Anal Bioanal Chem (2008) 390:53–59
DOI 10.1007/s00216-007-1691-3

ORIGINAL PAPER

Biosynthesis, isolation and characterization of ^{57}Fe-enriched *Phaseolus vulgaris* ferritin after heterologous expression in *Escherichia coli*

Matthias Hoppler · Leo Meile · Thomas Walczyk

Received: 17 August 2007 / Revised: 8 October 2007 / Accepted: 9 October 2007 / Published online: 20 November 2007

Abstract Ferritin is the major iron storage protein in the biosphere. Iron stores of an organism are commonly assessed by measuring the concentration of the protein shell of the molecule in fluids and tissues. The amount of ferritin-bound iron, the more desirable information, still remains inaccessible owing to the lack of suitable techniques. Iron saturation of ferritin is highly variable, with a maximum capacity of 4,500 iron atoms per molecule. This study describes the direct isotopic labeling of a complex metalloprotein in vivo by biosynthesis, in order to measure ferritin-bound iron by isotope dilution mass spectrometry. [^{57}Fe]ferritin was produced by cloning and overexpressing the *Phaseolus vulgaris* ferritin gene *pfe* in *Escherichia coli* in the presence of $^{57}FeCl_2$. Recombinant ferritin was purified in a fully assembled form and contained approximately 1,000 iron atoms per molecule at an isotopic enrichment of more than 95% ^{57}Fe. We did not find any evidence of species conversion of the isotopic label for at least 5 months of storage at −20 °C. Transfer efficiency of enriched iron into [^{57}Fe]ferritin of 20% was sufficient to be economically feasible. Negligible amounts of non-ferritin-bound iron in the purified [^{57}Fe]ferritin solution allows for use of this spike for quantification of ferritin-bound iron by isotope dilution mass spectrometry.

Keywords *Phaseolus vulgaris* · Ferritin · Molecular cloning · Iron · Isotope

M. Hoppler
Laboratory of Human Nutrition, ETH Zurich,
Schmelzbergstrasse 7,
8092 Zurich, Switzerland

L. Meile
Laboratory of Food Biotechnology, ETH Zurich,
Schmelzbergstrasse 7,
8092 Zurich, Switzerland

T. Walczyk (✉)
Department of Chemistry and Department of Biochemistry,
National University of Singapore,
Science Drive 4,
Singapore 117543, Singapore
e-mail: walczyk@nus.edu.sg

Introduction

Ferritin serves as an iron storage protein in virtually all living organisms from bacteria to plants and animals [1]. It consists of 24 subunits that are assembled into a spherical shell that can store up to 4,500 Fe(III) atoms in its inner cavity as an iron oxyhydroxide phosphate mineral [2]. Ferritin subunits of plant ferritin contain an N-terminal transit peptide (TP) responsible for the precursor targeting to plastids [3]. Accordingly, plant ferritin is exclusively located in plant cell plastids, while animal ferritin is found in various organelles [4]. In man, most ferritin is present in the liver for iron storage. If iron losses exceed dietary iron supply, iron is released from the liver to maintain hemoglobin concentrations in the blood for oxygen transport. In the case of excessive dietary iron uptake, iron is deposited in the liver as ferritin to protect the body from oxidative damage [5]. Free solubilized iron can catalyze the formation of hydroxyl radicals via Fenton's reaction [6].

If iron balance is persistently negative, iron stores are gradually emptied and anemia develops over time. An insufficient dietary iron supply and/or dietary iron with low bioavailability are major factors in the origin of this disorder. Biofortification, i.e., increasing the micronutrient content of food crops either by conventional plant breeding or by genetic engineering, is currently targeted in various multinational, multidisciplinary research projects in order to

improve iron nutrition in developing countries [7]. Ferritin is currently considered the most promising target molecule for triggering iron accumulation in food crops [8–11]. Lines of transgenic rice and maize which express ferritin from soybeans (*Glycine max*) or common beans (*Phaseolus vulgaris*) have already been developed [10, 12, 13]. Progress in biofortification with ferritin, however, is largely limited by the lack of techniques to quantify ferritin-bound iron in plants; it still remains unknown with what efficiency overexpressed ferritin in transgenic plants can be filled with iron.

