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Effect of the structure of gallic acid and its derivatives on their interaction with plant ferritin

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ABSTRACT

Gallic acid and its derivatives co-exist with protein components in foodstuffs, but there is few report on their interaction with proteins. On the other hand, plant ferritin represents not only a novel class of iron supplement, but also a new nanocarrier for encapsulation of bioactive nutrients. However, plant ferritin is easy to be degraded by pepsin in the stomach, thereby limiting its application. Herein, we investigated the interaction of gallic acid and its derivatives with recombinant soybean seed H-2 ferritin (rH-2). We found that these phenolic acids interacted with rH-2 in a structure-dependent manner; namely, gallic acid (GA), methyl gallate (MEGA) and propyl gallate (PG) having three HO groups can bind to rH-2, while their analogues with two HO groups cannot. Consequently, such binding largely inhibited ferritin degradation by pepsin. These findings advance our understanding of the relationship between the structure and function of phenolic acids.

1. Introduction

Phenolic acids as common food ingredients mainly exist in grains such as sorghum, wheat, rice, corn, and fruit such as grape and apples. There are two major phenolic acid compounds: hydroxy benzoic acid class (protocatechuic acid, vanillic acid, gallic acid, methyl gallate and propyl gallate) and hydroxy cinnamic acid class (cinnamic acid, caffeic acid, ferulic acid, chlorogenic acid). Among them, gallic acid and its derivatives are often used as antioxidant additives in both food and pharmaceutical industry, and they have similar structures and are rich in hydroxyl groups (Fig. 1A).

There is a considerable interest in phenolic acids because they have strong antioxidant properties, the obvious inhibition of cholesterol, and anti-cancer, anti-aging and bacteriostatic effects. Therefore, it has been proposed that absorbing a certain amount of phenolic acid can effectively prevent and curb the occurrence of diseases (Fiuza et al., 2004; Locatelli et al., 2008; Lu, Nie, Belton, Tang, & Zhao, 2006; Rice-Evans, Miller, & Paganga, 1996). Recently, studies have showed that plant polyphenols can interact with proteins to change the protein conformation, such as BSA (Soares, Mateus, & De Freitas, 2007). Moreover, it has been found that ferritin association induced by EGCG inhibits protein degradation by proteases (Wang, Zhou, Qi, & Zhao, 2014). However, there has been no report on the interaction of phenolic acid with ferritin, especially effect of their structure on such interaction.

Ferritin is a kind of iron storage protein and widely distributed in animal, plant, and bacteria. Ferritin is a hollow sphere consisting of 24 subunits in the 4-3-2 point symmetry (Harrison & Arosio, 1996; Ponka, Beaumont, & Richardson, 1998) (Fig. 1B). One ferritin molecule can accumulate up to 4500 iron atom within its inner

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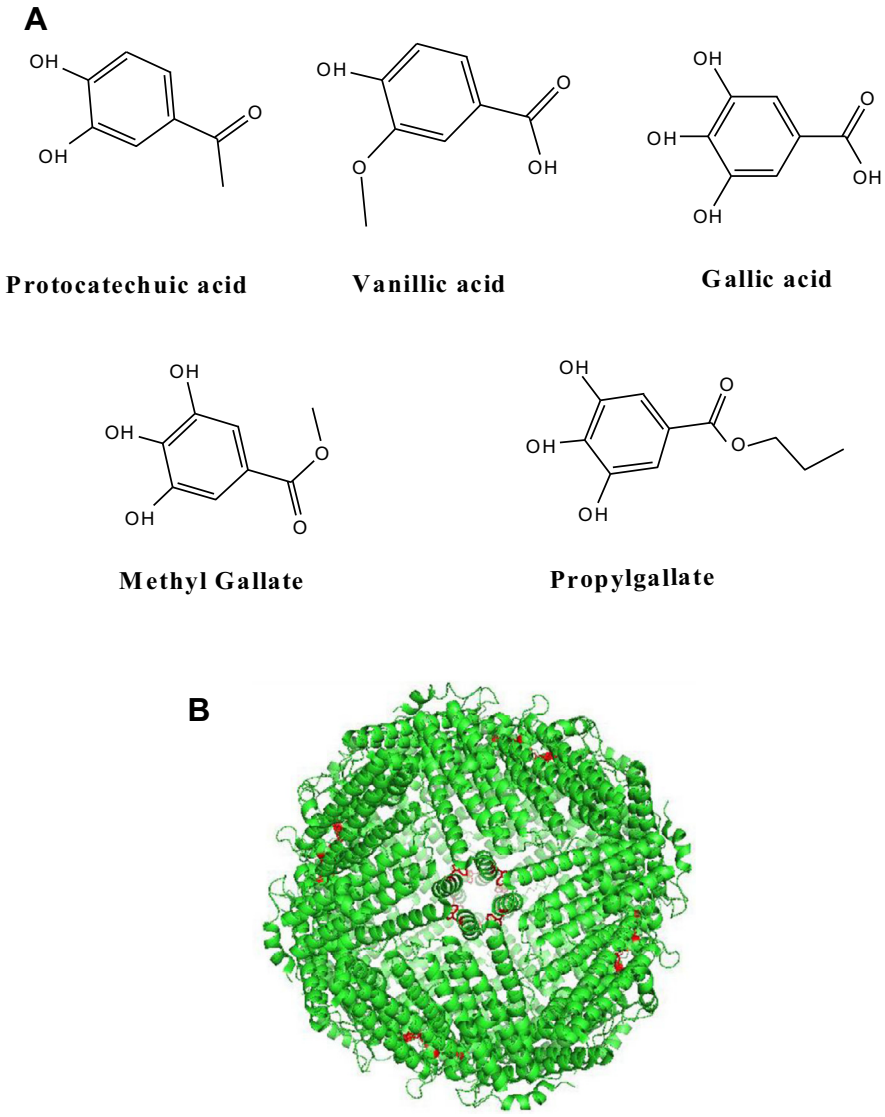

