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).

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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

widely used as an inert medium for improving image quality. The scans were performed using a JPK Nano Wizard II AFM (JPK Instruments Inc., Germany) mounted with a silicon tip (mMasch NSC21/NoAl, Spain; tip curvature Rc < 10 nm; typical force constant 17.5 N/m). For each sample, various scan areas were probed (10 μm × 10 μm, 5 μm × 5 μm, 2 μm × 2 μm, 1 μm × 1 μm, 500 nm × 500 nm) at intermittent contact mode, and images were processed using Nano Wizard® control software v.4. Average and RMS surface roughness of samples were determined basing on cross sections, performed on 1 μm × 1 μm height AFM measurements (values presented as mean ± SD, $n = 60$).

Also, the impact of FA on the colloidal properties of β-lg was investigated by studying the pH responsiveness of formed β-lg/FA complexes. These experiments included the preparation of 0.2% (0.11 mM) β-lg/0.05% (1.13 mM) FA complexes (molar ratio β-lg : FA was 1:10) at pH = 7 which was then adjusted to various pH conditions using NaOH or HCl before being studied in terms of size, by DLS, and appearance, by direct observation.

2.3. Simulated gastro-duodenal digestion and characterization of digesta

In order to gain evidence into the potential digestive fate of β-lg/FA complexes, an *in vitro* gastric model was used. In these experiments a water jacketed reactor (6.1418.250, Metrohm, Switzerland) was maintained at 37 °C and continuously stirred at 250 rpm while controlled by a dual auto titration unit (Titrando 902, Metrohm, Switzerland) which gradually varied reactor's pH from 4.5 to 1.5 using 0.5 M HCl during a 1 h gastric digestion phase and then elevated pH to 6.25 using 1 M ammonium bicarbonate and maintained pH for 1 h of duodenal digestion, as recently reported (Shani-Levi, Levi-Tal, & Lesmes, 2013). Timed changes in pH were pre-programmed using "TIAMO" software (Metrohm, Switzerland) which controlled the auto-titrators during the experiments. In each experiment, 20 mL samples of 0.2% β-lg or 0.2% β-lg and 0.05% FA were dissolved in 39 mL of simulated gastric fluid (SGF), and then the pH was adjusted to 4.5 prior to experiment initialization. Immediately at the beginning of the digestion program, 1 mL pepsin solution (71 mg/mL, pre-dissolved in SGF pH 4.5) and 15 μL of 2 M $CaCl_2$ were added to reach a final concentration of 1000U of pepsin per mL SGF and 0.2 mM $CaCl_2$. Following 1 h of gastric digestion, bioreactor's pH was elevated to 6.25 and 0.5 mL pancreatin solution (80 mg/mL, pre-dissolved in SDF pH 7) was added to reach a final concentration of 100U (based on protease activity) per mL of sample within the reactor vessel. Additionally, two bursts of glycodeoxycholate (80 mM) and taurocholic acid sodium salt hydrate (80 mM) dissolved in SDF (pH 6.5) were added to reach a final concentration of 4 mM in the duodenal phase. These bursts of 2 mL and 1 mL were added at the beginning of the duodenal phase and after 30 min, respectively, to account for physiological secretion of bile, according to previous work (Blanquet et al., 2004; Dupont et al., 2010). Digesta samples were aspirated after 0, 5, 30, 60 min during both digestion phases and were rapidly neutralized by elevating the pH to 7 using 1 M NH_4HCO_3 at the gastric phase or by adding 40 μL of 3.85 mM PMSF for duodenal digesta. Samples were then diluted with sample buffer (1:5 v/v) for SDS-PAGE analysis. Overall, digestion protocols and media relied on integration of recent publications of bio-relevant *in vitro* digestion models (Dupont et al., 2010; Kopf-Bolanz et al., 2012; Macierzanka, Sancho, Mills, Rigby, & Mackie, 2009a; Mandalari, Adel- Patient, et al., 2009; Shani-Levi et al., 2013).

