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Characteristics and functional analysis of a ficolin-like protein from the oyster Crassostrea hongkongensis

Zhiming Xiang a, Fufa Qu a, b, Fuxuan Wang a, b, Jun Li a, b, Yuehuan Zhang a, Ziniu Yu a, b

a CAS Key Laboratory of Tropical Marine Bio-resources and Ecology, Guangdong Provincial Key Laboratory of Applied Marine Biology, South China Sea Institute of Oceanology, Chinese Academy of Sciences, 164 West Xingang Road, Guangzhou 510301, China
b University of Chinese Academy of Sciences, 19A Yuquan Road, Beijing 100049, China

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ABSTRACT

Ficolins are a group of soluble animal proteins with multiple roles in innate immunity. These proteins recognize and bind carbohydrates in pathogens and activate the complement system, leading to opsonization, leukocyte activation, and direct pathogen killing, which have been reported in many animal species but might not be present in the shellfish lineage. In the present study, we identified the first ficolin-related protein from the oyster, Crassostrea hongkongensis. This novel ficolin-like protein contains a typical signal peptide and a ficolin-related domain (designated ChFNC) at the N and C termini, respectively, but does not contain the additional collagen-like domain of ficolins. The full-length cDNA of ChFNC is 1105 bp, encoding a putative protein of 297 amino acids with the molecular weight of 35.5 kD. ChFNC is ubiquitously expressed in selected tissues, with the highest expression level observed in the gills. The temporal expression of ChFNC following microbe infection shows that the expression of ChFNC in hemocytes increases at 3 h post-challenge. The ChFNC protein expression was also examined, and fluorescence microscopy revealed that deChFNC (truncated signal peptide) is located in the cytoplasm of HeLa cells. Full-length ChFNC was detected in the medium supernatant by western blot analysis. Recombinant ChFNC proteins with the molecular weight about 50 kD bind Saccharomyces cerevisiae, Staphylococcus haemolyticus or Escherichia coli K-12, but not those from Vibrio alginolyticus. Furthermore, the rChFNC protein could agglutinate Gram-negative bacteria E. coli K-12 and enhance the phagocytosis of C. hongkongensis hemocytes in vitro. These results indicate that ChFNC might play an important role in the immunity response of oysters.

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1. Introduction

Ficolins are fibrinogen-related proteins (FREPs) family proteins with key roles in the lectin pathway. The ficolin- or MBL (mannose-binding lectin)-MASP (MBL-associated serine protease) complex binds directly to carbohydrates present on the surface of a variety of Gram-negative or Gram-positive bacteria, and subsequently, the complex initiates the lectin pathway to activate the complement system, increasing the expression of the complement components, such as C3b and initiating the lysis of bacteria through the membrane attack complex [1]. In general, ficolins recognize the sugars present on microorganisms and enhance phagocytosis [2].

In mammals, ficolins are oligomeric proteins characterized by a short N-terminal segment, a collagen-like domain and a C-terminal fibrinogen-like (FBG) domain [3]. In the collagen-like domain sequence, a glycine residue occurs at every third position (GXX), and this domain associates with MASP to activate the lectin pathway. Furthermore, the C-terminal fibrinogen-like domain binds to the surface of pathogenic microorganisms [4]. Ficolins are important markers for the lectin pathway. Several ficolins have been identified in mammals, amphibians, birds and ascidians, but no ficolin proteins have been identified in bony fish or reptiles [5–7]. Recently, MBL and ficolin proteins have been identified in lamprey and ascidians, the most ancient ancestors of both proteins identified to date [7,8]. Although ficolins have not been described in the protostome lineage, FREPs have been universally detected in these animals, suggesting that a FBG domain must have existed before the divergence of protostomes and deuterostomes [7–9]. The origins of...
the complement component have puzzled people, as a complement system has been identified, although this system has been traced to the origins of multi-cellular animals, such as Caenorhabditis elegans and Drosophila melanogaster [10,11]. However, the phylogeny and evolution of complement remains unknown [12]. These results suggest that ficolins evolved from other proteins with FGB domains, such as tachylectins and tenasins, present in invertebrate and vertebrate animals, respectively, which have been studied to understand the evolution of ficolin genes [13].

