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Encapsulation of anthocyanin molecules within a ferritin nanocage increases their stability and cell uptake efficiency

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ABSTRACT

Anthocyanins are very spectacular plant pigments with many biological activities. However, because of their highly reactive nature, anthocyanins are unstable. We developed a novel strategy for improving the thermal stability and photostability of cyanidin-3-*O*-glucoside (C3G) molecules by encapsulating them within the inner cavity of apo recombinant soybean seed H-2 subunit ferritin (rH-2). C3G molecules were successfully encapsulated within protein cages with a C3G/protein ratio of 37.5 to 1. As expected, such encapsulation increased the thermal stability and photostability of C3G molecules by a factor of ~2. More importantly, Caco-2 cell monolayer absorption and adhesion analyses showed that C3G molecules encapsulated within apoferritin nanocages were more efficient in transport as compared to free C3G. These findings indicate that protein with a shell-like structure as a nanoplatform can play an important role in the field of nutrition.

Introduction

Anthocyanins are a major group of water-soluble plant pigments that are responsible for fruit and flower coloration either by themselves or in conjunction with other phytochemicals (carotenoids, etc.). As a consequence of customer preferences for natural food ingredients and the delisting of approved artificial dyes, the trend of replacing synthetic food colorants with natural anthocyanins in fruit juices, wines, jams and preserves is increasing (Cabrita, Fossen, & Andersen, 2000). Besides anthocyanin esthetic contributions, a renewed attention has been drawn to their potential health promoting properties as antioxidant, anticancer, antidiabetic, antiinflammatory, antiaging, and cardioprotective properties (Bagchi, Sen, Bagchi, & Atalay, 2004; Einbond, Reynertson, Luo, Basile, & Kennelly, 2004; Espin, Soler-Rivas, Wichers, & Garcia-Viguera, 2000; Lila, 2004; Zafra-Stone et al., 2007; Ziberna et al., 2012). Anthocyanins are gastric and intestinally absorbed, and then reach the systemic circulation in their native or metabolized forms, being available to exert their biological activities (Fernandes, Faria, Calhau, de Freitas, & Mateus, 2014).

Because of their highly reactive nature, anthocyanins readily degrade, or react with other constituents in the media, to form colorless or brown colored compounds (Jackman, Yada, Tung, & Speers, 1987). The basic structure of an anthocyanin is a glycosylated form of polyhydroxy and polymethoxy derivatives of 2-phenylbenzopyrylium or flavylium cation. Differences in structure between individual anthocyanins are the number of hydroxyl groups, the nature and number of sugars attached to the molecule and the position of the attachment, and the nature and number of aliphatic and aromatic acids attached to the sugars (Yan, Li, & Koffas, 2008). The presence of an oxonium ion makes the anthocyanins particularly susceptible to nucleophilic attack by various oxidants (Jackman et al., 1987). Additionally, anthocyanins are easily affected by a number of reactions occurring in food products, and the major problem associated with the storage of anthocyanins is their instability caused by temperature, oxygen, light, and some enzymes during processing and storage of the commodity. Therefore, many efforts have been invested in developing techniques for protecting anthocyanins against damage by the above-mentioned factors. For example, a certain degree of pigment stabilization may be conferred by acylation with various organic acids, copigmentation, self-association, and/or metal chelation (Francis, 1989; Jackman et al., 1987). Moreover, the chromophore is resistant to UV light in the presence of biopterin-α-glucoside (Saito et al., 2003). However, the anthocyanin-protective effects of these methods are still very limited.