2.3.1. Characterization of proteolysis products by SDS-PAGE

Comparison of the peptide breakdown profiles in the chyme samples collected during the gastro-duodenal digestions was based on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). All chemicals and gels used for SDS-PAGE analysis were purchased from Bio-Rad Ltd. (Israel). Acryl amide gels of 15% were run for 60 min at constant 150 V in a Mini-Protean Tetra Cell unit. A continuous buffer system was used, consisting of premixed electrophoresis buffer. The buffer contained 25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3 and was diluted 10 times with water before usage. Afterwards, gels were stained by Coomassie brilliant blue R250 and individual gels were imaged with a Microtek 9800XL Plus scanner (Microtek, Carson, CA).

2.3.2. Statistical analysis

All the determinations were made at least in duplicates and each value represents the mean of at least two measurements performed on different aqueous solutions. Specifically, all digestion experiments were run in triplicates. Statistical analyses (ANOVA and/or *t*-test) were performed using Microsoft Excel 2010 data analysis tool pack.

3. Results and discussion

3.1. Physicochemical characterizations of β-lg/FA nano-complexes

It is generally known that food proteins are susceptible to pronounced self-assembly and aggregation processes in aqueous media in response to changes in the environmental conditions and composition. Recently described in literature (Liang & Subirade, 2010), β-lg/FA nano-complexes have yet to be thoroughly investigated in terms of their size, ζ-potential and colloidal behavior using DLS. The sizing of pure FA solutions (data not shown) revealed that increasing FA concentrations beyond 0.1% (w/w) yielded increasingly bigger and polydisperse colloid samples, concurring with a recent report showing FA ability to form self-associated entities (Motkar et al., 2013). Here, the effect of increasing amount of FA on β-lg was evaluated in terms of size and ζ-potential using DLS (Fig. 1). These measurements revealed that FA addition affected sample particle size distribution curves and doubled the values of mean size from less than 1 nm for pure β-lg to about 2 nm for samples containing β-lg/FA nano-complexes (Fig. 1A).

ζ-potential measurements (Fig. 1B) showed that both FA and β-lg–FA mixtures had mild *z*-potential values (not exceeding −19.8 ± 4.4 mV). In the case of pure FA, fluctuations in both size and ζ-potential measurements could be explained by the ability of FA to self-assemble at concentrations of 0.1% (w/w) or higher, as recently shown *in vitro* and *in silico* (Motkar et al., 2013). In respect to β-lg–FA mixtures, literature indicates that electrostatically stabilized hydrocolloids commonly possess ζ-potentials exceeding absolute values of 40 mV (McClements, 2005). Thus, the physical stability of β-lg–FA mixtures does not seem to be rationally explained by electrostatic stabilization, suggesting other forces to dictate the systems stability. This could stem from hydrophobic interactions, van der Waals interactions or steric overlap interactions. At low FA concentrations in the nano-complex up to 0.03% (up to 1:6 M ratio of β-lg : FA), the nano-complex ζ-potential was significantly more negative than in FA alone, and was altered to less negative values at concentrations of 0.05% w/w FA (1:10 M ratio of β-lg : FA) or higher, drawing close to the values found for FA alone. At the FA concentration 0.05% w/w (molar ratio 1:10) the complex β-lg/FA was found to markedly change in its properties and approached its minimal absolute ζ-potential value, which was also very close to the value of the β-lg alone. In contrast to interactions of β-lg with anionic polysaccharides (Jones, Decker, et al., 2010; Jones, Lesmes, et al., 2010), in our experiments no charge reversal was observed, since FA, β-lg and their complexes were all found to be negatively charged. This excludes possible electrostatic

