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Phospholipid Interactions Protect the Milk Allergen α-Lactalbumin from Proteolysis during in Vitro Digestion

F. JAVIER MORENO,* ALAN R. MACKIE, AND E. N. CLARE MILLS

Institute of Food Research, Norwich Research Park, Colney Lane, Norwich NR4 7UA, United Kingdom

Interactions with food components may alter the resistance of food proteins to digestion, a property thought to play an important role in determining allergenic properties. The kinetics of breakdown of the bovine milk allergen α-lactalbumin during in vitro gastrointestinal digestion was found to be altered by interactions with physiologically relevant levels of phosphatidylcholine (PC), a surfactant that is abundant both in milk and is actively secreted by the stomach. Breakdown during gastric digestion was slowed in the presence of PC and accompanied by small alterations in the profile of resulting peptides, with little effect being observed during subsequent duodenal digestion. α-Lactalbumin was found to unfold at gastric (acid) pH, giving a CD spectrum similar to that obtained for the partially folded state it is known to adopt at pH values below its isoelectric point. Fluorescence polarization studies performed at low pH indicated that this partially unfolded form of the protein was able to penetrate into the PC vesicles. These interactions are probably responsible for the slowing of gastric digestion by reducing the accessibility of the protein to pepsin. These findings show that interactions with other food components, such as lipids, may alter the rate of breakdown of food proteins in the gastrointestinal tract. It underlines the importance of the food matrix in affecting patterns of food allergen digestion and hence presentation to the immune system and that in vitro digestion systems used for assessing digestibility of allergens must take account of surfactants.

KEYWORDS: α-Lactalbumin, digestion, food allergy, phosphatidylcholine, protein−lipid interaction

1. INTRODUCTION

Cow's milk allergy is one of the major food allergies (*1*) and is especially important in children during the first 3 years of life. The whey protein α-lactalbumin (α-La) is an important cow's milk allergen (*2*), comprising a water-soluble monomeric globular domain primarily α-helical in nature with some 3_{10} helix and β-sheet and a hydrophobic core well protected from the solvent in the interior by the rigid packing of side chains (*3*, *4*). It normally binds a single Ca^{2+} ion, with the calcium binding site being the most ordered part of the protein structure. Under acidic conditions the Ca^{2+} ion is lost and α-La adopts the so-called molten globule state, a well-defined and nativelike secondary structure, but with a complete loss of tertiary structure (*4*−*7*). Many food allergens are thought to sensitize individuals via the gastrointestinal tract (GIT) and hence their resistance to proteolysis maybe an important factor to consider since it may affect their allergenic potential (*8*−*10*). Thus for a food protein to sensitize an individual, sufficient immunologically active protein (or protein fragments) needs to survive the degradative environment of the GIT to interact with the mucosal immune system. Resistance to proteolysis by pepsin does appear to be a characteristic shared by many food allergens (*11*), but there are some notable exceptions, including the major milk allergens, the caseins and α-La, which are both very susceptible to proteolysis by both *endo*- and *exo*-peptidases (*2*, *11*). Thus, rapid degradation of α-La by pepsin within 30 s to 2 min has been observed following in vitro digestion (*8*, *12*).

Given such rapid breakdown of these proteins during digestion, how might they be able to sensitize the gut mucosal immune system? Many of the proteolysis studies of allergens give little information regarding the nature of proteins surviving digestion in the human GIT, not the least because of the excess of pepsin used. Thus, it has been estimated that pepsin secretion in adults is between 20 and 30 kUnits of enzyme activity/24 h at 37 °C (*13*) and from the activity in commercially available pepsin preparations used in digestion assays, this can be estimated to be equivalent to around 10 mg of pepsin secreted/24 h, whereas a typical adult dietary intake of protein could be estimated around 75 g/24 h (*14*). In addition, there are many other components in foods that may alter the course of proteolysis, including polysaccharides and lipids. Thus the susceptibility of β-lactoglobulin to proteolysis by trypsin and chymotrypsin is apparently retarded by polysaccharides such as pectins, gum arabic, and xylan (*15*). This paper reports an in vitro investigation into the effects of phosphatidylcholine (PC) on the proteolytic breakdown of α-La, since this lipid is

* Corresponding author. Current address: Instituto de Fermentaciones Industriales (CSIC), C/Juan de la Cierva 3, 28006 Madrid (Spain). Tel: +34 91 5622900. Fax: +34 91 5644853. E-mail: j.moreno@ifi.csic.es.

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abundant in milk itself, in addition to being secreted by the stomach itself.

