0.05$) among the groups.

3.4. Detection of the binding activity of EcC1INH-nonserpin to LPS

To investigate the possible binding of EcC1INH-nonserpin domain to LPS, LPS-ELISA assay was performed. As shown in Fig. 3A, the binding index in Trx protein are consistently lower from 4 μg/ml to 500 μg/ml, while the binding index in EcC1INH-nonserpin WT group are consistently higher among the groups. Binding index in EcC1INH-nonserpin WT group increased sharply in comparison with those of Trx groups when the protein concentration increased from 4 μg/ml to

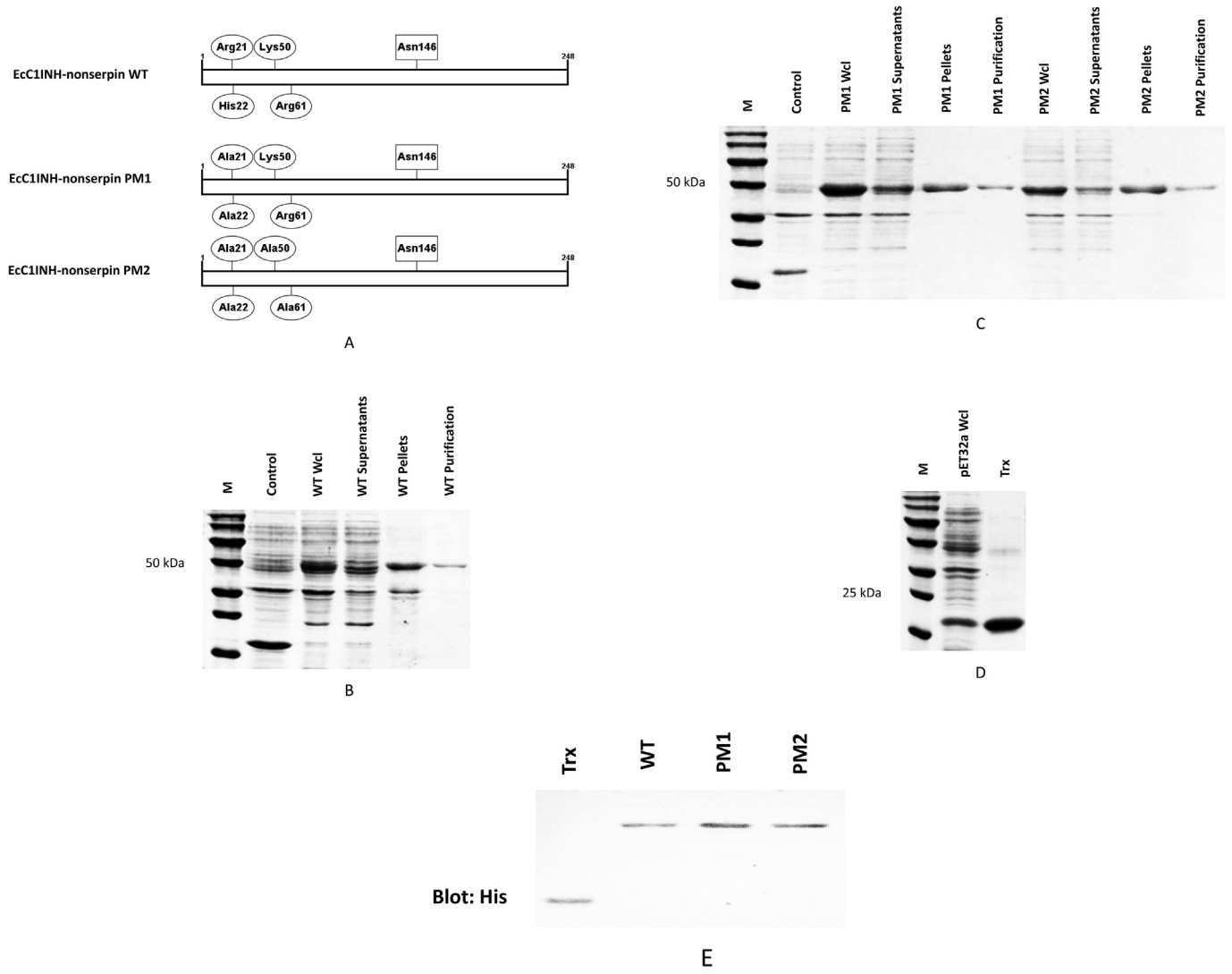
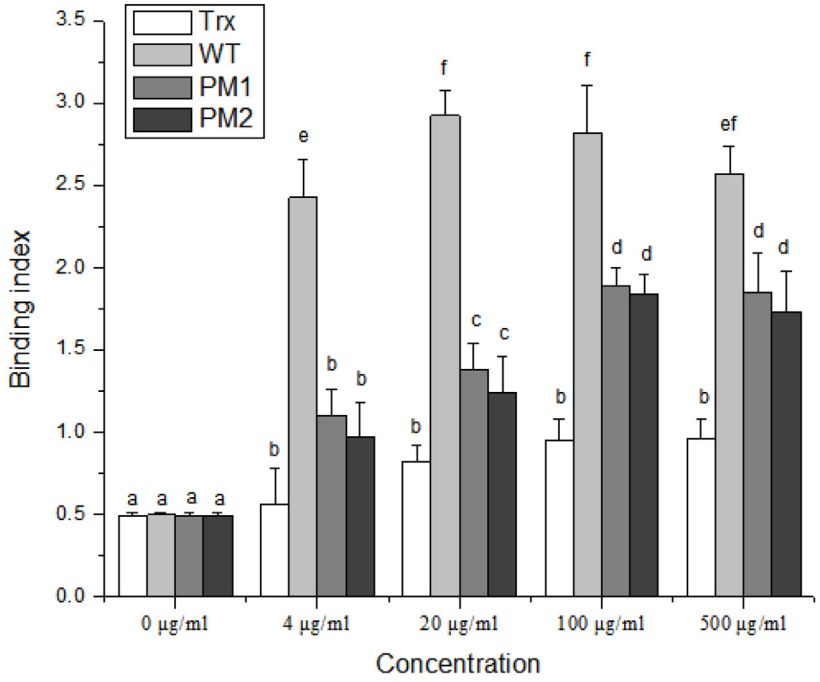


