Research Article

Physiological phosphatidylcholine protects bovine β-lactoglobulin from simulated gastrointestinal proteolysis

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We have investigated the effect of phosphatidylcholine (PC) on the resistance of bovine β-lactoglobulin (β-Lg) to simulated *in vitro* gastrointestinal proteolysis. Whilst addition of PC did not affect the resistance of β-Lg to gastric pepsinolysis, it protected the protein from subsequent degradation under duodenal conditions. The effect was dependent on the ratio of PC to β-Lg, 16% of the protein remaining intact in the presence of an equimolar ratio of PC/protein, which increased to 62% when a 60-fold molar excess of PC was included. PC also altered the pattern of digestion products observed by SDS-PAGE. Thermal denaturation of β-Lg abolished this effect showing that it was dependent on the native folded structure of the protein. Since neither of the β-Lg ligands retinol or palmitate exerted a protective effect, it is unlikely that PC is mediating its effect by occupying the central calyx. An alternative explanation may be that the lipids bind to a secondary fatty acid binding site in β-Lg, thus blocking the action of proteases for steric reasons. These data indicate how biomolecular interactions between proteins and lipids may alter patterns of proteolysis and need to be taken into consideration in any *in vitro* model of digestion.

Keywords: Food allergy / *In vitro* digestion / β-Lactoglobulin / Phosphatidylcholine / Protein – lipid interaction

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1 Introduction

β-Lactoglobulin (β-Lg) is a globular protein found in the whey fraction of the milk of most mammals apart from human, and belongs to the lipocalin family, possessing the β-barrel structure, characteristic of this group of proteins [1]. It is found in cows' milk at a level of around 2 – 5 mg/mL, although it is more concentrated in whey, comprising almost half of the protein in this fraction. The β-barrel forms a central calyx into which a variety of lipophilic ligands, including retinol, β-carotene, fatty acids and aliphatic hydrocarbons are known to bind [2], whilst a hydrophobic groove along the single strand of α-helix has been proposed as a secondary fatty acid binding site [3, 4]. The central calyx becomes closed off at low pH as protonation of Glu^{89} forces the E – F loop to move, with changes in protonation state allowing the E – F loop to open as the pH is raised to neutral [5, 6]. These observations explain both the pH-induced changes in structure known as the Tanford transition, and the fact that β-Lg is unable to bind ligands below pH 3 [7]. β-Lg is also highly resistant to pepsinolysis, leading to the proposition that this property, along with closing of the E – F loop, protects ligands in the calyx from the low pH conditions of the gastric compartment. These ligands are then released through opening of the E – F loop as the pH increases in the duodenal compartment, and degradation of the protein by pancreatic proteases. However, whether transport of lipophiles such as retinol is the real biological function of β-Lg is still a matter of conjecture, especially given the absence of lactoglobulins in the milk of certain mammal species, such as human.

In addition to binding lipids as ligands, β-Lg can also interact with phospholipid monolayers and bilayers, preferentially interacting with charged rather than neutral phos-

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Abbreviations: CD, circular dichroism; **GI,** gastrointestinal; **β-Lg,** β-lactoglobulin; **LPC,** lysophosphatidylcholine; **LPG,** lysophosphatidylglycerol; **PS,** phosphatidylserine

pholipids [8], with electrostatic forces dominating for anionic phospholipids such as phosphatidylserine (PS) [9]. The strength of interaction also partly depends on hydrophobic interactions, as reflected by the dependence of lipid association on lipid type and alkyl chain length [10 – 13]. Recent studies have also shown that β-Lg undergoes a structural transition on interacting with lysophospholipids (including lysophosphatidylcholine (LPC) and lysophosphatidylglycerol (LPG)) as indicated by a loss of β-sheet and an increase in α-helix, the extent of change to α-helix being more pronounced at neutral pH [14]. In addition to affecting such lipid interactions, pH also affects the oligomerisation of β-Lg which exists as a dimer at high and neutral pH, forming octomers at pH values between 4 and 6 before dissociating to give a mixture of monomers and dimers at pH values below 3 [15].

The resistance of β-Lg to digestion, particularly in the gastric compartment, coupled with its absence from human milk has been proposed to contribute to the allergenicity of bovine β-Lg in human [16]. Several other allergens belonging to the lipocalin family have also been identified, including inhalant allergens such as mouse urinary protein, and several dander allergens [17]. However, bovine β-Lg and its caprine and ovine homologues are the only lipocalin food allergens [18]. In addition to the intrinsic susceptibility of a given scaffold to proteolysis, the three-dimensional structure of a protein may facilitate interactions with other components found in foods or the gastrointestinal (GI) tract lumen, thus altering their stability to proteolysis. Indeed, polysaccharides, including pectins, gum arabic and xylan, may retard the susceptibility of β-Lg to degradation by trypsin and chymotrypsin [19]. We have previously shown that phosphatidylcholine (PC), a lipid abundant in milk and also secreted by the gastric mucosa, interacts with the milk protein α-lactalbumin slowing its breakdown during *in vitro* simulated gastric digestion [20] and has a slight protective effect on duodenal digestion of the LTP from grape [21]. The present paper reports an investigation into the effects of physiological surfactants on the simulated GI proteolysis of bovine β-Lg, showing that under certain conditions β-Lg is almost completely protected from simulated digestion.

2 Materials and methods

2.1 Materials

Bovine β-Lg (~90% PAGE), porcine gastric mucosa pepsin, bovine α-chymotrypsin, porcine trypsin, soybean Bowmann – Birk trypsin – chymotrypsin inhibitor, sodium taurocholate, sodium glycodeoxycholate, retinol and palmitate were all from Sigma Chemical, Poole, Dorset, UK. L-α-PC (egg lecithin grade 1, 99% purity) and L-α-PS (99% purity from egg) were purchased from Lipid Products (South Nutfield, Surrey, UK). L-α-LPC palmitoyl and L-α-LPG palmitoyl were obtained from Avanti (Alabaster, AL, USA). All other chemicals were of analytical reagent grade and purchased from Sigma Chemical unless otherwise stated.