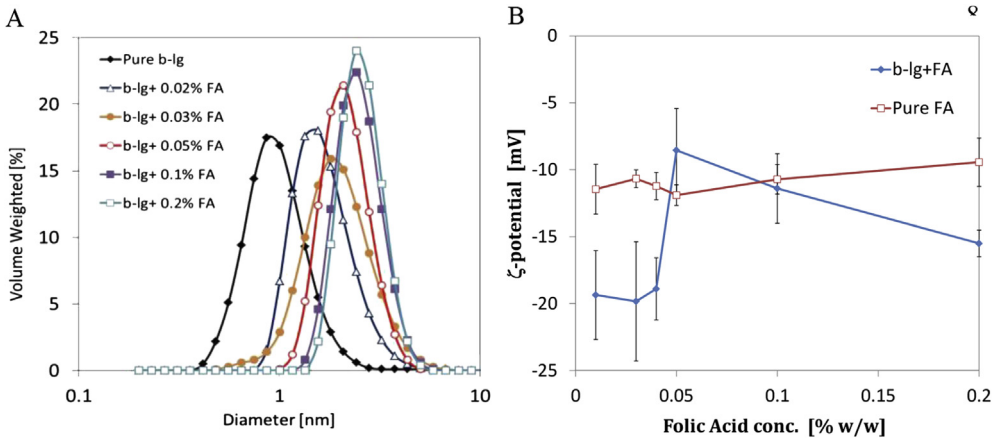

Fig. 1. (A) Particle size distribution curves of β-lactoglobulin (β-lg) and various β-lg mixtures with folic acid (FA) as determined based on DLS data. (B) ζ-potential values of pure folic acid at various concentrations from 0.02% w/w (0.45 mM) to 0.2% (4.5 mM) in comparison to corresponding samples also containing 0.2% w/w (0.11 mM) β-lg (molar ratios of β-lg:FA from 1:4 to 1:40).

interactions between oppositely charged species and raises the suggestion that the complexation is likely to be driven by other forces, e.g. hydrophobic interactions, as suggested by others (Liang & Subirade, 2012). Binding FA to β-lg is likely to induce conformational changes and may expose other charged areas to the protein surface, thus changing its electrophoretic mobility and consequently the derived ζ-potential values calculated through DLS measurements.

From these results, it can be seen that the maximum displacement in the particle size distribution for β-lg/FA mixed systems occurred at concentrations higher than 0.1% (w/w). Meanwhile, the ζ-potential measurements, increasing the FA concentrations (Fig. 2) indicated that at 0.2% (wt/wt) of the acid one could reach the maximum nanocomplex loading capacity.

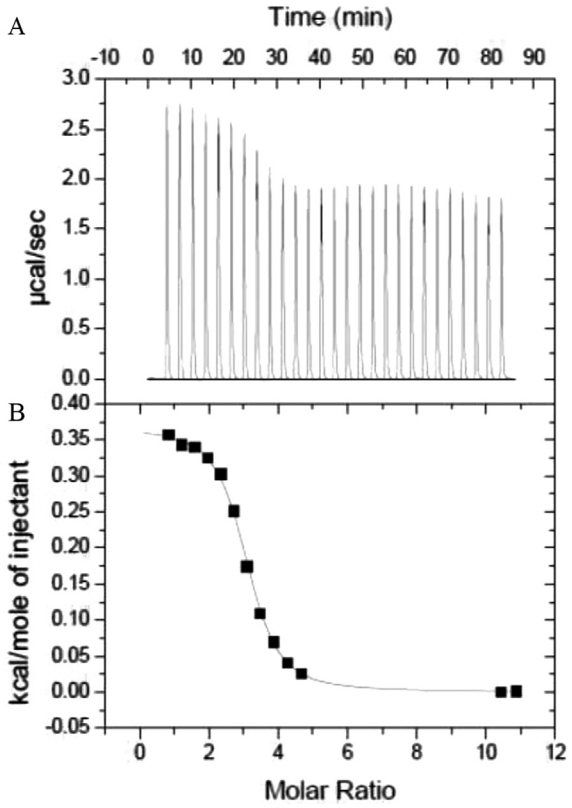

Fig. 2. Isothermal titration calorimetry results for addition of folic acid to a cell filled with 0.2% (w/w) β-lactoglobulin. (A) Thermogram and (B) binding isotherm corresponding to the titration of aqueous FA into an aqueous β-lg (0.2%, w/w) solution at 25 °C, pH 7.

