

Recombinant ferritin protein protects *Penaeus monodon* infected by pathogenic *Vibrio harveyi*

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ABSTRACT: Hemocytes of shrimp perform an essential role in defense against microbial pathogens, involving both cellular and humoral factors. The gene coding for ferritin in black tiger shrimp *Penaeus monodon* was cloned, sequenced and expressed using pQE-30-UA vector and SG13009 *Escherichia coli* host cells. The deduced amino acid sequence of *P. monodon* ferritin showed 32 to 95% similarity with ferritin proteins of other organisms. The recombinant protein was purified by nickel–nitrilotriacetic acid affinity chromatography. A single thick band of recombinant protein of approximately 21 kDa was observed in 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis. Following mild acid treatment, 2 bands of ca. 14 and 7 kDa were produced; aspartine and proline acid cleavage sites were found at amino acid residues 123–124. The purified recombinant ferritin helped in reducing the mortality in shrimp infected with *Vibrio harveyi*. However, no direct antimicrobial activity against pathogenic *V. harveyi* was observed.

KEY WORDS: Ferritin · Cloning and expression · *Penaeus monodon* · *Vibrio harveyi* · RT-PCR

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INTRODUCTION

Crustaceans such as penaeid shrimp are often confronted with infectious microorganisms. Black tiger shrimp *Penaeus monodon* is the most economically important penaeid shrimp cultured in many parts of the world. Commercial farming of *P. monodon* has been worst hit by viruses such as white spot syndrome virus and bacteria such as *Vibrio harveyi* and *V. alginolyticus* considered to be secondary and opportunistic pathogens. To fight such infectious agents, phagocytic activity of several haemocyte types in circulation and humoral factors are important (Roch 1999). Crustacean haemocytes play an important role in the host immune response. They have mechanisms to detect microbial components, such as β -glucans, lipopolysaccharides (LPS), which can directly activate defensive cellular functions such as phagocytosis, melanization, encapsulation and coagulation. Innate immunity is contributed by inflammatory processes, generation of radical oxygen interme-

diates, cytotoxic proteins and several families of antimicrobial peptides (Roch 1999).

Iron is an essential trace element in most living organisms and is regulated by specific transport and storage proteins, transferrin and ferritin, respectively (Park et al. 2005). Iron plays a central role in catalyzing reactive oxygen species production (superoxide radicals). Production of superoxide is an integral part of the crustacean immune system. Ferritin releases the stored iron molecules to a greater extent when blood iron is low, and to a lesser extent under high blood iron. A delicately balanced level of ferritin is required to maintain the iron level. Iron and oxygen chemistry under physiological conditions is corrosive, producing insoluble rust and soluble oxy radicals, and the expression of ferritin may differ according to environmental conditions such as pH stress (Zhou et al. 2008). Ferritin is widely distributed throughout the plant and animal kingdom in a highly conserved conformation. It helps in the storage of iron in a soluble, bioavailable and nontoxic form (Andrews et al. 1992). It also simultane-

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ously helps to arrange cellular defense against stress and inflammation (Torti & Torti, 2002). Ferritin stores excess iron and plays an important role in cellular homeostasis as the physiological source of iron for the cell (Durand et al. 2004). Ferritin is a multimeric protein consisting of a 24 subunit protein shell comprising 21 kDa heavy or 19 kDa light chains. Each fragment has a specific function and can bind about 4500 iron atoms within the central cavity (Theil 1987, Harrison & Arosio 1996). Ferritin molecules have been studied from microbial sources, including bacteria and fungi as well as higher plants and animals. Ferritin is responsible for resistance against oxidative stress in vertebrate systems and prevention of cell damage in both vertebrates and invertebrates (Orino et al. 2001). The increased level of ferritin also serves as a non-specific marker of inflammatory processes and neoplasms such as human breast cancer (Moroz et al. 1997) and renal cell carcinoma (Ozen et al. 1995). The protein plays a key role in the host defense response in animals and may be involved in the response against virus infection (Zhang et al. 2006). However, the potential role of ferritin gene in the defense of penaeid shrimp against bacterial infection remains unclear.

In the present study, ferritin gene from the tiger shrimp *Penaeus monodon* was cloned and expressed in *Escherichia coli*. The defense response of the recombinant *P. monodon* ferritin (rPmFer) protein was observed against pathogenic *Vibrio harveyi*. The nucleotide and deduced amino acid sequence of rPmFer was analyzed using bioinformatics based tools. Structural and functional studies of *P. monodon* ferritin will provide information on their role in defense response against *V. harveyi* in cultured shrimp.

MATERIALS AND METHODS

Shrimp. Healthy sub-adult *Penaeus monodon* weighing 15 to 20 g were used for the study. These individuals were collected from the southwest coast of India (Kundapur, Karnataka) and acclimatized in tanks of 500 l, containing seawater with 30 ppt salinity, under continuous aeration for 2 wk.