2.2 Preparation of phospholipid vesicles and ligand-β-Lg complexes

Phospholipid vesicles were prepared using a modification of a procedure described previously [20] using either PC or PS. Solvent was removed from a 0.94 mL aliquot of PC or PS stock solution (63.5 mM in chloroform) and dried under rotary evaporation at 5°C in order to make a thin film of phospholipids. The film of phospholipids was then suspended in 12.2 mL of warmed simulated gastric fluid (SGF, 0.15 M NaCl, pH 2.5) and the suspension sonicated for 7 min using a sonication probe (Status US 200, Avestin, Canada) at 5°C in a coolant-jacketed vessel. The liposomes were then covered with a cushion of argon before equilibrating at 37°C for 20 min in a shaking incubator.

Complexes of β-Lg with one among palmitic acid, retinol and lysophospholipids, were prepared using the following modification of the method of Ragona *et al*. [7]. The ligand was initially dissolved in chloroform and, after removing solvent by flushing with N_2, a solution of β-Lg in SDF buffer at pH 6.5 was added and the mixture equilibrated at 37°C for 2 h prior to use. Molar ratios of 60:1 or 10:1 with respect to the protein monomer were prepared. Additional ratios of 10:1, 5:1 and 1:1 were prepared for lysophospholipids with respect to the protein monomer as follows: the required amount of dried lipid was dissolved in chloroform and β-Lg was added after removing the solvent by flushing with N_2.

2.3 Circular dichroism (CD) spectroscopy

Samples (1 mg/mL protein) were dissolved in 10 mM sodium phosphate buffer pH 6.5 and spectra recorded with a J-710 spectropolarimeter (Jasco, Tokyo, Japan) using a 0.1 mm path length quartz cell. Spectra represent the average of four accumulations collected at 20 nm/min in the range between 180 and 260 nm, with a 4 s time constant, a 0.5 nm resolution and sensitivity of ±100 mdeg. They are presented, after the buffer blank has been subtracted, as molar CD. Analysis of the CD spectra was carried out using Selcon [22] with 29 reference spectra.

2.4 Simulated gastric and duodenal proteolysis

Proteolysis was performed essentially as previously described [20] using triplicate incubations at 37°C; control incubations were performed in the absence of proteases. Briefly, β-Lg was dissolved in SGF buffer pH 2.5 (1 mg/mL), mixed with any lipid addition and the pH was adjusted to 2.5 with 0.5 M HCl solution. Porcine gastric mucosa pepsin (EC 3.4.23.1, Sigma, activity: 3300 U/mg of protein calculated using haemoglobin as a substrate) was added to give

182 U of pepsin/mg of β-Lg (0.05 mM, final concentration). Aliquots (100 μL) were removed over the 60 min digestion time course. Pepsinolysis was stopped by raising the pH to 7.0 using 0.5 M ammonium bicarbonate (BDH, Poole, Dorset, UK). For those samples subsequently subjected to duodenal proteolysis the pH was adjusted to 6.5 by addition of 0.1 M NaOH and components added to give final concentrations as follows: 4 mM sodium taurocholate, 4 mM sodium glycodeoxycholate, 26.1 mM Bis-Tris buffer pH 6.5, 0.4 U/mg of β-Lg bovine α-chymotrypsin (activity 40 U/mg of protein using benzoyltyrosine ethyl ester, BTEE, as substrate), 34.5 U/mg of β-Lg porcine trypsin (activity 13 800 U/mg of protein using benzoylarginine ethyl ester, BAEE, as substrate). Aliquots (100 μL) were removed over the 30 min digestion time course, and proteolysis stopped by addition of a two-fold excess of soybean Bowmann–Birk trypsin–chymotrypsin inhibitor above that calculated to inhibit trypsin and chymotrypsin in the digestion mix.

2.5 SDS-PAGE analysis

SDS-PAGE was performed using 10% polyacrylamide NuPAGE Novex Bis-Tris precast gels (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. All samples were reduced using 0.5 M DL-DTT prior to analysis. Gels were fixed in 50% v/v methanol, 10% v/v acetic acid and after 30 min were rinsed in deionised water prior to staining with Colloidal Blue Staining Kit (Invitrogen). M_r markers comprised the following mix of proteins (Invitrogen): insulin α-chain (M_r 2500 Da), insulin β-chain (M_r 3500 Da), aprotinin (M_r 6000 Da), α-lactalbumin (M_r 14 200 Da), lysozyme (M_r 14 400 Da), trypsin inhibitor (M_r 20 000 Da), carbonic anhydrase (M_r 31 000 Da), lactate dehydrogenase (M_r 36 500 Da), ovalbumin (M_r 45 000 Da), glutamic dehydrogenase (M_r 55 400 Da), BSA (M_r 66 000 Da), BSA (M_r 66 300 Da), phosphorylase (M_r 97 400 Da), β-galactosidase (M_r 116 300 Da), myosin (M_r 200 000 Da).