Fig. 2. Purification and LPS binding ability of EcC1INH-nonpserin WT/mutant protein. (A) The gene mutagenesis of EcC1INH-nonserpin was shown as a diagram. WT: The obtained sequence of EcC1INH-nonserpin; PM1: Positively charged (PCRs) mutant sequence of EcC1INH-nonserpin and its mutant site was located at R21→A21 (the 21st Arg was replaced by Ala) and H22→A22 (the 22nd His was replaced by Ala); PM2: Positively charged (PCRs) mutant sequence of EcC1INH-nonserpin and its mutant site was located at R21→A21 (the 21st Arg was replaced by Ala), H22→A22 (the 22nd His was replaced by Ala), K50→A50 (the 50th Lys was replaced by Ala) and R61→A61 (the 61st Arg was replaced by Ala). (B) Production and purification of EcC1INH-nonpserin WT. Lane M: Protein molecular standard; Lane Control: Total protein from whole cell lysis of the induced pET-32a-BL21; Lane WT Wcl: Total protein from whole cell lysis of the induced pET-32a-EcC1INH-nonserpin WT-BL21; Lane WT Supernatants: Supernatants isolated from the induced pET-32a-EcC1INH-nonserpin WT-BL21 strains after sonication; Lane WT Pellets: Pellets isolated from the induced pET-32a-EcC1INH-nonserpin WT-BL21 strains after sonication; Lane WT Purification: Purified recombinant pET-32a-EcC1INH-nonserpin WT protein; (C) Production and purification of EcC1INH-nonpserin PM1 and PM2. Lane M: Protein molecular standard; Lane Control: Total protein from whole cell lysis of the induced pET-32a-BL21; Lane PM1 Wcl: Total protein from whole cell lysis of the induced pET-32a-EcC1INH-nonserpin PM1-BL21; Lane PM1 Supernatants: Supernatants isolated from the induced pET-32a-EcC1INH-nonserpin PM1-BL21 strains after sonication; Lane PM1 Pellets: Pellets isolated from the induced pET-32a-EcC1INH-nonserpin PM1-BL21 strains after sonication; Lane PM1 Purification: Purified recombinant pET-32a-EcC1INH-nonserpin PM1 protein; Lane PM2 Wcl: Total protein from whole cell lysis of the induced pET-32a-EcC1INH-nonserpin PM2-BL21; Lane PM2 Supernatants: Supernatants isolated from the induced pET-32a-EcC1INH-nonserpin PM2-BL21 strains after sonication; Lane PM2 Pellets: Pellets isolated from the induced pET-32a-EcC1INH-nonserpin PM2-BL21 strains after sonication; Lane PM2 Purification: Purified recombinant pET-32a-EcC1INH-nonserpin PM2 protein. (D) Production and purification of Trx protein. Lane M: Protein molecular standard; Lane pET32a Wcl: Total protein was isolated from whole cell lysis of the induced pET32a; Lane Trx: Purified Trx tag was isolated from whole cell lysis of soluble PET32a. (E) Determination of the purified recombinant proteins by western blotting. The His-tag antibody was used as the primary antibody.



