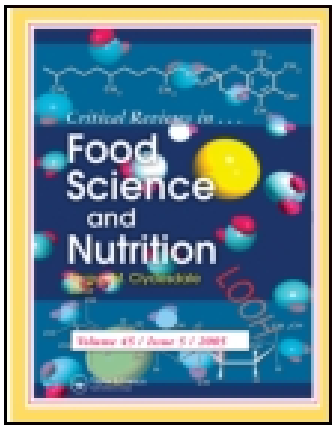

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Structure, Function, and Nutrition of Phytoferritin: A Newly Functional Factor for Iron Supplement

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Structure, Function, and Nutrition of Phytoferritin: A Newly Functional Factor for Iron Supplement

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Ferritins are members of the superfamily of iron storage and detoxification proteins present in all living organisms and play important roles in controlling cellular iron homeostasis. In contrast to animal ferritin, relatively little information is available on the structure and function of phytoferritin. Phytoferritin is observed in plastids whereas animal ferritins are largely found in the cytoplasm of cell. Compared to animal ferritin, phytoferritin exhibits two major distinctive features in structure. First, phytoferritin contains a specific extension peptide (EP) at the N-terminal while animal ferritin lacks it. The EP is located on the exterior surface of protein, which recently has been found to act as a second ferroxidase center for iron-binding and oxidation, and regulate iron release during the germination and early growth of seedlings. Second, only H-type subunit has been identified in phytoferritin, which is usually a heteropolymer consisting of two different subunits, H-1 and H-2, sharing ∼80% amino acid sequence identity. These two subunits in phytoferritin play a positively cooperative role in iron oxidative deposition in protein. Iron deficiency anemia (IDA) is the most common and widespread nutritional disorder in the world, so it is crucial to explore a safe and efficient functional factor for iron supplement. Fortunately, phytoferritin seems to be a suitable candidate. In legume seeds, more than 90% of iron is stored in the form of ferritin in amyloplasts. Recently, some studies at different levels have demonstrated that plant ferritin could be used as novel, utilizable, plant-based forms of iron for populations with a low iron status. This review focuses on recent progress in structure, function, and nutrition of phytoferritin.

Keywords Phytoferritin, EP domain, H-1 and H-2 subunits, iron supplement, iron deficiency anemia

INTRODUCTION

Iron is a vital element for almost all living organisms due to its essential role in numerous metabolic processes such as the tricarboxylic acid cycle, oxygen transport, DNA synthesis, nitrogen fixation, electron transport, and photosynthesis (Harrison and Arosio, 1996; Andrew, 1998). However, iron physicochemical properties make this element uneasy to be used by aerobic living organisms. In aqueous phase, at physiology pH, iron tends to precipitate as insoluble forms. Furthermore, its redox cycling participates in activation of reduced forms of oxygen through Fenton chemistry, leading to lipid peroxidation, protein oxidation, and DNA mutations, and consequently to cellular damage and possible cell death (Harrison and Arosio, 1996).

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In order to enable iron to be safely utilized by living organisms, a specific class of iron storage proteins, ferritin, has been evolved in living cell. Ferritins, as an iron buffer inside the cell, are ubiquitously found in bacteria, animals, and plants where they play important roles in maintaining iron level in a soluble, nontoxic bioavailable form (Lee et al., 2002; Theil, 2004). Animal ferritin is usually a heteropolymer that consists of H and L subunits, whereas plant ferritin is composed of H-type subunits. Additionally, the H subunit of mature phytoferritin contains extension peptide (EP) at its N-terminal extremity (Ragland et al., 1990; Masuda et al., 2010). Thus, there are 24 EP domains per molecule of mature phytoferritin. In the case of soybean seed ferritin (SSF), each EP domain is composed of ∼30 amino acid residues (Masuda et al., 2001). The difference in structure between animal and plant has a great effect on the structure and function of phytoferritin (see below). Different from other plant seeds, legume seeds store ∼90% of its total iron in ferritin, which was located in amyloplast. Therefore, phytoferritin from legume seeds is rich in iron.

On the other hand, iron is an essential nutrient for humans. Unfortunately, dietary iron deficiency is the most common and widespread nutritional disorder in the world. It affects about two billion people. The major consequences of such a disorder are poor pregnancy outcomes, reduced cognitive and motor development in infants, decreased immune function, and decreased work capacity. Supplementation with pharmaceutical iron preparations, food fortification, and dietary diversification are possible strategies to prevent iron deficiency (Theil, 2004; Lnnerdal, 2009). One promising approach to fighting iron deficiency is to explore phytoferritin as iron supplement. This review elucidates new progress in the relationship between the structure and function of phytoferritin, development as iron supplement, and challenges faced during this process.

FERRITIN MOLECULES

General Features of Ferritin

The structure of ferritin is highly conserved in all living kingdom, although its roles in the regulation of iron trafficking vary substantially. The typical ferritin molecule is usually composed of 24 identical or different subunits that are assembled into a hollow protein shell (outside diameter 12–13 nm, inside 7–8 nm) characterized by four-, three-, and two-fold symmetry (432 symmetry; Fig. 1). Up to 4500 iron atoms can be stored as an inorganic complex within its central cavity. The shape of the subunit is cylindrical, with a length of 5 nm and a width of 2.5 nm (Harrison and Arosio, 1996). Each subunit is composed of a four-α-helix bundle containing two antiparallel helix pairs (A, B and C, D) that is connected by a long nonhelical stretch of 18 residues (the BC-loop) between B and C helices. A fifth short helix (E helix) lies at one end of the bundle at about 60° to its axis. The E helix exists around the four-fold intersubunit symmetry axes of the protein shell and forms a hydrophobic pore (Sun et al., 1993; Crichton et al., 1996; Harrison and Arosio, 1996; Chasteen and Harrison, 1999; Masuda et al., 2010).