Immunochemical assays are widely used for quantification of ferritin in human serum in routine clinical practice as a surrogate measurement of ferritin iron stores in the liver [14]. Specific assays for plant ferritin are currently under development [15]. In both cases, immunoassays allow the quantification of ferritin protein in a sample but not the quantification of ferritin-bound iron, which is the supposedly more valuable information. The degree of iron saturation of the ferritin protein shell varies exceedingly between phenotypes and genotypes and possibly even between tissues of the same organism [16–22]. Factors which determine the iron saturation of ferritin in living organisms remain elusive and may provide valuable insights into mechanisms of iron homeostasis. Techniques to differentiate between ferritin-bound iron and other forms of iron in a sample are necessary. Monitoring the effectiveness by which iron is deposited in the ferritin molecule becomes vital in order to identify possible breeding strategies as well as to evaluate promising plant varieties for biofortification.

For elemental speciation analysis, isotope dilution mass spectrometry (IDMS) is widely referred to as a reference technique [23]. In principle it can also be used for the quantification of ferritin-bound iron in biological samples, including plant tissues. In this approach, a known amount of isotopically labeled ferritin-bound iron is added to the sample. After isolation of ferritin-bound iron from the spiked sample, the amount of native ferritin-bound iron can be derived from the induced changes in the iron isotopic composition, i.e., the degree by which isotopically labeled iron in the element species has been diluted with natural iron. To make use of this approach, a solution is needed in which the isotopically labeled iron is exclusively bound to ferritin iron.

Only few studies have been published up to now in which metalloproteins have been labeled for elemental speciation analysis by IDMS. This includes biosynthesis and isolation of [^{77}Se]selenomethionine as a relatively simple organometallic species by direct biosynthesis in yeast [24]. In two other experiments, complex metalloproteins such as transferrin and rusticyanin [25, 26] were labeled in vitro by adding stable isotopes to the apo protein.

In this paper we describe the first successful direct isotopic labeling of a complex metalloprotein in vivo, its purification and its characterization. Iron in recombinant ferritin was labeled with ^{57}Fe in *Escherichia coli* using a ferritin gene sequence from *P. vulgaris*, which is a potential target crop for biofortification.

Materials and methods

Materials

The complementary DNA of *P. vulgaris* ferritin [27] was kindly provided by Peter J. Lammers (Department of Chemistry and Biochemistry, New Mexico State University, Las Cruces, USA). ^{57}Fe-enriched iron in elemental form was purchased from Chemgas, Boulogne, France. The ^{57}Fe metal was dissolved in 5 M HCl in an ultrasonication bath, and water was evaporated by using a rotavapor (RE 111, Büchi Labortechnik, Flawil, Switzerland). Sodium citrate (1 M) and sodium ascorbate (0.1 M) were added to produce a [^{57}Fe]Cl_2 solution (10 mM), which was stable at pH 7. Recombinant [^{57}Fe]ferritin was characterized using an iron isotopic reference material (IRM-014, EU Institute of Reference Materials and Measurements, Geel, Belgium). Isolated recombinant [^{57}Fe]ferritin was compared with native *P. vulgaris* ferritin using a crude aqueous extract prepared from a commercial batch of red kidney beans.

Molecular cloning and culture conditions for bacterial growth

Complementary DNA of the ferritin gene of *P. vulgaris* seeds (*pfe*) [27] was inserted into an *E. coli* expression vector. Synthesis of recombinant plant ferritin in *E. coli* requires deletion of the sequence coding for the TP [28]; herefore, DNA fragments without the TP sequence were synthesized by polymerase chain reaction. An *Nco*I site and an ATG start codon were introduced downstream of the TP sequence of *pfe* [27] by using oligonlucleotide P1 (Table 1). Downstream of the stop codon of *pfe* an *EcoR*I site was added by using oligonucleotide P2 (Table 1). The resulting fragment was ligated to the *EcoR*I and *Nco*I sites on the expression vector pET-28a(+) to generate a plasmid containing the *pfe* gene but lacking the TP coding sequence (pPVF; Table 1). *E. coli*/pPVF transformants of strain BL21-CodonPlus(DE3)-RIPL were cultured at 37 °C in a modified M9 medium that was prepared from analytical grade reagents without addition of iron. To increase the iron uptake of *E. coli* during growth, the Mg^{2+} concentration of the M9 medium was decreased from 2 mM in the original recipe [29] to 0.5 mM (see later). The medium was supplemented with kanamycin sulfate (30 μg mL^{-1}) and

