JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2010, 58, 635–641 635
DOI:10.1021/jf903046u

Comparative Study on Iron Release from Soybean (*Glycine max*) Seed Ferritin Induced by Anthocyanins and Ascorbate

Jianjun Deng,[†] Jianjun Cheng,[‡] Xiayun Liao,[†] Tuo Zhang,[†] Xiaojing Leng,[†] and Guanghua Zhao*,[†]

[†]College of Food Science and Nutritional Engineering, China Agricultural University, Beijing 100083, People's Republic of China and [‡]The Key Laboratory of Soybean Biology of Education Ministry, Food Science College, Northeast Agricultural University, Harbin 150030, People's Republic of China

Anthocyanins have received much attentions due to their various activities. Phytoferritin represents a novel alternative for iron supplementation. In the present study, it was found that all tested anthocyanins such as cyanidin (Cy), delphinidin (Dp), delphinidin-3-*O*-glucoside (Dp3glc), malvidin (Mv), petunidin (Pt), and petunidin-3-*O*-glucoside (Pt3glc) had a strong interaction with SSF, respectively, resulting in iron release from soybean seed ferritin (SSF) just as for ascorbate. The order of iron release from SSF is as follows: Dp > Cy > Pt > Mv > Dp3glc > Pt3glc. Their ability to liberate iron from SSF is associated with the size of the molecules and the chemical structures but mainly depends on their chelating activity with Fe^{2+}. Interestingly, these pigments inhibited SSF degradation during the iron release to different extents while ascorbate did not. The difference in protective effects on SFF between ascorbate and the anthocyanins is in good agreement with their different Fe^{2+}-chelating activities.

KEYWORDS: Phytoferritin; anthocyanins; ascorbate; iron release; hydroxyl radical; interaction

INTRODUCTION

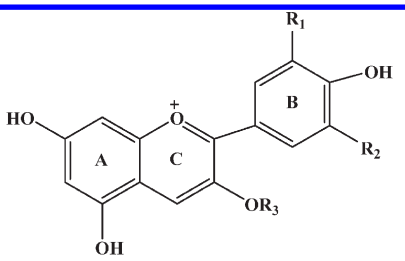
Iron is an essential element for living organisms because of its role in major biological reactions such as the tricarboxylic acid cycle, electron transport, nitrogen fixation, DNA synthesis, and detoxification reactions. Ferritin is a ubiquitous iron storage protein and plays a crucial role in intracellular iron homeostasis. Its three-dimensional structure is highly conserved among plants, animals, and bacteria (*1*). All ferritins have 24 subunits arranged in a 432 symmetry to give a hollow protein shell (the outside diameter is 12−13 nm and the inside diameter 7−8 nm) where ∼4500 Fe^{3+} atoms can be stored as an inorganic complex (*2*). Structural analyses indicate that each subunit consists of a four-α-helix bundle containing two antiparallel helix pairs (A, B and C, D) and a fifth short helix (E helix). The E helix lies at one end of the bundle at 60° to its axis and forms a hydrophobic pore (*2*).

Despite the probable common ancestry of plant and animal ferritins (*3*), plant ferritin exhibits various specific features as compared to animal analogues. First, plant ferritin is found in plastids such as amyloplasts in tubers and seeds, while animal ferritin occurs in the cytoplasm of the cell (*2*, *4*). Second, the expression of animal ferritin genes is mainly regulated at the translational level (*5*), whereas the expression of plant ferritin genes is controlled at the transcriptional level (*6*). Third, animal ferritin contains two distinct ferritin subunits known as H (∼21.0 kDa) and L (∼19.0 kDa) with about 54% identity. In contrast, only the H-type subunit has been found in plant ferritin (*7*). The H subunit has ferroxidase centers which usually consist of seven conserved amino acid residues and are responsible for the fast oxidation of Fe^{2+} by oxygen or H_2O_2. In contrast, the L subunit contains nucleation sites instead of the ferroxidase centers responsible for slower Fe^{2+} oxidation (*8*). Ferritins from dried soybean and pea seed are composed of two subunits of 26.5 and 28.0 kDa, which are termed H-1 and H-2, respectively, and share ∼80% amino acid sequence identity (*9*, *10*). These two subunits are synthesized as a 32 kDa precursor with a unique two-domain N-terminal sequence containing a "transit peptide" (TP) and an "extension peptide" (EP), but the mature plant subunits only contain the EP, whose function remains to be determined. From the standpoint of nutrition, biofortification of staple food with iron caged within phytoferritin from legumes is believed to be an effective strategy to prevent iron deficiency anemia, which affects ∼2 billion people in the world (*1*).

Fe^{2+} oxidation and mineral deposition in ferritin have been extensively studied (*2*, *8*). In contrast, there are relatively fewer 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. In addition, it is known that Fe^{2+} is much more toxic than its analogue Fe^{3+} because it induces the formation of $HO^{\bullet}$ through the Fenton reaction. Therefore, the safe removal of iron from ferritin is a goal of chelation therapies (*11*). Some reductants such as ascorbate (*12*, *13*), 6-hydroxydopamine (*14*), superoxide anion radical (*15*), 5-aminolevulinic

*To whom correspondence should be addressed. Tel: +86-10-62737761. Fax: +86-10-62737761. E-mail: gzhao1000@yahoo.com.

Published on Web 11/18/2009



1: $R_1 = OCH_3$, $R_2 = OCH_3$, $R_3 = H$ (malvidin, Mv)
2: $R_1 = OH$, $R_2 = H$, $R_3 = H$ (cyanidin, Cy)
3: $R_1 = OH$, $R_2 = OH$, $R_3 = H$ (delphinidin, Dp)
4: $R_1 = OCH_3$, $R_2 = OH$, $R_3 = H$ (petunidin, Pt)
5: $R_1 = OCH_3$, $R_2 = OH$, $R_3 =$ glycoside (petunidin-3-*O*-glucoside, Pt3glc)
6: $R_1 = OH$, $R_2 = OH$, $R_3 =$ glycoside (delphinidin-3-*O*-glucoside, Dp3glc)

Figure 1. Chemical structure of anthocyanins.

acid (*16*), 1,2,4-benzenetriol (*17*), and benzene metabolites (*18*) have been shown to induce iron release from ferritin. Several Fe^{3+} chelators such as 2,2′-bipyridine (*19*), nitrilotriacetate (*13*), salicylate (*13*), citrate (*13*), and desferrioxamine B (*11*) can also bring about iron release from ferritin, but at a much slower rate. However, so far, no report is available on the iron release from ferritin by anthocyanins and the effect of the iron release on the stability of ferritin.

