Food Chemistry 233 (2017) 282–289

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Efficient delivery of quercetin after binding to beta-lactoglobulin followed by formation soft-condensed core-shell nanostructures

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ARTICLE INFO

Article history:
Received 7 December 2016
Received in revised form 18 April 2017
Accepted 19 April 2017
Available online 21 April 2017

Keywords:
Complex coacervation
Quercetin
Nanoencapsulation
Core-shell
Controlled release
Soft condensation
Nanoparticle

ABSTRACT

Nature-made inherent transporting property of beta-lactoglobulin (BLG) was exploited to develop delivery systems for quercetin. After binding to BLG, quercetin was nanoencapsulated within soft-condensed nanostructures of BLG and sodium alginate (ALG). Fluorimetry results revealed that quercetin could bind to BLG even at acidic conditions. The amounts of stoichiometry binding (n) were 1.24 and 1.62 at pH values of 4 and 7, respectively. Formation of core-shell type nanostructures was confirmed by transmission electron microscopy. Quercetin was efficiently entrapped (>93%). The ejection from the carrier was very limited over time (<1% during 1 month). The protection of nanoencapsulated quercetin was at least 3 times better than that of free quercetin. Quercetin was not released (<3.5% during 6 h) in simulated gastric fluids (pH 1.2 and 4); while, a sustained release (77% during 12 h) was observed in simulated intestinal fluid (pH 7.4).

1. Introduction

Among various nutraceuticals, natural antioxidants have gained a great deal of attention due to a large number of health claims ascribed to them (Giampieri, Alvarez-Suarez, & Battino, 2014). Flavonoids as natural antioxidants have six subdivisions including flavones, flavonols, flavanones, flavan-3ols, anthocyanidins and isoflavones (Ross & Kasum, 2002). Quercetin, as a flavonol, is naturally present in vegetables (e.g., onions), fruits (e.g., apples), soft drinks (e.g., tea), alcoholic beverages (e.g., red wine) and medicinal plants (e.g., yellow sweet clover). Similar to other flavonoids, quercetin possess several potential health benefits such as antioxidant and antiviral activities (Zhang, Yang, Tang, Hu, & Zou, 2008) and may contribute to lowering the risk of cardiovascular disease, cancer, diabetes and obesity (Gutierrez, Prater, & Holladay, 2014). Most of quercetin present in plants is in glycosidic form; however, it can be also found as free aglycone.

Because of crystallinity and low water-solubility, quercetin has low bioavailability after oral administration (Aceituno-Medina, Mendoza, Rodríguez, Lagaron, & López-Rubio, 2015). Moreover, chemical instability, under the influence of oxygen, light and high temperature, makes the incorporation of quercetin into liquid functional foods very difficult.

Many efforts have been made to develop quercetin delivery systems using amorphous solid dispersions based on cellulose derivatives (Gilley et al., 2017), electrospun hybrid fibers synthesized from a mixture of amaranth protein isolate and pullulan (Aceituno-Medina et al., 2015), nanoparticles of chitosan oligosaccharide and β-lactoglobulin (Ha, Kim, Lee, & Lee, 2013), self-assembled lecithin and chitosan nanoparticles (Souza et al., 2013), poly-D, L-lactic acid (Kumari, Yadav, Pakade, Singh, & Yadav, 2010) and ionically cross-linked chitosan nanoparticles (Zhang et al., 2008).

Encapsulation systems based on protein-polysaccharide complexes offer many advantages such as increasing bioactivity, masking unpleasant tastes, targeted delivery and controlled release (Arroyo-Maya & McClements, 2015). Electrostatic complexes are formed at pH conditions between the isoelectric point (pI) of protein and the pK_a of ionic polysaccharide (Perez et al., 2015; Sanchez et al., 2002). Among various structures which can be formed in the mixture of oppositely charged biopolymers, spherical coacervates are the best choice for the encapsulation. However, the phase separation resulting from the coalescence of coacervates (Sanchez et al., 2002) limits their application as delivery system in clear beverages.

In recent years, the potential application of the soluble complexes in the encapsulation of bioactives has been extensively

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http://dx.doi.org/10.1016/j.foodchem.2017.04.126
0308-8146/

investigated (Arroyo-Maya & McClements, 2015; Hosseini, Emam-Djomeh, Van der Meeren, & Sabatino, 2015; Ilyasoglu & EI, 2014; Perez et al., 2015; Zimet & Livney, 2009). To our knowledge, binding of quercetin to beta-lactoglobulin (BLG) and then its delivery by means of mixed biopolymer nanocomplexes have not been studied yet. The hypotheses of this study were i) BLG (as a member of lipocalin protein family) can be used as a carrier for quercetin. However, its transporting properties may be influenced by pH; ii) the loaded quercetin may be better protected by deposition of a shell around the protein core via complex coacervation between BLG and an anionic polysaccharide; iii) the characteristics of the nanocarrier may be influenced by the ratio of both biopolymers; iv) the heat stability of mixed biopolymer nanocomplexes may be superior to individual BLG molecules.

Therefore, the objectives of the current work were i) the assessment of quercetin binding to BLG at various pH levels; ii) evaluating the protection conferred to quercetin after binding to BLG and then deposition of a sodium alginate (ALG) shell around the quercetin-loaded BLG (as the core); iii) evaluating the efficacy of the resultant delivery system (prepared at different biopolymer mixing ratios) in various aspects of entrapment, delivery and controlled release of quercetin; and iv) monitoring the effect of heat processing on the characteristics of the delivery system.