chloramphenicol (34 μg mL^{-1}). Bacterial growth was monitored by UV–vis spectrophotometry at 600 nm (Uvikon 940, Kontron Instruments, Eching, Munich, Germany). When the optical density of the culture solution reached 0.6, iron was added as [^{57}Fe]Cl_2 to a concentration of 0.2 mM together with isopropyl-β-D-thiogalactopyranoside (1 mM) to induct ferritin synthesis. Cells were harvested after 24 h by centrifugation and were washed three times with buffer A [20 mM tris(hydroxymethyl)aminiomethane–HCl, pH 7.4, 1 mM EDTA] and resuspended in 5 mL g^{-1} (wet weight) buffer A. Cells were disrupted by passing them through a chilled French pressure cell (SLM Aminco, SLM Instruments, Urbana, USA) three times at approximately 130 MPa and cell debris was removed by centrifugation at 20,000 *g*. Ferritin synthesis was confirmed by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) and native PAGE.

Protein purification

Cell extracts were desalted by gelfiltration using a Sephadex® G-25 column (GE Healthcare, Uppsala, Sweden) eluting with buffer A. In typical purification procedures, the samples were applied to a (diethylamino)ethyl Sepharose® column (10 cm×1.6 cm; GE Healthcare, Uppsala, Sweden). After equilibration of the column with buffer A, proteins were eluted using a linear gradient of 0–0.5 M NaCl in the buffer. Collected fractions were monitored for iron by atomic absorption spectrophotometry (Varian AA240Z, Malgrave, Australia). Iron-containing fractions, which were eluted at a NaCl buffer concentration of approximately 0.15 M, were pooled and concentrated 10–20-fold by ultrafiltration (Amicon stirred cell, Millipore, Billerica, USA). The isolate was further purified by size-exclusion chromatography using a Sephacryl® S-300 column (2.6 cm×60 cm; GE Healthcare, Uppsala, Sweden) and buffer B (50 mM phosphate, 0.15 M NaCl, 1 mM EDTA, pH 7) for elution. Ferritin-containing fractions were concentrated by ultrafiltration and stored at −20 °C in 50% glycerol. The purity of isolated recombinant [^{57}Fe]ferritin was assessed by SDS PAGE and assembly of the 24 subunits to multimeric ferritin molecules was monitored by native PAGE [30]. Iron in gels was stained with Prussian blue [2% $K_4Fe(CN)_6$ solution in 2% HCl] and protein was stained with Coomassie blue [21]. Protein concentrations were determined by the Bradford method.

Optimization of iron uptake by *E. coli*

Growth conditions of *E. coli*/pPVF were optimized for iron uptake and for subsequent ^{57}Fe loading of the recombinant ferritin. Bacteria were grown in conventional Luria–Bertani medium. Ferritin expression was induced as described earlier and iron was added in concentrations of 0–6 mM as divalent iron ($FeSO_4$) or trivalent iron ($FeCl_3$). The efficiency of iron uptake and incorporation into ferritin was monitored by native PAGE based on the density of the ferritin bands after iron staining of the gels. Additional experiments were conducted with M9 medium at a reduced Mg^{2+} concentration of 0.5 mM. Earlier studies have shown that magnesium deficiency in *E. coli* promotes an uptake of ferrous iron independent of the ferrous iron transport system Feo [31]. Purified ferritin isolated from *E. coli*/pPVF grown in Mg^{2+}-deficient M9 medium with $FeCl_2$ as the iron source was compared with ferritin isolated from *E. coli*/pPVF grown in conventional Luria–Bertani medium. Protein and iron concentrations in the purified ferritins were determined as described before.