Image analysis of SDS-PAGE gels was carried out using Totallab 120 (Nonlinear Dynamics, Newcastle, UK) using an automated process supplemented with occasional manual adjustments. Each lane was first defined, and the average intensity across the width of these lanes depicted as a function of the distance in pixels from the top of the image. The background was subtracted from each profile (parameters adjusted on a case by case basis), after which the bands were identified. Gaussian curves were fitted to the bands, which allowed overlapping bands to be deconvoluted. The lanes of M_r standards were then used to reference the position of the bands. For this, lanes corresponding to a given Rf or MW were drawn across the width of the gel, allowing the position of each band to be then estimated. The output of this process was a list of bands defined either by their Rf or by their MW, together with an estimate of the volume under the curve corresponding to each band (volume of fitted Gaussian curve).

2.6 RP-HPLC

Samples after *in vitro* duodenal digestion (70–80 μL) were applied to a Phenomenex Jupiter Proteo 90 Å pore size, 4 μm particle size (250 × 4.6 mm id) column and a Phenomenex Jupiter C4 300 Å pore size, 5 μm particle size (250 × 4.6 mm id) column attached to a Dionex HPLC system with a diode array detector following a modified method previously described [20]. Samples were eluted using 0.1% w/v TFA in purified water as solvent A and 0.085% w/v TFA in ACN as solvent B (gradient of 1 mL/min held at 5% solvent B for 2 min followed by linear gradient to 50% solvent B for 60 min).

3 Results

3.1 Effect of physiological phosphatidylcholine on simulated gastric and duodenal proteolysis of β-lactoglobulin

As described by others [23, 24], β-Lg was found to be completely resistant to pepsinolysis with the M_r 18 300 Da polypeptide remaining throughout the simulated gastric digestion (Fig. 1A). Subsequent incubation with duodenal enzymes, trypsin and chymotrypsin, resulted in gradual digestion of β-Lg, with only a trace of the parent protein remaining after 15 min, which completely disappeared after 30 min (Fig. 1B). Prominent intermediate digestion products of M_r 14 800, 12 900, 11 400, 5600 and 3700 Da were observed during the early stages of digestion (1–5 min), which were broken down completely after 30 min digestion. Only a trace of material running with a M_r 3700 Da was observed after this time.

Addition of physiologically relevant levels of the phospholipid PC [25] to the gastric digestion mix (final concentration of 3 mM) did not alter the resistance of β-Lg to pepsinolysis (results not shown) but did protect the protein from proteolysis by duodenal enzymes in a dose-dependent manner. Increasing the molar excess of PC/β-Lg to give a 60-fold molar excess, a more physiologically relevant concentration, resulted in the lipid being readily visible running at the dye-front of the SDS-PAGE (Fig. 1E). In the presence of PC the parent protein remained largely intact, the kinetics of appearance of digestion products also being altered (Figs. 1C–E). Thus, the $M_r \sim 14\,800$ Da polypeptide digestion product (indicated by the solid circle) was observed only transiently after 1–2 min digestion, only faintly at a 1:1 and 10:1 molar ratio of PC to protein, and not at all in incubations performed in the presence of a 60:1 molar excess. The $M_r \sim 12\,900$ Da polypeptide persisted for much longer (15 min digestion) in the presence of a PC/β-Lg molar ratio of 1:1 (Fig. 1C, indicated by the solid tri-

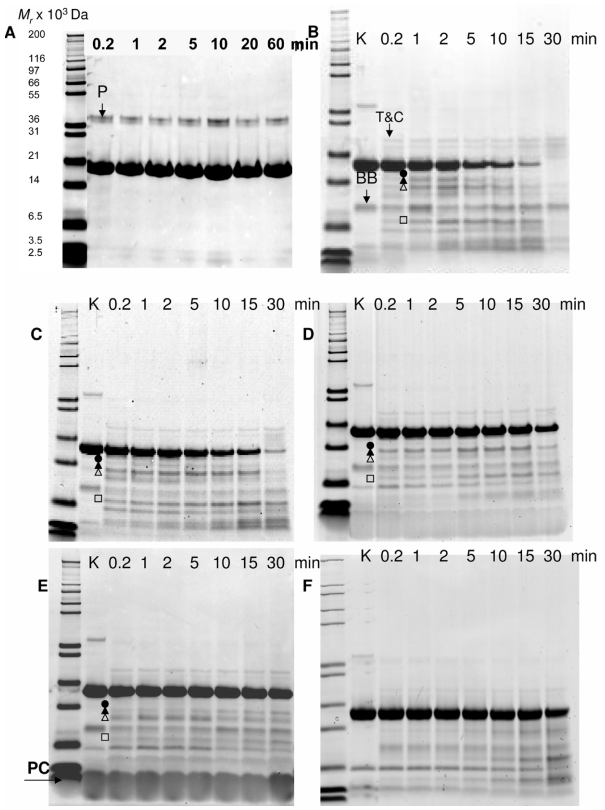

Figure 1. SDS-PAGE analysis of β-Lg subjected to gastric (A) and duodenal proteolysis (B – F) in either the absence (A, B) or presence of lipid (C – F) (C) PC 1; (D) PC 10; (E) PC 60. At either a 60:1 (PC 60), 10:1 (PC 10) or 1:1 (PC 1) molar ratio of PC to β-Lg. (F) LPC/β-Lg molar ratio of 1:1. SDS-PAGE was performed under reducing conditions. P, Pepsin; T&C, trypsin and chymotrypsin; BB, soybean Bowmann – Birk inhibitor; K, no enzyme added.

angle), and was further stabilised by 10-fold molar excess such that it persisted for 30 min digestion (Fig. 1D). In the presence of a 60:1 molar excess of PC the $M_r \sim 12\,900$ Da polypeptide was not so evident, with the $M_r \sim 11\,400$ Da polypeptide predominating early during digestion, both fragments being evident after 30 min digestion. The $M_r \sim 11\,400$ polypeptide (indicated by the open triangle) was also more abundant when digestions were performed in the presence of a PC/β-Lg molar ratio of 60:1. However, the appearance of $M_r \sim 7000$ Da polypeptide (indicated by the open square) was reduced in the presence of a 60:1 molar ratio of PC (Fig. 1E) although it, along with the poorly resolved M_r 3700 Da polypeptide, persisted until the end of digestion.

