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ARTICLE

Improved digestibility of β-lactoglobulin by pulsed light processing: dilatational and shear study.

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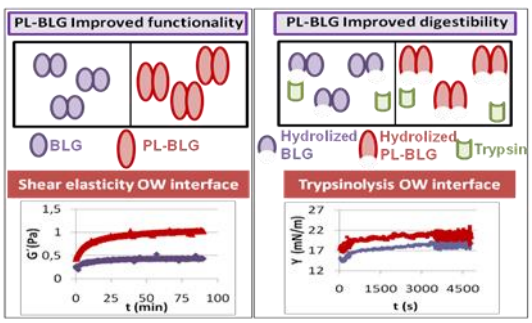
Modifying protein conformation appears to improve the digestibility of proteins in the battle against allergies. However, it is important not to lose protein functionality in the process. Light pulse technology has been recently tested as an efficient non-thermal process which alters the conformation of proteins while improves their functionality as stabilizers. Also, in order to rationally design emulsion based food products with specific digestion profiles, we need to understand how interfacial composition influences digestion of coated interfaces. This study has been designed to investigate the effects of pulsed light (PL) treatment on the gastrointestinal digestion of protein covered interfaces. We have used a combination of dilatational and shear rheology which highlights inter and intra-molecular interactions providing new molecular details on protein digestibility. The *in-vitro* digestion model analyses sequentially pepsinolysis, trypsinolysis and lipolysis of β-lactoglobulin (BLG) and pulsed light treated β-lactoglobulin (PL-BLG). The results show that the PL-treatment seems to facilitate digestibility of the protein network, especially regarding trypsinolysis. Firstly, PL treatment just barely enhances the enzymatic degradation of BLG by pepsin, which dilutes and weakens the interfacial layer, due to increased hydrophobicity of the protein owing to PL-treatment. Secondly, PL treatment importantly modifies the susceptibility of BLG to trypsin hydrolysis. While it dilutes the interfacial layer in all cases, it strengthens the BLG and weakens the PL-BLG interfacial layer. Finally, this weakening appears to slightly facilitate lipolysis as evidenced by the results obtained upon addition of lipase and bile salts (BS). This research allows identification of the interfacial mechanisms affecting enzymatic hydrolysis of proteins and lipolysis demonstrating an improved digestibility of PL-BLG. The fact that PL treatment did not affect the functionality of the protein makes it a valuable alternative for tailoring novel food matrices with improved functional properties such as decreased digestibility, controlled energy intake and low allergenicity.



Introduction

Healthier food products are becoming increasingly important to the food industry, which is investing in new food products with tailored functionality. In this regard, controlling the way we digest food can be crucial for the optimal design of food products with specific digestion profiles. To this end, we need to understand the details of the enzymatic and mechanical breakdown of food structures during digestion[1]. Applying physical and materials science to understand the fundamentals of nutrient release and the digestion of complex food structures is an emerging area of research as showed by the increase of new works published on this topic in the last decade[2-6].

The emergence of new allergies in the population is changing feeding habits. While food travels through the digestive tract during digestion, proteins in food are broken down into immunologically inactive fragments. Very small proportions of immunologically active material may escape digestion. This incomplete digestion of dietary proteins can be the origin of an inappropriate immune response in the gut[7]. If we are to rationally modify protein structure to affect digestibility it is important to improve the current understanding of the enzymatic breakdown of proteins through the digestive tract. One of the most important allergenic food nowadays is cows' milk, which affects approximately 2% of infants under 2 years of age in industrialized nations[7]. The globular protein β-lactoglobulin (BLG), present in whey fraction of the milk, is a major cause of allergenic response to cows' milk in humans owing to its rigid structure. This protein is resistant to pepsin digestion, which is associated to its complex structure characterized by hydrophobic pocket with stability in acidic pH[8]. Thus, BLG is able to cross the intestinal barrier and binds to the antibodies of sensitive individuals activating the immune response[9]. Besides its nutritional importance, milk proteins are magnificent emulsifiers[10] and hence, they are widely encountered in natural and prepared food, aggravating the allergic cases. The rate and extent of protein digestion is determined by the accessibility of the cleavage sites to enzymes and the local flexibility of the

substrate molecule. The strategy, then, would be to use a protein, which maintains its nutritional and functional quality, and try to reduce the allergenic potential by improving its digestibility. A common example of this strategy is the use of hydrolysed milk formula for infants[2].

Accordingly, food processing can alter the allergenic properties of proteins by altering their structure. Namely, hiding, destroying, or disclosing allergic epitopes through conformational changes in proteins besides improving the access of the hidden epitopes located within the protein to the gastrointestinal enzymes[9]. In this work we have modified the structural integrity of BLG by a new protocol patented by AZTI-Tecnalia based on pulsed light treatment (PL) (patent, PCT/ES2010/070163)[11]. PL is an effective process to inactivate a wide broad of microorganism involved in food products spoilage, solid as vegetables, eggs, meat and fishery products and transparent or clear liquids as water or juices[12, 13]. This treatment is not a thermal process and is able to increase the lifetime of the products avoiding negative changes in organoleptic properties and loss nutritional. The effects of PL-treatment on BLG have been studied in detail by Fernández and coworkers[14] demonstrating that the conformational changes induced by PL on BLG improved its functionality at the air-water interface.

