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Isolation and preliminary characterization of ferritin from clover seeds (*Trifolium subterraneum* L. cv. *Clare*)

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Ferritin from clover (*Trifolium subterraneum*) seeds was isolated and characterized. It was shown to contain polypeptide units of 28–26.5 kDa. The apparent molecular mass of the native protein was 560 kDa. The average iron cores were 4 nm in diameter and contained 1300 iron atoms, with Fe:P = 4:1. Purified clover apoferritin was shown to be functional by means of iron uptake experiments. Plots of initial velocities of iron uptake (at pH 6.7) into clover and horse spleen apoferritins were found to have similar profiles.

Keywords: characterization, ferritin, isolation, plant ferritin (clover seed)

Introduction

Ferritin, the iron storage protein in animals (Ford *et al.* 1984, Theil 1987, Crichton 1991) has a bacterial equivalent, bacterioferritin (Stiefel & Watt 1979, Chen & Crichton 1982) and a plant equivalent, phytoferritin (Crichton *et al.* 1978, Van der Mark *et al.* 1981, 1983, Bienfait & Van der Mark 1983, Van der Mark & Van Den Briel 1985, Sczekan & Joshi 1987, Laulhere *et al.* 1988, 1989, Lescure *et al.* 1990). All of these proteins consist of a spherical shell of 24 sub-units encompassing a cavity, about 8 nm in diameter, in which the non-heme-iron core is laid down (Theil 1987). Animal ferritins have been extensively studied, and much information is available on their structure and function (Theil 1987, Crichton 1991). Phytoferritins have not been investigated to the same extent, although they have been isolated from a variety of sources, e.g. bean seeds (Van der Mark & Van Den Briel 1985), pea (Laulhere *et al.* 1989) and soyabean (Sczekan & Joshi 1988, Lescure *et al.* 1990). Sequence comparisons indicate that phytoferritins are homologous with ferritins (Lescure *et al.* 1991, Theil & Hase 1993, Wade *et al.* 1993), though there is an extension peptide in phytoferritin with a molecular weight about 3 kDa.

Whilst animal ferritins consist of two types of subunit, the H and L subunits (Ford *et al.* 1984, Theil, 1987, Crichton 1991), phytoferritins studied to date only have one subunit type. The H and L subunits differ in size and sequence, and in their reactivities (Levi *et al.* 1988, 1989, Treffry 1992). Mechanistic work with ferritin has indicated that iron core formation *in vitro* requires the uptake of Fe(II) and its subsequent oxidation to Fe(III) at a ferroxidase site. A detailed model of this site has been constructed from X-ray studies coupled with site-directed mutagenesis experiments (Treffry *et al.* 1992). Central features of this site appear to be conserved in bacteroferritin and phytoferritin, leading to the proposal that ferroxidation is a common feature of iron uptake into all known classes of ferritin (Levi *et al.* 1989, Andrews *et al.* 1992, Grossman *et al.* 1992). However, there have been relatively few studies of the ferroxidase activity of phytoferritin and therefore this was one of the topics we investigated with clover ferritin.

Interest in clover comes from the widespread use of this important legume, which is grown for forage, to supply nitrogen to accompanying grasses, and to improve tilth and fertility of soils. Of all the cultivated annual clovers, *Trifolium subterraneum* currently makes the largest worldwide contribution to livestock feed production and soil improvement (McGuire 1985). Cultivar *Clare* is the variety cultivated in calcareous soils, where iron is not easily available. The molecular mechanisms that control iron uptake, storage and mobilization in plants have so far received relatively little attention (Emery 1978, Spiller *et al.* 1987), despite the fact that crop improvement can benefit from a better understanding of this aspect of the plant biochemistry. Therefore we are engaged on a study of iron metabolism in clover.