Densitometric analysis of residual intact β-Lg observed on SDS-PAGE gels (Table 1) indicated that whilst 96% of the β-Lg disappeared after 30 min duodenal digestion in the absence of PC, only 27% was degraded in the presence of PC at 60:1 molar ratio. These data were consistent with HPLC quantification of intact β-Lg, which eluted on a 300 Å pore size column at 54 min as a double peak, reflecting the presence of both variants A and B (Fig. 2A). Thus, HPLC analysis showed that 16% of the β-Lg remained intact at an equal molar ratio of PC to β-Lg monomer, increasing to 43% with a 10-fold molar excess, and 62% with a 60-fold excess of PC (Table 1). Qualitative changes in the abundance of certain peptides were also observed by HPLC, with species eluting at 13, 37, 38 min being reduced in abundance by increase in addition of PC. In addition the broad peaks running between 25 – 28 min became broader upon addition of PC. Peptides eluting at this time point are likely to represent smaller peptides which are less well retained on the column. Consequently, samples were also analysed by HPLC on a 90 Å pore size column which gives enhanced resolution of lower M_r material (Fig. 2B). This analysis showed residual intact β-Lg running as a poorly resolved peak at ~55 min, but showed the complexity of peptide digestion products, the presence of PC having a subtle effect on the peptide profile. Thus, several peptide peaks eluting at around 26, 33, 35, 37, 44 and 45.5 min were detected when β-Lg was digested in the absence of

Table 1. Residual β-Lg after *in vitro* gastric and *in vitro* gastric + duodenal digestion by HPLC and 1-D gel software analysis

Digestion conditions		Residual β-Lg (%)	
		HPLC	SDS-PAGE
Gastric	–PC	100	99.2
	+PC	100	99.7
Gastric + duodenal	–PC	9.7	3.5
	+PC 60	62.4	73.4
	+PC 10	43.1	58.8
	+PC 1	16.2	9.4
	PC 60 in absence of BS	68.4	73.1
	Heated β-Lg + PC 10	7.4	4.5

Residual β-Lg was calculated as a percentage of protein remaining after digestion relative to the starting material (average of two determinations). PC at 60:1 (PC 60), 10:1 (PC 10) or 1:1 (PC 1) molar ratio of PC/β-Lg. Heated β-Lg was incubated at 70 °C for 10 min. BS, bile salts mixture (sodium taurocholate and sodium glycodeoxycholate).

