



Stability of sunflower 2S albumins and LTP to physiologically relevant *in vitro* gastrointestinal digestion

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ABSTRACT

In order for a protein to elicit a systemic allergic response it must reach the circulatory system through the intestinal mucosa as a sufficiently large fragment with adequate structural integrity. Sunflower LTP and 2S albumins (SFA8 and three mixed fractions of Alb1 and Alb2) were digested in simulated gastric fluid (SGF) for 2 h and the conditions were then changed to mimic the intestinal environment for a further 2 h digestion. The effects of phosphatidylcholine (PC) and emulsification on the digestibility of the proteins were investigated. PC protected all of the proteins studied against both gastric and intestinal digestive enzymes but to different extents. Emulsification of SFA8 resulted in strong protection against digestion, which was further enhanced by the presence of PC in the SGF. These results highlight the importance of considering real food structures such as emulsified systems and also the gastrointestinal environment that proteins are exposed to once consumed when assessing allergenicity.

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1. Introduction

The 2S albumin storage proteins of sunflower (*Helianthus annuus* L.) are cysteine-rich, water-soluble proteins with molecular masses between 12 and 15 kDa. They belong to the prolamin superfamily, which also includes the non-specific lipid transfer proteins (nsLTPs). These proteins share structural compactness and high stability which are attributed to a highly conserved skeleton of eight cysteine residues (C–X_n–CX_n–CC–X_n–CXC–X_n–CX_n–C) (Breiteneder & Mills, 2005; Shewry, Napier, & Tatham, 1995) which form four intramolecular disulphide bonds (Monsalve, Villalba, Rico, Shewry, & Rodríguez, 2004).

Abbreviations: nsLTPs, non-specific lipid transfer proteins; SFAs, the total fraction of sunflower albumins; SFA8, sunflower albumin 8; Alb 1 and Alb2, albumin genes and proteins; RP-HPLC, reversed phase high performance liquid chromatography; SDS–PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; GI, gastrointestinal; IgE, immunoglobulin E; SGF, simulated gastric fluid; PC, egg 1-phosphatidylcholine.

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The sunflower 2S albumins comprise about 13 components, including SFA8 which has been studied in detail because of its high content of 16 methionine residues out of 103 residues (Pantoja-Uceda et al., 2004). Both SFA8 and sunflower nsLTP (LTP) are able to bind lipids, which in the case of SFA8 leads to significant emulsifying activity (Berecz et al., 2010; Burnett^a et al., 2002). All LTPs possess an internal hydrophobic cavity which is able to bind lipid molecules *in vitro* (Douliez, Michon, Elmorjani, & Marion, 2000; Luckett et al., 2001). This ability to bind lipid affects the stability of some LTPs and their resistance to hydrolysis (Breiteneder & Mills, 2005; Douliez et al., 2000; Vassilopoulou et al., 2006).

The 2S albumin and LTP protein families both include a number of major allergens, including SFA8 from sunflower (Yagami, 2010), Ara h 2 from peanut (Burks et al., 1991) and Ber e 1 from Brazil nut (Pastorello et al., 1998); and LTPs from peach (Pru p 1) (Wijesinha-Bettoni et al., 2010), barley (Wijesinha-Bettoni et al., 2010) apple (Sancho et al., 2005) and grape (Vassilopoulou et al., 2006).

Proteins must reach the circulatory system through the intestinal mucosa in an intact form or as a large fragment to elicit an allergic response. It is therefore not surprising that many allergens that sensitise through the GI tract possess high resistance to thermal denaturation and pepsin digestion (Astwood, Leach, & Fuchs, 1996). Allergenic proteins often also have an ability to bind lipids, to associate with membranes or are amphipathic in nature, these

properties allowing them to adsorb to gastric emulsions and providing further resistance to digestion (Macierzanka, Sancho, Mills, Rigby, & Mackie, 2009).

In vitro digestion is a useful method to mimic the gastrointestinal environment and digestion (Amigo-Benavent, Clemente, Ferranti, Caira, & Dolores del Castillo, 2011; Kopf-Bolan et al., 2012; Sirvent, Palomares, Cuesta-Herranz, Villalba, & Rodriguez, 2012). We have therefore determined the effects of emulsification and of the physiological surfactant phosphatidylcholine (PC) on the simulated gastric and intestinal digestion of sunflower 2S albumins and LTP, in order to understand whether specific food processing could change the allergenicity of sunflower proteins by affecting the resistance to proteolysis. The effect was measured in terms of both rates and patterns of digestion through *in vitro* simulated gastric and intestinal digestion.