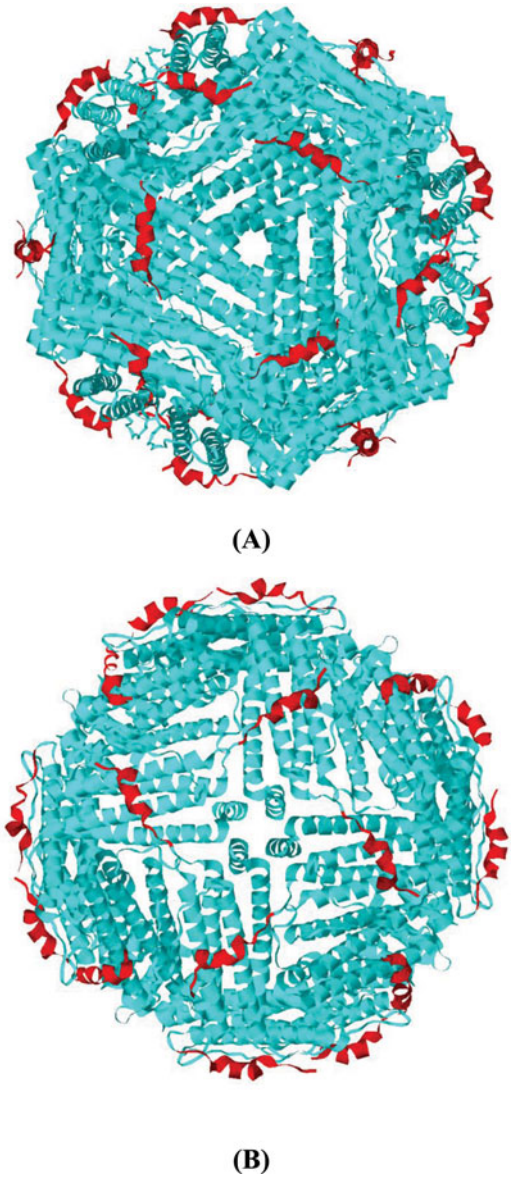

Figure 1 Plant ferritin shell with views down the (A) four-fold, and (B) three-fold axes (channels) of the protein shell (the EP peptide is shown in red). *Source*: Masuda et al. (2010). (Color figure available online.)

Special Features of Phytoferritin

Despite the common ancestry of plant and animal ferritins, phytoferritin exhibits various specific features as compared with its animal counterpart. Phytoferritin is observed in plastids (chloroplasts in leaves, amyloplasts in tubers and seeds, etc.) where iron is oxidatively incorporated into the ferritin shell to form the mineral core, whereas animal ferritin is usually found in the cytoplasm of cell (Waldo et al., 1995; Harrison and Arosio, 1996). The expression of animal ferritin is strictly regulated via the interaction between iron responsive elements and iron regulatory proteins at the translational level, but the expression of phytoferritin genes is controlled at the transcriptional level (Lescure et al., 1991; Harrison and Arosio, 1996). In animal ferritin, the three-fold channels are hydrophilic and their four-fold channels are hydrophobic, while these two kinds of channels are hydrophilic in phytoferritin (Treffry et al., 1989; Lobraux et al., 1992; Masuda et al., 2010). Although there is no report on the concentration of phosphate in the amyloplasts, it was established that the concentration of phosphate in the chloroplasts of spinach leaves reaches up to 12 mM (Bligny et al., 1990). This finding is in good agreement with the fact that phosphorus is an essential element for plant growth. As expected, phosphate has a pronounced effect on the formation of iron core within ferritin shell. It was found that phosphate is distributed throughout the core structure and this leads to an amorphous and much less

dense structure, compared to mammalian ferritin cores (Treffry et al., 1989; Bauminger et al., 1991).

In mammals, two distinct ferritin subunits (H and L) are found with ~55% sequence identity (Boyd et al., 1985). The ratio of the two kinds of subunits in the native protein varies according to the nature and the function of the tissue (Harrison and Arosio, 1996; Chasteen and Harrison, 1999). The H-subunit contains a dinuclear ferroxidase center necessary for fast oxidation of Fe^{2+} by O_2 or H_2O_2 followed by Fe^{3+} hydrolysis and mineralization to form the iron core within the protein interior, and consists of A and B iron-binding sites of conserved amino acid ligands Glu27, His65, Glu62, and Glu107. H-bonding residues Gln141 and Tyr34 are nearby the B-site (Harrison and Arosio, 1996; Zhao et al., 2003). Differently, the L-subunit lacks such a ferroxidase center but contains a putative nucleation site consisting of a cluster of negative residues Glu53, Glu56, and Glu57 that promotes nucleation of the iron core (Crichton et al., 1996). Similarly, the putative nucleation site (Glu61, Glu64, and Glu67) was considered to also occur in mammalian H-chain ferritin, which plays an important role in mineral core formation in this protein (Harrison and Arosio, 1996). However, a recent study has demonstrated that the putative "nucleation site" does not play an important role in iron uptake or mineralization in H-chain ferritin (Bou-Abdallah et al., 2004). Consistent with this observation, the NMR approach of combining solid-state experiments for partial sequence-specific assignment and solution ^{13}C-^{13}C NOESY spectra for side-chain observation of frog M ferritin has provided the identification of an iron channel that guides the directional transport of the multimeric iron (III) products from the ferroxidase centers toward the nanocage (Turano et al., 2010). Thus, the putative nucleation sites seem to play no role in the iron mineralization. These studies raise a question of the function of the nucleation site located in L subunit, so more studies are needed to address this question in future.

