TITLE: β-Lactoglobulin-linoleate complexes: In vitro digestion and the role of protein in fatty acids uptake

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INTERPRETIVE SUMMARY

β-lactoglobulin/Linoleate Complexes: In Vitro Digestion and Role of the Protein in Fatty Acids Uptake. **Le Maux et al.**

The dairy protein β-lactoglobulin is known to bind fatty acids such as the essential fatty acid, linoleate. We investigated how bovine β-lactoglobulin/linoleate complexes affect β-lactoglobulin digestion and linoleate transport into intestinal cells. β-lactoglobulin was digested more rapidly when complexed with linoleate. In contrast, /lineoleate transport into intestinal epithelial cells was reduced when complexed to β-lactoglobulin, compared to free linoleate. This highlights the importance of investigating food matrices to understand the digestion and bioaccessibility of individual components. Such research aims to optimize functional foods for bioactive delivery.

β-lactoglobulin/Linoleate Complexes:

In Vitro Digestion and Role of the Protein in Fatty Acids Uptake

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ABSTRACT

The dairy protein β-lactoglobulin (**βlg**) is known to bind fatty acids such as the salt of the essential long-chain fatty acid linoleic acid (*cis,cis*-9,12-octadecadienoic acid, n-6, 18:2). The aim of current study was to investigate how bovine βlg/linoleate complexes of various stoichiometry affect the enzymatic digestion of βlg and the intracellular transport of linoleate into enterocyte-like monolayers. Duodenal and gastric digestions of the complexes indicated that βlg was hydrolysed more rapidly when complexed with linoleate. Digested, as well as undigested, βlg/linoleate complexes reduced intracellular linoleate transport as compared to free linoleate. To investigate whether enteroendocrine cells perceive linoleate differently when part of a complex, the ability of linoleate to increase production or secretion of the enteroendocrine satiety hormone cholecystokinin (**CCK**) was measured. *CCK* mRNA levels and CCK secretion were not similar when linoleate was presented to the cells alone or as part of a protein complex. To conclude, understanding interactions between linoleate and βlg could help to formulate foods with targeted FA bioaccessibility and therefore aid in the development of food matrices with optimal bioactive efficacy.

Key words: β-lactoglobulin; Linoleate; In Vitro Digestion; Bioaccessibility; Cholecystokinin.

INTRODUCTION

Bovine β-lactoglobulin (**βlg**) is the major whey protein in bovine milk. It is a globular protein with a monomeric molecular weight of 18.4 kDa, consisting of 162 amino acids (Kontopidis et al., 2004). βlg is a member of the lipocalin family, which are able to bind small hydrophobic molecules, including fatty acids (**FA**) and hydrophobic vitamins (Perez and Calvo, 1995, Flower, 1996, Wang et al., 1997, Ragona et al., 2000, Kontopidis et al., 2002, 2004, Considine et al., 2007, Jiang and Liu, 2010). All members of the lipocalin family contain a β-barrel, shaped into a calyx, composed of eight antiparallel β-strands (Flower, 1996). It has been suggested that βlg binds hydrophobic ligands in its internal calyx, in a crevice near the α-helix on the external surface of the β-barrel and near the interface of βlg dimers (Lange et al., 1998, Wang et al., 1998, Wu et al., 1999, Muresan et al., 2001, Kontopidis et al., 2004, Forrest et al., 2005, Dong et al., 2006, Yang et al., 2008). However, the binding sites and stoichiometries of ligands have been controversial (Wang et al., 1998, Wu et al., 1999, Kontopidis et al., 2002). βlg may modify the transport, through the gastric tract of hydrophobic substances naturally present in bovine milk (Perez and Calvo, 1995, Ragona et al., 2000). βlg was reported to enhance pregastric lipase activity by binding FA that inhibit the enzyme (Perez et al., 1992). The binding of hydrophobic ligands has been shown to modify the proteins digestibility, as such interactions alter the accessibility of the protein to digestive enzymes (Puyol et al., 1993, Mandalari et al., 2009).

Previously, the salt of the essential long-chain fatty acid (**LCFA**) linoleic acid (**LA**, *cis*,*cis*-9,12-octadecadienoic acid, n-6, 18:2) was shown to bind βlg (Le Maux et al., 2012). LA represents 1-3 % (w/w) of the FA in milk fat (Jensen, 2002). The mechanisms by which LCFA are absorbed by cells remains controversial. Recent studies suggest that LCFA are taken up by intestinal epithelial cells by both active transport via specific FA transporters, and

by passive diffusion (Stremmel, 1988, Trotter et al., 1996, Abumrad et al., 1998). However, uptake and bioavailability of FA may be altered depending on the food matrix (Kushibiki et al., 2001, Mu, 2008, Singh et al., 2009). We observed that linoleate, the salt form of LA, bound to βlg was less bioaccessible compared to free linoleate (Le Maux et al., 2012). After uptake in enterocyte cells, FA are bound to proteins for their intracellular trafficking and thio-esterification (Niot et al., 2009). They are rapidly esterified into triacylglycerols in the endoplasmic reticulum. In the golgi, these triacylglcerols form chylomicrons (lipid droplets) before being released into the cytoplasm and finally into circulation for uptake by other cells (Niot et al., 2009). FA also trigger internal signals in the intestinal epithelium to allow the body to control food intake for example, secretion of satiety hormones by specialised entroendocrine cells.

Cholecystokinin (**CCK**) is a peptide hormone reported to have satiety effect as it influences the digestive processes in the gut (Gibbs et al., 1973). CCK has been associated with delay of gastric emptying, stimulation of gallbladder contraction, increases in pancreatic enzyme secretion, and reduced food intake (Purhonen, 2008). CCK is secreted by I enteroendocrine cells located predominantly in the proximal small intestinal mucosa in response to intraluminal nutrients such as FA and proteins (Liddle, 2000). Relatively little is known concerning the mechanisms whereby nutrients influence CCK synthesis and secretion. Several studies have shown that peptides and FA can use an external receptor to induce CCK secretions (Choi et al., 2007, Tanaka et al., 2008, Shah et al., 2011). In vivo and in vitro studies indicate that proteins increase CCK secretion (Beucher et al., 1994, Cordier-Bussat et al., 1997, Nishi et al., 2001). This effect is further improved when proteins are hydrolysed, however individual amino acids do not contribute to CCK release (Liddle et al., 1985). Secretion of CCK by FA is dependent on FA structure, chain length, degree of unsaturation

and whether it is in the free or bound form (Beard Shall et al., 1989, Douglas et al., 1990, Feltrin et al., 2007, Little et al., 2007, Pasman et al., 2008).

This study investigated whether βlg/linoleate complexes alter βlg digestion, linoleate bioaccessibility and intracellular transport of linoleate into intestinal epithelial cells Caco-2. To investigate whether enteroendocrine cells perceive linoleate differently when free or part of a complex, the production and secretion of the satiety hormone secretion CCK was measured in STC-1 cells.

MATERIALS AND METHODS

Materials

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

Complex Preparation

Linoleate/βlg complexes were prepared by mixing a solution of βlg and sodium linoleate adapted from Lišková et al. (2011) and described by Le Maux et al. (2012). Under these conditions complexes with 1, 2 and 3 linoleate bound to βlg are formed. βlg without FA was used as a βlg control.

In Vitro Digestion

In vitro adult digestion model was adapted from Dupont et al. (2009). Briefly, βlg samples were dissolved in simulated gastric fluid (0.15 M NaCl, pH 2.5) and the pH was adjusted to 2.5 with 0.5 M HCl solution. Porcine gastric mucosa pepsin (Sigma-Aldrich P7000, activity: 837 U/mg of protein calculated using haemoglobin as a substrate) was added to give 182 U of pepsin/mg of βlg. The final concentration of βlg was 0.05 mM in 20 ml solution. During this gastric digestion, aliquots (1 mL) were removed at regular intervals over a 60 min period. Pepsinolysis was stopped by raising the pH to 7.0 using 0.5 M NaOH. For duodenal proteolysis, the pH was adjusted to 6.5. Duodenal digestion components were dissolved in simulated duodenal fluid (0.15 M NaCl, pH 6.5). They were added to give final concentrations as follows: 4 mM sodium taurocholate, 4mM sodium glycodeoxycholate, 26.1 mM Bis-Tris buffer pH 6.5, 0.4 U/mg of βlg for α-chymotrypsin (Sigma-Aldrich C4129, activity 59 U/mg of protein using benzoyltyrosine ethyl ester as substrate) and 34.5 U/mg of βlg for trypsin (Sigma-Aldrich T0303, activity 14476 U/mg of protein using benzoylarginine ethyl ester as substrate). Aliquots of 1 mL were removed over a 30 min period of duodenal digestion. Proteolysis was stopped by addition of an excess of soybean Bowmann-Birk trypsin/α-chymotrypsin inhibitor (100 µL of 5 g/L in simulated duodenal fluid), which ensured inhibitor excess. The final solution was freeze-dried.

