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Isolation and characterization of ferritin from the hepatopancreas of the mussel *Mytilus edulis*

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Summary. The main iron-binding protein in the hepatopancreas of the mussel *Mytilus edulis*, which had been previously iron-loaded by exposure to carbonyl iron (spheres of elemental iron less than 5 μm diameter), has been isolated to electrophoretic purity and identified as ferritin. This ferritin has M_r of 480000, pI of 4.7–5.0 and is composed of two subunits, M_r 18500 and M_r 24600. Under the electron microscope, it appears as electron-dense iron cores of average diameter 5 nm surrounded by a polypeptide shell to a final average overall diameter of 11 nm. The purified protein contains, on average, 200 iron atoms/molecule protein. On immunodiffusion, *M. edulis* hepatopancreas ferritin gives a partial cross-reaction with antiserum to horse spleen ferritin and lamprey *(Geotria australis)* liver ferritin but does not react with antiserum to chiton *(Acanthopleura hirtosa)* haemolymph ferritin.

Key words: Ferritin — *Mytilus edulis* — Hepatopancreas — Fe metabolism — Biosynthesis

Introduction

The edible mussel, *Mytilus edulis*, has a widespread distribution and has been employed widely as an indicator of marine pollution, including globally comparable studies, e.g. the monitoring programme Mussel Watch (Goldberg 1980). Toxicological and pharmacological studies of the effect of heavy metals, such as zinc, cadmium, mercury and lead, on *M. edulis* have extended from the early identification of low-molecular-mass sulphur-rich cadmium-binding proteins known as metallothioneins (Nöel-Lambot 1976; Talbot and Magee 1978) to more recent physiological studies (e.g. Viarengo et al. 1985a, b, 1987). It is well recognised that interactions between metals (such as Cd and Cu, Zn and Fe) can be important in determining the toxicological response of an organism (Cunningham 1979). Recently, we reported on the association of the iron-binding protein ferritin with high-M_r zinc-binding components in tissues of the tropical rock oyster *Saccostrea cucullata* (Webb et al. 1985).

In the case of *M. edulis*, the kinetics of iron uptake and the distribution of iron among tissues have been investigated (Hobden 1967, 1969; Pentreath 1973; George et al. 1976, 1977; George and Coombs 1977; Lowe and Moore 1979). Particulate iron, as colloidal iron(III) hydroxide, is taken up via pinocytosis with iron eventually being transported to other tissues. Both the gills and alimentary canal are avenues for absorption with the gills operating as a sieve with a mesh size of approximately 3–4 μm (Owen 1974a, b; Jørgensen 1983).

Histological observations have been interpreted (Bottke 1986; Miksys and Saleuddin 1987) to indicate the presence of ferritin-like proteins in tissues, including oocytes, where they presumably act as an iron store. Comparatively little information, however, is available concerning the biochemistry associated with the metabolism of iron in *M. edulis*. We have developed procedures for challenging *M. edulis* with iron, using carbonyl iron, which is composed of particles of elemental iron less than 5 μm in diameter and which has been reported to produce iron loading in rats (Bacon et al. 1983). In a study of iron accumulation by *M. edulis*, the hepatopancreas was identified as the main organ for iron storage (Bootsma, unpublished results) and we report here the identifica-

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tion of the major iron-binding protein in this organ as a ferritin protein.

Ferritins are constructed generally as oligomeric proteins whose subunits form a spherical surface layer of polypeptide surrounding an inner core rich in iron (Aisen and Listowsky 1980; Ford et al. 1984). The three-dimensional structure of horse-spleen ferritin has been determined, revealing molecular and atomic detail of the above structural type (Ford et al. 1984). The subunit compositions and core structures for ferritins isolated from the haemolymph of several species of marine molluscs have been reported elsewhere (Webb and Macey 1983; Burford et al. 1986; Kim et al. 1986; St Pierre et al. 1986; Webb et al. 1986a). In the present study, the characteristics of *M. edulis* ferritin are compared with those of several invertebrate and vertebrate ferritins.