Accordingly, it this work we evaluate the digestibility of PL-BLG as compared to BLG by simulating *in-vitro* digestion in bulk and at the oil-water interface. Digestibility of BLG in solution has been reported in the literature[9] whereas digestibility at interfaces is much scarce[6, 15]. Proteins are emulsified along the gastrointestinal tract owing to the mixture with biosurfactants and peristaltic movements and therefore studying their enzymatic degradation at interfaces is very important[16]. In previous works we have studied the interfacial digestion of BLG by a single enzyme[17, 18] or in one compartment[1]. In the present work we simulate the passage through the whole digestive tract assessing the effects of gastric and duodenal enzymes and allowing cumulative and synergistic effects[19]. Another improvement of the present study is the combined use of dilatational and shear rheology to test the effect of digestion on interfacial protein layers. Dilatational techniques involve a change in interfacial area whilst simultaneously measuring the interfacial tension and tend to be more sensitive to the composition and structure of the surface film[20]. Shear methods involve inducing shear in the film without a change in area[20] and are sensitive to intermolecular interactions [21]. The combination of these experimental techniques generates new information about inter and intramolecular interactions of the protein adsorbed into interface. There are few works which combine dilatational and shear rheology to characterize interfacial layers and to our knowledge this is the first work reporting data on interfacial hydrolysis. This is indeed a very new area of research which offers many possibilities in the understanding of enzymatic breakdown of protein structures. In order to fully understand the whole picture, the results need to be extended to emulsified systems, but the experiments at interfaces already offer generic information which should be applicable to emulsions[1].

Experimental Section

Materials

Crystallized and lyophilized β-lactoglobulin from bovine milk (90% pure by PAGE) was purchased from Sigma-Aldrich® (cat n. L0130). It was stored at 4°C and used without further purification. β-lactoglobulin is a globular protein consisting of a single polypeptide chain composed of 162 amino-acid residues and a molecular weight of 18.4 kDa. β-lactoglobulin contains two disulfide bonds and one free cysteine group and an isoelectric point of 5.1[22]. Pepsin from porcine gastric mucosa (lyophilized powder, 4220 units/mg protein) was purchased from Sigma-Aldrich® (cat no P6887). Pepsin has a molecular weight of 34 kDa and an isoelectric point of 1.0[23]. Trypsin from bovine pancreas, (purchased from Sigma-Aldrich®, cat n. T1426) is a member of serine protease family and consists of a single chain polypeptide of 223 amino acid residues which is cross-linked by 6 disulfide bridges. It was used at 1:238 wt/wt. Alpha-chymotrypsin from bovine pancreas (purchased from Sigma-Aldrich®, cat n. C4129). This enzyme is a serine protease that hydrolyzes peptides bonds with aromatic or large hydrophobic side chain (Try, Trp, Phe, Met, Leu) on the carboxyl end of the bonds. The molecular weight of chymotrypsin is 25 kDa and the pH optimum is between pH 7.5-8.5. It is recommended to measure with 2 mM calcium chloride because the calcium functions as a stabilizer, and a possible activator of the enzyme[24]. The concentration of chymiotrypsin used in this study is 1:115 wt/wt. Pepsin, trypsin and chymiotrypsin were stored at -18°C and used as received. Lipase from porcine pancreas (Type II, 100-400 units/mg protein using olive oil was purchased from Sigma-Aldrich® (cat n. L3126), stored at 4°C and used as received. Bile salts, sodium glycodeoxycholate (NaGDC, >97% TLC, cat n. C9910), and sodium taurocholate (NaTC, >97% TLC, cat n. 86339) were obtained from Sigma-Aldrich®. Both bile salts are negatively charged, and their molecular weights are 537.68 Da (NaTC) and 471.6 Da (NaGDC) respectively. They were stored at room temperature and used as received.

Highly refined olive oil (Sigma-Aldrich®, cat n. 01514) was purified with Florisil® resins (Fluka, 60-10 mesh, cat n. 46385) prior to use by following the procedure used in previous studies[19, 25, 26]. Namely, a mixture of oil and Florisil® in proportion 2:1 w/w was shaken mildly for 2 h and then centrifuged at 14300 rpm for 30 min in a centrifuge from Kronton instruments (Centrikon T-124). The olive oil mixture was filtered with Millex® filters (0.1μm PDVF) and stored under nitrogen in the dark.