Iron isotopic analysis

Recombinant [^{57}Fe]ferritin was analyzed for its iron isotopic composition under chemical blank monitoring by negative thermal ionization mass spectrometry using FeF_4^-

Table 1 Bacterial strains, plasmids and oligonucleotides used in this study

Strain, plasmid or oligonucleotide	Relevant characteristics, construction or nucleotide sequence (5′→3′)[a]	Reference or source
Strains		
Escherichia coli BL21-CodonPlus(DE3)-RIPL	F$^-$ *ompT hsdS*(r_B^- m_B^-) *dcm*$^+$ Tet^r *gal* λ(DE3) *end*A Hte[*argU proL* Cam^r] [*argU ileY leuW* Strep/Spec^r]	Stratagene, La Jolla, USA
Plasmids		
pET-28a(+)	Kan^r LacI; expression vector; 5.4 kb	Novagen, San Diego, USA
pFPV	Kan^r; *pfe* gene lacking transit peptide sequence in pET-28a(+); 6.3 kb	This study
Oligonucleotides[b]		
P1	5′-*AAG**CCATGG***TGCCTCTTACTGGGGTG-3′ (402-419)[c]	This study
P2	5′-*CCA**GAATTC***GAAAAGCAGATATCG-3′ (1196-1210 reverse)[c]	This study

[a] Position in the nucelotide sequence according to the numbering in the GenBank database
[b] Oligonucleotides were synthesized by Microsynth, Balgach, Switzerland
[c] The *italicized sequences* correspond to an introduced tail for cloning purposes. Restriction sites are in **bold**.

molecular ions and a rhenium double-filament ion source. The evaporation filament as well as the ionization filament were coated with BaF_2 to promote the formation of negatively charged ions. Sample iron was loaded as FeF_3 in HF (40%) on top of the BaF_2 layer on the evaporation filament and coated with a solution of $AgNO_3$ in HF (20%). All mass-spectrometric measurements were carried out with a magnetic sector field mass spectrometer (MAT 262; Finnigan MAT, Bremen, Germany) equipped with a multi-collector system for simultaneous ion beam detection [32].

Samples were mineralized using a mixture of HNO_3 and H_2O_2 by microwave digestion (MLS ETHOS plus, MLS, Leutkirch, Germany). Sample iron was separated by anion-exchange chromatography and solvent–solvent extraction of eluted iron into diethyl ether [33]. IDMS was used to measure the concentration of total iron in isotopically labeled recombinant ferritin. The iron standard was prepared gravimetrically from an isotopic reference material (elemental iron, IRM-014; EU Institute of Reference Materials, Geel, Belgium) by dissolution of the metal in concentrated hydrochloric acid.

Single-step size-exclusion chromatography of isolated recombinant [^{57}Fe]ferritin and crude red kidney bean extract

An aqueous extract of red kidney beans was used to verify that the [^{57}Fe]ferritin produced and native ferritin would be coeluted during IDMS analysis. Red kidney beans were ground under liquid nitrogen with a rotar mill (ZM1, Retsch, Germany) using a titanium sieve (0.25-mm mesh). The sample was then suspended in ice-cold buffer B and treated in an ultrasonic bath for 30 min in ice-cold water. The suspension was centrifuged at 21,000 *g* for 30 min and the supernatant was separated. Proteins in the aqueous red kidney bean extract (approximately 40 mg mL^{-1}) and the prepared [^{57}Fe]ferritin solution were separated by size-exclusion chromatography using a Superdex® 200 column with a fractionation range of 10–600 kDa (1 cm×30 cm; GE Healthcare, Uppsala, Sweden) and using for elution buffer B, which contained EDTA (1 mM) to keep the iron dissolved. Protein was eluted over one column volume and monitored spectrophotometrically at 280 nm. Fractions of 1 mL were collected and iron content was measured in each fraction by atomic absorption spectrometry using a commercial iron standard (Titrisol; Merck, Darmstadt, Germany).

Results and discussion

A quantitative plant ferritin assay is currently lacking and is much needed for further progress in the iron biofortification of major plant food staples [34]. The quantification of ferritin-bound iron by IDMS requires the addition of the isotopically labeled species and its subsequent isolation together with the native species from the sample matrix. Provided that mixing is complete, advantages of isotope dilution can be fully exploited. No quantitative isolation of the isotope-diluted element species is necessary as the altered isotope ratio bears the desired information independent of separation yields. Furthermore, IDMS is one of the few analytical techniques which are considered to be definitive, i.e., all sources of uncertainty in the analysis can be identified and quantified, which allows the set-up of a full uncertainty budget based on error propagation analysis.