2. Materials and methods

2.1. General

2.1.1. Purification of 2S albumins from sunflower seed

Seeds of sunflower (*H. annuus*, L.) hybrid line PR A381 were obtained from Pioneer Ltd., Hungary. Purification was carried out as described by Berecz et al. (2010): a total sunflower albumin fraction was prepared by RP-HPLC and five fractions (LTP, SFA8 and three mixed 2S albumin fractions, called fractions A, B and C) were collected and analysed by SDS-PAGE. Preparative separation was carried out on a Phenomenex Jupiter 10 μm C₁₈ (250 $\times$ 10 mm) column. Peak fractions were collected, freeze-dried and stored at -20°C over silica gel. Purified fractions were reduced, pyridylethylated and subjected to N-terminal sequencing by automated Edman degradation using a model 494 Applied Biosystems protein sequencer.

2.1.2. Emulsion preparation

2 ml of n-hexadecane was homogenised with 5 mg of SFA8 dissolved in 1.8 ml Na-citrate buffer (pH 5) using a Status 200 sonicator. The sonication was carried out in a cooled jacketed vessel and the procedure (Burnett et al., 2002) consisted of a 0.1 s pulse (10% power) followed by 0.9 s relaxation repeated 20 times, which cycle was repeated after standing for 2 min. This procedure allows for heat dissipation therefore the emulsion temperature never exceeded 25°C . The emulsion was washed to remove the interstitial (non-absorbed) protein by centrifugation at 5000g for 10 min so that the emulsion separated into two phases (cream and subphase). The non-creamed subphase was then removed and analysed. Citrate buffer was added to the emulsion and it was shaken to redisperse the droplets. This process was repeated for a total of three washes (Burnett et al., 2002). No change in drop size was detected before and after the cleaning procedure indicating that coalescence had not occurred during centrifugation. The amount of protein associated with oil and the volume of the emulsion required to be added into the digesta to achieve the 2 mg ml^{-1} protein concentration used for the digestion were calculated. Drop-let size was measured by an LS-230 (Coulter, USA) light scattering instrument.

2.2. *In vitro* gastric and intestinal models

2.2.1. Preparation of phospholipid vesicles

Egg 1-PC, grade 1, was obtained from Lipid Products (South Nutfield, Redhill, Surrey, UK) at 99% purity. The storage solvent was removed by rotary evaporation and then under vacuum overnight in the absence of oxygen. It was stored under argon until further use. The dry PC was dispersed in SGF (simulated gastric fluid: 0.15 M

NaCl, pH 2.5, adjusted with 1 M HCl) by sonication at 5°C (10 min set at 30% full power, 9/10 power cycle) using a Status Ultrasonic (Avestin, Canada) US200 homogenizer fitted with a TT13 titanium flat tip. Phospholipid vesicles were collected and filtered through Millex-HA 0.45 μm mixed cellulose (Millipore, Billerica, MA, USA) to remove titanium particles.

2.2.2. *In vitro* gastric digestion (phase 1)

Digestions were performed in either the presence or absence of PC. In the former, the PC solution was replaced by SGF. Control samples, with no enzyme additions, were also analysed. Proteins were dissolved in SGF to the concentration of 1 mg ml^{-1} . For the experiments with the presence of PC, SGF was mixed with PC vesicle solution (1: 1.2, v/v) and the pH was adjusted to 2.5 with 1 M HCl if necessary. After incubation at 37°C for 15 min, a solution of pepsin (EC 3.4.23.1) 0.32% (w/v) in SGF, pH 2.5 (Sigma, Poole, Dorset, UK, P 6887), was added at an approximately physiological ratio of enzyme/substrate (1:20, w/w); 182 U pepsin U mg^{-1} of proteins. This resulted in a final volume of 3.5–4 ml and final concentrations of 6.3 mM PC and 2.5 mg ml^{-1} protein in the final phase 1 digestion mix. The digestion was performed at 37°C in an incubator with moderate agitation, and aliquots were taken at 0, 2, 4, 8, 16, 30, 60, 90 and 120 min for further analysis. The digestion was stopped by raising the pH to 7.5 by the addition of 40 mM ammonium bicarbonate (BDH, Poole, Dorset, UK).

2.2.3. *In vitro* intestinal digestion (phase 2)

In vitro intestinal digestion was performed on 120 min gastric digests. The pH was adjusted to 6.5 and the following were added (a) a bile salt mixture containing equimolar quantities (0.125 M) of sodium taurocholate (Sigma) and glycodeoxycholic acid (Calbiochem, La Jolla, CA, USA), (b) 1 M CaCl_2 (BDH), (c) 0.25 M Bistris, pH 6.5 (Sigma), (d) porcine pancreatic lipase (EC 3.1.1.3; 20 μL per 10 ml of total volume) (0.1% w/v; Sigma, L-0382), and (e) porcine colipase (40 μL per 10 ml of total volume) (0.055%, w/v; Sigma, C3028) (Moreno, Mellon, Wickham, Bottrill, & Mills, 2005). Finally, solutions of trypsin (EC 3.4.21.4; 0.1% w/v; Sigma, T 7418) and α -chymotrypsin (EC 3.4.21.1; 0.4% w/v; Sigma, C 7762) were prepared in SGF pH 7.0 and added at approximately physiological ratios of proteins (as denoted by the initial concentration in phase 1)/trypsin/chymotrypsin, 1:400:100 (w/w/w); $1\text{ mg}/34.5\text{ U}/0.44\text{ U}$. This resulted in the following final phase 2 digestion mix: 5.8 mM PC, 2.3 mg ml^{-1} protein, 7.4 mM bile salts, 9.2 mM CaCl_2 and 24.7 mM Bistris. Digestion was at 37°C and aliquots were taken 0, 2, 4, 8, 16, 30, 60, 90 and 120 min for further analysis (Kopf-Bolan et al., 2012), (Sirvent et al., 2012). The digestion was stopped by adding a solution of soybean Bowman-Birk trypsin-chymotrypsin inhibitor (Sigma, T9777), at a concentration calculated to inhibit twice the amount of trypsin and chymotrypsin present in the digestion mix.