Fig. 1. (A) Chemical structure of four phenolic acids (PCA, VA, GA, MEGA and PG). (B) Crystal structure of ferritin with views down the 4-fold axes (channels) of the protein shell. Trp residues in ferritin are highlighted in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

cavity, so ferritin, especially plant ferritin from legume seeds such as soybean seed, is considered as an alternative source for iron supplementation. Compared to other iron supplements, plant ferritin has two major advantages including the protection of protein shell from interaction with other dietary factors, and the higher safety form of iron stored as ferric cores than $FeSO_4$. On the other hand, upon removal of iron by reported methods, holoferritin becomes into apoferritin. Recently, apoferritin has been used to encapsulate lipophilic bioactive molecules such as β-carotene and curcumin in order to improve their water-solubility and thermal stability (Chen, Bai, Yang, Yang et al., 2014; Chen, Bai, Yang, Zang et al., 2014). However, ferritin is not stable enough against degradation by proteases existing in the gastrointestinal tract. Therefore, it is important to find edible compounds which have the ability to prevent phytoferritin from degradation by pepsin through their interaction with protein.

In the present study, we systematically investigated the interaction between gallic acid and its derivatives with phytoferritin for the first time, and found that the structure of these compounds has a pronounced effect on such interaction. Consequently, the strong interaction between gallic acid, methyl gallate or propyl gallate and plant ferritin can inhibit protein degradation by proteases *in vitro* to a great extent. In contrast, their analogues such as vanillic acid (VA) and protocatechuic acid (PCA) hardly interacted with protein, thereby resulting in no inhibitory activity.

2. Materials and methods

2.1. Chemicals

Gallic acid (GA), vanillic acid (VA), methyl gallate (MEGA), protocatechuic acid (PCA) and propyl gallate (PG) with a purity of 98% were obtained from J&K Chemical (Beijing, China). Pepsin, and trypsin were purchased from J&K Chemical (Beijing, China). Hemoglobin was purchased from Solarbio (Beijing, China). All other reagents used were of analytical grade or purer.

2.2. Preparation of recombinant soybean seed H-2 ferritin

Recombinant soybean seed H-2 ferritin (rH-2) was prepared as previously described (Deng et al., 2010; Masuda et al., 2007).

Recombinant vector was cultivated in LB medium with AMP, and protein expression was induced with IPTG. Finally, the protein solution was applied to an ion-exchange and a gel filtration column. The purity of rH-2 were analyzed by native and SDS PAGE. Determination of the apparent molecular weight of the rH-2 was achieved with native PAGE by using a 4–20% graded polyacrylamide gel run at 2 mA for 15 h at 4 °C. The buffer system of the gel was Tris–HCl (0.025 M, pH 7.8). Gels were stained with coomassie blue R-250. Electrophoresis of proteins under denaturing conditions was carried out in 15% SDS–polyacrylamide gel (Laemmli, 1970). Tested protein solution was mixed with equal volume 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 5 min, the supernatant was isolated upon centrifugation at 10,000×*g* for 10 min. Protein concentration was determined by Bradford Protein Assay Kit.

2.3. Fluorescence titration

Fluorescence titration experiments were performed using the Cary Eclipse spectrophotometer (Cary, Varian in USA). The concentration of rH-2 is 0.8 μM in 50 mM Tris-HCl, pH 7.5, at 25 °C. The titrations are conducted by adding 2 μL of GA, VA, PCA, and MEGA, respectively, to 1 mL rH-2 according to the reported method (Deng et al., 2010). Fluorescence spectra were scanned from 290 to 450 nm after each addition. The excitation wavelength is set at 280 mm.

