Purification and characterization of recombinant pea-seed ferritins expressed in *Escherichia coli*: influence of N-terminus deletions on protein solubility and core formation *in vitro*

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Plant ferritin subunits are synthesized as precursor molecules; the transit peptide (TP) in their NH_2 extremity, responsible for plastid targeting, is cleaved during translocation to this compartment. In addition, the N-terminus of the mature subunit contains a plant-specific sequence named extension peptide (EP) [Ragland, Briat, Gagnon, Laulhère, Massenet, and Theil, E. C. (1990) J. Biol. Chem. **265**, 18339–18344], the function of which is unknown. A novel pea-seed ferritin cDNA, with a consensus ferroxidase centre conserved within H-type animal ferritins has been characterized. This pea-seed ferritin cDNA has been engineered using oligonucleotide-directed mutagenesis to produce DNA fragments (1) corresponding to the wild-type (WT) ferritin precursor, (2) with the TP deleted, (3) with both the TP and the plant specific EP sequences deleted and (4) containing the TP but with the EP deleted. These four DNA fragments have been cloned in an *Escherichia coli* expression vector to produce the corresponding recombinant pea-seed ferritins. Expression at 37 °C led to the accumulation of recombinant pea-seed ferritins in inclusion bodies, whatever the construct introduced in *E. coli*. Expression at 25 °C in the presence of sorbitol and betaine allowed soluble proteins to accumulate when constructs with the TP deleted were used; under this condition, *E. coli* cells transformed with constructs containing the TP were unable to accumulate recombinant protein. Recombinant ferritins purified from inclusion bodies were found to be assembled only when the TP was deleted; however assembled ferritin under this condition had a ferroxidase activity undetectable at acid pH. On the other hand, soluble recombinant ferritins with the TP deleted and expressed at 25 °C were purified as 24-mers containing an average of 40–50 iron atoms per molecule. Despite the conservation in the plant ferritin subunit of a consensus ferroxidase centre, the iron uptake activity *in vitro* at pH 6.8 was found to be lower than that of the recombinant human H-ferritin, though it was much more active than the recombinant human L-ferritin. The recombinant ferritin with both the TP and the EP deleted (rΔTP/EP) assembled correctly as a 24-mer; it has slightly higher ferroxidase activity and decreased solubility compared with the wild-type protein with the TP deleted (rΔTP). In addition, on denaturation by urea followed by renaturation by dialysis the rΔTP/EP protein showed a 25% increase in core-formation *in vitro* compared with the rΔTP protein. These results demonstrate (1) that the TP must be deleted to produce assembled active recombinant pea-seed ferritin in *E. coli* and (2) that the specific sequence (EP) found at the N-terminus of the mature subunit of plant ferritin plays an active role in the structure/function properties of this protein, although it is not essential for obtaining assembled molecules active in iron-core formation.

INTRODUCTION

Ferritins are multimeric proteins (24-mers) present in animals, plants and microorganisms. They play an important role in controlling cellular iron homeostasis because of their ability to store iron atoms in a soluble, bioavailable non-toxic form [1]. Mammal ferritins are composed of two types of subunits, H and L, which assemble in various ratios according to the tissue in which they are found. H-subunits are necessary for rapid Fe(II)-oxidation and sequestration of Fe(III), avoiding iron-catalysed production of reactive oxy-radical species. The oxidase activity of the ferritin H-subunit depends on conserved amino acids [2–4]. Mutation of both the ferroxidase and nucleation-site ligands (Glu-27, Glu-61, Glu-62, Glu-64, Glu-67, His-65, Glu-107) in recombinant human H-ferritin (rHF) suppresses the Fe^{2+} oxidation whereas mutation of the nucleation-site ligands alone (Glu-61, Glu-64, Glu-67), which are conserved in both H- and L-subunits, decreases the activity only slightly [5]. Other residues may also be important in explaining the faster rates of iron biomineralization by H- compared with L-type ferritin, since an Fe(III)-tyrosinate intermediate has been shown to form during the first stages of iron uptake by recombinant H-ferritin from bullfrog red cells [6]. L-chains do not contain ferroxidase site ligands and core-formation is much slower than for H-chain ferritin [7,8]. L-subunits are however better than H-subunits in converting newly oxidized iron to iron-core nuclei, and this property could be due to the six conserved glutamate residues (Glu-49, 57, 60, 61, 64, 67) lying on the cavity-side of their B-helices; only three of these (61, 64 and 67) are present in mammalian H-chains [4].

Plant ferritins share an amino acid sequence identity with animal ferritins ranging from 39% to 49%. So far, only one type of plant-ferritin subunit has been described, which has both H- and L-subunit characteristics [4,9,10]. The amino acids involved in the definition of the ferroxidase centre are strictly conserved in all the plant ferritin sequences reported [9–12] except for the pea-seed ferritin PeSd1 [10]. In this case, a histidine residue is found at position 62 of the amino acid sequence; this has been

Abbreviations used: EP, extension peptide; TP, transit peptide; WT, wild type; IPTG, isopropyl β-D-thiogalactoside; rHF, recombinant human H-ferritin.

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The nucelotide sequence of Pesd2 appears in the EMBL Data Library under accession number X73369.

determined from the cDNA sequence [10] and the sequence of an internal peptide generated by CNBr cleavage of a purified pea-seed ferritin subunit [13]. Based on a computer modelling prediction [10], this single change does not affect the structure of the ferroxidase centre. A purified pea-seed ferritin has a ferroxidase activity *in vitro*, which is, however, slightly lower than that of the rHF [14]. In animal ferritins, the replacement of Glu-62 by another amino acid leads to a decrease in the ferroxidase activity of the protein [5,15]. Additional conserved glutamate residues characteristic of animal L-type ferritin are found on the cavity-side of their B-helices (Glu-57 and Glu-60) and these are also present in plant ferritin [10,13]. Furthermore, sequence comparison of plant and animal ferritins has revealed an extra sequence at the N-terminus of the plant protein subunit [13]. The first part of this sequence encodes a transit peptide (TP) responsible for the plastid targeting of plant ferritins; this TP is cleaved *in vivo* from the ferritin precursor during its translocation to plastids, giving the mature ferritin subunit which assembles in a 24-mer apoferritin within the plastids [9,16–18]. The second part of this sequence, named the extension peptide (EP), is specific to plant ferritins and is found in the mature subunit [13]. The EP sequence contains 24 amino acids and a 3-turn α-helix has been predicted within it [10]. The function of this peptide is unknown; however it has been suggested that it could be involved in the loss of protein solubility and stability during iron-exchange *in vitro* [19] and *in vivo* during the early steps of germination [20].