FREPs are a group of glycoproteins containing a FGB domain at the C-terminus but different domains at the N-terminus [14]. The innate immune response against pathogens [9,17,18]. Many FREPs have been identified in different animal species, including vertebrates, urochordates and invertebrates [7,14,16]. Only a few reports have described FREPs in mollusks, although these insects are the second most diverse group of animals (next to arthropods) with approximately 93,000 extant species [19]. Mediterranean mussel showed increased FREPs expression after bacterial infection or pathogen-associated molecular patterns (PAMP) treatment, and these proteins exhibited opsonic activities similar to mammalian ficolins [14]. In other shellfish mollusks, such as the scallop Argopecten irradians, AIRFREP was identified as a pattern recognition receptor in the immune response [20].

Economic shellfish, such as oysters, scallops, mussels, etc., which were the most common species, significantly contribute to the development of the marine economy, but the aquaculture of these species suffers great losses as a result of pathogen-causing diseases. Thus, it is important to understand the molecular mechanisms underlying innate immunity in the shellfish [21]. The oyster Crassostrea hongkongensis is an aquatic species with high commercial value along the coastal waters of the South China Sea. In the present study, we identified and cloned a cDNA encoding a FREP protein with high homology to ficolins (named ChFCN) from C. hongkongensis. To determine the immune function of ChFCN in marine bivalves, we conducted gene expression analyses following bacterial challenge and characterized the subcellular protein localization. The recombinant ChFCN protein can selectively bind pathogenic microorganisms and activate hemocyte phagocytosis in vitro.

2. Materials and methods

2.1. cDNA cloning and recombinant plasmid construction of ChFCN

We constructed an EST library from C. hongkongensis hemocytes, and 39,792 sequences were obtained following 454 sequencing and data analyses through a homolog search using the BLAST program (http://www.ncbi.nlm.nih.gov/blast). An EST was identified as homologous to a protein family in a homology search using the BLAST program. An EST was identified as homologous to a protein family in the origins of multi-cellular animals, such as Caenorhabditis elegans and Caenorhabditis elegans (http://www.ncbi.nlm.nih.gov/blast). An EST was identified as homologous to a protein family in the origins of multi-cellular animals, such as Caenorhabditis elegans and Caenorhabditis elegans (Table 1).

Table 1

| Primer name | Sequence (5’–3’)
|-------------|----------------
| 5’RACE-OU | GCCGATTCCACGGTATCACT
| 5’RACE-IN | TCTGTAATTTCACCGCTCCT
| 3’RACE-OU | TTGACACGTCTGCAATAG
| 3’RACE-IN | TGGTATAGAAGGGTCGATAG
| ORF-up | CGGCCATCGTCGCTTTGGGTT
| ORF-down | CCTAATTATATATATGCCTATAT
| ChFCN-qPCR F | CCGTCTGGCAGACATACA
| ChFCN-qPCR R | TTTGACGACTCGTCTCCGATAGA
| GAPDH-qPCR F | AAATCGAGATGCTAGAAGGCC
| GAPDH-qPCR R | GTATGATGCCCCTTTGTTGAGTC
| ChFCN His-HindIII F | TTTAGCTCCTAGTGCTTCTTTTG
| ChFCN His-XhoI R | TTTTCTGGATATCTGTGACATATA
| ChFCN GFP-Xhol F | TTTTCCGAGATGCTGCTTTGGGAT
| ChFCN GFP-HindIII R | TTTTGGGATGTGGTGGATTTG
| deChFCN GFP-Xhol F | TTTTGGGATGTGGTGGATTTG
| deChFCN GFP-HindIII R | TTTTGGGATGTGGTGGATTTG

The cDNA fragment encoding the mature ChFCN peptide was amplified using Promega Taq polymerase with the specific primers ChFCN His-HindIII Forward and ChFCN His-Xhol Reverse (Table 1). The HindIII and Xhol is the restriction enzyme site of the 5′ end of primers. The PCR fragment was digested with the appropriate restriction enzymes, and subsequently cloned into the HindIII/Xhol sites of expression vector pET-32a (Novagen). For subcellular localization of ChFCN, plasmids pEGFP-N1-ChFCN and pEGFP-N1-deChFCN were constructed for the expression of the full length or an expressed signal peptide of ChFCN, respectively. The primers for vectors are listed in Table 1, and the pEGFP-N1 vector was obtained from Clontech (CAT#6085-1) to generate GFP-ChFCN or –deChFCN fusion proteins for subcellular localization.

