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Oxidative stability and *in vitro* digestion of menhaden oil emulsions with whey protein: Effects of EGCG conjugation and interfacial cross-linking

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Genipin (PubChem CID: 442424)
EGCG (PubChem CID: 65064)
DPPH (PubChem CID: 2735032)
L-ascorbic acid (PubChem CID: 54670067)
Gallic acid (PubChem CID: 370)

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ABSTRACT

The goal of this study was to improve the chemical stability of menhaden oil and control the lipolysis in emulsions with whey protein during *in vitro* digestion through EGCG conjugation and genipin-mediated interfacial cross-linking (CL). WPI-EGCG conjugate was successfully synthesized, confirmed by SDS-PAGE, ESI-MS, and phenolic group quantifications (125.3 mg/g), and characterized with far UV CD and ATR-FTIR. Emulsion particle diameter with WPI-EGCG is lower than with WPI. Compared to the native emulsion, WPI CL increased particle diameter and physical stability. Higher oxidative stability was observed for emulsions stabilized with WPI-EGCG conjugate than that with interfacial cross-linking due to the great antioxidant activity. Whereas, WPI CL is more effective than WPI-EGCG conjugate in hindering the rate and extent of lipolysis. The combination of EGCG conjugation and interfacial CL showed both the highest protection of menhaden oil against degradation and highest inhibition on the rate and extent of lipolysis of menhaden oil.

1. Introduction

Menhaden oils comprise of high content of ω-3 fatty acids (eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)) which have been confirmed to have a variety of health-promoting properties including reducing heart disease risk, and advancing brain health (Kris-Etherton, Harris, & Appel, 2003). ω-3 fatty acids also play an important role in membrane fluidity, cellular signaling, gene expression, and eicosanoid metabolism (Horrocks & Yeo, 1999). However, the addition of fish oils into foods and beverages is problematic with various challenges owing to the oils' low water solubility, poor oxidative stability, and variable bioavailability. The oxidative deterioration can result in the destruction of nutritional values, off-flavors, and off-colors. Numerous food scientists attempted to increase the levels and stability of these bioactive lipids in food system by direct incorporation of fish oils into microcapsule, nanoparticle, microemulsion, and emulsion-based delivery system (Aghbashlo, Mobli, Madadlou, & Rafiee, 2013; Barrow, Nolan, & Holub, 2009; Jafari, Assadpoor, Bhandari, & He, 2008). Among these carriers, emulsion-based delivery systems provided a variety of potential benefits for incorporating fish oils into foods and beverages including great water dispersibility, relatively high stability, and easy preparation method (Walker, Decker, & McClements, 2015).

However, the physical and chemical stability of emulsions are highly relevant with the properties of interfacial layer (emulsifiers), such as emulsifier type, zeta-potential, interfacial layer thickness, and interface structure (Cornacchia & Roos, 2011; Walker et al., 2015). Thicker emulsifier coating can effectively inhibit lipid oxidation through hindering interactions between aqueous phase soluble prooxidants and lipids inside the emulsion droplet. Compared with gelatin alone, higher fish oil stability was observed for proanthocyanidin-gelatin colloidal complexes stabilized emulsion (Su et al., 2015). The oxidative stability of fish oil was dramatically enhanced in the presence of polyphenols (von Staszewski, Pizones Ruiz-Henestrosa, & Pilosof, 2014). Recently, protein-polyphenol conjugates (α-lactalbumin-catechin, β-lactoglobulin-catechin or chlorogenic acid, and gelatin-gallic acid) have been synthesized and used as effective stabilizer in delivery systems to improve the chemical stability of easily-oxidized nutraceuticals (Fan, Zhang, Yokoyama, & Yi, 2017; Spizzirri et al., 2009; Yi, Fan, Zhang, & Zhao, 2016; Yi, Zhang, Liang, Zhong, & Ma, 2015b). The improvement of oxidative stability was mainly due to the high free radical scavenging and metal ion chelating ability of grafted phenolic acids.

Genipin, a naturally occurring cross-linking (CL) agent, was obtained from the Gardenia fruit used in traditional Chinese medicine (Qi,

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Nuñez, & Wickham, 2012). Genipin can act with primary amine groups (such as lysine and arginine) of proteins, leading to the formation of inter- and intra-molecular covalent bonds (Aramwit, Siritientong, Kanokpanont, & Srichana, 2010). Compared with chemical cross-linker (e.g., glutaraldehyde), no cytotoxicity were observed for genipin (Sung, Huang, Huang, & Tsai, 1999). Casein micelles increases their physical stability against dissociating agents after CL by genipin (Casanova et al., 2017). The physical stability of emulsion stabilized with plant proteins during storage increased after CL (Johnston, Nickerson, & Low, 2015). CL can greatly inhibit Ostwald ripening or aggregation of the milk protein stabilized emulsions in the presence of ethanol. The main reason for the increase of physical stability was considered to be the increases in the viscosity/elasticity of the protein layer that prevented its collapse. Authors considered the increased viscosity/elasticity of protein layer that prevented its collapse may be the main reason (Færgemand, Otte, & Qvist, 1998). Interfacial CL by enzyme or high pressure homogenization can also enhance fish oil's oxidative stability in emulsions stabilized with sodium caseinate due to the inhibition of oxygen transfer (Phoon, Paul, Burgner, San Martin-Gonzalez, & Narsimhan, 2014). Furthermore, the lipolysis of oil droplets was restrained strikingly with sodium caseinate layer cross-linked by genipin (Hu et al., 2015).

Both protein-polyphenol conjugate and protein CL have been widely used as stabilizers in the food processing industry, however, little information is available on the effects of combine use of polyphenol conjugation and protein CL in food applications. Furthermore, few studies concerning the combination of polyphenol and protein cross-linking for hindering lipid oxidation and controlling lipolysis have been reported.