Bacterial culture and experimental infection. A virulent strain of *Vibrio harveyi*, isolated from moribund larvae, was grown in tryptone soy (TS) broth with 2% NaCl and used to stimulate the expression of immune-related genes, including ferritin. Overnight-grown bacterial cells were harvested by centrifugation (4000 *g* for 15 min) and washed twice with sterile phosphate saline buffer (PBS). Total viable count was done using different dilutions (10^4 to 10^7) on TS agar plates. The culture was inactivated by heating at 60°C for 1 h. An aliquot of 200 μ l bacterial suspension of 1.5×10^8

colony-forming units per milliliter (cfu ml⁻¹) was injected intramuscularly between the 3rd and 4th abdominal segments of the shrimp. Haemolymph was collected after 18 h with a syringe that had been rinsed and pre-loaded with 50 μ l of 2% EDTA solution. Haemocytes were separated by centrifuging at 5000 *g* for 10 min. Unchallenged healthy sub-adult *Penaeus monodon* were used as a control for the experiment.

Reverse transcriptase (RT)-PCR and cloning. Total RNA from haemocytes was extracted using TRIzol reagent (Invitrogen), and the concentration was determined at the optical density (OD)₂₆₀ using a UV-1601 spectrophotometer (Shimadzu). The RNA was reverse transcribed to cDNA from 2 μ g of RNA, using 2 μ l of oligo(dT) (100 ng μ l⁻¹) and 0.5 μ l of RevertAid H minus (MBI Fermentas) at 42°C for 1 h. A sequence of Pacific white shrimp *Litopenaeus vannamei* ferritin was retrieved from GenBank (Accession no. AY955373) and primers were designed from the flanking region of the gene. The sequences of primers were (5'–3') F-ATG GGG ATC CAA GTC CGC CAG and R-TTA GTG GAA TTC CTT ATC AAC. PCR was performed in a thermal cycler (MJ Research) in 30 μ l reaction volume containing 1.5 U *Taq* polymerase (Bangalore Genei), 3 μ l of 10 \times buffer (500 mM KCl, 100 mM Tris-HCl, pH 8.3, 20 mM MgCl₂), 50 to 100 ng cDNA, 200 μ M of each of the 4 dNTPs and 10 pmol of each primer. Thirty-five cycles were carried out with denaturation at 95°C for 1 min, annealing at 56°C for 1 min and extension at 72°C for 1 min. The cycling included an initial delay at 95°C for 5 min and a final delay at 72°C for 10 min. The PCR products were electrophoresed on 2% agarose gel. The product was purified using a PCR purification kit (Qiagen), ligated to commercial pQE30-UA linearized vector (Qiagen) at 16°C for 2 h, and finally transformed into chemically competent SG13009 *E. coli* by heat shock. The recombinant transformants were selected using ampicillin (100 μ g ml⁻¹) and kanamycin (25 μ g ml⁻¹) on Luria Bertani (LB) agar plates, and further confirmed by PCR using gene specific primers (conditions already described).

DNA sequencing and analysis. The cloned product was sequenced by M/s Genei Bangalore, India. The nucleotide and derived amino acid sequences were analyzed using the BLAST program of NCBI (www.ncbi.nlm.nih.gov). The program Sequence Quickie-Calc version 5.0 software was used for the prediction of molecular weight, isoelectric point (pI) and hydrophobic nature of the protein. The phylogenetic tree was constructed from CLUSTAL-generated alignment using the neighbor-joining method.

Expression and purification of ferritin. For testing the expression, overnight cultures of PCR-positive clones were inoculated into LB broth containing ampicillin (100 μ g ml⁻¹) and kanamycin (25 μ g ml⁻¹). Cul-

ture showing 0.6 to 0.7 OD₆₀₀ was induced with 1 mM of isopropyl β-D-1-thiogalactopyranoside (IPTG) for 4 h. Whole cell lysates of the bacteria were prepared and the expression of the recombinant protein was studied in 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970). Uninduced recombinant clone and *E. coli* SG13009 host cells were used as a control. The protein was purified by nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography (Qiagen). The cell pellet was obtained after IPTG induction of recombinants, which lysed by applying lysis buffer (6 M GuHCl; 0.1 M NaH₂PO₄; 0.01 M TrisCl; pH 8.0). Cell debris was separated and clear lysate was mixed with 50% Ni-NTA slurry. This mixture was passed through from the column (Qiagen) and then washed with wash buffer (8 M urea; 0.1 M NaH₂PO₄; 0.01 M TrisCl; pH 6.3 and 5.9). Finally the recombinant protein was eluted using elution buffer (8 M urea; 0.1 M NaH₂PO₄; 0.01 M TrisCl; pH 4.5). The purity of protein was examined by 15% SDS-PAGE and the concentration was estimated by Lowry's method (Lowry et al. 1951) using a UV-1601 spectrophotometer (Shimadzu).