The buffer used in all solutions was $NaH_2PO_4H_2O$ (Scharlau, SO0331) adjusted to the required pH with HCl. Control buffer consisted of: 1.13 10^{-3} M $NaH_2PO_4H_2O$, pH 7.0. Gastric buffer consisted of: 1.13 10^{-3} M $NaH_2PO_4H_2O$, 0.15 M NaCl, pH 2.0. And duodenal buffer consisted of: 1.13 10^{-3} M $NaH_2PO_4H_2O$, 0.15 M NaCl, 0.003 M $CaCl_2$, pH 7.0. Body temperature was adjusted to 37°C with an external temperature control directly in the measurement equipment. Native BLG solutions were prepared by dilution from a stock solution of 1 g/l prepared on the same day of use. Light pulsed treated BLG (PL-BLG) solutions were prepared at 1 g/l and stored in aliquots at -18°C, diluted on the same day of use. Pepsin samples were prepared immediately before use in the gastric buffer with a concentration that gives an enzyme:protein ratio of 1:20 (W/W) relevant to physiological studies and then stored in ice until use in order to restrict autolysis. Trypsin and chymotrypsin enzymes were dissolved at 0.42 10^{-3} gl^{-1} and 0.87 10^{-3} gl^{-1}, respectively just before use in the duodenal buffer. Bile salt solution was a mixture composed of 52.7% NaTC and 47.3% NaGDC with a total concentration of 1 mM dissolved in duodenal buffer. Lipase samples (0.16 g/l) were prepared immediately before use in a duodenal buffer and filtered before use with Millex® filters (0.1μm PDVF).

Ultrapure water, cleaned using a Milli-Q water purification system (0.054 μS), was used for the preparation of buffer solutions. All glassware was washed with 10% Micro-90 cleaning

solution and exhaustively rinsed with tap water, isopropanol, deionized water, and ultrapure water in this sequence. All other chemicals used were of analytical grades and used as received.

1. Light pulsed treatment

PL-treatments were performed using a SBS-XeMaticA-(L+L) device (SteriBeam Systems GmbH, Kehl, Germany). For the emission of light pulses, the electric power is stored in an energy storage capacitor and later released quickly to the Xenon lamps which emit then high intensity light pulses of 325 µs duration [27]. The emitted light spectrum includes wavelengths from 200 to 1000 nm with a considerable amount of light (approximately 40%) in the UV-C spectrum [28]. Samples at room temperature (20-23 ºC) were placed at 8 cm from the upper Xenon lamp and received between 1 and 10 light pulses of 0.4 $J.cm^{-2}$, up to a maximum total fluence of 4 $J.cm^{-2}$. The samples were named 1PL-BLG and 10PL-BLG after receiving 1 or 10 light pulses, respectively. 10 mL of BLG solutions were poured in a quartz trough (16.6 × 9.8 cm) and stirred between pulses. No significant temperature increase was found at the maximum total fluence.

2. SDS- gel electrophoresis essays of *in-vitro* proteolysis in solution

10 mg of native BLG were dissolved in 10 ml of gastric buffer. 10PL-BLG was diluted in control buffer, followed by 0.15 M $NaCl_2$ to lower the pH 2.0. Both samples were incubated in a bath at 37°C under agitation (170 rpm) for 10 minutes. 100 µl of pepsin (0.5 mg/ml) were then added to both samples and 200 µl of each time-point sample (0, 0.5, 1, 2, 5, 10, 20, 40, 60 minutes) were taken during pepsin digestion and mixed with 40 µl of ammonium bicarbonate 0.5 M to stop pepsinolysis. Pepsin digestion samples were stored in *eppendorfs* in the fridge until use. Diluted NaOH was added to the digestion sample after 60 minutes incubation with pepsin to increase the pH until 7.0 (in this step, the sample could be frozen at -20ºC).

This sample was then incubated at 37°C under mild agitation (170 rpm) in a bath for 10 minutes before trypsinolysis. Then, 0.003 M $CaCl_2$ was added to the sample and the reaction was started when trypsin and quimiotrypsin were added. 200 µl of each time-point sample (0, 0.5, 1, 2, 5, 10, 20, 40, 60 minutes) were taken during the trypsin digestion and mixed with 20 µl of 0.011M Peflaboc SC (Sigma-Aldrich®, cat n. 76307) to stop trypsinolysis. Trypsin digested samples were stored in *eppendorfs* in the fridge until use.

Progress of proteolysis (pepsinolysis and trypsinolysis) was evaluated by sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gel electrophoresis with the following protocol. 16 µl of each sample plus 4 µl 4x Laemmli sample buffer (Bio-Rad®, cat n. 161-0737) with β-mercaptoethanol (Sigma-Aldrich®, cat n. M3148) were heated in a water bath (70°C, 10 min) and subsequently centrifuged 5s until 5000 rpm. Precision Plus Protein Standard Dual color (Bio-Rad®, cat n. 161-0374) was used as molecular weight marker (250, 150, 100, 75, 50, 37, 25, 20, 15, 10 KDa). 10X Tris/Glycine/SDS buffer (Bio-Rad®, cat n. 161-0732) was diluted and used 1X and gels were run for 50 min at 120mA/gel and 200V in Mini-PROTEAN Tetra cell system (Bio-Rad). It was stained using Comassie Blue staining solution, 2.5 g of Coomassie Brillant Blue R-250 (Sigma-Aldrich®, cat n. 27816) in a mixture of acid acetic: methanol; distilled water (1:4:5) and stored during 15 min, at room temperature under mild agitation. Coomassie Blue destaining solution (acid acetic: methanol; distilled water, 1:4:5) was used for 15 min, changing solution at least 3 times. Finally, gels were photographed and visualized directly in the computer.