2. MATERIALS AND METHODS

Preparation of Phospholipid Vesicles. Egg L-α-phosphatidylcholine (PC) was obtained from Lipid Products (South Nufield, Surrey, UK) at 99% purity. Solvent was removed from a 3 mL (150 mg) aliquot of PC stock solution (63.5 mM in chloroform) under rotary evaporation at 5 °C in order to form a dry thin film of phospholipids. Residual solvent was removed under vacuum overnight and dried PC was then dissolved in 16.5 mL of warmed simulated gastric fluid (SGF, 0.15 M NaCl, pH 2.5) by addition of five glass beads. After flushing with argon, PC was placed in a shaking incubator (at 170 rpm, 37 °C) for 20 min to be resuspended. The suspension was then transferred to a water-jacketed sonication vessel and sonicated at 5 °C (30% full power, 9/10 power cycle) for 10 min using a sonication probe (Status US200, Avestin). Phospholipid vesicles were collected and filtered through Millex-HA 0.45 μm mixed cellulose (Millipore, Billerica, MA) to remove possible titanium particles (*16*).

With the aim to study a single species of phospholipid with a well-defined phase transition a 3 mL (150 mg) sample of 1-palmitoyl-2-myristoylphosphatidylcholine (PMPC, Avanti Polar-Lipids, Alabaster, AL) from a stock solution (70.8 mM in chloroform) was also sonicated as described above.

In Vitro Gastric and Duodenal Models. These digestion models have been previously described by Moreno et al. (*17*) and were based on in vivo data obtained by gastric and duodenal aspiration and from collection of effluent from illeostomy volunteers at the Institute of Food Research.

Gastric Digestion. Digestions were performed in either the presence or absence of PC. In the latter case, the PC solution was replaced by SGF at pH 2.5. Control samples, with no enzyme additions, were also analyzed. α-La from bovine milk (type I, Sigma, Poole, Dorset, UK) was dissolved in SGF (11.1 mg/mL) and mixed with phospholipid vesicle solution (1:1.2, v:v). The pH was adjusted to 2.5 with 1 M HCl if necessary. After incubation at 37 °C for 15 min, a solution of 0.32% (w:v) porcine pepsin (EC 3.4.23.1) in SGF, pH 2.5 (Sigma, activity: 3,640 U per mg of protein calculated using haemoglobin as substrate), was added at an approximately physiologically ratio of enzyme:substrate (1:20 w:w), giving 182 U of pepsin per mg of α-La. This gave a final concentration of 6.3 mM PC and 5 mg/mL α-La in the final gastric digestion mix. The digestion was performed at 37 °C in an incubator with moderate agitation, and aliquots, which were withdrawn from a single digestion mixture, were taken at 0, 2, 5, 15, 30, 60, and 120 min for further analysis. The digestion was stopped by raising the pH to 7.5 using 40 mM ammonium bicarbonate (BDH, Poole, Dorset, UK).

Duodenal Digestion. In vitro duodenal digestion was performed using 120 min in vitro gastric digesta as starting material. The pH was adjusted to 6.5 and the following added: (1) a bile salt mixture containing equimolar quantities (0.125 M) of sodium taurocholate (Sigma) and glycodeoxycholic acid (Calbiochem, La Jolla, CA); (2) 1 M $CaCl_2$ (BDH); (3) 0.25 M Bis-Tris, pH 6.5 (Sigma); (4) porcine pancreatic lipase (20 μL:10 mL of total volume) (0.1%, w:v, Sigma, activity 25 600 U/mg of protein); and (5) porcine colipase (40 μL:10 mL of total volume) (0.055%, w:v, Sigma). Finally, solutions of porcine trypsin (0.1%, w:v, Sigma, activity: 13 800 U/mg of protein using BAEE as substrate) and bovine α-chymotrypsin (0.4%, w:v, Sigma; activity, 44 U/mg of protein using BTEE as substrate) in water were prepared and added at approximately physiological ratios of α-La (as denoted by the initial concentration in gastric digestion):trypsin:chymotrypsin = 1:(1/400):(1/100) (w:w) or 1 mg:34.5 U:0.44 U. This gave a final duodenal digestion mix as follows: PC, 5.8 mM; α-La, 4.6 mg/mL; bile salts, 7.4 mM; $CaCl_2$, 9.2 mM, Bis-Tris; 24.7 mM. The digestion was performed at 37 °C, and aliquots were taken at 0, 2, 5, 15, 30, 60, and 120 min for further analysis. The digestion was stopped by adding a solution of Bowman−Birk trypsin−chymotrypsin inhibitor from soybean (Sigma) at a concentration calculated to inhibit twice the amount of trypsin and chymotrypsin present in the digestion mix.

SDS−PAGE Analysis. Samples taken at different stages of the digestion were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS−PAGE). Sample (20 μL) was added to 17.5 μL of ultrapure water and 12.5 μL of 4× NuPAGE LDS Sample buffer (Invitrogen, Carlsbad, CA) at 70 °C for 10 min. Finally, samples were loaded (10 μL) on a 12% polyacrylamide NuPAGE Novex Bis-Tris precast gel. A continuous buffer system was used consisting of 50 mL of 20× NuPAGE MES SDS running buffer with 950 mL of ultrapure water. Gels were run for 35 min at 120 mA/gel and 200 V and stained using a Colloidal Blue Staining Kit (Invitrogen). Molecular weight markers comprised the following mix of proteins: aprotinin (M_r 6500), α-La (M_r 14 200), trypsin inhibitor (M_r 20 000), carbonic anhydrase (M_r 29 000), ovalbumin (Mr 45 000), bovine serum albumin (M_r 66 000), β-galactosidase (M_r 116 000), myosin (M_r 205 000) (Sigma), and insulin B chain (M_r 3000), aprotinin (M_r 6500), α-lysozyme (M_r 16 000), myoglobin red (M_r 17 000), carbonic anhydrase (M_r 28 000), alcohol dehydrogenase (M_r 38 000), glutamic dehydrogenase (M_r 49 000), bovine serum albumin (M_r 66 000), phosphorylase (M_r 98 000), and myosin (M_r 188 000) (Invitrogen).

