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Methods paper

Molecular characterization of the iron binding protein ferritin in *Eisenia andrei* earthworms

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ABSTRACT

Ferritin is a storage protein that plays a key role in iron metabolism. In this study, we report on the sequence characterization of a ferritin-coding cDNA in *Eisenia andrei* earthworms isolated by RT-PCR using degenerated primers, and we suggest the presence of a putative IRE in the 5′-UTR of ferritin mRNA. The obtained ferritin sequence was compared with those of other animals showing sequence and structure homology in consensus sites, including the iron-responsive element (IRE) and ferroxidase centers. Despite the sequence homology in the *E. andrei* mRNA of ferritin with the sequences of other animals in consensus IRE sites, the presented cytosine in the IRE of *E. andrei* ferritin in the expected position does not form a conventional bulge. The presence of ferritin in the coelomic fluid of *E. andrei* was proven by iron staining assay. Moreover, aconitase activity in the coelomic fluid was assessed by aconitase assay, suggesting the presence of an iron regulatory protein. Quantitative analysis revealed changes in the gene expression levels of ferritin in coelomocytes in response to bacterial challenge, reaching the maximum level 8 h after the stimulation with both Gram-positive and Gram-negative bacteria.

1. Introduction

Iron is an essential element for all living organisms, and ferritin is the major iron-storage protein. It plays a key role in iron homeostasis, and it is ubiquitously present in animals, plants, fungi and bacteria. Ferritins from different organisms vary in size, distribution, and ways of regulation, despite sharing common features in their sequences and structures (Andrews et al., 1992; Harrison and Arosio, 1996). Most ferritins have a similar structure and consist of 24 subunits forming a hollow capable of storing up to 4500 iron atoms as a ferric inorganic complex (Aisen et al., 1999). In vertebrates, ferritin is composed of two types of polypeptides, heavy (H) and light (L) chain, that are encoded by different genes (Harrison and Arosio, 1996). The majority of invertebrate ferritins, including those of the sea star *Asterias forbesi* (Beck et al., 2002), Pacific giant oyster *Crassostrea gigas* (Durand et al., 2004), great pond snail *Lymnaea stagnalis* (von Darl et al., 1994), pearl oyster *Pinctada fucata* (Zhang et al., 2003), crayfish *Pacifastacus leniusculus* (Huang et al., 1996), dog tick *Dermacentor variabilis* (Mulenga et al., 2004) and marine polychaete *Periserrula leucophryna* ferritin (Jeong et al., 2006) are more similar to the vertebrate H-type subunit, while insect ferritins are more closely related to the vertebrate L-type subunit (Charlesworth et al., 1997; Dunkov et al., 1995; Nichol et al., 2002).

Iron exists in two oxidative states, ferrous (Fe^{2+}) and ferric (Fe^{3+}). In aqueous solution, the Fe is readily oxidized from Fe^{2+} to Fe^{3+}. It can also make iron toxic by the production of oxygen-derived radicals (Baker et al., 2003; Hentze and Kuhn, 1996). Therefore, iron regulation by iron-binding proteins is essential in all organisms. The molecules of ferritins are able to incorporate iron due to the presence of the ferroxidase centers (Andrews et al., 1992; Theil, 1987), which are also conserved in most invertebrates. The expression of ferritin is regulated at the posttranscriptional level by the interactions between an iron regulatory protein (IRP) and the iron-responsive element (IRE) in the 5′-untranslated region (UTR) of ferritin mRNA (Hentze et al., 1989). IRE-containing mRNAs encode proteins of iron storage and transport as well as proteins involved in iron utilization (Hentze and Kuhn, 1996). Two IRPs have been described in vertebrates so far. IRP1 can either bind an IRE site or function as a cytosolic isoform of aconitase, while IRP2 has only the IRE-binging activity (Guo et al., 1994). The conversion of IRP1 between an IRP-binding protein and aconitase is regulated by iron, through the folding or dissociation of a 4Fe–4S cluster (Caughman et al., 1988; Jaffrey et al., 1993; Theil, 1994). At low iron levels, IRP1 binds to the IRE and, thus, blocks ribosome binding and the subsequent translation of ferritin (Gray and

Abbreviations: PCR, polymerase chain reaction; IRE, iron responsible element; IRP, iron regulatory protein; UTR, untranslated region; LBSS, *Lumbricus* balanced salt solution; *Ea-Fer*, gene encoding *Eisenia andrei* ferritin.