2.4. Circular dichroism (CD) spectra

As previous described (Deng et al., 2010), protein samples were dissolved in 10 mM MOPS buffer, pH 7.5. CD spectra were recorded with a PiStar-180 spectrometer, using quartz cuvettes of 1 mm optical path length at 25 °C. CD spectra were scanned at the far UV range (190–260 nm) with 5 replicates at 50 nm/min, band width as 1 nm. The CD data were expressed in terms of mean residual ellipticity (θ), in deg cm^2 $dmol^{-1}$. The change in protein ellipticity induced by treatment with galic acid or its derivatives was defined as the ellipticity of the mixture minus that of galic acid or its derivatives alone under the same experimental conditions. Percentage of secondary structure was calculated using the web based program K2D2.

2.5. Transmission electron microscopy experiments

Transmission electron microscopy (TEM) experiments were performed at 80 kV with a Hitachi S-5500 scanning electron microscope according to earlier reports (Li, Jia, Yang, Deng, & Zhao, 2012). Liquid samples were diluted with 50 mM Tris-HCl prior to being placed on carbon-coated copper grids, and excess solution was removed with filter paper. Resulting samples were stained using 2% uranyl acetate for 2 min.

2.6. SGF digestion stability experiments

Simulated gastric fluid (SGF) digestion stability experiments was carried out as the United States pharmacopoeia (USP). All concentrations stated were final concentrations after mixing the two reagents. Briefly, the assay system (300 μL of total volume) contained 100 μL of protein and small molecule with a mass ratio of 1:10–10:1, 100 μL pepsin, and 100 μL trypsin. Reactions were carried out at 25 °C initially and set in a simulated gastrointestinal condition for 30 min. The pepsin activity was measured by UV spectrophotometry with the substrate of hemoglobin (Douglas & Stark, 2000).

2.7. Statistical analysis

All data analyses were performed using Origin 8.0 software and the structural formula was processed by ChemDraw 7.0. All experiments were carried out in triplicate.

3. Results and discussion

3.1. Purification and characterization of recombinant soybean seed H-2 ferritin (rH-2)

rH-2 was purified using a combination of IEC on a DEAE-Sepharose Fast Flow column, and GFC on a Sephacryl S-300 column. Native PAGE revealed that the rH-2 were single complex with an apparent MW of about 560 kDa (Fig. 2A) as previously described (Zhao, 2010). As expected, rH-2 contains only one subunit with the molecular weight of 28 kDa as suggested by SDS-PAGE (Fig. 2B). To confirm these results, TEM was used to visualize the morphology of ferritin, and revealed that this protein dispersed homogeneously with an outside diameter of ~12 nm (Fig. 2C), indicative of a successful preparation of ferritin.

3.2. Interaction between ferritin and gallic acid or its derivatives

Upon treatment with gallic acid or its derivatives, the electrophoretic behavior of rH-2 was not altered on SDS-PAGE (data not shown), indicating of no change in the primary structure of ferritin. Subsequently, the secondary structure of native apoferritin was studied with far UV-CD spectroscopy. Changes in far UV-CD correspond to alteration in the overall secondary structure of the protein (Zamorano et al., 2004). Apo rH-2 had two negative ellipticities in the far-UV spectrum at 208 and 222 nm (Fig. 1 in Supporting Information), being in good agreement with previous results showing that the secondary structure of soybean seed ferritin is rich in α-helix (Deng et al., 2011). Curve-fitting of apo rH-2 with program K2D2 produced a content of 58% α-helix, 15% β-sheet, and 27% random coil. Upon the addition of GA to apo ferritin, the CD spectrum of the protein was almost unchanged (Fig. 3), a result indicating that GA hardly caused a change in the secondary structure of the protein. Similarly, addition of gallic acid derivatives such as VA, PCA, MEGA and PG had little effect on protein secondary structure either (Fig. 1 in Supporting Information). These results indicated that the secondary structure of rH-2 was stable. Consistent with this idea, our recent observations have demonstrated that the secondary structure of naturally occurring soybean seed ferritin is also unchanged after treatment with either proanthocyanidins or high hydrostatic pressure (Deng et al., 2011; Zhang et al., 2012).

Proteins intrinsic fluorescence mainly contributed from Trp residues has been widely used for their interaction with small molecules because the fluorescence emission from Trp is sensitive to the microenvironment surrounding the fluorophore residue

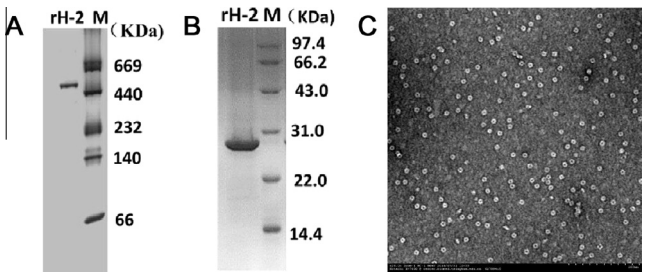

Fig. 2. (A) Native PAGE and (B) SDS-PAGE analyses of rH-2 ferritin. (C) TEM of purified rH-2 protein. Conditions: 0.08 μM apo-rH-2 ferritin in 50 mM Tris-HCl, pH 7.5, 25 °C.