RP-HPLC. Samples were diluted five times with solvent A and applied (50 μL) to a Phenomenex Jupiter Proteo 90 Å pore size, 4 μm particle size (250 × 4.6 mm i.d.) column attached to a Dionex HPLC system with a diode array detector. Samples were eluted using 0.1% (w/v) trifluoroacetic acid in double-distilled water as solvent A and 0.085% (w/v) trifluoroacetic acid in double-distilled water/acetonitrile (10:90, v/v) as solvent B following the method described by Moreno et al. (*18*).

Circular Dichroism Spectroscopy. Far- (195−260 nm) and near-ultraviolet (250−350 nm) CD spectra of α-La at pH 7 and 2.5 (dissolved in 10 mM sodium-phosphate buffer and SGF, respectively) were recorded with a J-710 spectropolarimeter (Jasco Corp., Tokyo, Japan) using a 0.2 and 0.1 cm path length quartz cell. The instrument was calibrated with ammonium d-10-camphorsulfonate. Spectra represent the average of four accumulations collected at 100 nm/min, with a 2 s time constant, 0.5 nm resolution, and sensitivity of ±100 mdeg. Spectra are represented as molar CD (with respect to moles of amide bond) and α-La was dissolved at 0.175 mg/mL for the analysis in the far-UV region and 2 mg/mL for the near-UV region. The buffer blanks were subtracted from each CD spectrum.

Fluorescence Polarization. To label the vesicle suspension (PC or PMPC), 0.4 μL of 0.5% (w:v) 1,6-diphenyl-1,3,5-hexatriene (Molecular Probes, Eugene, OR) was added per milligram of phospholipid. The mixture was incubated for 30 min at 37 °C. Then, the α-La solution (11.1 mg/mL in SGF pH 2.5) was mixed with labeled phospholipids vesicles (1:1.2, v:v). The pH of samples was adjusted to either 2.5 or 7 before incubating at 37 °C for 2 h with shaking. Samples were prepared in triplicate and the relative standard deviation was below 5% in all determinations.

Measurements of fluorescence polarization were performed with a LS 55 luminescence spectrometer (Perkin-Elmer, Wellesley, MA). 1,6-Diphenyl-1,3,5-hexatriene was excited at 366 nm and its emission detected at 430 nm (*19*, *20*). Excitation and emission slits were 2.5 nm, and the integration time was 2 s. The steady-state polarization of the fluorescence emission was measured in thermostated cells of 1 cm optical path length. An external water bath provided the means of temperature control for the cell, while a thermocouple measured the temperature in the cell's water jacket. Measurements were performed at 20, 25, 30, 35, 40, 45, 50, 55, and 60 °C.

3. RESULTS

In Vitro Gastric and Duodenal Digestion. SDS−PAGE analysis showed α-La to be rapidly degraded by pepsin and after 5 min only a faint band with a slightly faster electrophoretic mobility could be observed (**Figure 1A**). This was accompanied by the appearance of low molecular weight fragments running close to the gel dye front and of M_r < 6500. However, when PC was included at levels relevant to those found in the human stomach, proteolysis was retarded and a band of M_r slightly below 14 000, which might correspond to the partially trimmed

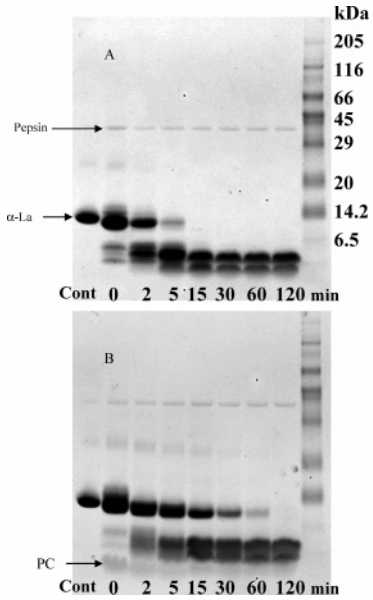

Figure 1. SDS–PAGE analysis under nonreducing conditions of α-La subjected to gastric digestion over 120 min in the absence (**A**) or presence (**B**) of phosphatidylcholine (PC).

