Purification and characterization of an iron-induced ferritin from soybean (*Glycine max*) cell suspensions

Anne-Marie LESCURE, Olivier MASSENET and Jean-François BRIAT*

Laboratoire de Biologie Moléculaire Végétale, Centre National de la Recherche Scientifique (Unité de Recherche Associée n° 1178) et Université Joseph Fourier, B.P. 53X, F-38041 Grenoble Cédex, France

Ferric citrate induces ferritin synthesis and accumulation in soybean (*Glycine max*) cell suspension cultures [Proudhon, Briat & Lescure (1989) *Plant Physiol.* **90**, 586–590]. This iron-induced ferritin has been purified from cells grown for 72 h in the presence of either 100 μM - or 500 μM -ferric citrate. It has a molecular mass of about 600 kDa and is built up from a 28 kDa subunit which is recognized by antibodies raised against pea (*Pisum sativum*) seed ferritin and it has the same *N*-terminal sequence as this latter, except for residue number 3, which is alanine in pea seed ferritin instead of valine in iron-induced soybean cell ferritin. It contains an average of 1800 atoms of iron per molecule whatever the ferric citrate concentration used to induce its synthesis. It is shown that the presence of 100 μM - or 500 μM -ferric citrate in the culture medium leads respectively to an 11- and 28-fold increase in the total intracellular iron concentration and to a 30- and 60-fold increase in the ferritin concentration. However, the percentage of iron stored in the mineral core of ferritin remains constant whatever the ferric citrate concentration used and represents only 5–6 % of cellular iron.

INTRODUCTION

Iron is an essential element for all living organisms. It is required by many functional molecules involved in a great variety of metabolic pathways, such as nitrogen fixation, ribonucleotide reduction, electron transfer, activation and transport of O_2 , as well as inactivation of reduced forms of O_2 . However, the presence of O_2 has two negative consequences for iron utilization by living organisms. Firstly, ferrous iron can be oxidized, and at a concentration greater than 10^{-18} M, ferric iron forms insoluble hydrous ferric oxides. Secondly, free ionic iron can be extremely toxic, catalysing the production of free-radical species, leading to cellular damage.

The storage of iron in a soluble, non-toxic and available form is achieved by ferritins, a class of proteins widely distributed in living organisms. Ferritins are multimeric proteins (24-mers) able to accommodate a few thousand atoms of iron inside their central cavity [for a review see Harrison *et al.* (1989)]. According to Theil (1987), the function of ferritin (i.e. iron storage) can be described in terms of three possible types: iron storage for other cells (specialized cell ferritin), iron storage for intracellular needs (normal housekeeping ferritin) and iron storage for intracellular protection from iron overload (stress housekeeping ferritin). Variations in the regulation of ferritin-gene expression and protein structure are known to occur between these different types (Theil, 1987).

In plants, most of the biochemical work concerning ferritin has been done with purified specialized cell ferritin isolated from seeds (Crichton *et al.*, 1978; van der Mark *et al.*, 1983a; Sczekan & Joshi, 1987; Laulhere *et al.*, 1989). Normal and stress housekeeping ferritins have been purified and partially characterized from French-bean (*Phaseolus vulgaris*) leaves. However, the isolation of such ferritins from leaves in a pure form presents problems because ribulose 1,5-bisphosphate carboxylase (RuBisCo), which represents more than 50 % of soluble proteins in this green tissue, shares comparable physicochemical properties with ferritin (van der Mark & van den Briel, 1985). We have recently reported that it was possible to induce ferritin synthesis and accumulation with ferric citrate in soybean cell

suspensions (Proudhon *et al.*, 1989). These undifferentiated cells are grown heterotrophically in the dark with sucrose as their carbon source. Obtention within a week of a few hundred grams of cells depleted of RuBisCo makes them good starting material to purify and characterize plant stress housekeeping ferritin. In the present paper we report this type of purification. We characterized the purified ferritin by native and denaturing PAGE, by reverse-phase h.p.l.c. and by *N*-terminal sequencing. Furthermore, we show that the iron concentration of the culture medium influences the intracellular concentration of both iron and induced ferritin, but it does not affect the number of iron atoms per purified ferritin molecule, which remains constant. The percentage of cellular iron stored inside the ferritin mineral core is found to be surprisingly low.