2.2. Cell culture, transient transfection and subcellular localization

HeLa cells were maintained and passaged using DMEM (Dulbecco’s modified Eagle’s medium; Gibco-BRL) supplemented with 10% fetal calf serum (FCS) and antibiotics (streptomycin and penicillin, Gibco) in a humidified atmosphere of 95% air and 5% CO2. Transient transfection was conducted using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. For the subcellular localization analysis, the cells were transfected with pEGFP-N1-ChFCN (or –deChFCN), and at 48 h after transfection, the HeLa cells were washed with PBS for 5 min, fixed with 4% paraformaldehyde for 10 min and subjected to nuclear staining with 4',6-diamidino-2-phenylindole (DAPI). The EGFP-ChFCN (or –deChFCN) fusion protein was detected using a Nikon inverted fluorescence microscope. For the secretory protein analysis, the secretory proteins were isolated using previously described methods [22], with modifications. Approximately 12 h before collection, the cell culture medium was replaced with culture medium containing no FCS. Subsequently, 10 mL of cell culture medium was harvested through centrifugation at 10,000× g for
10 min at 4 °C. The supernatant, containing the proteins secreted into the culture medium, was filtered through a 0.45 μm membrane to remove contaminating HeLa cells. A total of 25 ml of 100% cooled acetone was added to each 10 ml of collected medium, and the fractions were incubated at −70 °C overnight, followed by centrifugation at 12,000 × g for 10 min at 4 °C. The isolated secretory proteins were identified through western blot analysis.

2.3. Animals, tissue collection and immune challenge

Healthy C. hongkongensis (two years old, shell height 10.00 cm – 0.05 cm) were obtained from an oyster culture farm in Zhanjiang, Guangdong Province, China. The oysters were maintained at 24 ± 1 °C in tanks with circulating seawater for one week before the experiments and fed twice daily with the marine algae Tetraselmis suecica and Isochrysis galbana.

To determine the gene expression profiles, equal amounts of tissue from five healthy oysters were pooled for each tissue-specific expression analysis. The tissue samples were obtained from the gill, mantle, adductor muscle, heart, digestive gland, gonads and hemocytes. For bacterial challenge, the oysters were randomly divided into challenge and control groups. Vibrio alginolyticus (Gram-negative bacteria), Staphylococcus haemolyticus (Gram-positive bacteria) and Saccharomyces cerevisiae (fungus) were cultured separately. The bacteria or fungi were collected through centrifugation, followed by re-suspension in 0.1 M phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4 and 2 mM KH2PO4, pH 7.4) at 1.0 × 105 cells/L. Equal volumes of the three suspensions were generated as stock suspensions for injection, and the same volume of PBS was used as a control. The microbial suspension or PBS was injected into the adductor muscle of C. hongkongensis. After injection, the oysters were returned to the seawater tanks, pooled hemolymph from 5 individuals was used per each sampling point and treatment at 0, 3, 6, 12, 24 and 48 h post-injection. The samples were stored in liquid nitrogen until further use. Total RNA samples were extracted using TRizol (Invitrogen) according to the manufacturer’s instructions.

2.4. Isolation of total RNA and quantitative real-time PCR

The total RNA from each sample was treated with DNase I (Promega) and verified through agarose gel electrophoresis. The RNA concentration was determined after measuring the absorbance at 260 and 280 nm, and the purified RNA was diluted to a concentration of 1 μg/μL. A total of 2.5 μg of RNA was reverse-transcribed using random primers and the SuperScript III first-strand cDNA synthesis kit (Invitrogen, USA) according to the manufacturer's protocol. The cDNA mix was diluted to 1:10 and stored at −80 °C for subsequent expression analyses. PCR primers were designed for ChFCN and ChGAPDH (GenBank# JF919335, as a control), and the qPCR was performed using a Light-Cycler 480II System (Roche, USA) and a three-step RT-PCR protocol in a 20 μL reaction volume containing 10 μL of 2 × Master Mix (Roche, USA), 0.4 μL of each of the forward and reverse primers (10 μM), 1 μL of 1:10 diluted cDNA and 8.2 μL of PCR-grade water. The PCR conditions included initial denaturation at 95 °C for 3 min, followed by 45 amplification cycles of 95 °C for 15 s, 57 °C for 15 s and 72 °C for 10 s. A melting curve analysis was performed at the end of each PCR reaction to confirm the specificity of the amplicons. The data were quantified using the 2−ΔΔCt method based on the Ct values of ChFCN and ChGAPDH [23]. Dissociation curves were determined after the qRT-PCR to identify the specificity of PCR products. For each sample, experimental and control reactions were run in triplicate. The PCR efficiencies of the target and reference genes were approximately equal, and the differences were considered significant at P < 0.05.

