Food Hydrocolloids 25 (2011) 1270–1276

Contents lists available at ScienceDirect

Food Hydrocolloids

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

Re-assembled casein micelles and casein nanoparticles as nano-vehicles for ω-3 polyunsaturated fatty acids

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ARTICLE INFO

Article history:
Received 2 May 2010
Accepted 29 November 2010

Keywords:
Casein
Casein micelles
DHA
Omega-3
Nanoencapsulation
Nutraceuticals

ABSTRACT

Food enrichment with nutraceuticals is an important goal, but its effectiveness in preventing diseases depends on preserving the functionality and bioavailability of the bioactive nutraceuticals. Omega-3 polyunsaturated fatty acids, such as docosahexaenoic acid (DHA), are important nutraceutical lipids, providing protection against cardiovascular and other diseases. Caseins are the major milk proteins whose biological function is to transport calcium, protein and phosphate from mother to the neonate. Our goal was to harness the natural self-assembly properties of caseins for protecting and delivering this important, but sensitive nutraceutical, DHA. Using spectrofluorescence we have shown, apparently for the first time, that casein can bind DHA with a relatively high affinity ($K_b = (8.38 \pm 3.12) \times 10^6\ M^{-1}$), and the binding ratio was 3–4 DHA molecules per protein molecule on average. Moreover, DLS particle characterization experiments have shown the formation of nanoparticles upon addition of DHA (pre-dissolved in ethanol) to a casein solution. When calcium and phosphate were added (at 4 °C), DHA-loaded re-formed casein micelles (r-CM) with a size of 50–60 nm were obtained and there was no significant effect of the thermal treatment (74 °C, 20 s) on particle size. When casein nanoparticles (CNP) were prepared (at room temperature and without adding calcium and phosphate), DHA-loaded CNP with a diameter of 288.9 ± 9.6 nm were formed. Both the DHA-loaded r-CM and the DHA-loaded CNP systems showed a remarkable protective effect against DHA oxidation, demonstrating good colloidal stability and bioactive conservation throughout shelf life at 4 °C. These nanotechnologies may enable the enrichment of foods and beverages for promoting health of wide populations.

1. Introduction

With the growing public realization of the important role of food in disease prevention, new technologies are being introduced to enrich staple foods and beverages with health promoting substances and form functional foods. Fat-soluble nutraceuticals pose a particularly difficult challenge, especially in fat free and low fat foods, which are in growing demand. Most of these hydrophobic compounds, like fat-soluble vitamins and essential fatty acids are highly sensitive to oxidation, and thus require stabilization in an aqueous medium and protection against deteriorating factors. The formation of nano-vehicles for the delivery of hydrophobic nutraceuticals has been shown to facilitate their dissolution and protection. A particularly significant challenge is the delivery in aqueous food systems of omega-3 polyunsaturated fatty acids (ω-3 PUFA), of which the main representative is docosahexaenoic acid (DHA (22:6)). Many health benefits have been attributed to omega-3 fatty acids, mainly in reducing the risk of cardiovascular diseases and the metabolic syndrome. More specific attributed effects include antithrombotic, antiatherogenic and anti-inflammatory properties, lowering blood pressure, serum cholesterol and triglyceride levels (Lopez-Huertas, 2010; Ruxton, Calder, Reed, & Simpson, 2005; Yashodhara et al., 2009). Other reported beneficial impacts include prevention or delay of cognitive decline and dementia (Beydoun, Kaufman, Satia, Rosamond, & Folsom, 2007; Ruxton et al., 2005; van Gelder, Tijhuis, Kalmijn, & Kromhout, 2007), and reduction of bone mineral loss (Bhattacharya, Rahman, Sun, & Fernandes, 2007; Hogstrom, Nordstrom, & Nordstrom, 2007), as well as cancer-preventive effects (Berquin, Edwards, & Chen, 2008). Moreover, research indicates long-chain ω-3 polyunsaturated fatty acids (LC ω-3 PUFA) provision has an impact during development, and there is preliminary evidence that DHA supplementation during pregnancy could optimize brain and retina development in the infant (Ruxton et al., 2005).

Because of its polyunsaturated structure, DHA is highly prone to oxidation leading to undesirable off-flavors and odors in the enriched

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doi:10.1016/j.foodhyd.2010.11.025

food product. In addition, DHA is very hydrophobic and practically insoluble in water, even in the free fatty acid form (Serth, Lautwein, Frech, Wittinghofer, & Pingoud, 1991). Several reported approaches for omega-3 delivery in food and beverage systems include gum arabic-based emulsions (Zhang, Yan, May, & Barrow, 2009), micro-emulsions (Jakobsson & Sivik, 1994), multi-layered emulsions (Gudipati, Sandra, McClements, & Decker, 2010), Maillard reaction conjugates (Kosaraju, Weerakkody, & Augustin, 2009), and amylose based inclusion complexes (Zabar, Lesmes, Katz, Shimoni, & Bianco-Peled, 2010). Still, omega-3 oils and fatty acids pose a difficult challenge for delivery in food systems, while protecting their beneficial properties and using natural ingredients only.