In contrast, only one type of subunit has been identified as a functional subunit of phytoferritin, which shares ~40% sequence identity with the animal H-subunit (Laulhre and Briat, 1993; Masuda et al., 2001). The amino acids in the definition of the ferroxidase center are strictly conserved in all phytoferritin except for pea seed ferritin where a histidine residue is found instead of glutamic acid residue at position 62 of the amino acid sequence in the ferroxidase center (Lobraux et al., 1992; van Wuytswinkel et al., 1995). To date, all known naturally occurring phytoferritin from various plant seeds such as soybean, pea, black bean, maize, and alfalfa consists of two subunits of 26.5 and 28.0 kDa, which are designated H-1 and H-2, respectively, with high sequence identity (82% in SSF). Previous studies indicated that the H-1 subunit generated by degradation of the H-2 one by hydroxyl radicals (HO·) (Lobraux and Briat, 1991; Masuda et al., 2007). Inconsistent with the proposal, it is observed that the PMFs of the H-2 subunit of SSF, pea seed ferritin (PSF), and blackbean seed ferritin (BSF) are pronouncedly distinct from those of the H-1 subunit, suggesting that the H-1 subunit is not produced from the degradation of the H-2 one (Masuda et al., 2001; Li, Hu et al., 2009; Deng, Liao, Hu et al., 2010). Further support for this view comes from recent finding that the two subunits of SSF are encoded by two distinct genes, *SferH-1* (GneBank® accession number M64337) and *SferH-2* (GneBank® accession number AB062754), respectively (Masuda et al., 2001). Thus, it seems that phytoferritin seeds are controlled by multiple genes (Lobraux et al., 1992; Wicks and Entsch, 1993; Dong et al., 2007; Dong et al., 2008). Indeed, cowpea has at least four different ferritin genes, one encoding a protein with 97% sequence identity to soybean seed ferritin (Lescure et al., 1991). Recently, it has been demonstrated that a synergistic interaction exists between the H-1 and H-2 subunits in SSF in iron oxidation and translocation (see below; Deng, Liao, Yang et al., 2010).

The subunits of phytoferritin from soybean and other legume seeds are synthesized as 32 kDa precursor proteins that contain a unique two-domain N-terminal sequence: "transit peptide (TP)" and the following "EP" (Fig. 2). These N-terminal domains are not present in mammalian and bacterial ferritins. The TP is responsible for the precursor targeting plastids (Ragland et al., 1990). After being transported to the plastids, the TP is cleaved from the subunit precursor by an undefined mechanism, producing the mature subunit that assembles in a 24-mer ferritin within the plastids. Thus, in mature phytoferritin, 24 EP domains per molecule represent one major structural difference between animal and plant ferritin. In the case of SSF, each EP domain is composed of ~30 amino acid residues. The crystal structure of recombinant H-4 soybean seed ferritin shows that the EP is located on the exterior surface of protein and stabilizes the entire oligomeric conformation of phytoferritin by its interaction with a neighboring subunit on the shell surface, which is highlighted in red in Fig. 1 (Masuda et al., 2010). Recent studies reveal the role of the EP during iron oxidative deposition in PSF as the second binding and ferroxidase center that contributes to mineralization of the iron core at high iron loading of ferritin (>48 iron/shell; Li, Fu et al., 2009). On the other hand, the EP also exhibits a serine protease-like activity (Fu et al., 2010) that is responsible for protein auto-degradation during storage and seed germination (Lobraux and Briat, 1991; van Wuytswinkel et al., 1995; Fu et al., 2010). Associated with the degradation is faster iron release from ferritin to meet the requirements of seedling growth (see below; Fu et al., 2010).

IRON OXIDATION DEPOSITION IN PHYTOFERRITIN

Three Reaction Pathways Responsible for Iron Oxidative Deposition in Ferritin

Iron oxidative deposition in animal ferritin has been extensively studied in vitro. To date, it is found that at least three reaction pathways occur during iron oxidation/mineralization in human ferritin. At low flux of Fe^{2+} into ferritin (<48 Fe^{2+}/protein shell), Fe^{2+} oxidation by O_2 is processed completely by the ferroxidase site with an Fe^{2+}/O_2 stoichiometry of 2:1, leading to the quantitative production of H_2O_2 as in Equation (1) (Xu