2.5. Expression of recombinant ChFCN and purification of the fusion protein

The recombinant plasmid (pET-32a-ChFCN) was transformed into Escherichia coli BL21 (DE3) (Stratagene). The parent vector, without any inserts, was selected as a negative control. Positive transformants and negative controls were incubated at 37 °C in LB medium containing 50 mg/ml ampicillin with shaking at 220 rpm until the culture reached an OD600 of 0.5–0.7. IPTG was subsequently added to the medium at a final concentration of 1 mM, and the culture was incubated for another 4 h under the same conditions. The recombinant ChFCN (rChFCN) protein and negative control sample (designated as rTrx) were purified on a Ni2+ -chelating Sepharose column, washed with the wash buffer (8 M urea, 0.1 M NaH2PO4, 0.01 M Tris-Cl and 20 mM imidazole) and eluted with elution buffer (8 M urea, 0.1 M NaH2PO4, 0.01 M Tris-Cl and 400 mM imidazole). The purified protein was refolded at 4 °C in a gradient medium/TEA glycerol buffer (50 mM Tris–HCl, 50 mM NaCl, 10% glycerol, 2 mM reduced glutathione, 0.2 mM oxidized glutathione and 6, 4, 3, 2, 1 and 0 M urea in each gradient, pH 8.0). The purified proteins were subjected to reducing 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized using Coomassie brilliant blue R250. The concentrations of purified rChFCN and rTrx were quantified using the BCA method [20].

2.6. rChFCN binding to microbes

To characterize the recognition of the pathogens, the rChFCN was assessed for binding to V. alginolyticus, S. haemolyticus, E. coli K-12 and S. cerevisiae. Overnight cultures of the pathogens were killed after a 30 min incubation in 4% formaldehyde, followed by rinsing three times in TBS (pH 7.5) and treatment with 1% BSA in phosphate-buffered saline (PBS), pH 7.4, for 30 min at 37 °C. Subsequently, the cultures were washed and adjusted to 1 × 107 cells/ml with PBS buffer (contain 0.1 mmol Ca2+ or not). A total of 10 μl of rChFCN or rTrx (1 μg/μl) was incubated with the 200 μl of killed pathogens for 1 h at room temperature with gentle agitation, and rTrx was used as a negative control. The mixture was subsequently centrifuged at 6000 × g for 5 min. The microbial pellets were resuspended, rinsed with 1 ml TBS three times, and then the mixture were centrifuged, collected. The obtained deposits were resuspended in 2 × sample loading buffer (100 mM Tris-Cl (pH 6.8), 200 mM DTT, 4% SDS, 0.2% bromophenol blue, 20% glycerol), and subjected to SDS-PAGE and western blot analysis. To examine bacterial agglutination, 1 ml of E. coli K-12 bacteria (grown to OD600 = 0.5) were centrifuged at 5000 × g for 5 min. The pellets were washed and subsequently resuspended in 1 ml of TBS buffer (50 mM Tris–HCl and 50 mM NaCl, pH 7.5). A total of 50 μl of bacterial suspension was added to separate glass slides, and each slide sample was treated (or not) with 10 mM CaCl2 or 1 μl of rChFCN or rTrx (1 μg/μl) separately. The mixtures were incubated at room temperature for approximately 15 min, and the cells were subsequently observed using optical light microscopy (Carl Zeiss).