Methods

1. Interfacial tension/dilatational rheology: The OCTOPUS

All the interfacial tension and dilatational rheology measurements, including the *in-vitro* digestion, were made in The OCTOPUS. This a Pendant Drop Surface Film Balance equipped with a subphase multi-exchange device which has been fully designed and assembled at the University of Granada (patent *submitted* P201001588).[29] This device has been implemented on the basis of the single subphase exchange device, where the normal capillary tip was substituted by an arrangement of two coaxial capillaries, connected each to one of the channels of a specific micro-injector, which can operate independently[30] (Spanish Patent, registration number P9801626), to achieve a fully automated subphase multi-exchange device described in detail elsewhere[15, 19]. The OCTOPUS computer software DINATEN© has been also fully programmed at the University of Granada. The detection and calculation of surface area and surface tension is based on Axisymmetric Drop Shape Analysis (ADSA). The pendant drop is placed on a three axis micro-positioner and is immersed in a glass cuvette (Hellma) which is kept in an externally-thermostaticed cell at 37ºC for all the experiments.

Digestion step	Solute	Solvent	Time
Control	BLG (0.1 g/l)	pH 7, 37ºC	1 h
Pepsin (Pep)	Pepsin ($1.5 \cdot 10^{-8}$M)	pH 2, 150 mMNaCl, 37ºC	50 min
Trypsin (Tryp)	Trypsin($0.42\ 10^{-3}$ gl^{-1}) + Quimiotrypsin($0.87\ 10^{-3}$ gl^{-1})	pH 7, 150 mMNaCl, 3mM $CaCl_2$, 37ºC	50 min
Lipolysis (Lipo)	BS (1 mM) + lipase (0.16 $mgml^{-1}$)	pH 7, 150 mMNaCl, 3mM $CaCl_2$, 37ºC	50 min
Desorption (DES)	-	pH 7, 150 mMNaCl, 3mM $CaCl_2$, 37ºC	50 min

Table 1: Details of the digestion process designed including components of each digestion fluid and exposure time.

Drop images are captured by a CCD camera (Pixelink®) connected to an optical microscope (Edmund Optics®). The computer program DINATEN© fits experimental drop profiles, extracted from digital drop micrographs, to the Young–Laplace equation of capillarity by using ADSA, and provides as outputs the volume (V), the surface tension (γ), and the interfacial area (A) of the pendant drop. The adsorption process is recorded at constant interfacial area through a modulated fuzzy logic PID algorithm (proportional, integral, and derivative control). The dilatational rheology of the interfacial layers is measured by applying an oscillatory perturbation to the interface at the end of each adsorption step. The applied oscillations in interfacial area

were maintained at amplitude values of less than 5% variation, in order to avoid excessive perturbation of the interfacial layer, while the measurement frequencies (ν) were set to 0.01, 0.1 and 1 Hz. The system records the response of the surface tension to this area deformation, and the dilatational modulus (E) of the interfacial layer is calculated by image analysis program CONTACTO®[19].

2. *In-vitro* digestion in a single droplet.

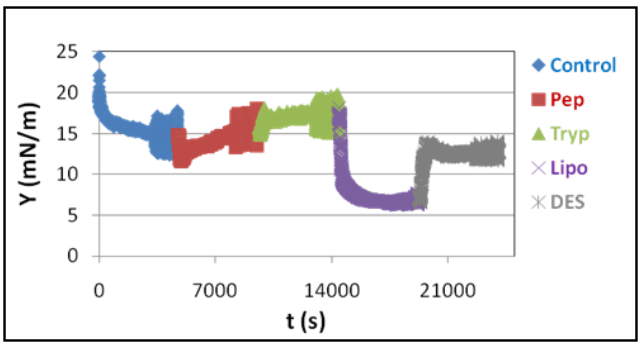


Figure 1: Representative curves of the *in-vitro* digestion of PL-BLG adsorbed layer at the olive oil-water interface. Conditions of each digestion step are met through subphase exchange of solutions shown in Table 1.

The OCTOPUS provides a sequential static digestion method to measure the effects of *in-vitro* digestion on a single droplet as described in detail in[19]. This device allows customization of the digestion model used, depending on the specific requirements of the experiments. In this work, we want to look into three different events within the gastrointestinal tract: pepsinolysis, trypsinolysis and lipolysis of protein-covered interfaces. To this end, we have designed the *in-vitro* digestion model consisting of 5 different solutions which are summarized in Table 1. This digestion model follows the standardized protocol given in the literature[31]. The different solutions are placed in *eppendorfs* which are connected directly with the pendant drop by each of the valves[19]. Initially, a protein layer is pre-formed at control conditions by adsorption of a protein solution onto the oil-water interface, which is then subjected to conditions of each compartment of the gastrointestinal tract. This is done by subphase exchange of the original bulk solution with those mimicking the different steps of the customized digestion process, contained in each *eppendorf*. The conditions of this compartment on the interfacial are monitored at constant interfacial area for 50-60 minutes and then, the drop is subjected to 10 cycles of deformation at three different frequencies 0.01, 0.1 and 1Hz, each one followed by constant interfacial area monitorization for one minute.