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The present paper describes the isolation of clover seed phytoferritin. This has not been previously reported. Characterization of the purified protein was performed by using gel electrophoresis, inductively coupled plasma (ICP) spectrometry, transmission electron microscopy (TEM) and energy-dispersive X-ray analysis (EDAX). Functional behavior of the purified apo-ferritin was demonstrated by means of iron uptake studies, which indicate that it does indeed possess ferroxidase activity. Whilst this manuscript was in preparation related work on pea seed phytoferritin was published (Wade *et al.* 1993). Our data are in broad agreement with theirs.

Materials and methods

Clover seeds (*T. subterraneum* L. cv. *Clare*) were obtained from a local dealer. Horse spleen ferritin was obtained from Sigma. All other chemicals used were of analytical grade, supplied by Sigma or Bio-Rad.

Isolation and characterization of phytoferritin

Isolation and purification of the plant protein was carried out by a modification of the procedure described by Laulhere *et al.* (1989). Dry seeds were allowed to swell in 4 volumes of distilled water for 2 h at room temperature. Swollen seeds were homogenized, at 4 °C, in 4 volumes of an aqueous buffer (50 mM Tris–HCl, pH 7.4, 1 mM EDTA, 0.1% phenylmethylsulfonyl fluoride, 1% polyvinylpyrollidene) using a waring blender. The slurry was filtered and centrifuged at 15000 *g* for 15 min. To the supernatant $MgCl_2$ was added to a final concentration of 50 mM and, after 20 min, trisodium citrate was added to a final concentration of 70 mM to complex Mg^{2+}. After 2 h at 4 °C, the mixture was centrifuged at 26000 *g* for 50 min. Pellets were resuspended in 50 mM Tris–HCl, pH 7.4, and centrifuged at 100000 *g* for 2 h. The brown pellets thus obtained were resuspended in the same buffer and centrifuged at 15000 *g* for 5 min to eliminate debris. The supernatant was then loaded on a Sephacryl S-300 gel-filtration column previously equilibrated in 50 mM Tris–HCl, 100 mM NaCl and 0.1 mM EDTA (pH 7.4), and phytoferritin eluted in the same buffer.

Protein concentration was determined according to the method of Bradford (1976). Purity of the protein extract was assessed by gel electrophoresis in a non-denaturing system (8–25% polyacrylamide gradient gel) and a denaturing system (10–15% polyacrylamide–SDS gradient gel) according to Laemmli (1970). The gels were stained for iron with Prussian blue (2% $K_4Fe(CN)_6$ solution in 2% HCl) and for protein with Coomassie blue stain.

For electron microscopy, the phytoferritin was stained with uranyl acetate and air-dried onto Formvar-coated copper electron microscope grids. Unstained samples were also prepared. Micrographs were obtained with a Hitachi H6000 transmission electron microscope, operating at 75 keV, which was equipped with EDAX facilities. Quantitative elemental analysis for iron and phosphorus were carried out by ICP spectrometry using a Perkin-Elmer Emission Spectrometer Plasma 2000 instrument and also by a colorimetric assay for phosphorus (Ames 1966).

Preparation of apoferritin

Iron was removed from purified clover ferritin by dialysis with 0.12 M thyoglycolic acid at pH 5.0, followed by dialysis against buffer 20 mM Tris–HCl, pH 7.4. For comparison, horse ferritin was treated in the same way to obtain the corresponding apoprotein.

Iron uptake

Iron uptake was investigated by the incubation of clover apoferritin in 0.1 M PIPES buffer, pH 6.7, with a 1500-fold molar excess of Fe^{2+} for 30 min and monitored by 6% polyacrylamide gel electrophoresis and Prussian blue stain. The kinetics of iron uptake, at 20 °C in the presence of atmospheric oxygen, was followed by monitoring the increase of optical density at 310 nm using a Perkin-Elmer 552 UV-vis spectrophotometer. Increasing amounts of freshly prepared ferrous ammonium sulfate solutions (up to 600 μM) were added to purified apo-phytoferritin samples of 0.3 μM in 0.1 M PIPES buffer, pH 6.0 and 6.7, and in 0.1 M MES buffer, pH 5.2. Comparative studies of horse spleen apoferritin were also carried out.