Materials and methods

Specimens of *M. edulis* (50–60 mm shell length) were collected from Fremantle Harbour, Western Australia (32°S, 116°E). They were maintained in well-aerated (≈ 9 mg l^{-1} O_2) seawater aquaria. Iron was supplied as carbonyl iron (10 mg l^{-1}) contained within food and was readily accepted by the mussels. In addition, some animals were kept as controls, i.e. they were held in aquaria under identical conditions but without the addition of carbonyl iron. For isolation of ferritin, iron loading was carried out for 21 days, after which time pooled samples of hepatopancreas (generally from 10 animals) were homogenised in borate buffer (0.025 M, pH 8.6) containing 0.15 M NaCl using a tissue homogeniser (Polytron). In initial experiments, the homogenate was gently filtered through Miracloth (Bio-Rad) before being applied to a column (45 × 1 cm) of Sephacryl S-300 (Pharmacia) equilibrated with the above buffer. Once the major iron-binding protein had been tentatively identified as ferritin, routine preparation of hepatopancreas ferritin employed heating (70° C, 10 min) and centrifugation (10000 *g*, 20 min) followed by chromatography on a column (45 × 2.6 cm) of Sephadex G-75 (Pharmacia). Chromatographic fractions were collected, their absorbance monitored for protein at 280 nm and, when necessary, their iron content determined by atomic absorption spectrophotometry (Perkin Elmer 505 spectrophotometer). The ferritin-containing fractions eluted at the void volume of the Sephadex G-75 column and were subsequently concentrated by ultrafiltration over an Amicon PM30 membrane. The concentrate was purified further by polyacrylamide gel electrophoresis (PAGE) in 5% gels using a Tris/glycine buffer, pH 8.6 (Fehrnström and Moberg 1977). The gel section containing the prominent brown band of ferritin was homogenised and clarified by centrifugation (10000 *g*, 20 min), yielding a colourless pellet and a brown supernatant. Final concentration of the supernatant was carried out by PM30 ultrafiltration.

The above procedures were monitored by analytical PAGE on 7.5% gels, pH 8.6 (Fehrnström and Moberg 1977). Duplicate gels were stained for protein (Coomassie brillant blue R-250) and for iron [$K_4Fe(CN)_6$, 2%, and HCl, 2%, mixed 1:1 (by vol.) immediately before use].

The molecular size of the purified ferritin was determined by molecular exclusion chromatography on the Sephacryl S-300 column calibrated with the following marker proteins: thyroglobulin (M_r 667000), horse-spleen ferritin (476000), catalase (232000) and bovine serum albumin (67000). The isoelectric point, pI, was determined by isoelectric focussing in polyacrylamide gels (5%) with 2.6% cross-linking (Winter and Andersson 1977). Ampholines (2%; LKB) over pH range 2–10 were used initially, with gels in pH range 4–6 being used for final pI determinations.

Ferritin was dissociated into subunits by heating (90° C, 3 min) in the presence of sodium dodecyl sulphate (SDS, 2%) and 2-mercaptoethanol (5%). Subunits were separated (Fehrnström and Moberg 1977) by SDS-PAGE (10%) using a M_r marker proteins bovine serum albumin (67000), egg albumin (45000), pepsin (34700), trypsinogen (24000), β-lactoglobulin (18400) and lysozyme (14300).

Purified preparations were assayed for protein using the modified Lowry assay (Hess et al. 1978). Electron microscopy was performed at 60 kV (Philips 301 electron microscope) on solutions air-dried onto Formvar-coated copper grids which were either untreated or negatively stained with phosphotungstic acid (2%, pH 6.0, 5 min). Immunological cross-reactivity was determined using the double-immunodiffusion method of Ouchterlony (Allen et al. 1977) in agarose gels (1%) incubated overnight at 37° C. Immunodiffusion was carried out between *M. edulis* hepatopancreas ferritin and antiserum to ferritin isolated (Kim et al. 1986) from the haemolymph of the chiton *Acanthopleura hirtosa*, syn. *Clavarizona hirtosa* (Ferreira 1986), antiserum to ferritin isolated (Macey, unpublished results) from the liver of the lamprey *Geotria australis* and antiserum to horse-spleen ferritin (Miles-Yeda).