Mild acid treatment. The cleavage activity of the recombinant ferritin protein was studied as described by Andrews et al. (1987) with minor modifications. The recombinant protein (2.5 mg ml⁻¹) was dissolved in 70% v/v formic acid and 5 mM-2-mercaptoethanol. The sample was flashed with nitrogen (N₂) vapour, sealed and incubated at 37°C for 60 h. The formic acid was evaporated under a stream of N₂. The digested protein was freeze-dried and analyzed on 15% SDS-PAGE.

Virulence assay. The virulence of *Vibrio harveyi* to *Penaeus monodon* was estimated and expressed as lethal dose (LD)₅₀ value (Reed & Muench 1938). An 18 h bacterial culture in brain heart infusion (BHI) broth was harvested by centrifuge and washed with sterile PBS. Ten-fold serial dilutions of the culture pellet were made in PBS; 100 μl of 4 consecutive dilutions (1.5 × 10⁴, 1.5 × 10⁵, 1.5 × 10⁶ and 1.5 × 10⁷) were injected intramuscularly to 10 shrimp each and mortality was observed up to 96 h.

Effect on lethality of *Vibrio harveyi* for *Penaeus monodon*. From the virulence study it was estimated that 1.5 × 10⁶ cfu ml⁻¹ *Vibrio harveyi* culture was lethal for *Penaeus monodon*. Three groups of 30 shrimp each were used for the experiment. Each group consisted of 3 replicates of 10 individuals each. In the experimental setup, 1 group was injected with purified rPmFer protein (1.5 μg g⁻¹ of shrimp), a second group with the mixture of rPmFer protein (1.5 μg g⁻¹ of shrimp) in 100 μl of virulent *V. harveyi* culture at 1.5 × 10⁶ cfu ml⁻¹ and a third group with 100 μl of *V. harveyi* culture (1.5 × 10⁶ cfu ml⁻¹) alone. The shrimp were observed

for mortality up to 96 h. Protection was measured as relative percentage survival (RPS, Amend 1981). Any significant difference in the mortality between different groups was determined by 1-way ANOVA (p < 0.05).

Study of antimicrobial activity against *Vibrio harveyi*. Direct antimicrobial activity of rPmFer against shrimp pathogenic *V. harveyi* was also studied by solid phase assay (Tyagi et al. 2007). For solid-phase assay, bacterial lawn was prepared on TS agar plates. After drying, 15 μg of purified rPmFer protein was added to a 3 mm well, punched at the centre. Plates were incubated overnight at 30°C. To study the effect of ferritin on viable count, 100 μl of overnight-grown *V. harveyi* was added to sufficient number of test tubes containing 4 ml of LB broth and allowed to grow until an OD₆₀₀ of 0.6. After this, 60 μl of recombinant ferritin (1 μg μl⁻¹ in PBS) was added to these cultures in triplicate, resulting in final concentrations of 15 μg ferritin ml⁻¹ of culture. The mixtures were incubated at 30°C and viable counts by surface spreading were determined at 0 min, 1 h and 2 h. *V. harveyi* cultures containing 60 μl of PBS were used as control.

RESULTS

Cloning and expression of shrimp ferritin

RNA from hemocytes of *Penaeus monodon* infected with *Vibrio harveyi* generated a 513 bp fragment gene coding for ferritin by RT-PCR. This purified product was cloned in pQE-30-UA expression vector. After antibiotic selection using ampicillin and kanamycin, positive clones were confirmed for the presence of insert by PCR. The recombinant protein was expressed after 4 h induction with 1 mM IPTG. The expressed recombinant protein tagged with 6_x-histidine had a slightly higher molecular weight of 21 kDa, as determined in 15% SDS-PAGE (Fig. 1), and a concentration of 7.5 mg/100 ml of culture.

Sequence analysis

BLAST analysis of deduced amino acid sequence of rPmFer showed presence of conserved eukaryotic ferritin domain. The predicted protein was acidic with a pI of 5.2 and an estimated molecular weight of 19337.6 Da. Phylogenic comparison of deduced amino acid sequence of *Penaeus monodon* ferritin (Fig. 2) with that of light and heavy chains of ferritin of other organisms revealed conserved amino acids among the shrimp ferritin. Multiple protein sequence analysis showed its closeness to ferritin of *Litopenaeus van-*

namei (95%) and *Fenneropenaeus chinensis* (94%). Distance relatedness was observed to *Pacifastacus leniusculus* ferritin (69%), *Artemia franciscana* ferritin (58%), *Mus musculus* ferritin (heavy chain) (58%), *Mus musculus* ferritin (light chain) (58%), *Danio rerio* ferritin (58%), and an A-chain of recombinant *Homo sapiens* H ferritin (58%).