Results and discussion

The purification procedure gave clover seed phytoferritin which was collected as a single and homogeneous fraction by gel filtration chromatography (Sephacryl S-300 column). Non-denaturing polyacrylamide gradient gel electrophoresis revealed a single Coomassie stained band, which also stained positive for iron with Prussian blue. The corresponding molecular mass was 560 kDa, as shown in Figure 1(A). Electrophoresis in denaturing 10–15% gradient polyacrylamide–SDS gels revealed (Figure 1B) a broad band in the 28–26.5 kDa range. However, when the protein sample was stored for a few days at 4 °C, faint bands corresponding to smaller subunits of 22–20 kDa (possibly due to proteolytic breakdown) were also observed as minor components. These results do not clearly indicate that clover phytoferritin consist of 24 subunits. However, ferritins run on non-denaturing gels often exhibit a lower molecular weight than they actually have and for phytoferritin an apparent molecular mass of about 500–600 kDa is generally considered (Crichton *et al.* 1978) to indicate 24 subunits.

The observed electrophoretic behavior of clover ferritin is similar to that found for other phytoferritins. Thus, soyabean (Crichton *et al.* 1978, Sczekan & Joshi 1987) and pea and maize seed ferritins (Van der Mark *et al.* 1981, Laulhere *et al.* 1988) were reported to have an overall apparent molecular mass of 540–572 kDa and a subunit molecular weight of 28 kDa. Smaller subunits, of 22–26 kDa, were also seen alongside the 28 kDa band (Sczekan & Joshi 1987, Laulhere *et al.* 1989). Sczekan and Joshi (1987) showed that, for soyabean ferritin, the 22 kDa

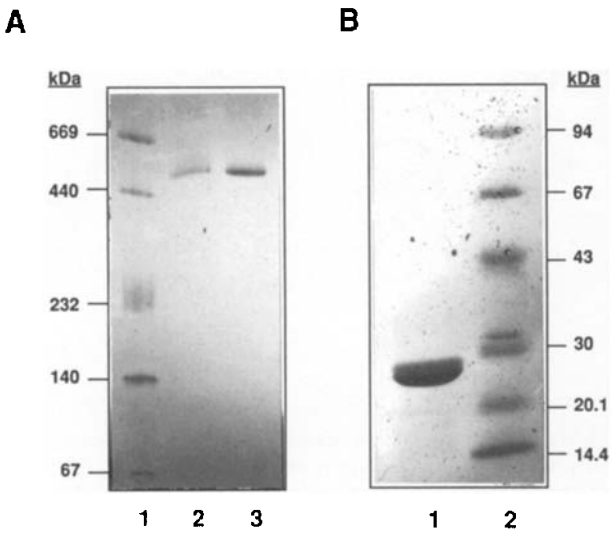

Figure 1. (A) Non-denaturing 8–25% gradient gel electrophoresis of clover ferritin. The gel was stained with Coomassie blue. Lane 1, molecular weight marker (HMW; Pharmacia). Lanes 2 and 3, 1 and 2 μg of phytoferritin. (B) Electrophoresis in denaturing 10–15% polyacrylamide–SDS gels. Coomassie blue stain. Lane 1, 1 μg of phytoferritin. Lane 2, low molecular weight marker (Pharmacia).

subunit could be formed by incubation of the 28 kDa monomer with subtilisin, a serine protease. This strongly suggests that the smaller subunits are the result of proteolytic processes occurring during isolation of the protein and that the mobility of the subunits in SDS gels is anomalous to that expected from the actual mass because of aberrant SDS binding (Theil & Hase 1993). In the case of pea ferritin, the fully preserved subunit contains an extension peptide and has a mass of 30 kDa, yet the subunit found in seeds runs on gels with an apparent mass of 26–28 kDa (Laulhere *et al.* 1989). Since we are uncertain of the actual molecular mass of the protein component of clover phytoferritin we have taken a value of 672 kDa for the intact molecule (24 × 28 kDa) in the analysis of iron content and iron uptake rates given below.