2.3. SDS PAGE analysis

Samples taken at different stages of the digestion were analysed by SDS PAGE. Digests (20 μL) were added to 17.5 μL of ultrapure water and to 12.5 μL of 4 $\times$ NuPAGE[®] lithium dodecyl sulphate sample buffer (40% (w/v) glycerol, 0.1 M Tris/HCl buffer, pH 8.5, 8% (w/v) lithium dodecyl sulphate, 0.075% (w/v) Serva Blue G250 and 0.025% (w/v) Phenol Red, pH 8.5; Invitrogen, Carlsbad, CA, USA) and heated at 70°C for 10 min. When required, samples were reduced with 0.5 M dithiothreitol. Samples (10 μL) were loaded onto a 12% polyacrylamide NuPAGE[®] Novex Bistris precast gel. A continuous buffer system (50 ml of 20 $\times$ NuPAGE[®] Mes SDS running buffer with 950 ml of ultrapure water) was used. Gels were run for 35 min at 120 mA per gel and 200 V and then stained using a Colloidal Blue Staining Kit (Invitrogen). Invitrogen SeeBlue[®] Plus2 and

Mark12™ molecular weight standards were used. SDS–PAGEs were analysed and molecular weights were assessed by using Phoretix software (TotalLab).

3. Results

An extract of total water-soluble proteins (albumins) from seeds of sunflower was separated by RP-HPLC to give fractions corresponding to LTP, SFA8 and three mixed albumin fractions (A, B and C). SDS–PAGE showed that fraction A contained components between 7 and 18 kDa, fraction B components of between 4 and 20 kDa and fraction C components of between 4 and 19 kDa. The identities of LTP and SFA8 were confirmed by N-terminal sequencing and by electrospray mass spectrometry, their molecular masses being approximately 9 and 12 kDa, respectively. MALDI-TOF analysis (results not shown) showed that the major bands in fraction A corresponded to Alb2, the N-terminal polypeptides of the precursor proteins encoded by the 2S albumin genes, while those in fractions B and C corresponded to Alb1, the C-terminal polypeptides of the precursor proteins. UniProt IDs of the studied proteins are: LTP: Q39950, Non-methionine rich sunflower albumin precursor (containing Alb1 and Alb2): Q8GUD8, SFA8: P23110.

3.1. LTP

3.1.1. Gastric digestion (phase 1)

The digestion of LTP in the absence and presence of PC was monitored by SDS–PAGE as shown in Fig. 1A and B, respectively. In the absence of PC, the 8.4 kDa band of the LTP decreased little even after 120 min digestion time. However, a second band of size 5.5 kDa appeared in the 2nd min and increased in intensity throughout the 2 h digestion. In the presence of PC this 5.5 kDa band was much less pronounced indicating that LTP was more resistant to pepsinolysis in the presence of PC.

3.1.2. Intestinal digestion (phase 2)

After 2 h of simulated gastric digestion, the pH was adjusted to 6.5 and trypsin and chymotrypsin were added with bile salts (sodium taurocholate and glycodeoxycholic acid) in order to simulate the intestinal environment (phase 2 digestion). Again the digestion in the absence and presence of PC was monitored by SDS PAGE as shown in Fig. 1C and D, respectively.

In the absence of PC (Fig. 1C) the LTP remained stable with only a small decrease in band density over the 120 min of digestion. The band at 5.5 kDa also persisted throughout phase 2 digestion. Further bands at 7 and 4.5 kDa appeared rather strongly after 2 min but gradually faded as digestion progressed. In the presence of PC the intensity of the main LTP band (8.4 kDa) decreased. The band at 5.5 kDa was degraded very quickly (in less than two min) and the two bands at 7 and 4.5 kDa that appeared in the absence of PC were also present but stained more strongly and were not subsequently degraded. The LTP and at least two smaller peptides were therefore more resistant to phase 2 digestion in the presence of PC.

The main LTP band (8.4 kDa) and the 5.5 kDa fragment were only observed on SDS–PAGE under reducing conditions with a broad smear being visible under non-reducing conditions (Fig. 1E). This suggests that the main band and the smaller fragment are still linked by disulphide bonds.