Anthocyanins are widely distributed in various plant foods such as fruits and vegetables (*20*), which are responsible for their colors. Three main anthocyanins, i.e., cyanidin-3-*O*-glucoside (Cy3glc), Dp3glc, and Pt3glc, also occur in black soybean seed coats (*21*). Previous studies showed that the anthocyanins exhibited powerful antioxidative activity (*22*), whereas ferric ions within phytoferritin shell had certain oxidative properties. Since the anthocyanins and phytoferritin coexist in plant foods in the diet, it is of special interest to know whether there is an interaction between them. If so, what is the consequence of the interaction?

In this paper, we determine the ability of iron release from SSF induced by anthocyanins containing Mv (**1**), Cy (**2**), Dp (**3**), Pt (**4**), Pt3glc (**5**), and Dp3glc (**6**) (**Figure 1**), with ascorbate as a control sample. It was found that all the anthocyanins could induce iron release from SSF, but to different extents. The ability of these pigments to induce iron release from SSF correlates with their chelating activity on Fe^{2+}. In comparison to ascorbate, these pigments exhibit a significant protective effect on SSF against oxidative damage by hydroxyl radicals produced during the iron release from ferritin.

MATERIALS AND METHODS

Materials. Dried soybean (*Glycine max*) seeds were obtained from the local market. The six anthocyanins Cy, Dp, Dp3glc, Pt, Pt3glc, and Mv were purchased from ChromDex Chemical Co. (Beijing, China) as chloride salts. The stock solutions of different anthocyanins were prepared in dilute HCl at pH 2.0 and stored at −80 °C. The ferrous iron chelator 3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine (ferrozine) was obtained from Sigma-Aldrich Chemical Co. (Beijing, China). Sephacryl S-300, DEAE Sepharose Fast Flow, native electrophoresis marker, and SDS electrophoresis marker were purchased from GE Healthcare Bio-Sciences AB (Beijing, China). 3-(*N*-Morpholino)propanesulfonic acid (Mops) was obtained from Amersco (Beijing, China). Ascorbate, terephthalic acid (TA), sodium citrate, and magnesium chloride hexahydrate were obtained from Beijing Chemical Reagents Co. (Beijing, China). All other reagents used were of analytical grade or purer.

SSF Purification. SSF was purified as previously described with some modifications (*23*). Typically, approximately 1 kg of soybean seeds was soaked in distilled water overnight and blended in three volumes of extraction buffer (0.05 M KH_2PO_4−Na_2HPO_4, pH 7.5, 1% polyvinylpolypyrrolidone). The resultant mixture was filtered through cheesecloth. The filtrate was incubated for 15 min at 55 °C and then was centrifuged at 4800*g* for 10 min to separate the insoluble material. The supernatant was adjusted to 0.5 M $MgCl_2$ and the mixture stood for 30 min at 4 °C followed by addition of sodium citrate (final concentration of 0.7 M) to complex the magnesium. After 8 h, the resultant supernatant was centrifuged at 12000*g* for 30 min at 4 °C. The brown pellet thus obtained was dissolved in 0.05 M KH_2PO_4−Na_2HPO_4 buffer (pH 7.5) and was dialyzed against the same buffer three times. The protein was further purified by ion exchange chromatography in a DEAE-Sepharose Fast Flow column previously equilibrated in 0.05 M KH_2PO_4−Na_2HPO_4 buffer (pH 7.5). Fractions containing SSF were pooled, concentrated, and finally loaded on a Sephacryl S-300 gel filtration column pre-equilibrated with 0.05 M KH_2PO_4−Na_2HPO_4 buffers (pH 7.5) containing 0.15 M NaCl. After verification of the purity of the ferritin using SDS-polyacrylamide gel electrophoresis (SDS-PAGE), the fractions were pooled, concentrated, and stored at 4 °C for later use. Protein concentrations were determined according to the Lowry method with BSA as standard.

Protein Gel Electrophoresis. Determination of the molecular weight of the native SSF was done using a 4−20% polyacrylamide gradient gel run at 25 V for 13 h at 4 °C. The buffer system of the gel was Tris-HCl (0.025 M, pH 8.3). Gels were stained with Coomassie Blue R-250. The electrophoresis of proteins under denaturing conditions was carried out with 15% SDS-polyacrylamide gel. Tested protein samples (∼20 μg) were suspended in 50 μL of water. To the solution was added 100 μL of sample buffer containing 25% glycerol, 12.5% 0.5 M Tris-HCl, pH 6.8, 2% SDS, 1% bromophenol blue, and 5% β-mercaptoethanol. After the solution was boiled for 10 min, the supernatant was isolated by centrifugation at 10000*g* for 10 min.

Kinetic Measurement of Iron Release. Iron release from SSF was investigated using the reported procedure (*24*). Briefly, the assay system (1 mL of total volume) contained 0.15 μM SSF, 500 μM ferrozine, 0.1 M NaCl, and the desired concentration of anthocyanins or ascorbate in 0.05 M Mops buffer, pH 7.0. Reactions were carried out at 25 °C against reference cuvettes containing all reactants and were initiated by the addition of anthocyanins or ascorbate. The development of $[Fe(ferrozine)_3]^{2+}$ was measured by recording the increase in absorbance at 562 nm using a Varian Cary 50 spectrophotometer and the iron released estimated, using $\varepsilon_{562} = 27.9\ mM^{-1}\ cm^{-1}$. The kinetic data were further analyzed with Origin 7.5 software (Microcal Inc.). The initial rate (v_0) of iron release measured as Fe^{2+}-ferrozine complex formation was obtained from the linear A_1 term of third-order polynomial fitted to the experimental data as described previously: namely, $Y = A_0 + A_1t + A_2t^2 + A_3t^3$ and $dY/dt = A_1 + 2A_2t + 3A_3t^2$ (at $t = 0$, $(dY/dt)_0 = v_0$). Here t is the time in seconds and Y is the concentration of $[Fe(ferrozine)_3]^{2+}$ at time t in seconds (*8*).