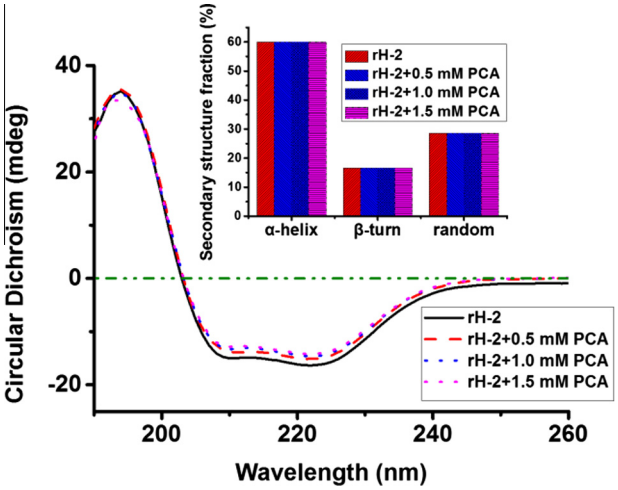

Fig. 3. CD spectra of rH-2 in the presence and absence of GA. Inset: Estimated secondary structure fractions of rH-2. Conditions: 0.3 μM rH-2 in 5 mM MOPS (pH 7.5) buffer.

(Lehrer & Kerwar, 1972; Singh & Krikorian, 1982; Zamorano et al., 2004). There are 24 tryptophan residues in the rH-2 oligomer, which were highlighted in red in Fig. 1B (Masuda, Goto, Yoshihara, & Mikami, 2010). Therefore, the changes in the tertiary structure of rH-2 could be determined by intrinsic emission spectroscopy. Since all phenolic acid compounds used in this study exhibited different fluorescence intensities in the region of 320–350 nm, their fluorescence must be deducted from each of apoferritin plus each of phenolic acids accordingly in order to observe the change in the fluorescence of protein itself. Firstly, the interaction between PCA and apo rH-2 was studied, and results were shown in Fig. 4A. In the absence of PCA, apo rH-2 exhibited a strong fluorescence emission peak at ~330 nm when excited at 280 nm, indicating that most of the observed fluorescence was contributed from the Trp residue (Deng et al., 2011; Zhang et al., 2012). Addition of PCA almost had little effect on the protein fluorescence over the concentration range of 0–120 μM, suggesting that there is no interaction between PCA and ferritin. Similarly, VA also hardly quenched the protein fluorescence under the present experimental conditions (Fig. 4B), indicative of no interaction between VA and ferritin.

