A novel plant ferritin subunit from soybean that is related to a mechanism in iron release

Taro Masuda, Fumiyuki Goto* and Toshihiro Yoshihara

Dep. Bio-Science, Central Research Institute of Electric Power Industry, 1646 Abiko, Abiko, Chiba 270-1194, Japan

*To whom correspondence should be addressed: Fumiyuki Goto, Dep. Bio-Science, Central Research Institute of Electric Power Industry, 1646 Abiko, Abiko, Chiba 270-1194, Japan, Telephone: +81 471 82 1181, Fax: +81 471 83 3347
E-mail: gotoh@criepi.denken.or.jp

Running Title
Iron release mechanism of soybean ferritin

The abbreviations used are:
TP; transit peptide
EP; extension peptide
Summary

Ferritin is a multimeric iron storage protein composed of 24 subunits. Ferritin purified from dried soybean seed resolves into two peptides of 26.5 and 28 kDa. To date, the 26.5 kDa subunit has been supposed to be generated from the 28 kDa subunit by cleavage of the N-terminal region. We performed amino acid sequence analysis of the 28 kDa subunit, and found that it had a different sequence to the 26.5 kDa subunit, thus rendering it novel among known soybean ferritins. We cloned a cDNA encoding this novel subunit from ten-days-old seedlings, each of which contained developed bifoliates, an epicotyl and a terminal bud. The 26.5 kDa subunit was found to be identical to that identified previously lacking the C-terminal 16 residues that correspond to the “E-helix” of mammalian ferritin. However, the corresponding region in the 28 kDa soybean ferritin subunit identified in this study was not susceptible to cleavage. We present evidence that the two different ferritin subunits in soybean dry seeds show differential sensitivity to protease digestions, and that the novel, uncleaved 28 kDa ferritin subunit appears to stabilize the ferritin shell by co-existing with the cleaved 26.5 kDa subunit. These data demonstrate that soybean ferritin is composed of at least two different subunits, which have co-operative functional roles in soybean seeds.
(Introduction)

The iron storage protein ferritin has a structure highly conserved among plants, animals and bacteria. Ferritin has 24 subunits that are assembled into a spherical shell characterized by 4-fold, 3-fold and 2-fold symmetry (432 symmetry). Up to 4500 Fe(III) atoms can be stored as an inorganic complex in the inner cavity of assembled ferritin, rendering these atoms non-toxic and biologically available (1). Structural analyses of vertebrate and bacterial ferritins indicate that each subunit consists of a four-helix bundle (helices A, B, C and D) and a fifth short helix (E helix). The E-helix exists around the 4-fold inter subunit symmetry axes of the protein shell, and forms a hydrophobic pore (1-4). In mammals, two distinct ferritin subunits (H and L) are found (5). These subunits have 50% identity and similar 3-dimensional structures. The H subunit has ferroxidase activity (6,7) and catalyzes oxidation of Fe(II), which is the first step in iron storage. The L subunit promotes nucleation of the iron core (8). The synthesis of ferritin in vertebrates is regulated during translation. It was suggested that both the 5’- and 3’- untranslated regions of ferritin mRNA contributed to translational control (9). The iron responsive element (IRE), which was first identified in the 5’- untranslated region of human ferritin mRNA (10), is highly conserved among mammals and other vertebrates. Despite the probable common ancestry of plant and vertebrate ferritins (12), expression of plant ferritin is regulated primarily at the transcriptional level, in response to iron administration (11). A sequence with similarity to the IRE is absent in the 5’-untranslated region of plant ferritin.
mRNA, however, Wei and Theil (2000) recently suggested the existence of an “iron regulatory element (FRE)” in the promoter region of the soybean ferritin gene, which controls the transcription together with a trans-acting factor (13).