α-La, could still be detected after 60 min of gastric digestion, although by 120 min it appeared to be completely broken down to fragments of the same mobility as those observed following gastric digestion in the absence of PC (**Figure 1B**). HPLC analysis confirmed this observation with some evidence that intact α-La (eluting at 57 min) was present at 5 and 30 min only in the presence of PC, although the peak shape changed and may be indicative of "trimming" of the intact protein by pepsin, as has been observed for other proteins (*17*). HPLC analysis revealed that the M_r < 6500 species observed by SDS PAGE did not comprise a single major product but was instead a mixture of peptides with retention times between 20 and 45 min (**Figure 2**). Some minor differences in peptide profiles were observed when gastric digestion was performed in the presence of PC, with a minor peptide eluting at 20 min (indicated by open arrows in **Figure 2**) being more abundant in the presence of PC.

After 2 h of gastric digestion, the pH was raised to 6.5, irreversibly inactivating pepsin and mimicking the transfer of gastric contents into the duodenal compartment. Digestion was followed for up to 120 min in the duodenal phase, although a time of 15 min was chosen as that most relevant, on the basis that this is approximately the transit time down the duodenum and into the jejunum/ileum to the site of the first Peyer's patch.

The M_r < 6500 species remaining after pepsinolysis was rapidly broken down during duodenal digestion by a combination of trypsin and chymotrypsin, and after 120 min all trace of α-La-derived peptides had gone, essentially identical digestion patterns being obtained in the presence (data not shown) or absence of PC (**Figure 3**). This was confirmed by HPLC analysis of digesta, which showed the presence of a complex mixture of peptides eluting between 15 and 42 min (**Figure 4**),

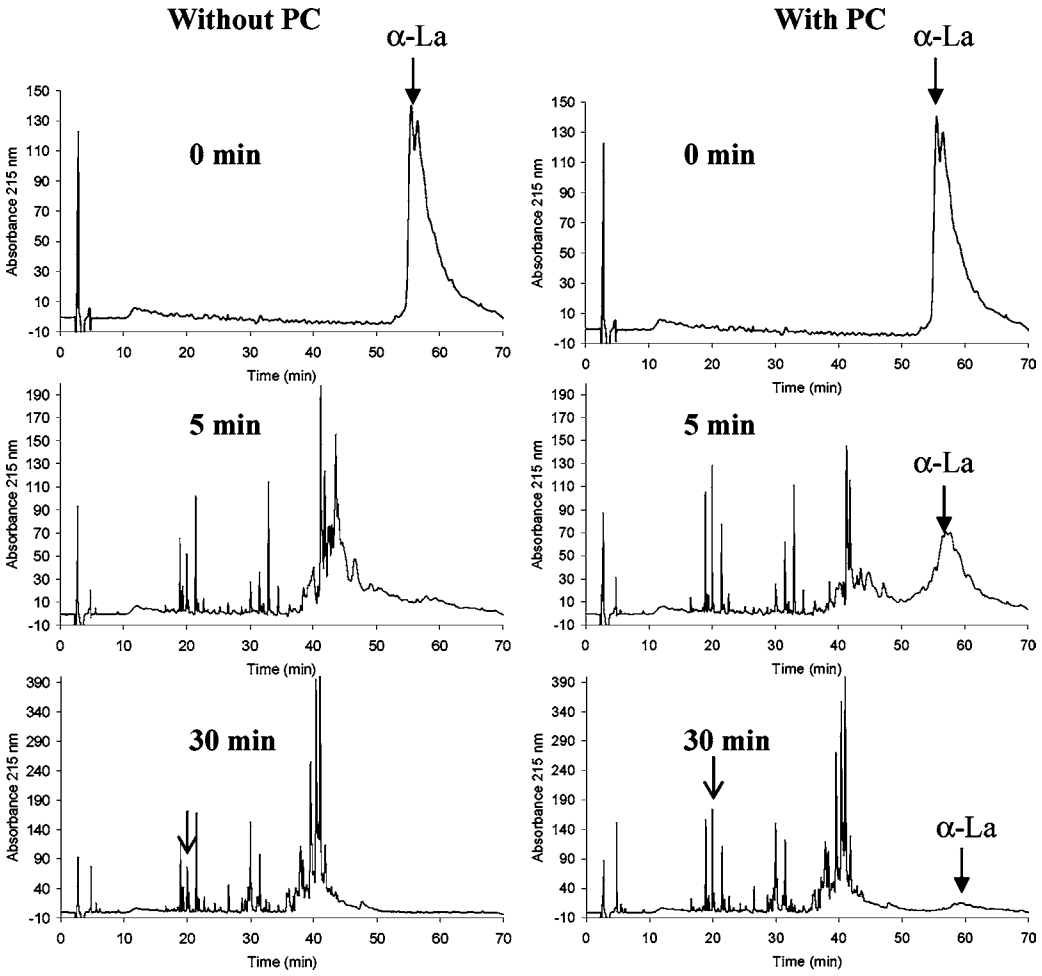

Figure 2. Gastric digestion of α-La analyzed by RP-HPLC in the presence or absence of phosphatidylcholine (PC) using a Jupiter Proteo column (90 Å pore size). Closed arrows indicate the presence of intact α-La and open arrows show minor differences in the peptide profile.