2.7. Western blot assay

Following SDS-PAGE, the proteins were transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, USA). The blot was incubated for 1 h at 4 °C in a blocking buffer (10 mM Tris, pH 8.0, 150 mM NaCl, 0.05% Tween 20, and 5% nonfat dry milk), followed by overnight incubation at 4 °C with mouse anti-GFP (Abcam, USA) or anti-His (ProteinTech, USA) antibody diluted
1:1000, and the blocking buffer was used to dilute all of the anti-bodies. The anti-GFP antibody was used in the subcellular localization analysis for detecting the expression of GFP fusion proteins. The cells were transfected with plasmids pEGFP-N1-ChFCN (or -deChFCN) and expressed the GFP tagged FCN fusion proteins, the full length of GFP-FCN fusion protein have a secreted signal peptide and should be detected in culture media, and GFP-deChFCN because of the truncated a secreted signal peptide was desected in intracellular of the cells. Furthermore, the anti-His antibody was used in bacterial binding assays, the anti-His antibody could recognized the both of the recombinant proteins rChFCN and rTrx because them have a HIS tag. The membranes were subsequently washed 3 times for 10 min in TBS containing 0.05% (v/v) Tween 20, followed by incubation with goat anti-mouse IgG peroxidase conjugates (Millipore, USA) diluted 1:4000 about 1 h at 37 °C. Detection was performed using DAB (diaminobenzidine) and H2O2 after incubation with a peroxidase-conjugated secondary antibody.

2.8. Phagocytosis assay of C. hongkongensis hemocytes

For the phagocytosis assays, hemolymph was extracted from 10 oysters on ice and simultaneously diluted 1:3 in an antiaggregant solution (Modified Alsever’s Solution, MAS; glucose: 20.8 g; sodium citrate: 8 g; EDTA: 3.36 g; NaCl: 22.3 g; and H2O: 1000 ml, pH 7.5). The cells were collected through centrifugation (1000 × g, 5 min, 4 °C; Eppendorf, Germany). Subsequently, the hemocytes were resuspended at 1 × 10^5 cell and aliquoted into six-well plates. For the analysis of phagocytosis, latex beads (L1030, Sigma) were treated with 1% BSA in phosphate-buffered saline (PBS, pH 7.4) for 30 min at 37 °C, followed by washing and incubation with final concentration of 1, 3, 5 μg/mL of rChFCN or rTrx purified proteins diluted in PBS or rTrx as a negative control. After incubation at 25 °C for 1 h, the treated latex beads were added to hemocytes at a 0.1% bead-to-hemocyte ratio, and the samples were incubated in the dark for an additional 4 h at 25 °C with gentle shaking. Subsequently, the redundant and suspending latex beads were discarded through centrifuging (1000 × g, 5 min, 4 °C), and phagocytosis was terminated after placing the test tubes on ice. A FACSCalibur flow cytometer was used to measure the fluorescence (FL-2), as an indicator of hemocyte phagocytosis.

2.9. Statistical analysis

The results are shown as the means ± SD of different biological samples. The significance of the data was examined using one-way ANOVA, and the differences were considered statistically significant at P < 0.05.

3. Results

3.1. Cloning and sequence analysis of ChFCN

A ChFCN cDNA (GenBank# KJ527586) was identified after screening a C. hongkongensis cDNA library using NCBI BLAST [24]. The complete nucleotide and deduced amino acid sequences are shown in Fig. 1(A). The full-length ChFCN cDNA is 1105 bp including an 19 bp 5′-untranslated region (UTR), an 894 bp open reading frame encoding 297 amino acids, and a 192 bp 3′-UTR. The deduced molecular weight is 33.55 kDa and isoelectric point (pI) is 9.03. The motif scan analysis revealed that ChFCN has a fibrinogen-related domain (FBC) at amino acid positions 86 through 295 (Fig. 1B), and include four conserved cysteine residue and a Ca^2+ binding site. These molecular structures demonstrated that ChFCN has characteristic features of known fibrinogen-related proteins (FREPs), indicating that ChFCN is a novel fibrinogen-related protein from the mollusk. Furthermore, we observed that the peptide sequence of the ChFCN is similar to that of ficolins. The C-terminal FBC was highly conserved; the deduced amino acid sequence of the domain shared the highest identity (68%) with that of ficolin B in Mus musculus (GenBank# AAC98781). The same genes likely encode the homologous ChFCN protein ficolin-2 (GenBank# EK42930) in C. gigas, as the identified amino sequence showed 94% similarity. The multiple sequence alignment was conducted using the deduced amino acid sequences of ChFCN and those of other known ficolins were retrieved through BLAST analysis using this sequence. In addition, a phylogenetic tree of ChFCN sequences was constructed using different members of the ficolin superfamily from both vertebrates and invertebrates (Fig. 2A and B). The results showed that ChFCN is located at the bottom of the branches, indicating that ChFCN is a conserved protein.