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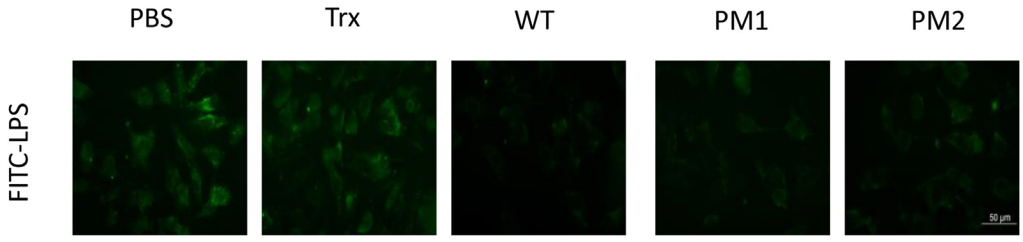

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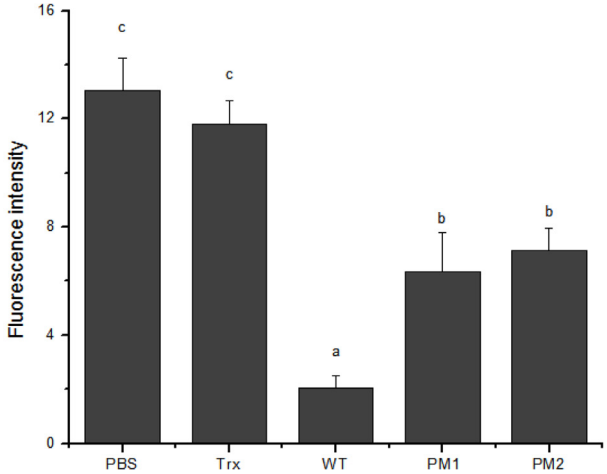

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Fig. 3. Determination of the binding activity of EcC1INH N-terminal domain to LPS. (A) ELISA assay. The calculated data (mean ± SD) with different letters were significantly different ($P < 0.05$) among the groups. The experiment was performed in triplicate. (B–C) Fluorescence microscopy. Cells were incubated FITC-LPS/PBS, FITC-LPS/Trx complex or FITC-LPS/EcC1INH-nonserpin WT/mutant complexes at 37 °C for 1 h and then detected by using a fluorescence microscopy. The fluorescence intensity analyses were measured by using image J program. The calculated data (mean ± SD) with different letters were significantly different

500 μg/ml. In contrast, although the binding index in EcC1INH-nonserpin mutant groups increased slightly from 4 μg/ml to 500 μg/ml, they were consistently lower than those of EcC1INH-nonserpin WT group, implying that EcC1INH-nonserpin WT can directly bind to LPS.

As shown in Fig. 3B and C, a strong fluorescence signaling was observed in PBS group and Trx group, while the fluorescence signaling in EcC1INH-nonserpin WT group decreased significantly with a value of 5.78- and 5.24-fold lower than PBS group and Trx group. In contrast, the fluorescence intensity in EcC1INH-nonserpin PM1 group decreased significantly with a value of 2.06- and 1.86-fold lower than PBS group and Trx group. The fluorescence intensity in EcC1INH-nonserpin PM2 group decreased significantly in comparison with PBS group and Trx group, suggesting that EcC1INH-nonserpin WT may abrogate the binding activity of LPS to cell surface.

3.5. Protective effect of EcC1INH-nonserpin in LPS-stimulated MyD88-dependent pathway

To investigate the protective effect of EcC1INH-nonserpin in LPS-stimulated inflammatory response in grouper, LPS challenge was performed *in vivo*.

As shown in Fig. 4A-C, *in vivo* injection of recombinant purified EcC1INH-nonserpin WT protein cannot activate the inflammatory signaling and induce the expressions of proinflammatory cytokines during the 24 h challenge experiment.

In Fig. 4A, the expression level of liver MyD88 (JF271883.1, Fig. 4A-1), IRAK4 (JX856139.1, Fig. 4A-2), TRAF6 (KF137656.1, Fig. 4A-3), IL-1β (EF582837.1, Fig. 4A-4), TNF1 (HQ011925.1, Fig. 4A-5) and TNF2 (HQ011926.1, Fig. 4A-6) in LPS + PBS group peaked at 24 h and its highest values were approximately 49.80-, 62.50-, 16.34-, 9.06-, 40.24- and 9.93-fold higher than EcC1INH-nonserpin WT + LPS group, respectively.

In Fig. 4B, the expression level of kidney MyD88 (Fig. 4B-1), IRAK4 (Fig. 4B-2), TRAF6 (Fig. 4B-3), IL-1β (Fig. 4B-4), TNF1 (Fig. 4B-5) and TNF2 (Fig. 4B-6) in LPS + PBS group peaked at 12 h and its highest values were approximately 6.87-, 3.63-, 5.49-, 4.03-, 2.43- and 1.99-fold higher than EcC1INH-nonserpin WT + LPS group, respectively.

