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Protection and delivery of mandarin (*Citrus reticulata* Blanco) peel extracts by encapsulation of whey protein concentrate nanoparticles

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ABSTRACT

Flavonoids rich in citrus peels have poor stability and bioavailability, which limits their commercial applications. In present study, citrus peel extracts were obtained from four citrus types and their stability and antioxidant activity were investigated in terms of pH conditions. Then, the extracts were encapsulated and protected by whey protein concentrate nanoparticles (WPC NPs). The results showed that the degradation rate of extracts decreased with higher pH values. At pH 2–3, the antioxidant activity of extracts was relatively lower than that at pH 4–7. Fourier transform infrared spectroscopy results indicated that extracts were encapsulated by WPC NPs via hydrophobic interaction or H-bond. Encapsulation efficiency (EE) and loading efficiency (LE) of extracts in WPC NPs ranged from 14.4 ± 0.7% to 27.9 ± 0.9% and from 11.0 ± 0.6% to 18.6 ± 0.9%, respectively, depending on the citrus types. Transmission electron microscope showed that all nanoparticles had spherical morphology. WPC NPs controlled the release of encapsulated flavonoids and kept their antioxidant activity under *in vitro* simulated gastrointestinal conditions. After 4 h, DPPH• scavenging activity of extracts from Guangxi red orange in simulated intestinal fluids was 34.8 ± 1.5% and 66.4 ± 2.4%, respectively. The obtained knowledge will be helpful to develop food protein-based carriers for flavonoids.

1. Introduction

Citrus fruits are one of the most important fruits in the world, and they are the third most traded agricultural products followed by corn and wheat (Lv et al., 2015). Citrus fruits possess lots of nutritional properties, due to the presence of numerous bioactive compounds. Citrus peels, by-products of citrus processing, are rich in natural antioxidants, such as phenolic compounds (e.g., flavonoids and phenolic acids) (Khan, Zill, & Dangles, 2014). Wang, Chuang, and Hsu (2008) found quercetin, hesperidin, diosmin, chlorogenic and p-coumaric acid in peel extracts. Our previous results showed the presence of narirutin, didymin, sinensetin, nobiletin and tangeretin in the mandarin peel extracts (Hu, Zhang, Ke, Li, & Zhou, 2017). Then, hesperidin is found as a major phenolic compound existed in mandarin and its peel extracts (Nipornram, Tochampa, Rattanatraiwong, & Singanusong, 2018). In recent years, citrus flavonoids are recognized for their antioxidant activity (Zou, Xi, Hu, Nie, & Zhou, 2016), anti-inflammatory activity (Chen, Tait, & Kitts, 2017; Gabriele et al., 2017), regulation of lipid metabolism (Toth et al., 2016), anticancer activity (Benavente-García & Castillo, 2008) and neuroprotective activity (Cirmi et al., 2016). Flavonoids are natural active compounds with high safety, and they have the potential value for the development of functional foods. However, some citrus flavonoids have poor bio-availability and are sensitive to diverse environment stresses (pH, heat and oxidation, etc.) (McClements & Xiao, 2017). The bioavailability of these active substances is mainly determined by three factors: bioaccessibility, absorptivity and bioconversion. While bioaccessibility is affected by the release of active substances from food substrates in gastric juice (McClements & Xiao, 2017). Thereby, the delivery and protection of these flavonoids are important challenges to promote their functional attributes.

Encapsulation for increasing the shelf-life of sensitive molecules and targeting the release of bioactive/nutritional substances has been applied in pharmaceutical, food and cosmetic industries. The produced capsules are divided into macro (> 5000 μm), micro (1.0–5000 μm), and nano (< 1.0 μm) grade (Jafari, Assadpoor, He, & Bhandari, 2008).

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Recently, nano-encapsulation systems have been taken into consideration owing to many advantages, such as high stability, high bioavailability, and good permeability (Faridi Esfanjani & Jafari, 2016; Katouzian & Jafari, 2016). Various food proteins have been utilized to produce nano-carriers, which could protect flavonoids and deliver them into the gut (Bohin, Vincken, van der Hijden, & Gruppen, 2012). Whey protein is one kind of the natural vehicles for bioactive components. This protein is structurally and chemically versatile and well adapted for several delivery systems (Livney, 2010). Whey protein-based carriers are physicochemical compatible with the food matrix, without side-effects and beneficial to the food applications.

Many studies have focused on the interactions between proteins and flavonoids. The solubility of some bioactive components, such as curcumin and resveratrol, is increased after binding to whey protein (Liang, Tajmir-Riahi, & Subirade, 2008; Sneharani, Karakkat, Singh, & Rao, 2010). Fang et al. (2011) found that quercetin decreased the diameter of bovine serum albumin nanoparticles. After encapsulation, quercetin had higher stability under intestinal condition, but its antioxidant activity was obscurely changed.

In this study, the stability and antioxidant activity of citrus peel extracts were first investigated under different pH conditions. Then they were encapsulated by whey protein concentrate nanoparticles (WPC NPs). Their stability passing through simulated gastrointestinal tract was evaluated. Finally, the release properties and antioxidant activity of citrus peel extracts after encapsulation were characterized. The obtained knowledge might provide the applications of plant extracts in the food and pharmaceutical fields.