The major limitation of the IDMS method for element speciation is the need to isotopically label the element in the species of interest. In the case of most inorganic element species this can be achieved by direct chemical synthesis using the isotopically enriched element. Isotopic labeling of metals in complex biomolecules, however, is difficult by conventional laboratory techniques as synthetic pathways are commonly much too complex or may even not exist. In earlier studies, labeling could be achieved in vitro by binding of the label to the apo protein for transferrin and rusticyanin [25, 26] or in vivo for selenomethionine, a relatively simple metal-containing biomolecule. In this study we show for the first time that a metal in a complex metalloprotein can be labeled directly in vivo by biosynthesis. For this purpose, the *P. vulgaris* ferritin gene *pfe* was cloned and overexpressed in *E. coli* for incorporation of the iron isotopic label added to the bacterial growth medium.

Expression of recombinant *P. vulgaris* ferritin in *E. coli*

SDS PAGE of *E. coli*/pPVF extracts showed the overexpression of an approximately 26-kDa protein (Fig. 1a), which corresponds well to the molecular mass of the subunits of *P. vulgaris* seed ferritin of 26.5 kDa [35]. Control extracts of *E. coli* host cells transformed with the empty vector did not show overexpression of any protein of similar molecular mass. Thus, we conclude that the accumulation of recombinant protein was due to the ferritin insert. A band with mobility to that for horse spleen ferritin was observed in native PAGE after staining for protein and iron (Fig. 1b,c lanes 2). The findings in native PAGE under nondenaturing conditions, which leaves the ferritin molecule intact, and SDS PAGE under denaturing conditions, which reveals the ferritin subunits, demonstrate that overexpressed ferritin subunits in *E. coli*/pPVF were effectively assembled into multimeric functional ferritin molecules (Fig. 1b,c, lanes 2). Positive iron staining showed that iron added to the growth medium was absorbed by the bacteria and incorporated into recombinant ferritin (Fig. 1c, lane 2).

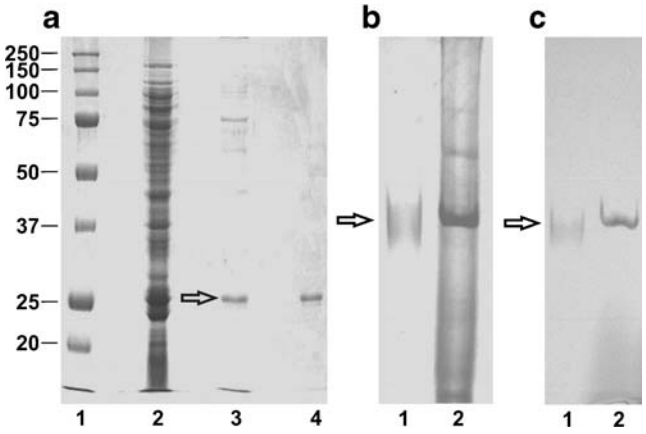

Fig. 1 Expression, purification and iron content of recombinant *Phaseolus vulgaris* ferritin from *Escherichia coli*. Ferritin was expressed by the recombinant plasmid pPVF in *E. coli* cells (see "Materials and methods"). Protein extracts were subjected to native polyacrylamide gel electrophoresis (PAGE) and sodium dodecyl sulfate (SDS) PAGE for qualitative analysis. **a** SDS PAGE (12% polyacrylamide gel) of extracts after progressive purification. Numbers indicate the molecular mass (in kilodaltons) of the standard proteins. *Lane 1*, protein standard; *lane 2*, *E. coli*/pPVF cell extract; *lane 3*, *E. coli* cell extract after ion-exchange chromatography using a (diethylamino)ethyl Sepharose® column; *lane 4*, *E. coli* cell extract after gel filtration using a Sephacryl® S-300 column. **b** Native PAGE (5% polyacrylamide gel) of protein extract stained by Coomassie blue. *Lane 1*, horse spleen ferritin; *lane 2*, *E. coli*/pPVF cell extract. **c** Native PAGE (5% polyacrylamide gel) with iron staining with Prussian blue. *Lane 1*, horse spleen ferritin; *lane 2*, *E. coli*/pPVF cell extract. Intact ferritin in **b** and **c** and ferritin subunits in **a** are marked with an *arrow*

