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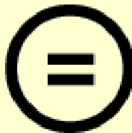

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A Thesis
FOR THE DEGREE OF MASTER OF SCIENCE

Ferritin subunit cDNA cloning, molecular characterization and expression analysis from manila clam *Ruditapes philippinarum*

Hyowon Kim

Department of Marine Life Science

GRADUATE SCHOOL

JEJU NATIONAL UNIVERSITY

2011. 12

Ferritin subunit cDNA cloning, molecular characterization and expression analysis from manila clam *Ruditapes philippinarum*

Hyowon Kim
(Supervised by professor Jehee Lee)

A thesis submitted in partial fulfillment of the requirement for the degree of Master of Science

2011 . 12 .

This thesis has been examined and approved.

Thesis director, Moon-soo Heo, Prof. of Marine Life Science

Jun-bum Jeong, Prof. of Marine Life Science

Jehee Lee, Prof. of Marin Life Science

Date

Department of Marin Life Scinece

GRADUATE SCHOOL

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요 약 문

모든 생명체에 있어 철 이온은 매우 중요한 미네랄 중 하나로 산소 운반, 전자 이동, DNA 합성, 면역 및 여러 효소 반응 등 생체 내 많은 반응에 관여한다.

그러나 이러한 철 이온이 체내에 과도하게 축적이 되면 Fenton 반응에 의해 활성산소종 (Reactive Oxygen species)을 생성하여 지질과 단백질 산화 및 DNA 파괴 등으로 이어진다. 따라서 생체는 이러한 부작용을 막기 위해 철 이온 농도를 조절하여 항상성 (homeostasis) 을 유지하고자 하는 시스템이 필요하다.

페리틴은 철 이온의 항상성을 유지하는 시스템 중 중요한 단백질 종류로, 포유류, 세균, 식물 등 넓은 범위의 생물에게서 발견할 수 있다. 페리틴은 체내의 남는 철 이온을 독성 형태 (Fe^{2+})에서 비(非)독성 형태 (Fe^{3+})로 바꾸어 장기간 저장하거나 철분이 부족 시 방출하는 역할을 한다.

페리틴은 약 4500개의 무기 형태의 철 이온을 저장 할 수 있는 공간을 중심으로 24개의 단량체들이 둘러싸인 형태를 지닌 단백질이다. 단량체는 분자량과 주로 분포된 조직에 따라 H subunit 과 L subunit으로 나뉠 수 있다. H subunit은 L subunit 보다 조금 더 무거운(Heavy) 분자량을 지녔고 심장(Heart)에서 비율이 높다. 반면, L subunit은 상대적으로 가벼운(Light) 분자량에 간(Liver) 조직에서 비율이 높다. 각각의 단량체는 특성과 기능이 다른데, H subunit은 ferroxidase라는 불리는 7개의 아미노산잔기가 있어 철 이온을 붙잡아 산화시킨다. L subunit은 산화된 철을 페리틴 핵 내부로 저장시키는 역할을 한다.

이번 연구에서는 바지락 *Ruditapes philippinarum* 에서 발현된 페리턴 subunit의 cDNA의 전체 염기서열과 아미노산 서열을 밝혀서 분자 유전학적으로 분석하고 mRNA 발현 및 단백질 발현 실험을 수행하였다. 그 결과, 이 실험에서 연구한 바지락 페리틴 subunit은 연체동물의 H subunit에 속하는 유전자임을 밝혔다. 바지락 H subunit의 mRNA발현량은 각 조직별로 다른 발현 분포도를 보였는데 아가미와 혈구 조직에서 높은 발현을 보였다. 또한 아가미 조직에서 세균 감염 시 시간 별로 발현되는 양상을 조사한 결과, 비교적 짧은 시간 내 유의적으로 발현됨을 밝혔다. 그리고 바지락의 H subunit의 재조합 단백질은 iron-chelating 실험을 통해 단백질 농도에 따라 철 이온의 결합능이 비례함을 보였다.

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Abstact

In all living organism, iron is essential element for wide range of physiologic processes, but excess free iron reacts with oxygen, it can produce reactive oxygen species (ROS) by Fenton reaction which have toxicity to DNA, protein and lipids. Thus living organisms have tight iron regulating system to protect those oxidative injury. Ferritin is a major iron regulating protein and one of a highly conserved conformation in a wide range of organisms.

Ferritin serves detoxification of the toxic form iron (Fe^{2+}) to safe form (Fe^{3+}) and store 4500 detoxified iron atoms in shell which is composed 24 subunits. When the biological is placed under iron scarcity conditions, the stored iron ion can be used.

There are two types of ferritin subunit distinguished by molecular weight and ratio in different cell type. One is H subunit, H means Heavy MW and most found in Heart while L subunit means Light MW and Liver.

In this study, ferritin subunit gene from manila clam *Ruditapes philippinarum* cDNA library was identified as a ferritin H subunit (RpFer). And molecular characterization and gene analysis results showed that also RpFer is belong to mollusk ferritin H subunit.

And mRNA expression experiment in different tissues was performed. Gill and hemocytes tissues were observed significantly higher relative mRNA expression level than in any other manila clam tissues. After bacterial challenge, RpFer was researched in gill tissues and the results was showed that the RpFer was up-regulated and significantly increased until 12 hr and rapidly decreased at 24 hr level after infection compare to unchallenged group using a negative control.

Recombinant protein was expressed and purified for ferrous-ion chelating assay. The recombinant clam ferritin H subunit protein was able to bind iron, and the iron concentration was decrease as more protein.

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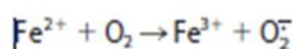
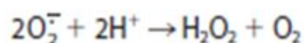
I. INTRODUCTION

In all living organism, iron is essential element for wide range of physiologic processes such as oxygen transport, acid cycle, electron transfer, DNA biosynthesis, immune and many enzymatic reactions. But excess free iron reacts with oxygen, it can produce reactive oxygen species (ROS) by Fenton reaction which have toxicity to DNA, protein and lipids [D. Lucana, M. Roscher, et al., 2010].



Reaction 1



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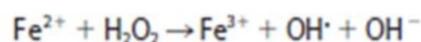

Reaction 3

Thus living organisms have tight iron regulating system to protect those oxidative injuries. Ferritin is a major iron regulating protein and one of a highly conserved conformation in a wide range of organisms, such as fungi, bacteria, plants, vertebrates and invertebrates [Harrison and Arosio, 1996]. This protein was isolated from horse spleen in 1937 by Laufberger and discovered that contained up to 23% by dry weight of iron.

Ferritin serves detoxification of the toxic form iron (Fe^{2+}) to safe쇼 form (Fe^{3+}) and store 4500 detoxified iron atoms in shell which is composed 24 subunits, called *apoferritin*. When the biological is placed under iron scarcity conditions, the stored iron ion can be used [W. Wang, M.A. Knovich et al, 2010].

There are evidences that ferritin located in cell nuclei can protect DNA from oxidative stress by binding DNA and oxidation from oxidative stress

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inducediron-H_2O_2 in *in vitro* experiment [K.J. Thompson, Michael G et al, 2002; N. Surguladze, Khristy M., et al., 2004].