Penaeus monodon ferritin showed least similarity with ferrin 1 subunit of *Drosophila melanogaster* (32%,

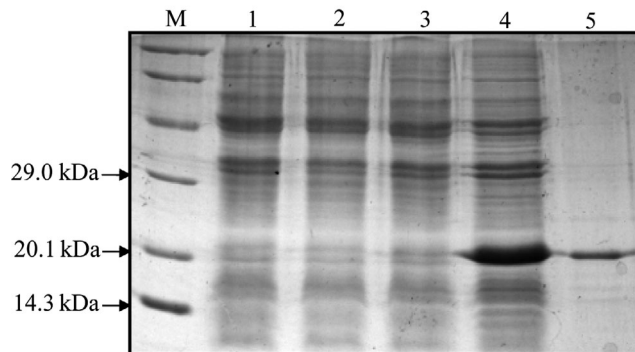


Fig. 1. M: PMW-M Protein Marker (GeNei™). Lane 1: SG *E. coli* cells without isopropyl β -D-1-thiogalactopyranoside (IPTG) induction; Lane 2: SG *E. coli* cells with final 1 mM IPTG induction; Lane 3: ferritin clone without IPTG induction; Lane 4: ferritin clone with final 1 mM IPTG induction; Lane 5: purified ferritin protein

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1  atggggatccaagtccgccgaactaccatgaggattgtgaagcc
   M G I Q V R Q N Y H E D C E A
46  tccattaacaagcagatcaacatggaactttacgccagctacggt
   S I N K Q I N M E L Y A S Y V
91  tacctttcgatggctcactacttcgaacgtgacgatgtagctctg
   Y L S M A H Y F E R D D V A L
136 cctggttttgccaagttcttcaaggaatcgagcgatgaggaaagg
   P G F A K F F K E S S D E E R
181 gaacatgccagattttcatgaagtaccagaacaagcgaggtggc
   E H A Q I F M K Y Q N K R G G
226 cgcacgtttctccagcagattgcagctccttccatgcaagaatgg
   R I V L Q Q I A A P S M Q E W
271 ggcactggtctggaagcccttcaggctgctcttgatctggagaag
   G T G L E A L Q A A L D L E K
316 caggtcaatcagtcctcctcctggaactccatggcactgctagtggc
   Q V N Q S L L E L H G T A S G
361 aacaatgatccatctcaccgaagcttcttgaagatgagtatctg
   N N D P H L T K L L E D E Y L
406 gaggaacaggtggattccatcaagaagattggtgacatgattacc
   E E Q V D S I K K I G D M I T
451 aagctgaagcgtgctggcccagctggtcttggagagtacttgttt
   K L K R A G P A G L G E Y L F
496 gatatgaattccacttaa 513
   D M N S T *

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Fig. 2. Nucleotide and predicted amino acid sequence of the *Penaeus monodon* ferritin. Bold font and asterisk (*) represents initiation and stop codon, respectively. Box shows aspartine (D) and proline (P) acid cleavage sites. Arrows indicates the primer binding portion, forward (→) and reverse primer (←)

Fig. 3). Analysis of the phylogenetic tree showed that *P. monodon* ferritin formed a separate cluster with other shrimp ferritin proteins (Fig. 4). In deduced amino acid sequence of *P. monodon* ferritin, residues involved in formation of ferroxidase centre and ferrihydrite nucleation centre were identified based on comparison with conserved eukaryotic ferritin domain (Euk_Ferritin, GenBank Accession no. CD01056) and ferritin of *Litopenaeus vannamei* and *Fenneropenaeus chinensis*. Four residues (Glu54, Asp57, Glu58, Glu61) were involved in formation of the ferrihydrite nucleation centre, while the ferroxidase centre consisted of 7 amino acid residues (Glu24, Tyr31, Glu58, Glu59, His62, Glu104, Gln138). The acid cleavage site of 2 amino acid residues, aspartine and proline (DP), was found to be at the 123–124 amino acid residue positions.

Biological activity of recombinant ferritin protein

With mild acid treatment, the rPmFer protein generated 2 bands of approximately 14 and 7 kDa in a 15% SDS-PAGE, whereas untreated ferritin gave a 21 kDa band (data not shown).

Protection study

Results of the experimental study of the protective nature of rPmFer are summarized in Fig. 5. Shrimp were protected when injected with the mixture of pathogenic *Vibrio harveyi* culture and the rPmFer protein, whereas >90% mortality was observed when injected with *V. harveyi* alone. There was a statistically significant difference in survival between the *V. harveyi* control and *V. harveyi* with ferritin (ANOVA, $p < 0.05$). However, the rPmFer protein did not show any direct antimicrobial activity against *V. harveyi* by solid phase assays and there was no significant difference in viable *V. harveyi* counts between the control and ferritin containing cultures after 0 min, 1 h and 2 h incubation (Mann-Whitney U -test, $p < 0.05$, data not shown).