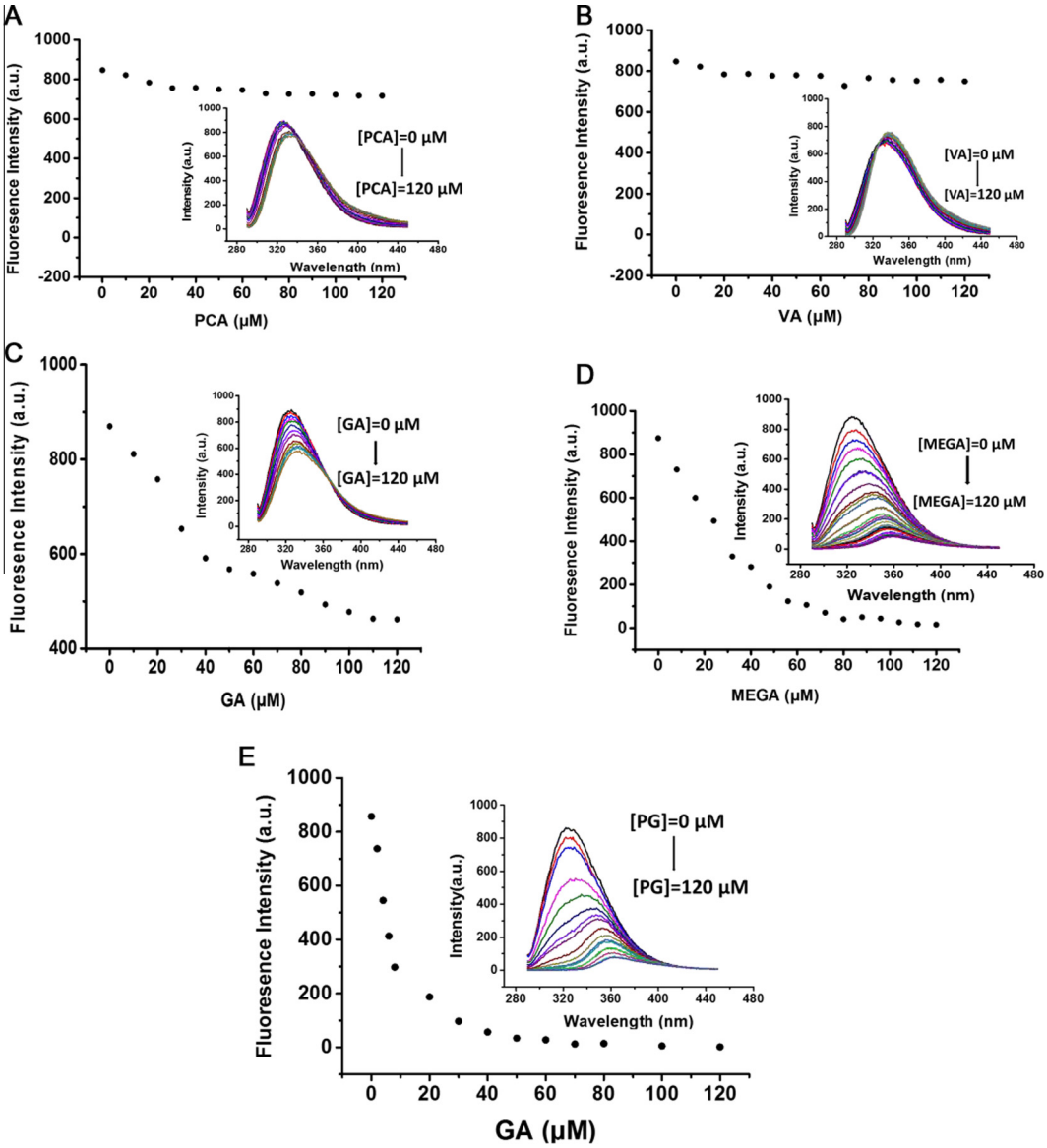

Fig. 4. Fluorescence spectra of apo rH-2 and apo rH-2 treated with (A) PCA, (B) VA, (C) GA, (D) MEGA and PG at different concentrations, respectively. Conditions: 0.8 μM rH-2 in 50 mM Tris-HCl, [phenolic acids] = 0–120 μM, pH 7.5, 25 °C. λ_{Ex} = 280 nm, slits for excitation and emission are 5 nm and 10 nm.

In contrast, with increasing GA concentration, the fluorescence intensity of apoferritin decreased sharply, accompanied by a slightly red shift of maximum emission wavelength (Fig. 4C, Inset), indicating that the microenvironment around Trp was changed on account of the addition of GA. When the concentration of GA reached 120 μM, approximately 50% of the protein fluorescence was quenched. This observation suggested that there is a strong interaction between GA and ferritin, consequently altering the tertiary structure of apoferritin around Trp residues. Compared to the structure of VA, GA has one more hydroxyl group located at *ortho*-position of carboxyl group (Fig. 1A). Thus, an increase in one hydroxyl group resulted in large difference in protein fluorescence quenching between PCA and GA, demonstrating that three adjacent hydroxyl groups on benzene ring are necessary for the interaction between this type of phenolic acids (hydroxy benzoic acid class) and ferritin. Agreeing with this conclusion, both MEGA and PG, which also have three hydroxyl groups at the same position of benzene ring, quenched ferritin fluorescence but to a much greater extent than GA as depicted in Fig. 4D and F. For example, about 95% of protein fluorescence was quenched by MEGA or PG at 120 μM. Therefore, the generation of acyl seems to facilitate the interaction between GA derivatives and protein. We believe that GA, MEGA and PG molecules can interact with apo ferritin through hydrogen bonds, at least partly, between their hydroxyl groups and the amide carbonyl of the peptide backbone or side chain of protein polar amino acid resides. Support for this view comes from recent studies showing that tannic acid which contains 10 GA units in its structure can easily form strong hydrogen bonds with the amide carbonyl of the peptide backbone (Li et al., 2012). In addition, electrostatic interactions between carboxyl group of GA and protein Lys or Arg resides might be also involved in the interaction of GA and apo rH-2. Except for pH 7.5, the above experiments were also carried out at pH 3.0, and similar results were obtained (Fig. 2 in Supporting Information); namely GA, MEGA and PG can quench protein fluorescence while their analogues PCA and VA cannot, again supporting for the conclusion that this type of phenolic acids with three HO groups are prerequisite for their interaction with ferritin.