Most ferritin subunits can be found at two different types termed H and L encoded by distinct genes. The name of H and L subunit is derived from their molecular weight and diverse ratio in different cell types [M.C. Sammarco, S. Ditch, et al,. 2008]. The H subunit is heavier chains than L subunits. Normally, in mammal, molecular weight of H subunit is 21 kDa, the L subunit 19 kDa. The function was different between H and L subunit. There is a site in H subunits which promotes iron binding and oxidization, called ferroxidase diiron center which has conserved iron metal ligands site. The ligands transform toxic Fe^{2+} iron into safety iron form, Fe^{3+} [S. Levi, B. Corsi, et al., 1994]. The L subunit even has lack of ferroxidase activity, several negative charged residues on inner surface of the ferritin shell can convert iron by ferrihydrited form and nucleate.

H subunits are most found in Heart while L subunits are most found in Liver [W. Wang, Mary A. Knovich et al, 2010]. Recently, new type ferritin subunit is reported named M subunit in lower vertebrates [X. Zhang, W. Wei et al., 2009] that subunit displays both features of H and L subunit.

Ferritin gene expression is regulated in transcription and translation levels by many different simulators including iron condition [G. Cairo, L. Bardella, et al., 1985; C. Jin, C. Li, et al., 2011], cooper [J. Zhang, F. Li et al., 2006], cytokines [S.V. Torti, E.L. Kwak, et al., 1988], hormones [L.G. Colucci-D'Amato, M.V. Ursini, et al., 1989], cAMP [M.A. Bevilacqua, M.C. Faniello, et al., 1994], pH stress [J. Zhou, W. Wang, et al., 2008], and oxidative stress [Y. Tsuji, H. Ayaki, et al., 2002].

In this study, we researched into isolation and characterization and expression level of manila clam *Ruditapes philippinarum*. Ferritin of some

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clam species were characterized in molecular level under iron exposure condition, thermal stress and different larval stages [X. Wang et al, 2009]. In this study, the manila clam is stimulated by clam disease bacteria, *Vibrio tapetis* for researching the mRNA expression level of *Ruditapes philippinarum* ferritin subunit by quantitative real-time PCR. And iron binding activity is tested to identify whether *Ruditapes philippinarum* ferritin subunit (RpFer) can bind iron.

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II. MATERIALS AND METHODS

2.1 Experimental animals

Manila clams (*Ruditapes philippinarum*) with average body length 3.5 ± 0.2 cm were captured from tidal land of Sung-san, Korea. The manila clam was acclimatized to laboratory conditions in 80 L flat bottom tanks at 21 ± 1 °C with recirculating seawater (salinity 34 ± 1 ‰) for one week before experiments.

For the research of immune responsive expression of RpFer, animals were exposed to *Vibrio tapetis* (clam diseases bacteria, gram-negative; KCTC no. 12728). Bacteria were diluted in phosphate buffer saline (PBS). Clams were injected intramuscularly with 100 µL of *V. tapetis* (3.2 X 10^7 cfu individual) into adduct muscle. PBS was also injected with same volume (100 µL) as a positive control. Healthy clam group was considered as a negative control.

2.2 Tissue sampling, RNA isolation, cDNA synthesis and normalization

Gill, adductor muscle, mantle, foot, siphon and hemocytes from manila clam were collected to determine mRNA expression level. Hemolymph (1~2ml each clam) was collected by 22 G syringes and centrifuged at 3000 g at 4 ℃ for 10 min to isolate hemocytes. All of tissues was immediately snap-frozen in liquid nitrogen and stored at -70 ℃ until use.

The total RNA was isolated from clam tissues using Tri Reagent™ (Qiagen), following the manufacturer's protocol. After that, mRNA was isolated by using FastTrack® 2.0 (Invitrogen, USA). The concentration of purified mRNA was determined at 260 nm in a UV-spectrometer (BioRad, USA) and diluted to 1 µg/µL for cDNA synthesis as a first strand. cDNA was synthesized by using Creator SMART cDNA library construction kit (Clontech, USA) and amplified with the 50 X Advantage 2 PCR kit

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(Clontech). Then, the resultant cDNA library was normalized by using the Trimmer-Direct cDNA normalization kit (Evrogen, Russia).

2.3 cDNA data base of clam and identification of RpFer

The Sequencing of cDNA library was performed by pyrosequencing technology on a Roche 454 Genome Sequencer FLX Titanium instrument (DNA linker, Korea). Subsequently, the Arachne whole-genome shotgun assembler program performed the processing and assembling for output data reads [S. Batzoglou, D.B. Jaffe et al., 2008]. The manila clam ferritin subunit cDNA sequence was identified by Basic Local Alignment Search Tool (BLAST) algorithm [S.F. Altchul, W. Gish, et al., 1990]

2.4 Cloning of clam ferritin RpFer into the pMal™-c2X

A set of primer (RpFer-Forward, RpFer-Reverse) (Table. 1) was designed to amplify the 513 bp coding open reading frame (ORF) of clam ferritin gene and also designed with *Pst* I and *Eco*R I restriction sites at the N-terminus and C-terminus to clone into the expression vector, pMal™-c2X (NEB, USA). Amplification of clam ferritin was conducted in a total of 50 μL of reaction volume using 2 μL of DNA template, 5 μL of 10X *Ex taq*™ buffer, 4 μL of 25 mM dNTP mix, 2 μL of both 20 pmol/μL primer and 0.25 μL (0.5 U/μL) of *Ex taq*™ polymerase enzyme. The PCR was carried out by a Takara thermos cycle with a denaturing step at 95 min for 5 min, followed by 30 cycles of 94 ℃ for 30 sec, 57 ℃ for 30 sec, 72 ℃ for 45 sec and then final extension followed by 72 ℃ for 5 min. To confirm amplification of the product after PCR, the PCR product was visualized on a 1 % agarose gel with a marker. Then, the PCR products were digested with *Pst* I and *Eco*R I, after purification of the PCR product using an Accuprep™ PCR purification kit (Bioneer Co., Korea). The digested PCR products were gel-purified using 1 % agarose gel and

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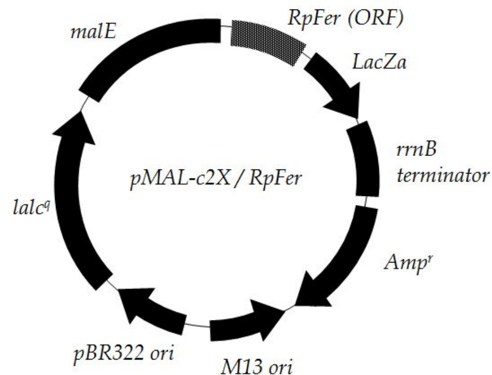
an Accuprep™ gel purification kit (Bioneer Co., Korea). The expression vector pMAL-c2X was digested with same restriction enzyme, *Pst* I and *Eco*R I. The purified PCR product of RpFer was ligated into the vector by incubating at 4 ℃ overnight. The recombinant plasmids were transformed into competent cells. Subsequently, the plasmids were extracted by using an Accuprep™ plasmid extraction kit (Bioneer Co., Korea).