DISCUSSION

Ferritin is a member of the diiron-carboxylate protein superfamily and functions as an iron binding protein in a wide variety

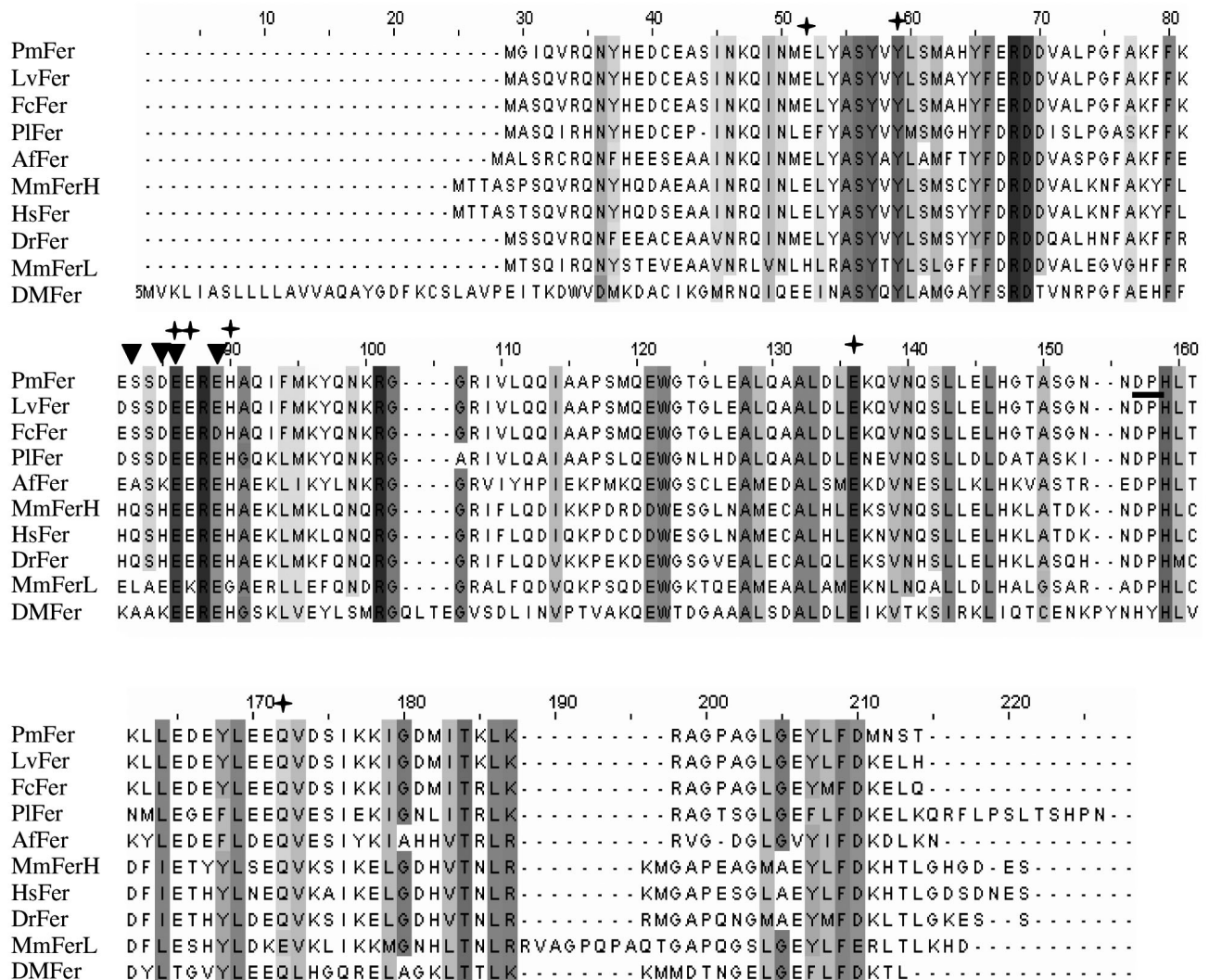


Fig. 3. Comparison of the amino acid sequences of ferritin. Similar intensity of grey shading shows the conserved amino acids. Symbols (+) and (▼) indicate the residues involved in formation of the ferroxidase center and ferrihydrite nucleation center, respectively. PmFer (*Penaeus monodon*, EF523241); LvFer (*Litopenaeus vannamei*, AAX55641); FcFer (*Fenneropenaeus chinensis*, ABB05537); PIFer (*Pacifastacus leniusculus*, CAA62186); AfFer (*Artemia franciscana*, AY062897); MmFerH (*Mus musculus* heavy chain, P09528); HsFer (*Homo sapiens* H ferritin A-chain, 2CE1A), DrFer (*Denio rerio*, NP571660); MmFerL (*Mus musculus* light chain, AAH83350); DmFer (*Drosophila melanogaster*, AAB70121). Underlined aspartine and proline (DP) residues for acid cleavage (Position 123–124)