Whey protein isolate (WPI) composes almost 20% of the total protein in bovine milk and β-lactoglobulin and α-lactalbumin comprise approximately 80% of whey protein by weight. WPI was widely used as a food-emulsifying agent because it can effectively adsorb to the surface of oil droplets and stabilized oil droplet from coalescence by electrostatic repulsion and steric hindrance.

In this study, (−)-Epigallocatechin-3-gallate (EGCG), the most active catechin of tea polyphenols, is used as a representative. WPI-EGCG conjugate was developed and characterized with SDS-PAGE, far UV CD, ATR-FTIR, and LC-MS, and used to encapsulate menhaden oil in emulsions. The antioxidant activity of WPI-EGCG was monitored. The effects of WPI-EGCG conjugate, interfacial WPI-CL with genipin, and the combination of EGCG conjugation and interfacial WPI CL on the physical and chemical stability as well as on *in vitro* digestion profile of menhaden oil emulsions were evaluated. The information obtained in this study may help design and develop emulsion-based delivery systems with desirable characteristics.

2. Materials and methods

2.1. Materials

Whey protein isolate (WPI) was supplied free by Davisco Foods International Inc. (Le Sueur, MN, USA). Menhaden oil (with palmitic and stearic acid content ≤30.0%; and ω-3 fatty acids content between 20.0 and 31.0%), pepsin, bile extract, pancreatin, gallic acid, L-ascorbic acid, H_2O_2 (30%, w/w), 2, 2′-azino-bis (3-ethyl- benzothiazoline-6-sulfonic acid) diammonium salt (ABTS, > 98%), AAPH, fluorescein, and Trolox were purchased from Sigma-Aldrich (St. Louis, MO) and used without further purification. EGCG (purity > 95% by HPLC) was purchased from Chuangsai Co., Ltd. (Shanghai, China). Genipin (> 98%) was obtained from Linchuan Zhixin Biotechnology Co., Ltd. (Jiangxi, China). All other analytical grade chemicals and reagents were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

2.2. Preparation of WPI-EGCG conjugates

Aforementioned method based on free radical grafting was employed to produce WPI-EGCG conjugates (Spizzirri et al., 2009). Briefly, 0.5 g WPI was dispersed fully in 50 mL ultrapure water, then, 0.5 mL of hydrogen peroxide (10.0 M) and 0.25 g L-ascorbic acid were added successively. The mixture was magnetically stirred at room temperature under atmospheric pressure for 2 h. Next, 0.25 g EGCG was added to the mixture and their reaction was allowed for the next 24 h. After that, free, unreacted EGCG was discarded by dialysis (MWCO: > 3 kDa cut-offs) for for 48 h at 4 °C with 10 times water changes. The absence of free EGCG or free L-ascorbic acid in the dialyzed conjugate solution was confirmed with RP-HPLC. The WPI-EGCG conjugate solution was freeze-dried with a lyophilizer (Labconco, MO) and stored in a freezer for further use.

2.3. Evaluation of phenolic groups by Folin–Ciocalteu reagent

The grafted EGCG amount in WPI-EGCG conjugate was monitored with aforementioned method (Fan et al., 2017). Briefly, 0.5 mL of WPI-EGCG conjugates was mixed with 1 mL of Folin–Ciocalteu reagent for 5 min avoiding light. Then, 2 mL of 20% sodium carbonate (Na_2CO_3) was added, and the sample was further vortexed and incubated 1 h at 25 °C. The absorbance was measured with a spectrophotometer (UV2600, Shimadzu, Japan) at a wavelength of 747 nm. Gallic acid was used as a standard. The grafted yield of WPI-EGCG conjugates was presented as milligrams of gallic acid equivalent per gram of conjugates.

2.4. Characterization of WPI-EGCG conjugates

2.4.1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE analysis was performed with aforementioned method in the presence of β-mercaptoethanol at a constant voltage of 120 V (Yi, Lam, Yokoyama, Cheng, & Zhong, 2015a). A home-made 4–20% gradient polyacrylamide gel was used for the separation of WPI (control), and WPI-EGCG conjugates. Samples (5 mg/mL) were mixed with SDS sample buffer and boiled for 5 min. Twenty μL volume of samples was loaded to designated wells for electrophoresis. The Colloidal Coomassie G-250 Staining protocols were used for the gels staining (Dyballa & Metzger, 2009). The gel was photographed with a common camera.

2.4.2. Far UV circular dichroism (CD)

CD spectra was obtained with a J-815 CD spectrometer (Jasco, Tokyo) and the secondary structure changes of WPI after conjugation was determined between 195 and 260 nm based on a method described previously (Yi, Lam, Yokoyama, Cheng, & Zhong, 2014). A cell with a 2.0 mm path length was used. Samples dispersed with 10 mM PB (pH 7.0) were used at a protein concentration of 0.2 mg/mL and PB solution was used as the blank. Scanning was performed at 50 nm/min at 20 °C. The recorded data were the average of ten scans.

2.4.3. ATR-FTIR

Infrared spectra were obtained with ATR-FTIR spectrophotometer (Nicolet iS10, Thermo-Scientific, Madison, WI) in the wavenumber range of 4000–650 cm^{-1} at room temperature. All data were collected with 4 cm^{-1} window and were the average results of 256 scans. A background spectrum was obtained for each sample. The powder samples (WPI or WPI-EGCG conjugate) were placed at the center of the crystal surface.

2.4.4. ESI-MS

The Mw of WPI, and WPI-EGCG conjugate was analyzed with a ESI-MS system with a Thermo Easy Nano-II (Thermo Fischer Scientific, MA) HPLC. Freeze-dried WPI, and WPI-EGCG was dispersed in ultrapure