Gel Permeation-HPLC

The relative proportion of protein monomers, aggregates and peptides in the digested samples were determined by gel permeation-HPLC (**GP-HPLC**) using in tandem a TSK G SW guard column (7.5 × 7.5 mm, Tosoh Bioscience GmbH, Stuttgart, Germany) and a TSK G2000 SW column 130 (7.5 × 600 mm, Tosoh Bioscience GmbH) connected to an HPLC system (Waters 2695 Separations Module, Waters 2487 Dual λ Absorbance Detector, at 214

and 280 nm) with Empower Pro software (Waters, Milford, MA). A quantity of 0.05 mg protein was injected using a solution of 30 % (v/v) acetonitrile (LabScan Analytical Sciences, Dublin, Ireland) and 0.1 % (w/v) trifluoracetic acid in Milli-Q water (Millipore, Carrigtwohill, Ireland) as an eluent, at a flow rate of 0.5 mL/min. The method was calibrated using a set of protein molecular-weight standards (Sigma-Aldrich). Digestion solutions without βlg or FA served as control chromatograms and were subtracted from chromatograms generated from test samples to reduce background noise. Chromatograms were integrated and peaks expressed by size (> 30 kDa, 30 to 10 kDa, 10 to 1 kDa and < 1 kDa) as a percentage of protein content.

Extraction and Determination of the Fatty Acid Content in Digested Samples by Gas Chromatography

FA concentration in samples was determined by gas chromatography (**GC**). To determine FA concentration in digested complexes, total FA were extracted and methylated according to the protocol of Palmquist and Jenkins (2003)(Palmquist and Jenkins, 2003) with some modifications described by Le Maux et al. (2012).

Fatty Acid Methyl Esters (**FAME**) was quantified using a CP-SELECT CB column (100 m, 0.25 mm, 0,25 μm film thickness, Varian BV, Middelburgh, the Netherlands) on a Varian 3400 GLC (Varian, Walnut Creek, CA, USA), which was fitted with a flame ionization detector as previously described by Coakley et al. (2003).

Cell Culture

Cell lines were cultured in a humidified 37°C incubator with a 5 % (v/v) CO_2 in air atmosphere.

Caco-2 Cell Line. The Caco-2 cell line was purchased from the European Collection of Cell Cultures (collection reference: ECACC 86010202) and was derived from human colonic adenocarcinoma cells.

Cells were routinely grown in 75 cm^2 plastic flasks in Dulbecco's modified Eagle medium (**DMEM**) containing 4.5 g/L glucose and 0.584 g/L L-glutamine. Media for subculture was supplemented with 10 % (v/v) foetal bovine serum (**FBS**), 100 U/mL penicillin and 100 mg/mL streptomycin. At 80 % confluency, cells were trypsinised with 0.25 % trypsin/EDTA, diluted 1:6 in media and reseeded. Media was changed three times a week. All cells used in these studies were between passage number 32 and 42.

STC-1 Cell Line. The STC-1 cell line was purchased from American Type Culture Collection (ATCC, Gaithersburg, MD). This enteroendocrine cell line originated from a double transgenic mouse tumour (Rindi et al., 1990).

Cells were routinely grown in 75 cm^2 plastic flasks in DMEM containing 4.5 g/L glucose and 0.584 g/L L-glutamine. Media for subculture was supplemented with 20 % (v/v) FBS, 100 U/mL penicillin and 100 mg/mL streptomycin. Cells were passaged at 80 % confluence. Cells at passage numbers of between 15 to 20 were used in this study.

Viability Assay

Cytotoxicity of test samples on Caco-2 and STC-1 cell proliferation was determined by MTS assay, using CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega Corporation Madison, Wisconsin, USA) according to the manufacturer's instructions.

Viability was defined as the ratio of absorbance of treated cells to untreated cells (cells incubated in media only) at 490 nm. Each cell exposure was repeated in triplicate, at a minimum.

For Caco-2 experiments, cells were seeded in 96-well plates, at a cell density of 2×10^4 cells/well, using serum-free media (DMEM only supplemented with 100 U/mL penicillin and 100 mg/mL streptomycin) 24 h prior to experiment. The Lethal Dose 50 (**LD_{50}**), concentration required to decrease the cell viability by 50 %, of free linoleate is 58 µM linoleate (Le Maux et al., 2012). As such, Caco-2 cells were exposed for 24 h to digested complexes (molar ratio of 1, 2 and 3 linoleate/βlg) containing 58 µM linoleate.

Cytotoxicity assays were also performed on Caco-2 monolayers. Caco-2 cells were seeded in 96-well plates, at a cell density of 6.5×10^4 cells/well, using complete media. Initially, media was changed after 6 h, then every two days up to 21 days. Complete media was change to serum-free media 24 h prior to experiment. Cells were exposed to 50 µM linoleate for 24 h.

For STC-1 experiments, STC-1 cells were seeded in 96-well plates, at a cell density of 2×10^5 cells/well, using complete media 24 h prior to experiment. STC-1 were treated with different concentrations of linoleate (0 to 200 µM) and βlg (0 to 200 µM) in serum-free media for 24 h. LD_{50} were determined using Graph-Pad Prism software 3.03 (GraphPad Software Inc., La Jolla CA, USA). The sigmoidal dose-response with variable slope was used to fit the measured curves and calculate LD_{50}.

Caco-2 Transepithelial Transport

The Caco-2 cell line is derived from human colonic carcinoma and has similar characteristic to small intestine epithelial cells (Yee, 1997). Cultured as a monolayer, these cells can differentiate into intestinal-like absorptive cells with tight junctions and a well-differentiated

brush border, which express nutrient transporters (Delie and Rubas, 1997). The apical side of these enterocyte-like cells, which contains the brush border, would be in contact with the lumen of the gut. The basal side of the cells would be in contact with the blood and lymphatic systems. As such, Caco-2 monolayers are a well-established intestinal barrier model to determine the bioavailability of substances and their transport into and out of the intestinal epithelium in vitro (Delie and Rubas, 1997, Yee, 1997).

For transepithelial transport experiments, Caco-2 cells were seeded at a density of 3×10^5 cells/well into permeable Transwell filter inserts (24 mm diameter, 0.4 μm pore size; Costar, Cambridge, MA). Cell culture media was changed every two days for 21 days, upon which time the cells are fully differentiated. Serum free media was changed 24 h prior to experimentation. In these conditions, Caco-2 cells were exposed to media devoid of FA in order to minimise the impact of exogenous FA, and maximise the impact of added linoleate.

Cell monolayer integrity was confirmed by measuring transepithelial electrical resistance (**TEER**) at 37°C using a Millicell-ERS meter (Millipore Corporation, Bedford, MA, USA) according to the manufacturer's instructions. Inserts with a TEER value ≤ 1300 $\Omega.cm^2$ were discarded.

Transepithelial transport studies were adapted from Hubatsch et al. (2007). Briefly, inserts were washed three times with serum-free media. Serum-free media (2.5 mL) was added to the basal side, 1.5 mL of tested compound (1, 2 and 3 linoleate/βlg complexes digested and undigested with a linoleate concentration of 50 μM) in serum-free media containing 5.3 mM fluorescein sodium salt was added to the apical side. Fluorescein acts as an indicator of paracellular transport across the Caco-2 monolayer (Gilman and Cashman, 2007). Plates were then incubated in a humidified 37°C incubator on an orbital shaker at 60 rpm to minimise the impact of unstirred layer. After 4 h, apical and basal media were removed for analysis. Six replicates of each experiment were performed.

