Food Chemistry 159 (2014) 333–342

Contents lists available at ScienceDirect

Food Chemistry

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

Cationic β-lactoglobulin nanoparticles as a bioavailability enhancer: Comparison between ethylenediamine and polyethyleneimine as cationizers

Zi Teng [a], Ying Li [a], Yuge Niu [b], Yuhong Xu [c], Liangli Yu [a,b], Qin Wang [a,*]

[a] Department of Nutrition and Food Science, University of Maryland, 0112 Skinner Building, College Park, MD 20742, United States
[b] Institute of Food and Nutraceutical Science, School of Agriculture and Biology, Shanghai Jiao Tong University, Shanghai 200240, China
[c] School of Pharmacy, Shanghai Jiao Tong University, Shanghai 200240, China

ARTICLE INFO

Article history:
Received 30 November 2013
Received in revised form 31 January 2014
Accepted 4 March 2014
Available online 14 March 2014

Keywords:
Cationic beta-lactoglobulin (CBLG)
Ethylenediamine (EDA)
Polyethyleneimine (PEI)
Nanoparticles
Mucoadhesive properties
Secondary structure
Protein digestion

ABSTRACT

Cationic β-lactoglobulin (CBLG) was synthesized by two strategies: extensive conjugation of ethylenediamine (EDA) and limited cationization with polyethyleneimine (PEI). Both methods provided CBLG with satisfactory water solubility and resistance to peptic digestion. Compared with EDA-derived CBLG (C-EDA), PEI-derived CBLG (C-PEI) exhibited a higher zeta potential (54.2 compared to 32.4 mV for C-EDA), which resulted in significantly elevated mucoadhesion (439% and 118% higher than BLG and C-EDA, respectively) in a quartz crystal microbalance (QCM) study. In addition, PEI caused reduced conformational disruption on BLG compared to EDA as evidenced by FTIR measurement. This character, together with the steric hindrance provided by PEI, caused a phenomenal reduction in tryptic digestibility by at least 75% compared to C-EDA. In the presence of aqueous acetone, C-PEI aggregated spontaneously into nanoparticles with average size of 140 nm and narrow size distribution. These merits made C-PEI a useful material that provides desirable solubility and protection for orally administrated nutraceuticals or drugs.

1. Introduction

In the past decades, nano-scaled encapsulation and delivery systems have attracted increasing attention as transporters for nutraceuticals or drugs (Augustin & Hemar, 2009; Jahanshahi & Babaei, 2008). Numerous nanoencapsulation strategies and systems have been developed by now, providing target compounds with desirable solubility (Luo & Wang, 2013), satisfactory stability (Guzey & McClements, 2006), controlled release properties (Teng, Luo, & Wang, 2013) and elevated bioavailability (Luo, Teng, Wang, & Wang, 2013). It is generally believed that the success of delivery is highly dependent on the surface properties of the encapsulant, such as charge and hydrophobicity (Futami, Kitazoe, Murata, & Yamada, 2007). Cationic polymers such as chitosan (Agnihotri, Mallikarjuna, & Aminabhavi, 2004), polylysine (Mislick, Baldeschwieler, Kayyem, & Meade, 1995) and lactoferrin (Bengoechea, Jones, Guerrero, & McClements, 2011), have demonstrated significantly higher mucoadhesive capacity and cellular internalization compared with anionic macromolecules. This phenomenon was largely attributed to two factors. The first factor is the affinity of the polycations to the negatively charged glycoproteins, the latter of which are abundant on the membrane of epithelia cells or tissues (e.g., small intestine wall) (Blau, Jubeh, Haupt, & Rubinstein, 2000). The second advantage for cationic encapsulants is their ability to acquire a negatively charged "corona" consisting mostly of serum proteins, which bind strongly to specific receptors on the cell membrane and thus promote the cellular internalization (Wang et al., 2013).