Figure 2 depicts representative electron micrographs of the purified clover ferritin, along with the results of EDAX spectroscopy which show the presence of iron and phosphorus. Selected area electron diffraction (not shown) gave two diffuse rings corresponding to *d* spacings of 0.322 and 0.197 nm, respectively, which can be assigned (Towe & Bradley 1967) to a ferrihydrite mineral core.

The unstained preparation (Figure 2A) shows discrete particles of rather uniform electron-optical contrast, which have an average diameter of about 4 nm. The stained preparation (Figure 2B) shows that these particles are surrounded by protein coats. Most molecules are partially filled (dark with a white halo), but some are unloaded. The simultaneous presence of loaded and unloaded molecules was also observed by researchers working with mammalian ferritins (Ford *et al.* 1984, Crichton 1991, Deighton *et al.* 1991). It seems that further capture of iron ions is favored once an initial mineral core is already

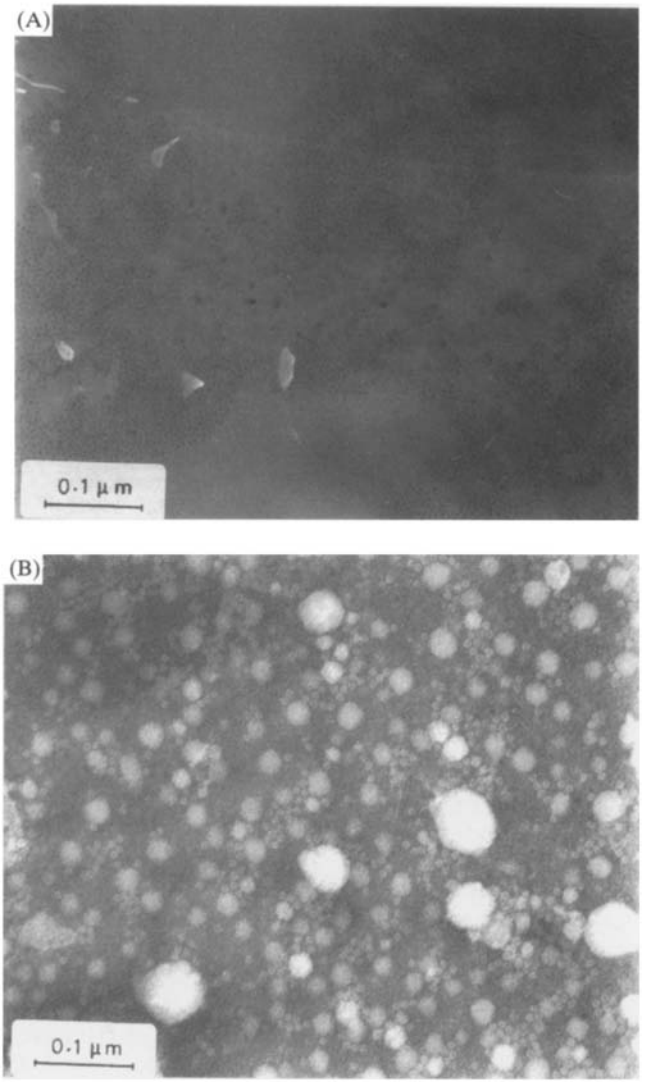

Figure 2. Electron micrographs (200 000 ×) of (A) unstained and (B) uranyl acetate-stained clover ferritin. (C) EDAX pattern showing iron and phosphorous peaks.

formed inside the protein shell. This could well be the result of ordinary crystal growth, with large crystals growing at the expense of smaller ones (Ford *et al.* 1984, Crichton 1991).