In order to contribute to the general knowledge of ferritin structure/function relationships, we decided to express recombinant plant ferritins in *Escherichia coli* and to characterize them. In this paper we report the cloning and sequencing of a pea-seed ferritin cDNA encoding a ferritin subunit with a consensus ferroxidase centre. This cDNA has been engineered and used to express recombinant pea-seed ferritins in *E. coli*. In particular, influence of the plant-specific N-terminus has been investigated by using a set of deletion mutants. The effects of the deletion of the TP and of the EP sequence on ferritin–protein assembly, solubility and *in vitro* core-formation have been determined.

MATERIALS AND METHODS

Cloning, sequencing and mutant synthesis

A pea-seed cDNA library already described [10] was screened with the *Eco*RI insert fragment from the ferritin cDNA clone PeSd1 [10] ^{32}P-labelled by random priming. Twelve positive phages were purified and further analysed by subcloning into pUC 18 followed by sequencing using the dideoxy chain termination method [21].

One of these clones, PeSd2 (Figure 1), was used as a template in PCR reactions in order to synthesize DNA fragments designed to be cloned in an *E. coli* expression vector. The PCR fragment corresponding to the wild-type (WT) ferritin sequence was synthesized by using the oligonucleotide P1 (5′-GCGCATATGGCTCTTTCTTCTTC-3′) and the oligonucleotide P6 (5′-CCCGGATCCTCAAGCACCATGTACTC-3′) hybridizing to the 5′ and 3′ ends of the PeSd2 template respectively. The PCR fragment corresponding to the pea-seed ferritin sequence with the sequence coding for the TP deleted (ΔTP) was synthesized by using the oligonucleotide P2 (5′-GCGCATATGACAACTGCTCCTTTGAC-3′) in the 5′ position and the oligonucleotide P6 in the 3′ position. The PCR fragment corresponding to the pea-seed ferritin sequence with the sequences coding for both the TP and the EP deleted (ΔTP/EP) was synthesized by using the oligonucleotide P3 (5′-GCGCATATGTCTGTTCCTCTTGTTTC-3′) in the 5′ position

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                                                                  *
PeSd1   MALSSSKFSSFSGFSLSPVSGNGVQKPCFCDLRVGEKWGSRKFRVCATTA
PeSd2   --------------------------------------------------
                                    1
        PLTGVIFEPFEEVKKDYLAVPSVPLVSLARQNFADECESVINEQINVEYN
        --------------------------------------------------

     30 ASYVYHSLFAYFDRDNVALKGFAKFFKESSEEEREHAEKLMKYQNTRGGR
        V------M----------------------------E----------------

     80 VVLHPIKDVPSEFEHVEKGDALYAMELALSLEKLTNEKLLNVHSVAERNN
        ----------------------H---------------------------

    130 DLEMTHFIEGEYLAEQVEAIKKISEYVAQLRRVGKGHGVWHFDQRLLHGV
        --------------------------------------------------

    180 HGA
        ---
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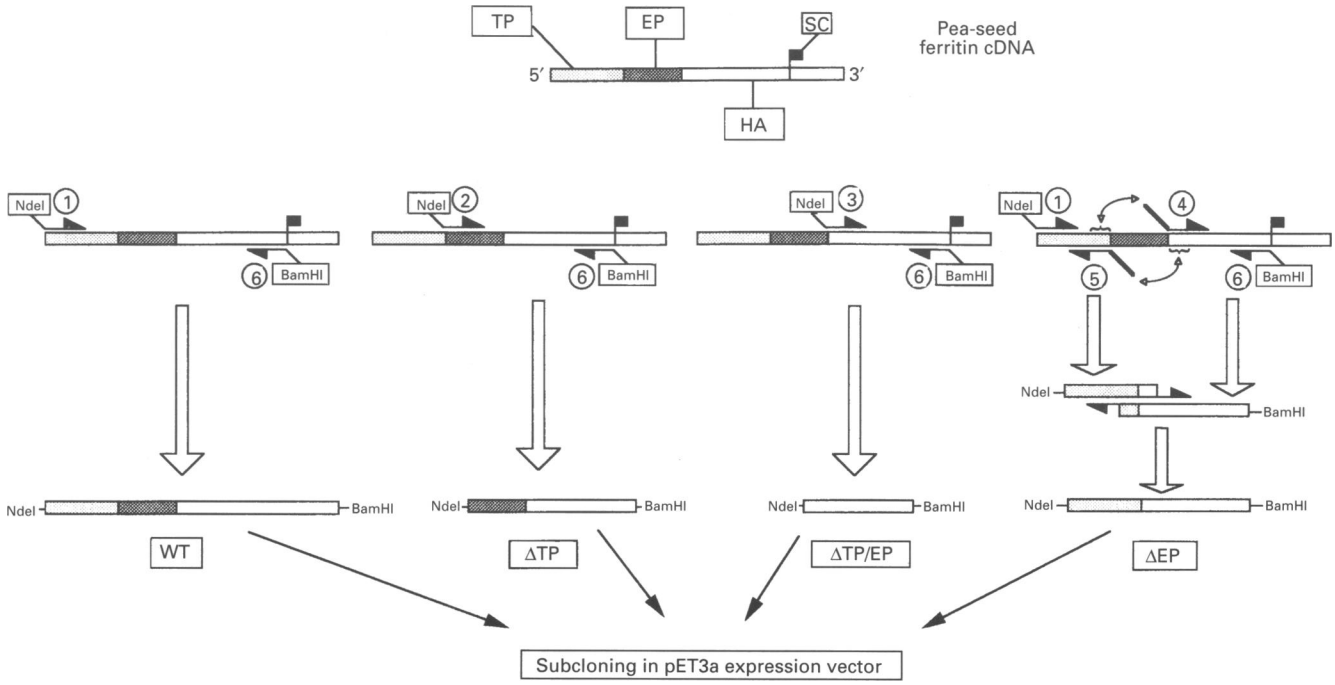
Figure 1 Amino acid sequence comparison of two different pea-seed ferritin subunits