MATERIALS AND METHODS

Soybean cell cultures

Soybean cells (*Glycine max* cv. Mandarin, line Sbe4) were grown at 30 °C in the dark in B5 medium with 5 mM-2,4-dichlorophenoxyacetic acid, 0.2 % casein hydrolysate and 100 μM - FeNa_2EDTA as described by Leguay & Jouanneau (1987). In order to induce ferritin synthesis, 200 ml of an 8-day-old culture was transferred into 1 litre of fresh medium containing 100 or 500 μM -ferric citrate (Proudhon *et al.*, 1989). Cells were harvested after 3 days of growth, washed with 20 mM-KCl/5 mM-EDTA, frozen in liquid N_2 and stored at -70 °C.

Purification of iron-induced ferritin from soybean cells

Ferritin was purified from soybean cells induced by 100 or 500 μM -ferric citrate as described for the purification of seed ferritin (Laulhere *et al.*, 1988), but modified as follows. Frozen cells (400–600 g) were ground in liquid N_2 with a pestle and mortar. The resulting powder was transferred into 800 ml of extraction buffer [500 mM-potassium phosphate (pH 7.0)/1 % polyvinylpyrrolidone/0.02 % *o*-phenanthroline/1 mM-phenyl-methanesulphonyl fluoride] and homogenized using a Polytron (PCU-2 Kinematica, Luzern, Switzerland) at maximum speed.

Abbreviations used: RuBisCo, ribulose-1,5-bisphosphate carboxylase; TFA, trifluoroacetic acid.

* To whom correspondence and reprint requests should be sent.

The homogenate was centrifuged for 10 min at 10000 rev./min in a Sorvall SS 34 rotor, and 50 mM-MgCl₂ was added to the supernatant. After a second 5 min centrifugation at 5000 rev./min, 70 mM-trisodium citrate and RNase A (10 mg/ml) were added to the supernatant at room temperature for 1 h. This suspension was then centrifuged through 5 ml of 50 % (v/v) glycerol in a Beckman Ti60 rotor at 59000 rev./min for 1 h. The brown pellets were resuspended in 10 ml of 10 mM-potassium phosphate, pH 7.0. The non-solubilized material was eliminated by a 5 min centrifugation at 5000 rev./min in a Sorvall SS 34 rotor. The supernatant containing ferritin was then loaded on a DEAE-cellulose column (8 cm × 2.7 cm) equilibrated in 10 mM-potassium phosphate, pH 7.0. After washing with the initial buffer, a 100 ml linear gradient was run from 0 to 500 mM-NaCl in the same buffer. Fractions (2 ml) were collected and used to determine protein and iron concentrations. Iron-rich fractions were pooled and centrifuged for 1 h at 80000 rev./min in a Beckman TLA 100-2 rotor. The ferritin pellets were dissolved in 100–200 µl of water and stored at –20 °C.

Iron concentration measurements

Total cellular iron concentrations were determined from dry-ashed cells as described by Beinert (1978). Ferritin iron concentration was obtained from purified ferritin. In both cases iron was estimated by measuring absorbance of Fe-*o*-phenanthroline at 510 nm, using thioglycolic acid as a reducing agent.

Protein analysis

Protein concentrations were determined as described by Bradford (1976). Native and denaturing PAGE, as well as reverse-phase h.p.l.c. and *N*-terminal sequencing, were performed as described by Laulhere *et al.* (1988, 1989). Briefly, 30 µg of iron-induced ferritin purified from soybean cells grown in the presence of 500 µM-ferric citrate for 72 h were pelleted by centrifugation at 100000 rev./min for 1 h in a Beckman TLA 100-2 rotor and resuspended in 30 µl of 10 mM-Tris/HCl (pH 7.6)/150 mM-LiCl/6 M-urea/0.4 M-mercaptoethanol before injection on to a 30 nm (300 Å)-pore-size C₈ silica column (Ultrapore RPSC; Beckman); after washing the column with 10 % (v/v) acetonitrile in 0.1 % trifluoroacetic acid (TFA), the column was eluted with a 10–70 % acetonitrile gradient in 0.1 % TFA; each fraction was monitored at 214 nm, and the fractions containing ferritin were freeze-dried before *N*-terminal sequencing.

Ferritin concentrations were estimated by immunodots, using a titration curve established from purified soybean seed ferritin, antibodies raised against pea seed ferritin and ¹²⁵I-Protein A (Proudhon *et al.*, 1989).