During the last two decades, with the development of biotechnology and material science, biomaterials have brought new opportunities for bioactive molecular stability and delivery technology. Various nanostructures have shown great promise in biomedical imaging, biosensing, drug delivery, and disease diagnostics (de la Rica & Matsui, 2010; Gao,

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Cui, Levenson, Chung, & Nie, 2004; Rosi & Mirkin, 2005). Protein cage architectures such as ferritins offer a good opportunity to improve the stability of anthocyanins by encapsulation nanotechnology (Mann & Ozin, 1996; Uchida, Kang, Reichhardt, Harlen, & Douglas, 2010; Yamashita, Iwahori, & Kumagai, 2010). Ferritin is a specific class of ubiquitous iron storage protein (Arosio, Ingrassia, & Cavadini, 2009), which represents the first shell-like protein used for nanomaterial preparation (Mann & Ozin, 1996). Ferritin consists of 24 subunits that self-assemble into a shell-like molecule (Fig. 1A) endowed with 432 symmetry (molecular mass is about 500 kDa; external and internal diameters: 12 and 8 nm) designed to oxidize and store iron as microcrystalline hydrated ferric oxide particle (Harrison & Arosio, 1996). The subunits related by 3- or 4-fold symmetry form at their junction pores that traverse the protein shell so as to allow the passage of ions and small molecules (Harrison & Arosio, 1996; Chasteen & Harrison, 1999). The native iron oxide particle may be easily removed from the protein cage by reduction of Fe(III) and subsequent chelation of Fe(II), resulting in the formation of apoferritin having an empty, intact protein cage. The interesting architecture of ferritins has attracted previous research interest in such purposes as biomimetic material synthesis, magnetic resonance imaging (MRI) contrast agents, gene therapy, drug encapsulation, cell specific targeting, and catalysis (Uchida et al., 2010; Yamashita et al., 2010). However, to the best of our knowledge, there is no report about the application of this protein with a nanocage in the field of nutrition.

Despite the rigid architecture, the association among the ferritin subunits is pH-dependent, which means that the nanostructure can be broken down in an acidic environment and can be restored at neutral pH (Kang et al., 2008; Lin et al., 2011). The protein shell of ferritin is highly resistant to chemical and physical denaturants, so the reassembly route could be used to encapsulate such unstable small species which could not entry the protein cavity through the subunit junction pores as anthocyanins to protect them from disturbance. Resulting anthocyanin-loaded ferritin nanoparticles could be absorbed by human intestinal Caco-2 cells through a receptor-mediated process and related with other factors (Bejjani, Pullakhandam, Punjal, & Nair, 2007; Kalgaonkar & Lonnerdal, 2009; San Martin et al., 2008). Based on these considerations, in this work, ferritin reassembling property was utilized to encapsulate Cyanidin-3-*O*-glucoside (C3G) into ferritin nanocage. We found that such encapsulation greatly enhanced the stability of anthocyanin, while promoting the transport of anthocyanin through Caco-2 cell monolayers.

Materials and methods

Materials

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. (Shanghai, China). Sephacryl S-300, DEAE Sepharose Fast Flow was purchased from GE Healthcare Bio-Sciences AB (Beijing, China). Cyanidin-3-*O*-glucoside (C3G) was obtained from BOMEI Biotechnology Co. (Hefei, China), and its purity is greater than 95%. 3-(N-Morpholino) propanesulfonic acid (Mops) was purchased from Amersco (Beijing, China). Methanol and methane acid were obtained from Beijing Chemical Reagents Co. (Beijing, China). All other reagents used were of analytical grade or purer. Protein concentrations were determined according to the Lowry method with BSA as standard.

