Gene
September 2004; 338(2) : 187-195
http://dx.doi.org/10.1016/j.gene.2004.04.027

Crassostrea gigas ferritin: cDNA sequence analysis for two heavy chain type subunits and protein purification[1]*

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Abstract: Ferritin has been shown as being the principal iron storage in the majority of living organisms. In marine species, ferritin is also involved in high-level accumulation of 210Po. As part of our work on the investigation of these radionuclides' concentration in natural environment, ferritin was searched at the gene and protein level. Ferritin was purified from the visceral mass of the oyster Crassostrea gigas by ion-exchange chromatography and HPLC. SDS-PAGE revealed one band of 20 kDa. An Expressed Sequence Tag (EST) library was screened and led to the identification of two complementary DNA (cDNA) involved in ferritin subunit expression. The complete coding sequences and the untranslated regions (UTRs) of the two genes were obtained and a 5′ Rapid Amplification of cDNA Ends (RACE) was used to obtain the two iron-responsive elements (IREs) with the predicted stem-loop structures usually present in the 5′-UTR of ferritin mRNA. Sequence alignment in amino acid of the two new cDNA showed an identity with Pinctada fucata (85.4–88.3%), Lymnaea stagnalis (79.3–82.2%) and Helix pomatia (79.1–79.1%). The residues responsible for the ferroxidase center, conserved in all vertebrate H-ferritins, are present in the two oyster ferritin subunits. Oyster ferritins do not present the special characteristics of other invertebrate ferritins like insect ferritins but have some functional similarities with the vertebrate H chains ferritin.

Keywords: Oyster; EST; IRE; Iron

1. Introduction

The ferritin molecule has two functions, iron detoxification and iron storage. By storing excess iron, this protein plays an important role in the cellular homeostasy as the physiological source of iron for the cell. Ferritin is widely distributed throughout the living kingdom in a highly conserved conformation, which is necessary to achieve the sequestration of iron in a soluble, bioavailable, and non-toxic form (Andrews et al., 1992). Additional roles have been described for this storing molecule. Indeed, early studies on subcellular distribution of transuranic elements such as ^{238}Pu and ^{241}Am in mammals tissues have shown also that this iron-carrier systems (ferritin and transferrin) have an important function in the transport and fixation of these radionuclides (Stover et al., 1970, Lehman et al., 1983) as well as in accumulation of the natural radionuclide ^{210}Po in marine organisms (Durand et al., 1999, 2002). The ferritin has also been suggested as being an acute phase protein responding to a nonlethal injury to the organism. This was shown by the variation of iron level in starfish with amebocytes stimulated with lipopolysaccharide or phorbol 12-myristate 13-acetate (Beck et al., 2002). Furthermore, other experiments demonstrated the coupling of nitric oxide with the neuronal expression of intracellular forms of ferritin in the snail *Helix pomatia* (Xie et al., 2001). More recently, the involvement of the ferritin and the iron distribution in the outer side of the mantle, thus playing a role in biomineralisation, has been described for the bivalve *Pinctada fucata* (Zhang et al., 2003).

The ferritin complex is formed by 24 subunits surrounding a central cavity with a capacity for storing up to 4500 atoms of iron. This core is a ferric-oxyhydroxyphosphate complex (Theil, 1987). Much of knowledge of ferritin structure stems from studies in vertebrates. In these organisms there are two types of subunits called H and L polypeptide chains encoded by distinct genes (Cairo et al., 1991; Orino et al., 1997). Tissues ferritins are composed of a variable proportion of the two types of subunits, the H subunit is predominant in heart ferritin, whereas the L subunit is predominant in liver ferritin. These subunits show distinct amino acids in some sites with different functions in metal oxidation and fixation. Obviously the same architecture is found among animals but with a very large variation in amino acid composition, with identity percentage as low as 14 % (Harrison and Arosio, 1996).

Ferritins have been studied in few invertebrates (Huang et al., 1996; Beck et al., 2002) and most of them share the characteristics of vertebrate ferritins although insect ferritins present unusual properties (Charlesworth et al., 1997). These insect ferritins include H-type secreted ferritin subunits, non-H type secreted ferritin subunits and H type cytosolic ferritin subunits (Nichol et al., 2002). Few studies were carried out on mollusk ferritins, but some cDNA sequences are now available from snails (Von Darl et

al., 1994; Xie et al., 2001), cephalopods (Zinovieva et al., 1999) and pearl oyster (Zhang et al., 2003). The majority of those ferritins share identities with the H-type subunit found in vertebrates.

The oyster *Crassostrea gigas* is one of the most cultivated shellfish worldwide and as such, is subject of a large range of research programmes, including their immunity mechanisms or bioaccumulation of various toxic compounds. We have previously shown that accumulation of radionuclide occurs in the core or in the protein coat of the ferritin of this bivalve (Durand et al., 1999, 2002). In this work, we report on the sequencing investigations on this mollusk ferritin subunits by two approaches i) the protein level by purification of the ferritin protein using ion-exchange chromatography, HPLC and SDS-PAGE and ii) at the nucleic acid level taking advantage of our recent works on an EST database of the oyster *Crassostrea gigas* (Gueguen et al., 2003).

The characterization of a ferritin and its encoding gene in the oyster *C. gigas* will allow for comparative analysis in functional studies with other genes, studies on population variations and constitutes a first approach toward the identification of conserved structures in ferritin which may ensure radionuclide or metal binding in this bivalve mollusk.

2. Materials and methods

2.1. Construction of a cDNA library

A *Crassostrea gigas* oyster cDNA library was used and partially sequenced to establish an Expressed Sequence Tag (E.S.T.) library as described (Gueguen et al., 2003). Briefly, this cDNA library was constructed from hemocyte mRNA of oysters in lambda Zap Express bacteriophages (Stratagene). The double strands cDNA were separated on a Sepharose CL-B column and fractions exceeding 300 bp were sorted for the construction of the library. Titer of this bank yielded $1.6\ 10^5$ on the primary basis and $1.2\ 10^9$ on the secondary amplified step.