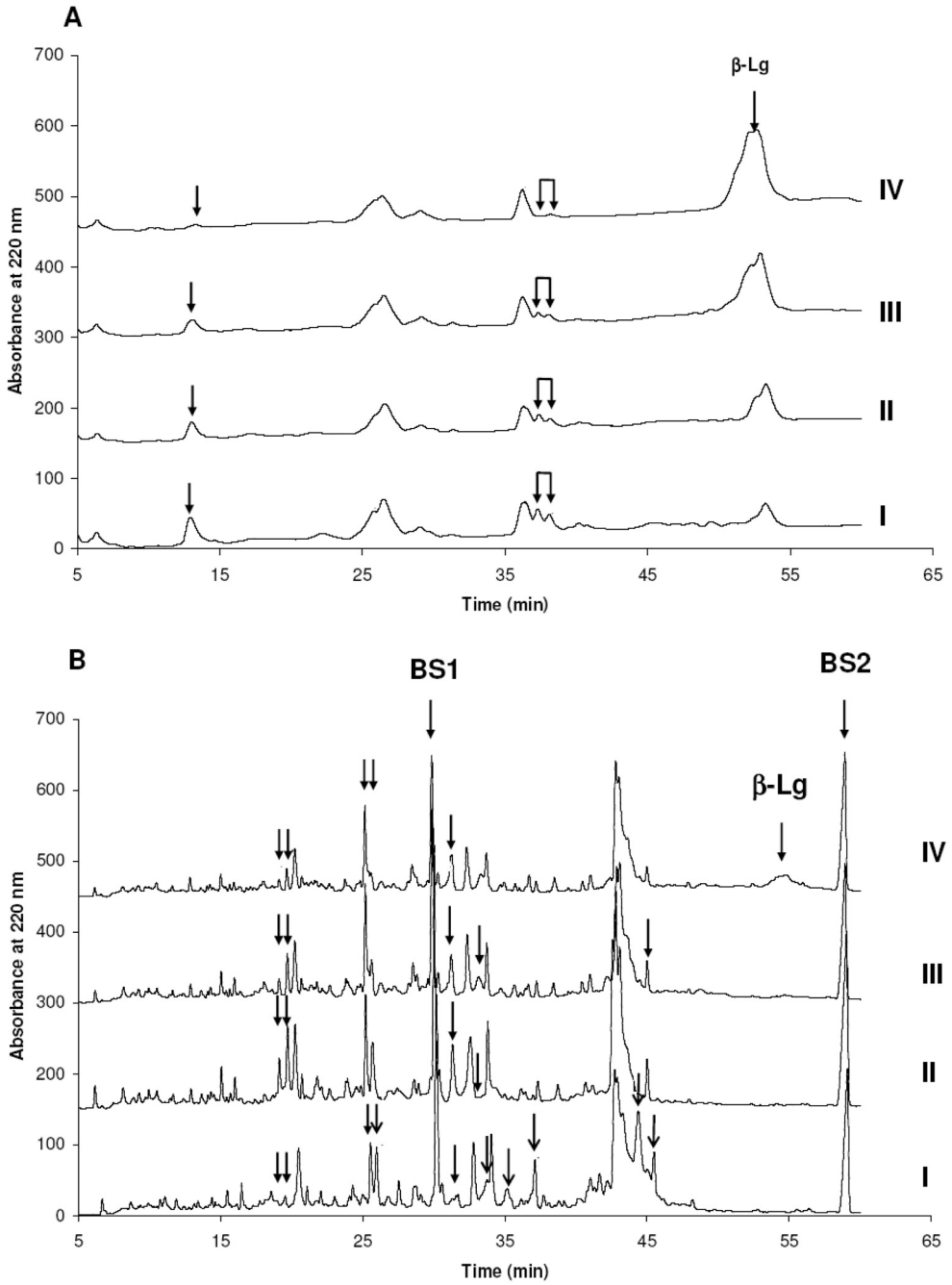

Figure 2. RP-HPLC analysis of β-Lg digestion products using (A) 300 Å and (B) 90 Å pore size column. Digestions were performed in the absence (I) or presence of PC (II – IV) at different molar ratios as follows: II, PC 1; III, PC 10; IV, PC 60. Peaks corresponding to sodium taurocholate (BS1) and sodium glycodeoxycholate (BS2) are indicated. Open arrows indicate peptide peaks decreasing in size on addition of PC; other peptide peaks changing in abundance are indicated by closed arrows.

PC, the abundance of which was reduced by increase in the molar excess of PC. Additionally, a peptide eluting at 34 min only appeared when the PC was added in 10 and 60-fold molar excess, two peptides running at 18 and 29 min increased in the presence of equimolar PC, but then decreased as the PC concentration increased. Two peptides eluting at 31 and 45 min showed a more complex behaviour, increasing in abundance when PC was added at a PC/β-Lg molar ratio of 1:1, then reducing in abundance when the molar excess of PC was increased. These data indicate that addition of PC both slowed proteolysis and altered the pattern of digestion products.

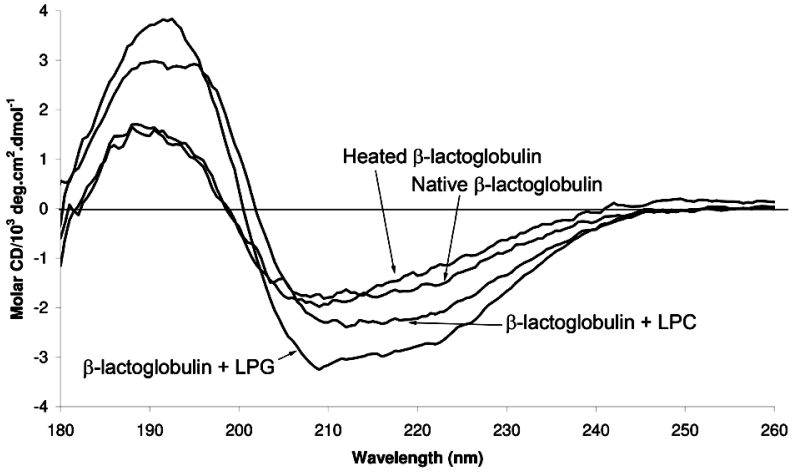

Figure 3. Effect of thermal denaturation and addition of lysolipid on the far-UV CD spectrum of β-Lg. Far-UV region of native β-Lg, heated β-Lg at 70°C for 10 min, β-Lg in the presence of LPC and LPG micelles at a molar ratio of lysolipids/β-Lg of 5:1.

3.2 Thermal stability of the β-lactoglobulin – phosphatidylcholine interactions

Milk proteins frequently undergo thermal treatment prior to consumption and consequently digestions were performed on β-Lg after heating to 70°C for 10 min. The effect of heating on β-Lg secondary structure was assessed by far-UV CD spectroscopy and showed a shift in wavelength of the broad negative minimum from 202–245 nm to 199–240 nm (Fig. 3). These data were consistent with previously reported heat-induced shifts in secondary structure [26, 27]. Previous spectra have been interpreted as a loss of all the α-helix and up to a fifth of the β-sheet at around 65°C which results in the formation of a partly unfolded state derived by destabilisation of the intermolecular β-strand 1 and the loss of the main helix [28]. When subjected to simulated gastric and duodenal proteolysis, it was found that the heat-treated protein was still stable to gastric digestion (results not shown). However, it abolished the protective effect of PC on duodenal digestion (Table 1), giving a pattern of proteolysis products identical to that obtained for the native protein in the absence of PC. These data indicate that the protective effect of PC required interaction with the native, folded protein.

3.3 Effect of ligands and surfactants on simulated gastroduodenal proteolysis

The bile salt (8 mM)-PC (3 mM) components in the duodenal digestion mix were present exclusively as mixed micelles, as indicated by a phase diagram at 37°C based on the data of Maser *et al*. [29] and Matsuoka *et al*. [30]. In order to assess how the form of PC may affect its protective effect on β-Lg, simulated gastroduodenal digestion was undertaken in the absence of bile salts, when the PC would maintain its vesicular form. This showed PC was able to protect β-Lg from proteolysis, whether present in a vesicular form or in mixed micelles with bile salts (Fig. 4). Changing the charge on the vesicles by replacement of the zwitterionic phospholipid PC with the negatively charged PS at a molar ratio of PS/β-Lg 60:1 only slightly decreased the protective effect lipids had on β-Lg degradation. In order to explore the nature of interactions between β-Lg and PC the effect of two lysophospholipids on digestion was studied. The critical aggregation concentration for PC is ~10^{-10} M under these conditions, while the critical micelle concentrations (CMCs) of LPC and LPG in 150 mM sodium chloride buffer are ~6 and ~7 μM, respectively. Digestions undertaken with these more soluble phospholipids present as mixed micelles showed that both LPC and LPG protected the protein from breakdown in a dose dependent manner to an even greater extent than with PC itself (Fig. 4). Interestingly, the negatively charged micelles of LPG had a more protective effect than the neutral micelles of LPC. Thus,