The fact that the mineral cores have an average diameter of only 4 nm indicates that the protein, with a probable internal diameter of about 8 nm, is not heavily loaded. This is in agreement with the results of ICP analysis which gave an average of 1300 iron atoms per protein molecule, assuming a molecular weight of 672 kDa. Ferritin molecules have a capacity to store up to 4500 iron atoms in their central cavity (Theil 1987), although as isolated such proteins are not usually so heavily loaded.

ICP spectrometry also showed a Fe:P ratio of about 4:1 (average of five determinations). For mammalian ferritins this ratio is usually 8:1 or higher (Treffry & Harrison 1978, Harrison *et al.* 1989), whilst bacterioferritins are characterized (Stiefel & Watt 1979, Moore *et al.* 1986) by their low Fe:P ratio (between 1:1 and 2:1). This difference is associated with a difference in core morphology. Mammalian ferritins have a crystalline ferrihydrite core whilst bacterioferritins have an amorphous core containing iron-phosphate (Stiefel & Watt 1979, St Pierre *et al.* 1986, Harrison *et al.* 1989, Theil & Hase 1993). However, small differences are apparent between bacterioferritin cores, e.g. that from *Azotobacter vinelandii* appears to contain small crystalline regions of ferrihydrite (St Pierre *et al.* 1989) whilst that from *Pseudomonas aeruginosa* (Moore *et al.* 1986, St Pierre *et al.* 1989) does not. A similar distinction may occur for the phosphate-rich phytoferritin cores. For example, electron microscopy (EM) studies of native pea seed phytoferritin containing an average 1800 iron atoms per molecule and with an FE:P ratio of 2.8:1 failed to identify any crystallinity (Wade *et al.* 1993), whilst in our clover phytoferritin EM studies there is some evidence for the presence of ferrihydrite. Additional EM studies and comparative Mössbauer studies are required to further characterize this difference.

Functional behavior of the purified clover apoferritin was demonstrated by the iron uptake experiments described above. Incubation of the apophytoferritin with a 1500-fold molar excess of Fe^{2+} for 30 min at pH 6.7, followed by electrophoresis on a non-denaturing 6% polyacrylamide gel, gave a band that stained with prussian blue, as shown in Figure 3. Progress curves of iron uptake into clover apo-phytoferritin at different iron:protein ratios monitored by the appearance of the 310 nm absorbance associated with Fe(III) are shown in Figure 4. It is clear from these curves that Fe(II) oxidation is faster in the presence of phytoferritin than in its absence, similarly to the behavior of horse spleen ferritin, and consistent with phytoferritin containing a ferroxidase center (Grossman *et al.* 1992, Treffy *et al.* 1992, Wade *et al.* 1993). The measurement of initial velocities of iron uptake into clover apo-phytoferritin and horse spleen ferritin (Figure 5) are consistent with this. The inset plot in Figure 5 showing that iron uptake is faster at pH 6.7 than at lower pH is also similar to the behavior of horse spleen ferritin (Ford *et al.*

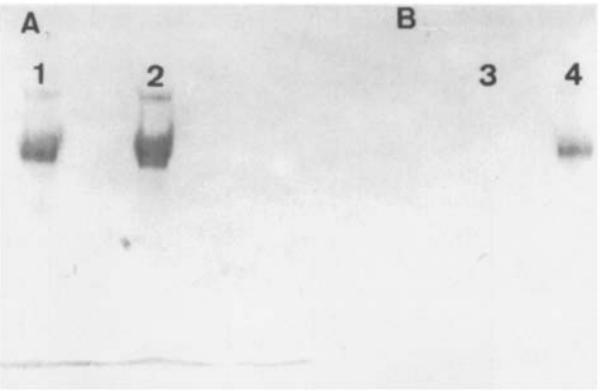

Figure 3. Polyacrylamide gel electrophoresis (6%) of clover ferritin. Lanes 1 and 3, apo-phytoferritin. Lanes 2 and 4, apo-phytoferritin after incubation with 1500-fold molar excess of iron for 30 min, in 0.1 M PIPES buffer at pH 6.7. (A) Coomassie blue stain. (B) Prussian blue stain.