2. Materials and methods

2.1. Materials and reagents

Fruits of *Citrus reticulata* Blanco 'Ponkan No 3', *Citrus reticulata* Blanco 'Taiwan Ponkan', *Citrus reticulata* Blanco 'Wulong sour orange' and *Citrus reticulata* Blanco 'Guangxi red orange' were collected from the National Citrus Germplasm Repository, Citrus Research Institute of Chinese Academy of Agricultural Sciences (Chongqing, China). Whey protein concentrate (WPC, 80% protein, Hilmar™ 8040) was purchased from Hilmar Ingredients (Turlock, CA, USA). DPPH (2, 2-diphenyl-1-picryl hydrazyl) was purchased from Sigma (St. Louis, MO, USA). Milli-Q water was used for all experiments. Pepsin from porcine gastric mucosa (enzymatic activity of 3000 units/mg protein) and trypsin from porcine pancreas (enzymatic activity of 250 units/mg protein) were purchased from the Aladdin Chemical Reagent Co., China. All other reagents were analytical grade.

2.2. Preparation of citrus extracts

Citrus peels were manually separated and dried to a constant weight in an oven at 50 °C. The dried peels were ground by a mechanical grinder. Then the obtained powders were sieved through a 40-mesh sieve before further treatment. Afterwards, the extraction for each sample was carried out according to our previous report (Hu, Li, Zhang, Kou, & Zhou, 2018). Briefly, dried citrus peel powder were mixed with 70% ethanol and the ratio of material to liquid is 1:20 (w/v). Then, the obtained suspension was kept in a shaking incubator (200 rpm, 40 °C) for 12 h. After that, the suspension was filtered and the residue was discarded. Next, the filtrate was condensed with a rotary evaporator at 50 °C water bath under reduced pressure. The concentrated extracts were freeze-dried into powder and stored at −20 °C before use. The obtained citrus peel extracts from different fruits were named as GX (from Guangxi red orange), WL (from Wulong sour orange), PS (from Ponkan No 3), and TP (from Taiwan Ponkan).

2.3. Determination of total flavonoids content (TFC)

Citrus peel extracts were dissolved in ultrapure water at a concentration of 30 mg/mL. The suspension was centrifuged (5000 ×g, 10 min) to separate and discard the undissolved residue. The supernatant was stored at 4 °C refrigerator before analysis. TFC of citrus peel extracts were determined by using the method described in previous report (Ramful et al., 2010). Briefly, 150 μL of $NaNO_2$ (5%, w/v) was added to 2.5 mL of diluted extracts solution. After reacting for 5 min, 150 μL of $AlCl_3$ (10%, w/v) was added. After 1 min, 1 mL of NaOH (1 mol/L) was added. Then the absorbance of the solution was measured at wavelength of 510 nm with a UV-visible spectrophotometer (UV-1800, Japan). For the quantification of TFC in citrus peel extracts, a standard curve of rutin in ultrapure water were constructed in the concentration range from 6 mg/L to 196 mg/L. TFC was expressed as rutin equivalents (mg/L).

2.4. Antioxidant activity

The DPPH• scavenging activity of citrus peel extracts was measured according to the previous method (Brand-Williams, Cuvelier, & Berset, 1995) with minor modifications. One hundred microliter of sample was mixed with 3900 μL of DPPH solution (0.1 μmol/L) and the mixture was kept under dark condition for 30 min. The absorbance of mixture was measured at a wavelength of 517 nm. The DPPH• scavenging percentage was calculated using the following formula.

$$Scavenging\ activity\ (\%) = \left(1 - \frac{As}{Ac}\right) \times 100\% \quad (1)$$

Where As was the absorbance of the sample and Ac was the absorbance of the control.

2.5. Influence of pH during storage on TFC and antioxidant activity

Citrus peel extracts solution was prepared and stored according to the methods in the section of 2.3. The pH of the obtained solution (5 mL) was adjusted to 2, 3, 4, 5, 6 and 7, by adding 1 mol/L HCl or NaOH and then the solutions were stored at 4 °C. The stability of flavonoids against pH was monitored within 25 days. At a particular time point, 100 μL of citrus peel extracts solution was sampled and used for the determination of TFC and the antioxidant activity.

2.6. Degradation kinetics of flavonoids in citrus peel extracts

The degradation kinetics of flavonoids was assessed by the first-order degradation model (Eq. (2)).

$$ln\ C = lnC_0 - kt \quad (2)$$

Where t is the storage time (day), C_0 and C are the TFC at time zero and time t, respectively. The degradation rate constant (k) is determined as the slope (absolute value) of the linear fit by Origin version 8.0 (OriginLab, USA).

2.7. Preparation of nanoparticles

2.7.1. WPC solution preparation

Initially, WPC solution (2%, w/v) was prepared by dissolving WPC powder in ultrapure water with magnetic stirring (500 rpm) for 2 h and pH was adjusted into 7.0. Then WPC solution was stored in a refrigerator (4 °C) for 12 h to ensure complete hydration. Next, the WPC solution was heated at 80 °C for 30 min and rapidly cooled down. The resultant WPC solution was centrifuged (6000 × g, 15 min) to separate the undissolved residue. The supernatant was collected and kept at 4 °C before use.