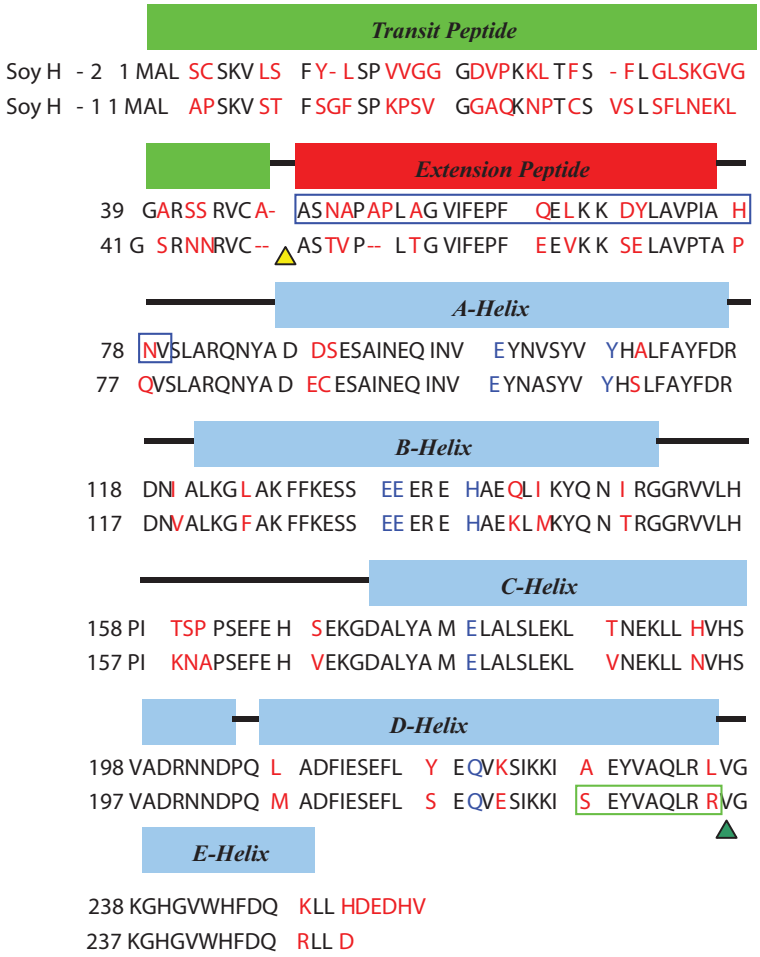

Figure 2 Amino acid sequences of soybean ferritin subunits. *Top*, amino acid sequence of the H-2 subunit. *Bottom*, amino acid sequence of the H-1 subunit. Conserved residues between the two subunits are shown in *black*. Residues in *blue* indicate the deduced ferroxidase center. The N-terminal 47 (TP) and 28 (EP) residues of the H-2 subunit are shown *boxed* in *green* and *red*. The cleavage sites of the TPs 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 to 26.5 kDa is indicated by a *green arrowhead*. Amino acid sequences of the H-1 and H-2 are from Masuda et al. (2001). (Color figure available online.)

and Chasteen, 1991; Treffry et al., 1993; Bou-Abdallah, 2010). Since this reaction occurs at the diiron ferroxidase centers of ferritin, it corresponds to protein catalysis model (Crichton and Roman, 1978).

$$2Fe^{2+} + O_2 + 4H_2O \rightarrow 2Fe(O)OH_{(core)} + H_2O_2 + 4H^+ \quad (1)$$

At medium iron loading (200 > iron/protein shell > 48), some of the H_2O_2 produced at the ferroxidase site rapidly reacts with further Fe^{2+} via the detoxification reaction by Equation (2) (Bou-Abdallah et al., 2002; Zhao et al., 2003). This reaction was called detoxification reaction because toxic reactants Fe^{2+} and H_2O_2 were converted to inert iron core with no production of hydroxyl radical.

$$2Fe^{2+} + H_2O_2 + 2H_2O \rightarrow 2Fe(O)OH_{(core)} + 4H^+ \quad (2)$$

When a larger flux of Fe into the protein is employed (>200 Fe^{2+}/protein) the mechanism changes the Fe^{2+}/O_2 stoichiometry approaches 4:1, and dioxygen is ultimately reduced to water according to the net reaction given by Equation (3). Since Equation (3) is identical to that for Fe^{2+} autoxidation and hydrolysis, it has been assumed that core mineralization at high Fe^{2+} fluxes likely occurs through iron deposition directly on the surface of the mineral according to the crystal growth model (Xu and Chasteen, 1991; Yang et al., 1998).

$$4Fe^{2+} + O_2 + 6H_2O \rightarrow 4Fe(O)OH_{(core)} + 8H^+ \quad (3)$$

A Novel Reaction Pathway for Iron Oxidative Deposition in Phytoferritin Regulated by the EP

The EP represents one major structural difference between plant and animal ferritins (Harrison and Arosio, 1996; Masuda et al., 2001, 2010). Although it has been almost 20 years since EP was first reported (Lescure et al., 1991), its function in phytoferritin has been elucidated until recently (Li, Fu et al., 2009).

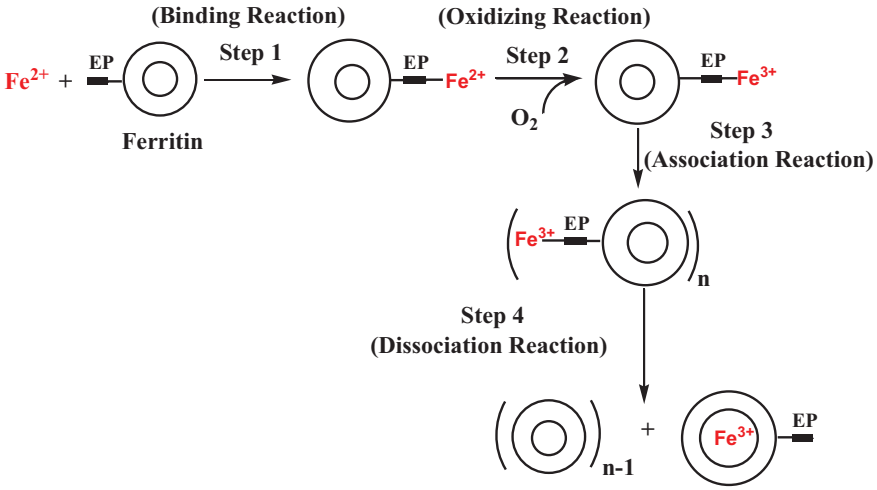

Figure 3 A new pathway of iron oxidative deposition in phytoferritin through protein association at high Fe^{2+} flux (more than 48 Fe^{2+}/shell) into ferritin. Figure is redrawn according to Li, Fu et al. (2009). (Color figure available online.)