Only one polypeptide chain type has been identified as a functional subunit of plant ferritins (11). Like the mammalian H subunit, with which it shares about 40 % sequence homology, this subunit has ferroxidase activity (14,15). During the last decade, evidence for ferritin multigene families in maize (16), cowpea (17,18) and soybean (18) was provided. Cowpea has at least four different ferritin genes, one that encodes a protein with 97% sequence identity to soybean ferritin (11,19). The peptides encoded by the other cowpea genes in the ferritin family are more divergent. Despite the evidence that multiple ferritin genes exist in plants, it is not clear whether the products of these different genes are functionally divergent. This is in contrast to the situation reported in pigs, frogs and salmon, where two functional H-type subunits showing different tissue specificity are found (20-22).

The ferritin subunits of soybean and many other legumes are synthesized as 32 kDa precursor proteins (23) which contain an unique two-domain N-terminal sequence (19). These N-terminal domains are not present in mammalian or other ferritins. The first domain, which consists of 40-50 residues and is known as the ‘transit peptide’ (TP), is presumed to facilitate transport of the ferritin precursor to plastids (14). The function of the second domain, which is a part of the mature protein
and is termed extension peptide (EP), is currently unclear. The ferritin subunit purified from soybean seed is 28 kDa, but it is apparently converted in significant amounts to a 26.5 kDa subunit (19,24). In 1991, Regland et al. (23) suggested that such truncation occurs by cleavage of the EP during germination or free radical damage. They considered the 28 kDa subunit to be an iron containing form, and the 26.5 kDa subunit to be an iron releasing form (23,25). This proposal has yet to be unsubstantiated by experimental evidence.

Here we report evidence that the iron conversion mechanism of soybean seed ferritin involves two distinct subunits. One of these is novel and presented here for the first time, while the other is identical to that previously identified. These data about soybean ferritin maturation allow us to hypothesize a novel mechanism by which iron is released from the assembled ferritin molecule.

EXPERIMENTAL PROCEDURES

Purification of native soybean ferritin

500g of soybean dry seeds (Glycine max Merr cv. Kita-no-shiki) were crushed into flour by a mill. The soybean seed flour was suspended in 50mM Tris-HCl buffer (pH7.5) containing 1mM EDTA and 10mM 2-mercaptoethanol (2-ME), homogenized, and centrifuged at 10,000 g for 10min. The supernatant was fractionated using 20% saturation of ammonium sulfate. An amber colored
precipitant was collected by centrifugation and dialyzed against 50 mM Tris-HCl (pH 7.5) buffer. The
diazyed sample was applied to a DEAE-Toyopearl (TOSOH, Tokyo, Japan) column previously
equilibrated by 50 mM Tris-HCl (pH 7.5) containing 1 mM EDTA. The column was washed with a
buffer containing 0.15M sodium chloride, and amber colored ferritin was eluted. The eluate was
again fractionated with 20% saturated ammonium sulfate. The supernatant was collected by
centrifugation, applied to a butyl-Toyopearl (TOSOH) column and eluted with a linear gradient of
20-0% saturated ammonium sulfate. Fractions containing soybean ferritin were pooled and dialyzed
against 50 mM Tris-HCl (pH 7.5) containing 1 mM EDTA and applied to Q-sepharose columns
(Amersham- Pharmacia). Proteins were eluted with a linear gradient of sodium chloride from 0 to
0.7 M. Fractions containing soybean ferritin were pooled and concentrated, and finally loaded to
Superdex 75 pg gel filtration columns (Amersham- Pharmacia) equilibrated before use in 10 mM
Tris-HCl buffer (pH 7.5) containing 0.15 M NaCl.