Results

Direct chromatography of the homogenate from *M. edulis* hepatopancreas on Sephacryl S-300 revealed a complex profile of ultraviolet absorbing material (Fig. 1a). Analysis of chromatographic fractions for iron indicated that the bulk of the iron eluted in high M_r fractions near the void volume (Fig. 1b).

This complexity was confirmed by electrophoresis of crude homogenates from test animals and from controls. Electrophoretic patterns from both groups of animals contained several bands but differed in that a slowly migrating band was distinctly more intense in the case of the homogenate from iron-loaded animals. Heat treatment of the hepatopancreas homogenate resulted in a considerably simplified pattern that was dominated by this slowly migrating band. This band was located somewhat closer to the origin than was that of horse-spleen ferritin which was used as an additional control, and gave, moreover, a positive reaction with the $K_4Fe(CN)_6$ iron stain (Fig. 2). A small iron-staining band was also observed just below the origin and was assigned to oligomers of the main iron-binding protein (Theil 1983). The use of preparative gel electrophoresis in subsequent preparations allowed these oligomers to be separated from the main component.

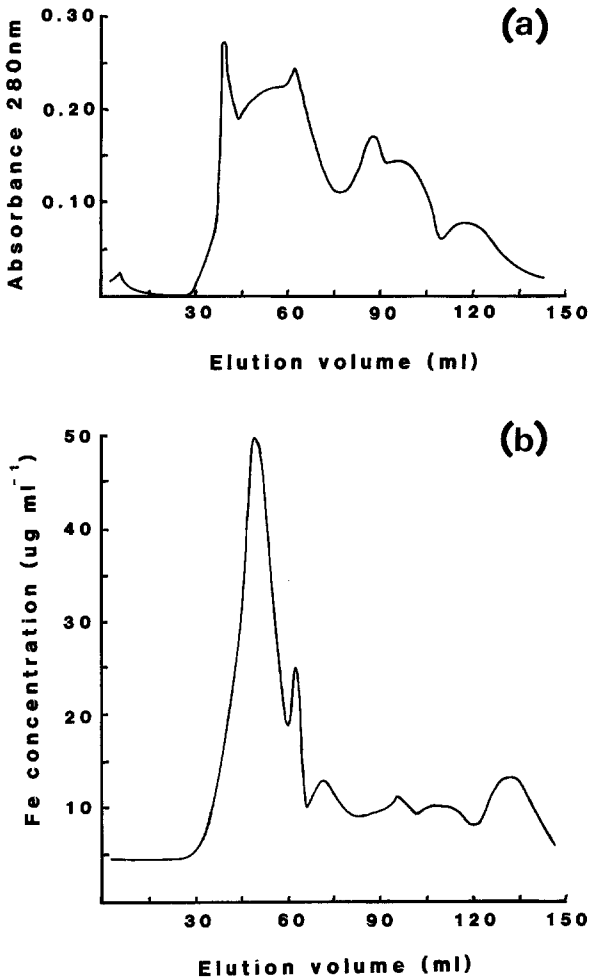

Fig. 1a, b. Elution profile of homogenate of hepatopancreas of iron-loaded *Mytilus edulis* on Sephacryl S-300: **a** absorbance at 280 nm; **b** iron concentration