PeSd1 is from [10]. Numbers correspond to those used for the recombinant human H-ferritin [3]. Between * and 1 is the plant specific sequence found in the NH_2 extremity of the mature subunit [13]. The sequence for the TP responsible for plastid targeting of plant-ferritin precursors corresponds to the region from the first Met residue to * [13]. The residue 62 (HisGlu) which restores the consensus ferroxidase centre in PeSd2 is in bold.

and the oligonucleotide P6 in the 3′ position. The last PCR fragment, coding for the pea-seed ferritin sequence with the EP deleted but containing the TP (ΔEP), in frame with the ferritin sequence downstream of the EP, was synthesized by using a two-step PCR method. The first step allowed two PCR sub-fragments with sequence identity at one of their extremities to be produced. Each sub-fragment was synthesized independently by using oligonucleotides P1 and P5 (5′-GAAACAAGAGGAACAG-AAGCGCAAACCCTAAATTTTC-3′) for the first one and oligonucleotides P4 (5′-GAAAATTTAGGGTTTGCGCTTCT-GTTCCTCTTGTTTC-3′) and P6 for the second one. It should be noted that the sequences of P4 and P5 were complementary and contained sequences coding for the C-terminal part of the TP and for the N-terminal part of the ferritin sequence homologous to animal ferritin found downstream of the EP sequence. These two sub-fragments were then used to perform a second PCR in which each sub-fragment could be hybridized to the other one due to the complementarity of one of their extremities, resulting from the first PCR. Amplification of this partial hybrid led to the synthesis of a PCR fragment with the internal sequence coding for the EP deleted (see Scheme 1). All the oligonucleotides used in the PCR reactions, with the exception of P4 and P5, were extended at their 5′-end with three non-hybridizing C- or G-nucleotides to allow good restriction of the PCR fragments by appropriate enzymes. Oligonucleotides P1, P2 and P3 contained an *Nde*I restriction site with the ATG translation initiation codon (underlined sequence). The P6 oligonucleotide contained the TGA stop codon (bold sequence). PCR reactions were conducted for 30 cycles including 30 s of denaturation at 94 °C, 2 min of hybridization at 60 °C and 2 min of DNA synthesis driven by the Taq polymerase (Boehringer-Mannheim) at 75 °C. Oligonucleotides were used at a concentration of 1 pM/μl. The PeSd2 template, linearized by *Pst*I, was used at a concentration of 0.2 pg/μl. After restriction by *Nde*I and *Bam*HI, the final PCR fragments were subcloned by conventional methods [22] into the pET3a vector [23] cut by the same enzymes (Scheme 1). This orientated cloning put DNA fragments under the control of the T7 promoter for their transcription. Plasmids harbouring the PCR-amplified fragments were selected based on restriction patterns. Both strands of the DNA inserts of each plasmid were sequenced in order to ensure that no mutations had been introduced during the course of PCR amplification. They were found to be identical with the corresponding sequence of the



Scheme 1 Schematic representation of the different DNA fragments engineered to express recombinant pea-seed ferritins in *E. coli*

The PeSd2 cDNA was used as a template to amplify, by PCR, DNA fragments coding for the pea-seed ferritin precursor with the EP deleted (ΔEP) or not (WT) and coding for the WT pea-seed, mature ferritin subunit with the EP deleted (ΔTP/EP) or not (ΔTP). The sequence homologous to animal ferritin is labelled HA. TP = transit peptide responsible for plastid targeting of ferritin precursors; EP = extension peptide found specifically in the N-terminus of plant ferritin subunit; SC = translational stop codon of pea-seed ferritin. Oligonucleotides (1, 2, 3, 4, 5 and 6) are described in Materials and methods. The DNA fragments were introduced into the pET3a vector. Expression and purification of recombinant ferritins was then performed according to Method I or II described in the Materials and methods section.

PeSd2 cDNA template (data not shown). *E. coli* BL21(DE3) [23] strain was then transformed with these constructs.

Culture conditions for expression in bacteria

Two different expression conditions were used varying in culture-broth composition and in the temperature of induction. In Method I, cultures in LB broth were grown at 37 °C until an A_{600} of 0.6–1 was reached. Expression was then induced by adding 0.4 mM isopropyl β-D-thiogalactoside (IPTG) [23]. Expression was maintained for 3 h at 37 °C until cells were harvested by centrifugation. In Method II, adapted from Blackwell and Horgan [24], a preculture at 37 °C in LB broth supplemented with 1 M sorbitol and 2.5 mM betaine/HCl was grown until an A_{600} of 0.6–1 was reached. The temperature of the cultures was then lowered to 25 °C and 0.4 mM IPTG was added. Expression was maintained at 25 °C for 3 h until cells were harvested.

Protein purification

Pea-seed ferritin was purified according to Laulhère et al. [19]. Purification of the recombinant ferritins was performed by two different procedures, depending on the conditions of culture that were used. After expression induced by Method I, cells were harvested by centrifugation and resuspended in 20 mM Tris/maleate buffer, pH 8.0. They were then lysed by sonication for 3 min and centrifuged for 5 min at 12100 *g* in a JA-20 Beckman rotor. The pellet containing inclusion bodies of expressed ferritin was washed with 20 mM Tris/maleate buffer, pH 8.0, 1 mg/ml deoxycholate, 1 mM EDTA and 0.2 mg/ml lysozyme and centrifuged again. The resulting pellet was washed twice with 20 mM Tris/maleate buffer, pH 8.0, 3 M urea and 0.2% Triton X-100. The final pellet was then solubilized in 6 M urea. After centrifugation under the same conditions as described above the supernatant was collected and dialysed for 20 h against large volumes of 20 mM Tris/maleate buffer, pH 8.0, containing decreasing concentrations of urea until its removal was complete.