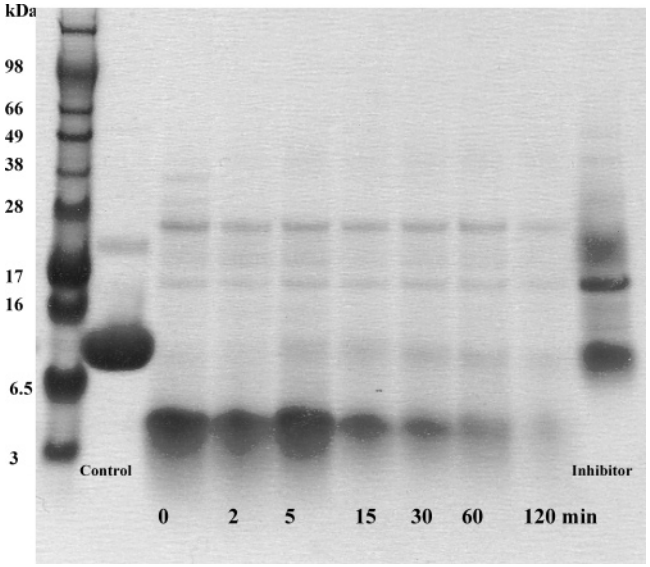

Figure 3. SDS–PAGE analysis under nonreducing conditions of α-La subjected to gastric digestion for 120 min followed by duodenal digestion over 120 min in the absence of phosphatidylcholine (PC).

although there appear to be differences in the proportions of peptides eluting at around 20 min, suggesting that while PC did not protect α-La peptides from further proteolysis, it did affect the fragmentation pattern.

Effect of Gastric Conditions on the Secondary and Tertiary Structure and Lipid-Binding Properties of α-La. To investigate the mechanism underlying the protective effects of PC on pepsinolysis of α-La, the effect of the low gastric pH on its structure and interaction with phospholipids was studied. The far-UV CD spectrum of native α-La (i.e. at pH 7.0) was typical for the protein, with a broad negative minimum between 210 and 230 nm (**Figure 5A**) but on lowering the pH to a gastric pH of 2.5, the negative minimum intensified at 206 nm and became more positive at 230 nm. Both spectra became positive at 200 nm. Such a spectrum is characteristic of the so-called "molten globule" structure of α-La induced at low pH (*7*, *21*). This partially folded state is also characterized by a loss of tertiary structure, which was evident from the near-UV CD spectrum at low pH, which is essentially featureless (**Figure 5B**).

The interaction of α-La with egg PC and the single chain lipid PMPC was then assessed as a function of temperature at