2.5 Analysis of cDNA and putative amino acid sequence

The open reading frame (ORF) of RpFer sequence and deduced amino acid sequence were derived using DNAassist (ver 2.2) and analyzed using the BLAST program (http://www.ncbi.nlm.nih.gov/blast). Searching for homologies, similarities with known ferritin genes from the NCBI database was used. Multiple alignments was performed on Clustal W software. On the basis of alignment, phylogenetic tree was analyzed by using MEGA 4.0 software based on Neighbor-joining (NJ) with 1000 bootstrap replicates. The identity and similarity percentage was calculated by EMBOSS pairwise alignment algorithms (http://www.ebi.ac.uk/Tools/emboss/align/). The molecular weight (MW) and isoelectric point (p*I*) was predicted by Protparam system (http://expasy.org/tools/protparam.html). To identify the signal peptide, SignalP program (http://www.cbs.dtu.dk/services/SignalP/) was performed. The PROSITE program (http://www.expasy.org/prosite/) identified the

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conserved domain in manila clam ferritin subunit amino acid sequences.

2.6 Over-expression of recombinant RpFer protein and purification

For recombinant protein over-expressions, recombinant plasmids, pMal-c2X/RpFer, were transformed into *Escherichia coli* BL21 (DE3). The recombinant RpFer was expressed in the *E. coli* cell by using iso-propyl β-thio-galactopyranoside (IPTG). Briefly, transformed *E. coli* BL21 (DE3) cells were incubated in LB broth containing ampicillin (100 μg/mL) at 37 ℃ overnight. The 250 uL of cultured cells were used to inoculate into 10 mL of LB broth in 2 % glucose with ampicillin, and then the cells were incubated at 37 ℃ for 3 h until cell density reached 0.6 at $O.D_{600}$. The cells were induced in 1 mM IPTG at 25 ℃ for 3 h. The induced cell was collected by centrifugation. After supernatant was discarded, collected pellets were washed by column buffer and repeated centrifugation. The cells stored at -80 ℃ overnight. Subsequently, the cells were thawed in ice and added column buffer then sonicated to lyse. The soluble RpFer protein was isolated by centrifugation at 13000 rpm at 4 ℃ for 30 min. Purification of protein was performed following protocols, pMal™-c2X fusion protein purification (New England Biolabs, USA). The results of purified proteins were analyzed by 10 % sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-page) with low-molecular-weight marker (Takara). The gel was stained with coomassie brilliant blue to detect the proteins. The concentration of purified protein was measured with Bradford method [Bradford MM, 1976].

2.7 mRNA expression of RpFer by Real-time PCR

Tissue-specific expression level of mRNA was analyzed in manila clam gill, adductor muscle, mantle, foot, siphon and hemocytes by Real-time PCR. RpFer mRNA expression in gill tissues from clam challenged with bacteria was determined at 3, 6, 12, 24, 48 h. The gene specific primers

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(Table. 1) for amplification of RpFer fragment. As an internal PCR control, β-actin was used.

The experiment was performed at the same time in a 15 μL reaction containing 4 μL of cDNA, 7.5 μL 2X SYBR Green Master Mix (Takara, Japan), 0.6 μL of a set of primer (10 and 2.3 μL of PCR grade water. The real-time PCR cycling was carried out following cycling: 95 °C for 10 sec; 35 cycles of 95 °C for 5 sec, 58 °C for 10 sec and 72 °C for 20 sec. The optical analysis was calculated by the 2-ΔΔT method.

2.8 Ferrous-ion chelating assay

The Ferrous-ion chelating assay of purified recombinant RpFer protein was determined based on our previous report [M. De Zoysa and J. Lee et al., 2007]. To examine iron binding is dependent on the protein concentration purified recombinant RpFer and maltose binding fusion protein was dissolved in column buffer to various concentrations.

All protein reaction was initiated to add 20 μL of $FeCl_2 \cdot 4H_2O$ (2 mM) into protein solutions and the solutions was mixed to combine protein. And by adding 40 μL of ferrozine (5 mM), the reaction was initiated. The mixture was incubated for 10 min at room temperature, with shaking. After incubation, absorbance at 562 nm was measured with spectrometer (BioRad, USA).

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III. RESULS

3.1 Sequence analysis of RpFer

The full length cDNA of RpFer was 776 bp, which exhibits an open reading frame (ORF) of 513 bp coding for 171 amino acids (Fig. 1). The molecular weight (MW) and isoelectric points (p*I*) of deduced amino acid sequence was calculated; 19.6 kDa and 5.23. The result of using the SignalP program, there are no signal peptide in N-terminal of RpFer. The deduced amino acid sequences were involved two iron-binding region signatures (IBRS) identified by the PROSITE program. IBRS 1 was ^{59}EEREHAEKLMKYQNKRGGR^{77} and IBRS 2 was ^{124}DAQMSDFIEEEFLNEQVESIK^{144}. The IRE loop structure, which can control the H subunit gene expression, was observed in the 5' UTR of RpFer, 5'-CAGTGA-3'. N-glycosylation site which is found in most ferritin subunit was also not observed. The seven amino acids which are conserved residues as ferroxidase center: Glu_{25}, Tyr_{32}, Glu_{59}, Glu_{60}, His_{63}, Glu_{105} and Gln_{139}. The amino acid sequences also have potential biomineralization residue $Tyr_{27,}$ Tyr_{30}, Tyr_{32}. A putative polyadenylation signal, AATAAA was exhibited in 595 nucleotide sequence.