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Hentze, 1994). Once the level of intracellular iron is increased, a 4Fe–4S cluster in IRP1 is assembled, resulting in the loss of high-affinity RNA binding capacity in the acquisition of aconitase activity and in the translation of ferritin. Aconitases are iron–sulfur enzymes that interconvert citrate to isocitrate through a stereospecific reversible isomerization, which requires the binding of the substrate to the 4Fe–4S cluster (Beinert et al., 1996; Walden, 2002). A putative IRE site is observed in the 5′UTR of ferritin mRNA containing a typical CAGUGN loop and unpaired or "bulged" C residue five nucleotides upstream from the loop, which appears to be important for the function of IREs (Henderson et al., 1994).

Here we report on the sequence characterization of a ferritin-coding cDNA in *Eisenia andrei* earthworms isolated by RT-PCR using degenerated primers. We aligned the sequence of *E. andrei* ferritin with those of other animals having structural and sequence homology in IRE sites and ferroxidase centers. We proved the presence of ferritin in the coelomic fluid by iron staining assay. Furthermore, enzyme assay confirmed aconitase activity in the coelomic fluid, suggesting the presence of an iron regulatory protein. Quantitative PCR analysis revealed the changes in the gene expression levels of ferritin in coelomocytes in response to bacterial challenge, suggesting a role of ferritin in earthworm defense.

2. Materials and methods

2.1. Isolation of coelomic fluid and coelomocytes

Adult *E. andrei* (*Oligochaeta, Annelida*) earthworms were maintained on moist paper towels without food for 2 days to lower gut load and avoid sample contamination during coelomocyte collection. Coelomic fluid containing free coelomocytes was harvested by puncturing post-clitellum segments of the coelomic cavity with a Pasteur micropipette. Coelomocytes were isolated by centrifugation (500 *g*, 10 min, 4 °C) and washed twice with a *Lumbricus* balanced salt solution [LBSS; 71.5 mM NaCl, 4.8 mM KCl, 3.8 $CaCl_2$, 1.1 mM $MgSO_4$, 0.4 mM KH_2PO_4, 0.4 mM NaH_2PO_4, 4.2 mM $NaHCO_3$; pH 7.3 (Stein and Cooper, 1981)] before being used in further experiments.

2.2. Stimulation of earthworms

Earthworms were stimulated with approximately 10^8 CFU of live Gram-negative bacteria *Escherichia coli* K12, live Gram-positive bacteria *Bacillus subtilis* W23, and a solution of 10 mM $FeCl_3$ (Sigma) in LBSS for 4, 8, 16, 24, and 48 h. Each experimental sample consisted of the pool of coelomic fluid of 5 individuals. Non-stimulated earthworms were used as negative controls.