RESULTS

Purification of iron-induced ferritin from soybean cell suspension

Centrifugation of a crude extract of iron-induced soybean cells through a 5 ml 50 %-(v/v)-glycerol cushion, as described in the Materials and methods section, produces a dark-brown pellet. When proteins contained in this pellet are chromatographed on a DEAE-cellulose column, a peak of protein is observed at 185 mM-NaCl if the column is eluted with a linear gradient of from 0 to 500 mM-NaCl. The iron content of the different fractions collected indicates that a peak of iron corresponds to the peak of proteins. An example of the elution profile obtained is given in Fig. 1. Central fractions of the iron and protein peak (fractions 17–22 in Fig. 1 for example) were collected and centrifuged at 80000 rev./min (280000 g) for 1 h in a Beckman TL 100-2 rotor. Dark-brown pellets were resuspended in water to a protein concentration of 1–2 mg/ml.

Fig. 1. DEAE-cellulose chromatography of iron-induced soybean cell ferritin

Elution profile showing the superimposition of the protein and iron peaks corresponding to the chromatography of protein extracted from cells induced with 100 µM-ferric citrate as described in the Materials and methods section.

Fig. 2. Determination of the molecular mass of iron-induced soybean cell ferritin by non-denaturing gradient-gel electrophoresis

Lane 1, 10 µg of horse spleen ferritin; lane 2, 8 µg of pea seed ferritin; lane 3, 8 µg of iron-induced ferritin from soybean cells grown in the presence of 500 µM-ferric citrate for 72 h. The gel was a linear 5–10 % polyacrylamide gradient in Tris/borate buffer. It was run at 50 V for 24 h, after which time no movement of the ferritin could be detected.

Identification and characterization of purified iron-induced ferritin

Four independent experiments were performed to identify and characterize the iron-induced ferritin purified from soybean cell suspensions. Firstly, native PAGE of the purified protein reveals a unique band of molecular mass of about 600 kDa, which is higher than that of the horse spleen and pea (*Pisum sativum*) seed ferritin (Fig. 2), but similar to that of the jack-bean (*Canavalia ensiformis*) seed ferritin (Briat *et al.*, 1989). This purified protein contains an average of 1800 atoms of iron per molecule (Table 1). Secondly, denaturing-gel electrophoresis indicates that this high-molecular-mass protein is built up from a 28 kDa subunit, as in the pea seed ferritin (Fig. 3a). Furthermore, an immunoblot experiment has shown that this 28 kDa polypeptide is recognized by antibodies raised against the pea seed ferritin (Fig. 3b). Thirdly, iron-induced ferritin purified from cells grown in the presence of 500 µM-ferric citrate was chromatographed by reverse-phase h.p.l.c. The protein is eluted at 48 % acetonitrile (Fig. 4), as are pea and maize seed ferritins (Laulhere *et al.*, 1988). Fourthly, the *N*-terminal part of this

Table 1. Influence of the extracellular concentration of ferric citrate on the total cellular iron, ferritin and ferritin iron concentrations in soybean cell suspension culture

All the analyses were made on cells grown for 72 h at 30 °C in the dark after adding ferric citrate. Proteins were extracted as described in the Materials and methods section. Iron and ferritin concentrations were determined in triplicate on aliquots of these extracts. Iron concentration was determined by reduction and spectrophotometric dosage of Fe(II)-*o*-phenanthroline complex at 510 nm. Ferritin concentration was estimated by immunodots on extracts of total protein using soybean seed ferritin, antibodies raised against pea seed ferritin and ¹²⁵I-Protein A to establish a titration curve. The number of iron atoms/ferritin molecule was calculated by using 600 kDa as the molecular mass of the iron-induced ferritin.

[Ferric citrate] in the culture medium (μM)	[Cellular iron] (pmol/mg of dry matter)	[Ferritin] (pmol/mg of dry matter)	Iron atoms/ ferritin molecule (n)	[Ferritin iron]	
				(pmol/mg dry matter)	(% of total cellular iron)
0	400	<0.005	—	—	—
100	4500	0.15	1800	270	6.0
500	10 500	0.30	1880	540	5.1

Fig. 3. Characterization of the subunit of the ferritin purified from iron-induced soybean cell suspension cultures

(a) Coomassie Blue R-250 staining of a 15%-polyacrylamide/SDS gel loaded with: 4 μg of marker (lane 1), 4 μg of pea seed ferritin (lane 2) or 4 μg of iron-induced ferritin from soybean cells grown in the presence of 500 μM-ferric citrate for 72 h (lane 3). (b) Immunoblot analysis of a 15%-polyacrylamide/SDS gel loaded with: 1.5 μg of concanavalin A (lane 1), 0.25 μg of pea seed ferritin (lane 2) and with 0.75 μg of iron-induced ferritin from soybean cells grown in the presence of 500 μM-ferric citrate for 72 h (lane 3). After electrophoresis, the proteins in the gel were electrotransferred on to nitrocellulose and detected with pea seed ferritin antibodies diluted to 1:500 and anti-(rabbit IgG) sheep immunoglobulins coupled with peroxidase (diluted to 1:600).