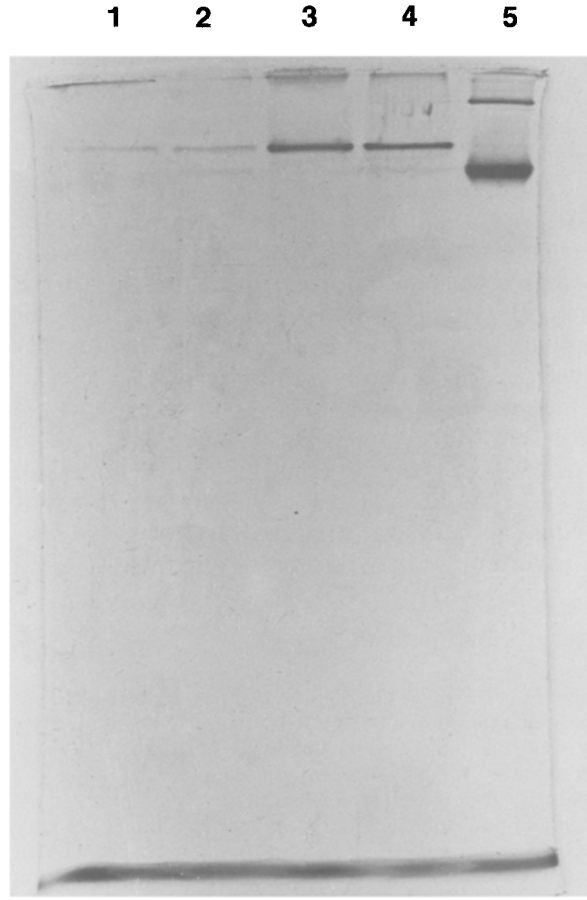

Fig. 2. Polyacrylamide gel electrophoresis profiles of *M. edulis* homogenates from control *(1, 2)* and iron-loaded *(3, 4)* animals. The gel has been stained for iron. Samples 1 and 3 were heat-treated, horse-spleen ferritin is on gel 5

Electron microscopy of unstained preparations of the main iron-binding protein revealed an electron-dense core of mean diameter 5 nm and, on negative staining with phosphotungstic acid, a halo of electron-poor material (i.e. polypeptide) around the core. These data, which are characteristic of ferritins (Aisen and Listowsky 1980; Ford et al. 1984), are shown in Fig 3.

When chromatographed on a Sephacryl S-300 column calibrated with proteins of known molecular size, *M. edulis* ferritin eluted at a position similar to that of horse-spleen ferritin, indicating a size corresponding to a globular protein with M_r approximately 480000. After dissociation of the oligomeric protein, SDS-PAGE revealed that the protein was composed of two subunits of distinctly different sizes (M_r 18500 and 24600) but present in approximately equal proportion. Isoelectric focussing yielded a complex pattern of bands in the pI range 4.7–5.0. Under the same conditions, horse-spleen ferritin yielded a simpler pattern dominated by two major bands, in the pI range 4.3–4.5. Analysis of purified preparations for protein and iron revealed that the average iron content of *M. edulis* ferritin was 200 atoms iron/molecule protein.

Immunological reactions of partial identity occurred between *M. edulis* hepatopancreas ferritin and antisera to horse-spleen ferritin and lamprey-liver ferritin, indicating some similarity in the antigenic sites on these ferritin molecules (Fig. 4). No reaction was observed between *M. edulis* ferritin and chiton-haemolymph ferritin antiserum. Interestingly, a strong cross-reaction occurred between lamprey-liver ferritin and horse-spleen ferritin antiserum.