Chelating Activity on Fe^{2+} Ion. The chelating activity of anthocyanins on Fe^{2+} was measured as previously reported (*25*) with some modifications. The assay system (1 mL of total volume) contained 20 μM $FeSO_4$, 60 μM ferrozine, and 60 μM anthocyanins or ascorbate in 0.05 M Mops buffer at pH 7.0. The reactions were carried out at 25 °C for 5 min, and then the absorbance at 562 nm was determined spectrophotometrically. A lower absorbance indicates a stronger Fe^{2+}-chelating ability. The ability to chelate the ferrous ion was calculated as follows: chelating activity (%) = $[(A_0 - A_1)/A_0] \times 100$, where A_0 is the absorbance of the control and A_1 is the absorbance in the presence of the anthocyanins or ascorbate. Doubly distilled H_2O was used instead of anthocyanins or ascorbate in a control sample.

Determination of $HO^{\bullet}$ Release. The production of $HO^{\bullet}$ was evaluated by monitoring the formation of the hydroxylated terephthalate (TA-OH) between TA and $HO^{\bullet}$, as described (*26*). SSF (0.15 μM) was incubated at 25 °C in 0.05 M Mops buffer (pH 7.0) with 0.1 M NaCl, 500 μM TA, and 25 μM anthocyanins or ascorbate for 48 h. The fluorescence intensity of TA-OH was measured at 326 nm as an excitation wavelength (Ex) and at 432 nm as a emission wavelength (Em) with a Cary Eclipse fluorescence spectrophotometer. $HO^{\bullet}$ concentration (nM) in the reaction medium was calculated from the following calibration curve, determined by linear regression ($R^2 = 0.999$): $Y = 0.1077X + 0.03341$,

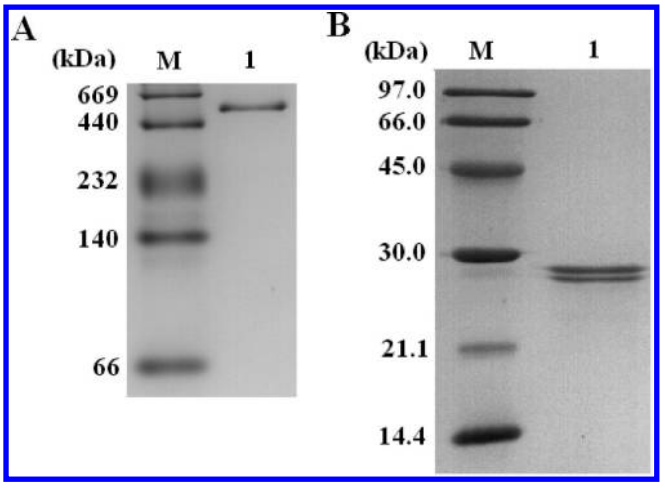


Figure 2. Native PAGE and SDS-PAGE analyses of purified ferritin from soybean seed. Samples were added after the final gel filtration chromatography on a Sephacryl S-300 column: (**A**) native PAGE; (**B**) SDS-PAGE; (lane 1) SSF; (lane M) protein markers and their corresponding molecular masses.

where Y is the fluorescence intensity of TA-OH and X is the concentration of HO•.

Assay of HO•-Scavenging Activity. The HO•-scavenging activity was determined by a reported method (*27*). The reaction system (3 mL of total volume) contained 1 mM $FeSO_4 \cdot 7H_2O$, 1 mM EDTA, 50 mM Mops (pH 7.0), 1 mM TA, and 25 μM anthocyanins or ascorbate. The reaction was started by addition of 1 mM H_2O_2. After incubation at 37 °C for 2 h, the fluorescence intensity of TA-OH was measured at Ex 326 nm and Em 432 nm. The HO•-scavenging activity (%) was calculated as $[(FIc - FIs)/FIc)] \times 100$, where FIc is the fluorescence intensity of TA-OH in the Fenton reaction system and FIs is the fluorescence intensity of TA-OH in the Fenton reaction system with anthocyanins or ascorbate.

Statisitial Analysis. The data were analyzed using the Statistical Analysis System (SAS 9.0) software package for analysis of variance (Duncan's test). All experiments were carried out in triplicate. The significance was established at $p \leq 0.05$.

RESULTS AND DISCUSSION

Isolation and Characterization of Ferritin from Soybean Seeds. Nondenaturing gel electrophoresis (native PAGE) resolved the purified SSF as a single complex, estimated to be about 560 kDa (**Figure 2A**). SDS-PAGE indicated that the purified SSF is composed with nearly identical 28.0 and 26.5 kDa subunits (**Figure 2B**), a result consistent with previous observations (*12*). Although pea seed ferritin (PSF) likewise consists of 28.0 and 26.5 kD subunits, the ratio of 28.0 kDa subunit to the 26.5 kDa species is around 2:1 (*28*). SSF as isolated contains ~1800 g atom of iron.

Iron Release from SSF Induced by Anthocyanins. To elucidate whether there is an interaction between anthocyanins and ferritin, six anthocyanins were allowed to incubate with SSF, respectively, followed by monitoring iron release from SSF by the formation of the Fe^{2+}-ferrozine complex. Among the anthocyanins, Dp exhibited the strongest activity to induce the iron release from SSF; therefore, it was chosen to study the rate of the iron release as a function of dose, and the result is shown in **Figure 3**. With an increase in the concentration of Dp, the rate increases linearly with the concentration of Dp (**Figure 3**, inset), indicating that the iron release is first order with respect to Dp concentration. This result implies that diffusion of anthocyanins into the ferritin shell may be the first step for the iron release from ferritin.

Subsequently, we determined the ability of all anthocyanins to induce the iron release from SSF with ascorbate as a positive

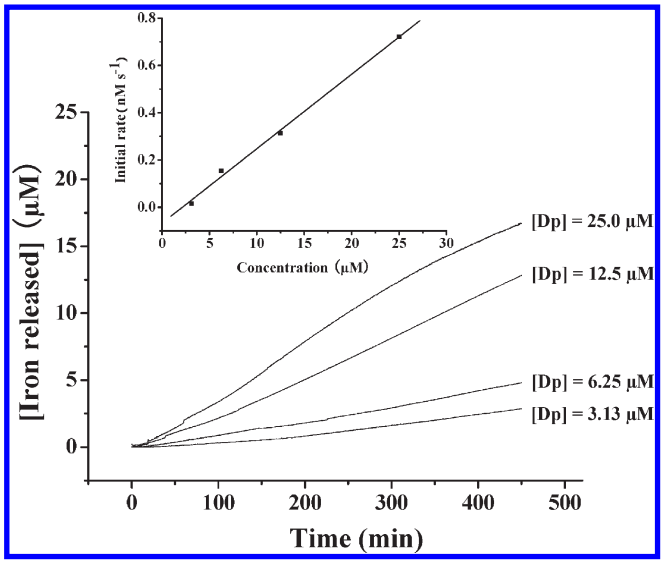


Figure 3. Dependence of iron release from SSF on Dp concentration. The correlation of the initial rate of iron release and Dp concentration is shown in the inset. Iron release from SSF in the presence of Dp was followed by measuring the increase in absorbance at 562 nm over 400 min due to the chelation of Fe^{2+} by ferrozine. The curve represents an average of three experiments. Conditions: 0.15 μM SSF, 3.13−25 μM Dp, 0.05 M Mops, pH 7.0, 0.1 M NaCl, 500 μM ferrozine, 25 °C.