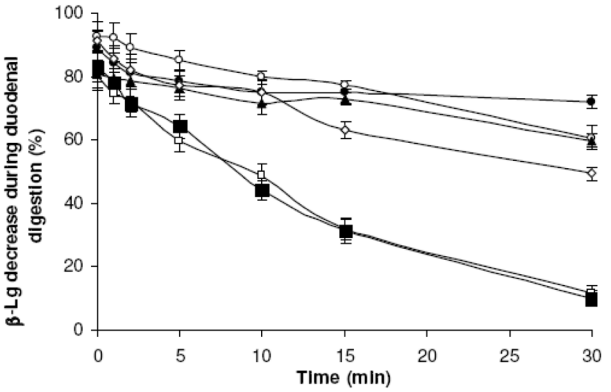

Figure 4. Effect of ligands and lysophospholipids on digestion of β-Lg by trypsin and chymotrypsin. Incubations were undertaken in the presence of retinol (□), palmitate (■), LPG at a molar ratio LPG/β-Lg 5:1 (●) and LPG/β-Lg 1:1 (○), LPC at a molar ratio LPC/β-Lg 5:1 (▲) and LPC/β-Lg 1:1 (◇). Digestion of β-Lg was determined by densitometric analysis of the intact β-Lg band on three replicate SDS-PAGE gels. Residual β-Lg was calculated as a percentage of protein remaining after 30 min digestion relative to the starting material. Data points represent means (n = 3) ± SD.

whilst only 20% of the protein was digested in the presence of LPC or LPG at a molar ratio of 10:1 (results not shown), at molar ratios of 5:1 and 1:1, LPC protected β-Lg with 40 and 50% of the protein being digested respectively compared with 28 and 40% respectively with LPG (Fig. 4). SDS-PAGE analysis indicated that inclusion of LPC at a molar ratio of 1:1 altered the kinetics and pattern of appearance of digestion products (Fig. 1F). Thus, polypeptides of $M_r \sim 13\,700$ and 9700 Da appeared early on during the digestion time course and persisted until the end of digestion. Lower M_r polypeptides of $M_r \sim 4700$ and 3600 Da were also detected, becoming more abundant towards the end of incubation.

Ligand binding is known to stabilise the protein structure [7]; therefore the effect of ligand type on stability of β-Lg to proteolysis was explored, since LPC and LPG could mediate their effects through such interactions. Retinol and palmitate are known to bind in the central calyx of β-Lg with affinities of $4.8 \times 10^7\,M^{-1}$ and $6.8 \times 10^5\,M^{-1}$ respectively [31]. Digestions were undertaken in the presence of retinol or palmitate in a $10\times$ or $60\times$ molar excess over the β-Lg monomer. Whilst ligands will not bind to β-Lg at gastric pH, they would occupy the calyx at the pH used to simulate the duodenal environment employed in this study (pH 6.5). Neither ligand protected β-Lg from simulated duodenal proteolysis (Fig. 4). These data indicate that ligand binding *per se* is not able to prevent proteolysis by trypsin and chymotrypsin. Alternatively, β-Lg may be protected from proteolysis as a consequence of penetration into the lipid vesicles.

In order to assess the effect of lipids on β-Lg secondary structure, far UV CD spectra of β-Lg alone and in the presence of either LPC or LPG at a molar ratio of 5:1 lipid/protein were recorded (Fig. 3). With the addition of neutral micelles of LPC and negatively charged LPG at pH 6.5, β-Lg exhibited a decrease in ellipticity between 208 and 240 nm and this feature was more significant with LPG (Fig. 3). Average secondary structure variation showed an increase in the level of α-helical structure from 10% in the native state to 19 and 28% in the presence of LPC and LPG micelles, respectively, whereas the β-sheet structure decreased from 37% in the native state to 25 and 19% in the presence of LPC and LPG micelles, respectively. No significant changes were observed in the turn (∼22%) and aperiodic (∼28%) structure in the presence of lysolipids. The lysolipid-induced increase in the level of α-helical structure has also recently been observed by Zhang *et al*. [14], where the negatively charged LPG micelles induced a more substantial β-to-α transition compared to the zwitterionic LPC micelles.

4 Discussion

We have shown that inclusion of physiologically relevant concentrations of vesicular PC in simulated gastroduodenal digestion had a protective effect against proteolysis of cows' milk β-Lg. Denaturation of the protein through heating abolished this effect, indicating that it required an intact native structure (Table 1). Reduction in proteolysis of β-Lg could occur through three potential mechanisms. One is through ligand binding in the calyx, which might stabilise the protein to digestion by altering polypeptide mobility. Alternatively, the protection could be through ligand binding in the secondary binding site alongside the single strand of α-helix, which might stabilise the protein to digestion by altering polypeptide mobility. Finally, the protein could insert into lipid vesicles, as we have previously shown for the other major whey protein, α-lactalbumin, which adopts a low pH molten globule state able to penetrate into the PC vesicles [20].

The greater protective effect of lysophospholipids compared to PC could be attributed to their greater solubility and ability to bind to the main calyx. However, since ligand binding with retinol or palmitate was not able to protect the protein from digestion (Fig. 4) and LPC binding is much weaker than retinol or palmitate, with an affinity of only $6.0 \times 10^3\,M^{-1}$ [31] it seems unlikely that ligand binding in the main calyx slows the protein digestion by trypsin and chymotrypsin. Studies on binding and penetration of β-Lg into lipid membranes have shown that zwitterionic phospholipid vesicles cause significantly smaller conformational changes than anionic phospholipid vesicles, the effect being enhanced at low pH as the protein has a net positive charge below pH 4.6 [9, 12].