A mass *in vivo* excision was performed and allowed isolation of 55 000 bacterial clones in 384 wells plates.

2.2. Sequence determination and analysis.

BLAST-X analysis was performed using the substitution matrix BLOSUM62 (Henikoff and Henikoff 1992). Comparison of 1142 ESTs against non-redundant Swissprot and Genbank databases revealed that 615 clones (53.9 %) were significantly similar (E value $< 10^{-3}$) to isolated genes. This information is located at http://www.ifremer.fr/GigasBase, where all the EST and their corresponding functional classification are available as well as a complete list of BlastX matches, and contigs assemblage. Contig search was performed using CAP3 assembly program. Pairwise alignments of amino acid sequences were calculated by the ALIGN program version 2 (Myers and Miller, 1988). Multiple alignments of amino acid sequences were performed using the

CLUSTAL W algorithm version 1.8 (Thompson et al., 1994). A phylogenetic tree was constructed from genetic distances calculated by the neighbor-joining method (Saitou and Nei, 1987) as implemented in the program MEGA version 2.1 (Kumar et al., 2001). The tree was constructed using the Poisson correction and the reliability of the phylogenetic topologies (branching patterns) was determined by the bootstrap resampling test with 1000 replicates. A consensus tree was obtained with a bootstrap cut-off value of 70%.

2.3. 5'RACE-PCR

A RACE-PCR (Rapid Amplification of 5' cDNA End) was performed as specified by the manufacturer (Roche Molecular Biochemical). Briefly, 2µg of total RNA from hemocytes were subjected to reverse transcription using 2 antisense primers derived from the cDNA sequence: F52 (5' CTG GTA GGT ATA GCA GGC ATA CAG 3') and F12 (5' ATG CCA GCT TCG CTC TCC TGG TGG 3'). After PCR amplification, the products were cloned in the Cloning Vector pCR-Script (Stratagene, La Jolla) and sequenced.

2.4. Northern blot

The probe was obtained by PCR amplification on one of the ferritin-containing phagemid using the universal primers T3 and T7. The amplification product was then radio-labeled by [^{32}P] dCTP using the random priming kit from Invitrogen^{TM}.

Total RNAs (35 µg) were fractionated by denaturing 1.2 % agarose/formaldehyde gel electrophoresis and blotted onto Hybond-N membrane (Amersham). Membranes were prehybridized for two hours at 65°C in 50% formamide, 5 X SSC, 8 X Denhardt's, 50 mM NaH_2PO_4 pH 6.5, 0.1% SDS and 100 µg/ml denatured salmon sperm DNA. Hybridizations were performed overnight at 42°C in prehybridization buffer containing the radio-labeled probe. After hybridization, membranes were washed twice for 15 min at room temperature in a solution of 2 X SSC containing 0.1% SDS, and twice for 20 min at 60°C in a solution of 1 X SSC containing 0.1% SDS.

2.5. Isolation and purification of oyster ferritin

The ferritin from the visceral mass of oysters (*Crassostrea gigas*) was purified from forty eight adult specimens collected during the spring of 2002 from Atlantic coast of France (Baie de Bourgneuf, Noirmoutier). Oysters were dissected and 80.8 g of visceral tissues were pooled and homogenized in 100 ml of 150 mM NaCl buffered at pH 7.5 with 10 mM (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] : HEPES) and containing 0.1 mM phenyl methyl sulfonyl fluoride (PMSF) to inhibit digestive proteases (buffer A). Homogenization was

performed with a Waring Blender (20 sec. bursts at high speed) and then in a Potter-Elvehjem homogenizer with a motor-driven teflon pestle (3 stokes at 200 rev/min). The homogenized tissue was heated at 65°C for 10 min in a water bath with continuous stirring. After centrifuging the homogenate at 10,000 *g* for 10 min, the pellets were discarded and the supernatant was brought to pH 4.8 with 2 M acetic acid. The solution was left overnight at 4°C. After a centrifugation at 10,000 g for 20 min the sediment was discarded and the supernatant was adjusted to pH 6.5 by addition of 2 M NaOH. Then PMSF and 2-mercaptoethanol were added to final concentrations of 0.1 mM and 0.02 % respectively. The solution was brought to 75 % saturation with ammonium sulfate. The suspension was further centrifuged at 1,600 g for 30 min and the pellet was dissolved in 30 ml of 1 mM HEPES, 2 mM 2-mercaptoethanol, and 0.1 mM PMSF at pH 7.5 (buffer B). The precipitation procedure, using 60 % ammonium sulfate, was repeated once more. The final pellet of ferritin was dissolved in buffer B. This protein solution was dialysed overnight against the same buffer to eliminate residual ammonium sulfate. The solution was then applied to a column of diethylaminocellulose DE 52 Whatman (25 x 120 mm) equilibrated with buffer B. The elution was performed initially by passing through 300 ml of buffer B at a flow rate of 82.5 ml h^{-1}, and then using a linear gradient of 0 to 0.3 M NaCl in buffer B (400 ml) at the same flow rate. The elution was monitored for protein and iron concentration. The fractions containing the crude ferritin were pooled and brought to 60 % saturation with ammonium sulphate. After centrifugation at 10 000 *g* for 10 min, the pellets were solubilized in 5 ml of 1 mM HEPES buffer at pH 7.5. This solution was centrifuged again at 10 000 *g* for 10 min and the supernatant was applied to a column of Sephacryl S300 (30 x 900 mm) eluted by 1 mM HEPES (pH 7.5) at a flow rate of 35.5 ml h^{-1}. This gel filtration gave a single peak of protein corresponding to an iron peak. The fractions of ferritin were pooled and concentrated in a Savant SpeedVac concentrator. A 5 ml section of this sample was purified by size-exclusion chromatography on a HPLC TSK G4000 SW column (21.5 x 600 mm), equilibrated and eluted by 1 mM HEPES, 100 mM NaCl, 2mM 2-mercaptoethanol, 0.1 mM PMSF, pH 7.5 at a flow rate of 1ml min^{-1}. The purified fraction of ferritin was concentrated and stored at - 20°C. The molecular weight of native oyster ferritin was estimated by chromatography on the HPLC TSK G4000 SW column calibrated with Pharmacia Biotech high molecular mass markers. Protein and iron concentrations were determined as previously described (Durand et al., 2002).