Determination of Paracellular Transport in Monolayer Experiments

Fluorescein concentration in the basal chamber was determined by detecting fluorescence in a 50 μL aliquot. Fluorescence was detected with a Synergy Biotek plate reader (BioTek Instruments Inc., Winooski, VT) at excitation and emission wavelengths of 485 nm and 535 nm respectively. Fluorescein concentration was determined by interpolating fluorescence readings from a fluorescein standard curve. The concentration of fluorescein in the basal chamber was expressed as a percentage of the total fluorescein added to the apical side of the inserts. Paracellular transport was always inferior to 0.1 %.

Extraction and Determination of the Fatty Acid Content in Monolayer Experiments by Gas Chromatography

FA concentration in the apical and basal chambers was determined by GC. Total FA were extracted according to the method of the International Organization for Standardization (**ISO**) standards 14156:2001 (ISO, 2001). C13:0 (0.75 mg) was employed as an internal standard. Following removal of the diethyl ether (Fischer Scientific, Pittsburgh, PA) by heating at 45°C under nitrogen, extracted FA were converted to FAME by base catalysed methylation with 2 mL of 0.5 N sodium methoxide at ambient temperature for 10 min. This was followed by acid catalysed methylation using an adaptation of the method of previously described by Palmquist and Jenkins (2003). The concentrated FAME was transferred to clean glass gas chromatography vials and quantified as described above.

The amount of linoleate remaining in the apical chamber and its transport in the basal chamber were corrected by the paracellular transport and the control (cells with media only).

The corrected linoleate data was then expressed as a percentage of the initial linoleate applied to the apical side cells (50 µM).

Confocal Fluorescence Microscopy

Intracellular lipid accumulation was imaged using confocal microscopy. Caco-2 cells were seeded into 8-well chambered glass coverslips (Labtek, Nunc) at a density of 5×10^4 cells/well with serum-free media 24 h prior to experimentation. Cells were then treated with 50 µM linoleate or 50 µM linoleate in a complex with a molar ratio of 3 linoleate/βlg. Following 4 h exposure, cells were gently washed with phosphate buffered saline (**PBS**) and stained with 4 µM Nile Red (prepared in PBS) to highlight intracellular lipid droplets (Greenspan et al., 1985). Cells were examined using a Nikon C1Si Laser Scanning Confocal Imaging System on inverted microscope TE2000-E (Nikon, Champigny-sur-Marne, France) equipped with a helium/neon laser emitting at 543 nm. Fluorescence emission was acquired with a 590/50 nm filter. Average fluorescence intensity was calculated from 9 images.

Cholecystokinin Experiments on STC-1 Cells

In order to assess the impact of samples on CCK secretion in vitro, STC-1 cell line was employed (Pasman et al., 2008, Hand et al., 2010).

The CCK assay was adapted from Hand et al. (2010). Briefly, STC-1 cells were seeded into 12-well plates at a cell density of 2×10^6 cells/well, in complete media and incubated overnight. On the day of the experiment, media was removed and cells were washed three times with serum-free media and then incubated for 30 min with serum-free media at 37°C. Media was removed and 400 µL of serum-free media containing 5 µM of linoleate, free or

complexed to βlg (molar ratio 1 to 3), either digested or undigested were added. All CCK experiments were performed in triplicate.

After a 4 hour incubation, 360 µL of the supernatant was removed, 40 µL of 10 % bovine serum albumin (prepared in PBS) was added and the solution centrifuged at 900 g for 5 min at 4°C to remove cellular debris. The supernatant was collected and stored at -80°C prior to analysis by ELISA.

For determination of *CCK* mRNA levels, cells were washed with PBS. Cells were lysed using QIAzol Lysis Reagent (QIAGEN Ltd., West Sussex, UK) and RNA was isolated from cell suspensions using the QIAGEN miRNeasy Mini kit (QIAGEN Ltd., West Sussex, UK) according to the manufacturers' instructions. RNA was quantified spectrophotometrically using the Nanodrop 1000 (Thermo Fisher Scientific, USA) and the integrity assessed by electrophoresis in a 1.5 % glyoxyl gel with 1X glyoxyl buffer (Ambion, Applied Biosystems, Foster City, USA). Complementary DNA synthesis was prepared from 1 µg of RNA using the QIAGEN QuantiTect reverse transcription kit (QIAGEN Ltd., West Sussex, UK).

Determination of cholecystokinin secretion using ELISA. A CCK (26-33, non sulphated) fluorescent ELISA immunoassay kit (Phoenix Pharmaceutical Inc., Burlingame, CA, USA) was used according to manufacturer's instructions. CCK content was determined using 25 µL of supernatant. Fluorescence (excitation 337 nm, emission 460 nm) was read using a FLUOstar Omega multi-mode microplate reader and analysed with the FLUOstar Omega software (BMG LABTECH GmbH, Ortenberg, Germany). Concentration of CCK in samples was determined by extrapolation to a standard curve. Data were expressed as a percentage of the control value (cells with media only). STC-1 cells do not produce detectable level of gastrin, so the detected signal was attributed to CCK (McLaughlin et al., 1998). Experiments were performed in triplicate.

Determination of Cholecystokinin mRNA using Real Time-PCR. CCK mRNA was quantified using Lightcycler and SyBr green technology (Roche Diagnostics Ltd, Mannheim, Germany) and by the principles of relative quantification using the housekeeping gene ubiquitin-conjugating enzyme (E2D2). Protocol and primers for murine CCK were previously described by Hand et al. (2010).

The target (*CCK*) to reference (*E2D2*) ratio expression was calculated for each FA treatment and normalised compared to untreated (media alone) controls. Experiments were performed in triplicate.

Statistical Analysis

Where appropriate, results were compared using R software package version 2.15.1 (R Foundation for Statistical Computing, Vienna, Austria) and the ANOVA system with a Tukey's least significant difference comparison. *P*-Values < 0.05 were deemed to be statistically significant.

RESULTS

Protein Digestion

Gastric and duodenal in vitro digestion was performed on native βlg, βlg control and linoleate/βlg complexes (linoleate/βlg molar ratio of 1, 2 and 3, Figure 1). As linoleate/βlg complexes had similar chromatograms, only the 3 linoleate/βlg molar ratio complex was compared to βlg control. The GP-HPLC chromatogram was divided into four fractions:

protein oligomers (molecular weight, $\mathbf{M_w} \geq 30$ kDa); protein monomers (30 kDa $\geq M_w \geq 10$ kDa); large peptides (10 kDa $\geq M_w \geq 1$ kDa); small peptides ($M_w \leq 1$ kDa).

The initial HPLC profiles (prior to in vitro digestion) of βlg control and complexes were different regarding oligomer and monomer categories. The proportion of oligomers in the initial samples was 15 % for βlg control and 31 % for complex with a molar ratio of 3 linoleate/βlg. These βlg oligomers were rapidly hydrolysed during the gastric digestion. Their proportion decreased from 31 % to 7 %, and from 15 % to 5 %, within the first minute of gastric digestion, for the βlg control and 3 linoleate/βlg complex, respectively. The proportion of monomers decreased from 85 % to 61 % for βlg control and from 65 % to 45 % for a complex with a molar ratio of 3 linoleate/βlg. Small and large peptides were produced during the gastric digestion for all the samples. They represent about 10 % for βlg control and 20 % for the 3 linoleate/βlg complex at the end of the gastric digestion process.

During the duodenal digestion, the proportion of βlg monomers decreased from 61 % to 53 % for the βlg control sample while it remained unchanged in samples with linoleate. A decrease in the proportion of small peptides (< 1 kDa) was observed for all the samples prior the addition of enzymes of the duodenal digestion. For example, a decrease from 20 % to 12 % of small peptides was observed for the 3 linoleate/βlg complex. Concomitantly, an increase of oligomers (M_w ~ 66 kDa) was detected. Since there was no change in monomer and large peptide populations, these oligomeric species were attributed to the aggregation of small peptides. This result was confirmed by SDS-PAGE experiments, which showed the formation of aggregated molecules that disappeared after chemical reduction (Data not shown).