of organisms (Andrews et al. 1992). In vertebrates, ferritin is a multimeric protein made up of 24 subunits of ~20 kDa consisting of heavy (H) and light (L) chains in various ratios (Harrison & Arosio 1996). Ferritin subunits H and L differ in rates of iron uptake and mineralization and have been characterized in some organisms. Fe (II) oxidation in the H subunits takes place initially at the ferroxidase center, which is a carboxylate-bridge diiron center located within the subunit four-helix bundle. H-ferritin may suppress proliferation of T cells (Matzner et al. 1979, 1985, Rosen et al. 1998), E-rosette formation (Wigginton 1995) and colony formation by human macrophages (Broxmeyer

et al. 1986). In a complementary role, negatively charged residues on the inner surface of the L-subunit protein shell promote ferrihydrite nucleation. Although numerous studies on ferritin have been performed in vertebrates, mammals in particular, very little is known about the sequence and distribution of the transcripts in decapod crustaceans. Results from real-time quantitative RT-PCR (Hsieh et al. 2006) suggest that the ferritin expression is approximately 3-fold more in haemocytes than the hepatopancreas. In the present study, the black tiger shrimp *Penaeus monodon* was challenged with inactivated bacteria to induce the non-specific immune response and haemo-

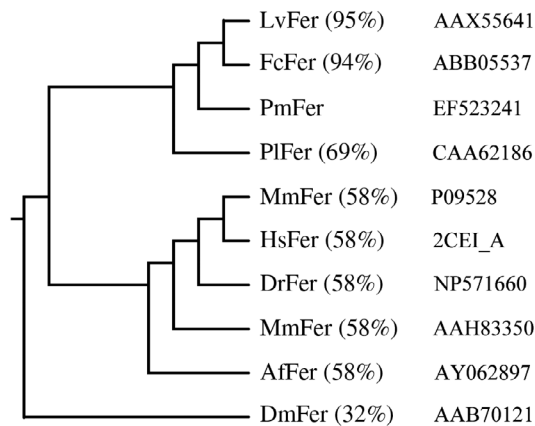


Fig. 4. Phylogenetic comparison of ferritin. A phylogenetic tree was constructed as described in experimental procedures from the deduced amino-acid sequences of ferritin from several other organisms. The various accession numbers used are indicated in the tree. Percentage similarity of *Penaeus monodon* ferritin obtained in this study (PmFer) with other ferritin proteins has also been indicated. PmFer (*Penaeus monodon*, EF523241); LvFer (*Litopenaeus vannamei*, AAX55641); FcFer (*Fenneropenaeus chinensis*, ABB05537); PIFer (*Pacifastacus leniusculus*, CAA62186); AfFer (*Artemia franciscana*, AY062897); MmFerH (*Mus musculus* heavy chain, P09528); HsFer (*Homo sapiens* H ferritin A-chain, 2CEI_A), DrFer (*Danio rerio*, NP571660); MmFerL (*Mus musculus* light chain, AAH83350); DmFer (*Drosophila melanogaster*, AAB70121)

cytes were collected for the RNA extraction. In the previous report on cDNA sequence of *Fenneropenaeus chinensis* (Zhang et al. 2006) and *Litopenaeus vannamei* (Hsieh et al. 2006), ferritin was cloned and sequenced. In *L. vannamei*, the preliminary full length cDNA sequence of 1249 bp was obtained, including 132 bp in the 5' untranslated region, 510 bp which encodes 170 amino acid residues and 607 bp in the 3' untranslated region (Hsieh et al. 2006).

In the present study, RT-PCR gave an amplicon of 513 bp of coding region of ferritin from *Penaeus monodon*. The PCR amplified fragment was cloned in pQE-30-UA vector and sequenced. The sequence has been deposited in GenBank (Accession no. EF523241). This protein was expressed in SG13009 *E. coli* with 1 mM IPTG induction. Purification was achieved by the guanidium hydrochloride denaturation method. Using the pQE-30-UA expression vector, the recombinant protein was obtained with N-terminal 6 \times -histidine fusion proteins, with high yields through single-step purification. The expressed protein obtained was estimated to be approximately 21 kDa by 15% SDS-PAGE; it has a slightly higher molecular weight than the native ferritin protein (~19 kDa) due to the presence of 6 \times -histidine residues including some portions of the vector. The nature of the rPmFer protein was determined by treatment with mild acid. The sequence data