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Fig. 1. Full-length nucleotide and deduced amino acid sequence of manila clam ferritin subunit cDNA. The start (ATG) and stop (TAG) codons are underlined. The iron response element sequence (IRE) in the 5' UTR is bolded. Putative ferritin iron-binding region signature (IBRS) 1 and 2 are double-underlined and thick underlined. The seven metal ligands in the ferroxidase center are boxed.

```
            A TCCTTAGTG  CGTCAGTGA  ACGAAAGAA  GGAAGGACA  CAAAGTAAC  GATAAC     -1

ATGGCTGAA  TCAAGACCT  CGCCAAAAT  TTTCACCAG  GAGAGTGAA  GCTGGATTA  AACAAA     60
M--A--E--  S--R--P--  R--Q--N--  F--H--Q--  E--S--E--  A--G--L--  N--K--     20

CAGATTAAC  ATGGAATTA  TATGCCAGT  TATGTCTAT  CAATCAATG  GCCTACTAT  TTTGAC    120
Q--I--N--  M--E--L--  Y--A--S--  Y--V--Y--  Q--S--M--  A--Y--Y--  F--D--     40

AGAGATGAC  GTAGCGTTG  CCGGGATTC  TCGAAATTT  TTCAAACAT  TCGGCAGAC  GAGGAG    180
R--D--D--  V--A--L--  P--G--F--  S--K-- F--  F--K--H--  S--A--D--  E--E--     60

AGGGAACAT  GCAGAAAAG  CTGATGAAG  TATCAGAAT  AAACGAGGT  GGCCGTGTT  GTCCTA    240
R--E--H--  A--E--K--  L--M--K--  Y--Q--N--  K--R--G--  G--R--V--  V--L--     80

CAAGCTATA  CAAAAGCCG  GACCGTGAC  GAGTGGGGA  TCAGGCCTT  GATGCGATG  AAAGCG    300
Q--A--I--  Q--K--P--  D--R--D--  E--W--G--  S--G--L--  D--A--M--  K--A--    100

GCGTTACAA  CTAGAAAAG  ACCGTGAAC  CAGGCGCTT  ATCGACCTT  CACAATGTT  GCCAGT    360
A--L--Q--  L--E--K--  T--V--N--  Q--A--L--  I--D--L--  H--N--V--  A--S--    120

GGTCATGGC  GATGCACAG  ATGAGCGAT  TTCATCGAG  GAAGAATTT  CTGAACGAA  CAAGTC    420
G--H--G--  D--A--Q--  M--S--D--  F--I--E--  E--E--F--  L--N--E--  Q--V--    140

GAGTCGATT  AAGGAAATC  AGCGATCAC  GTGACCACA  TTGACTCGT  CTAGGAAGC  GGACAC    480
E--S--I--  K--E--I--  S--D--H--  V--T--T--  L--T--R--  L--G--S--  G--H--    160

GGAGAATGG  CACTTCGAC  CAGAAACTT  CAGGGTTAG  GAGTTGTTA  TGGACTGAT  GGGCTG    540
G--E--W--  H--F--D--  Q--K--L--  Q--G--                                     171

GTCTCATCA  AGTTTGATA  TATAGAATG  GCCGTTTCA  ACAGATACA  AATATGTGT  AATAAA    600

CGTCAACGT  TGCTTCAGT  TAATAAAAT  GTACCTGTG  TTCCTTGTA  ATACCCGGA  CTTTGT    660

CATAAAATA  TGTTTACTA  AGTAACCTA  TTTATTCTT  CGTTTTTAA  CTCCGATCT  ACAATT    720

CAAAA                                                                         725
```


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3.2 Homology analysis of RpFer

BLAST was performed for pair-wise alignment between RpFer and other known amino acid sequence (Table 2). The manila clam ferritin subunit showed the highest degree of sequence similarity with other clam species, especially razor clam *Sinonova culaconstricta* ferritin (77.2 %). *Meretrix Meretrix* ferritin subunit and *Crassostrea gigas* GF1 and GF2 also exhibited high similarities between RpFer (76.6 %).

Multiple sequence alignment result showed that the ferroxidase center was highly conserved in RpFer amino acid sequence with other known ferritin H subunit amino acid sequences (Fig. 2).

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Table. 2. Homologies, similarities and gaps with known ferritin genes.

	Homology (%)	Similarity (%)	Gaps (%)	NCBI Accession number
Razor clam F	77.2	88.9	0.0	ACZ65230
Hard clam F	76.6	90.6	0.0	AAZ20754
Pacific oyster GF1	76.6	88.3	0.0	AAP83793
Pacific oyster GF2	76.6	89.5	0.0	AAP83794
pPacific oyster SF	76.0	87.7	0.0	CAD92096
T.granosa F	70.3	86.6	0.6	ADC34696
Bumblebee FH	26.8	42.2	32.0	EU14432
Xenopus laevis	59.9	77.4	4.0	AAB20316
B.tsingtauense F	72.7	83.7	0.6	AAN77903
Catfish F	60.1	76.4	4.5	AAY86949
Atlantic Salmon FH	59.6	77.0	4.5	AAB34575
Human FH1	55.7	73.2	6.6	AAH66341

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Fig. 2. Multiple alignment of RpFer with other selected ferritin amino acid sequences. The conserved ferroxidase sites (seven metal ligands) were boxed.

```
Manila_clamF         ---MAESRPRQNFHQESEAGLNKQINMELYASYVYQSMAYYFDRDDVALPGFSKFFKHSA 57
Razor_clamF          ---MAETMPRQNFHQDSEAGINKQINMELYASYVYQSMSFYFDRDDVALKGFAKFFKESS 57
Hard_clamF           ---MADSRPRQNFHKESEAGINRQINMELYASYCYMSMAYYFDRDDVALKGFSKFFKESS 57
Ark_shellF           ---MAQTQPRQNFHVESEAGINKQINMELYASYVYQSMYMYFDRDDVALPSFAKYFKHNS 57
CatfishFH            ----MSSQVRQNFHQDCEAAINRQINLELYASYVYLSMSYYFDRDDQALHNFAKFFRKQS 56
Human_FH1            MTTASTSQVRQNYHQDSEAAINRQINLELYASYVYLSMSYYFDRDDVALKNFAKYFLHQS 60
Rainbow_troutFH1     ----MESQIRQNYHHDCEAAINRMINLEMFASYTYTSMAFYFSRDDVALRGFAHFFKENS 56
                            :  ***:* :.**.:*: **:*::*** * **  **.*** ** .*:::* ..:

Manila_clamF         DEEREHAEKLMKYQNKRGGRVVLQAIQKPDRDEWGSGLDAMKAALQLEKTVNQALIDLHN 117
Razor_clamF          DEEREHAEKLMKYQNKRGGRIVLQPISKPDRDEWGSGLDAMKAALNLEKSVNQSLLELHK 117
Hard_clamF           EEEREHAEKLMKYQNKRGGRVVLQNIQKPDRDEWGSGLEAMETALQLEKTVNQSLLDLHK 117
Ark_shellF           EEEREHAEKLMKYQNKRGGRIVLQDIQKPDLDEWGSPLEAMQTTLALEKSVNQALLDLHK 117
CatfishFH            HEEREHAEKLMKIQNQRGGRIFLQDIKKPERDEWGSGVEALECALQLEKNVNQSLLDLHK 116
Human_FH1            HEEREHAEKLMKLQNQRGGRIFLQDIKEPDCDDWESGQNAMECALHLEKNVNQSLLELHK 120
Rainbow_troutFH1     DEEREHAEKLLSFQNKRGGRILLQDIKKPERDEWGNGLEAMQCALQLEKNVNQALLDLHK 116
                     .*********:. **:****:.** *.:*: *:* .  :*:: :* ***.***:*::**:

Manila_clamF         VASGHGDAQMSDFIEEEFLNEQVESIKEISDHVTTLTRLGS---GHGEWHFDQKLQG--- 171
Razor_clamF          VADSHGDAQMCDFLEGEYLEEQVEAIKDLSDRITNLNRVGK---GLGEWHYDQKLLS--- 171
Hard_clamF           IADTHGDAQMMDFLEGEYLKEQVDAVKDLSDRITNLKRVGA---GHGEWHYDRELQS--- 171
Ark_shellF           IADKHGDAQMMDFLEGEYLKEQVDAIEEISDHITNLKRVGT---GLGEYMYDKETMSS-- 172
CatfishFH            VSTDHNDPHMCDFIESRYLDEQVKSIKELSDWVTNLRRMGAPQNGMAEYLFDKHTLGSES 176
Human_FH1            LATDKNDPHLCDFIETHYLNEQVKAIKELGDHVTNLRKMGAPESGLAEYLFDKHTLGDSD 180
Rainbow_troutFH1     IASGKVDPHLCDFLETHYLNEQVEAIKKLGDHITNLTKMDAVKNKMAEYLFDKHTLGGQS 176
                     ::  : *.:: **:* .:*.***.:::.:.* :*.* ::.      .*: :*:.  .
```