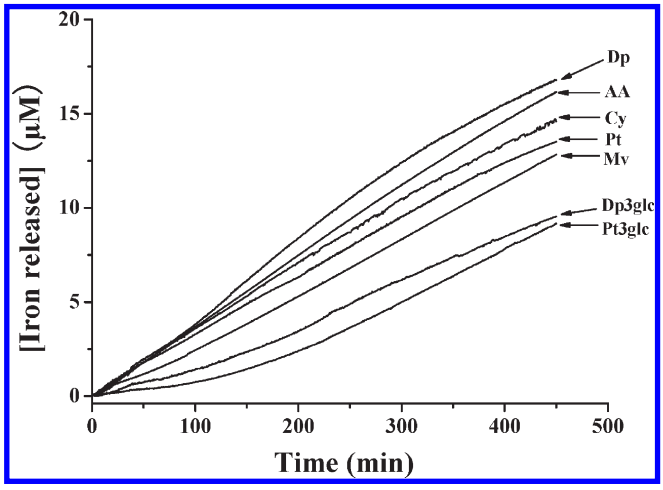


Figure 4. Kinetics of iron release from SSF induced by anthocyanins or ascorbate. Iron release from SSF in the presence of reductants was followed by measuring the increase in absorbance at 562 nm over 400 min due to the chelation of Fe^{2+} by ferrozine. The curve represents an average of three experiments. Conditions: 0.15 μM SSF, 25 μM anthocyanins or ascorbate, 0.05 M Mops, pH 7.0, 0.1 M NaCl, 500 μM ferrozine, 25 °C.

control at concentration of 25 μM, and the results are displayed in **Figure 4**. It was found that all of them were able to cause the iron release from protein shell but to different extents, again indicating that the anthocyanins could interact with SSF at physiological pH. This result is in accordance with a previous report showing that epigallocatechin, gallic acid methyl, sinapic acid, and ferulic acid can induce iron release from horse spleen ferritin (*24*). The rate of the iron release is Dp > Cy > Pt > Mv > Dp3glc > Pt3glc in Mops buffer (pH 7.0), at 25 °C (**Table 1**). Thus, the rate of the iron release seems to be inversely proportional to the size of these anthocyanins. This might be because the anthocyanins with a large size such as Dp3glc and Pt3glc enter into the protein shell with more difficulty as compared to their analogues such as Dp,

Table 1. Initial Rate of Iron Release (nM/s) from SSF Induced by Anthocyanins or Ascorbate[a]

Dp	Cy	Pt	Mv	Dp3glc	Pt3glc	AA
0.757 ± 0.032	0.643 ± 0.037	0.507 ± 0.024	0.369 ± 0.027	0.171 ± 0.015	0.127 ± 0.022	0.642 ± 0.018

[a] Values are the means ± standard deviations (n = 3).

Cy, and Pt. This finding is consistent with the above idea that the diffusion of the pigments into the protein shell represents the first step for the iron release from ferritin. In addition, the above observation raises the possibility that the glucosyl group of the C-ring on Dp3glc and Pt3glc blocks their interaction with SSF, resulting in slower iron release from the protein shell. Although all of them have comparable sizes, Dp and Cy induced markedly faster iron release from ferritin than Pt and Mv (**Figure 4**), suggesting that, except for the size of the reductants, other factors also play an important role on their interaction with SSF (see below).

Relationship between the Chelating Activity of Anthocyanins on Fe^{2+} and the Rate of Iron Release. To find other factors related to the iron release from SSF, the chelating activity of anthocyanins on Fe^{2+} was determined. As shown in **Figure 5A**, the order of the chelating activity of anthocyanins on Fe^{2+} is Dp > Cy > Pt > Mv > Dp3glc > Pt3glc, a result in agreement with a previous proposal that an *o*-dihydroxyphenyl structure was important in the iron-releasing mechanism from ferritin (*14*). Dp and Cy possess an *o*-dihydroxyphenyl moiety but lack a $-OCH_3$ group on the B-ring; these structural features could explain why these two compounds exhibit stronger chelating activity on Fe^{2+} than others. Thus, Dp and Cy easily act as Fe^{2+} chelators to help Fe^{2+} to find its way out of the molecule (*2*).

There is a good relationship between the chelating activity and the rate of the iron release (**Figure 5B**): namely, the stronger the chelating activities, and the faster the iron release. Therefore, as for anthocyanins, the size and the chelating activity of the anthocyanins have important effects on the rate of the iron release. Support for this conclusion comes from a previous discovery that the chelating activity of reductants on iron ion was an important factor responsible for iron release from the ferritin shell (*29*). Although the chelating activity of ascorbate is pronouncedly lower than that of Cy, which is larger than ascorbate, the initial rates of the iron release caused by ascorbate and Cy were nearly the same (**Table 1**), demonstrating that the mechanism of the iron release induced by ascorbate is significantly distinct from that by the anthocyanins. These results suggest that the mechanism of the iron release is complex and reductant-dependent.

Effect of Anthocyanin-Induced Iron Release on SSF. As a result of the interaction between the reductants and SSF, Fe^{3+} was reduced to Fe^{2+} followed by diffusion into a bulk solution. In the presence of oxygen, Fe^{2+} facilitated the production of $HO^{\bullet}$ through the Haber−Weiss reaction (*2*). Previous studies mainly focused on the effects of the iron release from ferritin on lipid peroxidation (*16*) and DNA degradation (*16*, *17*). However, there is no information on its effect on ferritin itself. To determine whether ferritin damage also occurs during the iron release induced by ascorbate and the anthocyanins, SDS-PAGE was run with all samples, and the results are shown in **Figure 6**. SSF was degraded into small peptides (which cannot be detected with SDS-PAGE) upon the interaction of ascorbate (500 μM) with SSF for 48 h, whereas such a degradation was much less or completely inhibited after incubation of all the anthocyanins with ferritin (**Figure 6A**). This result suggests that all the anthocyanins have significantly protective effects on ferritin, while ascorbate does not. The protective effects of the anthocyanins on SSF are similar to each other and cannot be distinguished under the present experimental conditions.