The EP in PSF was found to serve as a second center responsible for iron binding and oxidation at high Fe^{2+} flux into protein through a novel four-step pathway as shown in Fig. 3 (Li, Fu et al., 2009). Step 1 corresponds to the binding reaction of excess Fe^{2+} with the EP domain located on the exterior surface of protein at iron/protein ratios exceeding 48 Fe^{2+}/protein shell. Fe^{2+} oxidation by O_2 under the catalytic action of the EP represents the second step. The resulting bound Fe^{3+} triggers protein association that corresponds to Step 3. After Fe^{3+} is transferred from the outer surface of protein to the inner cavity, dissociation of the aggregate into monomer is the fourth step. However, this pathway is distinct from the previous mineralization reaction corresponding to Equation (3) where Fe^{2+} autoxidation directly occurs on the surface of the core. As the size of iron core increases, the mineral surface autoxidation reaction becomes increasingly important as previously described. The above finding represents the fourth pathway for iron oxidative deposition in ferritin that may occur only in phytoferritin (Li, Fu et al., 2009).

Role of H-1 and H-2 Subunits in Phytoferritin in Iron Oxidative Deposition in Protein

Another significantly structural difference between plant and animal ferritins is that naturally occurring phytoferritin is composed of H-1 and H-2 subunits (Masuda et al., 2001; Li, Hu et al., 2009). The ratio of these two subunits depends on plant source (Theil, 2004). This property is reminiscent of animal ferritin that consists of two different type subunits, H and L. There are now considerable evidences supporting the view that the H and L subunits have complementary functions (Harrison and Arosio, 1996). For example, in heteropolymers the dinuclear centers of the H subunits are responsible for the ferroxidase activity, whereas the negatively charged residues on the inner surfaces of the L subunits promote ferrihydrite nucleation (Wade et al., 1991; Levi et al., 1994; Bou-Abdallah, 2010). However, all subunits in phytoferritin characterized thus far contain both a typical H-type ferroxidase center and all the amino acid residues characteristic of an L-type subunit for efficient iron nucleation with the cavity. Based on these structural features, phytoferritin is considered an H/L hybrid ferritin (Lobraux et al., 1992). Therefore, it is of special interest to elucidate the role of H-1 and H-2 in plant ferritin for oxidative deposition of iron in protein. Recent studies demonstrate that the H-1 and H-2 subunits have markedly distinctive functions in oxidative deposition of iron in SSF (Deng, Liao, Yang et al., 2010). At low iron flux into the protein ($\leq$48 Fe^{2+}/shell), both the H-1 and H-2 subunits catalyze iron oxidation via the diiron ferroxidase center through a ferroxidase mechanism as previously proposed (Yang et al., 1998). Under conditions of moderate iron flux (48–200 Fe^{2+}/shell), the above mechanism likewise dominates iron oxidation in the H-1 subunit because of its stronger ability to remove Fe^{3+} from the inner cavity than that of H-2. However, in the H-2 subunit, this mechanism is gradually replaced by another mechanism by which the iron oxidation is processed by EP-2 (the EP of the H-2). At high iron loading of the protein (>200 Fe^{2+}/shell), EP-1 (the EP of the H-1) also appears and participates in the iron oxidation, but its ability to catalyze iron oxidation and transfer Fe^{3+} to the cavity is markedly weaker than that of EP-2. More interestingly, when more than 48 Fe^{2+} or Fe^{3+} per protein are loaded, there is a positively synergistic interaction between the H-1 and H-2 chains in SSF in iron oxidation and translocation, resulting in stronger catalyzing activity of both wild-type (WT) SSF and recombinant SSF. Thus, heteropolymeric ferritin may facilitate plant cell absorption of both ferrous and ferric ions from soil more effectively compared with a homopolymeric one, and this helps explain why naturally occurring phytoferritin is usually a heteropolymer.

IRON RELEASE IN PHYTOFERRITIN

A Novel EP-Involved Pathway for Iron Release from Phytoferritin

To date, Fe^{2+} oxidation and mineral deposition in ferritin have been extensively studied (Chasteen and Harrison, 1999; Zhao et al., 2003; Bou-Abdallah et al., 2005; Li, Fu et al., 2009). In contrast, there are relatively less studies carried out with iron release from ferritin, especially from phytoferritin. The iron release is of crucial importance because this process is involved in cell growth in both plants and animals, and is also related to phyotoferritin used as iron supplement in future. Iron in phytoferritin from legume seeds is required for the germination and early growth of seedlings (Lobraux and Briat, 1991). However, the mechanism by which phytoferritin regulates its iron complement to the physiological process remains unknown. Recent studies demonstrate that the EP domains on SSF possess a serine protease-like activity previously unrecognized, thus resulting in its auto-degradation (Fig. 4). Associated with the degradation is faster iron release from ferritin to meet the requirement of seedling growth, representing a novel pathway for how phytoferritin controls its iron complement through a specific domain in phytoferritin, the EP (Fu et al., 2010; Yang et al., 2010). Protein degradation was found to occur upon holoSSF during storage, an observation consistent with previous reports where holoPSF or holoSSF showed the same protein degradation taking place during seed germination (Lobraux and Briat, 1991; van Wuytswinkel et al., 1995). Thus, phytoferritin degradation seems to constantly occur in vitro and in vivo. Previous studies proposed that holoPSF degradation is due to damage by iron-induced hydroxyl radical (HO·) (Laulhre et al., 1989; Lobraux and Briat, 1991). However, the addition of iron chelators (*o*-phenanthroline or desferrioxamine B) to holoSSF has no effect on such degradation, and this degradation also occurs with apoSSF, which precludes the possibility that protein degradation is due to iron-induced HO·. Thus, protein shell, not iron, is responsible for the protein degradation. As expected, upon removal of the EP from holoSSF, resultant protein (consists of 23.5 and 21.0 kDa subunits with MW as ∼440 kDa) becomes stable with no degradation under the same experimental conditions, demonstrating that the auto-degradation of SSF actually corresponds to the degradation of the EP. Therefore, the EP is a protease-susceptible sequence possibly because its exposure on the outer surface of protein predicated the three-dimensional model (Masuda et al., 2010). Direct evidence for this view comes from protease activity measurements showing that the EP-1 exhibits significant serine protease-like activity while the activity of the EP-2 is very weak. Thus, the EP-1 is mainly responsible for the SSF degradation. The large difference between the hydrolyzing activity of the EP-1 and EP-2 may reside in their somewhat different amino acid residues. The EP-1 contains two serine residues at positions 50 and 68 while the EP-2 only having one serine residue at position 49 (Fig. 2). The above conclusion was further confirmed by the fact that rH-2 (recombinant H-2 soybean seed ferritin) is a stable protein molecule whereas its analogue rH-1 (recombinant H-1 soybean seed ferritin) is susceptible to degradation (Fu et al., 2010). These results unambiguously demonstrate that the auto-degradation WT SSF during storage is mainly derived from the serine protease-like activity of the EP-1. Consistent with present observation, previous studies showed that H-2 subunit was more resistant toward proteolysis as compared to H-1 subunit (Masuda et al., 2001). Interestingly, associated with the EP degradation is a considerable increase in the rate of iron release from SSF induced by ascorbate in the amyloplast (pH range, 5.8–6.1). This is because a reaction between a reducing regent such as ascorbic acid and dissociated holoferritin occurs more easily. Consistent with the idea, a recent study by Yang et al. demonstrated that the removal of the EP deleted protein association induced by low pH while the shell-like structure of phytoferritin kept intact (Yang et al., 2010).