3.2. Fraction A

3.2.1. Gastric digestion (phase 1)

The mixture of proteins in fraction A was digested by pepsin in phase 1 digestion and analysed by SDS–PAGE (Fig. 2A). All bands

decreased slightly in intensity as the digestion proceeded with the smallest measurable band (~3.4 kDa) being absent by the 16th min. The presence of PC did not have any apparent effects on the patterns of digestion (data not shown).

3.2.2. Intestinal digestion (phase 2)

The main bands degraded rapidly (<2 min) after adding the intestinal enzymes (Fig. 2B). In particular, a band at 4.0 kDa which was visible throughout the simulated gastric digestion became very pronounced after the intestinal enzymes were added and then decreased gradually. The addition of PC resulted in increased stability of this and the other bands (data not shown).

3.3. Fraction B

3.3.1. Gastric digestion (phase 1)

The mixture of the proteins in fraction B changed very little during gastric digestion with little observable effect of PC except that the faint 5.3 kDa fragment disappeared by the 16th min in the absence of PC (Fig. 2C) but was still detected at 30 min in the presence of PC (data not shown).

3.3.2. Intestinal digestion (phase 2)

The major protein bands in fraction B were digested quickly (within 2 min) after adding the intestinal enzymes, but further digestion occurred more slowly (Fig. 2D). The addition of PC appeared to slow down digestion, with more bands still being detected after 8 min digestion. Although an additional band (at 3.1 kDa) was present after 120 min in the digests carried out without PC compared with those with PC (data not shown), it is possible that this fragment was also present in the digest with PC but obscured by the PC itself which formed a smear on the gel. PC showed a slight protective effect, especially on the smaller components of fraction B (data not shown).

3.4. Fraction C

3.4.1. Gastric digestion (phase 1)

PC had a clear protective effect on the proteins present in fraction C throughout the 2 h gastric digestion. The 5.7 kDa band was observable only until 8 min without PC (Fig. 2E) but in the presence of PC it was still detected after 30 min (data not shown).

3.4.2. Intestinal digestion (phase 2)

The protein and peptides remaining after phase 1 digestion were rapidly degraded, with only minor changes in the intensities of bands under 10 kDa being observed after 2 min (Fig. 2F). PC did not have any apparent effects on the digestion, except that a small fragment (3.9 kDa) was detected at 2 min in the digests without PC only. However, it is possible that this 3.9 kDa polypeptide was also present in the digest with PC but was hidden by the smeared PC on the gel.

3.5. Native SFA8

3.5.1. Gastric digestion (phase 1)

Native SFA8 was digested by pepsin in 90 min and no protein was detected by SDS–PAGE after that time (Fig. 3A). A minor band with a molecular weight of 5.0 kDa appeared very quickly, 2 min after adding the pepsin to the SGF. This fragment was only detected under reducing conditions indicating that it remained bound to other fragment(s) by disulphide bond(s) (data not shown). Since all of the proteins were digested by 90 min in the gastric phase, no protein remained for digestion in the intestinal phase. PC had a slight protective effect on SFA8 (Fig. 3B), as smaller fragments were observed for longer times during the digestion.