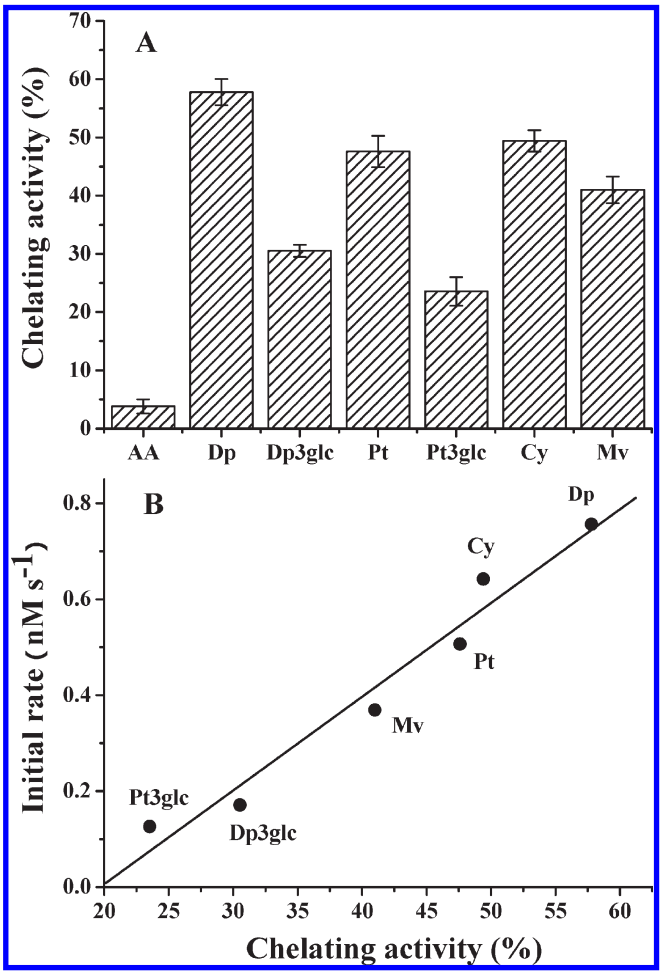


Figure 5. (**A**) Chelating activity of anthocyanins on Fe^{2+} ion. Vertical bars represent the standard error from the means of three separate tests. (**B**) Initial rate of iron release from SSF versus the chelating activity of anthocyanins on Fe^{2+} ion. Conditions: 20 μM $FeSO_4$, 60 μM ferrozine, 60 μM anthocyanins or ascorbate, 0.05 M Mops buffer, pH 7.0, 25 °C.

Subsequently, to better understand the disparity in the protective property between ascorbate and the anthocyanins, Dp was chosen to compare its protective activity against ferritin degradation with that of ascorbate in the concentration range 25−400 μM (**Figure 6B**). Generally, the protein degradation by ascorbate is dose-dependent. At different low concentrations (25−100 μM), the presence of ascorbate is capable of inducing protein degradation to a large extent. When its concentration was increased to 400 μM, SSF was completely degraded into unidentified small peptides. In contrast, such degradation was nearly completely inhibited by Dp at low and high concentrations, again confirming the protective effect of Dp on ferritin.

Determination of $HO^{\bullet}$ Release. To gain insight into the reason for ferritin damage during the iron release, $HO^{\bullet}$ was measured upon treatment of SSF with the anthocyanins and ascorbate for 48 h, respectively. TA can capture $HO^{\bullet}$, yielding fluorescent