In Fig. 4C, the expression level of spleen MyD88 (Fig. 4C-1) and IL-1β (Fig. 4C-4) in LPS + PBS group increased at 12 h and its highest values were 3.60- and 1.66-fold higher than EcC1INH-nonserpin WT + LPS group, respectively. The expression of spleen IRAK4 (Fig. 4C-2) and TRAF6 (Fig. 4C-3) in LPS + PBS group increased at 24 h and its highest values were about 8.33- and 25.91-fold higher than EcC1INH-nonserpin WT + LPS group, respectively. The expression of spleen TNF1(Fig. 4C-5) and TNF2 (Fig. 4C-) increased at 6 h and its highest values were approximately 2.16- and 3.15-fold higher than EcC1INH-nonserpin WT + LPS group, respectively.

4. Discussion

The activation of mammalian complement cascades is involved in the host defense mechanism against microorganism infection [49]. Generally speaking, microorganism infection can activate the human alternative pathway by targeting to C3 receptor-bearing cells [50]. Among the known complement cascades, alternative pathway is an antibody-independent complement cascade, which can be directly activated by LPS stimulation [51–53]. Recent findings demonstrate that mammalian C1INH plays a vital role in suppression of LPS-induced macrophage activation and cell injury [54]. Additionally, both inactive and active C1INH can inhibit LPS binding to macrophage surface and suppressing LPS-induced cytokines expression, playing an important role in the anti-inflammatory mechanism [41]. We found that both liver and kidney EcC1INH mRNA expression showed a sharp increase at 36 h post-challenge in this challenge study, which may be immune response to vibrio infection. Thus, the increased level of EcC1INH expression may be related to immune response to vibrio challenge.

Mammalian C1INH contains a reactive-site loop serving as a recognition element essential for binding to the catalytic groove of target proteases [55], which is responsible for its inhibitory activity [39]. In addition, its N-terminal domain may also harbor several NSS and PCRs, which can confer protection against LPS-induced shock and cytokine expressions by directly interacting with LPS [48]. Previous studies demonstrate that O-linked glycosylation and C-terminal residue in mammalian C1INH cannot exhibit LPS binding activity and the deletion of N-terminal domains can also strongly abrogate its LPS binding activity, but its LPS binding activity do not require intact reactive center loop in full-length C1INH architecture and the cleaved C1INH retains the inhibitory effect in LPS binding activity to cell surface, suggesting that the blockade of LPS binding may highly depend on the NSS and PCRs in C1INH N-terminal [41]. In teleost, previous studies indicate that N-terminal domains of teleostean C1INHs contain extended motifs lacking NSS by comparing with the counterparts in mammal [22], while serpin domain exhibits a higher conservation [21]. Although there are several studies focusing on the gene structure, expressions or protease inhibitory activity of teleostean C1INHs [21,22], protective effect of teleostean C1INH N-terminal domain against LPS stimuli is limited. Thus, we cloned the N-terminal domain of EcC1INH (EcC1INH-nonserpin) and performed the site-directed mutagenesis in order to investigate its possible interaction with gram-negative bacterial LPS. In this study, EcC1INH-nonserpin WT can bind to LPS *in vitro* by comparing with purified Trx (the control), while the mutagenesis on PCRs of EcC1INH-nonserpin can strongly abolish its binding activity to LPS. In addition, the administration of EcC1INH-nonserpin WT can also inhibit the binding of LPS to cell surface, while loss of PCRs in EcC1INH-nonserpin became less capable of blocking LPS binding to cell surface.

Broadly speaking, LPS stimulation can initiate the intracellular signaling by binding to toll-like receptors (TLRs) [56]. TLRs emerging as a cluster of pattern recognition receptors (PRRs) is a critical recognition constitute of microbial components and its cytoplasmic domain is able to activate the downstream signaling molecules such as MyD88 [57].

MyD88, previously described as a myeloid differentiation marker, is an adaptor protein involved in TLRs regulation [58]. MyD88 can recruit the downstream signaling molecules such as IRAK4 [59], whereas deletion of MyD88 can abolish the response to LPS stimulation [60].

IRAK4 belonging to IRAK family can participate in TLRs signaling and a majority of innate immune responses [61]. Previous studies indicate that macrophages and mouse embryo fibroblasts (MEFs) isolated from IRAK4 knockout mice exert a defective signaling transduction in response to TLRs ligands, leading to Nuclear Factor κB (NF-κB) suppression, the blockade of c-Jun and p38 phosphorylation, as well as a decreased level of TNFα, IL-1β and interleukin-6 (IL-6) [62].