2.6. SDS-gel electrophoresis and protein sequencing

SDS-PAGE of oyster ferritin was performed under reducing conditions in 15 % gel along with commercial Bio-Rad low molecular weight markers (phosphorylase b, 97.4 kDa; bovine serum albumin, 66.2 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa; soybean trypsin inhibitor,

21.5 kDa; lysozyme, 14.4 kDa) according to the method of Laemmli (1970). Gels were stained for the detection of protein using 0.25 % Coomassie brilliant blue R-250 in methanol (40 %), acetic acid (7 %) and water followed by destaining in methanol (5 %), acetic acid (7 %) and water. Ferritin subunits were electroblotted onto polyvinylidene difluoride membrane (PVDF-Plus MSI). The ferritin band was excised for determination of the N-terminal sequence. Internal peptide sequences were obtained after cleavage with trypsin of the excised band from SDS-PAGE, RP-HPLC separation of peptides and sequence analysis.

3. Results

3.1. Screening of the database

Screening of the 1142 ESTs, allowed the identification of 10 tags presenting homology with known ferritin (table 1). All the BlastX results using these raw sequences indicated a preferential alignment with the soma ferritin of *Lymnaea stagnalis* (great pond snail). The contig analysis using the Cap 3 assembly program confirmed however that two distinct groups (called A and B) seemed to emerge (table 1).

The DNA was extracted for all the 10 clones and submitted to an enzymatic hydrolysis with the restriction enzyme *Bss*HII that releases the insert in the cloning vector. Within the 2 groups, the longest insert was sorted out by agarose gel electrophoresis and the corresponding clone (called GF1 and GF2) fully sequenced.

3.2. Sequence analysis and comparison of sequences

The two sequenced cDNA are 688 and 677 base pairs in length and show both a 513 nucleotides open reading frame coding for a 171 amino acid protein. The sequences are highly homologous to various known ferritins (fig 1)

In nucleic acid, a databank search using again the Basic Local Alignment Search Tool (BLAST) yielded the highest homologies (82%) with the complete sequence of soma ferritin of the gastropod mollusk *Lymnaea stagnalis* (great pond snail) for the GF1 ferritin sequence, 82% with the prochordata *Branchiostoma belcheri tsingtaunes*, 82% with the rainbow trout *Oncorhynchus mykiss* ferritin and 81% with the medaka *Oryzias latipes* ferritin. The same results appear for the GF2 ferritin sequence with 81% identity with *Lymnaea stagnalis* soma ferritin. Taking all the UTRs into account alignment of the two new ferritins gives 67% identity wheras the ORF alignment gives 76.4%. During the first sequencing run, only the second clone (GF2) showed the complete IRE sequence. A RACE was employed to ascertain the integrity of the IRE for the first clone (GF1).

The two oyster ferritin cDNA contain a 5' untranslated region (5' UTR) with a highly conserved canonical Iron Responsive Element sequence (IRE). These IREs are present in the majority of ferritin mRNAs and alignment and prediction of loop structure of this new characterized IRE from the oyster *Crassostrea gigas* shows again a primary relevant consensus with the other available mollusks IREs, *Lymnaea stagnalis* and *Pinctada fucata.* (fig 2 and 3).

At the amino acid level (on the deduced sequence), a first BLASTp alignment showed 82% identity for the GF1 sequence and 83% for the GF2 sequence with the soma ferritin of the gastropod mollusk *Lymnaea stagnalis* (great pond snail), 76% and 73% with two different arthropod acari, 70% with the sea star *Asteria forbesi*, the identity with the other mollusk (*Octopus dofleini*) being 67%.

A pair wise alignment of the available sequences with the algorithm ALIGN version 2.0 confirmed the range of identities with the other invertebrates and showed 86% identities between the two new oyster ferritins (Fig 4a.). Phylogenetic analysis indicates a logical conservation within and between groups. The two new oyster ferritins fits in the same group with the pearl oyster and the mollusks constitute a relevant cluster (Fig 4b).

The amino acid residues responsible for the fixation on ferritin, oxidation and incorporation of iron in the hydrous iron(III) oxide mineral core are obviously conserved in oyster ferritin. The primary avenue by which Fe^{2+} gain access to the interior of ferritin is through the eight 3-fold hydrophilic channels with two important residues: D 131 and E 134 (human H ferritin sequence numbering system). These amino acids D 129 and E 132 are present in the two oyster ferritins. The other residues of this site are: C 130, H 118, L 114, L 138, R 76, D 126 (human H ferritin sequence numbering system). All these amino acids are present in the two oyster ferritin subunits: C 128, H 116, L 112, L 136, R 74, D 124. All the amino acid residues constituting the H-specific ferroxidase center where oxidation and nucleation are achieved (E 27, Y 34, E 61, E 62, E 64, H 65, E 67, E 107, Q 141) (human H ferritin sequence numbering system), are conserved in the oyster ferritin subunits (E 25, Y 32, E 59, E 60, E 62, H 63, E 65, E 105, Q 139). An other group of amino acids is conserved in vertebrate H-ferritin (Y 29, Y 32, Y 34, Y 137, L 138) (human H ferritin sequence numbering system) and these specific residues are also found in oyster ferritin (Y 27, Y 30, Y 32, Y 135, L 136).

3.3. Expression of ferritin mRNA

Northern blot analyses were carried out using total RNAs extracted from different tissues of the oysters (Fig. 5). This hybridization was aimed at identifying preferential spatial ferritin expression among the different oyster organs. Obviously, the ferritin is widely distributed throughout the oyster tissues. An apparent higher expression can be seen in hemocytes and the heart (containing

in majority hemocytes also). The digestive gland constitutes a logical place for expression when referring to the literature.