After expression induced by Method II the cells were collected and lysed as in Method I. The crude extract was centrifuged to eliminate the bacterial cell debris and the resulting supernatant was incubated at 75 °C for 10 min. After centrifugation for 5 min at 12000 *g* in a JA-20 Beckman rotor, the supernatant containing the expressed soluble recombinant ferritin was collected and loaded on a gel-filtration AcA 22 (IBF, Villeneuve la Garenne, France) column previously calibrated with purified pea-seed ferritin. Proteins contained in the fractions corresponding to the elution-profile of pea-seed ferritin were then concentrated on YM30 membranes (Amicon) and loaded on a MonoQ cation-exchange column (Pharmacia). The recombinant ferritin was eluted through a 0–0.5 M NaCl gradient in 20 mM Tris/maleate buffer, pH 8.0.

Protein determination and electrophoresis

Protein concentrations were determined by the method of Bradford [25]. Electrophoresis under denaturing conditions was performed as previously described [20]. Samples used for the analysis of total *E. coli* proteins were obtained by denaturing bacteria cell pellets at 100 °C for 10 min in 50 mM Tris/HCl, pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% Bromophenol Blue and 10% glycerol. Non-denaturing electrophoresis was performed on 4–25% polyacrylamide gradient gels in TBE

buffer [19]. Gels were stained with Coomassie Brilliant Blue R250.

Iron determination and uptake measurements

Iron content of purified ferritin was determined as previously described [19]. The kinetics of iron uptake by various ferritins were studied as described by Santambrogio et al. [26] using a molar apoferritin/iron ratio of 1:1000. Freshly prepared iron(II) sulphate was added to a final concentration of 0.1 mM to 0.1 μM of various recombinant apoferritins, in 20 mM Hepes buffer, pH 6.8, at room temperature. The formation of an amber colour resulting from iron(III) incorporation into ferritins was monitored by the increase in absorbance at 310 nm.

Denaturation–renaturation analysis

Recombinant ferritins were denatured by using deionized urea at various concentrations ranging from 1 M to 7 M during 10 min at room temperature. Renaturation was achieved by following the method reported by Santambrogio et al. [26]: denatured samples were diluted at least 10-fold in 20 mM Tris/maleate buffer, pH 8.0, and left for 2 h at room temperature. Core-formation by these renatured samples was then monitored by adding iron(II) sulphate under the same conditions as described above. pH was decreased to 6.8 by adding Hepes buffer to a final concentration of 100 mM. Measurement of the amber colour formation was performed at 310 nm after 40 min of iron uptake, which is enough time for the plateau of the kinetics to be reached.

RESULTS

Characterization of a pea-seed ferritin cDNA encoding a ferritin subunit with a consensus ferroxidase centre

By systematic sequencing of ferritin cDNAs isolated from a previously described pea-seed cDNA library [10], a novel pea-seed ferritin cDNA (PeSd2) was characterized. At the nucleotide level, six base differences were observed in comparison with PeSd1 (Table 1); at the amino acid level two of these changes have no effect. Among the four others (Table 1 and Figure 1) it is important to notice the change His-62→Glu-62, thus the PeSd2 pea-seed ferritin subunit has complete identity with the consensus ferroxidase centre found in animal H-type ferritin. Glu-57 and Glu-60, conserved in the L-type ferritin subunit, as well as Glu-61, Glu-64 and Glu-67, found both in H- an L-type ferritin subunits are also conserved in the PeSd2 cDNA. It can be concluded therefore, that there are different transcripts encoding pea-seed ferritin subunits with small amino acids variations; the significance of this remains to be determined.

Table 1 Differences in the nucleotide sequence and deduced amino acid sequences between the two pea-seed ferritin subunits PeSd1 and PeSd2

Position (bp)	Base change (PeSd1 to PeSd2)	Amino acid change
302	C to T	Ala to Val (30)
322	T to A	Leu to Met (37)
397	C to G	His to Glu
399	T to G	(62)
432	A to G	None
441	C to T	None
517	T to C	Tyr to His (102)

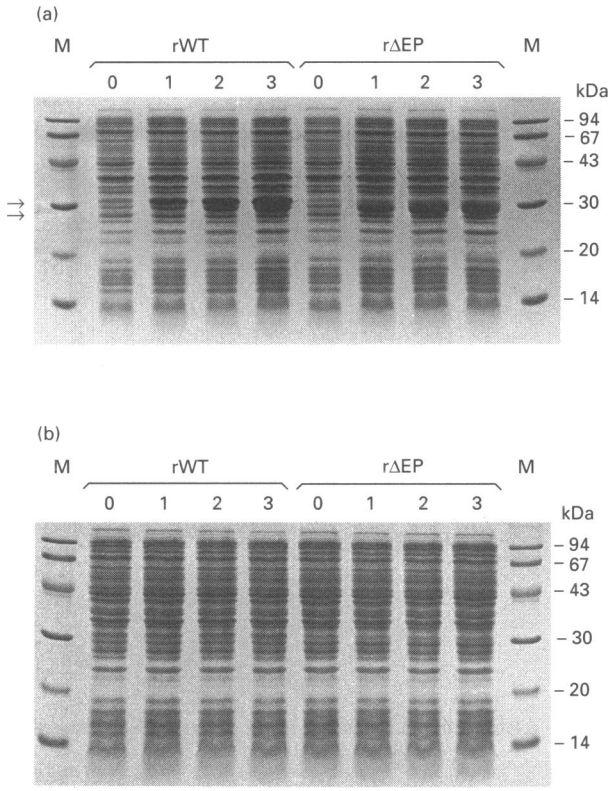


Figure 2 Expression of recombinant pea-seed ferritin precursors in *E. coli*

Recombinant pea-seed ferritin precursors were expressed in *E. coli* BL21 (DE3): (**a**) at 37 °C and (**b**) at 25 °C in the presence of 1 M sorbitol and 2.5 mM betaine. Induction was achieved by adding 0.4 mM IPTG to the culture broth when the absorbance at 600 nm was between 0.6 and 1. Induction was performed for 1, 2 or 3 h. Total proteins contained in the cells were analysed on SDS/PAGE. Arrows indicate the migration of the two recombinant precursors expressed at 37 °C. Note that no polypeptides corresponding to ferritin precursors are accumulated at 25 °C, whatever the constructs used. rWT = recombinant wild-type pea-seed ferritin precursor; rΔEP = recombinant pea-seed ferritin precursor with the EP deleted. M = molecular weight markers.

