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

N. Bootsma¹, D. J. Macey¹, J. Webb², and V. Talbot³

¹ School of Biological and Environmental Sciences and
² School of Mathematical and Physical Sciences, Murdoch University, Murdoch WA 6150, Australia
³ 60 Vincent Street, Nedlands WA 6009, Australia

**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*ₐ of 480 000, pI of 4.7–5.0 and is composed of two subunits, *M*ᵣ 18 500 and *M*ᵣ 24 600. 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* 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-
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 O₂) 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 (10 000 × g, 20 rain) 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 (Fehrström and Moberg 1977). The gel section containing the prominent brown band of ferritin was homogenised and clarified by centrifugation (10 000 × 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 (Fehrström and Moberg 1977). Duplicate gels were stained for protein (Coomassie brilliant blue R-250) and for iron [K₃Fe(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, 667 000), horse-spleen ferritin (476 000), catalase (232 000) and bovine serum albumin (67 000). The isoelectric point, pl, was determined by isoelectric focusing 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 (Fehrström and Moberg 1977) by SDS-PAGE (10%) using a M₉ marker proteins bovine serum albumin (67 000), egg albumin (45 000), peptic (34 700), trypsinogen (24 000), β-lactoglobulin (18 400) and lysozyme (14 300).

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. _Clavanzona 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 M₉ 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 K₃Fe(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.