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Complexes between linoleate and native or aggregated β-lactoglobulin: Interaction parameters and *in vitro* cytotoxic effect

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ABSTRACT

The dairy protein β-lactoglobulin (βlg) is known to form a complex with fatty acids (FA). Due to industrial processing, βlg is often in its non-native form in food products, which can modify the FA/βlg complex properties. We investigated the interaction of bovine βlg, in selected structural forms (native βlg, a covalent dimer and as nanoparticles), with linoleate (C18:2). Using fluorescence and Isothermal Titration Calorimetry, linoleate was found to bind βlg at two different binding sites. Regardless of the structural state of βlg, association constants remained in the same order of magnitude. However, the stoichiometry increased up to 6-fold for nanoparticles, compared to that of native βlg. The impact of these structural changes on linoleate uptake *in vitro* was measured by cytotoxicity assays on Caco-2 cells. The order of cytotoxicity of linoleate was as follows: free > complexed to dimers > complexed to nanoparticles > complexed to native βlg. Therefore, the *in vitro* cytotoxicity of linoleate could be modulated by altering the state of βlg aggregation, which in turn affects its binding capacity to the FA.

1. Introduction

β-Lactoglobulin (βlg), the major whey protein in bovine milk, is present in a large number of food products. βlg is a member of the lipocalin family, composed of 162 amino acids with a monomeric molecular weight of 18.4 kDa (Braunitzer, Chen, Schrank, & Stangl, 1973). It contains nine β-strands, labelled from A to I, and a three turn α-helices, that are arranged to form a globular protein structure (Creamer, Parry, & Malcolm, 1983; Sawyer & Kontopidis, 2000). Eight antiparallel β-strands are organised in a β-barrel, shaped into a hydrophobic calyx. Under physiological conditions, native βlg exists as a non-covalent dimer/monomer in equilibrium. However, the βlg structure is highly sensitive to processing conditions used in the food industries, especially heat treatments, that are applied during food manufacture to achieve specific food textures or to reduce the microbial load (Considine, Patel, Anema, Singh, & Creamer, 2007; de Wit, 2009). Such treatments denature native βlg, leading to the formation of non-native monomers and aggregates of βlg in food products (de Wit, 2009).

βlg is able to bind to small hydrophobic molecules, such as fatty acids (FA) (Sawyer and Kontopidis 2000), and the formation of such complexes modifies FA digestion (Perez et al., 1992). It has been suggested that native βlg binds to hydrophobic ligands in its internal calyx and on surface binding sites (Wu, Pérez, Puyol, & Sawyer, 1999; Yang et al., 2008). However, FA binding to βlg is sensitive to the physicochemical conditions of the medium. Several studies related the decrease of association constants between βlg and binding FA with a decrease in pH. Indeed, below pH 6.2, the calyx binding site is closed by the EF loop region, decreasing interaction with the hydrophobic components (Ragona et al., 2000). Additionally, Wang, Allen, and Swaisgood (1998) demonstrated that a decrease in the proportion of native βlg dimer increased the βlg affinity constant for the palmitate. A number of studies have assessed the interaction of ligands with heat treated βlg (O'Neill and Kinsella, 1988; Yang et al., 2008). However, these different studies have shown inconsistent changes in the binding constants of such ligands with heat treated βlg compared to the native form. This may be due to the nature of the ligand, or to differences in the applied heat treatments (O'Neill and Kinsella, 1988; Yang et al.,

Abbreviations: αla, α-lactalbumin; βlg, β-lactoglobulin; CLA, conjugated linoleic acid; CMC, critical micelle concentration; DMEM, Dulbecco's modified Eagle medium; FA, fatty acid; FAME, fatty acid methyl ester; FBS, foetal bovine serum; GC, gas chromatography; GP-HPLC, gel permeation high performance liquid chromatography; ITC, isothermal titration calorimetry; K_a, association constant; LA, linoleic acid; LCFA, long chain fatty acid; *n*, reaction stoichiometry; NATA, N-acetyl-tryptophanamide; PBS, phosphate buffered saline.