Optimization of iron uptake by *E. coli*

E. coli/pPVF grown in Luria–Bertani medium showed the highest iron incorporation into ferritin at a final concentration of 3 mM $FeSO_4$ or $FeCl_3$ in the medium according to the ferritin-band density in the stained native PAGE gels (not illustrated). Higher iron concentrations inhibited bacterial growth as indicated by a lower optical density of the bacterial suspension and a decrease in ferritin-band density. Iron saturation was approximately 400 iron atoms per ferritin molecule after 24-h incubation time and an iron concentration of 3 mM $FeCl_3$ in the bacterial growth medium (Table 2). Incubation time (8 h compared with 24 h) had no effect on iron saturation of the purified ferritin but showed a positive correlation with ferritin yield. Higher iron saturations could be achieved using the Mg-deficient M9 medium for cultivation. However, bacterial growth was strongly inhibited at the optimum iron concentrations for the Luria–Bertani medium. Lowering of the iron concentration in the medium to 0.2 mM $FeSO_4$ resulted in adequate bacterial growth and an iron saturation of approximately 1,100 iron atoms per ferritin molecule (Table 2). This contrasts with an iron saturation of 40–50 iron atoms per molecule in recombinant ferritin in an earlier experiment [28], in which iron uptake by *E. coli* was not optimized.

Purification and characterization of recombinant *P. vulgaris* [^{57}Fe]ferritin

E. coli/pPVF extracts yielded a single band at approximately 26 kDa (Fig. 1a, lane 4) in SDS PAGE after purification by ion-exchange and size-exclusion chromatography. To make use of recombinant ferritin as an iron isotopic marker, all of the iron in this marker has to be present as ferritin iron, i.e., no other forms of iron should be present. This was assessed by gel filtration chromatography and measuring iron in collected fractions. Recombinant [^{57}Fe]ferritin was eluted from the Superdex® 200 column as a single peak approximately 20 min after sample injection using UV–vis absorption at 280 nm for monitoring. More than 99% of the injected iron was found in corresponding fractions, i.e., more than 99% of the iron in the sample was ferritin-bound (Fig. 2, panel a). This elution pattern could be reproduced after 5 months of protein storage at −20 °C in 50% glycerol. Negative thermal ionization mass spectrometry was used to confirm that isotopic labeling of the recombinant ferritin was successful [32]. The isotopic abundance of ^{57}Fe in the ferritin solution was 95.07±0.04% (standard deviation, SD, n=5) which compares with 95.343±0.03% (SD, n=5) for the ^{57}Fe metal and 2.119±0.0065 (2 times SD) for natural iron [36]. IDMS yielded a total iron concentration of the ferritin solution of 160±0.002 μg g^{-1} (SD, n=5). Over 20%

Table 2 Effect of bacterial growth medium on iron saturation of ferritin

Growth medium	Iron form[a]	Fe concentration (mM)	Fe saturation of ferritin (Fe atoms/ferritin molecule)	Fe recovered in purified ferritin (% of added Fe)
Mg^{2+}-deficient M9 medium	Fe^{2+}	0.2	1100	20
Mg^{2+}-deficient M9 medium	Fe^{2+}	0.1	920	9
Luria–Bertani medium	Fe^{2+}	0.1	500	4
Luria–Bertani medium	Fe^{3+}	3	400	0.2

[a] In experiments with Fe^{2+} sodium ascorbate and sodium citrate were added to the iron solution as described in Materials and methods