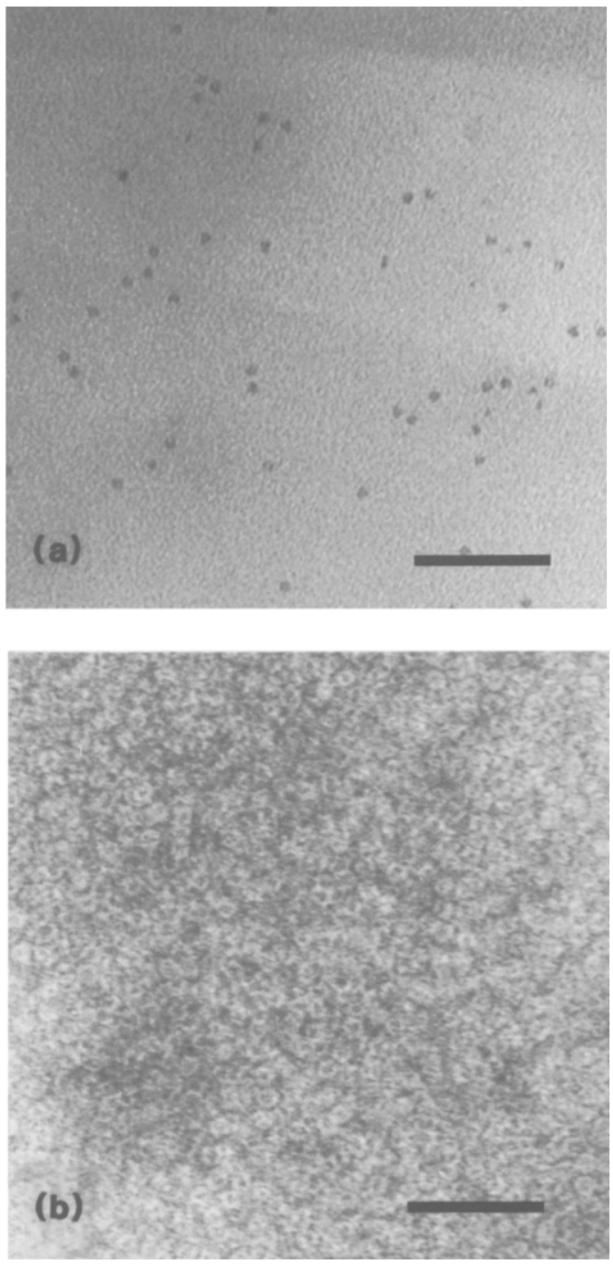

Fig. 3a, b. Transmission electron micrographs of ferritin from *M. edulis* hepatopancreas. Scale bar = 100 nm. **a** Unstained; **b** after staining with phosphotungstic acid

Discussion

Data presented here establish that ferritin is present as the main iron-binding protein in the hepatopancreas of mussels. Under conditions of iron loading, its biosynthesis appears to be increased, as has been observed in many mammalian systems (Bomford and Munro 1980). Most hepatopancreas proteins are heat-labile and hence purification of ferritin from this organ utilizes a fairly simple procedure involving heat denaturation, molecular exclusion chromatography and preparative gel electrophoresis. Mussel ferritin resembles horse-spleen ferritin in its size (M_r 480000) and its behaviour on isoelectric focussing but it should be noted that ferritins from many species have pI values in the range 4.5–5.5 (Aisen and Listowsky 1980). The microheterogeneity that is apparent on isoelectric focussing could be due to the presence of ferritin molecules made up of different proportions of the two subunits (differing in mass by 6 kDa). Alternatively, *M. edulis* ferritin may undergo post-translational modifications and/or be modified during isolation.

The size of *M. edulis* hepatopancreas ferritin is on the low end of the range of M_r values reported for ferritins from invertebrate sources. These range from 530000 and 520000 for haemolymph ferritins from the chiton *A. hirtosa* (Kim et al. 1986) and the limpet *Patella peronii* (Burford et al. 1986) respectively down to 460000 for the earthworm *Octolasium complanatum* (Arosio et al. 1984). The difference in subunit sizes for *M. edulis* hepatopancreas ferritin is rather striking. Horse-spleen ferritin has a single subunit, as does limpet hemolymph ferritin (Webb et al. 1986b),