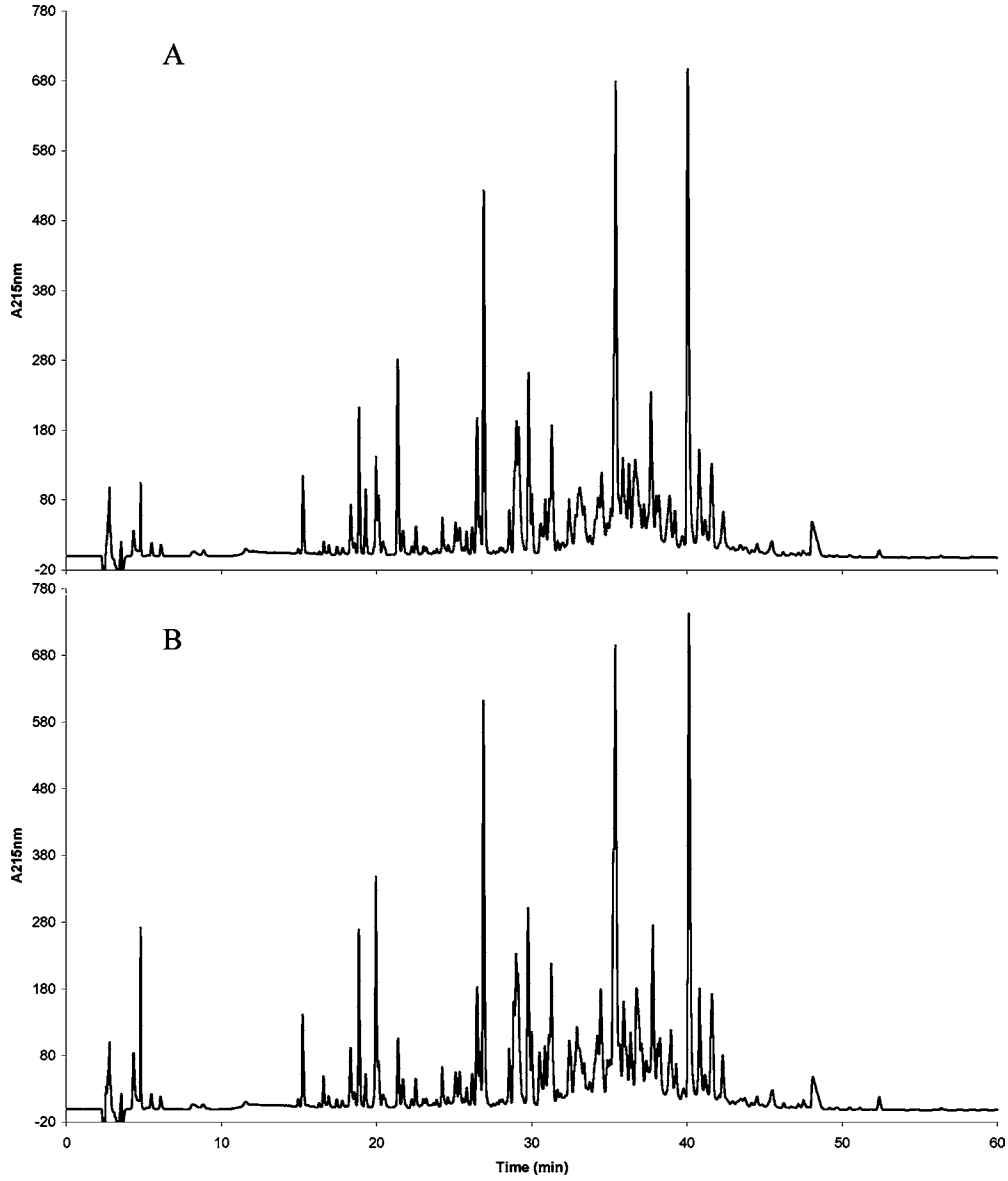

Figure 4. α-La subjected to gastric digestion for 120 min followed by duodenal digestion for 15 min analyzed by RP-HPLC in the absence (**A**) or presence (**B**) of phosphatidylcholine (PC) using a Jupiter Proteo column (90 Å pore size).

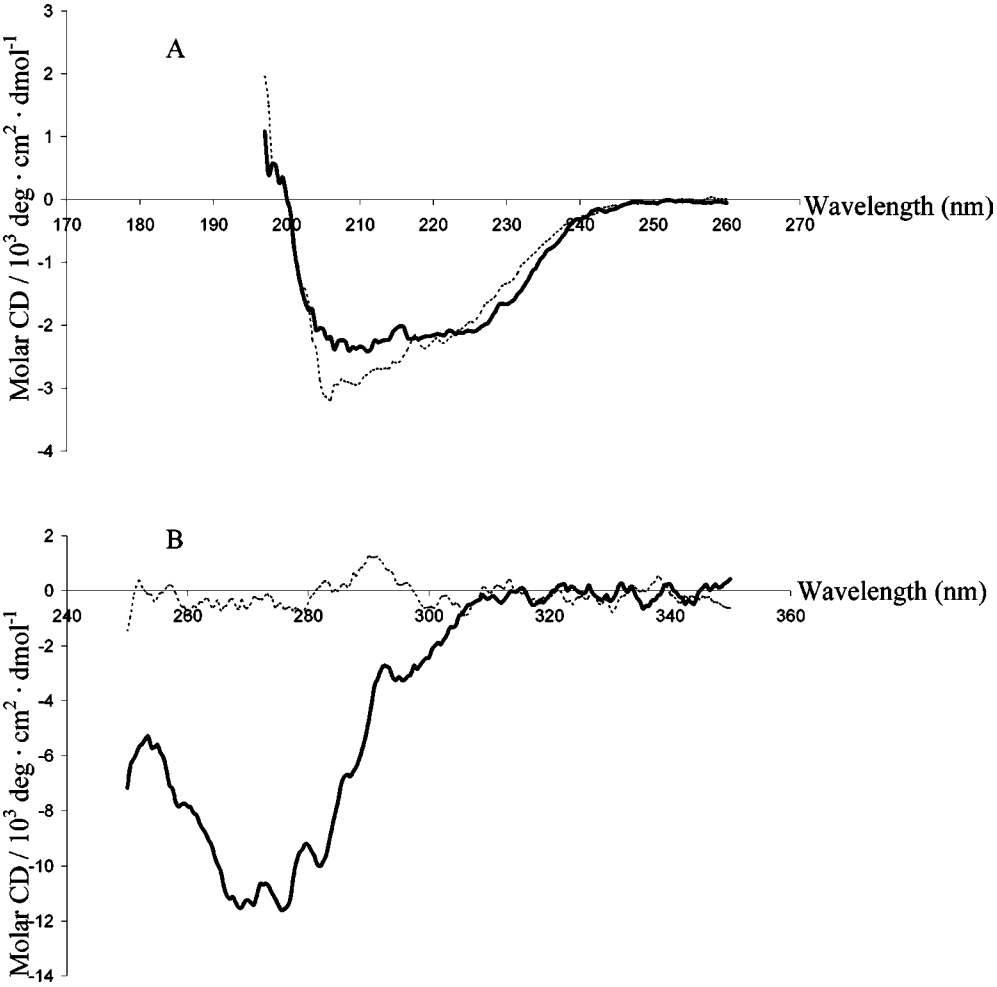

Figure 5. CD spectra in the far-UV (**A**) and near-UV (**B**) region of α-La at pH 7 (solid line) and pH 2.5 (dotted line).