3.2. Subcellular localization of ChFCN

The subcellular localization of ChFCN was examined after introducing pEGFP-N1-ChFCN and pEGFP-N1-deChFCN plasmids into HeLa cells through transient transfection. At 48 h after transfection, the localization of the deChFCN, but not ChFCN, fusion proteins was visualized through epifluorescence after labeling the nuclei with DAPI, as a control. Enhanced green fluorescent protein (EGFP) protein is detected in the nuclei and cytoplasm of the cells (Fig. 3A). As a secreted protein, ChFCN was identified in the supernatant of cell culture media through immunoblot analysis. These results suggested that ChFCN is a secreted protein (Fig. 3B).

3.3. Expression pattern variation and expression under microbes challenge

We investigated the basal level of ChFCN transcription in several tissues of unchallenged C. hongkongensis. The relative expression of each transcript was normalized to that of hemocytes. The constitutive expression of ChFCN was detected in all seven tissues analyzed, with relatively low ChFCN expression detected in hemocytes, mantle cells, muscle cells, the digestive glands and heart tissues. In contrast, ChFCN expression was relatively high in the gills (Fig. 4A). ChFCN mRNA expression was up-regulated during the first 6 h after V. alginolyticus and S. haemolyticus infection, which peaked at 6 h after challenge, showing increases in mRNA expression of 1038- and 804-fold, respectively, compared with the blank. The expression of the ChFCN transcript was downregulated, decreasing to basal levels of expression at 48 h after challenge (Fig. 4B and C). After S. cerevisiae infection, the variable ChFCN mRNA expression was not as obvious as that observed during the first 6 h after V. alginolyticus and S. haemolyticus infection. The ChFCN mRNA expression peaked (344-fold) at 12 h after infection and gradually decreased to basal levels at 48 hpi (Fig. 4D). The significant differences were observed in the ChFCN expression of tissue distribution and microbial challenge assays.

3.4. Expression and functional analysis of ChFCN

After IPTG induction, the whole cell lysate of E. coli BL21 (DE3) with pET-32a-ChFCN was analyzed through SDS-PAGE, and a distinct band with a molecular weight of approximately 50 kDa (Fig. 5A) was revealed, consistent with the predicted molecular mass of the rChFCN fusion protein. In addition, a unique protein of approximately 17 kDa, representing rTrx, was detected and purified from the IPTG-induced whole cell lysate. The proteins were purified on a Ni^2+-chelating Sepharose column and refolded at 4 °C in gradient urea/TBS glycerol buffer. The biological activity of the purified rChFCN was measured using a phagocytosis assay, and
after the hemocytes were incubated with purified rChFCN or rTrx as a control, the rate of phagocytosis of the fluorescent beads was increased approximately 13%, 23.8% and 34.1% compared with the control, respectively (Fig. 5B).

To further investigate microbial binding activity in vitro, rChFCN was incubated with formaldehyde-killed *S. cerevisiae*, *S. haemolyticus*, *V. alginolyticus* and *E. coli* K-12, to which rChFCN displayed the strongest binding activity. After stringent washes, the proteins bound to the microbes were eluted and subjected to Western Blot analysis, and the results indicated that *S. cerevisiae*, *S. haemolyticus* and *E. coli* K-12, but not *V. alginolyticus*, could specifically bind rChFCN when present of the Ca$^{2+}$, while there was no band detected in the negative control lane or deficiency of the Ca$^{2+}$ (data is not show) (Fig. 6A). In the presence of Ca$^{2+}$, rChFCN was incubated with *E. coli* K-12 at a concentration 0.02 mg/ml, and significant agglutination was observed; however, no agglutination was observed in the control groups which were treated (or not) with the same dose of Ca$^{2+}$ or rTrx, suggesting that rChFCN has agglutination activity (Fig. 6B).