The components of each digestive media are detailed in Table 1 and Figure 1 shows the evolution of the interfacial tension following *in-vitro* digestion process of BLG interfacial layer as an example of the experimental results obtained with the OCTOPUS. The successive artificial media mimicking the different compartments (mouth, stomach, small intestine) are applied sequentially on the control layer by exchange of the subphase. The interfacial tension is recorded *in-situ* throughout the whole *in-vitro* digestion process and the dilatational elastic modulus of the interfacial layer is computed at the end of each step/compartment. The reproducibility of the experiments was tested by performing at least three replicate measurements. The interfacial tension of the clean olive oil-water interface was measured before every experiment, in order to confirm the absence of surface-active contaminants, yielding values of 29.5±0.5 mN m^{-1} at 20°C.

3. Shear rheometer: *in-vitro* digestion at interfaces

Surface shear rheological measurements were carried out to study the mechanical and flow properties of adsorbed layers at fluid interfaces, which are sensitive to surface structure and composition. Experiments at the oil–water interface were made using a stress controlled rheometer, AR2000 Advanced Rheometer (TA Intruments) and an aluminum bicone (diameter 37mm, angle cone 4:59:13) as measuring geometry. The surface rheological response between 20 mL protein solution (1g/l) and 20 mL purified oil was tested by oscillation mode within the range of linear viscoelastic region at a frequency and strain of 0.5 Hz and 0.015, respectively. Measurements were monitored and five different solutions were incorporated to the water phase according to the conditions shown in Table 1. This successive artificial media was applied sequentially on the control layer by exchange of the subphase (using syringes) until conditions were reached for each digestion step.

Results and Discussion

In order to address accurately the *in-vitro* digestion profiles of BLG and PL-BLG adsorbed layers at oil-water interfaces, it is interesting to understand the *in-vitro* digestibility of these proteins in solution. To this end, we use a simplified digestion model and apply it to the proteins in solution. On the basis of this screening, we then move on to test the digestion oil-water interfaces which are the objective of the work. It is important to consider the events occurring at the interface if we are to modify digestion profiles. Owing to the mixture with biosurfactants along the gastrointestinal tract and peristaltic movement of the stomach, the substrate of lipid digestion is an emulsion.

In-vitro digestion of BLG and PL-BLG in solution

In-vitro proteolysis of BLG and 10PL-BLG (1g/l) in solution was hence, studied first by applying a simplified *in-vitro* digestion model composed only of steps 2 and 3 from Table 1. Accordingly, we evaluate sequentially, *in-vitro* pepsinolysis, trypsinolysis and lipolysis conditions on BLG and PL-BLG in solution. Due to the absence of oil in these samples we didn't check the effects of lipolysis. Time-dependent proteolysis of BLG and PL-BLG was followed by SDS gel electrophoresis for all samples. Aliquots were taken at different times during the 1 hour period of digestion to assess time-dependent degradation of each sample, and to record the rate and form of the hydrolysis of BLG and PL-BLG and assess the impact of PL-treatment in the enzymatic hydrolysis of the protein. A main innovation of this study is the application of sequential *in-vitro* digestion, which looks at the pepsinolysis followed by trypsinolysis and then lipolysis of protein solutions. This allows recording cumulative degradation effects and resulting enzymatic synergisms.

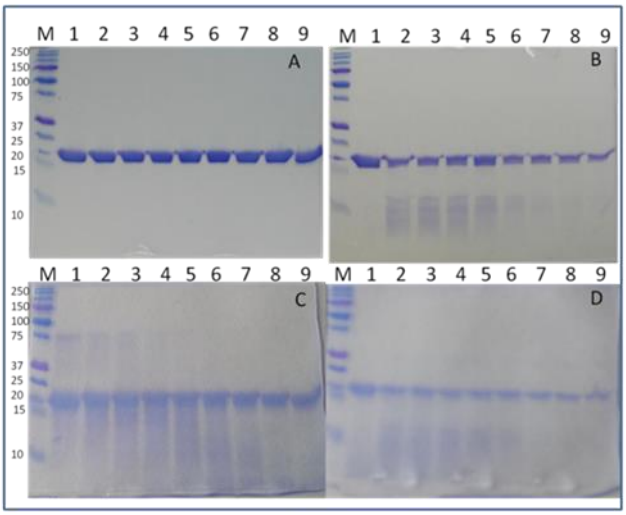


Figure 2: SDS-PAGE analysis of the time-dependent *in-vitro* pepsinolysis and trypsinolysis digestion of BLG and 10PL-BLG (1 g/L) in solution. A) pepsinolysis digestion of BLG, B) trypsinolysis digestion of BLG, C) pepsinolysis digestion of 10PL-BLG and D) trypsinolysis of 10PL-BLG. Lane M, molecular weigth marker (250, 150, 100, 75, 50, 37, 25, 20, 15, 10 KDa). Lane 1: control solution. Lanes 2–9: time point samples of pepsinolysis/trypsinolysis stopped at 0.5, 1, 2, 5, 10, 20, 40 and 60 minutes.