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2008). In fact, the aggregates differ in the parts of protein exposed and therefore differ in how they react to heat (de Wit, 2009).

The essential long-chain fatty acid (LCFA) linoleic acid (LA, *cis,-cis*-9,12-octadecadienoic acid, $n-6$, 18:2) constitutes 1–3% (w/w) of the total FA found in bovine milk fat (Jensen, 2002). LA serves as an essential precursor to a number of long chain metabolites (Mantzioris, James, Gibson, & Cleland, 1995; Russo, 2009). Its health benefits include anti-inflammatory effects, improvements in serum lipoprotein profiles and a reduced risk of cardiovascular coronary artery disease (Zhao et al., 2005; Zock & Katan, 1998). Furthermore, LA, at high concentrations, is cytotoxic to cancerous cells *in vitro* (Lu et al., 2010). However, the bioaccessibility of FA is altered according to the structure of the food matrix (Le Maux, Giblin, Croguennec, Bouhallab, & Brodkorb, 2012; Mu, 2008; Singh, Ye, & Horne, 2009). We previously demonstrated an interaction between the water soluble form of LA, linoleate, and native βlg (Le Maux et al., 2012). This binding alters the cytotoxicity of linoleate by decreasing its transport into the cell.

As βlg is often in a non-native form in food products, the aim of this present work was to determine whether βlg structural forms alter the βlg/linoleate interaction and consequently linoleate cytotoxicity, which is indication of its transport into the cell. Therefore, selected βlg aggregates of controlled size, covalent dimers and nanoparticles, were formed. The binding properties of native βlg, covalent dimers and nanoparticles with linoleate were measured by both isothermal titration calorimetry and intrinsic fluorescence. The cytotoxicity of linoleate either, free in solution or in complexes, was measured for a better understanding of the protein structure impacts on the FA transport.

2. Materials and methods

2.1. Materials

βlg (96% purity) was obtained from Davisco Foods International, Inc. (Eden Prairie, Minnesota) and sodium linoleate (purity $\geqslant$98%) from Sigma–Aldrich (St. Louis, MO). All other chemicals and solutions were purchased from Sigma–Aldrich unless stated otherwise.

2.2. Protein sample preparation and characterisation

2.2.1. Formation of β-lactoglobulin dimers and nanoparticles

Covalent dimers of βlg were formed using the protocol reported by Gulzar, Croguennec, Jardin, Piot, and Bouhallab (2009). Briefly, βlg was dissolved in a 5 mM Bis–Tris buffer (pH 6.7), the final protein concentration was 5 g/L. Copper chloride ($CuCl_2$) was added to the βlg solution, at a Cu^{2+}/βlg molar ratio of 0.6. The solution was heated at 80 °C for 30 min to form covalent dimers, then cooled on ice. Covalent dimers were first dialyzed against 10 mM NaCl (dialysis baths were changed every hour for 4 h) and then against distilled water for 48 h (the water bath was changed twice). Samples were then freeze-dried and stored at −20 °C prior to experiments.

Nanoparticles of βlg were formed according to the method of Schmitt et al. (2009) Briefly, βlg was dissolved in Milli-Q water (Millipore, Carrigtwohill, Ireland), to a final protein concentration of 10 g/L. The pH of the protein solution was adjusted to 5.9 using 1 M HCl, before heating the solution at 85 °C for 15 min, and then rapidly cooling on ice. Samples were dialysed for 48 h against an excess of distilled water, freeze-dried and stored at −20 °C prior to experimental use.

2.2.2. Characterisation of native β-lactoglobulin, covalent dimers and nanoparticles

2.2.2.1. Quantification of β-lactoglobulin concentration in reconstituted solutions. The concentration of native βlg and covalent dimers (expressed as monomer) were determined by optical density using the extinction coefficient of βlg at 278 nm (ε_{278} = 0.96 L/g/cm).