Cytotoxicity of In Vitro Digested Complexes on Caco-2 Cells

Exposure of Caco-2 cells to the digested complexes, containing 58 μM linoleate, the LD_{50} of linoleate on Caco-2 cells in these conditions(Le Maux et al., 2012), resulted in a significant decrease in Caco-2 viability after 24 h compared to digested βlg alone (Figure 2). Cells exposed to digested βlg control had a viability of 90.8 ± 1.8 % whereas cells exposed to digested complexes had a viability of 71.7 ± 2.6 %, 75.0 ± 1.7 % and 67.9 ± 3.2 % for 1, 2 and 3 linoleate/βlg, respectively. Digested complexes did provide a protective effect to the cells, compared to free linoleate. However undigested complexes, similarly to βlg alone had minimal effect on cell viability under the experimental conditions used (96.6 ± 5.6 %).(Le Maux et al., 2012) Consequently, the level of cell viability with digested complexes is intermediate between undigested complexes and free linoleate.

Uptake of Linoleate by Caco-2 Cells

Uptake by Caco-2 cells of linoleate free or bound to βlg was followed both by the use of Caco-2 cell monolayers, which mimic the intestinal barrier, and by confocal imaging.

Levels of linoleate in the apical and basal chamber of the Caco-2 monolayer was quantified by GC analysis (Figure 3). Preliminary cytotoxic assays demonstrated that a 4 h exposure of differentiated Caco-2 cells to 50 μM linoleate free or in complex did not disturb the Caco-2 monolayer (data not shown). Therefore, this sub-lethal concentration was selected for the Caco-2 transepithelial transport experiments. It was observed that the presence of βlg did not affect the transport of linoleate through the basal chamber as the basal level of linoleate was similar for all the samples (ie. 3.86 ± 0.7 %). However, the molar ratio of linoleate/βlg complexes affected the proportion of linoleate remaining in the apical chamber. As shown in Figure 3, 4.9 ± 0.9 % linoleate remained in the apical chamber when cells were exposed to 50

μM of free linoleate compared to 10.4 ± 0.4 %, 13.7 ± 0.3 % and 15.3 ± 2.5 % linoleate when cells were exposed to complexes of 1, 2 and 3 linoleate/βlg, respectively. Interestingly, the proportion of linoleate remaining in the apical chamber was similar for all digested samples (12.2 ± 0.6 % for 3 linoleate/βlg experiment). Hence, binding of βlg with linoleate significantly decreased the uptake of linoleate by the cells.

Lipid accumulation in Caco-2 cells was measured by Nile Red fluorescence via confocal imaging (Figure 4). Cells were incubated for 4 h with 50 μM linoleate free or in a 3 linoleate/βlg complex. A significant increase in the amount of lipid accumulated in the cells exposed to free linoleate compared to the cells exposed to linoleate complexed to βlg was observed. Fluorescence intensity was 333 ± 25 AU and 2203 ± 570 AU after 4 h of incubation with bound and free linoleate, respectively. For both treatments, the formation of lipid droplets was observed in the confocal images, with larger lipid droplets in cells exposed to free linoleate (Figure 4).

Impact of linoleate/β-lactoglobulin on the Regulation of the Satiety Hormone Cholecystokinin, Secretion and mRNA Level

To determine if linoleate bound to βlg can alter downstream cellular response, the enteroendocrine cell line STC-1 was incubated with free linoleate or complexes of βlg-linoleate. Preliminary experiments showed that βlg was not cytotoxic to STC-1 cells at the concentrations tested (0 to 200 μM) after 24 h, as measured by MTS assay (data not shown). In contrast, STC-1 cells were sensitive to linoleate with a LD_{50} of 12.68 ± 2.56 μM after 24 h (data not shown).

There was no significant difference in the levels of CCK peptide secreted from STC-1 cells exposed to untreated control, free linoleate, undigested and digested complexes (data not shown) for 4 h. In contrast, all the samples tested increased *CCK* mRNA transcripts levels compared to untreated control after 4 h (Figure 5). No significant difference was observed between the normalised mRNA response of undigested 1, 2 and 3 linoleate/βlg molar ratios used. In the same manner, digested complexes did not show significant difference between the three linoleate/βlg molar ratios. Normalised mRNA levels increased was of 1.007 ± 0.004 for free linoleate and increased to 1.041 ± 0.013 and 1.072 ± 0.014 when cells were exposed 3 linoleate/βlg complex undigested and digested, respectively ($P<0.05$). However, the normalised *CCK* mRNA levels were 1.027 ± 0.016 and 1.059 ± 0.009 for undigested and digested βlg control, respectively. Therefore, undigested linoleate/βlg complex (3 linoleate/βlg molar ratio) increased normalised *CCK* mRNA levels by 0.014 compared to the undigested βlg control. Digested complexes with a 3 linoleate/βlg molar ratio, increased normalised *CCK* mRNA level by 0.013 above those measured for cells exposed to digested βlg control. Linoleate control (5 µM linoleate) increased *CCK* mRNA level from 0.007 compared to the untreated control. As such, no significant difference were observed between increases of mRNA level due to linoleate free or bound to βlg.

DISCUSSION

Digested complexes of βlg/linoleate were found to be more cytotoxic to Caco-2 cells compared to undigested complexes but less cytotoxic than free linoleate (Le Maux et al., 2012). As linoleate has to be transported into the cell to be cytotoxic (Lu et al., 2010), this would infer that a portion of the linoleate remains bound to the protein or peptides after in vitro gastro-duodenal digestion. Even if it is assumed that linoleate dissociates from the

protein under gastric conditions (pH 2.5), complexes could reform under duodenal digestion conditions (pH 6.5) (Ragona et al., 2000) The interaction between FA and βlg is pH-dependent: at low pH (below pH 6), FA are released from the βlg but the interaction is reversible when the pH is increased. As up to 50 % of βlg monomers remained after digestion, it is highly probable that reformed complexes consisted of linoleate and undigested monomers.

In its native state, βlg conformation is resistant to pepsin hydrolysis (Guo et al., 1995). However, βlg was hydrolysed faster under gastric digestion conditions when FA/βlg molar ratio was increased. This is probably due to the increased proportion of oligomers in the sample (Le Maux et al., 2012). During the duodenal digestion, no hydrolysis was observed for linoleate/βlg complexes. Only the βlg control undergoes peptic hydrolysis during duodenal digestion suggesting a protective effect of linoleate. Puyol et al. (1993) demonstrated that the binding of palmitic acid to βlg has a protective effect on the protein against hydrolysis, whereas the binding of retinol does not. Mandalari et al. (2009) also demonstrated that phosphatidylcholine could protect βlg from hydrolysis possibly because the binding sites are located close to protease cleavage sites. Furthermore, Mandalari et al. (2009) observed that this protective effect was only effective if the protein was in its native form.

During duodenal in vitro digestion, we observed the appearance of aggregates greater than 30 kDa and a concomitant decrease in the population of small peptides less than 1 kDa. The proportions of large peptides and monomers remained constant. These aggregates disappeared under reducing conditions, suggesting the aggregation of a fraction of the low M_w peptides via disulphide bonds occurs under duodenal conditions. Aggregates were observed for all complexes and βlg control samples, suggesting that the presence of linoleate did not influence peptide aggregation. Jiang and Liu (2010) also observed the formation of aggregates of around 36 kDa during trypsin treatment of conjugated linoleic acid/βlg while Bateman

Bateman et al. (2011) showed formation of βlg fibrils after pepsin hydrolysis and a subsequent pH increase to 6.9.

Caco-2 cells are an appropriate model to investigate FA transport across the intestinal gut (Puyol et al., 1995, Riihimäki-Lampén, 2009). Our results showed a decrease in the transport of the linoleate, when bound to βlg, into and out of the Caco-2 monolayer. Confocal microscopy confirmed that more linoleate was accumulated in the cells when free, compared to when linoleate was complexed to βlg. This is in agreement with the results of Riihimäki-Lampén (2009) where the authors demonstrated that free retinol and free palmitic acid were transported more efficiency, from the apical to the basal side of Caco-2 monolayer, than when complexed to βlg. However, no such change was observed for cholesterol. Opposite results were found for conjugated linoleic acid (**CLA**) (Jiang and Liu, 2010). These authors showed that the level of CLA in cells was significantly greater when CLA was provided to the cells as a βlg/CLA complex compared to free CLA. The low solubility of CLA and the concentration range used in the various studies could explain the discrepancy.