GA strongly quenched ferritin fluorescence, suggesting that this small organic molecule could bind to apo rH-2. To confirm this idea, dialysis experiments were carried out wherein apo rH-2 (0.8 μM) was firstly incubated with GA (96 μM), followed by dialysis against the same 50 mM Tris-HCl buffer four times to remove free GA. It was found that after dialysis for 16 h, the fluorescence spectrum of apo rH-2 was pronouncedly different from that of untreated protein sample; namely, the maximal fluorescence emission intensity of apo rH-2 decreased by ~30% (Fig. 5A). Moreover, even dialysis time was prolonged to 50 h, resulting protein solution exhibited a nearly the same fluorescence spectrum as protein which was dialyzed against buffer for 16 h. These results indicated that GA binds to apo ferritin tightly, and thus dialysis cannot remove bound GA from apo rH-2.

By using identical dialysis method, the interaction of MEGA/PG and apo rH-2 was studied, and results were in Fig. 5B and C. Upon treatment with MEGA/PG followed by dialysis for 16 h, the fluorescence spectrum of apo rH-2 was also markedly distinct from that of untreated protein sample, and the maximal fluorescence emission intensity of apo rH-2 decreased by ~40%, indicating that MEGA and PG also can bind to apoferritin. Interestingly, it was observed that compared to an initial mixture of protein and MEGA/PG where only ~5% of the total protein fluorescence was left, the fluorescence of this mixture was recovered to ~60% upon dialysis for 16 h, and prolong dialysis time had no effect on further fluorescence recovery. In contrast, the fluorescence of protein and GA mixture at the beginning just slightly recovered after dialysis against buffer for 16 h (Fig. 5A). These results demonstrated that GA molecules bind to apoferritin more tightly than its derivative MEGA and PG. Thus, it appears that the carboxyl group also play an important role in the binding of GA to apo ferritin, while the acylation of carboxyl could prevent this class of phenolic acids from their binding to ferritin, agreeing with the above view that electrostatic interactions which might be partly responsible for the binding of GA to protein. These results demonstrated that the ferritin-binding activity of these phenolic acid compounds is structure-dependent; namely, three adjacent hydroxyl groups are prerequisite for the ferritin binding among the phenolic acids (hydroxy benzoic acid class).

3.3. Effect of GA and its derivatives on the digestive stability of ferritin

The above results demonstrated that the GA, MEGA and PG can bind to ferritin while their analogues PCA and VA cannot. This raises an interesting question of whether such binding can protect ferritin from degradation by proteases. Our previous studies showed that ferritin is unstable in simulated gastric fluid (SGF) while it is relatively stable in simulated intestinal fluid (SIF) (Deng et al., 2011; Li et al., 2012). To answer this question, the digestive stability of rH-2 ferritin in SGF upon treatment with these four compounds, respectively, was evaluated. Studies define the normal pH of stomach contents of adults and children respectively are 2.0 and 4.0, but the stomach pH would increase to a certain degree after eating, and thus the stomach pH of adults and infants may become 3.0 and 5.0 (Agunod, Yamaguchi, Lopez, Luhby, & Glass, 1969; Kalantzi et al., 2006; Simonian, VO, Doma, Fisher, & Parkman, 2005). So ferritin was mixed with these four compounds, respectively, with different mass ratios of protein to phenolic acid (1:10, 1:6, 1:3, 3:1, 6:1, 10:1) at pH 3.0, and then the mixtures were digested for 30 min in SGF. At pH 3.0, it was observed that rH-2 ferritin molecules were degraded completely by pepsin as shown in lane named "E + rH-2". Similarly, the presence of PCA did not inhibit protein degradation under the present experimental conditions except for at the protein/PCA ratio of 1–10 (Fig. 6A), suggesting that PCA had a much weak protective effect on ferritin degradation by pepsin. Nearly the same results were obtained with VA which also cannot prevent ferritin degradation by pepsin at pH 3.0 (Fig. 6B). In contrast, GA greatly inhibited ferritin from degradation in simulated SGF at a protein/GA mass ratio of 3 to 1 when it was incubated with apo ferritin at pH 3.0 (Fig. 6C). As this ratio was changed from 3/1 to 1/10, such inhibitory effect increased, reaching the maximum at the mass ratio of 1–10. Thus, GA inhibited ferritin degradation in a dose-dependent manner. Just like GA, MEGA also exhibited a strong protective effect on ferritin against degradation by pepsin. However, the inhibitory activity of MEGA seems to be stronger than that of GA based on the observation that MEGA exhibited a marked inhibitory activity at the protein/MEGA mass ratio of 6–1 while GA lacked under this condition. As expected, PG exhibited virtually the same inhibitory activity as its analogue, MEGA.