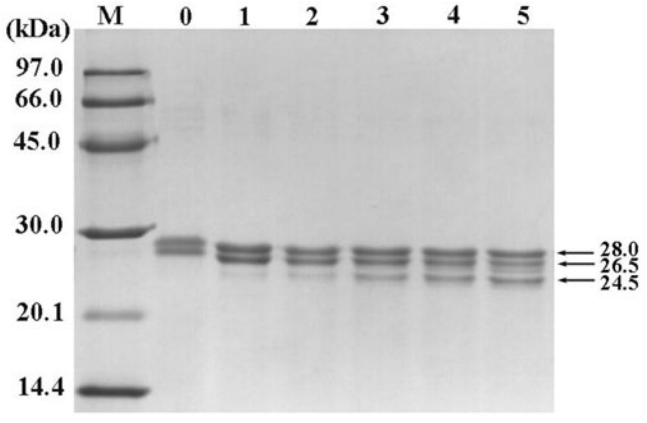

Figure 4 SDS-PAGE analysis of SSF degradation. The purified SSF was incubated in 50 mM phosphate buffer containing 150 mM NaCl at 4°C at different time intervals. Lanes 0–5 correspond to incubation times 0, 10, 20, 30, 40, and 50 days, respectively; Lane M, protein markers and their corresponding molecular masses (Fu et al., 2010).

The crystal structure analysis of the soybean ferritin indicates that the EP stabilizes the protein shell by maintaining the conformation of the C-helix. Therefore, it is likely that auto-degradation of the EP destabilizes the outer case of the ferritin structure, leading to the facile release of iron. This is reminiscent of the mechanism of iron release from animal ferritin, which is usually a cytosolic molecule. Many studies have shown that cytosolic ferritin gains entry into lysosomes by autophagy, and that ferritin degradation within lysosomes is responsible for iron release (Radisky and Kaplan, 1998; Kurz et al., 2008). Thus, there is something in common between the mechanism of iron release from plant and animal ferritins, namely, their degradation occurs prior to iron exit from protein. However, phytoferritin degradation is slightly partial degradation. As a result, the degraded phytoferritin still retains an intact protein shell as shown by TEM analysis (Wade et al., 1991). Thus, reductants, such as ascorbate, are still required for reducing Fe^{3+} caged in phytoferritin, and resultant ferrous ions diffuse out of ferritin shell through three- or four-fold channels. In contrast, complete

degradation of animal ferritin by hydrolases existing in the lysosomes results in iron release from protein without participation of the reductants (Radisky and Kaplan, 1998; Kurz et al., 2008).

Anthocyanins Induce Iron Release from Phytoferritin

Anthocyanins, a class of naturally occurring pigments in foodstuffs, receive great attention due to their various activities (Wrolstad, 2004). Previous studies showed that the anthocyanins exhibited powerful antioxidative activity (Wang et al., 1997) whereas ferric ions within phytoferritin shell had certain oxidative properties. Since these two bioactive substances (anthocyanins and phytoferritins) co-exist in plant foods in the diet, it is of special interest to know whether or not there is an interaction between them. If so, what is the consequence of the interaction? Recent studies demonstrate that anthocyanins have a strong interaction with SSF, resulting in iron release from SSF (Deng, Cheng et al., 2010). The ability of anthocyanins to liberate iron from SSF is associated with the size of molecules, the chemical structures, and their chelating activity on Fe^{2+}. Interestingly, these anthocyanins completely inhibit ferritin from degradation by HO· during the iron release, while ferritin was degraded into small peptides during the ascorbate-induced iron release from SSF under the same experimental conditions. Possible reasons for the protective effects of the anthocyanins on ferritin come from their stronger ability to complex Fe^{2+} and HO·-scavenging activities than ascorbate (Deng, Cheng et al., 2010).