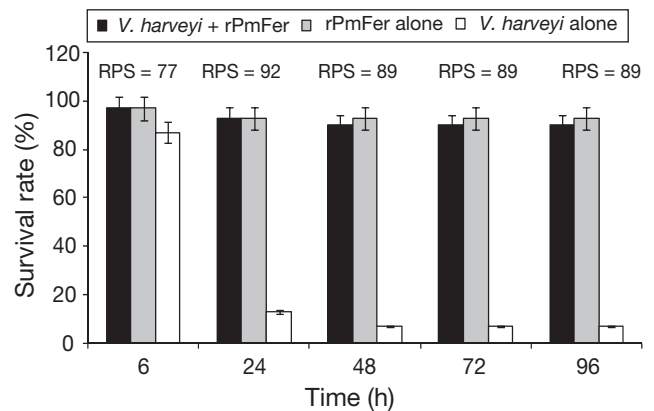


Fig. 5. *Penaeus monodon*. Error bar diagram (with 5% value) for relative percentage survival rate (RPS) of shrimp after injection with a mixture of rPmFer protein with *Vibrio harveyi*, purified rPmFer protein, or *V. harveyi* alone. Three groups of 30 shrimp (i.e. n = 30) each were used. Significant difference in RPS obtained for different groups ($p < 0.05$). RPS = $[1 - (\text{mortality of shrimp challenged with } V. \text{harveyi with rPmFer} / \text{mortality of } V. \text{harveyi alone})] \times 100\%$

obtained in the present study (Fig. 2) showed the presence of an Asp-Pro bond at amino acid residues (123–124) at the expected acid cleavage site (Piszkievicz et al. 1970) which split the protein into 2 fragments of approximately 14 and 7 kDa. Two similar fragments were obtained when rat liver ferritin was treated with acid (Leibold et al. 1984). The *P. monodon* ferritin amino acid sequence deduced in the present study showed high similarity to the deduced ferritin amino acid sequences of other animals: 95% to *L. vannamei*, 94% to *Fenneropenaeus chinensis*, 69% to *P. leniusculus*, 58% to *Artemia franciscana*, 58% to both light and heavy chains of *Mus musculus*, 58% to *Danio rerio*, and 58% to the A-chain of human H ferritin. However, *P. monodon* ferritin showed little similarity (32%) with *Drosophila melanogaster* ferritin (Fig. 3). During phylogenetic analysis, *P. monodon* ferritin formed a separate cluster with other shrimp ferritin proteins (Fig. 4) and showed a closer relationship with vertebrate ferritin than *D. melanogaster* ferritin. This indicates that *Drosophila* ferritin is unique to this organism and has evolved separately from other crustacean ferritins. The deduced amino acid sequence of *P. monodon* ferritin also had residues involved in the formation of the ferroxidase centre and ferrihydrite nucleation centre. Iron storage by a ferritin protein involves the uptake of Fe (II) at the protein shell, its oxidation by the molecular oxygen at dinuclear ferroxidase centre and movement of Fe (III) into the cavity for deposition as ferrihydrite. Thus, the presence of these ferroxidase and ferrihydrite nucleation centers in *P. monodon* ferritin indicates its oxidase and iron storage functions and possible role in iron detoxification and intracellular iron transport.

The availability of iron has been shown to be a critical factor in the pathogenicity of microorganisms invading living hosts (Bullen 1981). Haemin, haemoglobin and all other iron sources found in homeothermic and/or poikilothermic hosts increase the lethal potential of pathogenic organisms such as *Vibrio anguillarum* (Nakai et al. 1987), *V. damsela* (Fouz et al. 1994) and *V. vulnificus* (Stelma et al. 1992, Magarinos et al. 1994). In the present study, when shrimps were injected with virulent *V. harveyi* and ferritin, there was a high level of protection offered, possibly due to a reduction in the lethality of the organism (Fig. 5). There was no mortality observed when ferritin alone was injected to shrimp. *V. harveyi* alone resulted in >90% mortality. However, recombinant ferritin did not show any direct antimicrobial activity against *V. harveyi*, and its addition did not reduce viable counts of *V. harveyi*. These results suggest that the PmFer protein may be involved in the defense response against *V. harveyi*, possibly by restricting the availability of iron, which is required for the organism to survive and multiply inside the host body.