Milk proteins have important functional properties such as the ability to bind hydrophobic molecules, interact with other biopolymers, stabilize emulsions, form gels, and to some extent retard oxidation. Because of these properties, milk proteins are ideal materials for the entrapment and delivery of bioactive compounds (Livney, 2010). In a previous study, we have successfully entrapped DHA within nanocomplexes of a milk protein, beta-lactoglobulin, and a polysaccharide, pectin. The new process allowed the delivery of DHA in clear aqueous systems and effectively retarded DHA oxidation (Zimet & Livney, 2009). Caseins comprise about 80% of milk protein and consist of four principal proteins: α_{s1}-, α_{s2}-, β- and κ-casein, accounting for about 38, 10, 35 and 15% of whole casein, respectively (Fox, 2003). Owing to their serine-phosphate clusters, the two α_s-caseins and the β-casein are Calcium (Ca^{2+})-sensitive, meaning that they are easily precipitated by mM concentrations of Ca^{2+}, whereas κ-casein is insensitive to Ca^{2+} (Fox, 2003; de Kruif & Holt, 2003). The biological function of caseins in the mammary gland is to transport calcium, phosphate and protein to the neonate (de Kruif & Holt, 2003). Caseins have a very strong tendency to associate, which is very applicable for nanoencapsulation purposes. About 95% of the casein in milk exists as colloidal particles known as micelles. These are generally spherical with a diameter ranging from 50 to 500 nm (150 nm average) and a mass ranging from 10^6 to 3×10^9 Da (average 10^8 Da) (Fox & Brodkorb, 2008). Over the past 50 years, a variety of models has been proposed to describe the structure of casein micelles, based on the chemical and physical properties of casein. Many of these models emphasize the roles of hydrophobic interactions between the caseins as well as the role of calcium and phosphate (and their nanoclusters) in bridging between the serine-phosphate groups of the caseins and forming the micelle (Horne, 2003; de Kruif & Holt, 2003; Liu & Guo, 2008; McMahon & McManus, 1998; Walstra, 1990).

Casein micelles may be reassembled in vitro (Knoop, Knoop, & Wiechen, 1979), presenting similar properties to those of the naturally occurring casein micelles. In a study done in our laboratory, it was suggested for the first time, that these re-assembled casein micelles (r-CM) can be loaded with a hydrophobic nutraceutical, such as Vitamin D_2, and used as a nanodelivery system, inspired by their natural role as nutrient delivery nano-vehicles. These r-CM, that had average diameters similar to those of natural casein micelles, stabilized the hydrophobic vitamin in an aqueous medium and provided significant protection against UV-light-induced degradation to vitamin D_2 (Semo, Kesselman, Danino, & Livney, 2007).

The aim of the present research was to study the binding of DHA to casein and then, entrap DHA within re-assembled casein micelles. This may allow the delivery of this important and sensitive hydrophobic nutraceutical in food products, ideally without affecting their sensory properties, and provide an effective protection to DHA.

Our approach for the formation of these nano-vehicles included first the binding of DHA to casein, by pre-dissolving DHA in a water-miscible food grade solvent such as ethanol, then adding it to a caseinate solution while stirring. The final ethanol concentration is merely ~1.25%, a rather negligible amount, which may be evaporated or dialyzed-out if necessary in the unlikely case where even this trace amount would be deemed intolerable. We evaluated two different ways to achieve the formation of casein nano-vehicles, i.e. with and without the addition of calcium and phosphate. We hypothesize that the co-assembly of casein particles and DHA without the presence of calcium and phosphate would rely predominantly on hydrophobic interactions.

2. Materials and methods

2.1. Materials

Sodium Caseinate (93.5% protein) was obtained from Meggle (Molkerei Meggle Wasserburg GmbH & Co. KG). DHA, *N*-acetyl-tryptophanamide (NATA), Trizma Base and tri-potassium citrate were purchased from Sigma–Aldrich, (Rehovot, Israel). Ethanol (absolute) and HCl were obtained from Bio-Lab (Jerusalem, Israel). K_2HPO_4 was purchased from Spectrum Chemical MFG. Corp. (Gardena, CA, USA). $CaCl_2$ was purchased from Loba Chemie Pvt. Ltd. (Mumbai, India). NaOH was purchased from Merck KGaA (Darmstadt, Germany). Ethylene diamine tetra-acetic acid (EDTA) was obtained from Acros (NJ, USA).

2.2. Methods

All measurements were performed at ambient temperatures (22–25 °C), unless otherwise stated.

2.2.1. Binding of DHA to casein

The binding reaction was studied using fluorescence quenching, based on the method described by Cogan, Kopelman, Mokady, and Shinitzky (1976). Measurements of the binding-induced quenching of the intrinsic fluorescence of tryptophanyl (TRP) residues found in the hydrophobic domains of all caseins (Swaisgood, 2003), were made using excitation and emission wavelengths of 287 and 332 nm respectively. Protein solutions in a 5 mM phosphate buffer, at pH 7.0 were titrated with 3–12 μl incremental aliquots of DHA dissolved in absolute ethanol. An *N*-acetyl-tryptophanamide (NATA) blank was used to eliminate the effect of non-binding-induced quenching, as NATA fluoresces similarly to tryptophan, but does not bind DHA (Cogan et al., 1976).

Measurements were performed in duplicate. To determine the parameters of the binding reaction of DHA to caseinate, the mass law equation (equation (1)) (Cogan et al., 1976) was used

$$K'_d = \frac{1}{K'_b} = \frac{n[P][L]}{[PL]} \tag{1}$$

where K'_d is the apparent dissociation constant of a single site, K'_b is the apparent binding constant of a single site, n is the number of independent binding sites with the same (or higher) binding affinity for DHA as the site(s) near the fluorescent TRP residues (sites with higher affinity are saturated earlier, thus are also accounted for by "n" during gradual titration), $[P]$ is the free protein concentration, $[L]$ is the free ligand (DHA) concentration and $[PL]$ represents the concentration of ligand bound to the protein. Equation (1) had been further derived into

$$[P]_0\alpha = \frac{1}{n}\frac{[L]_0\alpha}{1-\alpha} - \frac{1}{nK'_b} \tag{2}$$

where α represents the fraction of free binding sites on the protein molecules, $[P]_0$ is the total protein concentration and $[L]_0$ is the total DHA concentration. The fraction of free binding sites, α, may be