Our results suggest that a considerable quantity of FA was metabolised or stored by the cell. Riihimaki et al. (2008) and Puyol et al. (1995) showed that palmitic acid-bovine βlg complex was principally stored in the cell (more than 90 % after 24 h in Puyol et al. (1995)) and its transport in the basal chamber was low (ie. 5 % to 7 % in the presence of bovine βlg Riihimaki et al. (2008) and Puyol et al. (1995)). However, our confocal analysis showed a substantial difference in the lipid accumulation in the cells compared to the monolayer experiment which showed up to 10 % difference between free and bound linoleate in the apical chamber. This might be accounted for by our use of Nile Red dye, as this Nile Red dye fluoresces better in lipid droplets (Greenspan et al., 1985). This confocal result suggests that linoleate from the βlg/linoleate complexes is slower to form lipid droplets.

When in complexes, an increase in linoleate uptake was observed with increasing concentrations of βlg content using Caco-2 monolayer. As linoleate concentration was constant (50 µM linoleate), βlg concentration was three times smaller for a linoleate/βlg molar ratio of 3 compare to a molar ratio of 1. Native βlg has been shown to be transported into the cells (Caillard and Tome, 1995). This could explain why the kinetics of linoleate transport from the medium to the cells is affected by βlg concentration. However, some authors reports the existence of another mechanism involving a pH dependant dissociation of the ligand bound to βlg immediately prior to transport into the cell, as the pH of the environment close to the cells is more acidic (pH<5) (Ragona et al., 2000, Niot et al., 2009).

Digestion of linoleate/βlg complexes seems to impact linoleate uptake by Caco-2 cells. Linoleate remaining in the apical chamber increased when the linoleate/βlg complex with a molar ratio of 1 was digested compared to undigested. This could be explained by the fact that after digestion, the βlg monomer concentration was reduced by a factor of 2. Under these conditions, the concentration of residual βlg monomers was sufficient to load linoleate (as indicated for complex with linoleate/βlg molar ratio of 2). However, transport of FA to the cells facilitated by βlg would be lower in the digested sample as the amount of βlg monomer was lower. Interestingly, the concentration of linoleate remaining in the apical chamber decreased when the complex with linoleate/βlg molar ratio of 3 was digested compared to undigested. Under this condition, it is possible that the concentration of residual βlg monomer after digestion was too low to bind all the linoleate molecules in the sample increasing the amount of free linoleate. As free linoleate is quickly transported to the cells an overall faster transport is observed for digested complex than undigested.

The ability of STC-1 cells to produce and secrete CCK is thought not to require internalisation of the FA as an external receptor seems to induce CCK secretions (Choi et al., 2007, Tanaka et al., 2008, Shah et al., 2011). After 4 h exposure to the studied protein/FA

complexes, no effect on CCK secretion was detected, whereas mRNA *CCK* levels differed between samples. This supports that CCK synthesis and secretion are distinct. This was highlighted by Hand et al. (2010) who showed that after STC-1 cells were exposed to 100 µM linoleic acid, there were differences between the mRNA *CCK* levels and CCK content in the cell or secreted after either 30 min or 72 h. We found that digested samples induced higher *CCK* mRNA levels than undigested <samples. This is probably due to the protein digestion, as it is already reported that protein hydrolysis leads to higher CCK response compared to undigested protein (Beucher et al., 1994). Digested or undigested linoleate/βlg complexes did not show modification of the linoleate impact on *CCK* mRNA levels, which may be caused by the high signal of the protein compare to the linoleate signal. However, the *CCK* mRNA data seem contradictory with the cytotoxicity and the transport experiments. Indeed, these experiments demonstrated that less linoleate was free in presence of βlg. Thus, we would expect less linoleate to be in contact with the cell membrane and therefore a lower CCK response. However; the denaturation of the βlg is likely to have exposed unfolded proteins, which could increase the *CCK* mRNA response compared to the native protein.

This study has demonstrated that βlg modifies linoleate transport and FA metabolism in Caco-2 cells by altering FA bioaccessibility. Binding of linoleate to βlg induces protein oligomerisation and consequently influences protein susceptibility to digestion. Digested linoleate/βlg complexes delayed the cytotoxicity of the FA suggesting that part of FA is still in a complexed form. Binding of linoleate to βlg potentially alters linoleate uptake by altering levels of free FA. However, no effect on CCK secretion and *CCK* mRNA level was observed when linoleate was free or bound to βlg. Nevertheless, because the FA transport into the intestinal cell is slower in presence of βlg, the FA remains longer into the gut. This slower gut transport may increase the time between meals (Maljaars et al., 2007) and have a positive

effect on satiation. This work highlights the importance of investigating food matrices to understand the digestion and bioaccessibility of individual components. Such research aims to optimize foods for bioactive delivery.

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REFERENCES

Abumrad, N., C. Harmon, and A. Ibrahimi. 1998. Membrane transport of long-chain fatty acids: Evidence for a facilitated process. J. Lipid Res. 39(12):2309-2318.

Bateman, L., A. Ye, and H. Singh. 2011. Re-formation of fibrils from hydrolysates of beta-lactoglobulin fibrils during in vitro gastric digestion. J. Agric. Food Chem. 59(17):9605-9611.

Beard Shall, K., Y. Morarji, S. R. Bloom, G. Frost, J. Domin, and J. Calam. 1989. Saturation of fat and cholecystokinin release: Implications for pancreatic carcinogenesis. Lancet 334(8670):1008-1010.

Beucher, S., F. Levenez, M. Yvon, and T. Corring. 1994. Effects of gastric digestive products from casein on CCK release by intestinal cells in rat. J. Nutr. Biochem. 5(12):578-584.

Caillard, I. and D. Tome. 1995. Transport of beta-lactoglobulin and alpha-lactalbumin in enterocyte-like Caco-2 cells. Reprod. Nutr. Dev. 35(2):179-188.

Choi, S., M. Lee, A. L. Shiu, S. J. Yo, G. Halldén, and G. W. Aponte. 2007. GPR93 activation by protein hydrolysate induces CCK transcription and secretion in STC-1 cells. Am. J. Physiol-Gastr. L. 292(5):G1366-G1375.

Coakley, M., R. P. Ross, M. Nordgren, G. Fitzgerald, R. Devery, and C. Stanton. 2003. Conjugated linoleic acid biosynthesis by human-derived Bifidobacterium species. J. Appl. Microbiol. 94(1):138-145.

Considine, T., H. A. Patel, H. Singh, and L. K. Creamer. 2007. Influence of binding conjugated linoleic acid and myristic acid on the heat-and high-pressure-induced unfolding and aggregation of beta-lactoglobulin B. Food Chem. 102(4):1270-1280.

Cordier-Bussat, M., C. Bernard, S. Haouche, C. Roche, J. Abello, J. A. Chayvialle, and J. C. Cuber. 1997. Peptones stimulate cholecystokinin secretion and gene transcription in the intestinal cell line STC-1. Endocrinology 138(3):1137-1144.

Delie, F. and W. Rubas. 1997. A human colonic cell line sharing similarities with enterocytes as a model to examine oral absorption: advantages and limitations of the Caco-2 model. Crit. Rev. Ther. Drug Carrier Syst. 14(3):221-286.

Dong, S. Y., Z. W. Zhao, and H. M. Ma. 2006. Characterization of local polarity and hydrophobic binding sites of beta-lactoglobulin by using N-terminal specific fluorescence labeling. J. Proteome Res. 5(1):26-31.

Douglas, B. R., J. B. Jansen, A. J. de Jong, and C. B. Lamers. 1990. Effect of various triglycerides on plasma cholecystokinin levels in rats. J. Nutr. 120(7):686-690.

Dupont, D., G. Mandalari, D. Molle, J. Jardin, J. Léonil, R. M. Faulks, M. S. J. Wickham, E. N. C. Mills, and A. R. Mackie. 2009. Comparative resistance of food proteins to adult and infant in vitro digestion models. Mol. Nut. Food Res. 54(6):767–780.

Feltrin, K. L., T. J. Little, J. H. Meyer, M. Horowitz, T. Rades, J. Wishart, and C. Feinle-Bisset. 2007. Effects of lauric acid on upper gut motility, plasma cholecystokinin and peptide YY, and energy intake are load, but not concentration, dependent in humans. J. Physiol. 581(2):767-777.

Flower, D. R. 1996. The lipocalin protein family: Structure and function. Biochem. J. 318(1):1-14.

Forrest, S. A., R. Y. Yada, and D. Rousseau. 2005. Interactions of vitamin D3 with bovine beta-lactoglobulin A and beta-casein. J. Agric. Food Chem. 53(20):8003-8009.