Expression in *E. coli* and characterization of the recombinant pea-seed ferritin precursors rWT and rΔEP

The plasmids containing inserts corresponding to the pea-seed ferritin precursor with the EP sequence deleted (pΔEP) or not (pWT) (see Materials and methods section and Scheme 1) were used to express wild-type pea-seed ferritin precursor (rWT) and its related EP deletion mutant (rΔEP) in the *E. coli* strain BL21(DE3). Two different conditions were tested to express rWT and rΔEP proteins. At 37 °C in LB broth (Method I), good expression is obtained for both rWT and rΔEP (Figure 2a). The two recombinant proteins have a molecular weight expected for ferritin precursors whose TP is uncleaved. However, the recombinant proteins are insoluble due to their localization in inclusion bodies. This could explain the unprocessing of these precursors in *E. coli*. Expression at 25 °C in LB broth complemented with sorbitol and betaine (Method II), known to favour expression of soluble protein in *E. coli* [24], was therefore performed to try to obtain soluble forms of the recombinant precursors. Under this condition, after a 3 h induction the recombinant precursors were not accumulated (Figure 2b). Inclusion bodies containing ferritin precursors expressed at 37 °C were purified according to Method I (see Materials and methods

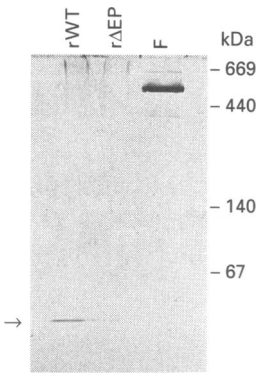


Figure 3 Characterization by non-denaturing gel electrophoresis of recombinant pea-seed ferritin precursors

Recombinant pea-seed ferritin precursors expressed at 37 °C were purified according to Method I. Purified recombinant proteins were analysed on a non-denaturating 4%–25% polyacrylamide gel gradient (8 cm × 6.5 cm × 0.1 cm). The arrow indicates the migration of non-assembled subunits. Electrophoresis was performed in TBE buffer at a constant 10 mA for 4 h. rWT = recombinant wild-type pea-seed ferritin precursor; rΔEP = recombinant pea-seed ferritin precursor with the EP deleted; F = purified pea-seed ferritin.

section) and analysed by non-denaturing PAGE (Figure 3). The solubilized precursors were found in a non-assembled form, even when the EP was deleted. It can therefore be concluded that it was not possible to express assembled recombinant pea-seed ferritin in *E. coli* because unprocessing of the TP from precursor molecules led to unassembled proteins. These proteins are insolubilized into inclusion bodies at 37 °C and probably degraded under conditions of expression at 25 °C. It appears therefore that the deletion of the TP is necessary before trying to express functional recombinant pea-seed ferritin in *E. coli*.

Expression in *E. coli* of the recombinant pea-seed ferritins rΔTP and rΔTP/EP, with the TP deleted

The plasmids containing inserts corresponding to pea-seed ferritin sequence with the TP deleted (pΔTP) and with both the TP and the EP deleted (pΔTP/EP) (see Material and methods section and Scheme 1) were used to express WT pea-seed ferritin (rΔTP) and its related EP deletion mutant (rΔTP/EP) in the *E. coli* strain BL21(DE3). Two different conditions were tested with overexpressed rWT and rΔEP proteins by addition of IPTG to the culture medium (see Materials and methods section): at 37 °C (Method I) and at 25 °C in the presence of 1 M sorbitol and 2.5 mM betaine (Method II). Both methods led to expression of either rΔTP or rΔTP/EP proteins as shown in Figure 4(a) and 4(b). After 3 h of induction, rΔTP and rΔTP/EP proteins represented respectively 13% and 12.5% of total protein using Method I and 10% and 9% total protein using Method II. The rΔTP protein subunit migrates with the same mobility as the subunit of ferritin purified from pea seed (Figure 4a). The difference in mobility of the rΔTP/EP mutant compared with