3.4. Purification of the protein

Taking advantage of thermostability and resistance to proteolysis of ferritin, the purification of oyster ferritin was performed after denaturation at 65°C of the visceral masses homogenate to remove other proteins. The ferritin was purified by salt fractionation and ion exchange chromatography followed by Sephacryl S300 gel filtration. In calibrated TSK G4000 chromatography, native oyster ferritin eluted in the same range of volume as horse ferritin with a molecular weight from 420 to 440 kDa. The purified oyster ferritin contained around 29 mg Fe/ g of ferritin. Under reducing conditions in SDS/PAGE the oyster ferritin migrated as one band with a molecular mass of about 20 kDa, which is similar to the size of ferritin subunits of other organisms (Fig. 6).

The N-terminal sequence of the purified oyster ferritin was blocked, suggesting an acetylation as in other ferritins, but two internal part of tryptic peptide fragment was sequenced, corresponding to a region in the deduced amino acid sequence of the ferritin GF1 and not GF2 thus authenticating the obtained cDNA sequence of oyster ferritin (fig 1). The deduced molecular masses from computer analysis are 19.9 kDa with a calculated pI of 5.4 for the two subunits. These masses and pI are in the range observed for other vertebrate and invertebrate ferritins.

4. Discussion

We clearly identified two different oyster ferritins belonging to the same type showing two 20 kDa subunits. An internal peptide sequence of the oyster ferritin electrophoretic band has confirmed it to be one of the sequenced ferritins.

The two new genes show some similarities with H-type ferritins mainly by the presence of the ferroxidase center in the two types of subunits and confirmed by the alignment identities (62% and 63% with human H-ferritin and 50% and 51% identities with human L-ferritin). It seems probable that the common ancestor of all ferritins may have been of the H-type and that differentiation into H and L-types occurred late in vertebrate evolution (Dietzel et al., 1992). The higher similarity of oyster ferritin to the H vertebrate isoform is simply due to a faster evolutionary rate of L ferritins.

Only one other bivalve ferritin sequence has been published so far (*Pinctada fucata*) with only one cDNA (Zhang et al., 2003). In this species only one type of subunit was described. The two cDNA of *Crassostrea gigas* ferritins contained ORFs encoding 171 amino acids. The *Pinctada fucata* ferritin cDNA encodes a 206 amino acid polypeptide. The sequence of the first 171 amino acids of the *Pinctada fucata* ferritin shows a very high percentage of identity with the sequences of the ferritin subunits of *Crassostrea gigas* (85.4% with GF1 and 88.3% with GF2). Surprisingly, the percentage of similarity obtained in these conditions between GF2 and *Pinctada fucata* ferritin is higher than the percentage obtained between GF1 and GF2 (86%). The main difference between *Crassostrea gigas* ferritin and *Pinctada fucata* ferritin is the extension of 35 amino acids at the carboxylic end of *Pincata fucata* ferritin. Most ferritins of mollusks and echinoderm have a number of amino acids very close to those of *Crassostrea gigas* ferritins (*Lymnaea* 174, *Helix* 172, *Octopus* 172, *Asteria* 171), only *Liolophura japonica* has a higher number of amino acids (223) with two insertions and one N-terminal extension.

Similarly, the number of amino acid residues in vertebrate ferritin subunits is very close to those of *Crassostrea gigas* ferritins, generally in the range of 170 to 185 amino acids. Other ferritins are relatively different. The plant ferritins contain a N-terminal extension that is removed from the mature ferritin. This part of the polypeptide targets the protein for the transport from the cytoplasm to plastids (Plastid Targeting Sequence) (Harrison and Arosio, 1996, Bruce, 2000). Some other ferritins such as yolk *Lymnaea* ferritin or *Liolophura* ferritin have insertions of amino acids. They may lead to special conformations or important functions (Von Darl et al., 1994). Special characteristics of the N-terminal polypeptide were also observed in insect secreted ferritins and mitochondrial ferritin (Levi et al., 2001, Nichol et al., 2002).

The translational control of ferritin synthesis by iron is managed by iron regulatory proteins (IRPs). In mammals, IRP1 and IRP2 regulate translation by binding to iron responsive elements (IREs) located in the untranslated regions (UTRs) of mRNA encoding ferritin subunits. When cellular iron levels are low, IRP/IRE interaction represses ferritin translation; if iron levels increase, a decline in IRP/IRE interaction results in increased synthesis of ferritin for iron storage (Theil, 1998). The analysis of cDNA encoding the two oyster ferritin subunits revealed the presence of "IRE-like elements" in the 5'UTRs.

The nucleotide sequence analysis shows high similarity with IRE of other mollusks, in particular with the only one other studied bivalve: *Pinctada fucata*. This sequence contains all the structural characteristics necessary for smooth functioning of the IRE (stem-loop structure needed to IRP fixation). The presence of these IRE-like elements indicates that oyster ferritin synthesis is probably post-transcriptionnally regulated by iron. It therefore remains to find the IRPs as well as to show that the IREs are functional.

The main role of oyster ferritin is obviously as in all other organisms to store and regulate the available iron at the cellular level. The characteristic sites of cytosolic H type ferritin are indeed present in the oyster ferritin subunits: the site of fixation of Fe^{2+}, the ferroxidase center and the nucleation site (Harrison and Arosio, 1996). These specific Fe-binding sites are probably involved in radionuclide fixation and accumulation in oyster ferritin (Durand et al., 2002). As L type subunits are not present in oyster ferritin they may be the major sites of radionuclide binding on the ferritins of different species. These oyster ferritins do not show the special features of secreted ferritins as it may be found in insect ferritins (Nichol et al., 2002).

The level of ferritin expression is however quite high in all the oyster tissues. This can be compared to the observations of different authors who suggested other functions related to the presence of iron in various tissues of other organisms. The ferritin may be involved in shell formation from the pearl oyster (Zhang et al., 2003). It was suggested that echinoderm ferritin is an acute phase protein and that iron sequestration is important in invertebrate immune response (Beck et al., 2002). Several results also support a role of ferritin as a protectant against oxygen free radical-mediated damage (Orino et al., 2001). Other investigations suggested that ferritin may be a gene regulator or may be involved in cellular proliferation (Le and Richardson, 2002).