4. Discussion

Ficolins are responsible for complement activation through the lectin pathway [25]. The mammalian ficolins have been well studied, but similar invertebrate ficolins have only been identified in chordates, echinoderms and arthropods [14]. As no ficolins have been identified in other invertebrates, fibrinogen-related proteins have been suggested to function as ficolins for the recognition of invading microorganisms, acting as pattern recognition receptors in innate immunity [26]. Although the oyster genome project [27] has been recently completed, no ficolins have been detected in mollusks. Based on the results obtained in the present study, we identified a homologous ficolin protein from the oyster, *C. hongkongensis*.

The typical ficolin comprises several triple-helix structures containing collagen- and fibrinogen-like domains, and these proteins have been associated with MBL-associated serine proteases, thereby facilitating the activation of the complement system. The fibrinogen-like domain and Ca$^{2+}$ binding sites are also involved in carbohydrate binding [3]. In the present study, the full-length ChFCN cDNA was cloned from oyster *C. hongkongensis*. The 894 bp ORF of ChFCN cDNA encodes a 297 amino acid polypeptide. The protein structure of ChFCN is similar to that of other known ficolins, but this protein lacks a collagen-like domain. The four conserved cysteines present at the C-terminus of ChFCN were treated (or not) with the same dose of Ca$^{2+}$ or rTrx, suggesting that rChFCN has agglutination activity (Fig. 6B).

**Fig. 1.** Nucleotide/amino acid sequences and phylogenetic analysis of ChFCN: (A) the poly(A) signal “aataaa” is underlined, and the predicted positions of the Ca$^{2+}$ binding site “DND” are indicated with dots. The 20-amino acid signal peptide is indicated with a box, and the predicted FGB domain is shaded. (B) The domain organization of ChFCN. The number on the right indicates the nucleotide and amino acid sequences.
FBG domain in ChFCN was 64% homologous to that of *M. musculus* filcolin (GenBank# AAC98781). Moreover, the phylogenetic analysis showed that ChFCN was located at the bottom of the branch. These results suggest that ChFCN is a novel member of the FREP family, which might be the origin of the known filcols.

The bioinformatics analysis revealed that ChFCN, with a signal peptide at the N-terminus, is a secreted protein. Analysis of the subcellular localization of ChFCN using epifluorescence microscopy or western blotting revealed that the fusion protein deChFCN-GFP was detected in the cytoplasm when the signal peptide was
truncated, but full length of ChFCN-GFP was not detected in the cytoplasm through epifluorescence microscopy. We detected the secreted protein in the supernatant culture media using western blotting, and the results further indicated that ChFCN is a secreted protein.

We observed that ChFCN is ubiquitously expressed in adult tissues, suggesting an important role for ChFCN in Crassostrea hongkongensis immune defense. The gills represent the primary interface between the organism and the surrounding external environment, which is the first line of defense against bacterial infection [28]. The predominant expression of ChFCN mRNA in the gills suggests that ChFCN might be involved in the innate immune response. In mollusks, hemocytes also play an important role in the host defense against microorganism invasion. Several shellfish FREPs, but not collagen-like domains, have been shown to play important roles in innate immunity, such as those in A. irradians and Mytilus galloprovincialis, as these FREPs have been demonstrated to act as pattern recognition receptors in the immune response, contributing to innate immunity in invertebrates [14,20]. To explore the potential function of ChFCN in hemocytes of C. hongkongensis, oysters were challenged with V. alginolyticus, S. haemolyticus and S. cerevisiae. We subsequently observed acutely increased ChFCN expression in hemocytes following pathogen infection. V. alginolyticus and S. haemolyticus infection increased ChFCN expression, peaking at 6 hpi, and S. cerevisiae infection increased ChFCN expression, peaking at 12 hpi. Gram-negative and Gram-positive bacteria or fungi provoked significant increases in ChFCN expression (804-, 1038- and 344-fold increases, respectively). Similar expression patterns were observed in Mediterranean mussel FREPs [14], suggesting that ChFCN is involved in host immune responses.