pH 2.5 and 7 (**Figure 6**). No significant shift in the fluorescence polarization of the probe, diphenylhexatriene, was observed when native α-La (i.e. at pH 7) was incubated with PC, while a noticeable increase occurred at pH 2.5 at all temperatures (**Figure 6A,B**). Since egg PC comprises a complex mixture of phospholipids of various chain lengths, a broad phase transition was obtained. Therefore, experiments were repeated using a single-chain phospholipid, PMPC (**Figure 6C,D**). Using this more defined lipid, an increase in fluorescence polarization was observed at neutral pH at 25−35 °C, indicating that α-La was interacting with the PC vesicles. At acid pH, a more significant increase in fluorescence polarization was obtained at all temperatures with a great shift in the phase transition of the phospholipid whose melting temperature is 30.7 °C (*22*). The increase in the transition temperature suggests that α-La is able to penetrate the vesicle and to interact hydrophobically with the phospholipids fatty acid chains, reducing their mobility and, therefore, increasing the fluorescence polarization of the probe (*19*).

Finally, an association between the pepsin with the PC vesicles which could reduce its activity can be ruled out, since certain proteins (such as caseins) are unaffected by the presence of PC during their pepsin digestion (N. M. Rigby, personal communication).

4. DISCUSSION

While there are several reports on the in vitro digestion of α-La by proteases, such as pepsin (*8*, *12*, *23*), none has, to date, taken account of the presence of physiological surfactants found in both milk and in the gastric environment of the GIT. We have shown, for the first time, that the interaction between PC and α-La at gastric pH is strong enough to retard the breakdown of the α-La during the gastric digestion, with traces of the protein partially trimmed still evident after 1 h of digestion. There are also indications that PC altered the fragmentation pattern of α-La.

At pH 7.0, which is well above the isoelectric point of α-La of 4.8, flouresence polarization studies indicated a weak interaction between the protein and PC vesicles and is probably indicative of the protein being more loosely associated with the outer surface of the vesicle, probably as a consequence of electrostatic interactions. However, at the acid pH of the stomach, α-La adopted a partially unfolded state, a conformational state that would be more able to penetrate into the PC vesicles by virtue of hydrophobic interactions between hydrophobic residues revealed on the surface of the partially unfolded protein and the alkyl chains of the PC (*20*). It is well-known that the molten globule state possesses substantial conformational flexibility (*4*, *24*) and a higher surface hydrophobicity than the native state (*7*, *25*, *26*). Indeed, Semisotnov et al. (*4*) showed the stronger affinity of a fluorescent hydrophobic probe (ANS) to the molten globule state of α-La as compared with the native form. The adoption of the molten globule conformation is likely to be a prerequisite for the penetration of α-La into phospholipid vesicles, as it will be dependent on the more flexible structure of the molten globule state in addition to the