The samples were incubated in a bath at simulated body temperature (37ºC) and kept under mild agitation, mimicking transfer of the gastric *digesta* into duodenal conditions and subsequent degradation. The pepsin digestion started when pepsin enzyme was added into the BLG or PL- BLG sample and we took each time point sample in which the pepsinolysis was stopped by adding ammonium bicarbonate. After time points were taken for 1 hour, we stopped the gastric digestion in the protein sample by pH increment to pH 7.0. Pepsin enzyme is inactivated above pH 6.0. For the trypsin digestion, the sample was incubated simulating body conditions in the digestion, and the trypsin step started when trypsin and quimiotrypsin enzymes were added into the samples. Trypsin digestion was stopped by addition of Pefabloc SC (4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride, AEBSF), which is an irreversible inhibitor of serine proteases like trypsin and quimiotrypsin enzymes and inhibits by acylation of active site of enzyme. *In-vitro* proteolysis of BLG, 1PL-BLG and 10PL-BLG (1g/l) in solution were assayed but Figure 2 shows only the gels corresponding to BLG and 10PL-BLG since we did not see significant changes in the proteolysis of 1PL-BLG.

Hence, Figure 2 shows the electrophoretic profiles of BLG (A, B) and PL-BLG (C, D) in solution after simulated gastric pepsinolysis and subsequent trypsinolysis, let us comment briefly the results.

Pepsinolysis of native protein BLG (Figure 2A) showed a single band corresponding to BLG monomer (18.4 kDa), indicating that this protein is resistant to pepsinolysis. This was already well established in the literature[7, 8, 32, 33] and corroborates previous findings[1]. Native BLG is characterized by a hydrophobic pocket with high stability in acidic pH[34] and a highly structured β-sheet core[35]. It is hence very rigid and resists pepsin cleavage (see figure 2A). Differently, the electrophoretic profile of *in-vitro* trypsin digestion of BLG following pepsin digestion (Figure 2B), showed diffuse bands at the first 5 minutes, with the appearance of an increasing number of peptides of decreasing size with time, which suggested a partial digestion of BLG. This agrees with previous studies of Sakuno and coworkers[36] described that BLG in solution (in water at pH 7.0) was barely attacked by the trypsin digestion. Macierzanka et al[37] studied BLG digestion after heat 70°C, 24h at different pH 6.5, 5.2, 4.8, 2.5 and in each case a fraction of the sample was very quickly degraded in the simulated duodenal digestion.

Before evaluating the effects of *in-vitro* digestion on 10PL-BLG protein let us comment the conformational changes of PL-BLG with respect to BLG as regards the SDS gel electrophoresis bands in Figure 2. The control layer of 10PL-BLG (lane 1 in Figure 2C) displays a band corresponding to BLG monomer (18.4 kDa) and some diffuse bands below, corresponding to less molecular weight segments (see figure 2C). This already suggests that the PL-treatment is degrading the BLG native structure due to the some loss of secondary and tertiary structures, increase of random coil and partial denaturation of the BLG protein after PL-treatment[14].

The electrophoretic profile of pepsin digestion products from 10PL-BLG (Figure 2C) showed a reduced intensity of the major band, accompanied by the simultaneous appearance of diffuse lower molecular weight bands, especially after 5, 10 and 20 minutes. This indicates that the change of BLG structure produced by the PL-treatment, allowed pepsin enzyme to hydrolyze the protein (see figure 2C). Pepsin is a nonspecific enzyme with broad substrate specificity. It is most efficient in cleaving peptide bonds between hydrophobic amino acids and preferably aromatic amino acids such as phenylalanina, tryptophan and tyrosine and will not cleave at bonds containing valine, alanine or glycine[38]. Literature works have demonstrated that modification of the native structure of BLG by using thermal and chemical treatments exposes susceptible peptide bonds and decreases resistance of BLG to peptic cleavage [39, 40]. Accordingly, the pepsin degradation of 10PL-BLG shown in Figure 2C indicates that the conformational change induced by PL-treatment in BLG exposes hydrophobic amino acids, hence pepsin susceptible sites. This agrees with the increase in surface hydrophobicity reported for PL-BLG in the literature [14]. Hence, 10PL-BLG was degraded by pepsinolysis owing to the laxer structure and enhanced unfolding of PL-BLG in a way that pepsin susceptible sites become accessible [14]. The pulsed light treatment changes the globular structure of the BLG and appears to improve the *in vitro* pepsin digestion in solution.

Next, the duodenal proteolysis was carried out by adding trypsin and chymotrypsin enzymes to the pepsin digested sample. Trypsin is a serine protease that cleaves peptides chains mainly at the carboxyl side of the amino acids lysine or arginine, except when either of them is followed by proline[41]. The main substrates of chymotrypsin include tryptophan, tyrosine, phenylalanine, leucine and methionine[24]. We observed *in-silico* that trypsine enzyme has 17 cleavages in the amino acid sequence of BLG and chymotrypsin has 37 cleavages. This information shows that both enzymes are able to hydrolyze BLG in multiple sites. The C- and N-terminal areas of native BLG protein are easily digested by trypsin enzyme, the internal part of the BLG shows more resistance to hydrolysis because the structure of the protein is globular and the release of final peptides within this region passes through the formation and subsequent degradation of intermediate peptides[42]. 10PL-BLG had been partially digested by pepsin owing to the PL-treatment which exposed hydrophobic sites as discussed above. This again seems to facilitate the trypsin digestion. Figure 2D shows how 10PL-BLG was degraded in the first 10 minutes by trypsin conditions after pepsinolysis digestion. This resulting synergism between pepsin and trypsin digestion in

the PL-BLG sample proves the importance of considering sequential *in-vitro* digestion models to record cumulative degradation effects[19].