TRAF6 functions downstream of IRAK4 [63] and is involved in the regulation of TNF receptor signaling and interleukin 1 receptor/toll-like receptor (IL1R/TLR) signaling [64]. As is well known, the induction of proinflammatory cytokines may require the activation of IκB kinases/nuclear factor κB (IKKs/NF-κB) pathway or mitogen-activated protein kinase/activator protein-1 (MAPK/AP-1) pathway [58,63,65], which is all converged on the transforming growth factor-β–activated kinase 1 (TAK1) activation by forming the complex of TARF6 and ubiquitin-conjugating enzymes [66].

In general, IL-1β and TNF can modulate the systemic responses and direct the adaptive immunity [67]. As is known, IL-1β is a proinflammatory cytokine that can significantly modulate organic anion transporters [68] and participate in the regulation of chronic disease processes [69], while TNF can participate in modulation of systemic defense, homeostasis and a number of disease states [70,71]. Thus, in order to investigate the protective effect of EcC1INH-nonserpin against LPS-induced inflammatory signaling in grouper, we performed LPS challenge experiment *in vivo*. In this study, MyD88, IRAK4, TRAF6, IL-1β, TNF1 and TNF2 mRNA expression increased sharply in LPS + PBS group in comparison with those of EcC1INH-nonserpin WT + LPS

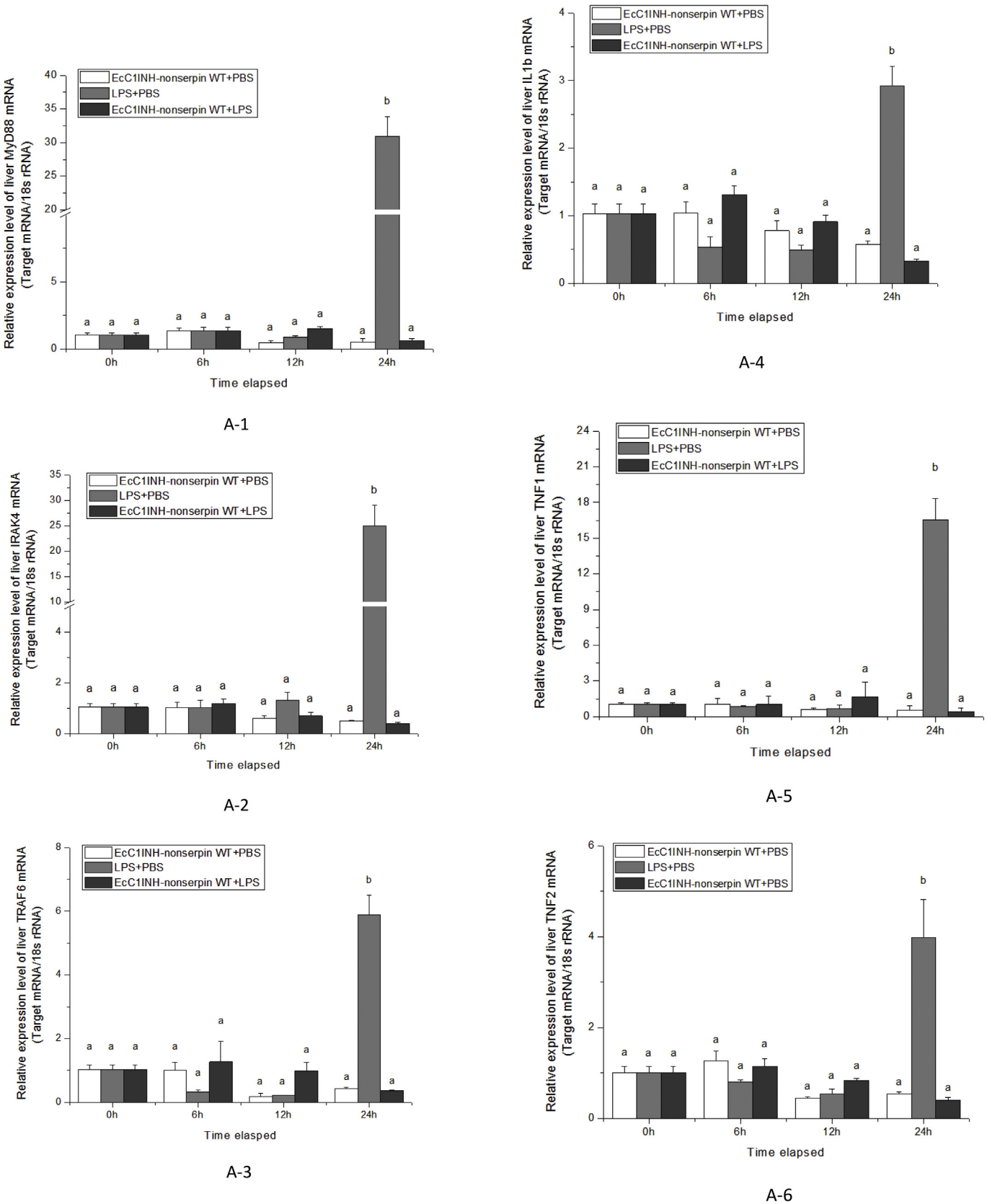
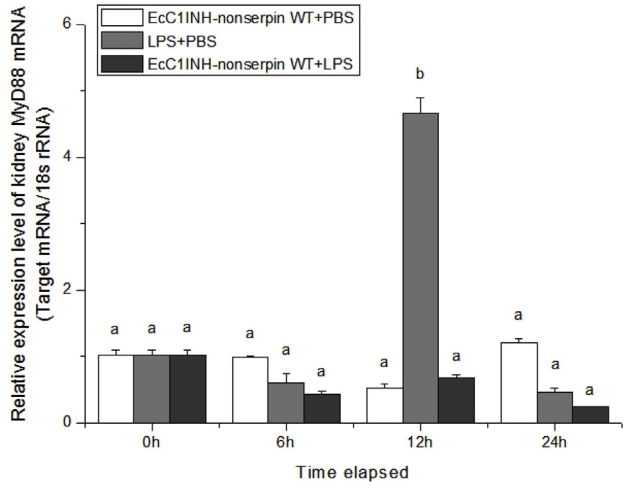
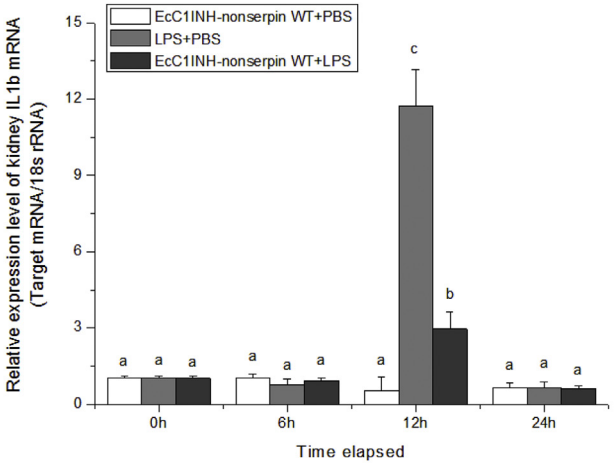