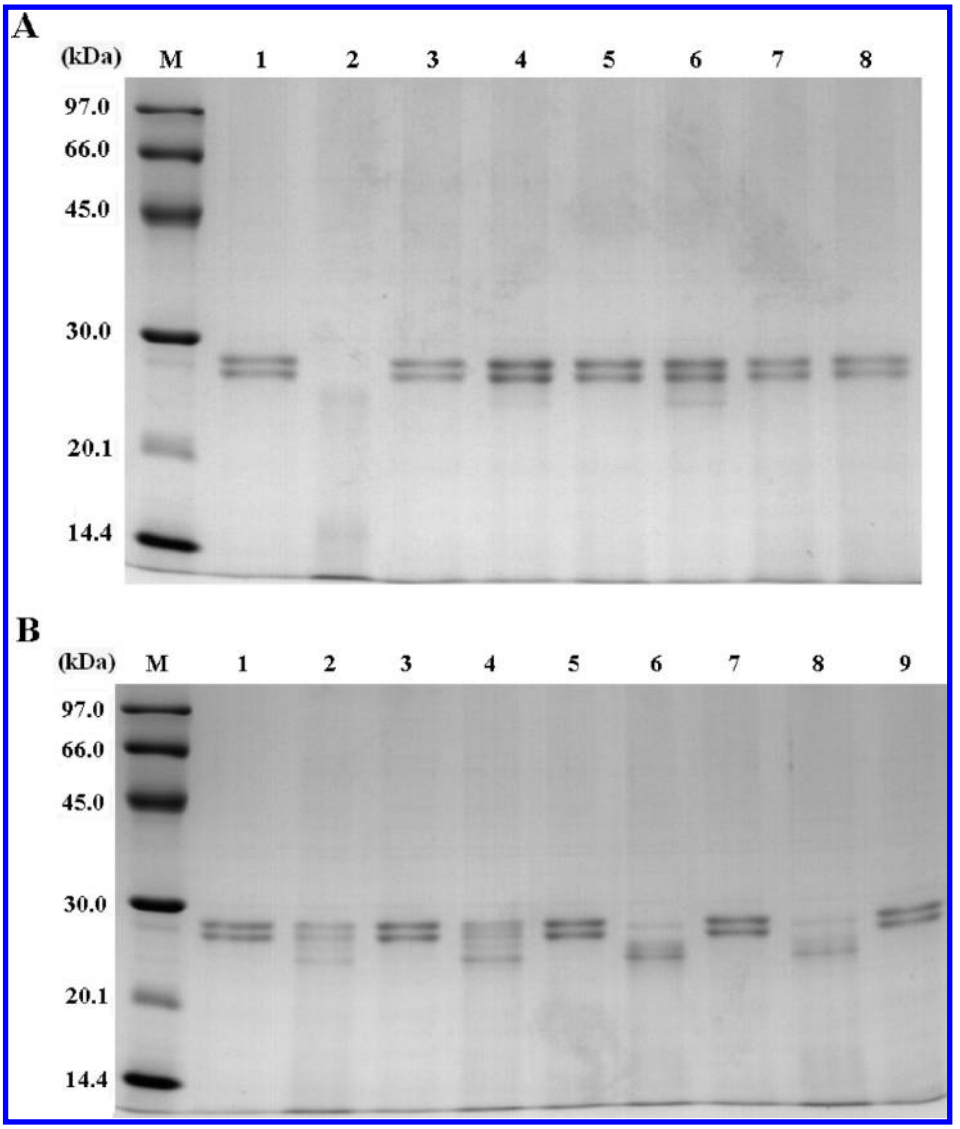


Figure 6. SDS-PAGE analyses of interaction between anthocyanins and SSF for 48 h, with ascorbate as a positive control sample. The reaction system contained 0.4 μM SSF, 25−500 μM anthocyanins or ascorbate, 0.05 M Mops buffer, pH 7.0, 25 °C. (**A**) Results of 500 μM anthocyanins or ascorbate with SSF: (lane M) protein markers and their corresponding molecular masses; (lane 1) SSF; (lane 2) SSF + ascorbate; (lane 3) SSF + Dp; (lane 4) SSF + Dp3glc; (lane 5) SSF + Pt; (lane 6) SSF + Pt3glc; (lane 7) SSF + Cy; (lane 8) SSF + Mv. (**B**) Results of 25−400 μM Dp or ascorbate with SSF: (lane M) protein markers and their corresponding molecular masses; (lane 1) SSF; (lane 2) SSF + 25 μM ascorbate; (lane 3) SSF + 25 μM Dp; (lane 4) SSF + 100 μM ascorbate; (lane 5) SSF + 100 μM Dp; (lane 6) SSF + 200 μM ascorbate; (lane 7) SSF + 200 μM Dp; (lane 8) SSF + 400 μM ascorbate; (lane 9) SSF + 400 μM Dp.

TA-OH, which is a stable reaction product; therefore, this reported fluorescent method (*26*, *27*) was used to determine the concentration of $HO^{\bullet}$ (**Figure 7A**). In general, $HO^{\bullet}$ was generated during all reactions between the tested anthocyanins or ascorbate and SSF. Consistent with this is evidence that $HO^{\bullet}$ is also formed upon incubation of ascorbate with PSF, thereby facilitating the protein degradation (*9*). Although the mechanism by which the radicals are formed cannot be unambiguously elucidated on the basis of the present results, these $HO^{\bullet}$ radicals are most likely produced from the iron-catalyzed Haber−Weiss reaction. Support for this view comes from a recent study showing that $HO^{\bullet}$ is likewise produced during the reduction of Fe^{3+} to Fe^{2+} in ferritin by neurotoxin 6-hydroxydopamine through the Haber−Weiss reaction, resulting in cell damage in vivo, and finally causing the progression of Parkinson's disease (*14*).

It is worth noting that the amount of $HO^{\bullet}$ (less than 600 nM) (**Figure 7A**) produced is much less than the concentration of Fe^{2+} (10−20 μM) released from holoSSF (**Figure 4**), suggesting that the $HO^{\bullet}$ formation is a side reaction. A control system only containing holoferritin (in absence of anthocyanins and ascorbate) did not detect hydroxyl radical generation (data not shown), indicating that the formation of $HO^{\bullet}$ comes from the iron release from ferritin. The concentration of $HO^{\bullet}$ generated from the system consisting of ascorbate and SSF is significantly higher than those of the anthocyanins and SSF. This might be one of the important reasons why ferritin was completely damaged when ascorbate was used a reducing agent. Among all the reactions between the anthocyanins and SSF, Dp induces the production the least amount of $HO^{\bullet}$ followed by Pt, and Cy. These observations are consistent with their chelating activity (**Figure 5A**). The correlation coefficient between the $HO^{\bullet}$ production and their chelating activity is 0.8779 (**Figure 7B**), further confirming this idea.

In addition, it has been established that, when ascorbate is used to reduce ferric ions in the presence of oxygen, it acts as a prooxidant rather than an antioxidant and is converted to an