Further studies will have to considered isolation of the IRPs and to investigate the distribution of ferritin subunits in the different oyster tissues. It will be also important to undertake experiments to ensure identification of sites of fixation of metals or radio-elements on ferritin subunits. It appears that ferritin is a very important molecule for cellular safety in all animals.

Acknowledgment

We thank D. Mazzocut (IBCP Lyon, France) for technical support, M.A. Cambon-Bonavita (IFREMER Brest, France) for discussions on evolution of ferritins and K. Czerwinski (MIT USA) for help in the preparation of the manuscript.

References

Andrew, S.C., Arosio, P., Bottke, W., Briat, J.F., Von Darl, M., Harrison, P.M., Laulhère J.P., Levi, S., Lobreaux, S., Yewdall, S.J., 1992. Structure, function, and evolution of ferritins. J. Inorg. Biochem. 47,161-174.

Beck, G., Ellis, T.W., Habicht, G.S., Schluter, S.F., Marchalonis, J.J., 2002. Evolution of the acute phase response: iron release by echinoderm (*Asterias forbesi*) coelomocytes, and cloning of an echinoderm ferritin molecule. Dev. Comp. Immunol. 26, 11-26.

Bruce, B.D., 2000. Chloroplast transit peptides: structure,function and evolution. Trends Cell Biol. 10, 440-447.

Cairo, G., Rappocciolo, E., Tacchini, L., Schiaffonati, L., 1991. Expression of the genes for the ferritin H and L subunits in rat liver and heart: evidence for tissue-specific regulations at pre- and post-translational levels. Biochem. J. 275, 813-816.

Charlesworth, A., Georgieva, T., Gospodov, I., Law, J.H., Dunkov, B.C., Ralcheva, N., Barillas-Mury, C., Ralchev, K., Kafatos, F.C., 1997. Isolation and properties of *Drosophila melanogaster* ferritin. Molecular cloning of a cDNA that encodes one subunit, and localization of the gene on the third chromosome. Eur. J. Biochem. 247, 470-475.

Dietzel, J., Hirzmann, J., Preis, D., Symmons, P., Kunz, W., 1992. Ferritin of *Schistosoma mansoni*: sequence comparison and expression in female and male worms. Mol. Biochem. Parasitol. 50, 245-254.

Durand, J.P., Carvalho, F.P., Goudard, F., Pieri, J., Fowler, S.W., Cotret, O., 1999. ^{210}Po binding to metallothioneins and ferritin in the liver of teleost marine fish. Mar. Ecol. Prog. Ser. 177, 189-196.

Durand, J.P., Goudard, F., Barbot, C., Pieri, J., Fowler, S.W., Cotret, O., 2002. Ferritin and hemocyanin: ^{210}Po molecular traps in marine fish, oyster and lobster. Mar. Ecol. Prog. Ser. 233, 199-205.

Gueguen, Y., Cadoret, J.P., Flament, D., Barreau-Roumiguière, C., Girardot, A.L., Garnier, J., Hoareau, A., Bachère, E., Escoubas, J.M., 2003. Immune gene discovery by expressed sequence tags generated from hemocytes of the bacteria-challenged oyster, *Crassostrea gigas.* Gene 303, 139-145.

Harrison, P.M., Arosio, P., 1996. The ferritins: molecular properties, iron storage function and cellular regulation. Bioch . Biophys. Acta. 1275, 161-203.

Henikoff, S., Henikoff, J.G., 1992. Amino acid substitution matrice from protein blocks. Proc. Natl. Acad. Sci. USA 89 (22), 10915-10919.

Huang, T.S., Law, J.H., Söderhäll, K., 1996. Purification and cDNA cloning of ferritin from the hepatopancreas of the freshwater crayfish *Pacifastacus leniusculus*. Eur. J. Biochem. 236, 450-456.

Kumar, S., Tamura, K., Jacobsen, I.B., Nei, M., 2001. MEGA2: Molecular Evolutionary Genetics Analysis software. Bioinformatics 17(12), 1244-1245.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.

Le, N.T.V., Richarson, D.E., 2002. The role of iron in cell cycle progression and the proliferation of neoplastic cells. B.B.A. 1603, 31-46.

Lehmann, M., Culig, H., Taylor, D.M., 1983. Identification of transferrin as the principal plutonium-binding protein in the blood serum and liver cytosol of rats: immunological and chromatographic studies. Int. J. Radiat. Biol. 44(1), 65-74.

Levi, S., Corsi, B., Bosisio, M., Invernizzi, R., Volz, A., Sanford, D., Arosio P., Drysdale J., 2001. A human mitochondrial ferritin encoded by an intronless gene. J. Biol. Chem. 270 (27), 24437-24440.

Myers, E.W., Miller, W., 1988. Optimal alignments in linear space. Comput. Appl. Biosci. 4, 11-17.

Nichol, H., Law, J.H., Winzerling, J.J., 2002. Iron metabolism in insects. Ann. Rev. Entomol. 47, 535-559.

Orino, K., Eguchi, K., Nakayama, T., Yamamoto, S., Watanabe, K., 1997. Sequencing of cDNA clones that encode bovine ferritin H and L chains. Comp. Biochem. Phys. 118B(3), 667-673.

Orino, K., Lehman, L., Tsuji, Y., Ayaki, H., Torti, S.V., Torti, F.M., 2001. Ferritin and the response to oxidative stress. Biochem. J. 357, 241-247.

Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406-425.

Stover, B.J., Bruenger, F.W., Stevens, W., 1970. Association of americium with ferritin in the canine liver. Radiat. Res. 43, 173-186.

Theil, E.C., 1987. Ferritin: structure, gene regulation, and cellular function in animals, plants, and microorganisms. Ann. Rev. Biochem. 56, 289-315.

Theil, E.C., 1998. The iron responsive element (IRE) family of mRNA regulators. Regulation of iron transport and uptake compared in animals, plants and microorganisms. in Dekker, (Ed.), Metal ions in biological systems, New-York, USA, 35, pp.403-434.