All of these results demonstrated that these five compounds have different inhibitory activities, being in good agreement with their binding activities to ferritin. Thus, it is reasonable believe that the ferritin protective activity of GA, MEGA and PG against degradation by pepsin is derived from their ability to bind to ferritin. Support for this idea came from the fact that PCA and VA had a much weak binding activity to ferritin, resulting in a loss of the inhibitory activity (Fig. 6A and B). Although all of the GA, MEGA and PG can bind to ferritin, they did not induce protein association because TEM analyses showed that ferritin in the presence of GA or MEGA (Fig. 3 in Supporting Information) dispersed as homogeneously as ferritin alone (Fig. 2C). Thus, the inhibitory mechanism of ferritin degradation by GA, MEGA or PG is different from that by tannic acid which induced ferritin aggregation to a great extent. The difference in the inhibitory mechanism of protein degradation

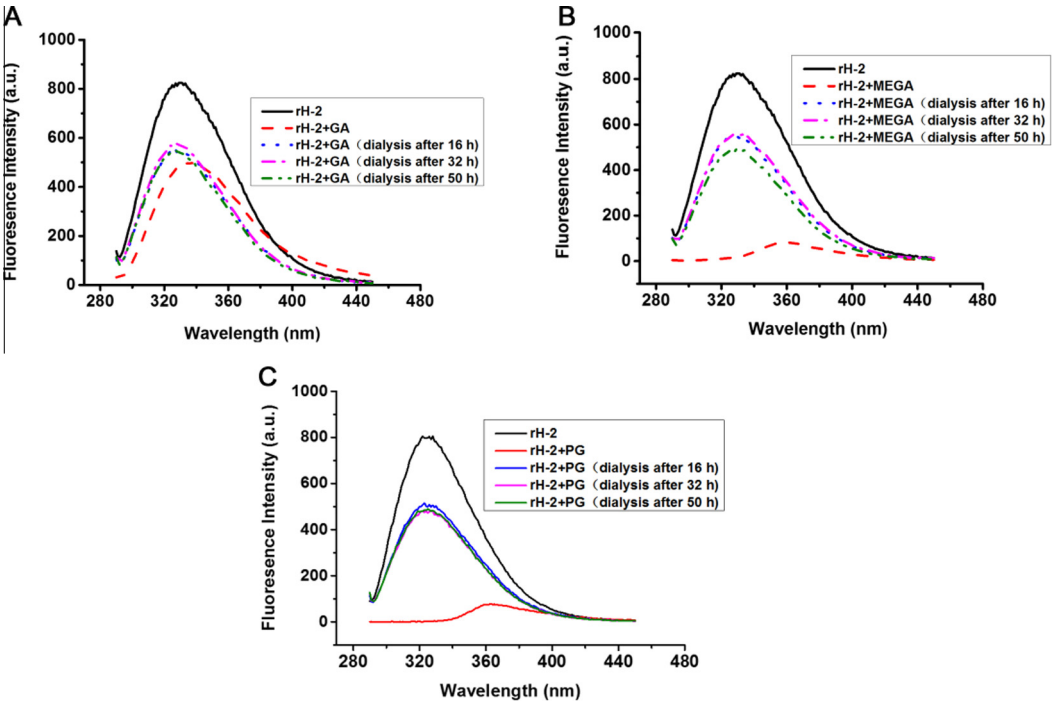

Fig. 5. Fluorescence intensity of ferritin treated with (A) GA, (B) MEGA and (C) PG after dialysis for different times. Conditions: 0.8 μM rH-2 in 50 mM Tris-HCl, [GA, MEGA or PG] = 96 μM, pH 7.5, 25 °C. λ_{Ex} = 280 nm, slits for excitation and emission are 5 nm and 10 nm.