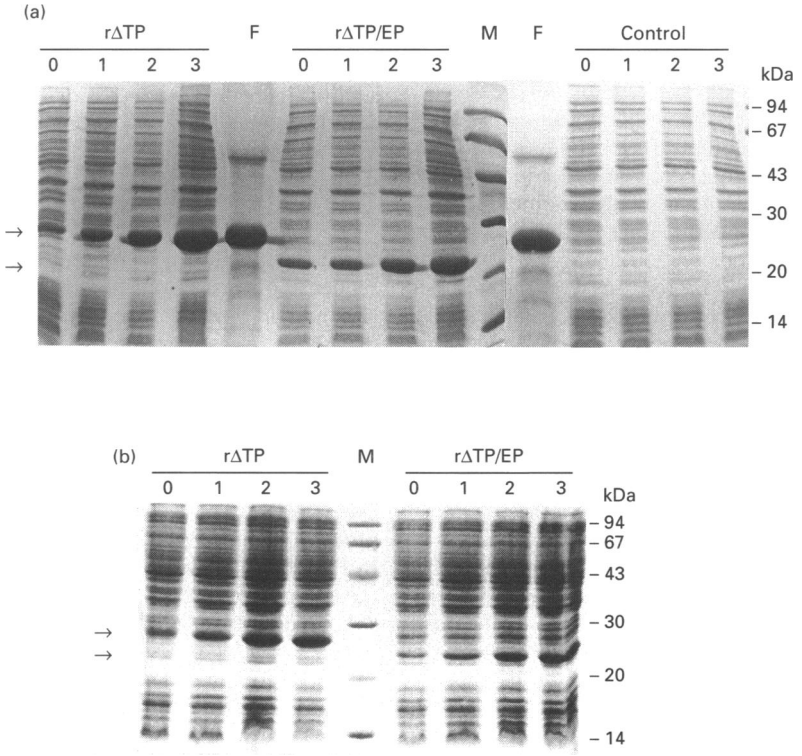


Figure 4 Expression of recombinant pea-seed ferritins in *E. coli*

Recombinant pea-seed ferritins were expressed in *E. coli* BL21(DE3): (**a**) at 37 °C and (**b**) at 25 °C in the presence of 1 M sorbitol and 2.5 mM betaine. Induction was obtained as in Figure 2. rΔTP = recombinant WT pea-seed ferritin; rΔTP/EP = recombinant pea-seed ferritin with the EP deleted; F = purified pea-seed ferritin; M = molecular mass markers; Control = *E. coli* BL21(DE3) transformed with the vector pET3a.

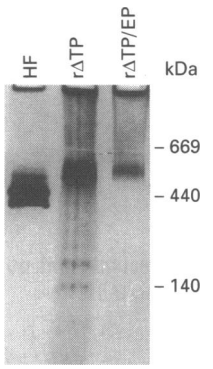


Figure 5 Native gel-electrophoresis of recombinant pea-seed ferritin purified from inclusion bodies

Electrophoresis was performed on a 4%–25% polyacrylamide gel gradient (8 cm × 6.5 cm × 0.1 cm) in TBE buffer at a constant 10 mA for 15 h. HF = horse spleen ferritin (Boehringer); rΔTP = recombinant wild-type pea-seed ferritin; rΔTP/EP = recombinant pea-seed ferritin with the EP deleted.

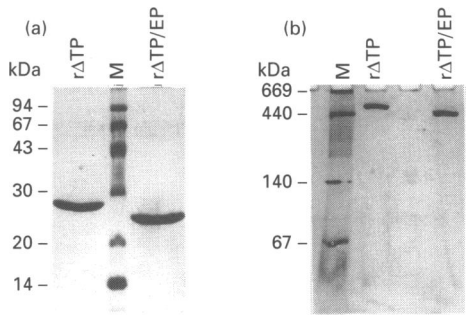


Figure 6 Characterization of purified soluble recombinant pea-seed ferritins by gel electrophoresis

(**a**) Under denaturing conditions (SDS) and (**b**) under native conditions. Electrophoresis was performed as described in Figure 5. A 10 μg amount of each sample of recombinant pea-seed ferritin purified according to Method II was loaded on the gels; rΔTP = recombinant wild-type pea-seed ferritin; rΔTP/EP = recombinant pea-seed ferritin with the EP deleted; M = molecular weight markers

rΔTP protein was clearly visible (Figure 4a and b). Both rΔTP and rΔTP/EP proteins were recognized by antibodies raised against pea-seed ferritin and not by antibodies raised against bacterioferritin (data not shown). Accumulation of the recombinant proteins was confirmed to be due to the ferritin inserts in plasmids pΔTP and pΔTP/EP since no expressed proteins were visualized when *E. coli* cells were transformed with the vector pET3a (Figure 4a).

Purification and characterization of the recombinant pea-seed ferritins rΔTP and rΔTP/EP, with the TP deleted

When Method I was used to express recombinant pea-seed ferritins, rΔTP and rΔTP/EP ferritins were localized within inclusion bodies, while using Method II resulted in soluble recombinant ferritins. As a consequence, two procedures were used to purify these proteins: one using urea to solubilize the ferritins in the inclusion bodies and the other to purify soluble ferritins (see Materials and methods section).

Using Method I, rΔTP and rΔTP/EP ferritins were purified as high-molecular weight proteins assembled in 24-mers, as judged by native gel electrophoresis (Figure 5) and molecular weight determination by gel filtration (not shown). rΔTP and rΔTP/EP proteins were almost pure as judged by SDS/PAGE (Figure 8). However, it seems that fewer molecules of rΔTP/EP were assembled as 24-mers compared with rΔTP molecules (Figure 5). Furthermore, ferroxidase activity of both rΔTP and rΔTP/EP ferritins expressed and purified according to Method I was detected only at pH 8 (Figure 7a) and not at pH 6.8, which is abnormal compared with the ferroxidase activity of ferritin purified from pea seed [14] and more generally with H-type ferritin [7]. We therefore concluded that solubilization by urea of recombinant pea-seed ferritins from inclusion bodies, followed by renaturation by dialysis, results in the purification of poorly active proteins.

Using Method II soluble recombinant rΔTP and rΔTP/EP ferritins were purified to homogeneity and found to be correctly assembled as judged respectively by SDS/PAGE and by native gel-electrophoresis (Figure 6a and 6b). rΔTP and rΔTP/EP proteins both contained between 40 and 50 atoms of iron per molecule. Ferroxidase activity of rΔTP and rΔTP/EP recombinant apoferritins was estimated at pH 6.8 by measuring the kinetics of core-formation at 310 nm compared with those for recombinant human H- and L-apoferritins (Figure 7b). Recombinant pea-seed apoferritins were both found to have a ferroxidase activity reminiscent of that determined with apoferritin purified from pea seed [14]. This activity is clearly higher than that measured for recombinant human L-apoferritin but lower than that of recombinant human H-apoferritin (Figure 7b). It is also interesting to note that the rΔTP/EP ferritin appears to have slightly increased activity compared with the rΔTP protein. We therefore concluded that recombinant pea-seed ferritins expressed in *E. coli* and purified according to Method II are correctly assembled and take up iron *in vitro* at pH 6.8. In addition, deletion of the EP plant-specific sequence slightly increases the ferroxidase activity of recombinant pea-seed ferritin.