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W : improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673-4680.

Von Darl, M., Harrison, P.M., Bottke, W., 1994. cDNA cloning and deduced aminoacid sequence of two ferritins: soma ferritin and yolk ferritin, from the snail *Lymnaea stagnalis* L. 222, 353-366.

Xie, M., Hermann, A., Richter, K., Engel, E., Kersch baum, H.H., 2001. Nitric oxide up-regulates ferritin mRNA level in snail neurons. Eur. J. Neurosci.13, 1479-1486.

Zhang, Y., Meng, Q., Jiang, T., Wang, H., Xie, L., Zhang, R., 2003. A novel ferritin subunit involved in shell formation from the pearl oyster (*Pinctada fucata*). Comp. Biochem. Phys. B 135, 43-54.

Zinovieva, R.D., Piatigorsky, J., Tomarev, S.I., 1999. O-crystallin, arginine kinase and ferritin from the octopus lens. B.B.A. 1431, 512-517.

Legends

Table 1: Origin and identification number of the 10 ferritin ESTs, as shown on the database http://www.ifremer.fr/GigasBase, The 2 contig group are indicated A or B.

Figure 1 : Nucleotide sequence and deduced amino acid sequence of the two *Crassostrea gigas* ferritin cDNA. Sequences in small letters correspond to UnTranslated Regions; Primers used for Race and sequencing are underlined; the putative iron response element in the 5'UTR is indicated by the double lines. In bold underlined on the GF1 sequence, the exact match between the sequences in nucleic and amino acids.

Figure 2 : IRE alignement of several different species. After Huang et al. 1996; Beck et al. 2002.

Figure 3 : Predicted IRE structure of the three known bivalve ferritin cDNA.

Figure 4 : a) Table indicating the percentage identities of several invertebrate ferritin amino acids. Alignment were performed pair wise using ALIGN version 2 (Myers and Miller 1989). b) Phylogenetic analysis of the two new oyster ferritin GF1 and GF2 showing relationship with other proteins. Alignment of amino acid sequences with CLUSTALW, consensus Neighbor-Joining tree with MEGA 2.1 (Kumar et al. 2001), bootstrap value ≥70% are indicated.

Figure 5 : Northern blot analysis of *Crassostrea gigas* ferritin gene expression using total cellular RNA isolated from adductor muscle (AM), mantel margin (Mm), mantel inner surface (Mi), heart (Ht), gills (G), labial palps (LP), digestive gland (DG), stomach (S) and hemocytes (He). Blot containing 35 µg of total RNA per lane was hybridized with radio labeled ferritin and ribosomal probe.

Figure 6 : SDS gel electrophoretic pattern of purified ferritin. Lane 1: oyster (*Crassostrea gigas*) ferritin; Lane 2 : low molecular weight markers (Bio-Rad).

Ferritin number	**1**	**2**	**3**	**4**	**5**
Contig	A	A	B	A	A
ID number :	012-A-02	012-E-10	012-E-24	012-N-01	017-B-05
GenBank number :	BQ426319	BQ426414	BQ426427	BQ426605	BQ426697
dbEST ID :	12530373	12530468	12530481	12530659	12530751
Ferritin number	**6**	**7**	**8**	**9**	**10**
Contig	A	A	A	B	B
ID number :	017-D-03	017-M-04	027-H-22	027-I-20	027-E-22
GenBank number :	BQ426741	BQ426944	BQ427190	BQ427210	BQ427126
dbEST ID :	12530795	12530998	12531244	12531264	12531180

Table 1

GF1

```
                                    Iron Response Element
                                ttacttgtcttgctgcgtcagtgaacgtacggac -63
aaaaatcttactcctaaatccatccctacttgagagattatttcaccatcggaactttcacc  -1
ATGGCCGAATCCCAATGTCGCCAAAATTACCACCAGGAGAGCGAAGCTGGCATCAACCGCCA  62
M  A  E  S  Q  C  R  Q  N  Y  H  Q  E  S  E  A  G  I  N  R  Q
AATCAACATGGAACTGTATGCCTGCTATACCTACCAGTCCATGGCCTACTACTTCGATAGAG 124
 I  N  M  E  L  Y  A  C  Y  T  Y  Q  S  M  A  Y  Y  F  D  R  D
ATGATGTGGCCCTTCCTGGATTCAGCAAGTTCTTCAAGAATTCATCAGATGAAGAACGGGAA 186
  D  V  A  L  P  G  F  S  K  F  F  K  N  S  S  D  E  E  R  E
CATGCTGAAAAACTGATGAAGTACCAGAACAAGAGAGGAGGGCGTGTCGTGCTCCAAGACAT 248
H  A  E  K  L  M  K  Y  Q  N  K  R  G  G  R  V  V  L  Q  D  I
CAAGAAACCCGACCGTGATGAGTGGGGCACCGGCTTGGACGCCATGCAGGTGGCGCTACAGC 310
 K  K  P  D  R  D  E  W  G  T  G  L  D  A  M  Q  V  A  L  Q  L
TGGAGAAGACTGTGAACCAGTCTCTGCTTGACCTTCACAAGGTCGCTGACAGTCACAAGGAT 372
  E  K  T  V  N  Q  S  L  L  D  L  H  K  V  A  D  S  H  K  D
GCACAGATGTGCGATTTCCTTGAGACCCACTACTTGGAGGAGCAAGTGAACGCCATCAAGGA 434
A  Q  M  C  D  F  L  E  T  H  Y  L  E  E  Q  V  N  A  I  K  E
GATATCGGACCACATCACTCAGTTGAAGAGAGTGGGCAGTGGGCTGGGAGAGTATGAATACG 496
I  S  D  H  I  T  Q  L  K  R  V  G  S  G  L  G  E  Y  E  Y  D
ATCGCCGCCTCGACTCCtaaagctcacacagagactgttacacccagcaactagtgtttagt 558
  R  R  L  D  S  *
gtcaaaatttacttttctgttaattgaattatggtggacatttatagttaaattttgtttt 620
gaatcgataacggagtttttaaaaaaaaaaaatgggtgtatataattatgaaaaaaaaaaaa 682
aaaaaa                                                         688
```

