Food Hydrocolloids 38 (2014) 40–47

Contents lists available at ScienceDirect

Food Hydrocolloids

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

Milk protein–vitamin interactions: Formation of beta-lactoglobulin/folic acid nano-complexes and their impact on *in vitro* gastro-duodenal proteolysis

Oscar E. Pérez [a,*], Tatyana David-Birman [b,c], Ellina Kesselman [b], Sharon Levi-Tal [b], Uri Lesmes [b,c]

[a] Departamento de Industrias, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires and Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Ciudad Universitaria, 1428 Buenos Aires, Argentina
[b] Faculty of Biotechnology & Food Engineering, Technion – Israel Institute of Technology, Haifa 32000, Israel
[c] Russel Berrie Nanotechnology Institute, Technion – Israel Institute of Technology, Haifa 32000, Israel

ARTICLE INFO

Article history:
Received 8 April 2013
Accepted 8 November 2013

Keywords:
β-lactoglobulin
Folic acid
Nano-complexes
Nano-particles
In vitro digestion

ABSTRACT

Growing attempts are being made to rationally utilize foods for human health improvement and disease prevention. Milk proteins are well suited for this purpose, since they are widely consumed, offer nutritional benefits and have been shown to be potentially suitable carriers for bioactive ingredients, such as vitamins and nutraceuticals. This work characterizes the interactions between β-lactoglobulin (β-lg) and folic acid (FA) at different load ratio and their functional implications, in terms of colloidal behavior and digestibility. Dynamic light scattering, isothermal titration calorimetery and atomic force microscopy were used to investigate β-lg/FA nano-complexes (mean size < 10 nm) formed at protein:vitamin molar ratio 1:10, whereas three FA molecules were found to be bound to one protein molecule. Colloidal stability tests ($3 < pH < 10$) revealed that nano-complexes formation improved β-lg dissolution near its isoelectric point and at low pH-values. This was also found to be accompanied by a shift in zeta-potential values at $pH = 5$ for pure β-lg (0.95 ± 0.09 mV) versus β-lg/FA nano-complexes (-20.13 ± 1.29 mV). SDS-PAGE analysis of digesta, collected from gastric and duodenal *in vitro* digestion of β-lg and its nano-complexes, revealed no marked alterations in the proteolytic susceptibility of β-lg. The study findings show the interactions of FA and β-lg in the formation of nano-complexes may be harnessed for delivery of FA in clear beverages with minimal effects to the protein's sensitivity to proteolysis.

1. Introduction

Food manufacturers and consumers increasingly seek strategies and products that optimally utilize food to delineate positive health outcomes beyond food's nutritional value. Thus, many recent studies look into natural ways to harness macronutrients, i.e. proteins, carbohydrates and lipids for efficient delivery of micronutrients and bioactive compounds (Benshitrit, Levi, Tal, Shimoni, & Lesmes, 2012). To this end, significant advances have been made in respect to the formation and rational design of particulate-based delivery systems (Augustin & Hemar, 2009; Chen, Remondetto, & Subirade, 2006; Dickinson, 2012; Jones & McClements, 2011; Lesmes & McClements, 2009; Matalanis, Jones, & McClements, 2011; Velikov & Pelan, 2008). Milk proteins are widely accepted as alimentary elements appropriate for delivery of bioactives, since milk is a remarkable component of human diet. Thus, various milk proteins have received considerable attention, both as potential delivery vehicles and as precursors of bioactive peptides that may form even during digestion (Agyei & Danquah, 2012; Livney, 2010; Nagpal et al., 2011; Relkin & Shukat, 2012; Zimet & Livney, 2009).

Milk contains various proteins that can bind and interact with a variety of biopolymers, molecules and ions to varying extent and ramifications. Among them there is β-lactoglobulin (β-lg), a major constituent of whey, a globular protein with a hydrodynamic radius of about 2 nm, a molar mass of 18.2 kg mol^{-1}, containing two disulfide bridges and one free thiol (Hambling, Alpine, & Sawyer, 1992). All the structural data concerning β-lg suggest that this protein is a member of a lipocalin structural family of hydrophobic molecule transporters (Monaco et al., 1987; Papiz et al., 1986). Its actual biological function is still unclear; however, it has been

* Corresponding author. Tel.: +54 11 4576 3377; fax: +54 11 4576 3366.
E-mail addresses: uri_lesmes@hotmail.com, operez@di.fcen.uba.ar (O.E. Pérez).