More quantitative look into the interactions between β-lg and FA was gained through isothermal titration calorimetry (ITC), as recently reviewed (Ghai, Falconer, & Collins, 2012). These enabled recording the heat flux versus time upon controlled addition of aliquots of FA to 0.2% (w/w) β-lg solution and generating the corresponding binding isotherm (Fig. 2). Through non-linear fitting of the experimental data, the affinity constant (K) for the FA binding to β-lg, the number of the acid molecules (n) bound per protein molecule and the thermodynamic functions: ΔH and ΔS, were determined and are summarized in Table 1. These parameters suggest β-lg/FA binding involves three FA molecules tightly bound to a β-lg molecule with high affinity in an endothermic process, since the determined K values were in the order of 10^5 M^{-1} and $\Delta H >> 0$. This could be due to specific binding accompanied by some structural changes in the protein, perhaps through some hydrophobic interactions, as some studies suggest to occur between some proteins and certain alimentary polyphenols (Ghai et al., 2012). This provides another support to the notion that b-lg–FA complexation is through hydrophobic interactions, as also suggested by others (Liang & Subirade, 2012).

Structural characterization of the β-lg/FA nano-complexes was performed using intermittent AFM imaging in a butanol environment, as described recently (David-Birman, Mackie, & Lesmes, 2013; Jones, Lesmes, et al., 2010; Shimoni, 2008). AFM images for pure β-lg, pure FA and β-lg/FA nano-complexes were obtained (Fig. 3). It has to be noted that the samples were prepared by drying on a molecularly flat support base (mica), thus revealing flattened structures, which underwent some degree of aggregation upon drying. In spite of these unavoidable structural changes, the samples appear different after the same drying procedure. This permits us to conclude they reflect real differences existing between the samples studied. These images supported the suggestion of nano-complexation and elucidated morphological differences between

Table 1
Thermodynamic parameters derived from the ITC analysis for the interaction of beta-lactoglobulin and folic acid. Data presented as mean ± SD ($n = 4$).

n	K (1/M)	ΔH (Kcal/mol)	ΔS (Kcal/(mol K))
3.0 ± 0.1	$2.3*10^5 \pm 1.8*10^3$	396.7 ± 19.4	24.32 ± 1.04

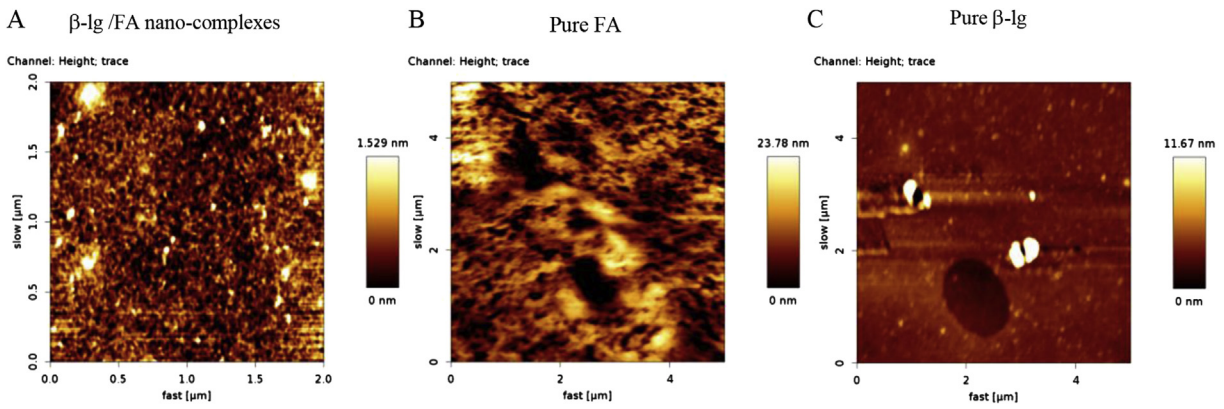

Fig. 3. Atomic Force Microscopy (AFM) scans (5 μm × 5 μm) of samples performed under butanol at intermittent contact mode. [A] Complex of 0.2% (w/w) β-lg and 0.05% (w/w) of FA; [B] sample of 0.05% (w/w) of pure FA; [C] Sample of 0.2% (w/w) pure β-lg.