However, as the bile salt-PC components in our duodenal digestion system were present exclusively as mixed micelles, we suggest that even if the β-Lg inserts into phospholipid vesicles during the simulated gastric phase of digestion this is not the cause of the protective effect seen during the duodenal phase.

Alternatively, surface association of the protein with lipid vesicles, which does not discriminate between the vesicular form of PC or mixed-micelles of PC and bile salts found in the duodenal digestion mix, may be responsible for protecting β-Lg from proteolytic attack. Such interactions could take place *via* the secondary fatty acid binding site in the hydrophobic groove along the single strand of α-helix found in the protein [3, 4] (Fig. 5). This hypothesis is consistent with our observation that thermally induced loss of the α-helical structure abolishes the protective effect of PC. The slight reduction in the effect observed using the negatively charged phospholipid, PS, indicates that electrostatic effects do not dominate the interaction. Together with the greater effect of LPC and LPG, this would support the evidence of more hydrophobic interactions required to protect β-Lg from proteolysis. However, the vesicle-based surface effect would also likely require some electrostatic and hydrophilic interactions. In addition, β-Lg is known to exhibit a high level of non-native helical structure in the presence of alcohols [32], ionic surfactants [33] and lipids

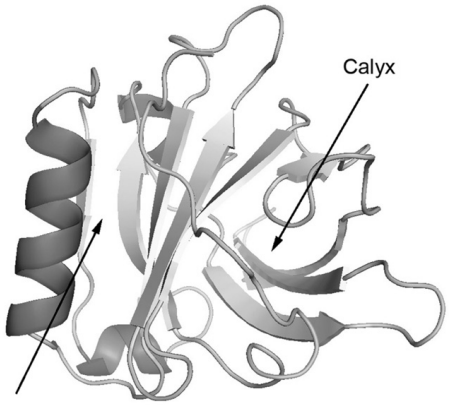

Figure 5. 3-D structure of β-Lg (PDB code: 3blg) showing the position of putative secondary lipid binding site.

[7], with a β-to-α conformational transition with lysophospholipids [14]. Our observation that these same conformational changes take place under the duodenal digestion conditions employed in this study are consistent with the hypothesis that the β-to-α conformational change may also play a role in determining the protective effect exerted by lysophospholipids (Fig. 3). Such interactions with lipids may impair attack by trypsin and chymotrypsin for stearic reasons and they clearly altered the pathway of proteolysis, as indicated by differences in the pattern of proteolysis products observed by SDS-PAGE.

The substantial protective effect of PC during duodenal digestion indicates that lipid components in the gut may allow more protein to reach the mucosal immune system, thus determining the allergic reaction. Other studies performed with simulated gastric or intestinal fluid have also shown that β-Lg was almost completely digested by pancreatic enzymes [23, 24]. This study showed that β-Lg is resistant to both gastric and duodenal digestion when physiologically relevant levels of PC are included in the digestion mix. We believe that PC protected β-Lg from digestion *via* monomeric lipid binding in the secondary fatty acid binding site along the single strand of α-helix present in the protein. The data presented in this study draw attention to interactions between allergens and other food components, especially lipids, which may affect the allergenicity of protein sensitising *via* the GI tract and proteolytic fragments generated during digestion. Therefore, further studies of allergen digestibility need to be performed, taking into account lipid interactions in foods and in the GI tract.

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5 References

[1] Brownlow, S., Cabral, J. H. M., Cooper, R., Flower, D. F., *et al*., Bovine β-lactoglobulin at 1.8 Å resolution – still an enigmatic lipocalin, *Structure* 1997, *5*, 481 – 495.

[2] Kontopidis, G., Holt, C., Sawyer, L., The ligand-binding site of bovine beta-lactoglobulin: Evidence for a function? *J. Mol. Biol.* 2002, *318*, 1043 – 1055.

[3] Frapin, D., Dufour, E., Haertle, T., Probing the fatty acid binding site of beta-lactoglobulins, *J. Protein Chem.* 1993, *12*, 443 – 449.

[4] Dufour, E., Genot, C., Haertle, T., beta-Lactoglobulin binding properties during its folding changes studied by fluorescence spectroscopy, *Biochim. Biophys. Acta* 1994, *1205*, 105 – 112.

[5] Qin, B. Y., Bewley, M. C., Creamer, L. K., Baker, H. M., *et al*., Structural basis of the Tanford transition of bovine beta-lactoglobulin, *Biochemistry* 1998, *37*, 14014 – 14023.

[6] Ragona, L., Fogolari, F., Catalano, M., Ugolini, R., *et al*., EF loop conformational change triggers ligand binding in beta-lactoglobulins, *J. Biol. Chem.* 2003, *278*, 38840 – 38846.

[7] Ragona, L., Fogolari, F., Zetta, L., Perez, M. D., *et al*., Bovine β-lactoglobulin: Interaction studies with palmitic acid, *Prot. Sci.* 2000, *9*, 1347 – 1356.

[8] Brown, E. M., Caroll, R. J., Pfeffer, P. E., Sampugna, J. J., Complex formation in sonicated mixtures of β-lactoglobulin and phosphatidylcholine, *Lipids* 1983, *18*, 111 – 118.

[9] Lefevre, T., Subirade, M., Interaction of β-lactoglobulin with phospholipid bilayers: A molecular level elucidation as revealed by infrared spectroscopy, *Int. J. Biol. Macromol.* 2000, *28*, 59 – 67.