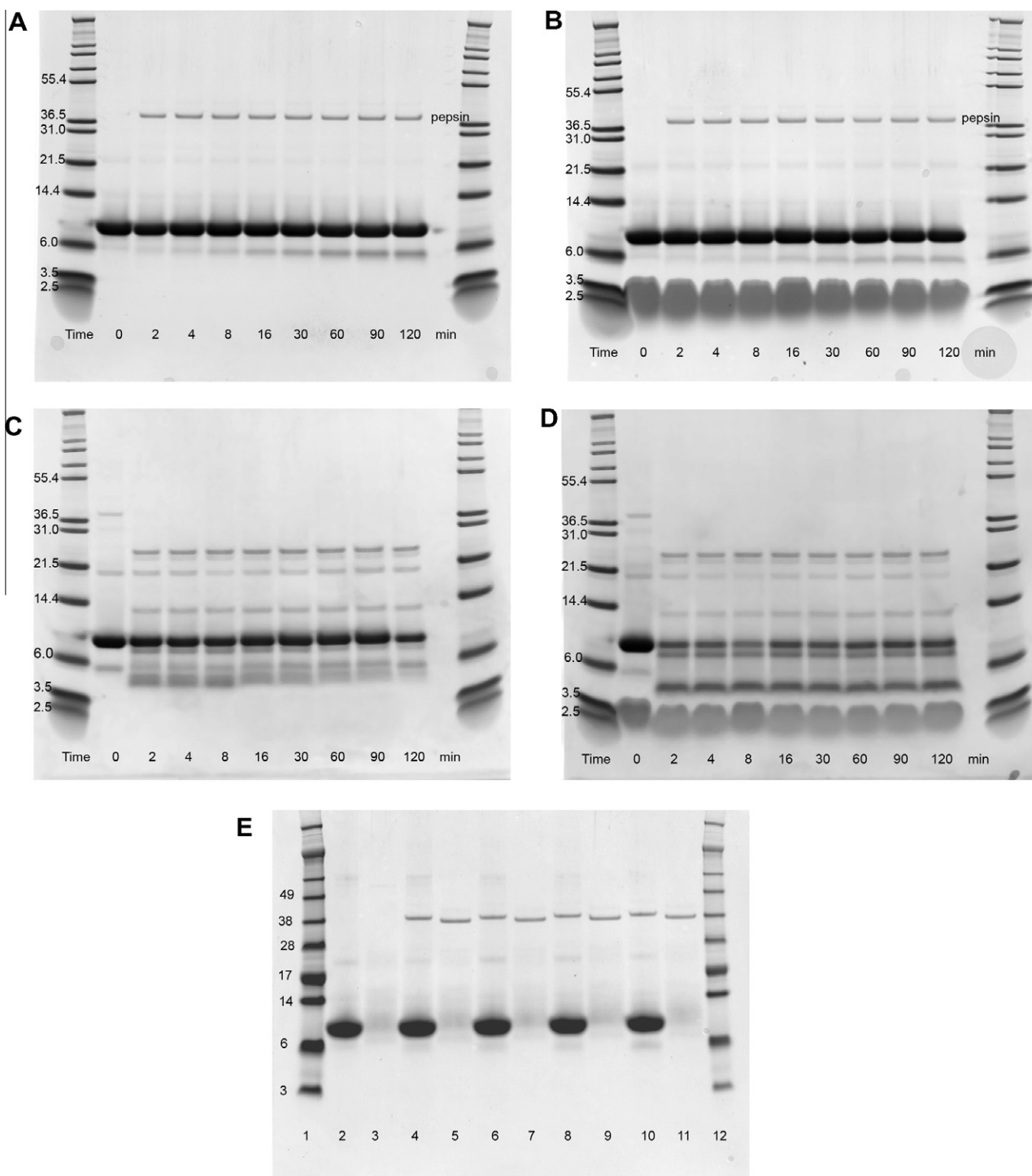


Fig. 1. SDS-PAGE analysis of the digestion of sunflower LTP with and without phosphatidylcholine (PC) in simulated gastric (phase 1) and intestinal (phase 2) digestions (A) LTP –PC phase 1, (B) LTP + PC phase 1, (C) LTP –PC phase 2, (D) LTP + PC phase 2, (E) LTP –PC phase 1, under reduced (R) and non-reduced (N) conditions; lane 1: molecular weight marker, lane 2: time 0, (R), lane 3: time 0 (N), lane 4: 2 min (R), lane 5: 2 min (N), lane 6: 4 min (R), lane 7: 4 min (N), lane 8: 8 min (R), lane 9: 8 min (N), lane 10: 16 min (R), lane 11: 16 min (N), lane 12: molecular weight marker. The tracks on the left and right show marker proteins with molecular weights of 2.5, 3.5, 6.0, 14.4, 21.5, 31.0, 36.5 and 55.4 kDa.

3.6. Emulsified SFA8

3.6.1. General

The effect of emulsification on digestibility was investigated. The average diameter of the oil droplets ($D_{3,2}$) in the emulsions was 1.85 μm .

3.6.2. Gastric digestion (phase 1)

Comparison of non-emulsified and emulsified SFA8 showed that while the former was digested in 90 min, the latter only partially degraded with smaller fragments being observed throughout the digestion (Fig. 4A). PC also had a slight protective effect on the digestion of emulsified SFA8 with the smaller fragments being observed for longer (data not shown).