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3.3 Phylogenetic analysis

To determine the evolutionary position of the RpFer, a phylogenetic tree was constructed using Clustal W and Neighbor-joining (NJ) algorithm with MEGA 4.0. The clusters were divided into two distinct branches significantly, vertebrate and invertebrate ferritin groups. Phylogenetic tree result showed that RpFer was further grouped into invertebrates. Razor clam and hard clam ferritin subunit was clustered into RpFer (Fig. 3)

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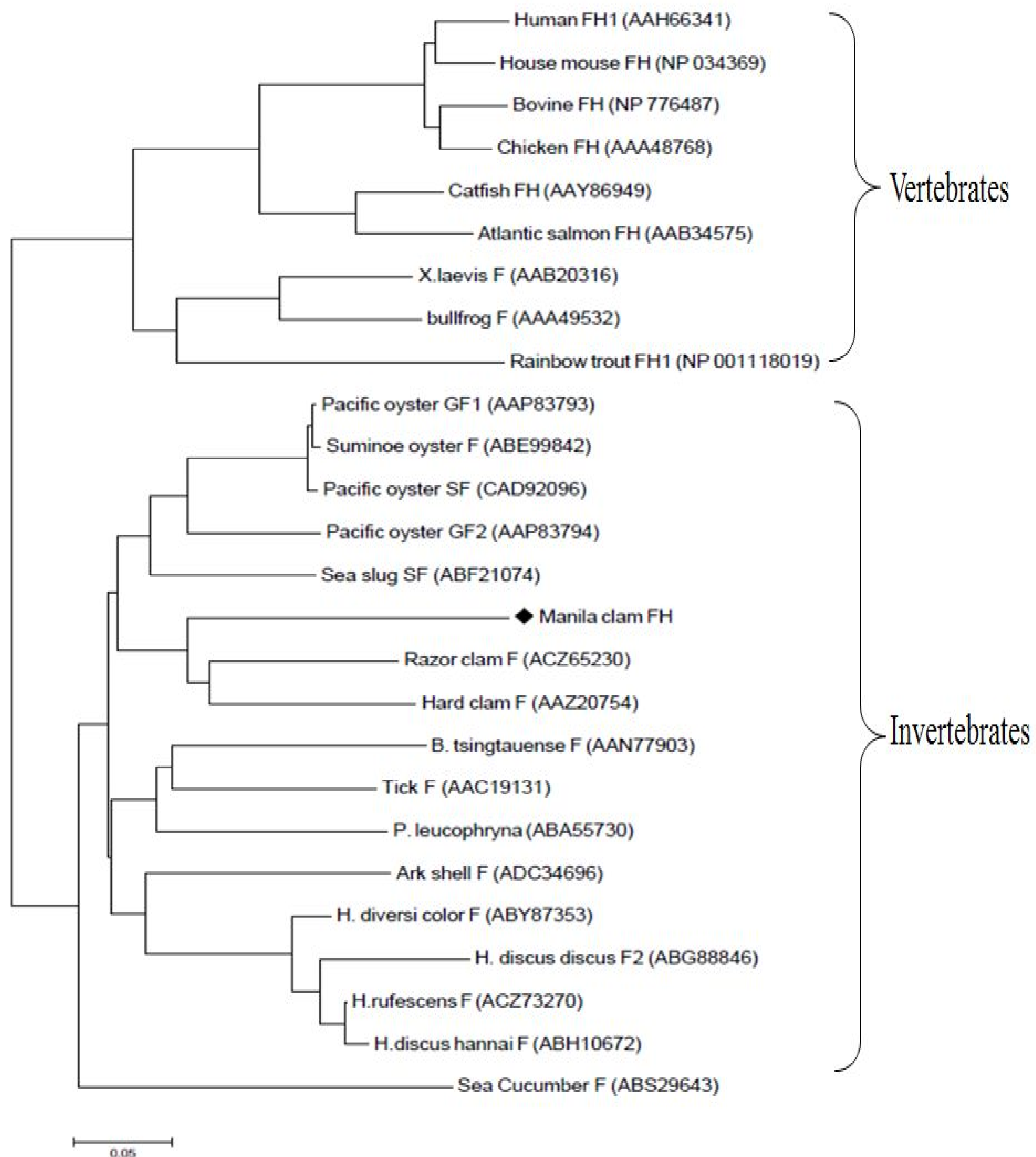
Fig. 3. Phylogenetic analysis of the clam ferritin RpFer. Based on amino acid sequence, the tree was constructed. The number of branches are bootstrap values for 1000 replication. The GenBank accession numbers are as follow:
X. Laevis F (AAB20316); bullflog (Rana catesbeiana) F (AAA49532), rainbow trout (Oncorhynchus mykiss) FH1 (NP 001118019); catfish (*Ictalurus punctatus*)FH (AAY86949); atlantic salmon(*Salmo salar*)FH (AAB34575); bovine (*Bos taurus*) FH (NP 776487); chiken (*Gallus gallus*) FH (AAA48768); human (*Homo sapiens*) F (AAH66341); house mouse (*Mus musculus*) F (NP_034369); polychaeta (*Periserrula leucophryna*) F (ABA55730); tick (*Ixodes ricinus*) F (AAC19131); amphioxus (*Branchiostoma belcheri*) F (AAN77903); ark shell (*Tegillarca granosa*) F (ADC34696); razor clam(*Sinonovacula constricta*) F (ACZ65230); hard clam (*Meretrix meretrix*) F (AAZ20754); pacific oyster (*Crassostrea gigas*)GF1 (AAP83793), GF2 (AAP83794), SF(CAD92096); suminoe oyster (*Crassostrea ariakensis*) F (ABE99842); sea slug (*Aplysia californica*) SF (ABF21074); sea cucumber (*Holothuria glaberrima*) F (ABS29643); *Haliotis diversicolor* F (ABY87353); *Haliotis discus discus* F2 (ABG88846); *Haliotis rufescens* F (ACZ73270)



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3.4 Expression and purification of rRpFer in *E. coli* BL21(DE3) cells

RpFer clones were transformed into *E.coli* BL21(DE3) cells and over-expressed in cells by IPTG induction, and purified as a recombinant clam ferritin subunit with MBP fusion protein, rRpFer. The 10 % SDS-page result clearly showed that the purified clam ferritin subunit protein was highly induced compared to un-induced cells (Fig. 4). The size of the purified recombinant manila clam subunit protein was 62.1 kDa which is contained maltose binding fusion protein, 42.5 kDa.