the samples. β-lg/FA complexation (Fig. 3A) showed small uniform nanoparticles with peak-to-valley differences (scan height scale) of less than 2 nm, in coincidence with the DLS results (Fig. 1A). In comparison, morphology of FA alone assemblies (Fig. 3B) indicated a considerably more heterogeneous and more rough array of aggregated matter with peak-to-valley differences exceeding 20 nm. Unlike the fine and homogenous dispersions of β-lg/FA nano-complexes, β-lg alone appeared as small particles that had a tendency to aggregate upon drying during the sample preparation into flat sporadic clusters of protein molecules having hundreds of nm in diameter, with peak-to-valley differences exceeding 10 nm (Fig. 3C). Because of aggregation upon drying, this observation contrasted to the literature data (de Wit, 2009), showing the presence of different structural units in β-lg solutions, from monomers to octamers, from 3 to 8 nm of diameter. Thus, FA addition to β-lg was found to limit protein aggregation on AFM slides upon drying and provided evidence on the formation of uniform complexes having nano-dimensions. Overall, these experiments showed that FA addition to β-lg solutions alters the colloidal properties of β-lg, in terms of size, ζ-potential and morphology, and ITC revealed some of the quantitative aspects of these molecular interactions.

Additional experiments provided more insights into the implication of FA to β-lg colloidal behavior under varying pH conditions. According to the literature (de Wit, 2009), at low pH the hydrophobic ligand-binding site of a β-lg molecule is in the "closed" position, and binding is inhibited or impossible, whereas at high pH it is open, allowing ligands to penetrate into the binding site. Fluorescence quenching supports the notion that folic acid binding occurs at neutral pH in a hydrophobic pocket rather than at the protein's outer rim (Liang & Subirade, 2010, 2012). As this study's ITC results implied β-lg–FA nano-complexation involves hydrophobic interactions, it can be postulated that this complexation could be mediated by a hydrophobic cleft which could accommodate up to three FA molecules.

In terms of ζ potential (Fig. 4A) and appearance (Fig. 4B), it was found that the β-lg/FA nano-complexes underwent significant changes at pH values of 3–5. Generally, β-lg has been shown to be highly liable to aggregation around the protein's isoelectric point, as studies have shown to predominantly occur in the range of $4 < pH < 5$ (Jones, Lesmes, et al., 2010; Yan et al., 2013). Such studies have shown that this aggregation is highly linked to protein charge and electrostatic interactions (Yan et al., 2013). This also concurs with the explanation to the observed instability of β-lg stabilized emulsions to pH (Lesmes & McClements, 2012). This was affirmed by the ζ-potential measurements (Fig. 4A), showing that at $pH = 5$ zeta-values of β-lg solutions were practically nullified ($\zeta = 0.95 \pm 0.09$ mV). Interestingly, at $pH = 5$ β-lg/FA nano-complexes had significantly altered ζ-potential, than pure β-lg ($p < 0.001$), lowered to a negative value. These nano-complexes were found to possess $\zeta = -20.13 \pm 1.29$ mV at $pH = 5$ and did not show notable differences in solution appearance or turbidity in comparison to the higher pH values (Fig. 4B). Thus, loading of β-lg with FA appear to modify ζ