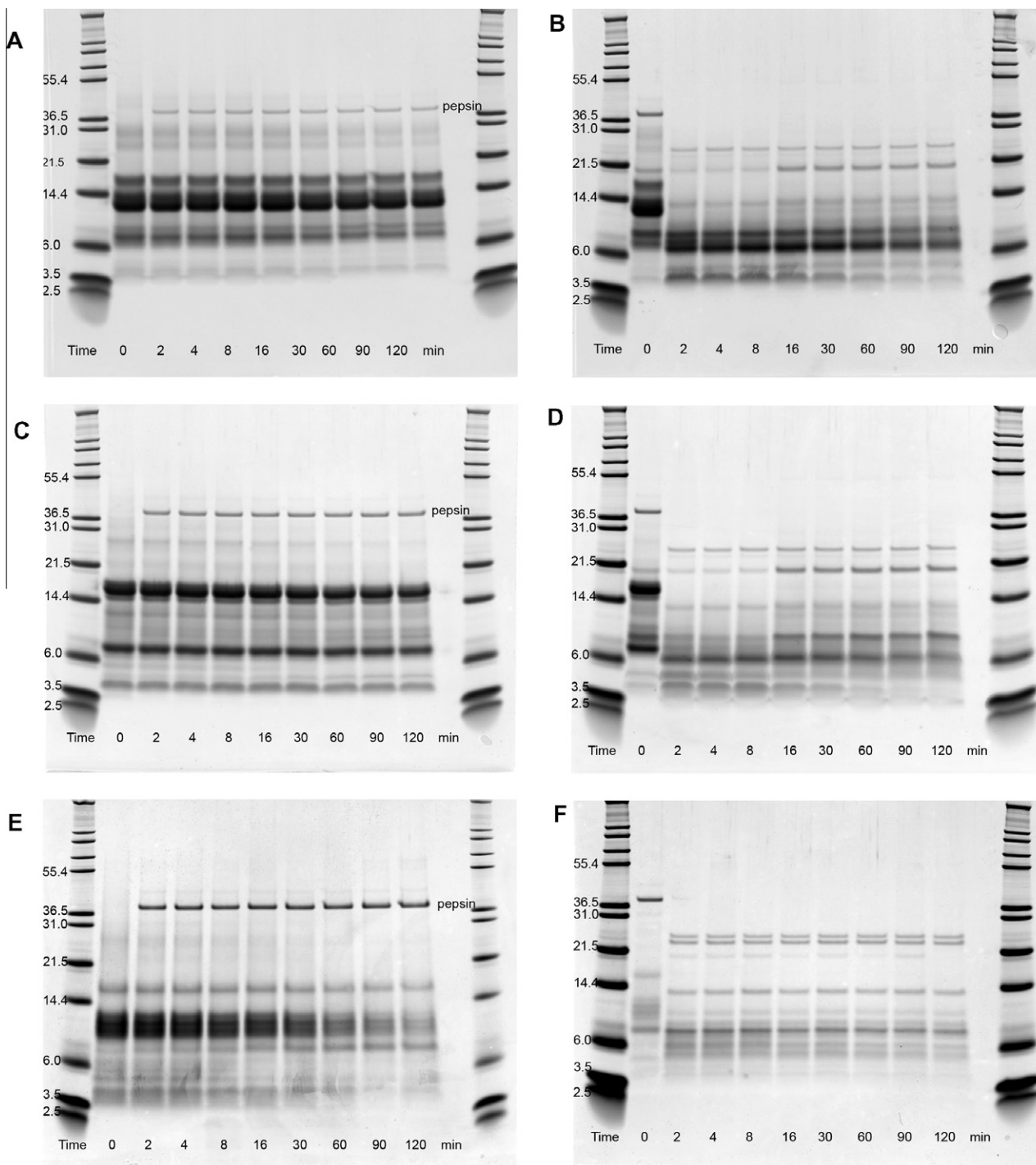


Fig. 2. SDS-PAGE analysis of the digestion of sunflower albumin fractions (A, B and C) without phosphatidylcholine (PC) in simulated gastric (phase 1) and intestinal (phase 2) digestions (A) fraction A –PC phase 1, (B) fraction A –PC phase 2, (C) fraction B –PC phase 1, (D) fraction B –PC phase 2, (E) fraction C –PC phase 1, (F) fraction C –PC phase 2. The tracks on the left and right show marker proteins with molecular weights of 2.5, 3.5, 6.0, 14.4, 21.5, 31.0, 36.5 and 55.4 kDa.

3.6.3. Intestinal digestion (phase 2)

Addition of intestinal enzymes resulted in dramatic changes in the patterns of bands during the first two min but little change thereafter except that a band (4.8 kDa) appeared in the 120 min digest in the presence of PC (data not shown). The emulsified SFA8 was less stable without PC, with a smaller band (size 3.8 kDa) being present which was not observed in the presence of PC or if the SFA8 was not emulsified (Fig. 4B).

In general, the emulsified SFA8 was digested slower than the non-emulsified and the presence of PC enhanced this effect.

4. Discussion

Resistance to proteolysis is a common feature shared by many, although not all, food allergens causing systemic reactions. The ability of food allergens to reach the intestinal mucosa in an immunogenic conformation (with adequate structural integrity or as large fragment) is essential to allergenicity. This ability implies the survival of the protein during cooking and gastric digestion under low pH and the subsequent hydrolysis by intestinal proteases and binding by PC and bile salts.