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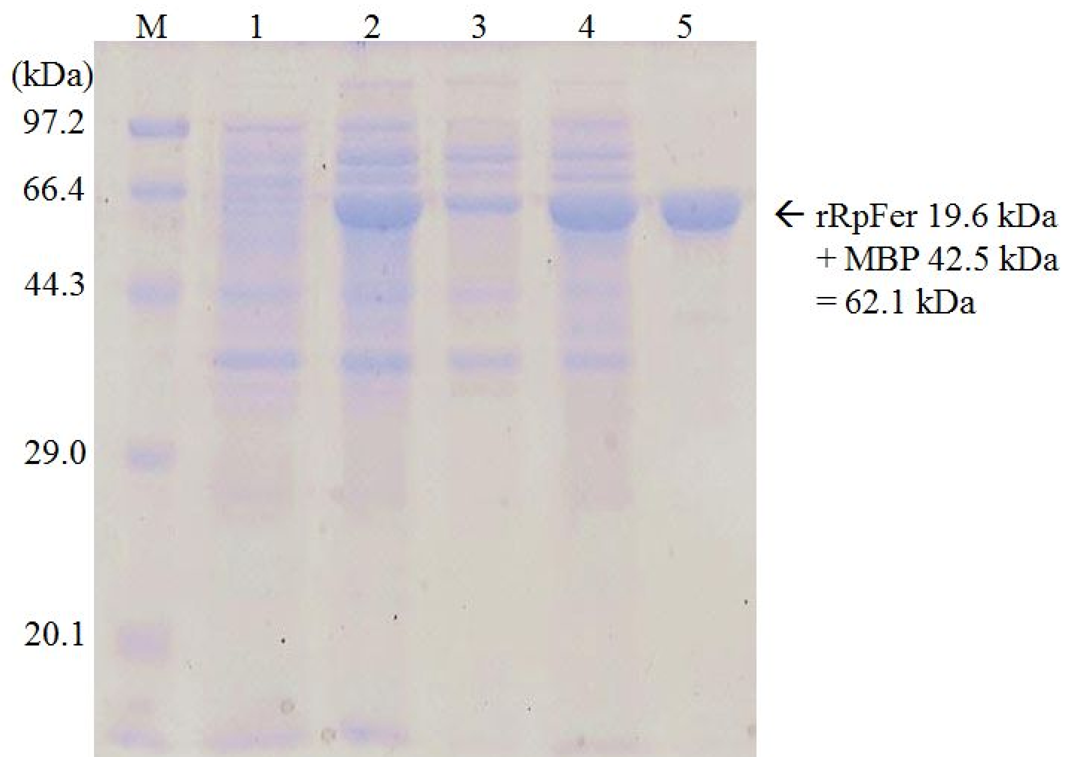
Fig. 4. SDS-page result of over-expressed recombinant RpFer in *E. coli* BL21 (DE3) cells and purified recombinant fusion protein. M : marker ; Lane 1: un-induced cells ; Lane 2: induced cell (whole lysated) ; Lane 3: induced cell (pellet) ; Lane 4: induced cell (supernatant) ; Lane 5: purified recombinant protein.



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3.5 mRNA expression pattern of RpFer in different tissues

mRNA expression pattern of RpFer in different tissues was analyzed by real-time PCR with RpFer gene specific primers (Table. 1). And as an internal PCR control, manila clam β-actin gene was amplified under the same PCR reaction conditions. RpFer mRNA were expressed in some tissues namely, gill, hemocyte, mantle and foot. Relative RpFer gene expression levels were normalized by adduct muscle which was shown the lowest expression. Gill and hemocyte tissues were observed significantly higher relative mRNA expression level than in any other manila clam tissues.

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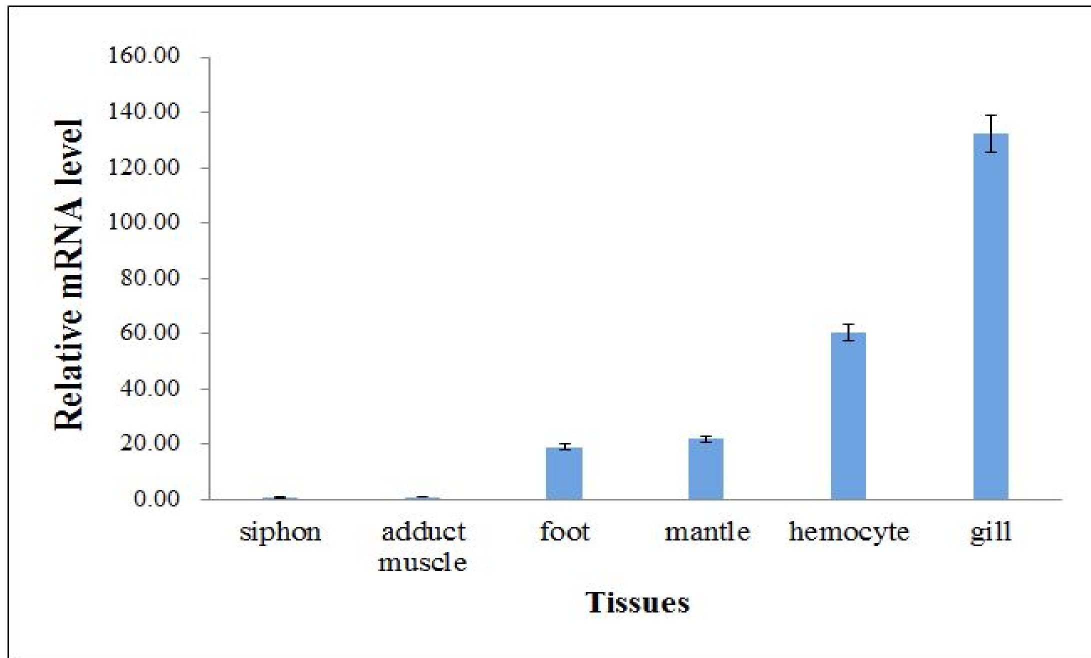
Fig. 5. Tissue-specific expression of RpFer mRNA. Expression levels of the RpFer mRNA in different tissue of manila clam were detected by real-time PCR. RpFer transcript level in adductor muscle, mantle, siphon, gill, foot and hemocyte was optimized to that of adduct muscle. The expression fold was calculated by the 2-ΔΔT method using clam actin as a reference gene.



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3.6 mRNA expressions pattern of RpFer in gill after bacterial challenges

To detect whether the RpFer mRNA is regulated by bacteria infection, manila clam was challenged gram -negative bacteria- *Vibrio tapetis*. PBS was a control as a dissolvent for immunogen. cDNA from gill tissue was used for real-time PCR. The real-time PCR results showed that RpFer gene was up-regulated and significantly increased until 12 hr and rapidly decreased at 24 hr level after infection compare to unchallenged group using a negative control (about 5 fold) (Fig. 6).