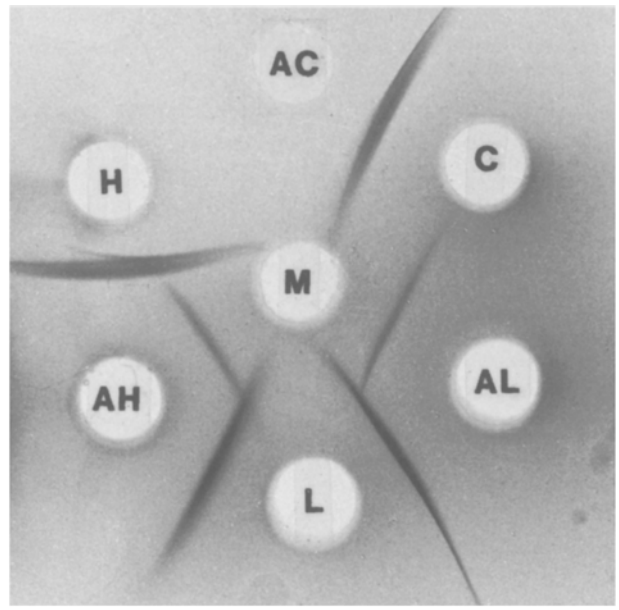

Fig. 4. Double immuno-diffusion of *M. edulis* hepatopancreas ferritin in 1% agarose gel. *M, M. edulis* hepatopancreas ferritin; *C,* chiton *(Acanthopleura hirtosa)* haemolymph ferritin; *AC,* anti-chiton haemolymph ferritin antiserum; *L,* lamprey *(Geotria australis)* liver ferritin; *AL,* anti-lamprey liver ferritin antiserum; *H,* horse-spleen ferritin; *AH,* anti-horse-spleen ferritin antiserum

while chiton haemolymph ferritin (Kim et al. 1986) and earthworm ferritin (Arosio et al. 1984) have two subunits with fairly similar M_r values. An interesting situation has been reported for the polypeptides of ferritin from the snail *Lymnaea stagnalis*, which have M_r values of 19000 and 24000 (Bottke 1982). The 19000-M_r polypeptide is widely distributed among tissues while the 24000-M_r polypeptide occurs only in the yolk platelets of vitellogenic oocytes (Bottke 1986). The origin and functional significance of these differences are as yet unknown.

The immunological reactions of *M. edulis* hepatopancreas ferritin are difficult to interpret. Cross-reactivity occurred between ferritins from the mussel and organisms as diverse as the horse and lamprey (a member of the Agnatha or jawless fish), while there was no reactivity with ferritin from another mollusc, the chiton. More detailed measurements are required, however, including a comparative analysis of the antigenic sites on various ferritin molecules. The antigenic sites on horse-spleen ferritin have been identified (Addison et al. 1984).

A rather surprising result is the observation that *M. edulis* hepatopancreas ferritin contains, on average, only 200 iron atoms/molecule protein but the iron core can be visualised by electron microscopy. Chemical analyses were performed on purified preparations which, however, had not been subjected to density gradient centrifugation to resolve the population of molecules according to their iron content. For haemolymph ferritin from *A. hirtosa*, this latter technique revealed that the molecular population was dominated by molecules of apoferritin (with no iron) and molecules of ferritin with at least 1000 iron atoms (Kim et al. 1986). If this were the case for *M. edulis*, then the average iron content of ferritin molecules containing iron could readily exceed 200 iron atoms/molecule.

The core (6–6.5 nm) of ferritin from *Pseudomonas aeruginosa* bacterial cells has been reported to have a low iron content of 900 iron atoms/molecule (Mann et al. 1986). Calculations (noting that the volume of a sphere is proportional to the cube of the diameter) indicate that the 5-nm core of the *M. edulis* ferritin would have a volume of approximately half that of the core from *P. aeruginosa* ferritin. Thus, to match this bacterial ferritin in density of iron atoms for microscopic visualization. *M. edulis* ferritin would need to contain only about 500 iron atoms. Furthermore, it is possible that the core structure in this *M. edulis* ferritin is rather more open than in other ferritins. Its phosphate content could also affect this structure (Treffry et al. 1988) which then would be expected to exhibit somewhat novel magnetic properties when studied by Mössbauer spectroscopy (St. Pierre et al. 1986). We are continuing studies of this ferritin including the effects of other metals, such as zinc, on its biosynthesis.

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