A wide array of cationic polymers have been exploited as novel encapsulating systems (Agnihotri et al., 2004; Bengoechea et al., 2011; Qi et al., 2012). However, these polymers are either insoluble at neutral pH (e.g., chitosan) or susceptible to digestion by pepsin or trypsin (e.g., polylysine). Therefore, the protection provided by these materials would be easily diminished when they enter the

Abbreviations: BLG, beta-lactoglobulin; CBLG, cationic BLG; PEI, polyethyleneimine; EDA, ethylenediamine dihydrochloride; C-EDA, EDA-derived CBLG; C-P600 and C-P1200, CBLG synthesized with PEI-600 and PEI-1200; EDC, *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide; PSM, porcine stomach mucin; TCA, trichloroacetic acid; PBS, phosphate buffer saline; QCM, quartz crystal microbalance; MALDI-TOF, matrix assisted laser desorption/ionization time-of-flight mass spectrometry; SEM, scanning electron microscopy; FT-IR, Fourier transform infrared spectroscopy; FSD, Fourier self deconvolution.

* Corresponding author. Tel.: +1 (301) 405 8421; fax: +1 (301) 314 3313.
E-mail address: wangqin@umd.edu (Q. Wang).

http://dx.doi.org/10.1016/j.foodchem.2014.03.022
0308-8146/

gastrointestinal tract, which is one of their major drawbacks. β-lactoglobulin (BLG), a 162-residue globulin, makes up approximately 60% of bovine whey protein (Kontopidis, Holt, & Sawyer, 2004). BLG is highlighted for its high surface charge and abundance of rigid β-sheet secondary structures. The first characteristic ensures the dispersion stability of BLG-based encapsulating systems even near its isoelectric point (~pH 5.0). The second property endows BLG with remarkable resistance against peptic digestion (Ko & Gunasekaran, 2006), thus providing maximal stability and controlled release for orally administrated bioactives.

To take advantage of both BLG and cationic polymers, our lab synthesized cationic BLG (CBLG) by grafting ethylenediamine (EDA) to the glutamic acid (Glu) or aspartic acid (Asp) residues of BLG (Teng, Li, Luo, Zhang, & Wang, 2013). The products exhibited highly positive surface charge, resulting in significantly improved mucoadhesion. In addition, they inherited the resistance to pepsin from BLG, whilst the digestion by trypsin was significantly promoted. A possible explanation for this phenomenon is the formation of Glu-/Asp-EDA conjugates (Teng, Li, Luo, Zhang, & Wang, 2013), which showed similar geometry and electric charge status to lysine. Since lysine is a known substrate for trypsin (Mattarella & Richardson, 1983), generation of Glu-/Asp-EDA conjugates might result in a significant increase in tryptic digestibility. Although increased tryptic digestibility provided EDA-derived CBLG with a certain degree of controlled release property, such change was unfavourable for delivering bioactive compounds that require prolonged protection after leaving the digestive tract and entering the circulatory system. Furthermore, the disintegration of EDA-derived CBLG implied the inability to gain the serum protein corona during circulation (Wang et al., 2013). This could not only compromise the efficacy of cellular uptake, but also lead to considerable cytotoxicity, since the corona plays a crucial role in counterbalancing the toxicity of cationic polymers (Lundqvist, 2013). Increased digestibility of CBLG, therefore, might be detrimental for its function as a bioavailability enhancer for certain nutraceuticals or drugs.

Polyethyleneimine (PEI, structure shown in Supplementary Fig. S1) is a cationic polymer whose repeating units consist of an amine and two methylene groups (–NH–CH_2–CH_2–). Owing to its low toxicity (Benjaminsen, Mattebjerg, Henriksen, Moghimi, & Andresen, 2012; Wiegand, Bauer, Hipler, & Fischer, 2013), PEI is permitted by the FDA to be added as an enzyme immobilizing agent in the beer industry (Carpenter, 1996). Amongst all PEI categories, branched PEI contains abundant primary amino groups that are reactive for cationization. Because of the high density of charge (one unit of positive charge in a mass unit of 43), conjugation of only a few PEI molecules is sufficient to introduce a significant amount of positive charges to the protein, without greatly altering its conformation (Futami et al., 2005). By far, PEI has been used for cationizing various proteins (Futami et al., 2007), resulting in significantly improved cellular uptake and low cytotoxicity.