Apo-ferritin was obtained using methods described by Chasteen and Theil (1982) (26). Purified
ferritin was dialyzed against 50 mM HEPES/NaOH buffer (pH7.0) containing 1 % thioglycolic acid,
followed by successive changes of HEPES buffer with (0.1 %) or without thioglycolic acid. Protein
was then dialyzed against HEPES buffer containing 13g/litre of Chelex-100 (Bio-Rad) and 0.2M
NaCl, and finally dialyzed against deionized water. The concentration of purified proteins was
determined using a ‘protein assay kit’ (Bio-Rad) and densitometer (Amersham- Pharmacia).
Amino acid sequence analysis

Soybean ferritin subunits were separated electrophoretically using a 12.5% SDS-polyacrylamide gel (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was stained with 0.1% Ponceau-S (SIGMA) in 2%(v/v) acetic acid and two distinctive bands of 28.0 and 26.5 kDa were independently cut from the membrane. N-terminal amino acid sequence analysis was performed by automatic Edman degradation using an Applied Biosystems’ model 477A pulse-liquid sequencer system.

C-terminal amino acid sequence analysis of the 26.5 kDa subunit was performed using p-phenylene diisothiocyanate controlled-pore glass (DITC-CPG Sigma) (27). Briefly, the 26.5 kDa subunit was digested with lysyl endopeptidase, and the resulting peptide fragments were covalently bound to DITC-CPG. Only the C-terminal fragment, which contained no lysyl residue, was detached by cleavage of the first residue with trifluoroacetic acid (TFA) (27). The sequence of the C-terminal fragment was determined by automatic Edman degradation, as described above.

Cloning of a novel ferritin gene cDNA from soybean.

Primers for amplification of the subunit containing a novel N-terminal amino acid sequence were designed using the expressed sequence tag (EST) sequences (AW185525, AI966037, AW397605,
AI443722, AI900240) of soybean registered in GenBank. 5'-rapid amplification of cDNA ends (RACE) and 3'-RACE was carried out using a ‘SMART RACE cDNA Amplification kit’ (Clontech) according to the manufacturer’s instructions; total RNA extracted from 10 days-old seedlings contained developed bifoliates, an epicotyl and a terminal bud was used as a template. About 10 candidate sequences of a target sequence coding the novel subunit were determined.

**Preparation of recombinant soybean ferritin**

DNA sequences encoding the mature region of soybean ferritin, previously reported by Lescure et al. (11), were amplified by PCR using the primers -TP (5'-GCGCATATGTCACGGTGCTCTCAC-3') and C (5'-GCGGGATCTGTAATCAAGAAGTCATCTCG-3'). -TP and C contained Nde I and BamH I restriction sites, respectively. The resulting fragments, which were missing the TP sequence, were ligated to the Nde I and BamH I sites on the expression vector pET3a (Novagen) to generate pESF. The expression plasmid pESF was transformed into the *E. coli* strain BL21(DE3)pLys. An *E. coli* strain harboring the expression plasmid was cultured in LB medium supplemented with ampicillin (50 µg/ml) at 37 °C. Bacterial growth was monitored with a spectrophotometer at 600 nm. When an absorbance of 0.6 was reached, isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 1mM. The cells were harvested by centrifugation, and proteins were extracted using ‘BugBuster’ protein extraction reagent (Novagen). Recombinant ferritin was purified using the same
methods described above for native ferritin.

Soybean ferritin degradation analysis

Soluble protein was extracted from 200 mg of soybean leaves by homogenization with 1 ml of extraction buffer [50 mM K₂PO₄, 10 mM 2-ME, 0.1% TritonX-100, 0.1% Sarcosine] and sea sand followed by centrifugation at 12000 rpm. The supernatant was used for degradation experiments of soybean ferritin. Recombinant ferritin or native soybean ferritin (150 ng each) were added to 20 µl of the leaf extract and incubated at room temperature for 0, 1, 10, 30, 60 and 120 minutes, respectively. After SDS-PAGE and electroblotting to a PVDF membrane, protein-immunodetection was performed. Antiserum was raised against the soybean ferritin as previously described (28). Probing of blots with soybean ferritin antibody was carried out using anti-rabbit IgG sheep immunoglobulin coupled with biotinylated horseradish peroxidase (Vectastain ABC kit, Vector Labs. USA). Immunostain HRP-1000 (Konica, Japan) was used for visualizing the signal (28,29).

