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Effects of flavonoid glycosides obtained from a *Ginkgo biloba* extract fraction on the physical and oxidative stabilities of oil-in-water emulsions prepared from a stripped structured lipid with a low omega-6 to omega-3 ratio

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ABSTRACT

In this study, we have produced a structured lipid with a low ω6/ω3 ratio by lipase-catalysed interesterification with perilla and grape seed oils (1:3, wt/wt). A *Ginkgo biloba* leaf extract was fractionated in a column packed with HP-20 resin, producing a flavonoid glycoside fraction (FA) and a biflavone fraction (FB). FA exhibited higher antioxidant capacity than FB, showing 58.4 mmol gallic acid equivalent (GAE)/g-of-total-phenol-content, 58.8 mg quercetin equivalent (QUE)/g-of-total-flavonoid-content, 4.5 mmol trolox/g-of-trolox-equivalent antioxidant capacity, 0.14 mg extract/mL-of-free-radical-scavenging-activity (DPPH assay, IC_{50}), and 2.3 mmol $Fe_2SO_4{\cdot}7H_2O$/g-of-ferric-reducing-antioxidant-power. The oil-in-water emulsion containing the stripped structured lipid as an oil phase with FA exhibited the highest stability and the lowest oil globule diameters (d_{43} and d_{32}), where the aggregation was unnoticeable by Turbiscan and particle size analyses during 30 days of storage. Furthermore, FA was effective in retarding the oxidation of the emulsions.

1. Introduction

Structured lipids (SLs) are triacylglycerols (TAGs) that have been modified by chemical or enzymatic reactions to change the fatty acid composition and/or their location in the glycerol backbone (Hamam & Shahidi, 2004). SLs provide the ability to create custom-made lipids with health benefits through esterification. Perilla oil is one of the major sources of plant omega-3 fatty acids (C18:3, ω-3, ALA). ALA has been proven to prevent cardiovascular disease, help manage chronic disorders, and decrease blood cholesterol levels (Sanders et al., 1997). However, western diets are usually deficient in ω-3 fatty acids and have excessive amounts of omega-6 (ω-6) fatty acids (Simopoulos, 2002). It has been claimed that the intake of fats with a low ω6/ω3 ratio would help in preventing coronary heart and atherosclerotic diseases (Simopoulos, 2002; Harris et al., 2009), and the use of SLs is an alternative method for obtaining such benefits.

Flavonoids are a class of plant secondary metabolites, the dietary consumption of which might be related to protection against some diseases (Hertog, Feskens, Kromhout, Hollman, & Katan, 1993). Furthermore, as an additive, flavonoids can retard lipid oxidation in emulsion systems (Roedig-Penman & Gordon, 1998). Recently, studies have shown that flavonoids can act as stabilizers of oil-in-water (O/W) emulsions through pickering stabilisation (Luo et al., 2011a, 2011b). This finding indicates that while the flavonoids exist as insoluble particles in the aqueous phase, they tend to adsorb at the oil-water interface. The adsorbed layer could provide an efficient steric barrier against the coalescence of emulsion oil globules. *Ginkgo biloba* leaf ethanol extracts (GBEs) have been widely investigated because of their possible beneficial effects on human health, such as alleviating short-term memory loss and disturbances in vigilance and mental concentration (Kobus et al., 2009). Furthermore, several studies have shown the antioxidant activity of GBEs in vitro and in vivo (Kobus et al., 2009). Since flavonoids are claimed to be the main bioactive compounds in GBEs, it is important to know if the fractionated extracts, which mainly contain flavonoid glycosides and biflavones, still

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retain their bioactivity after being incorporated in the emulsions and how they may interfere with the physical and chemical stabilities of the emulsions. Specifically, recent studies (Atarés, Marshall, Akhtar, & Murray, 2012; Luo et al., 2011a, 2011b) have found that some flavonoids show good emulsifying activity, indicating that the GBE particles may help maintain the physical and chemical stabilities of O/W emulsions.

To date, only a few methods have been developed for monitoring the destabilisation process of emulsions, such as optical analysis with microscopy, spectroscopy, turbidity analysis, and particle size analysis (McClements, 2007). However, these methods involve some form of dilution, which is not suitable for investigating the destabilisation process. Turbiscan optical analyzer can be used for real-time monitoring of the turbidity profile of an emulsion along the height of a glass tube filled with the emulsion (Liu et al., 2011; Mengual, Meunier, Cayre, Puech, & Snabre, 1999; Pan, Tomás, & Añón, 2004; Álvarez Cerimedo, Iriart, Candal, & Herrera, 2010). It provides a method for quick and simple detection of changes in the physical stability of the emulsion (creaming, sedimentation, clarification, coalescence, and flocculation), by recording the transmission and backscattering variations in the emulsion over time (Palazolo, Sorgentini, & Wagner, 2005; Huck-Iriart, Pizones Ruiz-Henestrosa, Candal, & Herrera, 2013).

Our previous study (Yang et al., 2013) showed that 80% GBE, containing both flavonoids and biflavones, could act as a good antioxidant in an O/W emulsion prepared from SLs and physically blended lipids. In the current study, we obtained samples with different GBE fractions containing either flavonoid glycosides or biflavones, and we examined these fractions for their antioxidant activity with regards to inhibiting lipid oxidation in the O/W emulsions prepared from stripped structured lipids (SSLs). Finally, the physical and oxidative stabilities of the O/W emulsions were monitored during 30 days of storage.