Preparation of C3G-loaded rH-2 nanoparticles

Recombinant soybean seed H-2 subunit ferritin (rH-2) was prepared as previously described (Deng et al., 2010; Masuda, Goto, & Yoshihara, 2001). Ferritin reassemble was carried out as previously reported with little modification (Liu, Wu, Wang, & Lin, 2006; Yan, Zhang, Kim, Yuan, & Vo-Dinh, 2010). Briefly, apoferritin was dissociated into subunits at pH 2.0, and then reconstituted at pH 7.5. During this process C3G was loaded within protein shell as shown in Fig. 1. The pH value of rH-2 in distilled water (5 mL, 2 μM) was gradually adjusted to 2.0 by slow addition of HCl (1.0 M) under magnetic stirring. Approximately 350 μL of C3G (10 mM) was slowly added into the rH-2 solution. The resulting solution was maintained for about 20 min in the dark. Then the pH value was slowly raised to 7.5 by the addition of NaOH (0.1 M). The resulting solution was stirred at 7 °C for 2 h in the dark and exhaustively dialyzed for 24 h to remove any free C3G against several changes of phosphate buffer using a dialysis bag with a molecular weight cutoff (MWCO) of 12,000 Da. Finally, the C3G-loaded rH-2 nanoparticles solution was applied to a disposable PD-10 desalting column with an exclusion limit of 5000 for purification. The eluent was 50 mM phosphate buffer, pH 7.4. Briefly, apoferritin was dissociated into subunits at pH 2.0, and then reconstituted at pH 7.5. During this process C3G was loaded within protein shell as shown in Fig. 1.

Dynamic light scattering (DLS) and MALDI-TOF/MS analysis

DLS experiments were performed at 25 °C using a Viscotek model 802 dynamic light scattering instrument (Viscotek, Europe) as described previously (Yang et al., 2010). The OmniSIZE 2.0 software was used to calculate the size/hydrodynamic radius (R_H) distribution of prepared solutions. All samples (0.5 μM) were allowed to stand for 24 h prior to DLS measurement to ensure that the reactions were complete. The exact molecular weight was obtained by MALDI-TOF/MS on an ABI 4800 Plus MALDI TOF/TOF (Applied Biosystems, USA) in refractive positive-ion mode. The sample was prepared in water/trifluoroacetic acid (100:0.1 v/v). Data were collected between 10,000 and 100,000 Da. The theoretical molecular weight of rH-2 subunit was 23,833.7 Da.

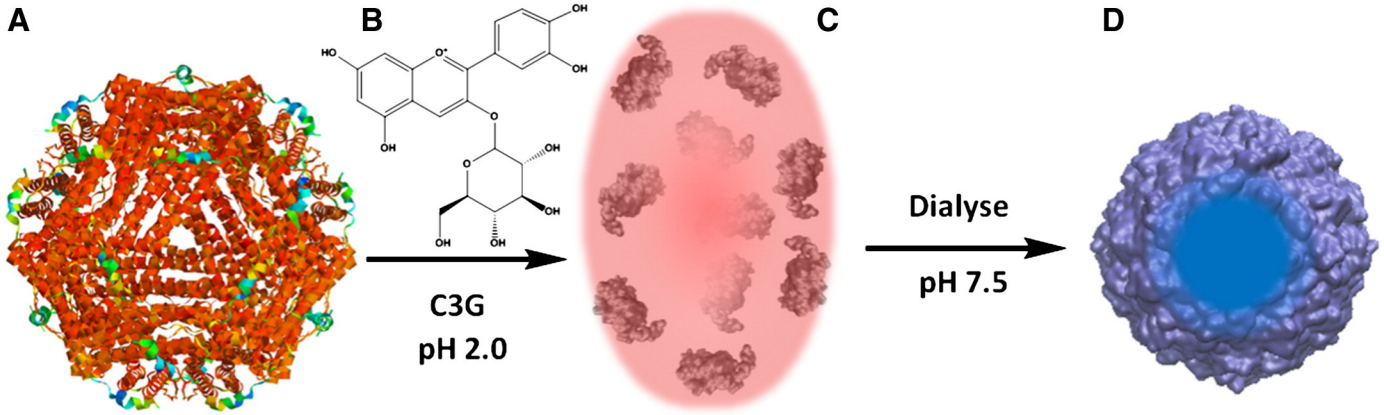

Fig. 1. A schematic of the synthesis of C3G-loaded ferritin nanoparticles. (A) Structure of recombinant H-2 subunit ferritin (rH-2). (B) Chemical structure of C3G. (C) Ferritin dissociated into subunits due to low pH (2.0), followed by incubation with C3G. (D) The formation of C3G-loaded ferritin nanoparticles upon increasing pH of the mixture to 7.5.