For nanoparticles, the concentration of the βlg monomers was quantified on a reduced sample by the Bradford test following the manufacturer's instructions (Sigma–Aldrich). For reduction, 470 µl of the nanoparticle sample (1 mg of powder/ml) was dissolved in phosphate buffered saline (PBS; 0.01 M phosphate buffer, 2.7 mM KCl, 137 mM NaCl, pH 7.4), 5 µl of 10% SDS and 25 µl β-mercaptoethanol, and the mixture was heated at 95 °C for 5 min.

2.2.2.2. Characterisation of β-lactoglobulin samples using gel permeation-HPLC. The proportion of monomers, dimers, oligomers and aggregates in βlg samples were determined by gel permeation-HPLC (GP-HPLC) using a TSK G SW guard column (7.5 × 7.5 mm, Tosoh Bioscience GmbH, Stuttgart, Germany) and a TSK G2000 SW column (7.5 × 600 mm, Tosoh Bioscience GmbH) connected to an HPLC system, consisting of a Waters 2695 Separations Module (Waters, Milford, MA) and a Waters 2487 Dual λ Absorbance Detector (Waters) working at 280 nm, using Empower Pro software (Waters), to acquire and analyse data. Solvent with 30% (v/v) acetonitrile (LabScan Analytical Sciences, Dublin, Ireland) and 0.1% (w/v) trifluoracetic acid in Milli-Q water was used for protein elution at a flow rate of 0.5 ml/min. The molecular-weight of the different molecular entities, in the samples, was determined using a protein molecular-weight standard calibration set (Sigma–Aldrich).

The molecular entities present in each βlg sample were determined as follows: solutions of native βlg, covalent dimers and nanoparticles were prepared at 1 g/L in PBS. Nanoparticle solutions were centrifuged at 12,000×*g* in order to separate the nanoparticles (pellet) from smaller molecular entities (supernatant). Solutions of native βlg, covalent dimers and the supernatant of nanoparticle solutions, were filtered (0.22 µm filter) prior to injection onto the GP-HPLC. The proportions of monomers, dimers and higher size oligomers of βlg were determined from their relative GP-HPLC chromatographic peak area obtained using Apex Track integration, and the sample total chromatographic peak area. The proportion of monomers and aggregates in the nanoparticle samples were determined from their chromatographic peak area in the supernatant of the nanoparticle sample and the total chromatographic area of a solution of native βlg prepared at 1 g/L. The proportion of the different molecular entities for each of the βlg samples (native βlg, covalent dimers and nanoparticles) and of α-lactalbumin (αla, impurity) were calculated. The native βlg sample contains 84.6 ± 1% monomers, 5.4 ± 0.5% dimers, 5.4 ± 0.4% oligomers and 4.6 ± 0.4% of αla. The covalent dimer sample has 74.4 ± 3.1% of dimers, 15.5 ± 1.4% of residual monomers, 6.5 ± 1.6% of oligomers and 3.6 ± 0.4% of αla. The nanoparticle sample has 77.6 ± 1.4% of aggregates and 22.4 ± 1.4% of monomers.

2.2.2.3. Mean hydrodynamic diameter of nanoparticles. To check the homogeneity of the preparation, the mean hydrodynamic diameter of the aggregates in the nanoparticle sample was measured by dynamic light scattering using a Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, Worcestershire, UK), equipped with a 4 mW helium/neon laser at a wavelength output of 633 nm. Particle sizing was performed at 25 °C at 10 s intervals in a particle-sizing cell, using backscattering technology at a detection angle of 173°. Results were the mean of 13 runs. The intensity of light, scattered from the particles, was used to calculate the mean hydrodynamic diameter (*z*-average mean), based on the Stokes–Einstein equation, assuming the particles to be spherical. The mean hydrodynamic diameter of the aggregates (nanoparticles) was centered around 130 nm (data not shown).