h.p.l.c.-purified iron-induced ferritin was sequenced (Fig. 5). It was found to show total sequence identity with the *N*-terminal extremity of the specialized cell ferritin from pea seeds which has previously been reported (Laulhere *et al.*, 1989), except for position 3, which is occupied by alanine in the pea seed ferritin sequence and valine in the soybean cell ferritin sequence; in neither sequence has it been possible to determine the first residue at the *N*-terminus without ambiguity, and it could be alanine, threonine or serine.

Fig. 4. Analysis of iron-induced soybean cell ferritin using reverse-phase h.p.l.c.

Iron-induced ferritin purified from soybean cells grown in the presence of 500 μM-ferric citrate for 72 h (30 μg) was analysed on a 30 nm (300 Å)-pore-size C₃ silica column (Ultrapore RPSC; Beckman) as described in the Materials and methods section.

X-T-A-P-L-T-G-V-I-F-E-P-F-E- Pea seeds
X-T-V-P-L-T-G-V-I-F-E-P-F-E- Iron-induced soybean cells

Fig. 5. Comparison of the *N*-terminal sequence of iron-induced soybean cell ferritin with that of pea seed ferritin

The *N*-terminal sequence of iron-induced soybean cell ferritin was determined from h.p.l.c.-purified protein by automatic Edman degradation. The pea seed ferritin sequence is from Laulhere *et al.* (1989). X is the first amino acid of both sequences, which has not been determined without ambiguity; it could be alanine, threonine or serine.

Influence of the extracellular iron concentration on the ferritin, ferritin iron and total cellular iron concentration

After 72 h, cells grown in the presence of 100 μM- and 500 μM-ferric citrate contain 11 and 28 times more iron respectively than

cells depleted of iron. Over the same period of time, ferritin concentrations increase by at least 30- and 60-fold respectively (Table 1). On the other hand, ferritin purified from cells induced with 100 μM - and 500 μM -ferric citrate respectively has the same amount of iron per molecule and is involved, in both cases, in the storage of no more than 6% of total cellular iron (Table 1).

DISCUSSION

We have previously reported that ferric citrate induces ferritin synthesis and accumulation in soybean cell suspensions and that the control of this induction is transcriptional (Proudhon *et al.*, 1989), thus confirming the results obtained by van der Mark *et al.* (1983b) with bean leaves. A transcriptional control also applies to animal housekeeping ferritin, as reported by Dickey *et al.* (1987), who used tadpole cells. On the other hand, synthesis of animal specialized-cell ferritin is controlled at the translational level (Zähringer *et al.*, 1976; Schull & Theil, 1982), and the molecular details of this mechanism have recently been elucidated [for a review, see Harford & Klausner (1989)]. The level of control of the synthesis of plant specialized-cell ferritin (i.e. seed ferritin) is so far unknown. Since in animal cells the control of the synthesis of housekeeping and specialized-cell ferritins does not occur at the same level, it would seem that it is important to study any possible differences in the respective structures and functions of these proteins which are involved in iron storage.

The soybean cell suspensions used provide a good system with which to develop our understanding of the molecular mechanisms involved in the transcriptional control of housekeeping ferritin synthesis. In order to use this system as a model to study also the structure and function of a stress housekeeping ferritin, we decided to purify and to characterize this iron-induced protein from soybean cells. The highest ferric citrate concentration used to induce ferritin synthesis in soybean cells led to the accumulation of 1 mg of ferritin/100 g wet weight of cells (as determined by immunodots). After purification, 0.1 mg of ferritin/100 g of cells was recovered. Improvement of this 10% recovery may be possible by employing better conditions in the early steps of our purification procedure (i.e. re-extraction of the paste after the first low-speed centrifugation should increase the amount of ferritin in the supernatant, which is then loaded on to the 50%-glycerol cushion).

The iron-induced ferritin which we purified is built up from a 28 kDa subunit similar to the 28 kDa subunit of pea seed ferritin (specialized-cell ferritin). This conclusion is based on migrations in denaturing polyacrylamide gels, cross-immunoreactivity, chromatographic properties on reverse-phase h.p.l.c. and *N*-terminal sequence similarity. However, the native molecular mass of the iron-induced ferritin is slightly higher than that of the pea seed ferritin. So far we have no explanation for this difference. The complete amino acid sequence and structural analysis of seed and plant iron-induced ferritin are required to determine whether there are any differences between specialized-cell and stress housekeeping ferritins in plants.