Gibbs, J., R. C. Young, and G. P. Smith. 1973. Cholecystokinin decreases food intake in rats. J. Comp. Physiol. Psychol. 84(3):488-495.

Gilman, J. and K. D. Cashman. 2007. The effect of marine oil-derived n-3 fatty acids on transepithelial calcium transport in Caco-2 cell models of healthy and inflamed intestines. Br. J. Nutr. 97(2):281-288.

Greenspan, P., E. P. Mayer, and S. D. Fowler. 1985. Nile red: a selective fluorescent stain for intracellular lipid droplets. J. Cell Biol. 100(3):965-973.

Guo, M., P. Fox, A. Flynn, and P. Kindstedt. 1995. Susceptibility of beta-lactoglobulin and sodium caseinate to proteolysis by pepsin and trypsin. J. Dairy Sci. 78(11):2336-2344.

Hand, K. V., C. M. Bruen, F. O'Halloran, L. Giblin, and B. D. Green. 2010. Acute and chronic effects of dietary fatty acids on cholecystokinin expression, storage and secretion in enteroendocrine STC-1 cells. Mol. Nut. Food Res. 54(S1):S93-S103.

Hubatsch, I., E. G. E. Ragnarsson, and P. Artursson. 2007. Determination of drug permeability and prediction of drug absorption in Caco-2 monolayers. Nat. Protoc. 2(9):2111-2119.

ISO. 2001. Milk and milk products Extraction methods for lipids and liposoluble compounds. ISO 14156:2001. ISO Publications, International Organization for Standardization, Geneva, Switzerland.

Jensen, R. G. 2002. The composition of bovine milk lipids: January 1995 to December 2000. J. Dairy Sci. 85(2):295-350.

Jiang, H. R. and N. Liu. 2010. Self-assembled beta-lactoglobulin-conjugated linoleic acid complex for colon cancer-targeted substance. J. Dairy Sci. 93(9):3931-3939.

Kontopidis, G., C. Holt, and L. Sawyer. 2002. The ligand-binding site of bovine beta-lactoglobulin: Evidence for a function? J. Mol. Biol. 318(4):1043-1055.

Kontopidis, G., C. Holt, and L. Sawyer. 2004. Invited review: Beta-lactoglobulin: Binding properties, structure, and function. J. Dairy Sci. 87(4):785-796.

Kushibiki, S., K. Hodate, J. Kurisaki, H. Shingu, Y. Ueda, A. Watanabe, and M. Shinoda. 2001. Effect of beta-lactoglobulin on plasma retinol and triglyceride concentrations, and fatty acid composition in calves. J. Dairy Res. 68(04):579-586.

Lange, D. C., R. Kothari, R. C. Patel, and S. C. Patel. 1998. Retinol and retinoic acid bind to a surface cleft in bovine beta-lactoglobulin: A method of binding site determination using fluorescence resonance energy transfer. Biophys. Chem. 74(1):45-51.

Le Maux, S., L. Giblin, T. Croguennec, S. Bouhallab, and A. Brodkorb. 2012. Beta-lactoglobulin as a molecular carrier of linoleate: Characterisation and effects on intestinal epithelial cells in vitro. J. Agric. Food Chem. 60(37):9476–9483.

Liddle, R. A. 2000. Regulation of cholecystokinin secretion in humans. J. Gastroenterol. 35(3):181-187.

Liddle, R. A., I. D. Goldfine, M. S. Rosen, R. A. Taplitz, and J. A. Williams. 1985. Cholecystokinin bioactivity in human plasma. Molecular forms, responses to feeding, and relationship to gallbladder contraction. J. Clin. Invest. 75(4):1144-1152.

Lišková, K., M. A. E. Auty, V. Chaurin, S. Min, K. H. Mok, N. O'Brien, A. L. Kelly, and A. Brodkorb. 2011. Cytotoxic complexes of sodium oleate with beta-lactoglobulin. Eur. J. Lipid Sci. Tech.:1207-1218.

Little, T. J., A. Russo, J. H. Meyer, M. Horowitz, D. R. Smyth, M. Bellon, J. M. Wishart, K. L. Jones, and C. Feinle-Bisset. 2007. Free fatty acids have more potent effects on gastric emptying, gut hormones, and appetite than triacylglycerides. Gastroenterology 133(4):1124-1131.

Lu, X., H. Yu, Q. Ma, S. Shen, and U. N. Das. 2010. Linoleic acid suppresses colorectal cancer cell growth by inducing oxidant stress and mitochondrial dysfunction. Lipids Health Dis. 9(106):1-11.

Maljaars, J., H. P. F. Peters, and A. M. Masclee. 2007. Review article: the gastrointestinal tract: neuroendocrine regulation of satiety and food intake. Aliment. Pharmacol. Ther. 26:241-250.

Mandalari, G., A. M. Mackie, N. M. Rigby, M. S. J. Wickham, and E. N. Mills. 2009. Physiological phosphatidylcholine protects bovine beta-lactoglobulin from simulated gastrointestinal proteolysis. Mol. Nut. Food Res. 53(S1):S131-S139.

McLaughlin, J., R. Lomax, L. Hall, G. Dockray, D. Thompson, and G. Warhurst. 1998. Fatty acids stimulate cholecystokinin secretion via an acyl chain length-specific, Ca2+ dependent mechanism in the enteroendocrine cell line STC-1. J. Physiol. 513(1):11-18.

Mu, H. 2008. Bioavailability of omega-3 long-chain polyunsaturated fatty acids from foods. Agro Food Ind. Hi Tec. 19(4):24-26.

Muresan, S., A. van der Bent, and F. A. de Wolf. 2001. Interaction of beta-lactoglobulin with small hydrophobic ligands as monitored by fluorometry and equilibrium dialysis: Nonlinear quenching effects related to protein-protein association. J. Agric. Food Chem. 49(5):2609-2618.

Niot, I., H. Poirier, T. T. T. Tran, and P. Besnard. 2009. Intestinal absorption of long-chain fatty acids: Evidence and uncertainties. Prog. Lipid Res. 48(2):101-115.

Nishi, T., H. Hara, T. Hira, and F. Tomita. 2001. Dietary protein peptic hydrolysates stimulate cholecystokinin release via direct sensing by rat intestinal mucosal cells. Exp. Biol. Med. 226(11):1031-1036.

Palmquist, D. L. and T. C. Jenkins. 2003. Challenges with fats and fatty acid methods. J. Anim. Sci. 81(12):3250-3254.

Pasman, W. J., J. Heimerikx, C. M. Rubingh, R. Van Den Berg, M. O'Shea, L. Gambelli, H. F. Hendriks, A. W. Einerhand, C. Scott, and H. G. Keizer. 2008. The effect of Korean pine nut oil on in vitro CCK release, on appetite sensations and on gut hormones in post-menopausal overweight women. Lipids Health Dis. 7:1-10.

Perez, M. D. and M. Calvo. 1995. Interaction of beta-lactoglobulin with retinol and fatty acids and its role as a possible biological function for this protein: A review. J. Dairy Sci. 78(5):978-988.

Perez, M. D., L. Sanchez, P. Aranda, J. Ena, R. Oria, and M. Calvo. 1992. Effect of beta-lactoglobulin on the activity of pregastric lipase. A possible role for this protein in ruminant milk. BBA-Lipid Lipid Met. 1123(2):151-155.

Purhonen, A. K. 2008. Signals arising from the gastrointestinal tract that affect food intake. in Department of Biotechnology and Molecular Medicine. Vol. PhD Thesis. University of Kuopio, Mediteknia Auditorium, Mediteknia building, University of Kuopio.

Puyol, P., M. Dolores Perez, L. Sanchez, J. M. Ena, and M. Calvo. 1995. Uptake and passage of beta-lactoglobulin palmitic acid and retinol across the Caco-2 monolayer. BBA Biomembranes 1236(1):149-154.

Puyol, P., M. D. Perez, L. Mata, J. M. Ena, and M. Calvo. 1993. Effect of retinol and fatty acid binding by bovine beta-lactoglobulin on its resistance to trypsin digestion. Int. Dairy J. 3(7):589-597.

Ragona, L., L. Zetta, F. Fogolari, H. Molinari, D. M. Pérez, P. Puyol, K. D. Kruif, F. Löhr, and H. Rüterjans. 2000. Bovine beta-lactoglobulin: Interaction studies with palmitic acid. Protein Sci. 9(7):1347-1356.