GF2

```
                                    Iron Response Element
                                gttttgctgcgtcagtgaacgtacggacgggat -63
tgtcacagaagtctttcacgttctcttttaacgttttacaaactcaacaacatccgaaaaa  -1
ATGTCTCAGAGTCAGCCTCGCCAGAACTTCCACGAGGAAAGTGAAGCAGGGATCAACCGACA  62
M  S  Q  S  Q  P  R  Q  N  F  H  E  E  S  E  A  G  I  N  R  Q
GATCAACATGGAGCTGTACGCCTCGTACACATACCAGTCCATGGCTCTCTACTTCGATCGGG 124
 I  N  M  E  L  Y  A  S  Y  T  Y  Q  S  M  A  L  Y  F  D  R  D
ACGATGTGGCTTTGCCAGGATTTCATAAGTTCTTCAAGCACTCGTCTGACGAGGAACGTGAG 186
  D  V  A  L  P  G  F  H  K  F  F  K  H  S  S  D  E  E  R  E
CATGCCGAGAAGTTGATGAAATACCAAAACAAAAGAGGAGGCCGTATTGTGCTGCAAGACAT 248
H  A  E  K  L  M  K  Y  Q  N  K  R  G  G  R  I  V  L  Q  D  I
CAAGAAACCTGACCGTGATGAGTGGGGAACAGGACTTGACGCCATGCAGATAGCCCTCCAAC 310
  K  K  P  D  R  D  E  W  G  T  G  L  D  A  M  Q  I  A  L  Q
TGGAGAAGAGTGTCAACCAATCCCTGCTTGACCTTCACAAGTTGGCAGACGTTCACCGCGAT 372
L  E  K  S  V  N  Q  S  L  L  D  L  H  K  L  A  D  V  H  R  D
GCTCAGATGTGTGATTTCATCGAGTCGGAATTCCTTGAGGAGCAGGTTAATGCCATCAAGGA 434
 A  Q  M  C  D  F  I  E  S  E  F  L  E  E  Q  V  N  A  I  K  E
GATTTCTGACCACGTCACCCAGCTGAAGCGCGTGGGGGCAGGTCTGGGCGAGTATGAGTATG 496
  I  S  D  H  V  T  Q  L  K  R  V  G  A  G  L  G  E  Y  E  Y
ACAAACAACTCCAGAGCtgaaccaatctccacagctgccagtccatgttataatccggctct 558
D  K  Q  L  Q  S  *
attgccctgtgtttccagttttggacttctattgtgcatttaaattactaactctgttattt 620
gactggcatttactcagtttattgatcaaaaaacaaataaaaaaactgtaaaacgtt      677
```

Figure 1

```
Oyster GF1:         tcttgctgcgtcagtgaacgtacggac
Oyster GF2:         ttttgctgcgtcagtgaacgtacggac
Pinctada f.:        gcttgctgcgtcagtgaacgtacgggc
Lymnea S:           tcttgctgcgtcagtgaacgtacagac
Crayfish:           tccggg-tcgccagtgtgtgaacgagc
Rana:               tcttgcttcaacagtgttgaacggaa
ChickenH:           tcctgcgtcaacagtgcttggacggaa
RatH:               tcctgcttcaacagtgcttgaacggaa
HumanH:             tcctgcttcaacagtgcttggacggaa
HumanL:             tcttgcttcaacagtgttgacgaaca
MouseL:             tcttgcttcaacagtgttgaacggaa
```

Figure 2

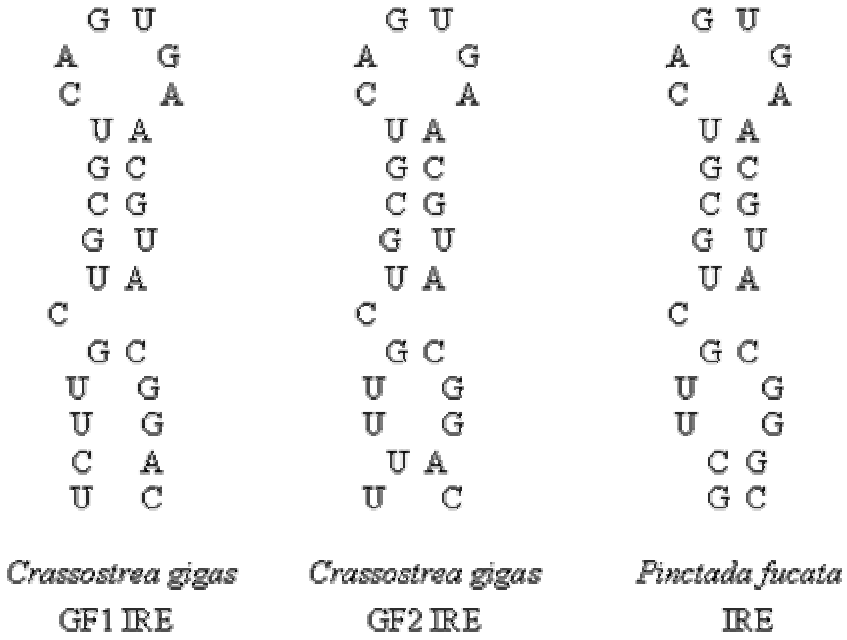
G U
A G
C A
U A
G C
C G
G U
U A
C
G C
U G
U G
C A
U C
Crassostrea gigas
GF1 IRE
G U
A G
C A
U A
G C
C G
G U
U A
C
G C
U G
U G
U A
U C
Crassostrea gigas
GF2 IRE
G U
A G
C A
U A
G C
C G
G U
U A
C
G C
U G
U G
C G
G C
Pinctada fucata
IRE

Figure 3

a)

	GF2	Snail S	Helix	Pinctada	Octopus	Liolophura	Starfish
GF1	86	79.30	79.1	85.4	64	42.3	67.6
GF2	-	82.2	79.1	88.3	66.9	40.7	68.8

b)