Iron incorporation and iron release measurement

Reactions examining iron uptake by native soybean and recombinant ferritin were performed in 100mM HEPES/NaOH buffer (pH7.0) with a Fe²⁺/ferritin molar ratio of 1000:1 (0.1 mM ferrous sulfate and 0.1 µM ferritin) at room temperature. Iron incorporation by each type of ferritin was
monitored by measuring the absorbance at 310 nm (30,31) using an UV spectrophotometer (U3000, Hitachi, Tokyo, Japan).

For iron-release experiments, native soybean and recombinant apoferritins (2µM each) were mineralized using freshly prepared ferrous sulfate (1mM) in 0.1 M MOPS containing 0.2 M NaCl (32,33) for 2 hours at room temperature and then over night at 4 °C. The excess iron which was not incorporated into the ferritin shell was precipitated by the centrifugation and separated from the mineralization mixture. External iron was then removed using ‘Econo-Pac 10 DG’ (Bio-Rad). Iron release was initiated by addition of 1 mM sodium ascorbate and 4 mM ferrozine (32); the former. was previously reported to enhance the reductive release of iron from the ferritin shell (32,34). Exogenous Fe\(^{2+}\) was measured by the absorbance of the Fe\(^{2+}\)/ferrozine complex at 560 nm using the same spectrophotometer described above. The data of iron uptake and release were obtained from 3 different preparations. The levels of reductive iron release by the two subunits were compared statistically using t-tests (p = 0.05).

**RESULTS**

*Isolation of soybean ferritin from seeds*

Soybean ferritin was extracted from dry seeds and isolated using the following sequential
chromatographic purification steps: anion exchange, hydrophobicity and gel filtration. Soybean ferritin was eluted in a single peak after each chromatographic step. Purified ferritin subunits were separated as two peptides (28 and 26.5 kDa) by SDS-polyacrylamide gel electrophoresis (Fig.1A). Densitometric analysis indicated that the 28 and 26.5 kDa subunits are present in purified native ferritin in nearly equivalent amounts (28:26.5 kDa = 1:1.09). Non-denaturing gel electrophoresis (Native PAGE) resolved purified soybean ferritin as a single complex, estimated to be about 550-560 kDa (Fig1B).

*Amino acid sequence analyses of ferritin subunits*

Comparisons between the N-terminal sequences of the 28 kDa subunit (Ala-Ser-Asn-Ala-Pro-Ala) and the 26.5 kDa subunit (Ala-Ser-Thr-Val-Pro-Leu) revealed that the 28 kDa subunit was different to any previously reported. Further determination for the N-terminal sequence verified that the 28 kDa subunit was a novel subunit (Fig.2). The N-terminal sequence of the 26.5 kDa subunit was identical to that of the EP region of the sequence reported by Lescure et al. (11). Our analyses revealed that the transit peptide is cleaved at the carboxyl side of the 48th cysteine in the 26.5 kDa subunit, which contrasts to the previously reported cleavage site at position 49 (alanine) (Fig.2) (11,23).

To detect the site of cleavage for hypothesized conversion of the 28 kDa subunit to the 26.5 kDa
subunit (11,13), the C-terminal amino acid sequence of the 26.5 kDa subunit was determined. The sequence of peptide fragment containing the C-terminal residue was Ser-Glu-Tyr-Val-Ala-Gln-Leu-Arg-Arg (Fig.2), which was the same as that previously reported (11). The C-terminal residue was determined to be the 234th arginine, which is situated 17 residues upstream from the C-terminus. These data demonstrated that the 26.5 kDa subunit in the soybean seed ferritin is generated as a result of proteolytic cleavage of the C-terminal 16 residues of the previously described subunit.