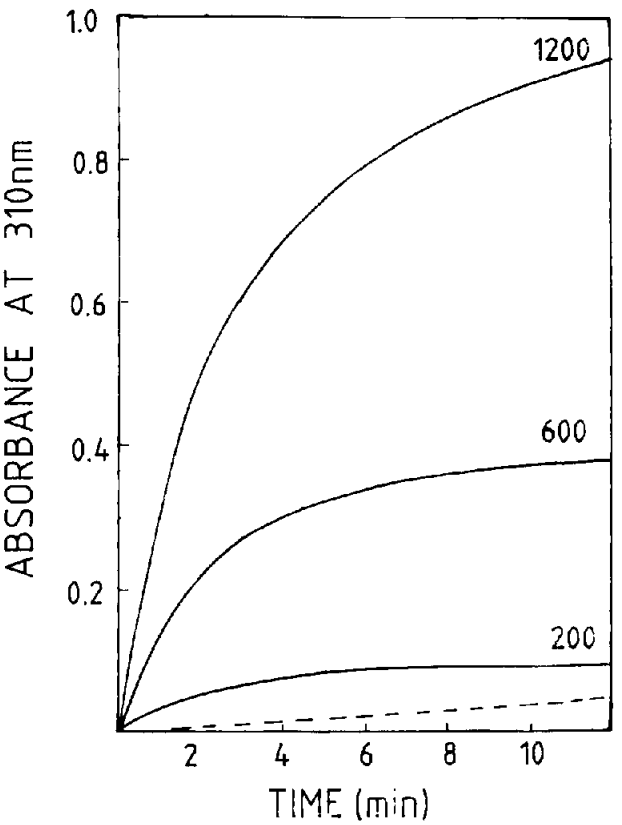

Figure 4. Time-dependent iron uptake by clover apoferritin (0.3 μM) in 0.1 M PIPES buffer, pH 6.7, at 20 °C. The dashed line is an iron oxidation control (320 μM) in the absence of protein. The ratio of iron atoms per protein molecule (MW = 672 kDa) is shown on each curve.

1984, Harrison *et al.* 1989). The H subunits of horse spleen ferritin each contain a single ferroxidase center, though the more abundant (85–90%) L chain subunits do not. Thus the similar profiles for clover phytoferritin and horse spleen ferritin indicate that either the phytoferritin sites are less active than the ferritin sites or that not all the