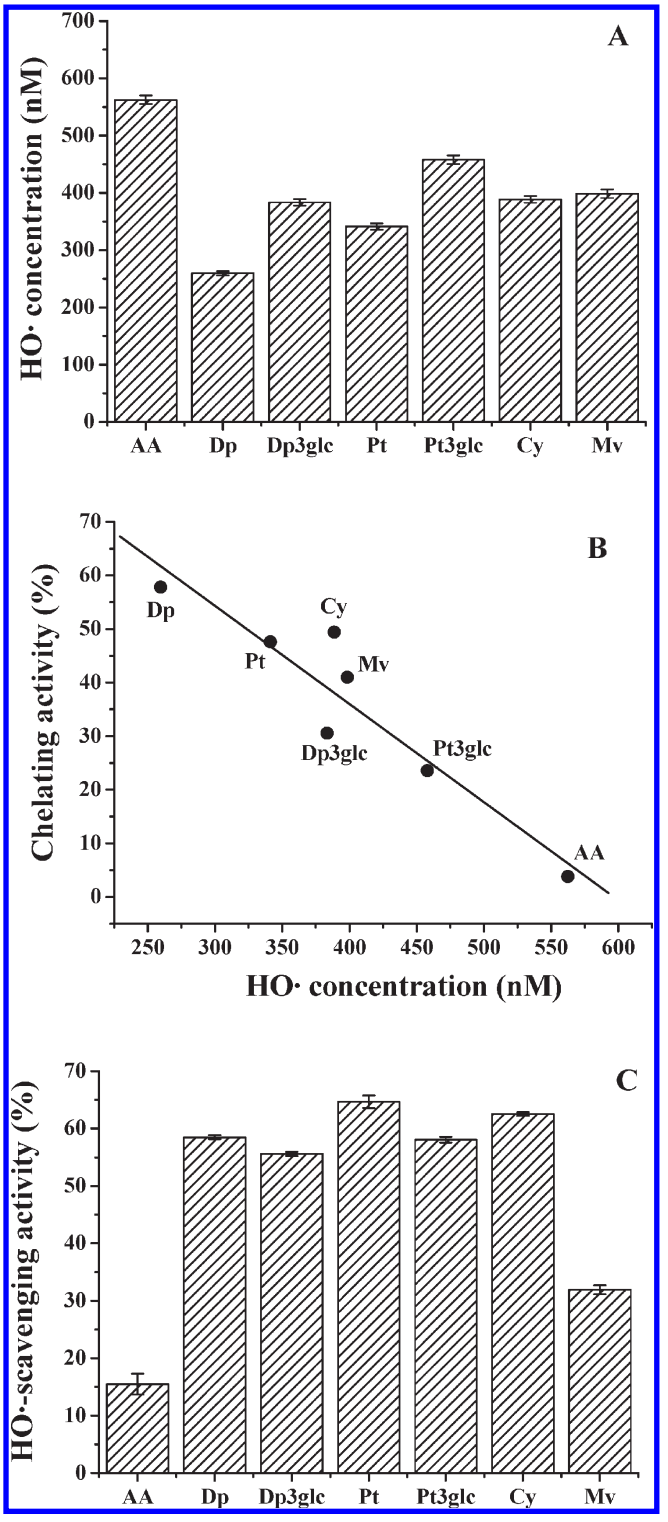


Figure 7. (**A**) Determination of HO• release. Vertical bars represent the standard errors from the means of three separate tests. Conditions: SSF (0.15 μM) was incubated at 25 °C in 0.05 M Mops buffer (pH 7.0) with 500 μM TA, 0.1 M NaCl, and 25 μM anthocyanins or ascorbate for 48 h. (**B**) Correlation of HO• production during the iron release from SSF with the chelating activity of the anthocyanins. (**C**) Assay of HO•-scavenging activity. Vertical bars represent the standard errors from the means of three separate tests. Conditions: the system contained 1 mM $FeSO_4$, 1 mM EDTA, 0.05 M Mops (pH 7.0), 1 mM TA, and 25 μM anthocyanins or ascorbate. The reaction was started by the addition of 1 mM H_2O_2. After incubation at 37 °C for 2 h, the fluorescence intensity of TA-OH was measured at Ex 326 nm and Em 432 nm.

ascorbate radical (*30*). We believe that, in addition to hydroxyl radicals, the ascorbate radical may also contribute to the damage of ferritin during the iron release. This could be a second important reason why the addition of ascorbate induces the complete degradation of phytoferritin, although the amounts of hydroxyl radical generated by the addition of ascorbate were comparable with those of the anthocyanin species (**Figure 7A**).

Among all the reactions between the anthocyanins and SSF, Dp induces the production the least amount of HO•, followed by Pt and Cy. These observations are consistent with their chelating activity (**Figure 5A**). The correlation coefficient between the HO• production and their chelating activity is 0.8779 (**Figure 7B**), further confirming this idea.

Assay of Scavenging HO• Activity. The above results show that Dp and its counterparts have the ability to inhibit the formation of HO•, whereas ascorbate is lacking in this regard. To elucidate the reason for this, the HO•-scavenging activity of the anthocyanins and ascorbate was determined (**Figure 7C**). Of all tested compounds, Pt exhibits the strongest ability to scavenge HO• (the rate of HO• scavenging is 64.6%), followed by Cy and Dp. Consistent with this is evidence showing that anthocyanins are capable of capturing free radicals and have strong antioxidative activity (*20*,*24*). In contrast, the HO•-scavenging rate of ascorbate is the lowest (15.5%). These results can explain why larger protein degradation occurs during the iron release induced by ascorbate as compared to the anthocyanins.

Dp exhibits a lower HO•-scavenging activity than Cy and Pt, but it has much stronger protein protective property against damage induced by HO• than other anthocyanins, indicating that the HO•-scavenging activity may be not an important factor affecting their protective activity. In contrast, the chelating activities of these compounds are consistent with their protective activities, a result indicating that the chelating activity is more important than the free-radical-scavenging capability for the protection of ferritin from oxidative damage. Support for this conclusion came from previous observations that the presence of either *o*-phenanthroline or desferrioxamine B completely inhibited the ascorbate-induced PSF degradation due to the ability to interrupt the Fenton cycle by complexing the iron (*9*). In addition, it is likely that some anthocyanins such as Dp and Cy can chelate pro-oxidant Fe^{2+}, thus preventing free radical formation from Fe^{2+} while simultaneously retaining their own HO•-scavenging capability. The observed ability of the compounds to inhibit protein from degradation during the iron release from SSF is actually a combination of their Fe^{2+}-chelating and HO•-scavenging activities.

In conclusion, the present study demonstrates that anthocyanins, a class of naturally occurring pigments, can induce iron release from SSF for the first time. Generally, Dp, Cy, and Pt without the glucosyl group of C-ring exhibit a comparable rate of iron release with ascorbate, whereas the rate of iron release induced by both Pt3glc and Dp3glc is markedly lower than that by ascorbate. Thus, the existence of the glucosyl group of the C-ring significantly prevents the interaction between anthocyanins and ferritin. Interestingly, these anthocyanins completely inhibit ferritin from degradation by HO• during the iron release. In contrast, ferritin was degraded into small peptides during the ascorbate-induced iron release from SSF under the same experimental conditions. This might be due to the stronger ability of the anthocyanins to chelate pro-oxidant Fe^{2+} as compared to ascorbate.

ABBREVIATIONS

AA, ascorbate; Cy, cyanidin; Cy3glc, cyanidin-3-*O*-glucoside; Dp, delphinidin; Dp3glc, delphinidin-3-*O*-glucoside; EP,

extension peptide; HO•, hydroxyl radical; Mops, 3-(*N*-morpholino)propanesulfonic acid; Mv, malvidin; Pt, petunidin; Pt3glc, petunidin-3-*O*-glucoside; PSF, pea seed ferritin; SDS, sodium dodecyl sulfate; SSF, soybean seed ferritin; TA, terephthalic acid; TA-OH, hydroxylated terephthalate; TP, transit peptide.

ACKNOWLEDGMENT

This project was supported by the China High-Tech (863) project (No. 2007AA10Z311), the Key Laboratory Open Fund of Soybean Biology of Education Ministry, and the National Natural Science Foundation of China (No. 30972045).