Increased insolubility and storage capacity of the rΔTP/EP ferritin after its denaturation followed by renaturation

We have previously reported that cleavages within the EP sequence of ferritin purified from pea seed can be mediated, *in vivo* and *in vitro*, as a consequence of free-radical formation during the release of the ferritin iron [19,20]. Such an event leads to aggregation of pea-seed ferritin and to its insolubility, although radical damages are likely to be responsible for hidden breaks within the molecules without loss of the EP sequence and without disorganization of the 24-mers; indeed the damaged molecules are still able to load iron in their mineral core, as evidenced by ^{59}Fe-labelling *in vitro* of these molecules [19]. In order to determine directly the influence of the EP sequence on the solubility of pea-seed ferritin we measured by SDS/PAGE the amount of rΔTP and rΔTP/EP ferritin subunits that remained soluble after their denaturation by 6 M urea followed by their renaturation by dialysis. As can be seen in Figure 8 almost all of the rΔTP protein subunit is soluble after renaturation by dialysis. On the other hand, only 50% of the rΔTP/EP protein subunit is found in the supernatant with the remainder being recovered in the pellet, indicating that half of the protein formed insoluble aggregates. Therefore, deletion of the EP sequence in recombinant pea-seed ferritin results in decreased solubility of this protein on renaturation.

In order to understand more precisely the influence of the EP sequence on the structure/function relationship of pea-seed

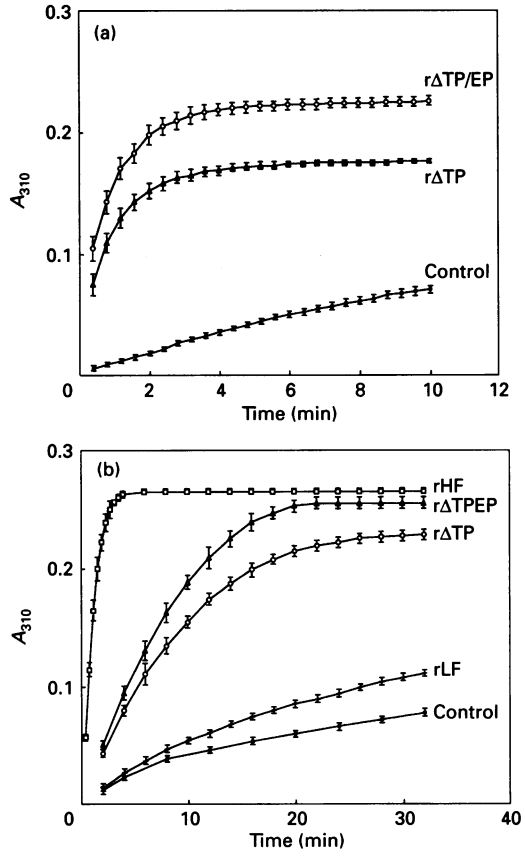


Figure 7 Kinetics of iron uptake by various recombinant apoferritin homopolymers *in vitro*

(**a**) Progress curves of iron-core formation in recombinant pea-seed apoferritin (rΔTP) and in its corresponding EP deletion mutant (rΔTP/EP) purified according to Method I. Fe(II) atoms, 300 per molecule, were added as $FeSO_4$ to the proteins at pH 8 in Tris/maleate buffer. (**b**) Progress curves of iron-core formation in recombinant pea-seed apoferritin (rΔTP) and in its corresponding EP deletion mutant (rΔTP/EP) purified according to Method II, and in recombinant human H-apoferritin (rHF) and in recombinant human L-apoferritin (rLF) provided by Dr. Arosio, Milan, Italy. Fe(II) atoms, 1000 per molecule, were added as $FeSO_4$ to the proteins at pH 6.8 in Hepes buffer. Controls were performed by replacing recombinant apoferritins by BSA. Mean values were obtained from three independent experiments.

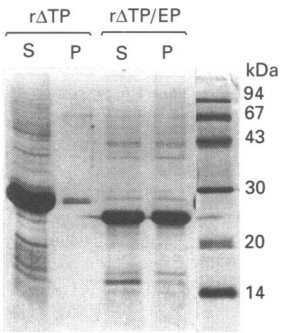


Figure 8 Influence of the N-terminus deletion on the solubility of renatured recombinant pea-seed ferritins

Inclusion bodies containing recombinant pea-seed ferritins, after expression according to Method I, were solubilized by 6 M urea and dialysed as described in the Materials and methods section. At the end of the dialysis period the same amount of each recombinant protein (rΔTP and rΔTP/EP) was centrifuged for 5 min at 12100 ***g*** in a JA-20 rotor (Beckman). Resulting pellets (P) and supernatant (S) were analysed by SDS/PAGE.

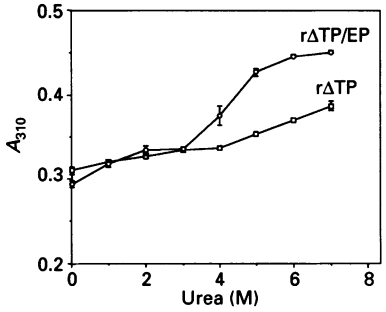


Figure 9 Influence of the N-terminus deletion on the core-formation of renatured recombinant pea-seed ferritins

Recombinant pea-seed ferritins purified according to Method II were denatured by increasing concentrations of urea and renatured by dilution according to Santambrogio et al. [26]. Fe(II) atoms, 1000 per molecule, were added as $FeSO_4$ to the proteins at pH 6.8 in Hepes buffer. After 30 min of iron uptake, corresponding to the plateau of the progress curves described in Figure 6(b), absorbance was measured at 310 nm. Mean values were obtained from three independent experiments.

ferritin we measured core-formation by rΔTP and rΔTP/EP proteins after treatment with increasing concentrations of urea followed by renaturation according to Santambrogio et al. [26]. Treatments with 4 M urea and lower concentrations did not reveal changes in core-formation by the two renatured proteins (Figure 9). However, an increase in the absorbance at 310 nm, after treatments up to this urea concentration, is observed for the rΔTP/EP protein, with a maximum difference of 25% compared with the core-formation obtained with the rΔTP protein (Figure 9). Therefore, it can be concluded that deletion of the EP sequence affects core-formation by increasing the iron storage-capacity of the rΔTP/EP protein after renaturation.