cDNA isolation and the deduced amino acid sequence of a novel ferritin subunits

A cDNA encoding a newly identified subunit (28 kDa) of soybean ferritin was cloned using a PCR-based strategy. The predicted amino acid sequence was compared with the previously reported sequence of ferritin subunit (11), and several differences were found (Fig.2). Based on the conservation of several residues, thought to comprise the ferroxidase site (6,7), we defined the 26.5 and the 28 kDa subunits as H-1 and H-2, respectively. The mature region of the H-2 subunit, in which the TP is detached from the precursor, is composed of 209 residues. It is seven residues longer than the H-1 subunit (202 residues). The amino acid sequence of the mature region of the H-2 subunit has 82 % identity with that of the H-1 subunit. In contrast to a high identity in the mature regions, low sequence identity between the TP sequences of H-1 and H-2 is found (41 %). In the sequence of the mature region, the deduced helical regions (A to E helices) (4) are highly conserved
between the H-1 and H-2 subunits, while the loop region between the B and C helices, as well as the EP region, are relatively variable. Specifically, the amino acid sequence identities of the helical, loop and EP regions are 90 %, 81 %, and 63 %, respectively (Table1). The H-2 subunit has C-terminal extension consisting of five residues (four of them charged). A comparison between the C-terminal cleavage site of H-1 (α-amino side of position 234) with the putatively homologous position in H-2 (position 235) revealed the presence of an arginine in H-1 and a leucine in H-2 (Fig.2). The unsusceptibility of the H-2 subunit to C-terminal cleavage is likely due to the presence of leucine instead of arginine at this position.

*Degradation of the soybean ferritin H-1 subunit*

The full mature region of the soybean ferritin H-1 subunit was expressed in *E. coli*. Native-PAGE (Fig.1B) and gel filtration (data not shown) analysis showed that the recombinant soybean ferritin subunit assembles into a 24-mer, presumably in a similar fashion to native ferritin. We abbreviated the recombinant H-1 subunit as ‘rH-1’ and native soybean ferritin purified from seeds as ‘seed H-1/H-2’. The rH-1 was composed of only the H-1 subunit in the original, uncleaved form (28 kDa), while the seed H-1/H-2 included both the H-2 and cleaved H-1 (26.5 kDa) subunits in nearly equal amounts (Fig.1 (A)). The rH-1 was incubated with soybean leaf extract (Fig.3). Prior to the incubation with the soybean leaf extract, the rH-1 subunit remained in its 28 kDa form, however it
was degraded quickly following addition of leaf extract. About half the amount of the rH-1 subunit was degraded to the cleaved form (26.5 kDa) in ten minutes, and after one hour of incubation, the original 28 kDa form had completely disappeared; only a small amount of the cleaved 26.5 kDa form was detected. Subsequently, the cleaved form degraded completely after two hours of incubation. In contrast, the seed H-1/H-2 was still detectable after two hours incubation, although the 28 kDa form was not present. Since we used an anti-serum raised against the H-1 subunit, specific detection of the H-2 subunit in seed H-1/H-2 was not expected to occur; this may explain the absence of a 28 kDa band in Fig.3.

Effect of cleavage on iron uptake and release

To investigate the effects of C-terminal cleavage in H-1 on the multimeric complex, we compared iron uptake and release activities between seed H-1/H-2 and rH-1. A recombinant form of the cleaved H-1 subunit (whose C-terminus was deleted) did not assemble into a 24-mer (data not shown). Both rH-1 and seed H-1/H-2 showed iron uptake activity (Fig.4A). Progression plots indicated that the uptake rate of the seed H-1/H-2 was slower than that of the rH-1, despite the presence in all the subunits of ferroxidase sites. Both the uptake rates were faster than that of Fe(II) autoxidation (control).

The rate of reductive release of iron from seed H-1/H-2 and rH-1 was assessed (Fig4B). The level
of ferrous atoms released from the ferritin shell was calculated via absorbance measurement of the Fe(II)-ferrozine complex. At every assessed time, the amounts of the Fe(II)-ferrozine complex were significantly larger with seed H-1/H-2 than with rH-1. This result suggested that cleavage of C-terminal 16 residues accelerated iron release from the protein shell.