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LITERATURE CITED

- Amend DF (1981) Potency testing of fish vaccines. *Dev Biol Stand* 49:447–454
- Andrews SC, Treffry A, Harrison PM (1987) A new form of ferritin heterogeneity explained: isolation and identification of a nineteen-amino-acid-residue fragment from sideosomal ferritin of rat liver. *Biochem J* 245:447–453
- Andrews SC, Arosio P, Bottke W, Briat JF and others (1992) Structure, function, and evolution of ferritins. *J Inorg Biochem* 47:161–174
- Broxmeyer HE, Lu L, Bicknell DC, Williams DE and others (1986) The influence of purified recombinant human heavy-subunit and light-subunit ferritins on colony formation *in vitro* by granulocyte-macrophage and erythroid progenitor cells. *Blood* 68:1257–1263
- Bullen JJ (1981) The significance of iron in infection. *Rev Infect Dis* 3:1127–1138
- Durand JP, Goudard F, Pieri J, Escoubas JM, Schreiber N, Cadoret JP (2004) *Crassostrea gigas* ferritin: cDNA sequence analysis for two heavy chain type subunits and protein purification. *Gene* 338:187–195
- Fouz B, Toranzo AE, Biosca EG, Mazoy R, Amaro C (1994) Role of iron in the pathogenicity of *Vibrio damsela* for fish and mammals. *FEMS Microbiol Lett* 121:181–188
- Harrison PM, Arosio P (1996) The ferritin: molecular properties, iron storage function and cellular regulation. *Biochim Biophys Acta* 1275:161–203
- Hsieh SL, Chiu YC, Kuo CM (2006) Molecular cloning and tissue distribution of ferritin in Pacific white shrimp (*Litopenaeus vannamei*). *Fish Shellfish Immunol* 21:279–283
- Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680–685
- Leibold EA, Aziz N, Brown AJ, Munro HN (1984) Conservation in rat liver of light and heavy subunit sequences of mammalian ferritin. Presence of unique octopeptide in the light subunit. *J Biol Chem* 259:4327–4334
- Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the folin phenol reagent. *J Biol Chem* 193:265–275
- Magarinos B, Romalde JL, Lemos ML, Barja JL, Toranzo AE (1994) Iron uptake by *Pasteurella piscicida* and its role in pathogenicity for fish. *Appl Environ Microbiol* 60:2990–2998
- Matzner Y, Hershko C, Polliack A, Konjin AM, Izak G (1979) Suppressive effect of ferritin on *in vitro* lymphocyte function. *Br J Haematol* 42:345–353
- Matzner Y, Konjin AM, Shlomai Z, Ben-Bassat H (1985) Differential effect of isolated placental iso-ferritins on *in vitro* T-lymphocyte function. *Br J Haematol* 59:443–448
- Moroz C, Chetrit A, Kahn M, Modan B (1997) FBL blood test as a predictive marker of breast cancer in high risk women. *Med Oncol* 14:39–42
- Nakai TT, Kanno ER, Cruz Muroga K (1987) The effects of iron compounds on the virulence of *Vibrio anguillarum* in Japanese eels and ayu. *Fish Pathol* 22:185–189
- Orino K, Lehman L, Tsuji Y, Ayski H, Torti SV, Torti FM (2001) Ferritin and the response to oxidative stress. *Biochem J* 357:241–247
- Ozen H, Uygur C, Sahin A, Tekgul S, Ergen A, Remzi D (1995) Clinical significance of serum ferritin in patients with renal cell carcinoma. *Urology* 46:494–498
- Park RY, Sun HY, Choi MH, Bai YH, Shin SH (2005) *Staphylococcus aureus* siderophore-mediated iron-acquisition system plays a dominant and essential role in the utilization of transferrin-bound iron. *J Microbiol* 43:183–190
- Piszkiwicz D, Landon M, Smith EL (1970) Anomalous cleavage of aspartyl-proline peptide bonds during amino acid sequence determinations. *Biochem Biophys Res Commun* 40:1173–1178
- Reed LJ, Muench H (1938) A simple method of estimating fifty percent end points. *Am J Epidemiol* 27:493–497
- Roch P (1999) Defense mechanisms and disease prevention in farmed marine invertebrates. *Aquaculture* 172:125–145
- Rosen HR, Ausch C, Reinerova M, Zaspin E and others (1998) Activated lymphocytes from breast cancer patients express the characteristic of type 2 helper cells – a possible role for breast cancer-associated p43. *Cancer Lett* 127: 129–134
- Stelma GN, Reyes AL, Peeler JT, Johnson CH, Spaulding PL (1992) Virulence characteristics of clinical and environmental isolates of *Vibrio vulnificus*. *Appl Environ Microbiol* 58:2776–2782
- Theil EC (1987) Ferritin: structure, gene, regulation and cellular function in animals, plant and microorganisms. *Annu Rev Biochem* 56:289–315
- Torti FM, Torti SV (2002) Regulation of ferritin genes and proteins. *Blood* 99:3505–3516
- Tyagi A, Khushiramani R, Karunasagar I, Karunasagar I (2007) Antiviral activity of recombinant lysozyme expressed from black tiger shrimp, *Penaeus monodon*. *Aquaculture* 272:246–253
- Wigginton JM (1995) Reversal of ferritin-mediated immunosuppression by levamisole: a rationale for its application to management of the acquired immune deficiency syndrome (AIDS). *Med Hypotheses* 44:85–88
- Zhang J, Li F, Wang Z, Zhang X, Zhou Q, Xiang J (2006) Cloning, expression and identification of ferritin from Chinese shrimp, *Fenneropenaeus chinensis*. *J Biotechnol* 125:173–184
- Zhou J, Wang WN, Ma GZ, Wang AL and others (2008) Gene expression of ferritin in tissue of the Pacific white shrimp, *Litopenaeus vannamei* after exposure to pH stress. *Aquaculture* 275:356–360