DISCUSSION

We have cloned and sequenced a pea-seed ferritin cDNA which contains a consensus ferroxidase centre (Figure 1), unlike a previously reported clone [10]. This result confirms that various transcripts from the same plant variety, with small differences within their coding sequences code for different ferritin subunits, as reported in the case of maize [11]. Whether these differences have any functional significance remains to be determined. Evidence for more than one type of ferritin transcript suggests the existence of plant ferritin heteropolymers *in vivo*. Therefore, purification of expressed recombinant plant ferritins as active homopolymers is of primary importance to facilitate structure/function studies of these proteins. Such an approach has been used with success for animal ferritins which contain at least two types of subunit [3,6,7,27,28].

Ferritin is synthesized in the cytoplasm of plants as a precursor whose N-terminal TP is removed upon chloroplast uptake [16,18]. Deletion of the TP and engineering of an initiation ATG codon should therefore be considered as a prerequisite for expression of recombinant plant ferritins. However, there is at least one case in the literature reporting the correct processing in *E. coli* of a recombinant chloroplast protein precursor, namely the pea ferredoxin $NADP^+$ oxidoreductase [29]. Therefore, it was worth trying to express recombinant pea-seed ferritin precursor in *E. coli*, hoping for processing of the TP and correct assembly of the

multimeric protein shell. However, the recombinant pea-seed ferritin precursor was not processed in *E. coli* and this behaviour was unchanged when the EP was deleted (Figure 2a). Unprocessing of the pea-seed ferritin precursor in *E. coli* is therefore dependent on the TP sequence itself. Interestingly, the pea-seed ferritin precursor and its related internal deletion mutant accumulated only at 37 °C as insoluble material embedded in inclusion bodies (Figure 2a). At 25 °C, in the presence of betaine and sorbitol, the precursor molecules were undetectable (Figure 2b) while under this condition recombinant ferritins expressed from constructs with the TP deleted accumulated in the soluble fraction (Figure 4b). In addition, solubilization with urea followed by renaturation by dialysis of recombinant pea-seed ferritin precursors found in inclusion bodies at 37 °C does not allow the recovery of assembled multimeric ferritin molecules (Figure 3). It is therefore very likely that pea-seed ferritin precursors are unable to assemble in multimeric molecules in *E. coli*. This result is consistent with the observation that plant ferritin purified as 24-mers from various sources were built up from subunits whose TP was removed [17]. In other words, no plant-ferritin build-up from its precursor as the monomer of the multimeric shell has been characterized. As a result of this unassembly in *E. coli*, soluble pea-seed ferritin precursors are probably synthesized and degraded at 25 °C, while they are protected against degradation at 37 °C by aggregating in inclusion bodies. Deletion of the TP and engineering of an ATG codon for translation initiation appeared therefore to be necessary in an attempt to express recombinant plant ferritin correctly assembled in *E. coli*.

We have therefore engineered the pea-seed ferritin cDNA PeSd2 in order to remove its TP responsible for plastid targeting and for cloning into an *E. coli* expression vector (Scheme 1). The conditions of this expression were found to be critical for further purification of active recombinant pea-seed ferritin molecules. Inclusion bodies were obtained when cells were induced at 37 °C and these required urea for solubilization of the recombinant pea-seed ferritin (Figure 5). This method, although successful for the purification, gave recombinant ferritin with a very poor ferroxidase activity, not detected at acidic pH (Figure 7a). This observation can be explained by inefficient renaturation and/or assembly of the protein during the dialysis step. On the other hand, purification of recombinant pea-seed ferritin expressed at 25 °C in *E. coli* in the presence of sorbitol and betaine gave perfectly assembled and functional proteins (Figures 6a and 7b).

The purification of an active recombinant pea-seed ferritin led us to start a study of the structure/function relationships of this protein, and in particular to investigate the role of the specific sequence found at the N-terminus of plant-ferritin subunit [13]. To do so, we expressed and purified a recombinant protein lacking this N-terminus sequence (rΔTP/EP). This protein was correctly assembled as 24-mers (Figure 6a) and was able to take up iron *in vitro* (Figure 7b). These results are consistent with those obtained previously with ferritin purified from pea seeds, this latter being found assembled as 24-mers and able to load iron *in vitro* even when cleaved in its N-terminus sequence in response to iron release [19]. In addition, it was also shown in the previous study that pea-seed ferritin, when cleaved at its N-terminus, had a tendancy to aggregate and become insoluble [19]. This property is confirmed in the work presented here, as deletion of the EP sequence strongly reduces the solubility of the rΔTP/EP protein compared with rΔTP protein (Figure 8). This clearly demonstrates that the EP sequence plays an important role in plant-ferritin structure. The WT recombinant pea-seed ferritin (rΔTP), which contains a consensus ferroxidase centre, has, however, a lower ferroxidase activity than that of recombinant human H-ferritin (Figure 7b). The structural basis of the difference in the ferroxidase activity of rΔTP compared with rHF will require further investigation. Interestingly, rΔTP/EP ferritin, which lacks the EP sequence, and is therefore more similar to animal ferritin, has an increased capacity for core-formation compared with rΔTP on renaturation (Figure 9). This observation could be related to changes in protein conformation for two reasons. First, as mentioned above, deletion of the EP sequence decreases plant-ferritin solubility, suggesting structural alterations. Secondly, computer-modelling predictions have highlighted interactions between some amino acids of the EP sequence and some of those lying in the downstream sequence [10]; these interactions are destroyed by deleting the EP sequence. Nevertheless, deletion of the EP sequence is not sufficient to obtain a ferroxidase activity of the recombinant pea-seed ferritin identical with that of the recombinant human H-ferritin (Figure 7b). Other structural parameters are therefore involved in this difference and will need to be determined in the future.

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