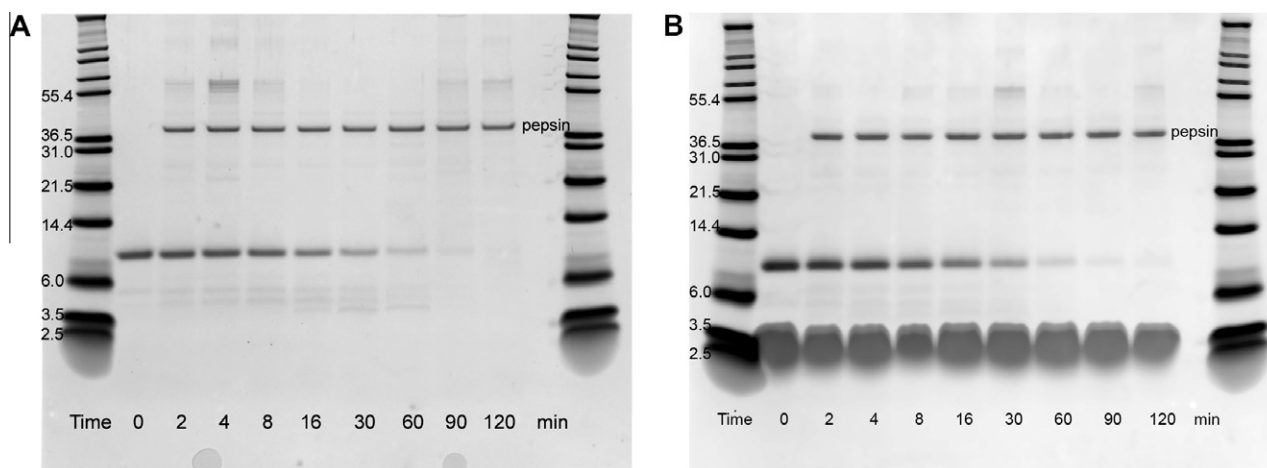


Fig. 3. SDS-PAGE analysis of the digestion of native sunflower albumin SFA8 with and without phosphatidylcholine (PC) in simulated gastric (phase 1) digestion (A) SFA8 – PC phase 1, (B) SFA8 + PC phase 1. The tracks on the left and right show marker proteins with molecular weights of 2.5, 3.5, 6.0, 14.4, 21.5, 31.0, 36.5 and 55.4 kDa.

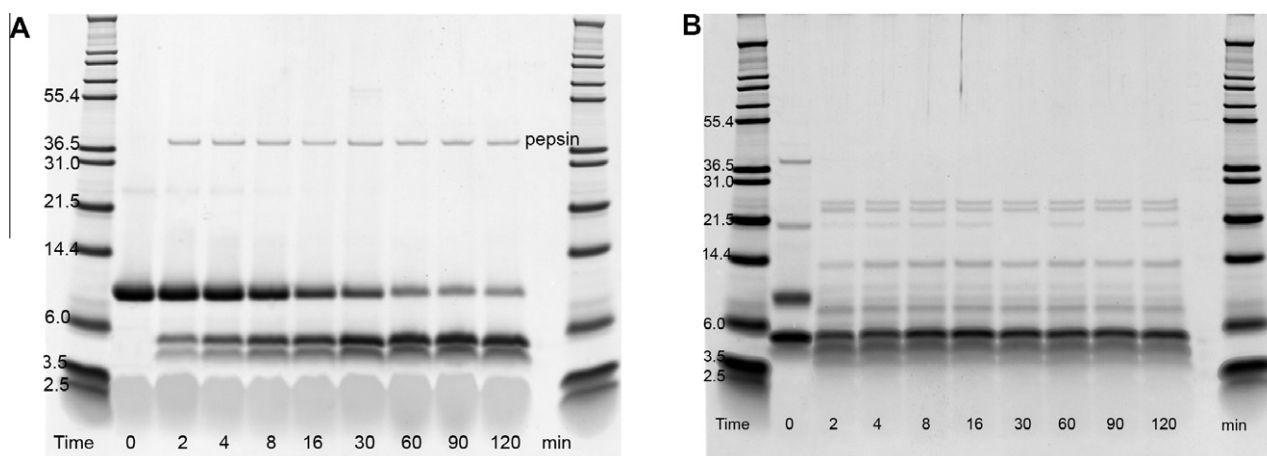


Fig. 4. SDS-PAGE analysis of the digestion of emulsified sunflower albumin SFA8 without phosphatidylcholine (PC) in simulated gastric (phase 1) and intestinal (phase 2) digestions (A) emulsified SFA8 – PC phase 1, (B) emulsified SFA8 – PC phase 2. The tracks on the left and right show marker proteins with molecular weights of 2.5, 3.5, 6.0, 14.4, 21.5, 31.0, 36.5 and 55.4 kDa.

We have demonstrated that sunflower LTP, three 2S albumin fractions and SFA8 were degraded by digestive enzymes to different extents. LTP showed high resistance against digestion by both gastric and intestinal enzymes, which was enhanced by the addition of PC. The main polypeptide chain and the digested fragments were linked by disulphide bonds, resulting in a stable complex which had a size close to that of the undigested protein.

LTPs from many species including peach (Wijesinha-Bettoni et al., 2010), barley (Wijesinha-Bettoni et al., 2010), mustard (Sirvent et al., 2012) apple (Sancho et al., 2005), grape (Vassilopoulou et al., 2006) and kiwifruit (Bernardi et al., 2011) show high stability. Sunflower LTP also showed high stability to pepsinolysis during simulated gastric digestion in this study, although less than reported for the grape LTP under similar conditions (Vassilopoulou et al., 2006). This high stability is consistent with the fact that many of these proteins are capable of eliciting an allergic response (Asero et al., 2000; Sancho et al., 2005). Some LTPs have even been shown to survive the digestive tract as fully IgE-reactive structures that can trigger mast cells in the vicinity of the epithelial monolayer (Asero et al., 2000). Furthermore, many LTPs share sequence motifs and structural features, resulting in allergenic clinical cross-reactivity (Pastorello et al., 2001).