2. Materials and methods

2.1. Materials

Block copolymer sodium alginate (ALG, 200 kDa) with a mannuronate:guluronate (M:G) ratio of 0.6 was obtained from BDH Co. (Poole, UK). The carbohydrate, moisture and total ash contents of ALG were 66.3, 14.2 and 9.5 wt%, respectively. Bovine β-lactoglobulin (BLG, product number L0130, 18.4 kDa, composition (wt%): 93% BLG, 5.4% moisture and 1.6% ash), pepsin (product number P7125, activity: 600–1800 unit/mg protein extracted from porcine gastric mucosa), pancreatin from porcine pancreas (product number P1625, >3 x USP specifications), glucono-δ-lactone (GDL, product number G4750, Purity ≥99.0%) and quercetin (analytical grade, product number Q4951, MW: 302.24 Da, Purity ≥95%) were obtained from Sigma Chemical company (St. Louis, MO, USA). Citric acid, analytical grade hydrochloric acid, sodium azide and absolute ethanol were purchased from Merck Co. (Darmstadt, Germany). In this study, all materials were used directly from the sample containers without further purification taking into account their purity.

2.2. Dispersion preparation

ALG (0.2 wt%) and BLG (0.1 wt%) stock dispersions were prepared in distilled water containing 0.03 wt% sodium azide as preservative. The dispersions were stirred at 250 rpm and 25 °C for 4–5 h and then stored at 4 °C overnight to complete the hydration process.

2.3. Quercetin binding to BLG

Fluorescence spectroscopy was utilized to study the binding of quercetin to BLG by measuring the fluorescence quenching of a tryptophanyl residue (Trp19) of BLG. To determine the effect of pH on the binding of quercetin to BLG, freshly prepared BLG stock dispersions were prepared in either 10 mM phosphate buffer (pH 7) or acetate buffer (pH 4). Insoluble particles, which were present particularly at pH 4, were removed by centrifugation at 10000*g* for 20 min. The concentration of clear BLG dispersion was determined using a UV–visible spectrophotometer at 278 nm and a molar absorptivity of 17600 M^{-1} cm^{-1} (Liang, Tajmir-Riahi, & Subirade, 2007). BLG concentrations were adjusted to 5 μM at both pH values. The quercetin stock solution was prepared freshly in the absolute ethanol at a concentration of 0.2 mM (mM). The stock solutions were prepared in 100-ml volumetric flasks. To avoid the minor errors during the weighing process, the final concentration was adjusted using the molar absorptivity of quercetin. The quercetin solution was then transferred into 2-ml Eppendorfs and covered with aluminum foil. To perform fluorimetry experiment, 1 ml of BLG dispersion was added into the quartz cell and the first emission spectrum recorded (using a Varian Cary Eclipse fluorescence spectrophotometer) at 290–550 nm and 25 °C. The excitation wavelength was 278 nm (Cogan, Kopelman, Mokady, & Shinitzky, 1976). The band slit was 5 nm for both excitation and emission. To determine the binding characteristics, 2-μl aliquots of quercetin stock solution were sequentially added into the protein dispersion. After mixing for 2 min, new emission spectra were recorded. The injections were continued until the ethanol concentration reached to ≈2.7%.

2.4. Quercetin nanoencapsulation

The alcoholic solution of quercetin was added into BLG dispersion (0.025 wt%) to reach a quercetin:BLG molar ratio of 1:1. The dispersion was then stirred for 0.5 h. Nanoencapsulation process was continued by incorporating different amounts of ALG dispersion so that to obtain two different total biopolymer concentrations (TBCs), namely 0.075 and 0.044 wt% (corresponding to mixing ratios (MRs) of 0.5 and 1.33, respectively). Distilled water was then added to reach constant final volumes. The process was finalized by adjusting the pH to 4 and stirring for an additional 0.5 h. Nanoencapsulated quercetin was equilibrated overnight at 25 °C before analysis. The concentration of protein (0.025 wt%) and hence quercetin remained constant in both samples; however, the ALG concentration was different. For each sample, two blanks including a distilled water (biopolymer-free) solution containing similar concentration of quercetin and a quercetin-loaded BLG (ALG-free) dispersion were also prepared and treated in a similar manner. Moreover, quercetin-free mixed biopolymer dispersion was also prepared.

2.5. Evaluation of the encapsulation process

2.5.1. Determination of the encapsulation parameters

To determine the efficiency of quercetin entrapment, free (unloaded) quercetin was separated from quercetin-loaded nanocomplexes using Amicon Ultra centrifugal filter units (MW cut off 10 kDa, Merck Millipore Ltd., Ireland). After centrifugation (4000*g*, 30 min, 25 °C), the concentration of the free quercetin in permeate was determined from a standard curve developed by quercetin standard solutions. Before developing the standard curve, quercetin was dissolved in various water-ethanol mixtures and then subjected to high-speed centrifugation to check for possible precipitation. The results showed that quercetin remained completely soluble in a 35:65 water:ethanol mixture. Therefore, standard solutions of quercetin were prepared by dissolving quercetin in this mixture. After that, the absorption spectrum of quercetin was measured at 300–650 nm. λ_{max} was determined as 373 nm. The standard curve was then generated by measuring the absorbance values of a set of quercetin standard solutions with concentrations ranging from 0.002 to 0.04 mM. The obtained equation was $Abs. = 26.678 \times [quercetin] - 0.0144$. The correlation coefficient was 0.9996. After generating the standard curve, absolute ethanol was added into the permeate so that to reach a 35:65 water:ethanol ratio, followed by measuring the absorbance at 373 nm and then finding the concentration from the standard