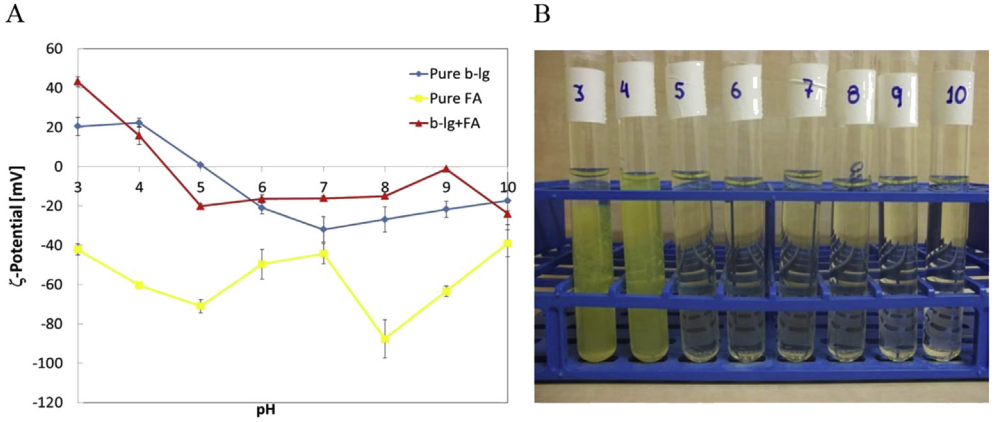

Fig. 4. Colloid pH responsiveness of β-lg and FA nano-complexes compared to the individual components. [A] ζ-potential values determined through DLS; [B] direct observation of β-lg and FA nano-complexes (label numbers correspond to sample pH).

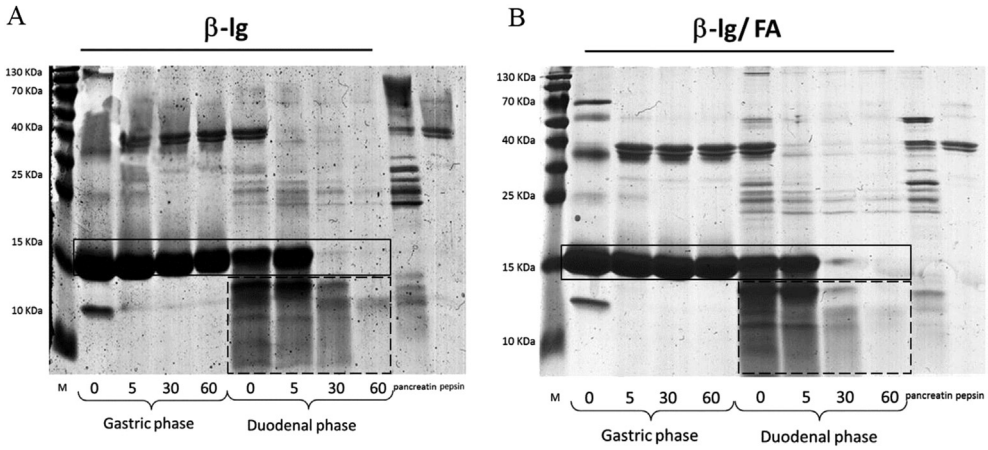

Fig. 5. SDS-PAGE gels of gastric and duodenal digestion of [A] pure β-lg and [B] β-lg/FA nano-complexes. The numbers 0, 5, 30, and 60 represent the time of the sampling (minutes). M represents Mw marker.

potential value of the nano-complexes around the isoelectric point of the protein, improving its solubility at this point. The overall findings of this study showed β-lg–FA nano-complexes increased β-lg stability at moderately acid conditions but showed poor stability at low pH values concurring concurs with a recent study which suggested β-lg–FA interactions to be abolished only at low pH values of 2 (Liang & Subirade, 2012).

The results of physicochemical characterization brought us to look at the system β-lg/FA not only as the simple binding of three FA molecules per one protein molecule, but as a more sophisticated structure. We have found most interesting behavior of our system at pH-range 6–7, when it turns from turbid when using solely FA (not shown) to transparent (see Fig. 4B for the translucency of samples of nano-complexes at these pH values), revealing solubilization of FA, when the mass ratio of β-lg/FA reached to 0.2%/0.05%, which is 10-times molar excess of FA. Thus, in the mixed systems turbidity appeared just at pH value equal to 4 or lower but not at pH of 6 or 7 like that noted in pure FA solutions (data not shown). Taking into account ITC-results that showed binding of only three FA molecules per one protein molecule, we tend to consider the studied complex as a system held by hydrophobic forces, where three FA-molecules are tightly bound to the protein, and others are in proximity, surrounding them. This stabilizes FA in solution and prevents its sedimentation. The nano-complex had improved aggregation properties when dried, forming uniform nanoparticles of tens nm of size, as was shown by AFM.