In summary, analysis of the electrophoretic profile of BLG and PL-BLG following *in-vitro* pepsinolysis and subsequent trypsinolisys already demonstrates that PL-treatment facilitates BLG proteolysis in solution. This is a very important finding which can have enormous implications in the rational control of protein digestibility. Accordingly, we now look into the *in-vitro* digestion of BLG and PL-BLG adsorbed layers at the olive oil-water interface in order to extend these findings to emulsified systems.

Interfacial properties of control layers of BLG and PL-BLG

Before addressing the potential effects in digestibility induced by the PL of BLG as compared with native BLG, we need to establish the control conditions. Hence, as a first step we discuss the characteristics of PL-BLG adsorbed layer in control conditions to then apply the different digestion media. The surface properties of PL-BLG (at the air-water interface) were already studied as compared to BLG in a previous work[14], as regards foaming behavior. However, the properties of PL-BLG adsorbed at oil interfaces, as regards emulsification, have not been addressed so far. Hence, we evaluate first the properties of the interfacial layers as a function of their interfacial tension, the dilatational and the shear rheology under control conditions (Table 1).

Protein	BLG			1PL-BLG			10PL-BLG		
Frequency (Hz)	0.01	0.1	1	0.01	0.1	1	0.01	0.1	1
Viscosity (Pa s)	17±3	2.4±0.7	0.4±0.1	19.9±0.8	2.5±0.1	0.4±0.1	12.4±1.1	2.2±0.1	0.4±0.1
Elasticity (mN/m)	22±3	26±3	32±3	22.7±0.9	27.1±1.3	32.5±1.6	17.0±0.1	23.0±1.3	30.2±0.8

Table 2: Dilatational viscosity (Pa s) and dilatational elastic modulus (mN/m) of BLG (0.1 g/l), 1PL-BLG (0.1 g/l) and 10PL-BLG (0.1 g/l) control layer. Values are obtained as mean of at least three replicate measurements.

1. Interfacial Tension of BLG and PL-BLG

Proteins are amphiphilic molecules that have generally a tertiary structure in aqueous solution where the hydrophobic parts are protected from the solvent by the surrounding hydrophobic parts. At the interface formed between two immiscible liquids, in our case olive oil and water, proteins adsorb and change their conformation depending on its thermodynamic stability, flexibility, amphipathicity, molecular size and charge[43, 44].

The interfacial tension decreases when proteins are being adsorbed onto an interface and attains a plateau level after several minutes or hours depending on the protein and the conditions of the measurement[45]. Normally, the adsorption is faster for flexible proteins having more hydrophobic surface[46]. The hydrophobic amino acids are hidden into the protein core and after the adsorption process, they occupy the interface. This rearrangement, termed interfacial denaturation, allows forming new interfacial covalent and non-covalent bonds and disulfide bonds realign at the interface. The adsorption of proteins onto oil-water interfaces is an spontaneous and generally irreversible process probably because the hydrophobic amino acid adsorbed have a greater affinity for the oil interface and hence, the conformational stability of the protein increases in the adsorbed state[47].

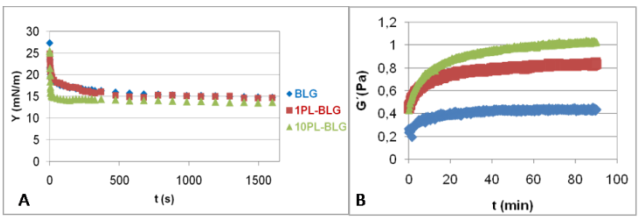


Figure 3: A) Dynamic interfacial tension of BLG (0.1 g/l, rhomboids), 1PL-BLG (0.1 g/l, squares) and 10PL-BLG (0.1 g/l, triangles) adsorbed layer at the olive oil-water interface in control conditions. B) Interfacial shear elasticity of BLG (1 g/l, rhomboids), 1PL-BLG (1 g/l, squares) and 10PL-BLG (1 g/l, triangles) adsorbed layer at the olive oil-water interface in control conditions. Curves are obtained as mean of at least three replicate measurements (standard deviation <2%).

Figure 3A shows the evolution of the interfacial tension of BLG and PL-BLG at the olive oil-water interface under control conditions (Table 1). All the curves follow similar kinetics. We observe two different regions: a very rapid reduction of the interfacial tension within the first 50 seconds followed by a plateau. The final interfacial tension reached by BLG and PL-BLG appears very similar, so that the PL-treatment does not seem to affect the final interfacial coverage. The major difference appears in the faster kinetics shown by 10PL-BLG. This behavior correlates with the increased surface hydrophobicity of the PL-BLG reported in[14] and the loss of secondary and tertiary structures owing to the PL-treatment[14]. Flexible proteins are known to adsorb faster[47] and produce lower interfacial tensions[47-49] in agreement with the trend observed in Figure 3A. Accordingly, 10PL-BLG hydrophobic groups are more exposed and therefore adsorbed better and faster onto the olive oil-water interface, thus decreasing the interfacial tension faster than BLG (see Figure 3A). This trend corroborates the findings at the air-water interface where PL-BLG showed faster kinetics and similar final surface tension values for the higher concentrations[14].