PHYTOFERRITIN AS A SOURCE OF IRON SUPPLEMENT

Iron deficiency anemia (IDA) is the most common and widespread nutritional disorder in the world, affecting more than two billion people (Theil, 2004). Women who are pregnant or lactating and young children are the most affected, especially in the developing countries (Beard et al., 1996; Kapur et al., 2002). Inadequate dietary intakes and low bioavailability of iron are the major factors contributing to IDA (Forbes et al., 1989). Iron supplementation, such as ferrous iron salts (ferrous sulfate and ferrous gluconate), is considered the most common strategy currently to combat IDA. However, since these iron supplementations have some negative consequences and side effects to human body (Theil, 2004), an alternative strategy will need to be developed to solve the problem of IDA. One promising approach to fighting iron deficiency is to find new functional factors for iron supplement that overcome the above-mentioned shortcomings, e. g., plant ferritin (see below).

Nutritional iron is classified as two types: heme iron of animal origin and nonheme iron of plant origin. The former is absorbed as stable porphyrin complex unaffected by other food components such as phytates and tannins. In contrast, these dietary components are capable of capturing iron from the nonheme iron in plant foods and form insoluble compounds in the intestinal lumen, resulting in inhibition of iron absorption (Theil, 2004). However, phytoferritin iron, as a novel and alternative dietary iron source, is masked by a protein coat, so it is less sensitive to chelators (phytates and tannis) present in the diet, representing an alternative strategy to the eradication of global deficiency in the 21st century (Beard, 2001; Murray-Kolb et al., 2003; Zhao, 2010).

In legume seeds, ∼90% of the total iron is in ferritin (Ambe et al., 1987; Theil, 2004). Differently, most of the iron is complexed by phytate as monoferricphytate in wheat grain, and is relatively unavailable (May et al., 1980). Therefore, plant ferritin from legume seeds is considered as an alternative strategy for iron supplementation, and has received great attentions recently (Theil, 2004; Lnnerdal, 2009). Using radioisotope, different results have been obtained from ferritin. This may be due to the difference in the sources of ferritin and in the labeling techniques. Extrinsic labeling of partially iron-saturated ferritin hardly accounts for iron in the insoluble ferritin mineral. Extrinsic iron added to ferritin involves a phase transition that causes much slow equilibration with endogenous iron. Early study with extrinsic labeling of legumes showed poor bioavailability of ferritin iron (Lynch et al., 1984). This is different from the reported high bioavailability of iron from soybeans using intrinsic labeling in a human study (Beard et al., 1996). Two types of meals (soup and muffins) were used and each subject received a reference dose of labeled $FeSO_4$ to assess inherent capacity to absorb iron. Results showed that the mean ^{55}Fe absorption from both soup and muffins was 27%, confirming that soybeans are a good source of nutritional iron in marginally iron-deficient individuals (Beard, 2001). Purified SSF was also used to evaluate iron absorption. Healthy, nonanemic women were fed a standardized meal (bagel, cream cheese, and apple juice) containing 1 μCi ^{59}Fe/meal as $FeSO_4$ or (extrinsically labeled) as iron-free soybean ferritin reconstituted with the high phosphate characteristic of plant ferritin. It was found that whole-body iron absorption from soybean ferritin and $FeSO_4$ was nearly the same with no significant difference between groups, again strongly demonstrating that iron from soybean ferritin is well absorbed (Lnnerdal et al., 2006). Moreover, a recent study in rats also demonstrated that SSF crude was as efficient as FeSO4 in restoring red blood cell, hemoglobin, serum ferritin, and serum iron levels (Yun et al., 2011). Thus, plant ferritin may represent a model for novel, utilizable, plant-based forms of iron for populations with a low iron status.

CHALLENGES FOR PHYTOFERRITIN AS AN IRON SUPPLEMENT SOURCE

On the other hand, some studies have been carried out about the stability of ferritin in the stomach. Upon digestion of ferritin by pepsin at pH 2.0, iron uptake from HoSF and $FeSO_4$ are nearly identical within experimental uncertainty, suggesting that most of the ferritin might be degraded by pepsin in the

stomach (Kalgaonkar and Lnnerdal, 2008). This finding is in good agreement with another study showing that, during in vitro gastric digestion of recombinant bean ferritin and red kidney bean extract, ferritin-iron was fully released from the protein and dissolved at pH 2.0 (Hoppler et al., 2008). Moreover, this study also found that ferritin iron was no longer detectable after boiling the legumes for 50 minutes in excess water, while some ferritin-iron from recombinant bean ferritin remained measurable. Thus, iron core in ferritin might be released during cooking and at gastric pH. This raises a question as to whether iron uptake from the iron core is the same as that from the other nonheme iron in food. To date, there is no report on the mechanism by which the iron core is absorbed. However, it cannot be excluded that a small amount of ferritin from legume escapes from the human stomach. Phytoferritin is usually composed of H-1 (26.5 kDa) and H-2 (28.0-kDa) subunits. Previous studies showed that H-2 is more resistant toward proteolysis compared with H-1 (Masuda et al., 2001). It has been established that PSF contains more H-2 subunits than SSF (Li, Hu et al., 2009). Therefore, it is possible that the ferritin consisting of either higher amounts of H-2 subunits or only H-2 subunits likely shields more ferritin from digestion. Recently, an alternative strategy to improve the stability of phytoferritin has been reported, namely, binding of proanthocyanidins (PAs) to phytoferritin could prevent protein from degradation by the protease(s) (Deng et al., 2011). Such binding can significantly inhibit the degradation of SSF by the protease(s) in simulated gastric fluid (SGF) at pH 4.0. Similarly, the stability of SSF in simulated intestinal fluid (SIF) was also increased upon treatment with PAs. These findings raise the possibility that the bioaccessibility of phytoferritin to the gastrointestinal tract was improved in the presence of PAs (Deng et al., 2011). However, another animal study with rats has showed that PAs appear to exhibit an inhibitory effect on iron uptake from ferritin by rats (Yun et al., 2011). The reason for this observation is unclear. Moreover, in this study, it was found PAs are toxic for rats with IDA, as suggested by the observation that all rats in Pas' group died at the eighth week while the rats in iron deficiency control group were all alive. This may be due to the strong ability of PAs to chelate metal ions including iron ion (Yun et al., 2011).