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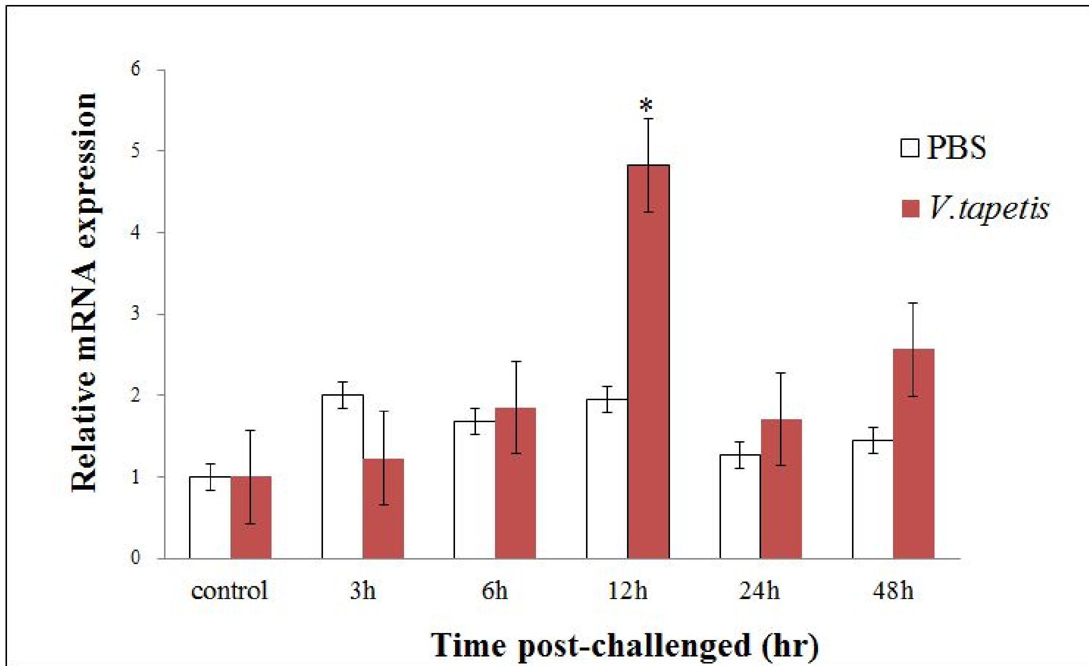
Fig. 6. Relative RpFer mRNA expression in clam gill tissue after bacterial challenge. Clam was challenged with *Vibrio tapetis* and PBS (control). RpFer expression in gill was determined by quantitative real-time PCR at various times post-challenged. The mRNA level of RpFer was normalized to that of β-actin. Values are shown as means ± SE (N=3). Significance between PBS-challenged clam and bacteria challenged clam is indicated with asterisks. * $p<0.05$.



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3.7 Ferrous-ion chelating assay of rRpFer

The purified recombinant protein, rRpFer, was examined to know whether this recombinant protein has iron binding activity. Ferrozine with ferrous ion has a strong absorbance at 562 nm, the higher the iron binding ability of the test protein gives the lower absorbance [J. Guo, H. Lee, et al., 2001].

Iron binding capacity of rRpFer exhibited that was significantly dependent on protein concentrations and reached at highest concentration 7.2 μg/mL. Maltose binding protein (MBP) as a control was purified and carried out iron chelating experiment under same condition with rRpFer, but it was not shown any specific iron binding capacity (Fig. 7).

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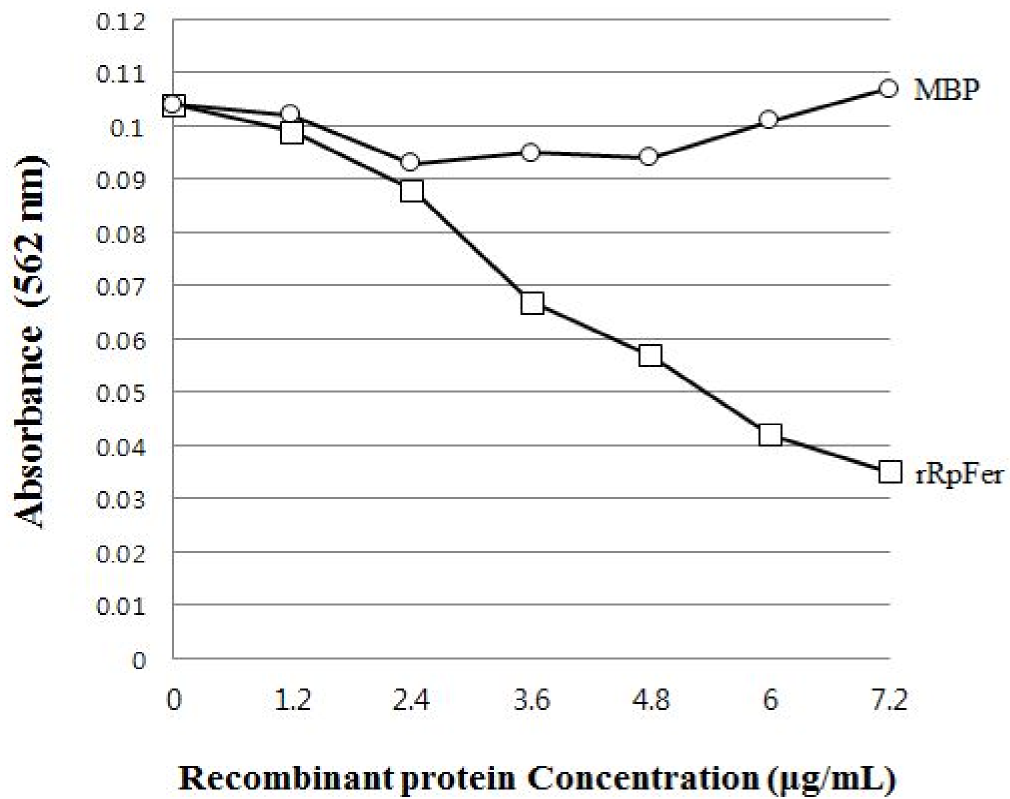
Fig. 7. Analysis of the ferrous-ion chelating assay of purified recombinant RpFer. FeCl2 was incubated with different concentrations of recombinant RpFer and MBP. Ferrozine was then added to the mixture, and absorbance at 562 nm was measured.



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IV. DISCUSSION

In this study, ferritin H subunit cDNA from manila clam *Ruditapes philippinarum* was successfully isolated and cloned cDNA. The RpFer gene was described characterization with other known ferritin subunit genes from NCBI data. Tissue specific relative mRNA expression and immune challenge response of the RpFer gene was analyzed after infection with clam disease bacteria *Vibrio tapetis*. And the recombinant RpFer protein was purified to identify its iron chelating activity.