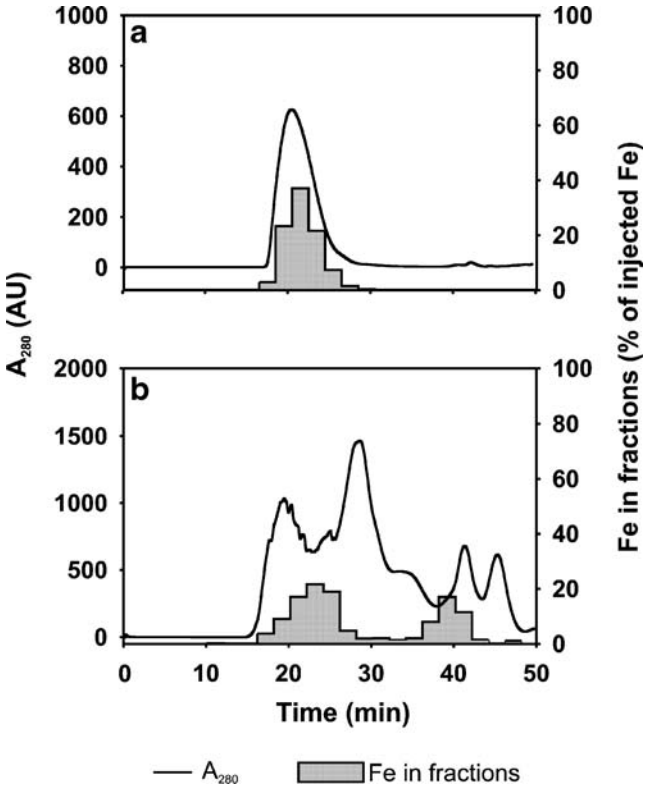

Fig. 2 Size-exclusion chromatograms of recombinant *P. vulgaris* [^{57}Fe]ferritin and red kidney beans using a Superdex® 200 column. Size-exclusion chromatograms were obtained by monitoring for protein ($A_{280\ nm}$) and measuring iron content in collected 1-mL fractions. **a** Purified recombinant [^{57}Fe]ferritin. **b** Red kidney bean extract

of total isotopic iron added to the bacterial growth medium was recovered in the purified recombinant ferritin (Table 2). The protein content of the [^{57}Fe]ferritin solution was 1.43± 0.02 mg g^{-1} (SD, n=5). Plant ferritins were reported to have an overall mass of 540–580 kDa [18–21, 37, 38]. When using an average molecular mass of 560 kDa for the apo protein, we found an iron saturation of approximately 1,100 iron atoms per ferritin molecule.

Size-exclusion chromatography of crude red kidney beans extract

Size-exclusion chromatography showed that approximately 65% of the iron in the red kidney bean extract was eluted from the column 20 min after sample injection. This is in good agreement with the iron elution pattern for the recombinant ferritin (Fig. 2). Approximately 35% of the iron in the red kidney bean extract was eluted 40 min after sample injection. This represents low molecular mass bound iron in the extract. Using the extraction procedure described above, we could extract approximately 60% of total seed iron into the extraction buffer in the presence of EDTA (data not shown). The iron distribution in the extract (Fig. 2, panel b) indicates that water-soluble ferritin iron represents approximately 40% of total red kidney bean iron.

Conclusions

The [^{57}Fe]ferritin spike obtained fulfills the necessary requirements to be used for quantification of ferritin-bound iron by IDMS:

1. Virtually all of the iron in the spike solution is ferritin-bound (Fig. 2, panel a).
2. Retention times for recombinant [^{57}Fe]ferritin and native ferritin from red kidney beans were found to be very similar during size-exclusion chromatography (Fig. 2). This allows the simultaneous collection of labeled and native ferritin from the spiked sample for iron isotopic analysis.
3. Iron remains bound to [^{57}Fe]ferritin in the spike solution for at least 5 months when kept in glycerol at −20 °C without any evidence of species conversion during storage.
4. At a recovery rate of 20% of the isotopic label in the purified recombinant ferritin, transfer efficiency of enriched iron into [^{57}Fe]ferritin is high enough to make the procedures described applicable from an economic point of view.

In conclusion, the ferritin protein content of a plant per se is only of limited predictive value for the amount of ferritin-bound iron in a sample. Direct quantification of ferritin-bound iron by IDMS will provide the necessary information and, in combination with immunochemical techniques for protein quantification, will open up the possibility to accurately assess the filling grade of ferritin in tissue samples. This will possibly help to better understand mechanisms that can potentially lead to iron accumulation in plants.

Acknowledgements The authors wish to express their gratitude to Peter J. Lammers (Department of Chemistry and Biochemistry, New Mexico State University, Las Cruces, USA) for providing the *pfe* gene. Richard Hurrell (Laboratory of Human Nutrition, ETH Zurich) and Christophe Lacroix (Laboratory of Food Biotechnology, ETH Zurich) are kindly acknowledged for providing their laboratory infrastructure for this research. We also thank Gottfried Dasen and Gabriel Hugenschmidt (Laboratory of Food Biotechnology, ETH Zurich) for their advice regarding the molecular cloning. This study was supported by a research grant from ETH Zurich.

