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Characterization of catechin-α-lactalbumin conjugates and the improvement in β-carotene retention in an oil-in-water nanoemulsion

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ABSTRACT

The goal of this study was to prepare and characterize α-lactalbumin (ALA)-catechin conjugates as a novel emulsifier in improving the retention of β-carotene (BC) in nanoemulsions via a free radical method. Covalent modification was observed and at least one catechin molecule was binding with ALA according to ESI–MS results. Far-UV CD indicated that the secondary structure of ALA was changed after conjugation. The *Z*-average particle diameters of nanoemulsions stabilized with ALA and ALA-catechin conjugates were 158.8 and 162.7 nm, respectively. The increase of mean particle size and the degradation of BC at 50 °C were both larger than at 25 °C during 30 days storage. BC retention stabilized with ALA-catechin conjugates was appreciably greater than ALA (control), which was attributed to the increase of ALA's radicals-scavenging and free metal ion binding ability after grafting with catechin. The chemical antioxidant activities of ALA-catechin conjugates were increased with increasing concentrations from 0.1 to 1.0 mg/ml. In general, labile phytochemicals, like BC, can be protected against oxidation during storage by proteins-polyphenols conjugates without any side effects.

1. Introduction

β-Carotenes (BC) are naturally occurring and the most abundant lipophilic carotenoid precursors of vitamin A (Lobo, Amengual, Palczewski, Babino, & von Lintig, 2012). Vitamin A deficiency (VAD) is a serious problem in developing countries, where it is estimated that 190 million preschool age children and 19.1 million pregnant women are VAD (retinol <70 μmol) (Geneva, 2009). One BC molecule can be broke up into two precursors of vitamin A in theory. Fortification of BC in food is beneficial to human health to overcome VAD. Besides high pre-vitamin A activities, BC was also reported to possess strong antioxidant activities (Paiva & Russell, 1999). Lipid peroxidation in diabetic rats tissues was suppressed by BC. *In vitro* study shows that the glucose tolerance was also improved by BC, indicating the ability of dietary BC on suppression of diabetic symptoms (Furusho, Kataoka, Yasuhara, Wada, & Innami, 2002). BC is also widely used as a colorant in foods and beverages. However, the utilization of BC as a functional food in the food industry is currently limited because of its poor water solubility, high chemical instability, and low *in vivo* bioavailability. Encapsulation of BC can be used to improve the aqueous solubility, physicochemical stability, and bioavailability (Mensi et al., 2014; Soukoulis & Bohn, 2015; Yi, Li, Zhong, & Yokoyama, 2014).

Oil-in-water nanoemulsion refers to a delivery system mainly for protecting lipophilic nutraceuticals from degradation and oxidation, with the mean particle radius defined as below 100 nm (Huang, Yu, & Ru, 2010). A wide variety of studies suggest that nanoemulsion have a number of potential advantages due to its small particles diameter, including high stability against flocculation or aggregation, high optical clarity, and increased bioavailability of encapsulated lipophilic bioactive molecules (Huang et al., 2010; McClements & Rao, 2011). In contrast, compared to conventional emulsions, smaller particle size and higher surface area increase nanoemulsion contact with prooxidants, such as metal ions, oxygen, and free radicals, resulting in encapsulated BC degradation and oxidation (Yi et al., 2014). Novel emulsifiers with great antioxidant activities are in demand.

Abbreviations: BC, β-carotene; ALA, α-lactalbumin; BLG, β-lactoglobulin; VAD, vitamin A deficiency; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; CD, circular dichroism; ESI–MS, electrospray Ionization–mass spectrometry; HPLC, high-performance liquid chromatography; PDI, polydispersity index.

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Recently, protein and nutraceuticals complexes were formed by mainly non-covalent bonds, including hydrophobic interaction, van der Waals, and hydrogen bond (Bandyopadhyay, Ghosh, & Ghosh, 2012; Delavari et al., 2015; Kanakis et al., 2011). Compared to covalent bonds, the interactions are relatively weak and unstable.

Milk proteins are widely used to stabilize functional components for their excellent emulsifying and carrying abilities (Livney, 2010; Yi, Fan, Yokoyama, Zhang, & Zhao, 2016; Yi, Lam, Yokoyama, Cheng, & Zhong, 2015; Yi, Zhong, Zhang, Yokoyama, & Zhao, 2015). α-Lactalbumin (ALA) is a small globular protein (123 amino acids) with a molecular mass of 14.2 kDa, making up approximately 20–25% of the whey proteins (Delavari et al., 2015). The secondary structure of ALA consists of a large α-helical domain and a small β-sheet domain, which is connected by a loop (Delavari et al., 2015). The cost-effective and good emulsifying abilities make ALA a good stabilizer. Catechin is one kind of highly aqueous soluble polyphenols found in tea and fruits, which has been extensively reported to show a strong cancer preventive ability and antioxidant activities (Yang, Wang, Lu, & Picinich, 2009).

The conjugates formed with proteins and polyphenols through covalent conjugation have already been illustrated to enhance milk proteins' antioxidant activities. β-lactoglobulin (BLG)-catechin conjugates were previously prepared using a free radical grafting approach previously and the results showed that BC retention was improved by BLG-catechin conjugates (Yi, Zhang, Liang, Zhong, & Ma, 2015). In this study, ALA-catechin conjugates were prepared using a free radical grafting approach, which used a hydrogen peroxide−ascorbic acid pair as a radical initiator system without the generation of toxic reaction byproducts (Curcio et al., 2009; Spizzirri et al., 2009). ALA-catechin conjugates were supposed to provide better protection of easily oxidized lipophilic bioactive molecules against oxidation when used in O/W delivery system. However, to date, no information was observed about ALA-catechin conjugates.