The amount of iron-induced ferritin doubles when the intracellular iron concentration doubles. However, the amount of stored iron in the mineral core remains constant and represents

only 5–6% of cellular iron (Table 1). This percentage is low in comparison with that of iron-induced ferritin from bean leaves. This latter ferritin also only contains 4% of total cellular iron (1200 atoms of iron/molecule) 24 h after iron induction, but it reaches 30% (2300 atoms of iron/molecule) 72 h after induction (van der Mark *et al.*, 1982). Concerning this point, it is important to be reminded that, in plants, ferritins are localized within plastids (Seckback, 1982; van der Mark *et al.*, 1983a). In green leaves, the number of chloroplasts per palisade cell amounts to a few hundred (Scott & Possingham, 1980). On the other hand, in soybean cell suspensions grown heterotrophically in the dark, we observed by microscopy that, 72 h after iron induction, the number of amyloplasts and proplasts was low (between 10 and 15/cell) and that most of the volume of each cell was occupied by a very large vacuole (results not shown). This difference in plastid number/cell in the two systems could explain the difference in percentage of cellular iron contained in ferritin. Furthermore, plant vacuoles, which were very large in our system, could be involved in iron detoxification. This would be consistent with the observation that, in baker's yeast (*Saccharomyces cerevisiae*), iron was in part chelated and stored as small undetermined molecules inside the vacuole (Raguzzi *et al.*, 1988).

REFERENCES

- Beinert, H. (1978) *Methods Enzymol.* **54**, 435–445
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- Briat, J. F., Massenet, O. M. & Lahlhere, J. P. (1989) *J. Biol. Chem.* **264**, 11550–11553
- Crichton, R. R., Ponce-Ortiz, Y., Koch, M. H. J., Parfait, R. & Stuhlmann, H. B. (1978) *Biochem. J.* **171**, 349–356
- Dickey, L. F., Sreedharan, S., Theil, E. C., Didsbury, J. R., Wang, Y. H. & Kaufman, R. E. (1987) *J. Biol. Chem.* **262**, 7901–7907
- Harford, J. B. & Klausner, R. D. (1989) *Science* **246**, 870–872
- Harrison, P. M., Artymiuk, P. J., Ford, G. C., Lawson, D. M., Smith, J. M. A., Treffry, A. & White, J. L. (1989) in *Biomimetalization: Chemical and Biochemical Perspectives* (Mann, S., Webb, J. & Williams, R. J. P., eds.), pp. 257–294, VCH, Weinheim
- Lahlhere, J. P., Lescure, A. M. & Briat, J. F. (1988) *J. Biol. Chem.* **263**, 10289–10294
- Lahlhere, J. P., Laboure, A. M. & Briat, J. F. (1989) *J. Biol. Chem.* **264**, 3629–3635
- Lahlhere, J. P., Laboure, A. M. & Briat, J. F. (1990) *Biochem. J.* **269**, 79–84
- Leguay, J. J. & Jouanneau, J. P. (1987) *Dev. Gene* **8**, 351–364
- Proudhon, D., Briat, J. F. & Lescure, A. M. (1989) *Plant Physiol.* **90**, 586–590
- Raguzzi, F., Lesuisse, E. & Crichton, R. R. (1988) *FEBS Lett.* **231**, 253–258
- Schull, G. E. & Theil, E. C. (1982) *J. Biol. Chem.* **257**, 14187–14191
- Scott, N. S. & Possingham, J. V. (1980) *J. Exp. Bot.* **31**, 1081–1092
- Sczekan, S. R. & Joshi, J. G. (1987) *J. Biol. Chem.* **262**, 13780–13788
- Seckback, J. (1982) *J. Plant Nutr.* **5**, 369–394
- Theil, E. C. (1987) *Annu. Rev. Biochem.* **56**, 289–315
- van der Mark, F. & van den Briel, W. L. (1985) *Plant Sci.* **39**, 55–60
- van der Mark, F., van den Briel, W. L., van Oers, J. W. A. M. & Bienfait, H. F. (1982) *Planta* **156**, 341–344
- van der Mark, F., van den Briel, W. L. & Huisman, H. G. (1983a) *Biochem. J.* **214**, 943–950
- van der Mark, F., Bienfait, H. F. & van der Ende, H. (1983b) *Biochem. Biophys. Res. Commun.* **115**, 463–469
- Zähringer, J., Baliga, B. S. & Munro, H. N. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 857–861