References

1. Harrison PM, Arosio P (1996) Biochim Biophys Acta Bioenergetics 1275:161–203
2. Harrison PM, Treffry A, Lilley TH (1986) J Inorg Biochem 27:287–293

3. Ragland M, Briat JF, Gagnon J, Laulhere JP, Massenet O, Theil EC (1990) J Biol Chem 265:18339–18344
4. Seckback J (1982) J Plant Nutr 5:369–394
5. Theil EC (2003) J Nutr 133:1549S–1553S
6. Nappi AJ, Vass E (2002) Dev Neurosci 24:134–142
7. Bouis H (1996) Nutr Rev 54:131–137
8. Theil EC (2004) Annu Rev Nutr 24:327–343
9. Lucca P, Hurrell R, Potrykus I (2001) J Sci Food Agric 81:828–834
10. Goto F, Yoshihara T, Shigemoto N, Toki S, Takaiwa F (1999) Nat Biotechnol 17:282–286
11. Theil EC, Burton JW, Beard JL (1997) Eur J Clin Nutr 51: S28–S31
12. Drakakaki G, Marcel S, Glahn RP, Lund EK, Pariagh S, Fischer R, Christou P, Stoger E (2005) Plant Mol Biol 59:869–880
13. Lucca P, Hurrell R, Potrykus I (2001) Theor Appl Genet 102:392–397
14. Alfrey CP (1978) Crit Rev Clin Lab Sci 9:179–208
15. Spinks A, Fairweathertait SJ (2006) In: Biovailability 2006. Optimizing dietary strategies for better health in developing countries. Institute of Nutrition, Mahidol University, Chiang Mai, p 91
16. Vandermark F, Vandenbriel W (1985) Plant Sci 39:55–60
17. Korcz A, Twardowski T (1993) J Plant Physiol 141:75–81
18. Barcelo F, Miralles F, Arean CO (1997) J Inorg Biochem 66:23–27
19. Laulhere JP, Lescure AM, Briat JF (1988) J Biol Chem 263:10289–10294
20. Sczekan SR, Joshi JG (1987) J Biol Chem 262:13780–13788
21. Barcelo F, Arean CO, Moore GR (1995) Biometals 8:47–52
22. Crichton RR, Ponceortiz Y, Koch MHJ, Parfait R, Stuhrmann HB (1978) Biochem J 171:349–356
23. Clough R, Truscatt J, Belt ST, Evans EH, Fairman B, Catterick T (2003) Appl Spectrosc Rev 38:101–132
24. Reyes LH, Sanz FM, Espilez PH, Marchante-Gayon JM, Alonso JIG, Sanz-Medel A (2004) J Anal Atom Spectrom 19:1230–1235
25. Busto MED, Montes-Bayon M, Sanz-Medel A (2006) Anal Chem 78:8218–8226
26. Harrington CF, Vidler DS, Watts MJ, Hall JF (2005) Anal Chem 77:4034–4041
27. Spence MJ, Henzl MT, Lammers PJ (1991) Plant MolBiol 17:499–504
28. Van Wuytswinkel O, Savino G, Briat JF (1995) Biochem J 305:253–261
29. Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Woodbury
30. Laemmli UK (1970) Nature 227:680–685
31. Hantke K (1997) J Bacteriol 179:6201–6204
32. Walczyk T (1997) Int J Mass Spectrom 161:217–227
33. Walczyk T, Davidsson L, Zavaleta N, Hurrell RF (1997) Fresenius J Anal Chem 359:445–449
34. Theil EC, Briat JF (2004) Plant ferritin and non-heme iron nutrition in humans. HarvestPlus technical monograph 1. Harvest-Plus, Washington
35. Vandermark F, Vandenbriel W, Huisman HG (1983) Biochem J 214:943–950
36. Taylor PDP, Maeck R, Debievre P (1992) Int J Mass Spectrom 121:111–125
37. Masuda T, Goto F, Yoshihara T (2001) J Biol Chem 276:19575–19579
38. Oh SH, Cho SW, Kwon TH, Yang MS (1996) J Biochem Mol Biol 29:540–544