3.2. Simulated gastric and duodenal digestion

It is well known that β-lg is a major whey protein whose digestion is of nutritional and allergenic importance. Its unique globular features are thought to contribute to its stability to proteolysis and to explain its altered digestibility in infants (Breiteneder & Mills, 2005; Dupont et al., 2010). Moreover, the colloidal properties of proteins are well established to play a role in their digestive fate (Mackie & Macierzanka, 2010). Thus, the observed implications of FA on β-lg colloidal properties reported herein were thought to have possible ramifications to β-lg susceptibility to gastric proteolysis. For this purpose, a dynamic *in vitro* digestion model mimicking the stomach and duodenum of a healthy adult (Dupont et al., 2010; Shani-Levi et al., 2013) was used to evaluate the digestive fate of β-lg/FA nano-complexes compared to pure β-lg solutions. Samples collected during these *in vitro* digestion experiments were subjected to SDS-PAGE analysis to ascertain any possible differences in the rate and extent of β-lg breakdown (Fig. 5). In line with previous reports (Dupont et al., 2010; Macierzanka, Sancho, Mills, Rigby, & Mackie, 2009b; Shani-Levi et al., 2013), these findings showed β-lg was poorly degraded during simulated gastric digestion but did exhibit rapid breakdown during duodenal digestion (closed box in Fig. 5). Comparison between the proteolysis of pure β-lg (Fig. 5A) and that of β-lg–FA nano-complexes (Fig. 5B) showed no marked differences in the persistence of β-lg during gastric and duodenal digestion. In the later samples, experiments showed possible differences in the apparent patterns of breakdown products for pure β-lg versus β-lg/FA nano-complexes (dashed boxes in Fig. 5). However, these require confirmatory experiments, e.g. HPLC or HPLC-MS to affirm such differences and if such to identify such peptides. In light of the possible bioactive peptides that may be derived from β-lg proteolysis (Agyei & Danquah, 2012; Nagpal et al., 2011), future work should look into the possible ramifications of these observations to β-lg digestibility and the bio-accessibility of FA and of β-lg derived peptides.

4. Conclusions

The findings of the present study demonstrate that β-lg can form nano-complexes with FA under specific conditions, which alters both FA and β-lg physicochemical properties. The nature of β-lg–FA interactions is believed to be mainly hydrophobic with ITC findings suggesting the direct complexation of three FA molecules to each β-lg molecule. However, according to the DLS-measurements, the nano-complex grows in size upon increasing FA-loading, suggesting the potential association of additional FA molecules to the β-lg–FA nano-complex. Nano-complexes of β-lg/FA have also been found to alter the responsiveness of β-lg to pH, but do not significantly alter β-lg resistance to gastric or duodenal proteolysis, implying no deleterious effects to the protein's bio-accessibility. From a physiological point of view, the complexation between FA and β-lg might tweak the bio-accessibility of FA or β-lg derived peptides and in turn affect consumer health. From a technological point of view, β-lg/FA nano-complexes could offer improved techno-functional properties, e.g. solubilization of FA and stabilization of β-lg in a wider range of pH. Since there is an interest in harnessing natural ingredient interactions to manage and deliver health to consumers, scientific research should continue to carefully screen

novel food formulations for possible beneficial or deleterious implications.

Acknowledgments

This research was supported by Consejo Nacional de Investigaciones Científicas y Técnicas de la República Argentina, University of Buenos Aires and the Rusell Berrie Nanotechnology Institute at the Technion – Israel Institute of Technology. Prof. Lesmes would also like to acknowledge the scientific stimuli of EU COST Action FA1005 INFOGEST regarding the application of biorelevant digestion models.

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