LITERATURE CITED

(1) Theil, E. C. Iron, ferritin, and nutrition. *Annu. Rev. Nutr.* **2004**, *24*, 327–343.
(2) Harrison, P. M.; Arosio, P. The ferritins: molecular properties, iron storage function and cellular regulation. *Biochim. Biophys. Acta* **1996**, *1275*, 161–203.
(3) Proudhon, D.; Wei, J.; Briat, J. F.; Theil, E. C. Ferritin gene organization: differences between plants and animals. *J. Mol. Evol.* **1996**, *42*, 325–336.
(4) Waldo, G. S.; Wright, E.; Whang, Z. H.; Briat, J. F.; Theil, E. C.; Sayers, D. E. Formation of the ferritin iron mineral occurs in plastids. *Plant Physiol.* **1995**, *109*, 797–802.
(5) Hentze, M. W.; Kühn, L. C. Molecular control of vertebrate iron metabolism: mRNA-based regulatory circuits operated by iron, nitric oxide, and oxidative stress. *Proc. Natl. Acad. Sci.* **1996**, *93*, 8175–8182.
(6) Lescure, A. M.; Proudhon, D.; Pesey, H.; Ragland, M.; Theil, E. C.; Briat, J. F. Ferritin gene transcription is regulated by iron in soybean cell cultures. *Proc. Natl. Acad. Sci.* **1991**, *88*, 8222–8226.
(7) Boyd, D.; Vecoli, C.; Belcher, D. M.; Jain, S. K.; Drysdale, J. W. Structural and functional relationships of human ferritin H and L chains deduced from cDNA clones. *J. Biol. Chem.* **1985**, *260*, 11755–11761.
(8) Zhao, G.; Bou-Abdallah, F.; Arosio, P.; Levi, S.; Janus-Chandler, C.; Chasteen, N. D. Multiple pathways for mineral core formation in mammalian apoferritin. The role of hydrogen peroxide. *Biochemistry* **2003**, *42*, 3142–3150.
(9) Laulhere, J. P.; Laboure, A. M.; Briat, J. F. Mechanism of the transition from plant ferritin to phytosiderin. *J. Biol. Chem.* **1989**, *264*, 3629–3635.
(10) Masuda, T.; Goto, F.; Yoshihara, T. A novel plant ferritin subunit from soybean that is related to a mechanism in iron release. *J. Biol. Chem.* **2001**, *276*, 19575–19579.
(11) Castruita, M.; Elmegreen, L. A.; Shaked, Y.; Stiefel, E. I.; Morel, F. M. M. Comparison of the kinetics of iron release from a marine (*Trichodesmium erythraeum*) Dps protein and mammalian ferritin in the presence and absence of ligands. *J. Inorg. Biochem.* **2007**, *101*, 1686–1691.
(12) Baader, S. L.; Bill, E.; Trautwein, A. X.; Bruchelt, G.; Matzanke, B. F. Mobilization of iron from cellular ferritin by ascorbic acid in neuroblastoma SK-N-SH cells: an EPR study. *FEBS Lett.* **1996**, *381*, 131–134.
(13) Laulhère, J. P.; Briat, J. F. Iron release and uptake by plant ferritin as affected by pH, reduction and chelation. *Biochem. J.* **1993**, *290*, 693–699.
(14) Double, K. L.; Maywald, M.; Schmittel, M.; Riederer, P.; Gerlach, M. *In vitro* studies of ferritin iron release and neurotoxicity. *J. Neurochem.* **1998**, *70*, 2492–2499.
(15) Topham, R. W.; Walker, M. C.; Calisch, M. P. Liver xanthine dehydrogenase and iron mobilization. *Biochem. Biophys. Res. Commun.* **1982**, *109*, 1240–1246.
(16) Oteiza, P. I.; Kleinman, C. G.; Demasi, M.; Bechara, E. J. H. 5-Aminolevulinica acid induces iron release from ferritin. *Arch. Biochem. Biophys.* **1995**, *316*, 607–611.
(17) Ahamad, S.; Singh, V.; Rao, G. S. Release of iron from ferritin by 1,2,4-benzenetriol. *Chem. Biol. Interact.* **1995**, *96*, 103–111.
(18) Agrawal, R.; Sharma, P. K.; Rao, G. S. Release of iron from ferritin by metabolites of benzene and superoxide radical generating agents. *Toxicology* **2001**, *168*, 223–230.
(19) Crichton, R. R.; Roman, F.; Roland, F. Iron mobilization from ferritin by chelating agents. *J. Inorg. Biochem.* **1980**, *13*, 305–316.
(20) Wrolstad, R. E. Anthocyanin's pigments−bioactivity and coloring properties. *J. Food Sci.* **2004**, *69*, C419–C421.
(21) Choung, M. G.; Baek, I. Y.; Kang, S. T.; Han, W. Y.; Shin, D. C.; Moon, H. P.; Kang, K. H. Isolation and determination of anthocyanins in seed coats of black soybean (*Glycine max* (L.) Merr). *J. Agric. Food Chem.* **2001**, *49*, 5848–5851.
(22) Wang, H.; Cao, G.; Prior, R. L. The oxygen radical absorbing capacity and anthocyanins. *J. Agric. Food Chem.* **1997**, *45*, 304–309.
(23) Laulhere, J. P.; Lescure, A. M.; Briat, J. F. Purification and characterization of ferritins from maize, pea and soybean seeds. *J. Biol. Chem.* **1988**, *263*, 10289–10294.
(24) Hynes, M. J.; Coinceanainn, M. Ó. Investigation of the release of iron from ferritin by naturally occurring antioxidants. *J. Inorg. Biochem.* **2002**, *90*, 18–21.
(25) Yen, G. C.; Wu, J. Y. Antioxidant and radical scavenging properties of extracts from Ganoderma tsugae. *Food Chem.* **1999**, *65*, 375–379.
(26) Freinbichler, W.; Colivicchi, M. A.; Fattori, M.; Ballini, K.; Linert, W.; Corte, L. D. Validation of a robust and sensitive method for detecting hydroxyl radical formation together with evoked neurotransmitter release in brain microdialysis. *J. Neurochem.* **2008**, *105*, 738–749.
(27) Banerjee, A.; Dasgupta, N.; De, B. *In vitro* study of antioxidant activity of Syzygium cumini fruit. *Food Chem.* **2005**, *90*, 727–733.
(28) Li, C.; Hu, X.; Zhao, G. Two different H-type subunits from pea seed (*Pisum sativum*) ferritin that are responsible for fast Fe(II) oxidation. *Biochimie* **2008**, *91*, 230–239.
(29) Boyer, R. F.; McCleary, C. J. Superoxide ion as a primary reductant in ascorbate mediated ferritin iron release. *Free Radical Biol. Med.* **1987**, *3*, 389–395.
(30) Theil, E. C.; Briat, J. F. *Plant Ferritin and Non-heme Iron Nutrition in Humans*; Harvestplus: Washington, DC, 2004; Technical Monograph 1.

Received for review August 30, 2009. Revised manuscript received November 4, 2009. Accepted November 09, 2009.