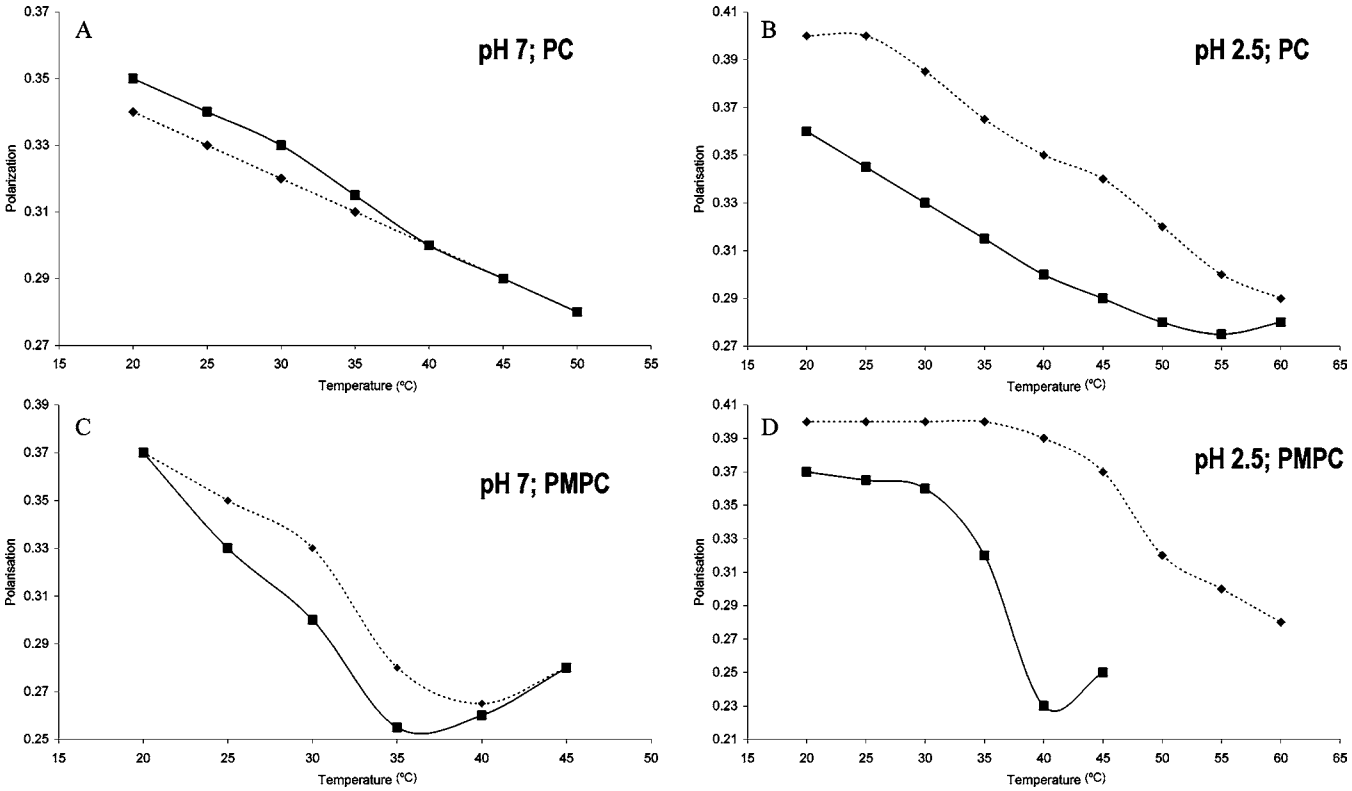

Figure 6. Fluorescence polarization of diphenylhexatriene in egg L-α-phosphatidylcholine (PC; **A**, **B**) and 1-palmitoyl-2-myristoylphosphatidylcholine (PMPC; **C**, **D**) as a function of temperature at pH 7 (**A**, **C**) and 2.5 (**B**, **D**), in the presence (dotted line) or absence (solid line) of α-La.

exposure of hydrophobic amino acid side chains (*25*, *27*, *28*). A similar interaction has been observed before for α-La, but for phosphatidylserine/phosphatidylethanolamine vesicles, the interaction of which was found to increase as a function of decreasing pH, with practically no binding at pH 6 and 7 (above the p*I* α-La), interactions below pH 6 were hydrophobic in nature (*27*).

Aspartic proteinases, such as pepsin, cleave substrate sequences which are forced to adopt an extended *β*-strand conformation (*29*), and hence, as might be expected, in the absence of PC α-La in its molten globule state proved to be a good substrate for pepsin. In the presence of PC, penetration of the α-La into the lipid vesicles was probably responsible for the reduced rate of pepsinolysis under gastric conditions, for steric reasons and possible repulsion by the charged PC headgroups. However, it is unlikely that α-La will have become completely enclosed in the vesicles, allowing pepsinolysis to proceed along exposed regions of the protein, albeit at a reduced rate. Previous studies (*27*, *30*, *31*) have demonstrated that a fragment of bovine α-La from residues 80−108 became embedded into phosphatidylserine/phosphatidylethanolamine and phosphatyldicholine vesicle bilayers under acidic conditions, which protected it from proteolysis by trypsin, chymotrypsin, and carboxypeptidase Y. There are five cleavage sites, according to the Peptide Cutter tool of ExPASy (http://us.expasy.org/tools/peptidecutter/), located in this region, which would be inaccessible to the pepsin once α-La is embedded in phospholipid vesicles.

The protective effect of PC was lost in subsequent duodenal digestion, with the α-La being rapidly broken down. This is probably because the bile salts in the duodenal digestion mix disrupt the vesicular structure of the PC as well as a possible mild detergent action on the α-La peptides. Burnett et al. (*32*) observed the desorption of several protein allergens from model stomach emulsions with the addition of bile salts.

The substantial protective effect of PC on gastric digestion indicates that lipid components in the gut may allow more protein to reach the mucosal immune system in the small intestine than would otherwise be the case. The in vitro model used in this study does not take account of the flow of material down the GIT. Thus, while α-La was subjected to 120 min of gastric digestion, in vivo it is likely that gastric emptying will begin after around 45 min. The extended lifetime of the α-La observed due to interactions with PC vesicles may therefore be of physiological significance and has potential to alter the amounts of intact or large fragments of proteins able to reach the mucosal immune system further down the GI tract. In addition, although stability is clearly important for a protein sensitizing via the gastrointestinal tract, as it will allow retaining sufficient three-dimensional structure, it might be that stabilization of proteolytic fragments generated during digestion also plays a role in sensitization.

While the structure of foods is known to influence the release of other nutrients during digestion, interactions between allergens and other food components, particularly lipids, have been largely neglected. However, the potential interaction of food allergens with cell membranes or lipid structures has been recently described as one of the properties that might promote allergenicity (*33*). The data presented here indicate that studies of allergen digestibility need to take account of lipid interactions either in foods or in the gut itself. A knowledge of such component interactions may help us begin to understand why, for example, milk proteins such as α-La and caseins, some of the most digestible protein sources known, are important milk allergens.

ABBREVIATIONS USED

α-La, α-lactalbumin; GIT, gastrointestinal tract; PC, phosphatidylcholine; PMPC, 1-palmitoyl-2-myristoylphosphatidylcholine; SGF, simulated gastric fluid.

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