In this study, the identification of the conjugates was conducted with far UV CD, SDS-PAGE, and ESI–MS. The antioxidant activities of the conjugates was evaluated *in vitro* by reducing power and 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) as well as hydroxyl scavenging assays. The effects of ALA alone and ALA-catechin conjugates on the retention of BC under storage were also evaluated.

2. Materials and methods

2.1. Chemicals and reagents

α-lactalbumin (>93%, purity) was supplied by Davisco Foods International Inc. (Le Sueur, MN, USA). Corn oil was obtained from a local market (Albany, CA). 2,2-diphenyl-1-picryl-hydrazil (DPPH), β-carotene (⩾97%, UV), (+)-catechin (⩾98% HPLC), potassium ferricyanide, L-ascorbic acid, hydrogen peroxide (30%, w/w), and 1,10-phenanthroline, were purchased from Sigma–Aldrich (St. Louis, MO). Precision Plus Protein unstained standards were obtained from Bio-Rad (Hercules, CA). All solvents used for ESI–MS were Optima LC/MS grade (Fisher Scientific, PA). Solvents used for BC determination were HPLC-grade and was purchased from Sigma–Aldrich (St. Louis, MO). All other chemicals and reagents used were analytical grade and obtained from Fisher Scientific (PA, USA). Ultrapure water was used in all experiments.

2.2. Preparation of ALA-catechin conjugates

ALA-catechin conjugates were produced using a free radical method as previously reported (Spizzirri et al., 2009) with slight modification. In brief, 0.5 ml of 10.0 M H_2O_2 (5.0 mmol) and 0.25 g L-ascorbic acid (1.4 mmol) were added dropwise into 50 ml of ALA solution (1 mg/mL) in a 100 ml glass flask, and the mixture was stirred at 25 °C under atmospheric air. After 2 h, 0.35 mmol of catechin was added to solution and reacted overnight at 25 °C. Dialysis was used to eliminate the free unreacted catechin in the reaction solution against ultrapure water, using 10 times the volume of the reaction solution (dialysis bag with 2000 molecular weight cutoffs) for 48 h at 4 °C in a freezer with 10 changes. RP-HPLC results showed that no free catechin was found in the dialyzed conjugate solution. The remaining ALA-catechin solution was frozen, dried and stored in a freezer. ALA (control) was obtained using the same procedure previous in the absence of catechin.

2.3. Characterization of ALA-catechin conjugates

2.3.1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE)

SDS–PAGE analysis under reducing condition was performed at a constant voltage of 120 V according to an established protocol (Yi et al., 2015). Native ALA, ALA (control), and ALA-catechin conjugates (5 mg/mL) were mixed with the same volume of ultrapure water and two times volume of 2X SDS sample buffer (pH6.8) containing 100 mM Tris–HCl, 4% SDS, 0.2% bromophenol blue, 20% glycerol, and 200 mM 2-mercaptoethanol and loaded on a 4–20% gradient polyacrylamide gel. Loading volume of samples was 15 μL (protein content was 18.75 μg). The Colloidal Coomassie G-250 Staining protocols were used for the gels staining (Dyballa & Metzger, 2009). An ImageQuant LAS 4000digital imaging system was used to scan the stained gel.

2.3.2. Far ultraviolet circular dichroism (CD)

The secondary structure changes of the protein after conjugation was determined by a J-815 CD spectrometer (Jasco, Tokyo) between 195 and 260 nm, based on a method described previously (Yi, Lam, Yokoyama, Cheng, & Zhong, 2014b). A cell with a 2.0 mm path length was used. Samples (protein concentration is 0.2 mg/mL) prepared with 10 mM PB (pH 7.0) were used and the PB solution (10 mM, pH 7.0) was used as the blank for all samples. The scan rate was 50 nm/min at 20 °C. Ten scans were averaged to obtain one spectrum. The obtained results were displayed as mean residue ellipticity (degrees • cm^2/dmol) using 123 for the average number of amino acid residues per molecule of ALA (Brew, Castellino, Vanaman, & Hill, 1970).

2.3.3. Electrospray Ionization-mass spectrometry (ESI–MS)

ESI–MS was used to analyze the molecular weights of ALA and ALA-catechin conjugates (Yi et al., 2015). Samples were injected into the ESI–MS system with a Thermo Easy Nano-II (Thermo Fischer Scientific, MA) HPLC. ALA or ALA-catechin conjugates (protein content is 1 mg/mL) dispersed in ultrapure water were filtered with a 0.22 μm filter, then diluted 10-fold with 0.1% formic acid solution. Solvent A was 0.1% formic acid in water and solvent B was 0.1% formic acid in acetonitrile. A 2 μL aliquot of sample solution mixed with 8 μL solvent A was injected and separated with a New Objectives PicoChip C_{18} column (100 mm, 3 μm) at a flow rate of 200 nL/min. The solvent gradient program was: 0 min, 98% A and 2% B; 0 to 7 min, linear gradient from 2% B to 16% B; 7 to 27 min, linear gradient from 16% B to 35% B; 27 to 31 min, linear gradient from 35% to 80%. ESI–MS was performed in positive-ion mode and data were collected on a Thermo Orbitrap Elite. The molecular mass results were determined based on ES data that was deconvoluted by the program MagTran (Zhang & Marshall, 1998).