**DISCUSSION**

In this study, we demonstrated that two polypeptide chains with different sequences are found in soybean ferritin purified from seeds. Previously, it was hypothesized that only one type of polypeptide chain, which was in either a cleaved or uncleaved form, was present in the multimer (11). We found that the two different subunits exist in nearly equal amounts in the ferritin of soybean seeds. Two kinds of subunits with different amino acid sequences were also detected previously in clover seed (35), though one of them existed only at very low levels, and its amino acid sequence was not unambiguously identified. From amino acid sequence and cDNA analysis, we designated the two soybean ferritin subunits as H-1 and H-2. H-1 had a sequence identical to that previously described (11), and experiments involving rH-1 and leaf extracts revealed that this 28 kDa subunit could be readily converted to 26.5 kDa by cleavage of the 16 residues at the C-terminus. In the amino acid sequence profiles, no contaminating peaks were detectable, indicating that the H-1 subunit was perfectly converted to the cleaved 26.5 kDa form. The H-2 subunit had a novel amino
acid sequence, and unlike H-1, appeared unsusceptible to cleavage, remaining in its 28 kDa mature form. These data are in conflict with the previous hypothesis that the 26.5 kDa subunit is generated by cleavage of the N-terminal extension peptide (EP) of the 28 kDa subunit, and that the 28 kDa and 26.5 kDa subunit are identical apart from this cleavage (23).

In degradation experiments using the extract of soybean leaves, the homo 24-mer of rH-1 was unstable compared with the seed H-1/H-2 (Fig.3). The instability of the H-1 multimer is likely due to cleavage of the C-terminal 16 residues in each subunit. In support of this hypothesis, a mutant recombinant form of H-1, which lacked the C-terminal 16 residues, could not assemble into a native-ferritin like complex. Luzzago and Cesareni (1989), and Levi et al. (1989) also remarked on the importance of the C-terminal region for shell stability in human H-chain ferritin (36,37). Notably, the H-2 subunit was not susceptible to cleavage of the C-terminal region, and was stable during incubation with the leaf extraction. These data indicate that the H-2 subunit stabilizes the native ferritin 24-multimer in soybean seeds.

In vertebrate ferritins, the 3-dimensional structures of the single subunit and assembled 24-mer have been analyzed in detail (2,3,7). Ferritin subunits in the spherical 24-mer are related by 432 symmetry. There are narrow channels around the 3-fold and the 4-fold symmetry axes (1). Residues around the 3-fold channels are mainly hydrophilic and the channels are proposed as the main entrance for iron atoms (38-40). In contrast, four subunits are tightly packed around the 4-fold
channels, with hydrophobic interactions occurring among the non-polar residues in the E-helices (1-3). Our results indicate that conversion of the H-1 subunit from 28 kDa to 26.5 kDa is due to the cleavage of C-terminal 16 amino acid residues. From amino acid sequence alignment data and the deduced three dimensional structure of plant ferritin subunits (41), the cleaved 16 residues correspond to the “E-helix” of vertebrate ferritin, which is a short helix forming the narrow channels around the 4-fold intersubunit interaction axes. In the case of amphibian red-cell L-chain ferritin, it was reported that the diameter of this channel is about 1.5 Å, while that of the 3-fold channel is about 3.7 Å at its narrowest point (2). Thus, the channel around the 4-fold axes does not appear to have enough pore size to allow ions to move freely (2). What might the functional significance of cleavage of the E-helices in soybean ferritin be? It is likely that the pore size of the channel around the 4-fold axes would be expanded drastically. Even though half of the subunits (H-2) in soybean ferritin are not susceptible to proteolysis, the pore generated by cleavage of the H-1 E-helices is expected to be large enough to allow iron atoms to pass freely. Indeed, the rate of iron release from seed H-1/H-2 was faster than that from the rH-1 (uncleaved 28 kDa form) (Fig.4B). These data suggest that the rate of iron release is accelerated by cleavage of the E-helix. In contrast to the case of iron entrance in mammalian ferritin, few studies have been performed on iron release pathways in either plant and animal ferritin (42). Therefore, the large pore generated by the cleavage of the E-helix provides a novel hypothesis for iron release from ferritin. Conversely, the iron uptake
activity of seed H-1/H-2 was relatively low compared with the rH-1, despite the fact that both the H-1 and H-2 subunits possess the predicted ferroxidase center. The apparent difference in the rate of iron uptake is probably due to the C-terminal cleavage in H-1, which facilitates reductive release of incorporated iron from native ferritin. C-terminal mutation in the human H-chain ferritin has also been reported to affect iron incorporation and ferroxidase activity (36).