[10] Cornell, D. G., Caroll, R. J., Miscibility in lipid-protein monolayers, *J. Colloid Interface Sci.* 1985, *108*, 226 – 233.

[11] Krinstensen, A., Nylander, T., Paulsson, M., Carlsson, A., Calorimetric studies of interactions between β-lactoglobulin and phospholipids in solutions, *Int. Dairy J.* 1997, *7*, 87 – 92.

[12] Lefevre, T., Subirade, M., Conformational rearrangements of β-lactoglobulin upon interaction with an anionic membrane, *Biochim. Biophys. Acta* 2001, *1549*, 37 – 50.

[13] Zhang, X., Keiderling, T. A., Lipid-induced conformational transitions of β-lactoglobulin, *Biochemistry* 2006, *45*, 8444 – 8452.

[14] Zhang, X., Ge, N., Keiderling, T. A., Electrostatic and hydrophobic interactions governing the interaction and binding of β-lactoglobulin to membranes, *Biochemistry* 2007, *46*, 5252 – 5260.

[15] Uhrinova, S., Smith, M. H., Jameson, G. B., Uhrín, D., *et al*., Structural changes accompanying pH-induced dissociation of the beta-lactoglobulin dimer, *Biochemistry* 2000, *4*, 3565 – 3574.

[16] Wal, J. M., Structure and function of milk allergens, *Allergy* 2001, *56*, 35 – 38.

[17] Mantyjarvi, R., Rautiainen, J., Virtanen, T., Lipocalins as allergens, *Biochim. Biophys. Acta* 2000, *1482*, 308 – 317.

[18] Jenkins, J. A., Breiteneder, H., Mills, E. C. N., Evolutionary distance from human homologs reflects allergenicity of animal food proteins, *J. Allergy Clin. Immunol.* 2007, *120*, 1399 – 1405.

[19] Mouecoucou, J., Villaume, C., Sánchez, C., Mejean, L., Beta-lactoglobulin/polysaccharide interactions during *in vitro* gastric and pancreatic hydrolysis assessed in dialysis bags of different M_r cutoffs, *Biochim. Biophys. Acta* 2004, *1670*, 105 – 112.

[20] Moreno, F. J., Mackie, A. R., Mills, E. C. N., Phospholipid interactions protect the milk allergen α-lactalbumin from proteolysis during *in vitro* digestion, *J. Agric. Food Chem.* 2005, *53*, 9810 – 9816.

[21] Vassilopoulou, E., Rigby, N., Moreno, F. J., Zuidmeer, L., *et al*., Effect of *in vitro* gastric and duodenal digestion on the allergenicity of grape lipid transfer protein, *J. Allergy Clin. Immunol.* 2006, *118*, 473 – 480.

[22] Sreerma, N., Woody, R. W., A self-consistent method for the analysis of protein secondary structure from circular dichroism, *Anal. Biochem.* 1993, *209*, 32 – 44.

[23] Fu, T.-J., Abbott, U. R., Hatzos, C., Digestibility of food allergens and nonallergenic proteins in simulated gastric fluid and simulated intestinal fluid – a comparative study, *J. Agric. Food Chem.* 2002, *50*, 7154 – 7160.

[24] Schmidt, D. G., Meijer, R. J., Slangen, C. J., van Beresteijn, E. C., Raising the pH of the pepsin-catalysed hydrolysis of bovine whey proteins increases the antigenicity of the hydrolysates, *Clin. Exp. Allergy* 1995, *25*, 1007 – 1017.

[25] Carey, M. C., Hernell, O., Digestion and absorption of fat, *Semin. Gastrointest. Dis.* 1992, *3*, 189 – 208.

[26] Qi, X. L., Brownlow, S., Holt, C., Sellers, P., Thermal denaturation of β-lactoglobulin: Effect of protein concentration at pH 6.75 and 8.05, *Biochim. Biophys. Acta* 1995, *1248*, 43 – 49.

[27] Iametti, S., De Gregori, B., Vecchio, G., Bonomi, F., Modifications occur at different structural levels during the heat denaturation of β-lactoglobulin, *Eur. J. Biochem.* 1996, *237*, 106 – 112.

[28] Qi, X. L., Holt, C., McNulty, D., Clarke, D. T., *et al*., Effect of temperature on the secondary structure of β-lactoglobulin at pH 6.7, as determined by CD and IR spectroscopy: A test of the molten globule hypothesis, *Biochem. J.* 1997, *324*, 341 – 346.

[29] Maser, N. A., Benedek, G. A., Carey, M. C., Quasielastic light scattering of aqueous biliary lipid systems. Mixed micelle formation in bile salt-lecithin solutions, *Biochemistry* 1980, *19*, 601 – 615.

[30] Matsuoka, K., Maeda, M., Moroi, Y., Characteristics of conjugate bile salt-phosphatidylcholine-cholesterol-water systems, *Coll. Surf.* 2004, *33*, 101 – 109.

[31] O'Neill, T. E., Kinsella, J. E., Binding of alkanone flavours to beta-lactoglobulin: Effects of conformational and chemical modification, *J. Agric. Food Chem.* 1987, *35*, 770 – 774.

[32] Sarker, D. K., Wilde, P. J., Clark, D. C., Competitive absorption of L-α-lysophosphatidylcholine/β-lactoglobulin mixtures at the interfaces of foams and foam lamellae, *Coll. Surf.* 1995, *3*, 149 – 356.

[33] Mendieta, J., Folqué, H., Tauler, R., Two-phase induction of the nonnative α-helical form of β-lactoglobulin in the presence of trifluoroethanol, *Biophys. J.* 1999, *76*, 451 – 457.