2.3. RNA isolation, cDNA synthesis, PCR, and rapid amplification of cDNA ends (RACE)

Total RNA was isolated from the coelomocytes using the TRIZOL reagent (Invitrogen) according to the manufacturer's protocol. DNAse I-treated total RNA (2 μl) was reverse-transcribed using Oligo(dT)$_{12–18}$ primer and Superscript II RNase H$^-$ Reverse Transcriptase (Invitrogen) and then used in a PCR reaction. A set of degenerated primers FR1/FR2-5′-GCNCTNCCNGGNTTYGCNAAR-3′ and 5′-TCYTTDATNGARTCNACYT-GYTC-3′ was designed based on a sequence homology with ferritin of the oyster *C. gigas*, resulting in a specific PCR product of about 300 bp that was amplified using the following cycling parameters: 2 min at 94 °C, followed by 40 cycles of 30 s at 94 °C, 40 s at 56 °C, and 120 s at 72 °C and a final extension for 10 min at 72 °C. The PCR product was ligated in pCR2.1-TOPO cloning vector (Invitrogen) and sequenced. The 3′ end of the ferritin cDNA was obtained using the 3′ RACE System (Invitrogen). The total RNA (2 μg) was reverse-transcribed using an adapter primer (5′-GGCCACGCGTCGACTAGTACTTTTTTTTTTTTTTTT-3′). A universal amplification primer (5′-CUACUACUACUAGGC-CACGCGTCGACTAGTAC-3′) and the ferritin-specific internal sense primer Fer1 (5′-GATGAGGAAAGAGAACATGC-3′) were used in subsequent PCR reactions. Similarly, the 5′ end of the ferritin cDNA was obtained using the 5′ RACE System (Invitrogen). Reverse transcription was carried out using 2 μg of the total RNA and the ferritin-specific internal antisense primer Fer2 (5′-TGCTCCTCCAGGAAATCTGT-3′). Terminal deoxynucleotidyl transferase was used to add homopolymeric oligo-dC tails to the 3′ end of purified cDNA. The primers used in subsequent PCRs were a sense-abridged anchor primer (5′-GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG-3′) and the ferritin-specific internal primer Fer3 (5′-GGCAACCTTATGCAGATCCA-3′). Both 3′ and 5′ RACE products were cloned in pCR2.1-TOPO and sequenced (as described below).

2.4. Sequencing and structural analysis

Isolated and purified plasmid DNA was sequenced using the ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The chain termination reaction (Sanger et al., 1977) was performed by the cycle sequencing technique (Murray, 1989) according to the manufacturer's protocol. Finally, sequences were determined using an ABI PRISM 3100 DNA sequencer (Applied Biosystems). The nucleotide sequence of *E. andrei* ferritin (*Ea-Fer*) was submitted to the GenBank with accession no. FJ516398. The deduced amino acid sequence of the *E. andrei* ferritin and the ferritin molecules of other invertebrates were aligned using the CLUSTALW program (Thompson et al., 1994). The protein sequence was analyzed by ExPaSy for the prediction of the molecular mass and the pI of the *E. andrei* ferritin. The Mfold program was used for the design and comparison of the secondary structure of ferritin 5′UTR (Zuker, 2003).

2.5. Polyacrylamide gel electrophoresis

Native gel electrophoresis of the *E. andrei* coelomic fluid and of horse ferritin (Sigma), used as a positive control, was performed in 8% polyacrylamide gel. Gels were subsequently either stained with Coomassie blue or subjected to iron staining, with the gel immersed in 0.75 mM Ferene S (Sigma) and 15 mM thioglycollic acid (Sigma) in 2% acetic acid (Chung, 1985). The native holoferritin was visible after approximately 30 min.

2.6. Enzymatic in-gel digestion and mass spectrometry

The protein band was excised from the gel, washed, and digested with trypsin as described previously (Kuzelova et al., 2010). Mass spectra were acquired using an Ultraflex III MALDI-TOF/TOF instrument (Bruker Daltonics, Bremen, Germany) equipped with the LIFT™ technology for MS/MS analysis. The mass spectra were searched against the NCBInr 20110527 database subset of eukaryotic proteins using the in-house MASCOT search engine.

2.7. Electroelution of active proteins

Following the separation of coelomic fluid in native gels, bands visible after the iron staining were cut and either subjected to MALDI analysis or electroeluted by Biotrap (Schleicher & Schuell) electroelution buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3) at 200 V for 8 h. The eluted proteins were boiled and separated in 12% polyacrylamide gel. Dithiothreitol (10 mM) was used for the reduction of the sample.

2.8. Real-time PCR

The DNAse I-treated total RNA (2 μl) isolated from coelomocytes from the control and stimulated earthworms was reverse-transcribed using Random Hexamers (Invitrogen) and Superscript II RNase H$^-$