In plant ferritin, some functional genes have already been identified (16-18). Here, we have identified a novel soybean ferritin subunit (H-2), whose maturation process is different to that of the originally described subunit (H-1). The novel subunit proved to be one of the major subunits of soybean seed ferritin. The primary structure of the novel ferritin subunit was closely related to that of the known (11) subunit (82% identity). However, the difference in the maturation process of H-2 appears to promote the novel function of “iron release”. The 28 kDa (presumably H-2-like) and partially cleaved 26.5 kDa (presumably H-1-like) subunits are also found in pea and other legume ferritins (22,23,35). Notably, a leucine is found at a similar position in one of cowpea ferritin subunits to that of the leucine at position 235 in H-2 (Fig.2) (18). Thus, like the case for soybean, it is possible that the different sized subunits observed in many legume ferritins derive from different genes, and play co-operative roles in the storage and release of iron atoms.

In agreement with the results of Lobréaux and Briat (43), concentration of ferritin subunits in soybean cotyledon decreased gradually during germination and finally disappeared after about 10
days (data not shown). To date, many studies have examined expression of plant ferritin in legume plants and maize. While ferritin accumulates in developing nodules, cotyledons and embryo axes (43-45) of soybean and pea, it has not been detected in green leaves in bean (43). In conflict with the latter result, van der Mark and colleagues reported that ferritin was detectable in normal green leaves of bean (46,47). Interestingly, these authors provided preliminary evidence for the existence of multiple subunits of ferritin in bean (48). A consensus on patterns of ferritin expression in plants has not yet been reached, and we suggest that a contributing factor to this has been the exclusive study of the H-1 gene and its product during analysis. We are currently analyzing the expression and tissue distribution of the newly identified H-2 subunit, as well as the H-1 subunit, in order to elucidate their respective functions and to obtain information on the significance of the multi gene family of soybean ferritin.

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Figure legends
Fig. 1 SDS-PAGE and native PAGE analysis of purified soybean ferritin. Samples were added after the final gel filtration step (see methods).

(A) Purified soybean ferritin was analyzed by SDS-PAGE and stained with CBB (lane 1).

(B) Native PAGE analysis of soybean ferritin. Lane 1: native soybean ferritin purified from seed; lane 2: recombinant soybean ferritin expressed in *E. coli*.

M: protein markers and their corresponding molecular masses.

Fig. 2 Deduced amino acid sequences of soybean ferritin subunits.

Upper: amino acid sequence of the novel, H-2 subunit identified during this study.

Lower: amino acid sequence of the H-1 subunit, which is identical to the sequence previously reported by Regland *et al.* (1991).

Conserved residues between the two subunits are shown in black. Residues in green indicate those which have been suggested to form the deduced ferroxidase center.

The N-terminal 32 residues of the H-2 subunit determined during amino acid sequence analysis are shown boxed in green.

The peptide fragment containing the C-terminal residue of the cleaved 26.5 kDa form of the H-1 subunit is shown boxed in red.
The cleavage sites of the transit peptides (TP) in both the H-2 and H-1 subunits are indicated by a yellow arrowhead. The mature regions of both subunits are downstream from here.

The cleavage site for conversion of the H-1 subunit from 28 kDa to 26.5 kDa is indicated by a green arrowhead.