2. Materials and methods

2.1. Materials

Grape seed oil (GSO) and perilla oil (PO) were purchased from the local market (Daejeon, South Korea). The GSO contained 6.6% C16:0, 4.8% C18:0, 17.4% C18:1, 70.9% C18:2 (ω-6), and 0.2% C18:3 (ω-3) fatty acids, whereas the PO contained 6.3% C16:0, 1.9% C18:0, 21.0% C18:1, 10.4% C18:2 (ω-6), and 60.5% C18:3 (ω-3). The SL contained 6.0% C16:0, 3.3% C18:0, 17.3% C18:1, 57.6% C18:2 (ω-6), and 15.8% C18:3 (ω-3), with an ω6 to ω3 ratio of 3.6. *G. biloba* leaf was locally collected in early October. TBA (4,6-dihydroxy-2-mercaptopyrimidine), trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), ABTS (2,2′-azinobis-3-ethylbenzothiazoline-6-sulphonic acid), TPTZ (2,4,6-tripyridyl-s-triazine), ferrous chloride, sodium acetate trihydrate, gallic acid, Folin–Ciocalteu's phenol, 2,2 diphenyl-1-picryihydrazyl, quercetin (Que), bis[2-hydroxyethyl]amino-tris-[hydroxymethyl]methane (bis-tris), Diaion® HP-20, alumina, silicic acid, charcoal, lecithin, and chloroform-d (99.9 atom% D, containing 0.1% v/v TMS) were purchased from Sigma–Aldrich (St. Louis, MO, USA). All the chemicals and solvents were HPLC-grade and were obtained from Fisher Scientific (Norcross, GA, USA). Lipozyme TLIM was purchased from Novozyme A/S (Bagsvaerd, Denmark).

2.2. Production and purification of SLs

The SLs were produced in a 250 mL flask with a screw cap using the optimal reaction conditions for GSO and PO (3:1, w/w, 24 g). Lipozyme TLIM (10% of the total substrate) was used for the reaction at 55 °C for 6 h (Yang et al., 2013). The tocopherols, β-carotene, diacylglycerol (DAG), monoglycerols (MAG), and free fatty acids (FFA) were removed from the SLs with some modifications (Boon et al., 2008). The SSLs were prepared by diluting 20 g of SLs with 10 mL hexane. This mixture was passed through a chromatographic column (3.5 cm in diameter and 20 cm in length). The bottom layer of the column was packed with 4 g of charcoal, and middle and top layers were filled with 20 g aluminium oxide and 20 g silicic acid, respectively. The SSLs were eluted with 200 mL hexane, and the hexane was subsequently removed under vacuum using a rotary evaporator (RE 111, Büchi, Flawil, Switzerland) at 38 °C. Traces of hexane were further removed by flushing with nitrogen, and the SSLs obtained were stored at −80 °C until use. The SLs and SSLs were analysed by HPLC, TLC, and ^{1}H NMR to confirm the removal of tocopherols, β-carotene, DAG, MAG and FFA (Yang et al., 2012).

2.3. DAG, MAG, and TAG analysis by ^{1}H NMR

SLs or SSLs (50 mg) were dissolved in 700 μL $CDCl_3$ and then placed in an NMR tube (5 mm in diameter) for further analysis. ^{1}H NMR spectroscopy were performed on a Bruker Avance III 600 spectrometer operated at 600.13 MHz. All the spectra were processed using ACDlabs NMR Processor, version 10.0. The chemical shifts (δ) are reported with reference to tetramethylsilane (TMS) at $\delta = 0$ ppm.

2.4. Preparation of G. biloba extracts and fraction

G. biloba leaves (100 g) were refluxed with 95% ethanol twice, followed by 70% ethanol twice [sample/solvent (w/v) = 1/6; 2 h each time]. The combined extract was concentrated using a rotary vacuum evaporator, and dried in a vacuum freeze dryer to prepare the final extract (GBE, 15 g). A part of the GBE (2 g) was fractionated using a column packed with HP-20 (50 g), and eluted with a gradient of polar solvents [distilled water to ethanol (100:0, 80:20, 50:50, 30:70, to 0:100)] to obtain five fractions (F1-F5). The distilled water fraction (F1) containing the pigments was discarded. The other four collected fractions were then combined to yield two fractions [FA (F2-F3) and FB (F4-F5)], according to their HPLC patterns, and concentrated using a rotary vacuum evaporator. After drying in a vacuum freezing dryer, FA (450 mg, yield 22.5%) and FB (90 mg, yield 4.5%) were prepared. The compounds in FA and FB were identified by HPLC-UV, according to the retention time as described in our previous study (Yang et al., 2013). The extracts were stored in a desiccator at room temperature until use.

2.5. Determination of antioxidant activity

In order to evaluate the antioxidative activity of GBE, the total phenol content (TPC), free radical scavenging activity (DPPH assay, IC_{50}), trolox equivalent antioxidant capacity (TEAC), and ferric reducing antioxidant power (FRAP) were measured, according to the methods described by Yang et al. (2013). The total flavonoid content (TFC) was measured, as described by Abdel-Hameed (2009).

2.6. Preparation of emulsion

Oil-in-water emulsion (100 g) was prepared with 10% (wt.%) SSL in a 20 mM bis–tris buffer solution (pH 7). Lecithin was used as an emulsifier at a concentration of 3 wt.% of the SSLs. FA or quercetin was added at a final concentration of 100 ppm in the mixture. The chelation of ferric ions by FA or quercetin was also investigated. Ferric chloride (50 μM) was added into the mixture. The mixture was kept in warm water until pre-homogenised by a