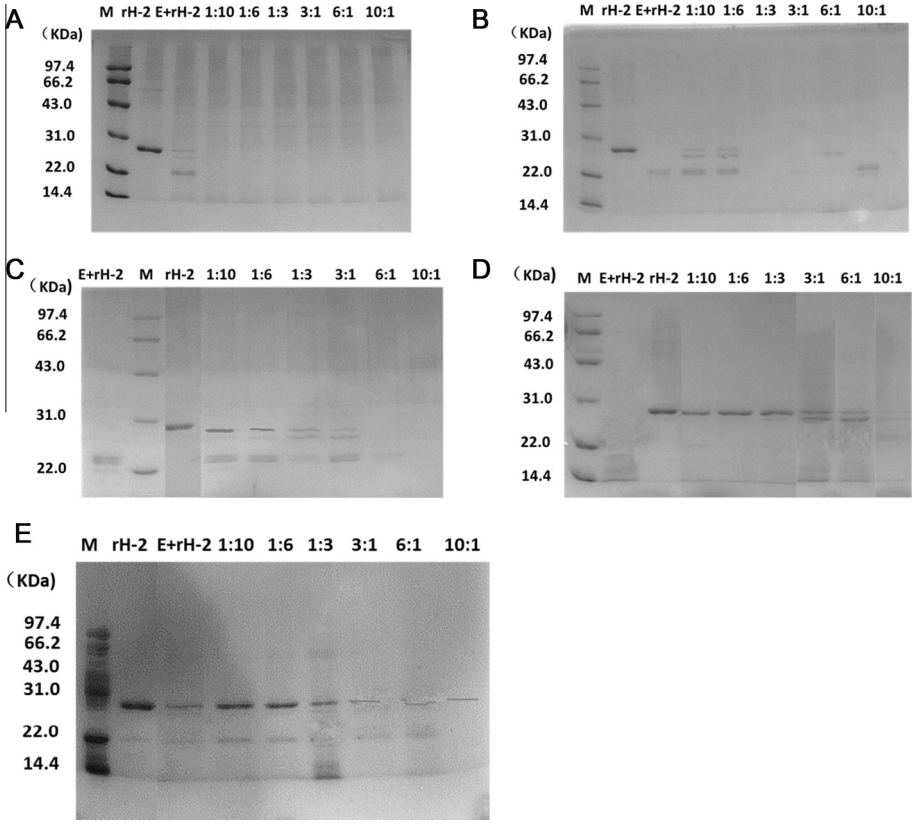

Fig. 6. SDS-PAGE analyses of the digestive stability of rH-2 in the absence and presence of PCA (A), VA (B), GA (C) MEGA (D) and PG (E) at different concentrations in simulated gastric fluid (pH 3.0). Conditions: [rH-2] = 0.5 μM, the ratio of protein to phenolic acid is over the range of 1:10, 1:6, 1:3, 3:1, 6:1, and 10:1. "E + rH-2" represents a mixture of pepsin and ferritin in the absence of phenolic acids.

between GA, MEGA or PG and tannic acid is stemmed from their different structures. It is possible that one tannic acid molecule can link two or more protein molecules together through hydrogen bonds because it contains much more hydroxyl groups than GA, MEGA or PG. In contrast, GA, MEGA or PG can only bind to single ferritin molecule. The detailed mechanism is under investigation.

Iron deficiency anemia is a major public health problem in the world, affecting more than 2 billion people (Zhao, 2010). Women who are pregnant or lactating and young children are its main victims, especially in developing countries. Plant ferritin has been considered as a promising and alternative dietary iron supplement. On the other hand, ferritin is emerging as a novel vehicle for encapsulation and deliver of bioactive nutrients (Chen, Bai, Yang, Yang, Zhao et al., 2014; Chen, Bai, Yang, Zang et al., 2014). However, ferritin is not stable enough against degradation by pepsin existing in the stomach. The present results *in vitro* raise the possibility that the binding of GA, MEGA or PG to ferritin could be an effective way to prevent protein degradation by pepsin in the stomach. After escaping from the stomach as an intact molecule in the presence of GA, MEGA or PG at pH 3.0 or higher, the whole ferritin molecules can be taken up via a receptor-mediated endocytic process in the intestine (San Martin et al., 2008).

However, we must realize the compromise that we make in the accuracy and ease of utilization of any *in vitro* digestion model. For example, we used single enzyme in SGF, but the digestion of one nutrient is often influenced by the digestion of other nutrients (Boisen & Eggum, 1991). Moreover, there are many other factors that are equal important for SGF such as sequential secretion of enzymes in physiological concentrations, removal of the digestion products, appropriate mixing at each stage of the digestion and physiological transit time for each step of the digestion (Minekus, Marteau, & Havenaar, 1995; Yang, 2011). Additionally, it should be noted that, unlike the *in vitro* digestion model, gastric digestion and emptying are dynamic processes *in vivo*. Therefore, animal experiments should be further carried out to explore the effect of the interaction between GA/MEGA/PG and rH-2 ferritins on the digestive stability of protein. Whether the binding of GA/MEGA/PG to ferritin has an effect on the absorption of protein or its degraded products (amino acids) also should be evaluated in future study.

4. Conclusion

All results presented in this study demonstrated for the first time that gallic acid and its derivatives interacted with ferritin in a structure-dependent manner. The number of hydroxyl groups in the benzene ring of this class of phenolic acids determines their ability to bind to ferritin; namely, the phenolic acids with three adjacent hydroxyl groups such as GA, MEGA and PG are able to bind to ferritin tightly, whereas their analogues having two hydroxyl groups cannot. In contrast, effect of the carboxyl group of this class of phenolic acids on such binding is not as large as that of hydroxyl group. Interestingly, the ferritin binding activity of these phenolic acids is consistent with their effect on digestive stability of this protein. For example, the presence of GA, MEGA or PG strongly inhibited protein degradation by pepsin, while PCA or VA lacked such activity. These findings demonstrated that the binding activity of the phenolic acids to protein is closely associated with their function, which advance our understanding of the relationship between structure and function of phenolic acids.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2016.06.085.

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