PC is a natural ligand of LTPs and had a slight protective effect on the protein, retarding proteolysis. This was probably as a

consequence of binding to the protein and reducing molecular flexibility (Wijesinha-Bettoni et al., 2010), therefore altering the allergenic activity (Breiteneder & Mills, 2005). Limited protection to proteolysis by PC has also been reported for the grape LTP (Vassilopoulou et al., 2006).

The three 2S albumin fractions differed in their properties. Fraction A was rapidly digested by pepsin with no significant effect of PC; however, PC did reduce the digestibility of the remaining fragments in the intestinal phase. PC also protected fraction B in phase 1 digestion and reduced the rate of digestion in phase 2. In case of fraction C, PC showed a strong protective effect in the gastric phase but no effect was observed on digestion in the intestinal phase.

A previous study showed that only SFA8 out of the sunflower albumins and LTP formed stable emulsions (Berez et al., 2010) and therefore SFA8 was studied in both native and emulsified forms. When not emulsified, SFA8 was digested in 90 min in the gastric phase leaving no detectable protein fragments. When emulsified, SFA8 was far more resistant to pepsinolysis. In the intestinal phase, most digestion of emulsified SFA8 occurred in the first 2 min followed by little further digestion. PC also had protective effects on both native and emulsified SFA8.

Resistance to peptic hydrolysis has also been reported for 2S albumins from several species including peanut (Vissers et al., 2011), mustard (Sirvent et al., 2012), Brazil nut (Moreno et al.,

2005; Murtagh et al., 2003), sesame seed (Orruno & Morgan, 2011) and sunflower (Murtagh et al., 2003). However, neither recombinant SFA8 nor the major 2S albumin from Brazil nut was fully denatured even at 95 °C, and both proteins largely regained their original structures upon cooling (Murtagh, Dumoulin, Archer, & Alcocer, 2002).

In common with the LTPs, 2S albumins have been described as highly stable to proteolysis (Murtagh et al., 2003) and thermal denaturation owing to their compact 3D structures, stabilised by disulphide bonds (Vissers et al., 2011). However, changes in the secondary structure can be introduced by emulsification (Burnett et al., 2002) which could explain why the emulsified SFA8 was more resistant to digestion than the non-emulsified protein. However, in the case of β -lactoglobulin and β -casein, adsorption to the interface resulted in a pepsin-susceptible form (Macierzanka et al., 2009).

Raising the pH from 2.5 to 6.5 and adding the pancreatic mixture (including lipase, colipase and bile salts) to mimic the intestinal environment resulted in changes in accessibility of the proteins to the intestinal digestive enzymes, trypsin and chymotrypsin. Furthermore, the addition of bile salts removes 90% of the adsorbed proteins from the SFA8 emulsion, resolubilising the protein into the continuous aqueous phase (Burnett et al., 2002). Resolubilisation eliminates the protective effect of being adsorbed to an interface, exposing the protein to the pancreatic proteases. It is probable that the desorbed protein undergoes denaturation and binds to the surfactants present in the intestines, which will also affect digestion.

Other food protein allergens, such as those belonging to the cereal inhibitor family and fruit LTPs, may also associate with lipids (Asero et al., 2000). Proteins associated with lipids may be protected from the proteolytic environment in the gut, and they also have enhanced thermal stability (Taneva, Donchev, Dimitrov, & Muga, 2000). Binding of lipids may also facilitate allergen absorption by the gut mucosa, either through association with cell membranes, as has been suggested for 2S albumins (Onaderra et al., 1994), or by co-adsorption to the surface of lipid droplets.

5. Conclusions

In summary, PC exhibited protective effects on both native and emulsified sunflower LTP and albumins (mixed Alb1 and Alb2 fractions and pure SFA8) in the gastric as well as in the intestinal phase of digestion. In the case of SFA8, significant protection was provided by emulsification, which was further enhanced by the addition of PC. It is generally accepted that resistance of proteins to pepsin digestion in the stomach is a marker for potential allergenicity. Thus, our results indicate that lipid-protein interactions and emulsification may have the potential to modulate allergenic activity. Protein digestibility (measured as resistance not only to pepsin but also to trypsin and chymotrypsin) can be used as a criterion for assessing the allergenic potential of novel proteins. Although the digestive model used in this study was a static *in vitro* method lacking the complexity of the *in vivo* digestive system, the results nevertheless clearly demonstrate the importance of considering the complex multi-phase nature of the gastro-intestinal environment when assessing the allergenic potential of a protein.

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