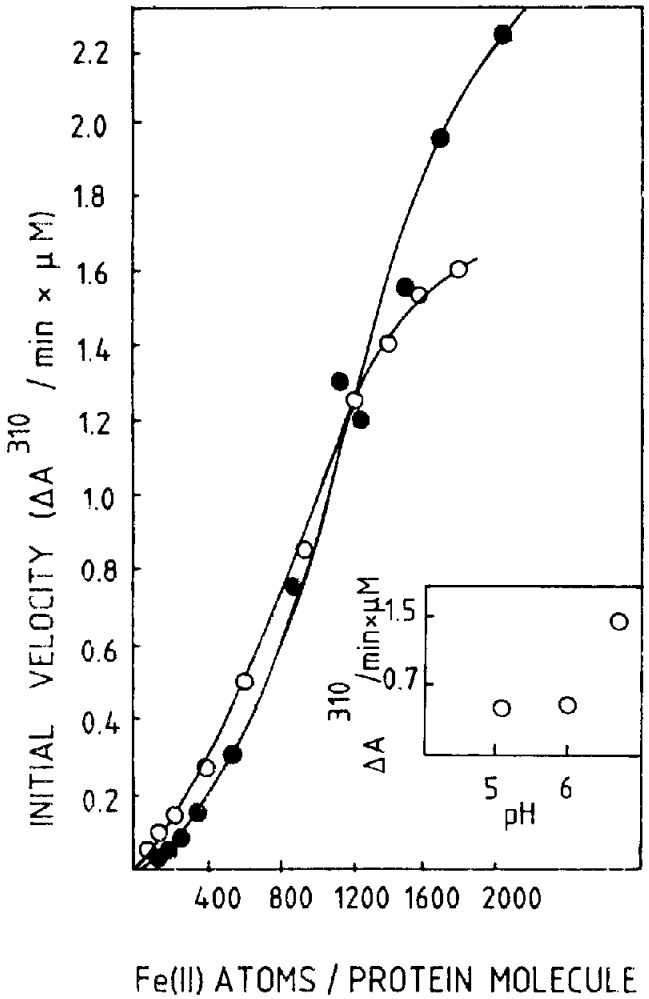

Figure 5. Initial velocities of iron uptake into clover apo-ferritin (○) and horse spleen apo-ferritin (●) as a function of the number of Fe(II) ions per protein molecule. Experiments were carried out in 0.1 M PIPES buffer at pH 6.7. The inset shows the initial velocity of iron uptake into clover apo-phytoferritin at various pH values; pH 6.1 and pH 6.7 (0.1 M PIPES buffer) and pH 5.2 (0.1 M MES buffer).

phytoferritin subunits contain functional ferroxidase sites. This is in contrast to pea seed phytoferritin which is substantially more active than horse spleen ferritin (Wade *et al.* 1993).

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References

Ames BN. 1966 Essay of inorganic phosphate, total phosphate and phosphatases. *Methods Enzymol* **8**.

Andrews SC, Arosio P, Bottke W, *et al.* 1992 Structure, function, and evolution of ferritins. *J Inorg Biochem* **47**, 161–174.

Bienfait HF, Van Der Mark F. 1983 Phytoferritin and its role in iron metabolism. *Ann Proc Phytochem Soc Eur* **21**, 111–123.

Bradford MM. 1976 A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**, 248–254.

Chen M, Crichton RR. 1982 Purification and characterization of a bacterioferritin from *Azotobacter chrococcum*. *Biochim Biophys Acta* **707**, 1–6.

Crichton RR. 1991 *Inorganic Biochemistry of Iron Metabolism*. New York: Ellis Horwood.

Crichton RR, Ponce-Ortiz Y, Koch MHJ, Parfait R, Staurhmann HB. 1978 Isolation and characterization of plant ferritins. *Biochem J* **171**, 349–356.

Deighton N, Abu-Ragabah A, Rowland IJ, Symons MCR, Peters TS, Ward RJ. 1991 Electron paramagnetic resonance studies of a range of ferritins and haemosiderins. *J Chem Soc Faraday Trans* **87**, 3193–3197.

Emery T. 1978 The storage and transport of iron. In: Siegel H, ed. *Metal Ions in Biological Systems*, vol. 7. New York: Dekker.

Ford GC, Harrison PM, Rice DW *et al.* 1984 Ferritin: design and formation of an iron-storage molecule. *Phil Trans R Soc London* **B304**, 551–560.

Grossman MJ, Hinton SM, Minak-Bernero V, Slaughter C, Stiefel EI. 1992 Unification of the ferritin family of proteins. *Proc Natl Acad Sci* **89**, 2419–2423.

Harrison PM, Artymiuk PJ, Ford GC, *et al.* 1989 Ferritin: function and structural design of an iron storage protein. In: Mann S, Webb J, Williams RJP, eds. *Biomineralization*. Weinheim: VCH.

Laemmli UK. 1970 Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature* **227**, 680–685.

Laulhere J-P, Lescure AM, Briat J-F. 1988 Purification and characterization of ferritins from seeds of maize, pea and soya bean. Distribution in various pea organs. *J Biol Chem* **263**, 10289–10294.

Laulhere J-P, Labouré AM, Briat J-F. 1989 Mechanism of the transition from plant ferritin to phytosiderin. *J Biol Chem* **264**, 3629–3635.