0268-005X/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.foodhyd.2013.11.010

reported to bind small hydrophobic ligands, such as curcumin, retinol, fatty acids, protoporphyrin IX, triacylglycerols, aliphatic ketones, aromatic compounds, tocopherols, cholesterol and calcium (Liang & Subirade, 2010; Liang, Tremblay-Hebert, & Subirade, 2011; Madureira, Pereira, Gomes, Pintado, & Malcata, 2007; Perez & Calvo, 1995; Relkin & Shukat, 2012; Sneharani, Karakkat, Singh, & Rao, 2010).

The interactions of β-lg with various ligands may modulate its physicochemical properties and colloidal properties, which in turn can have implications to the biological and digestive fate of the β-lg/ligand complexes. For example, β-lg interactions with physiological surfactants, namely phosphatidylcholine, has been reported to affect its susceptibility to gastrointestinal proteolysis (Mandalari, Mackie, Rigby, Wickham, & Mills, 2009). In addition, soluble miniaturized complexes of β-lg with alpha-tocopherol, resveratrol, curcumin or folic acid (FA) have been shown to delay to different extents the degradation of these liable bioactives (Liang & Subirade, 2010; Liang, Tajmir-Riahi, & Subirade, 2008; Liang et al., 2011; Sneharani et al., 2010). This is part of a spur of interest in milk proteins as vehicles for the controlled and targeted delivery of nutraceuticals (Benshitrit et al., 2012; Livney, 2010).

Nowadays, it is widely known that FA and its bioequivalent folates are essential dietary components. This vitamin has been suggested to be effective in decreasing the risk for cardiovascular diseases (Adank, Green, Skeaff, & Briars, 2003), colon cancer (La, Negri, Pelucchi, & Franceschi, 2002), neurological illnesses such dementia and Alzheimer's disease (Miller, 2003; Reynolds, 2002). It is most commonly known for its key role in women nutrition before conception, during pregnancy and lactation (Madziva, Kailasapathy, & Phillips, 2006). Moreover studies show that in spite of the hydrophilic nature of FA, it may self-assemble into unique fine structures even at low concentrations such as 0.1% (w/w) through hydrogen bonds and stacking interactions (Bonazzi, Demorais, Gottarelli, Mariani, & Spada, 1993; Ciuchi et al., 1994; Motkar, Lonare, Patil, & Mohanty, 2013). Functionally, bovine milk has been found to contain various proteins that naturally bind FA (Elkanat & Ratnam, 2004; Nygren-Babol & Landtröm Karonem, 2009).

In light of this, the staple milk protein β-lg was selected as a potential natural vehicle for FA at low concentrations, which can expand to a potential milk enrichment study in the future. Adapting a biomimetic approach, this work was aimed to produce β-lg/FA nano-complexes and to characterize some of their physicochemical attributes. Particularly, this work sought to characterize β-lg interactions with FA and their impact on colloidal size, morphology, and *in vitro* proteolysis of β-lg under conditions, modeling an adult stomach and duodenum.

2. Materials and methods

2.1. Materials and enzymes

BioPURE β-lg powder was provided by DAVISCO Foods International, Inc. (Le Sueur, MN, USA). According to the manufacturer protein content was 97.8% (w/w dry basis) and β-lg making up 93.6% (w/w) of total proteins, 0.3% (w/w) fat, 1.8% (w/w) ash and 5.0% (w/w) moisture. FA (>97%) was purchased from Sigma–Aldrich Chemical Company (Rehovot, Israel). Both β-lg and FA were used without further purification.