Hereby, we propose the synthesis of CBLG using branched PEI as a cationizer. The anticipated structures of EDA-derived CBLG (C-EDA) and PEI-derived CBLG (C-PEI) are illustrated in Fig. 1. We hypothesised that C-PEI would exhibit similar resistance against pepsin, with superior mucoadhesion compared to BLG or C-EDA. Meanwhile, the product was expected to maintain its integrity under simulated intestinal conditions, because of the absence of Asp-/Glu-EDA conjugate, as well as steric hindrance provided by the PEI backbone. The product was compared in parallel with BLG and C-EDA, with respect to its conjugation degree, surface charge, secondary structure, *in vitro* digestibility and mucoadhesion. Finally, C-PEI-based nanoparticles were fabricated by organic solvent desolvation method, and the unique particle forming behaviours of C-PEI were reported.

2. Materials and methods

2.1. Materials

The following chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA): bovine BLG (90% purity), ethylenediamine dihydrochloride (EDA, 98% purity), branched polyethyleneimine (PEI600 and PEI1200, with average MW of 600 and 1200 Da, respectively), *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide (EDC, 97% purity), sinapinic acid, pepsin (3200–4500 units/mg), trypsin (10,000 BAEE units/mg), and trichloroacetic acid (TCA). Porcine stomach mucin (PSM, type III, containing 0.5–1.5% sialic acid) was obtained from Himedia Co., India. All other reagents (trifluoroacetic acid, simulated digestive fluids, etc.) were of analytical grade.

2.2. Preparation of cationic beta-lactoglobulin (CBLG)

CBLG was prepared by grafting cationic EDA or PEI moieties to the anionic carboxyl groups on the Asp and Glu residues of native BLG (Teng, Li, Luo, Zhang, & Wang, 2013). This was achieved via a previously reported EDC-aided reaction (Lu et al., 2005). BLG and the cationizers (EDA or PEI) were dissolved in deionized water at 20 and 100 mg/ml, respectively. The pH was then adjusted to 4.75 using 1 M HCl for BLG solution or concentrated HCl for cationizer dispersions. Thereafter, 5 ml of BLG solution was added slowly to 30 ml cationizer dispersion under mild stirring, and the mixture was incubated at room temperature for 15 min. Cationization was initiated by the addition of 30 mg EDC and terminated by adding 108 μl sodium acetate buffer (4 M, pH 4.75) after 4 h. The resultant dispersion was subjected to dialysis centrifugation (5000*g*, 30 min), using a Macrosep® centrifuge tube (Pall Corp., Ann Harbor, MI) with a built-in filtering membrane. Tubes with different MW cutoff values (10,000 for EDA and PEI-600-derived CBLG and 100,000 for PEI-1200-derived CBLG) were chosen for different samples in order to maximise the removal of unreacted chemicals, without compromising protein yield significantly. The retentate obtained after centrifugation was further dialyzed against deionized water at 4 °C for at least 48 h and freeze dried. The moisture content (Labuza, Tannenbaum, & Kerel, 1970) of the final product was less than 5%, and the protein content was above 90% according to a Bradford assay calibrated with BSA. The samples were designated as C-EDA for EDA-derived CBLG and C-P600/C-P1200 for CBLG synthesized with PEI-600/PEI-1200.

2.3. Determination of molecular weight and net charge

The molecular weights (MW) of BLG and CBLG were determined by matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry in linear mode (Dai, Whittal, & Li, 1999). The samples were dispersed at 25 mg/ml in deionized water, which contained 1 μl/ml trifluoroacetic acid (TFA). The matrix solution was prepared by dissolving 10 mg sinapinic acid in a mixture of 200 μl pure acetonitrile and 200 μl water (containing 0.2 μl TFA). Prior to analysis, 1 μl matrix solution was spotted onto the target plate, and 1 μl of the sample solution was doped above the matrix layer. A second matrix layer (0.5 μl) was then dripped on top of the aforementioned two layers. Each layer was air dried at room temperature before doping the next one. The target plate was then loaded into a Shimadzu Axima-CFR MALDI-TOF spectrometer (Shimadzu North America, Columbia, MD, USA). Spectra were obtained by illuminating the samples with a N_2 laser beam (130 mJ per shot, 2 shots per second) and analysed with the Kompact software. For each sample, at least 150 spot spectra were