Riihimäki-Lampén, L. 2009. Interactions of natural products with beta-lactoglobulins, members of the lipocalin family. Vol. PhD Thesis. University of Helsinki.

Riihimaki, L., A. Galkinab, M. Finel, J. Heikura, K. Valkonen, V. Virtanen, R. Laaksonen, J. P. Slotte, and P. Vuorela. 2008. Transport properties of bovine and reindeer beta-lactoglobulin in the Caco-2 cell model. Int. J. Pharm. 347(1-2):1-8.

Rindi, G., S. Grant, Y. Yiangou, M. Ghatei, S. Bloom, V. L. Bautch, E. Solcia, and J. Polak. 1990. Development of neuroendocrine tumors in the gastrointestinal tract of transgenic mice. Heterogeneity of hormone expression. Am. J. Pathol. 136(6):1349-1363.

Shah, B. P., P. Liu, T. Yu, D. R. Hansen, and T. A. Gilbertson. 2011. TRPM5 is critical for linoleic acid-induced CCK secretion from the enteroendocrine cell line, STC-1. Am. J. Physiol. Cell Ph.:C210-C219.

Singh, H., A. Ye, and D. Horne. 2009. Structuring food emulsions in the gastrointestinal tract to modify lipid digestion. Prog. Lipid Res. 48(2):92-100.

Stremmel, W. 1988. Uptake of fatty acids by jejunal mucosal cells is mediated by a fatty acid binding membrane protein. J. Clin. Invest. 82(6):2001-2010.

Tanaka, T., S. Katsuma, T. Adachi, T. Koshimizu, A. Hirasawa, and G. Tsujimoto. 2008. Free fatty acids induce cholecystokinin secretion through GPR120. N.-S. Arch. Pharmacol. 377(4):523-527.

Trotter, P. J., S. Y. Ho, and J. Storch. 1996. Fatty acid uptake by Caco-2 human intestinal cells. J. Lipid Res. 37(2):336-346.

Wang, Q., J. C. Allen, and H. E. Swaisgood. 1997. Binding of vitamin D and cholesterol to beta-lactoglobulin. J. Dairy Sci. 80(6):1054-1059.

Wang, Q. W. Q., J. C. Allen, and H. E. Swaisgood. 1998. Protein concentration dependence of palmitate binding to beta-lactoglobulin. J. Dairy Sci. 81(1):76-81.

Wu, S. Y., M. D. Pérez, P. Puyol, and L. Sawyer. 1999. Beta-Lactoglobulin binds palmitate within its central cavity. J. Biol. Chem. 274(1):170-174.

Yang, M. C., H. H. Guan, M. Y. Liu, Y. H. Lin, J. M. Yang, W. L. Chen, C. J. Chen, and S. J. T. Mao. 2008. Crystal structure of a secondary vitamin D3 binding site of milk beta-lactoglobulin. Proteins 71(3):1197-1210.

Yee, S. 1997. In vitro permeability across Caco-2 cells (colonic) can predict in vivo (small intestinal) absorption in man-fact or myth. Pharm. Res. 14(6):763-766.

Figure 1, Le Maux et al.

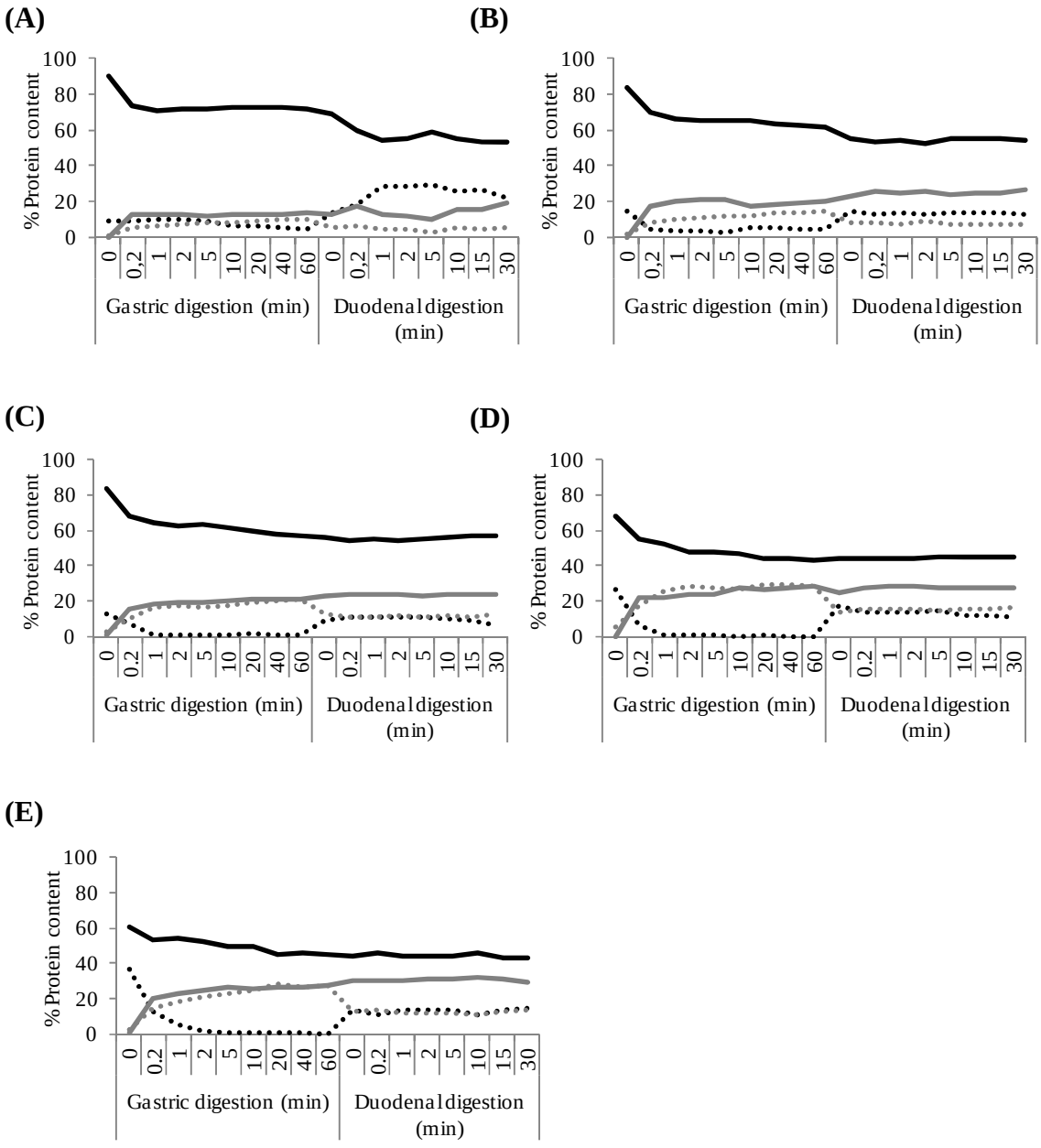

Figure 2, Le Maux et al.

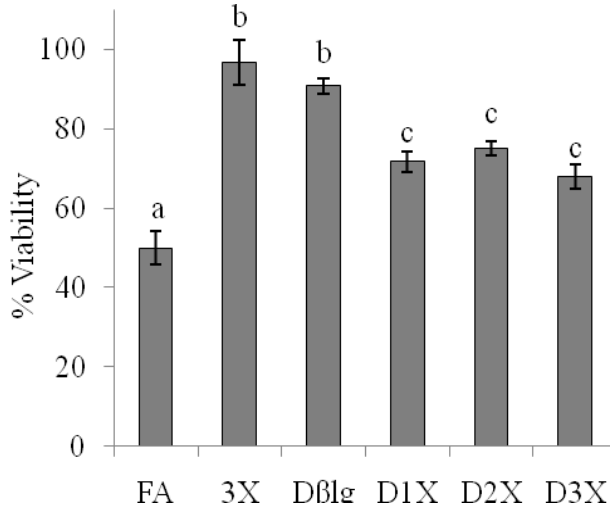

Figure 3, Le Maux et al.