Simulated gastric fluid (SFG) consisted of 35 mM KCl, 1.125 mM KH_2PO_4, 13 mM $NaHCO_3$, 40 mM NaCl, 0.6 mM $MgCl_2$, 1 mM NH_4Cl and 0.225 mM urea. Simulated intestinal fluid (SDF) consisted of 6.25 mM KCl, 1 mM KH_2PO_4, 85 mM $NaHCO_3$, 32 mM NaCl, 0.27 mM $MgCl_2$ and 1.8 mM urea. All simulated fluids were compiled based on previously described bio-relevant digestion models (Hur, Lim, Decker, & McClements, 2011; Kopf-Bolanz et al., 2012).

2.1.1. Preparation of protein–vitamin complexes

Powder samples of β-lg and FA were dissolved separately in Milli-Q ultrapure water at room temperature under agitation. The solutions were prepared freshly, filtered through 0.45 μm and 0.22 μm microfilters (Whatman International Ldt, Maidstone, England) and kept 24 h at 4 °C to achieve the complete hydration of the molecules. Sample pH was adjusted to 7.0 using 1 M HCl or 1 M NaOH (analytical grade materials). The β-lg/FA nano-complexes were produced by mixing the appropriate volume of the double concentrated solutions at pH 7 to give the required final concentrations of the protein and vitamin ligand in the bulk solution.

2.2. Physicochemical characterizations

2.2.1. Characterization of key colloidal properties

Particle size (*Z*-average diameter) and ζ-potential of the various samples were determined using a combined DLS and particle electrophoresis instrument (Nano-ZS, Malvern Instruments, Worcestershire, UK). Samples were diluted with filtered double distilled water, equilibrated for 1 min inside the instrument before dynamic light backscattering (detection angle = 173°). Data was collected over at least 12 sequential readings to determine the electrophoretic mobility of the samples. The ζ-potential of the particles was calculated using the Huckel model based on the rational that for the investigated nano-complexes and soluble assemblies are close to the dimensions of the globular protein (for which the Debye length at the experimental conditions was estimated as ~0.3 nm and the size 2–2.6 nm based on previous studies (Rabiller-Baudry, Bouguen, Lucas, & Chaufer, 1998)), thereby, rendering the Huckel model more suitable than the Smoluchowski model. The *Z*-average particle diameter was also determined through DLS measurements, whose data was translated into *z*-average sizes using the Stokes–Einstein equation (Stepanek, 1993). These experiments monitored FA bulk concentrations of 0.02, 0.03, 0.04, 0.1 and 0.2% (w/w).

2.2.2. Characterization through Isothermal Titration Calorimetry (ITC)

In order to better characterize the interactions between β-lg and FA the enthalpy change arising from their association was monitored by isothermal titration calorimeter, VP-ITC instrument (MicroCal, Inc.) at 298 K, controlled by Origin software. In these experiments a stainless steel cell filled with β-lg solution (2.2 mg/mL) was injected with controlled volumes of FA stock solution. This titration was carried out by sequential injections of FA titrant solution (2.75 mg/mL of FA) from a 250 μL injection syringe. Each injection took 5 min, and there was an interval of 20 min between every successive addition and the energy dissipation was recorded. The solution in the reaction cell was stirred at 60 rpm. The results are reported as triplicate averages with heat of dilution of pure ingredients into double distilled water, subtracted from all the other curves.

2.2.3. Morphological study of β-lg/FA complexes using atomic force microcopy (AFM)

In order to study fine morphological characteristics of β-lg/FA complexes, atomic force microscopy was applied. In practice, 2 μL of sample solution were placed on a freshly cleaved mica slide (SPI-Chem TM Mica, Grade V-4, 9.9 mm discs of 0.15 mm thickness; West Chester, PA). Specimen slides were then stored in a desiccator before scanning. The air dried mica specimens were immersed in butanol for scanning, as described before in literature (Ikeda, Morris, & Nishinari, 2002; Roesch, Cox, Compton, Happek, & Corredig, 2004). Butanol repulses all the bound water from the sample surface, and protects hydrophilic structures from swelling atmospheric water vapors. Due to these properties, butanol is