In vertebrates and invertebrates, ficolins act as PRRs in the innate immune response, and these proteins play important roles in pathogen recognition [29]. There is considerable evidence indicating that ficolins are lectins that specifically bind N-acetylglycosamine (GlcNAc). Furthermore, the ficolin-like domain of ficolins is likely responsible for the carbohydrate binding lectin activity of these proteins [29]. This activation is required for calcium binding in both invertebrates and vertebrates. Several Ca$^{2+}$-binding sites in this domain are involved in carbohydrate binding [30]. In shellfish, such as mussels, the ficolin-like domains of FREPs contain conserved Ca$^{2+}$ binding sites that likely bind carbohydrates in a calcium-dependent manner [14]. In the bay scallop, the AfFREP agglutinated the Gram-positive bacterium Micrococcus luteus and Gram-negative bacteria Entamoeba coli JM109 and Listonella anguillarum in a calcium-dependent manner.

To characterize the molecular functions of ChFCN, the recombinant rChFCN was expressed, and this protein displayed increased binding to S. haemolyticus, S. cerevisiae and E. coli K-12, but not V. alginolyticus, in the presence of Ca$^{2+}$. The outer membrane of the S. cerevisiae is primarily comprised of protein polysaccharides, and biochemical analyses have indicated that these protein polysaccharides are mainly modified by glucose, N-acetylglycosamine (GlcNAc), and mannose [31]; similar structures have been identified in staphylococcus [32]. As all known ficolins recognize GlcNAc [33], ChFCN might recognize the GlcNAc modified–protein on the outer membranes of S. cerevisiae and S. haemolyticus. Although both E. coli K-12 and V. alginolyticus are Gram-negative bacteria with a complex mixture of carbohydrates, lipopolysaccharides, lipids and protein polysaccharides on the cell surface, the outer membrane protein composition of these bacteria are different. These differences might reflect the selective binding of ChFCN to E. coli K-12 but
not *V. alginolyticus*. The similar results were found in freshwater crayfish *Pacifastacus leniusculus*, the crayfish ficolin-like proteins were found as pathogen-associated molecular patterns (PAMPs)-binding molecules through 2-DE and MS analysis, the proteins exhibited agglutination activity of Gram-negative bacteria *E. coli* and *Aeromonas hydrophila* in the presence of Ca\(^{2+}\). Furthermore, these proteins could bind to *A. hydrophila*, *E. coli* as well as *Staphylococcus aureus* [34]. To confirm the interaction between ChFCN and *E. coli* K-12, an agglutination assay was performed in the presence of Ca\(^{2+}\), and the results indicated that ChFCN interacts with *E. coli* K-12.

Current studies have suggested that FREPs increase the efficiency of the immune system. However, exposure to FREPs did not positively affect the rate of hemocyte phagocytosis in mollusks [14]. When hemocytes were incubated with ChFCN, the phagocytic rate increased approximately 13%, as previously described in *M. galloprovincialis* [14]. These results indicated that the ChFCN acts as an opsonin in hemocyte phagocytosis.

In conclusion, we have described a ficolin-like protein, ChFCN, in oyster, likely associated with mammalian ficolins. ChFCN is homologous to known ficolins, containing a FGB domain and a potential Ca\(^{2+}\) binding site, but lacking a collagen domain. The ChFCN molecule shows primitive characteristics of more evolved ficolins. The expression of the ChFCN gene increased after bacterial infection. As the invertebrate immune system depends on PRRs (pattern recognition receptor) to recognize molecular patterns (PAMPs) present on pathogen surfaces, rChFCN selectively bound to several pathogenic bacteria and agglutinated *E. coli* K-12, and as an opsonin, rChFCN increased the phagocytic ability of hemocytes; these activities are similar to those of known ficolins. The evolution of the components and pathways of the complement system remain unclear. Thus, the results from studies concerning the ChFCN identified in oysters will provide useful clues for elucidating the evolution of the component pathway. However, further studies...
are needed to elucidate a role for ChFCN in the evolution of these components.

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Fig. 6. Binding and agglutination of bacteria. (A) Western blot analysis of rChFCN binding to pathogenic bacteria. rChFCN showed binding to S. cerevisiae, S. haemolyticus and E. coli K-12, but not V. alginolyticus. rTrx was incubated with each bacteria as a negative control. (B) Agglutination of bacteria through rChFCN. The agglutination of E. coli K-12 was observed after incubation with rChFCN in the presence of Ca\(^{2+}\). rTrx was incubated with the bacteria as a negative control.

References