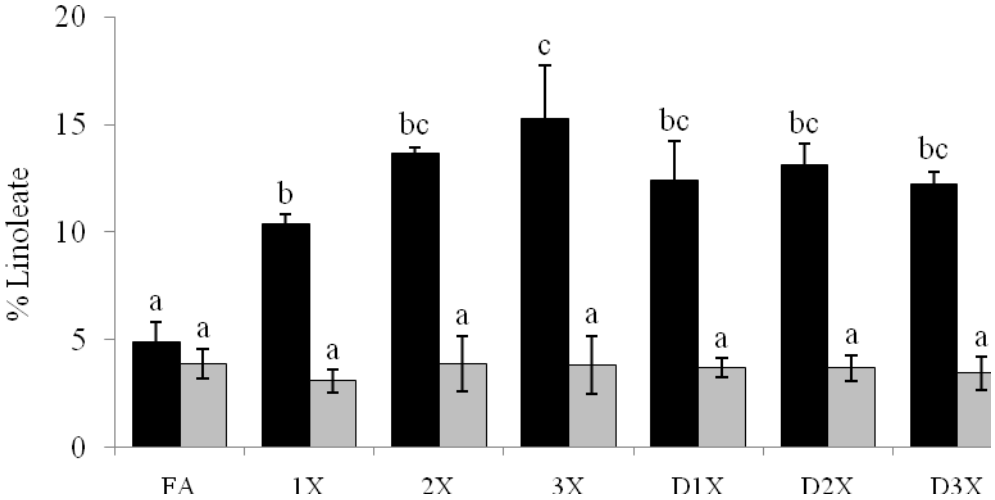

Figure 4 Le Maux et al.

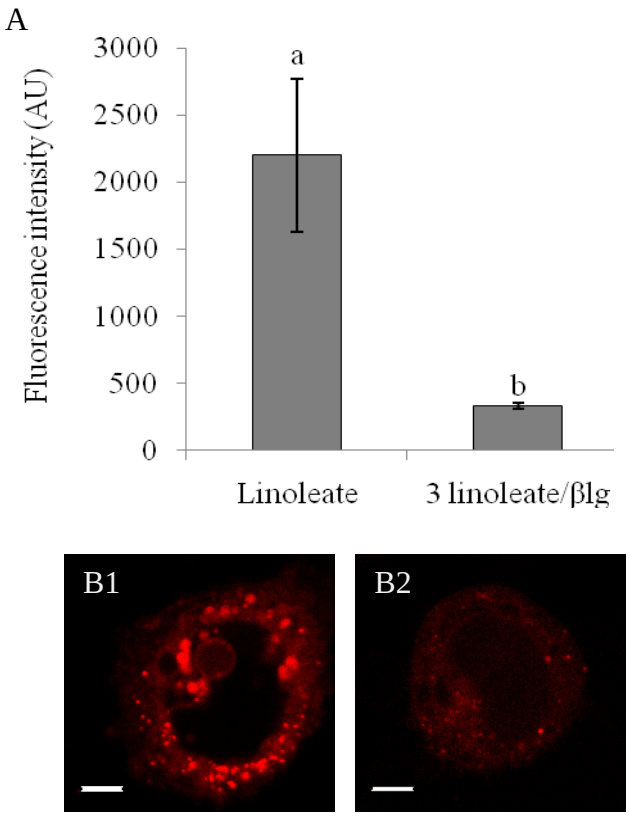

Figure 5, Le Maux et al.

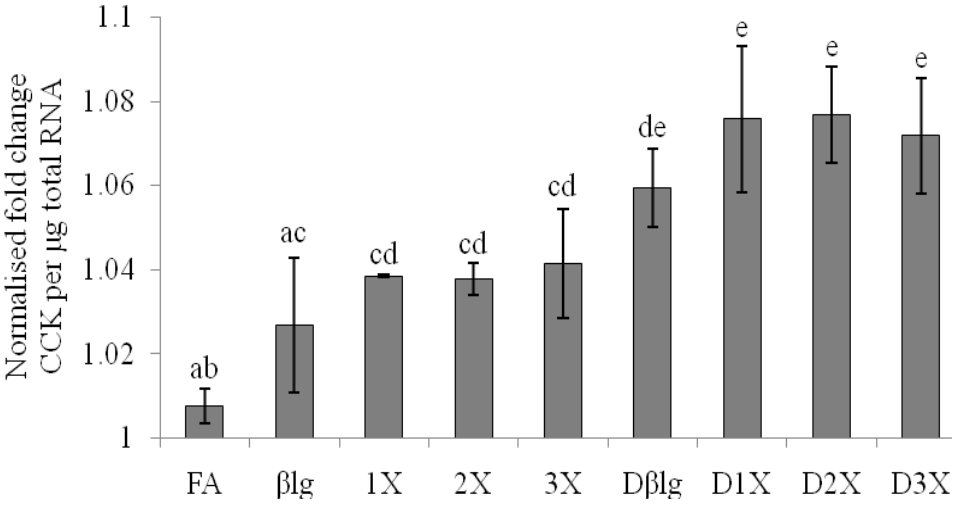

FIGURE CAPTIONS

Figure 1: Characterisation of in vitro gastric and duodenal digestion of βlg alone or in complexes with linoleate determined by GP-HPLC. Proportion of βlg monomers, aggregates, peptides (1-10 kDa), and small peptides (< 1 kDa) are represented by the black line, the black dotted line, the grey line and the grey dotted line, respectively. (A) native βlg, (B) βlg control, (C) 1 linoleate/βlg, (D) 2 linoleate/βlg and (E) 3 linoleate/βlg.

Figure 2: Viability of Caco-2 cells exposed to digested βlg /linoleate complexes for 24 hours. Cells ($2x10^4$ cells/well) were treated with digested complexes (molar ratio of 1, 2 and 3 linoleate/βlg each of which contained 58 µM linoleate). Cell viability is calculated as a percentage of viability of cells incubated in media alone. FA, linoleate; 3X, 3 βlg /linoleate complex; Dβlg, digested βlg control; D1X, D2X, and D3X, linoleate/βlg digested complexes with a molar ratio of 1, 2 and 3, respectively. Results represent mean ± SD (n=6). Multiple Comparisons of means was performed using Tukey contrasts ($p<0.001$).

Figure 3: Transport of linoleate across the Caco-2 monolayer after 4 h of exposure to 50 µM linoleate free or bound to βlg (undigested and digested complexes with a molar ratio of 1, 2 or 3 linoleate/βlg). Proportion of linoleate in apical or basal chamber was expressed as the percentage of the initial linoleate content exposed to cells. Linoleate was measured by GC in the apical chamber (black bars) and in the basal chamber (grey bars). FA, linoleate; 1X, 2X and 3X, undigested linoleate/βlg complexes with a molar ratio of 1, 2 and 3, respectively; D1X, D2X, and D3X, digested linoleate/βlg complexes with a molar ratio of 1, 2 and 3, respectively. Results represent mean ± SD (n=6). Multiple Comparisons of means was performed using Tukey contrasts ($p < 0.05$).

Figure 4: Confocal analysis of Caco-2 cells (5 x 10^4 cells/well) exposed to 50 µM linoleate free or bound in a complex with a 3 linoleate/βlg molar ratio for 4 h. Lipid droplets were stained with Nile Red dye. (A) Fluorescence intensity of Nile Red in Caco-2 cells. (B) confocal imaging depicting the difference in the level of fluorescence from cells exposed to free (B1) or bound (B2) linoleate (bar, 5 µm). Results represent mean ± SD (n=9 pictures). Multiple Comparisons of means was performed using Tukey contrasts ($p < 0.01$).

Figure 5: CCK mRNA levels in 2 x 10^6 STC-1 cells/well exposed to linoleate/βlg complexes with a linoleate concentration of 5 µM for 4 h. Normalised fold change in CCK mRNA levels in STC-1 cells exposed to test samples are normalised to levels of mRNA of the housekeeping gene, E2D2, and to CCK and E2D2 mRNA levels in untreated cells. FA, linoleate; βlg, βlg control; 1X, 2X and 3X, undigested linoleate/βlg complexes with a molar ratio of 1, 2 and 3, respectively; Dβlg, digested βlg control; D1X, D2X, and D3X, linoleate/βlg digested complexes with a molar ratio of 1, 2 and 3, respectively. Results represent mean ± SD (n=3). Multiple Comparisons of means was done using Tukey contrasts ($p<0.05$).