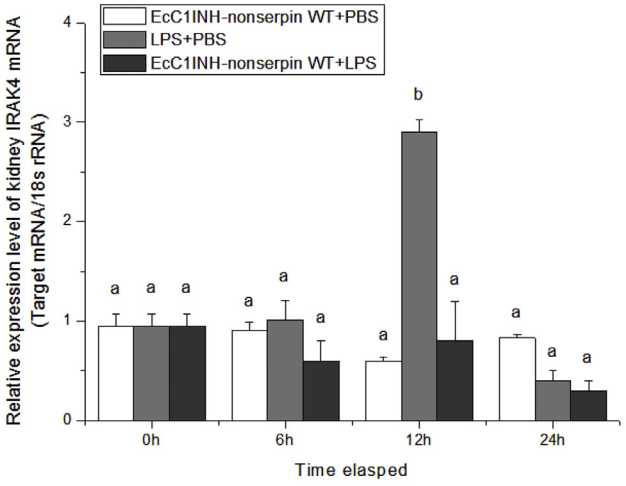
Fig. 4. Protective effect of EcC1INH-nonserpin WT in LPS-mediated inflammatory signaling and the expressions of proinflammatory cytokines in liver (A), kidney (B) and spleen (C) following LPS challenge. qRT-PCR analysis of MyD88 (JF271883.1), IRAK4 (JX856139.1), TRAF6 (KF137656.1), IL-1β (EF582837.1), TNF1 (HQ011925.1) and TNF2 (HQ011926.1) mRNA expression in liver at 0, 6, 12 and 24 h post-injection. The relative expressions of target genes were calculated by the $2^{-\Delta\Delta Ct}$ method using 18S rRNA as a reference gene. The calculated data (mean ± SD) of six individuals (n = 6) with different letters were significantly different ($P < 0.05$) among the groups.