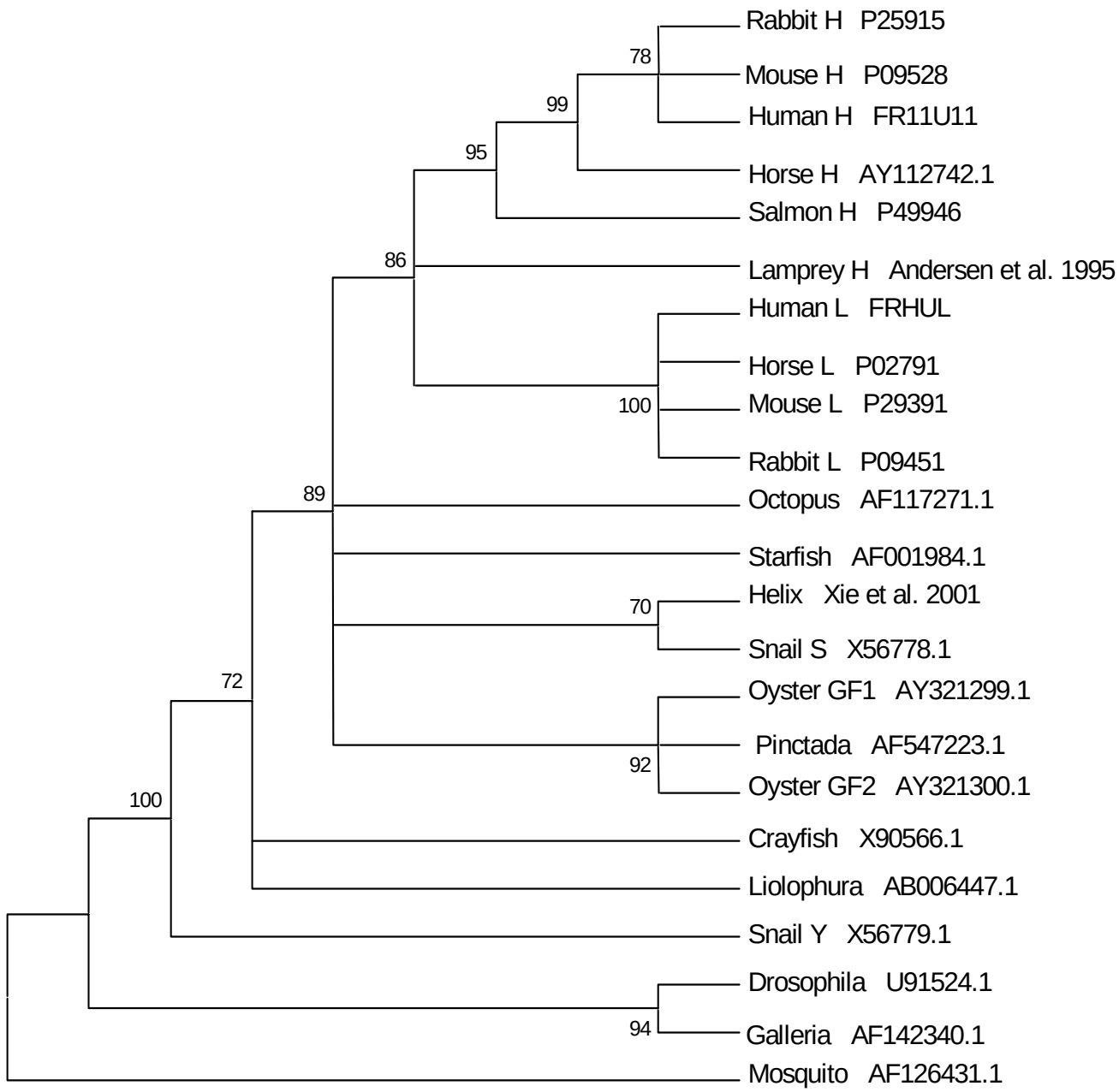

Figure 4

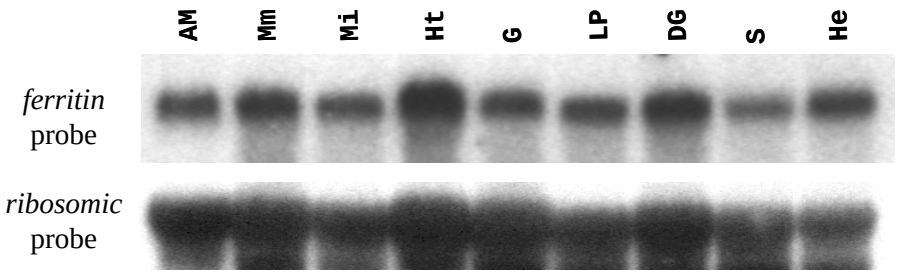

Figure 5

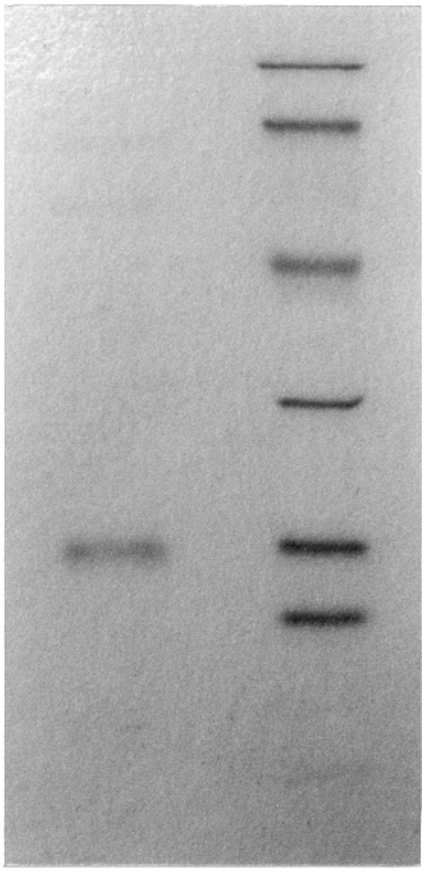

Figure 6