The RpFer cDNA full-length was 776bp and it had 513 bp ORF coding 171 amino acids. Amino acid sequence of the RpFer was found seven amino acid, Glu_{25}, Tyr_{32}, Glu_{59}, Glu_{60}, His_{63}, Glu_{105}, and Gln139, almost identical residues are conserved in the clam *Meretrix meretrix* H subunit which are in ferroxidase center [X. Wang, B. Liu and J. Xiang, 2009]. And potential biomineralization residue Tyr_{27}, Tyr_{30}, Tyr_{32} residues presence means that RpFer can accelerate binding Fe(II) ion, oxidation and incorporation by this H subunit specific ferroxidase. In 5' UTR of RpFer cDNA, there are six nucleotides which can form IRE stem-loop structure. All animal organisms respective have IRE site in 5' UTR of H subunit ferritin gene, IRE can induce ferritin synthesis in response to iron administration [Harrison and Arosio, 1996]. Moreover, there no well conserved signature residues associated with iron nucleation properties of L or M subunit [W. Zheng, Y. Hu et al, 2010]. Besides, the RpFer gene has significant homologies with molluscan H type ferritin, especially razor clam (*Sinonova culaconstricta*). Thus, RpFer is most likely an H subunit ferritin based on those factors and proposed that has a capacity of fast iron binding and oxidation.

Further functional analysis is needed to confirm RpFer characterization and thus recombinant protein purification and assay was carried out.

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The recombinant RpFer protein molecular weight was deduced 19.6 kDa by Protparam system (http://expasy.org/tools/protparam.html) and SDS-page result was also shown same pattern. Normally, H subunit ferritin protein molecular weight is approximately 20 kDa in many Bivalve species [C.Jin, C.Li et al, 2011; X. Wang et al, 2009; C. Li et al, 2011].

Tissue distribution results showed that the RpFer was shown highest expression in gill. Those results are supposed that relative with function of gill tissue.

Tissue distribution results showed that the RpFer was shown high expression in gill and hemocytes. In clam *Tegillarca granosa* and abalone *Haliotis discus hannai* ferritin gene was high expressed in gill and hepatopancreas, but in *T.granosa* hemocyte was shown high expression while abalone hemocyte was lower expression than other tissues [C. Jin, C. Li, et al. 2011; C. Wu, W. Zhang, et al. 2010]. The pacific white shrimp ferritin mRNA was expressed in various tissues, midgut gland, brain ganglion, gill, hepatopancreas, abdominal ganglion, eyestalk, muscle, thoracic ganglion and heart [S. Hsieh, Y. Chiu and C. Kuo, 2006].

In this study, RpFer mRNA was also up-regulated after immunogen challenged by Real-time PCR. Previous reports suppose that ferritin plays important role in invertebrate immune system, for example, pacific white shrimp [Y. Ruan, C. Kuo, et al., 2010], beetle [SR Kim, KS Lee, et al., 2004] and bumblebee [D. Wang, BY Kim, et al., 2009] ferritin expressed after virus challenged, respectively. In soft-shell clams, *Mya arenaria*, ferritin gene also up-regulated after bacteria challenge and it was considered that ferritin maybe involved in the early stages of hemocytes *Vibrio* infection [M.T. Araya, F. Markham, et al,. 2010]. Ferritin is researched as an acute protein that is up-regulated by microbacterial infections in human [Konijin AM, Hershko C, 1977], fish [X. Zhang, W. Wei et al., 2010; G. Beck, TW Ellis et al., 2002], crab[DST Ong, L.

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Wang,, 2005] and so on. It is considerable one of host defense strategies to make hostile environment for pathogens by reducing the cellular pool of biologically available iron [W. Zheng et al, 2010].

Therefore, RpFer is suggested that it is an important function in immune system. In human, p53 is regulated in oxidative stress and ferritin is relative with oxidative stress regulation [JH Lee and H. Jang et al., 2009].

And also ferritin is considered that it is involved in TNFα-induced apoptosis [A. Cozzi, S. Levi et al, 2003] and affects on cell proliferation rates in an iron independent manner [A. Cozzi, B. Corsi et al, 2004].

In mollusk, the ferritin has other functions, for example, shell initiation [X.Wang et al, 2009] and formation [Y. Zhang, Q. Meng, et al., 2003]. Dietary iron treatment is also one of element that up-regulate ferritin expression [C. Wu et al, 2010] and [C. Li, H. Li, et al. 2011]. And other heavy metals such as Zn^{2+} and Cu^{2+} also stimulate ferritin up-regulated expression [J. Zhang et al, 2006]. Thus, this study supposed that manila clam ferritin H subunit also could be increased under those conditions.

The recombinant RpFer was found that rRpFer is an iron chelator by ferrous-ion chelating assay. This result suggests that RpFer is likely to be a functional protein and participate in iron homeostasis in manila clam.

In conclusion, the results of this study identify that RpFer has sequence features of H ferritin and exhibits an expression pattern that is tissue specific and regulated by bacterial infection. This study result suggests that RpFer is likely to play a role in both iron storage and immune system.

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VI. ACKNOWLEDGEMENT

학부 졸업 무렵에 실험실에 들어온 지 벌써 2년이 넘는 시간이 지났습니다. 처음에는 그저 아무 것도 모르고 그저 하고 싶다는 열정만으로 실험실 생활을 하였는데 어느 새 석사를 졸업할 시간이 지났습니다. 아무 것도 몰랐던 저를 그저 믿고 받아주시고 이끌어 주셔서 무사히 석사 졸업을 마치게 도와주신 지도 교수님이신 이제희 교수님께 감사의 말씀을 드립니다. 바쁘신 와중에 저의 논문 심사를 해주신 허문수 교수님, 정준범 교수님과 석사 과정 동안 저에게 많은 조언과 가르침을 주신 김기영 교수님, 여인규 교수님, 전유진 교수님께도 감사의 글을 올립니다.

항상 힘들 때 의지가 되었던 실험실 식구인 황일선 박사님, 영득오빠, 숙경언니, 민영이, 유철오빠, 원보, 성도, Niroshana, Uma, Sanjaya, Saranya, Ajith, Anushka, 완창과 철홍오빠, Mahanama, Chamilani에게 감사의 인사를 드립니다.

올해 같이 졸업하며 고생한 석사동기인 혜미, 창익오빠, 지혁오빠, 창희오빠, 병훈오빠, 창수오빠, 익수오빠, 승민오빠와 해양생물이용공학연구실, 무척추동물연구실, 면역학연구실, 어류생리생태연구실, 미생물연구실, 양어사료학연구실, 유전육종연구실, 해양바이러스연구실, 해양과환경연구소의 선배님들 및 후배들께 감사드립니다.

옆에 있을 때나 멀리 있을 때나 늘 자기의 꿈처럼 저를 응원해주고 지지해준 친구들 은정, 윤주, 민신이, 지희, 규비, 유정이, 효경언니, 영화, 현재, 은혜, 기철, 비라와 비록 지금은 곁에 없지만 언제나 제게 용기를 북돋아 주었고 하늘에서도 지켜볼 은진이에게도 감사합니다.

마지막으로, 언제나 늘 제 편이 되어주시는 사랑하는 우리 가족들, 아빠,엄마, 태하, 태림이에게 감사를 드리며, 이 논문을 저를 사랑하고 아껴주신 모든 분들에게 바칩니다.

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