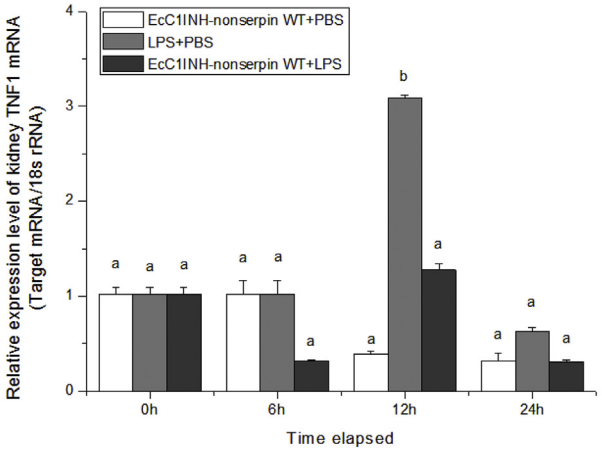
B-1



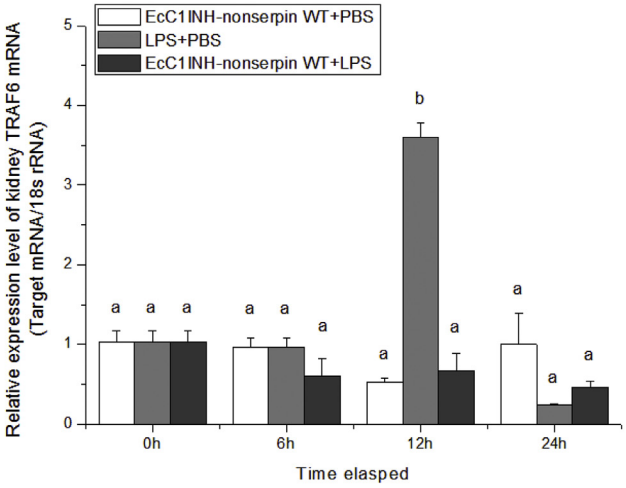
B-4



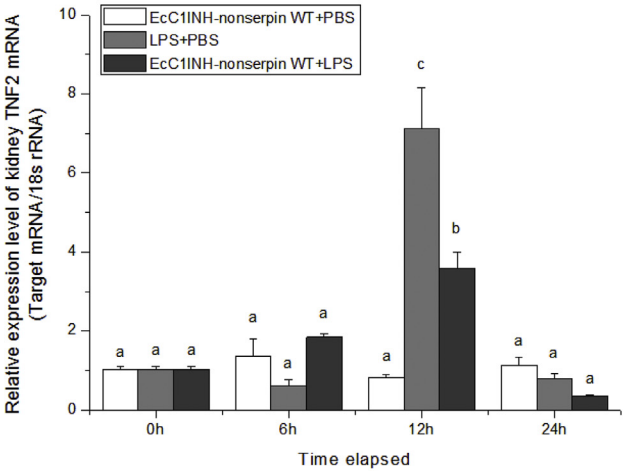
B-2



B-5

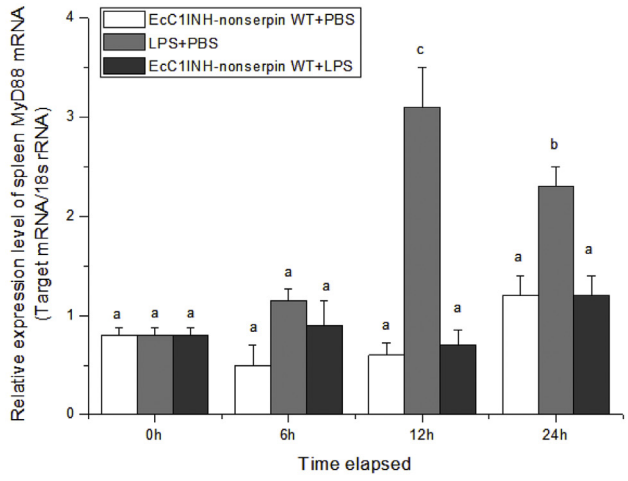


B-3

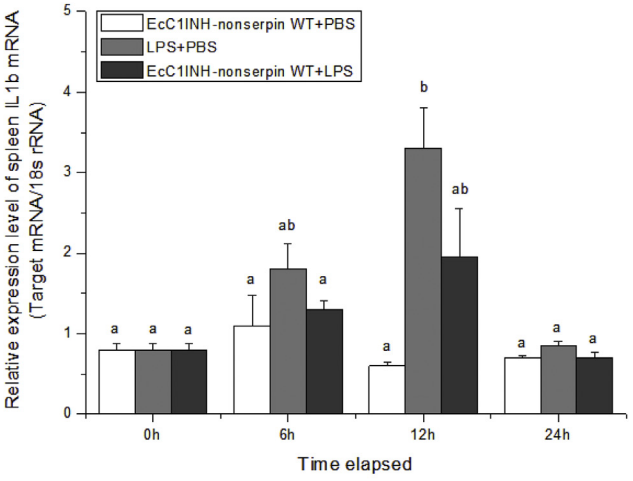


B-6

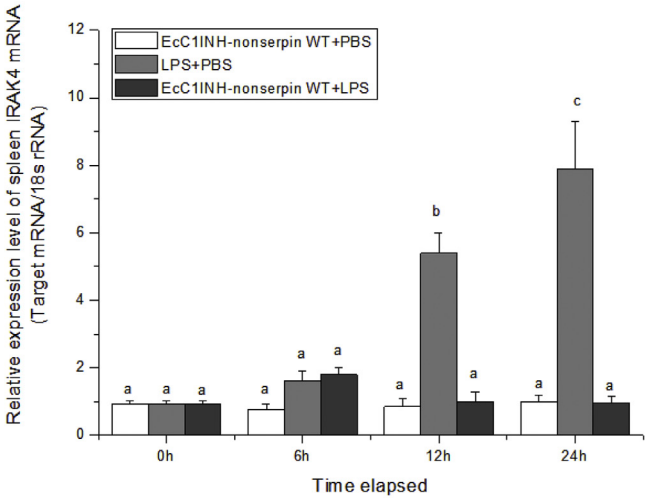
Fig. 4. (*continued*)



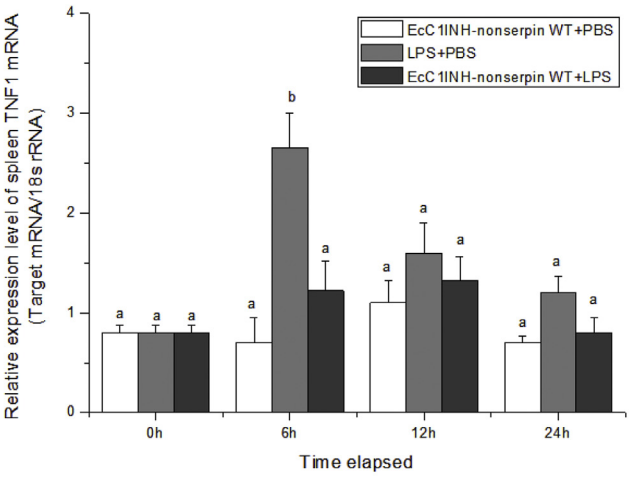
C-1



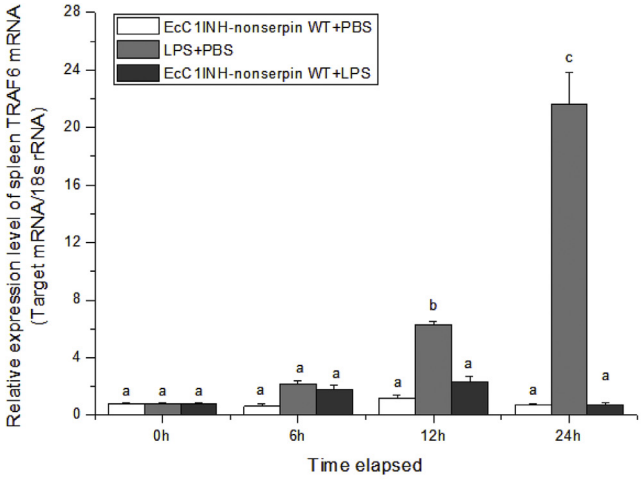
C-4



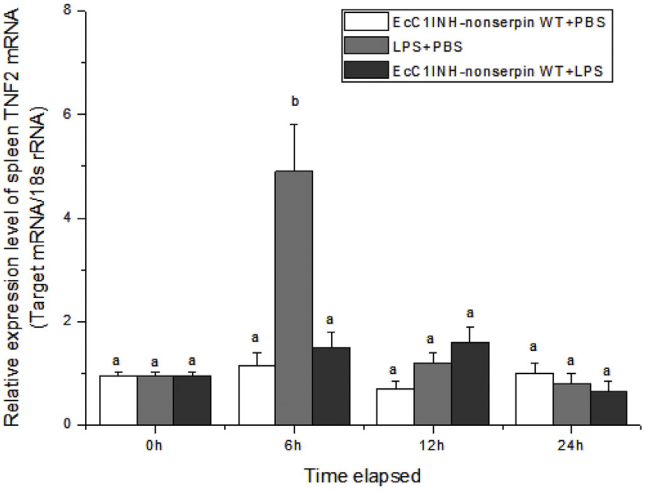
C-2



C-5



C-3



C-6

Fig. 4. (*continued*)

group. These results indicated that N-terminal of EcC1INH can abrogate the LPS-induced inflammatory signaling and inhibit the expressions of proinflammatory cytokines.

In summary, we cloned and characterized EcC1INH cDNA and studied up-regulated expressions of EcC1INH mRNA after the exposure to vibrio stimulation. Furthermore, N-terminal domain of EcC1INH can bind to LPS, attenuate LPS-induced inflammatory signaling and diminish the production of proinflammatory cytokines, while the mutagenesis on R21, H22, K50 and R61 can reduce the binding activity of EcC1INH N-terminal to LPS. Our results indicated that N-terminal residues of EcC1INH can exhibit a downregulatory effect on the LPS-induced inflammatory response by directly interacting with LPS.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2018.09.063.

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