Lescure AM, Massenet O, Briat J-F. 1990 Purification and characterization of an iron-induced ferritin from soyabean (*Glycine max*) cell suspensions. *Biochem J* **272**, 147–150.

Lescure AM, Proudhon D, Pessey H, Ragland M, Theil EC, Briat J-F. 1991 Ferritin gene transcription is regulated by iron in soybean cell cultures. *Proc Natl Acad Sci USA* **88**, 8222–8226.

Levi S, Luzzago A, Cesareni G, *et al.* 1988 mechanism of ferritin iron uptake: activity of the H-chain and deletion mapping of the ferro-oxidase site. *J Biol Chem* **263**, 18086–18092.

Levi S, Luzzago A, Franceschinelli F, Santambrogio P, Cesareni G, Arosio P. 1989 Mutational analysis of the channel sequences of human ferritin H-chain. *Biochem J* **264**, 381–388.

McGuire WS. 1985 Subterranean clover. In: Taylor NL, ed. *Clover Science and Technology*. Madison WI: American Society of Agronomy.

Moore GR, Mann S, Bannister JV. 1986 Isolation and properties of the complex nonheme-iron-containing Cytochrome b_{557} (Bacterioferritin) from *Pseudomonas aeruginosa*. *J Inorg Biochem* **28**, 329–336.

Sczekan SR, Joshi JG. 1987 Isolation and characterization of ferritin from soyabeans (*Glycine max*). *J Biol Chem* **262**, 13780–13788.

Spiller SC, Kaufman WF, Thompson WF, Briggs WR. 1987 Specific mRNA and rRNA levels in greening pea leaves during recovery from iron stress. *Plant Physiol* **84**, 409–414.

Stiefel EI, Watt GD. 1979 Azotobatter cytochrome $b_{557.5}$ is a bacterioferritin *Nature* **279**, 81–83.

St Pierre TG, Bell SH, Dickson DP, *et al.* 1986 Mossbauer spectroscopy studies of the cores of human, limpet and bacterial ferritins. *Biochim Biophys Acta* **870**, 127–134.

St Pierre TG, Webb J, Mann S. 1989 Ferritin and hemosiderin: structural and magnetic studies of the iron core. In: Mann S, Webb J, Williams RJP, eds. *Biomineralization*. Weinheim: VCH.

Theil EC. 1987 Ferritin: structure, gene regulation, and cellular function in animals, plants, and microorganisms. *Annu Rev Biochem* **56**, 289–315.

Theil EC, Hase T. 1993 Plant and microbial ferritins. In: Barton LL, Hemming BC. eds. *Iron Chelation in Plants and Soil Microorganisms*. New York: Academic Press.

Towe KM, Bradley WF. 1967 Mineralogical constitution of colloidal 'hydrous ferric oxides'. *J Colloid Interface Sci* **24**, 384–392.

Treffry A, Harrison PM. 1978 Incorporation and release of inorganic phosphate in horse spleen ferritin. *Biochem J* **171**, 313–320.

Treffry A, Hirzman J, Yewdall SJ, Harrison PM. 1992 Mechanism of catalysis of iron II oxidation by ferritin H chains. *FEBS Lett* **302**, 108–112.

Van der Mark F, De Lange Y, Bienfait HB. 1981 The role of ferritin in developing primary heavy bean leaves under various light conditions. *Planta* **153**, 338–342.

Van der Mark F, Van Den Briel W, Huisman G. 1983 Phytoferritin is synthesized *in vitro* as a high-molecular-weight precursor. *Biochem J* **214**, 943–950.

Van der Mark F, Van Den Briel W. 1985. Purification and partial characterization of ferritin from normal and iron-loaded leaves of *Phaseolus vulgaris*. *Plant Sci* **39**, 55–60.

Wade VJ, Treffry A, Laulhere J-P, *et al.* 1993. Structure and composition of ferritin cores from pea seed (*Pisum sativum*). *Biochim Biophys Acta* **1161**, 91–96.