**Fig.3** Degradation of recombinant and native soybean ferritin.

The rH-1 and seed H-1/H-2 were incubated separately in soybean leaf extract. Ferritin subunits were detected by anti H-1 subunit anti-serum. Lane 1: soybean leaf extract, lane 2: 150 ng of the rH-1 subunit, lane 3 to 8: 150 ng of rH-1 subunit after incubation with leaf extract for 0, 1, 10, 30, 60 and 120 minutes, respectively, lane 9: 150 ng of seed H-1/H-2, lanes 10 and 11: 150 ng of seed H-1/H-2 with leaf extract for 60 and 120 minutes, respectively.

**Fig.4 (A)** Progression plots of iron uptake in soybean ferritin.

Experiments were performed in 0.1 M HEPES-Na, pH 7.0, containing 0.1 μM of each ferritin type (rH-1 and seed H-1/H-2) and 0.1 mM ferrous sulfate. The control indicates the rate of Fe(II) auto-oxidation.

(B) Rates of iron release from assembled ferritin shells.

Seed H-1/H-2 and rH-1 (2 μM) were mineralized in vitro by mixing with 1 mM ferrous sulfate in 0.1
M MOPS (pH 7.0) and 0.2 M NaCl. Iron release from mineralized ferritins was initiated by the addition of 1 mM ferrozine and 4 mM sodium ascorbate. Released iron was detected in the Fe^{2+}/ferrozine complex form by monitoring the absorbance at 560 nm. The results were obtained from three experiments.

**Table 1** The partial amino acid sequence identity between the H-1 and H-2 subunit.

The amino acid sequences of both subunits were compared with each other in eight parts.

TP: transit peptide. EP: extension peptide
Fig. 1 Masuda et al.
**Transit Peptide**

**Soy H-2**
1. MALSCSKVLS FY-LSPVVG GDVPKKLTFS -ELGLSKGVG

**Soy H-1**
1. MALAPSKVST FGSEPKPSV GGAQKNPTCS VSLSFLNEKL

**Extension Peptide**

39. GARSSRVCA- ASNAPALLAC-VIFEPPQELK-KDYLAPVIPA-
41. GSRNNRVC-- ASTRP--LTG VIFEPEEVEK KSELAVPTAP

**A-Helix**
78. NQLARQNYA DDSESAINEQ INVEYNVSYV YHALFAYFDR
77. QVQLARQNYA DECESAINEQ INVEYNASYV YHSLFAYFDR

**B-Helix**
118. DNTALKGLAK FFKESEBEEER EHAELIKYQ NIIRGGRVVLH
117. DNVALKGEAK FFKESEBEEER EHAELKLKQ NTRGGRVVLH

**C-Helix**
158. PITSPESEFE HSEKGDALYA MELALSLLEKL TNEKLLHVHS
157. PIKNAPSEFE HVEKGDALYA MELALSLLEKL VNEKLLNVHS

**D-Helix**
198. VADRNNDPQL ADFIESSEFLY EQVSIIKKA EYVAQLRLVG
197. VADRNNDPOQM ADFIESSEFLS EQVESIKKA EYVAQLRLVG

**E-Helix**
238. KGHSVWHDQ KLLHDDEDHV
237. KGHSVWHDQ RLLD

**Fig. 2** Masuda et al.
Fig. 3 Masuda et al.
Fig. 4 Masuda et al.
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<th>Region</th>
<th>Identity (%)</th>
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<tbody>
<tr>
<td>TP</td>
<td>41</td>
</tr>
<tr>
<td>EP</td>
<td>63</td>
</tr>
<tr>
<td>Helix A</td>
<td>90</td>
</tr>
<tr>
<td>Helix B</td>
<td>86</td>
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<td>Helix C</td>
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<td>Helix D</td>
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<tr>
<td>BC loop</td>
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<td><strong>82</strong></td>
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Table 1 Masuda et al.
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Taro Masuda, Fumiyuki Goto and Toshihiro Yoshihara

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