FERRITIN RECEPTORS AND POSSIBLE CELL UPTAKE PATHWAYS

Increasing attentions have been paid to elucidate possible ferritin receptors recently. The Tim-2 receptor is specific for H ferritin (Chakravarti et al., 2005; Chen et al., 2005; Todorich et al., 2008). However, no human analog has been found. More recently, a human ferritin receptor has been identified to be transferrin receptor-1 (TfR-1; Li et al., 2010). It was observed that TfR-1 is specific for H ferritin but not for L ferritin. Interestingly, transferrin only partially inhibited H ferritin binding to TfR-1 indicating the ferritin and transferrin-binding sites do not overlap. Ferritin enters the endosome or lysosome by endocytosis. Since H-type subunits in phytoferritin only share ~40% sequence identity with the animal H-subunit, and contain 24 extra EP domains, so future work is needed to investigate whether or not TfR-1 is also suitable for plant ferritin. Another study indicated that the existence of a specific ferritin receptor in Caco-2 cells and inhibitors of endocytosis reduced iron uptake by the cells. The results support a model of SSF endocytosis through the apical cell membrane by an AP2-mediated endocytic pathway, followed by protein cage degradation, mineral reduction, and iron entry to the cytosolic iron pool (San Martin et al., 2008). It is obvious that endocytosis of iron as the ferritin mineral is a much more efficient transport event than the transport of individual iron atoms across the cell membrane in the Fe^{2+} form via DMT1 or in the heme form via HCP1 because the ferritin mineral usually contains hundreds to thousands of iron atoms. Taken together, there are at least two pathways correlated with iron uptake from ferritin. The first one is the normal pathway, as previously proposed for divalent ions. The iron core was released from ferritin by pepsin and the low pH (2.0) in the stomach, and then it was reduced to Fe^{2+} under the action of reducing agents, such as ascorbate, followed by absorption in the gut via DMT1 (Theil, 2004; Lnnerdal, 2009). However, whether the iron core could be taken up directly by a certain pathway remains to be determined. If a small amount of ferritin can escape from the stomach, it could be absorbed by Caco-2 cells in the gut by an AP2- or TfR-1-mediated endocytic pathway, corresponding to the second pathway. Because of possibly direct absorption of phytoferritin as a whole molecule, an interesting question arises as to whether the EP on the outer surface of plant ferritin plays a role in this pathway. If so, it is of special interest to elucidate whether or not the EP has an interaction with the receptor located in the Caco-2 cell membrane in future study.

CONCLUSIONS AND PERSPECTIVES

Since the subunit composition, amino acid sequence, and crystal structure of plant ferritin have been established recently, all these information facilitate the establishment of the relationship between the structure and function of phytoferritin. Phytoferritin exhibits distinctive features from animal and bacterial ferritin in structure. Phytoferritin usually consists of two types of subunits, H-1 and H-2, whose N-terminal contains a specific EP domain that animal subunits lack. These differences in structure confer special properties on phytoferritin. Indeed, a novel pathway of iron oxidative deposition was found in phytoferritin, in which the EP serves as the second ferroxidase center at high iron flux into ferritin. It has been elucidated that the H-1 and H-2 subunits of plant ferritin play different roles in iron mineralization in protein, and a synergistic interaction exists between the H-1 and H-2 subunits in SSF during iron mineralization. Moreover, the EP domain of phytoferritin has protease-like activity that facilitates the degradation of the EP. Consequently, plant ferritin becomes more soluble during storage, finally

resulting in a faster iron release from ferritin. However, there are some questions that remain to be answered in future studies: (1) Plant ferritin is located in plastids where other components such as DNA and RNA coexist, and iron oxidative corporation into protein occurs, so does phytoferritin have a DNA-protective function during this oxidative process? (2) Do other components that are also compartmentalized in plastids influence iron deposition and release? Answering all these questions will advance our understanding of the structure and function of phytoferritin.

Iron is taken up from SSF by either DMT1 as ferrous iron released from digested ferritin or receptor-mediated endocytosis of intact ferritin. Whether there are other mechanisms related to iron uptake from phytoferritin remains to be determined. As compared to other nonheme iron, the iron inside plant ferritin is well protected by a protein shell from interaction with other dietary factors, such as phytate rich in food. Second, ferritin iron might be safer than $FeSO_4$ because free ferrous ion can trigger the formation of radicals under aerobic conditions through the known iron-catalyzed Haber–Weiss process. Third, plant ferritin from legume seeds is likewise suitable for vegetarians with a low iron status. Finally, legume seeds as sources for the preparation of phytoferritin are plentiful because legume has been widely planted in the world. However, the stability of ferritin needs to be improved to escape from gastric digestion. Generally, plant ferritin represents a novel alternative dietary iron source for treatment of IDA.

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ABBREVIATIONS

EP = extension peptide
EP-1 = extension peptide of 26.5 kDa subunit of soybean seed ferritin
EP-2 = extension peptide of 28.0 kDa subunit of soybean seed ferritin
TP = transient peptide
EcBFR = *Escherichia coli* bacterioferritin
HoSF = horse spleen ferritin
HuHF = human H-subunit ferritin
IDA = iron deficiency anemia
PSF = pea seed ferritin
rH-2 = recombinant soybean seed ferritin H-2 subunit assembles
rH-1 = recombinant soybean seed ferritin H-1 subunit assembles
rH-1H-2 = recombinant H-1 and H-2 soybean seed ferritin
WT SSF = wild-type soybean seed ferritin
SSF = soybean seed ferritin
TEM = transmission electron microscopy

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