2. Interfacial rheology of BLG and PL-BLG adsorbed layers: shear and dilatational flow

Once the system has equilibrated (Figure 3A) we measure the dilatational parameters of the interfacial layer formed by BLG and PL-BLG at the olive oil-water interface under control

conditions. This is done by subjecting the interface to 10 cycles of area deformation whilst simultaneously recording the interfacial tension, at three different frequencies 0.01, 0.1, 1 Hz. From these data we extract the dilatational elasticity and viscosity of the interfacial layer. The dilatational parameters contain information about the strength of the protein network including inter and intra molecular interaction within the interfacial layer and depend also on the nature of the non-polar phase[1, 34]. The dilatational parameters obtained for the control layer are displayed in Table 2. The dilatational viscosity is only measurable at the lowest frequency considered (0.01 Hz) where the values do not seem to follow a clear trend. If anything, the value seems only significantly lower for the 10PL-BLG. This suggests that the relaxation processes occurring within the interfacial layer could be somehow hindered by the PL-treatment[34]. Early adsorbing proteins tend to exhibit a large loss of activity and are poorly exchangeable with the bulk phase after adsorption. At longer times, proteins develop attractive interactions and cross-links upon partial unfolding. Late adsorbing proteins tend to retain more activity, and can also participate in loosely held multilayers[48]. Hence, the lower viscosity recorded for 10PL-BLG correlates with the faster adsorption kinetics and the majority of the proteins being adsorbed at the interface, where the interfacial unfolding prevents exchange and relaxation process, and not in loose multilayers.

Concerning the dilatational elasticity of the interfacial layer in Table 2, again, it seems minimally affected by the PL-treatment. Only the 10PL-BLG shows a small, but significant, decrease recorded for the highest frequency (1 Hz) as compared to BLG. This slightly lower value correlates with the loss of secondary and tertiary structure of the protein which could well result in a lower elastic response of the network[49]. Also, as the PL-treated protein has a laxer structure, the thiol groups are more exposed and could react with each other forming aggregates which again prevents the formation of an elastic network at the interface[14]. Anyway, the dilatational parameters contain information on both intermolecular and intramolecular bonds formed at the interface and hence, it is interesting to compare the results with the interfacial rheology in shear flow.

Figure 3B shows the shear elastic moduli recorded for BLG and PL-BLG adsorbed layers at the olive oil-water interface under control conditions (Table 1). The concentration used is higher here due to experimental requirements but still allows comparison of the effect of PL-treatment on the properties of the interfacial layer formed. Contrary to the effect on the dilatational modulus (Table 2), the shear elasticity of BLG adsorbed at the oil-water interface seems importantly affected by the PL-treatment, even with only 1PL-treatment. This correlates with the findings at the air-water interface where the shear surface elasticity also increase substantially [14]. Fernández and coworkers relate the improvement of the elastic modulus at the air-water interface to the drastic structural changes in the secondary and tertiary structures induced by the PL treatment. Conformational changes and increase in surface hydrophobicity as well as molecular flexibility are closely related to the increase in the shear viscoelasticity of the interface. The rapid increase of the shear elastic constant indicates the presence of faster and probably different intermolecular associations between proteins[44]. Normally, globular proteins display small shear parameters during the first adsorption stages and reach large values (a few tens of mN/m) only after many hours, probably because they are associated to the third adsorption stage of surface gelification where the protein crosslinks owing to interfacial unfolding [44, 48]. In view of the data in Figure 3B we can conclude that this process is clearly enhanced by the PL-treatment where the interfacial layer displays substantially higher shear elastic moduli.

The fact that the PL-treatment reduces the dilatational modulus of the BLG whilst increases the shear response can be explained in terms of the impact on inter and intramolecular interactions[50]. The shear measurements are a direct mechanical measurement of the interfacial film, and are sensitive to intermolecular interactions[51] whereas dilatational measurements are a response to a compression expansion stress, and tend to be more sensitive to the composition and structure of the surface film. In view of the results, the PL-treatment clearly favors the formation of an elastic network owing to the enhancement of intermolecular bonds at the interface which result in an increase of the shear response as shown in Figure 3B. Conversely, the intra-molecular flexibility of the protein is greatly diminished by the PL-treatment resulting in a slightly lower dilatational response (Table 2). The impact of PL in dilatational parameters is less noticeable than the impact on shear elasticity because the dilatational response reflects both inter- and intra-molecular. The increase in intermolecular bonds compensates for the loss in intra-molecular structure and hence the interfacial layer just barely decreases its dilatational response. Therefore, only the combined analysis of shear and dilatational response allows discerning between inter and intra molecular bonds providing hence new aspects of the resulting mechanical properties at interfaces.

The fact that the interfacial coverage and the dilatational modulus remain practically unchanged whilst the shear elasticity increases with the PL-treatment represents an enormous technological benefit in emulsion technology. The PL-treatment modifies substantially the conformation and structure of BLG but maintains intact its functional properties (interfacial coverage, dilatational response) or even improves them (shear flow) at the oil-water interface.

In-vitro digestion of BLG and PL-BLG adsorbed layers at the olive oil-water interface

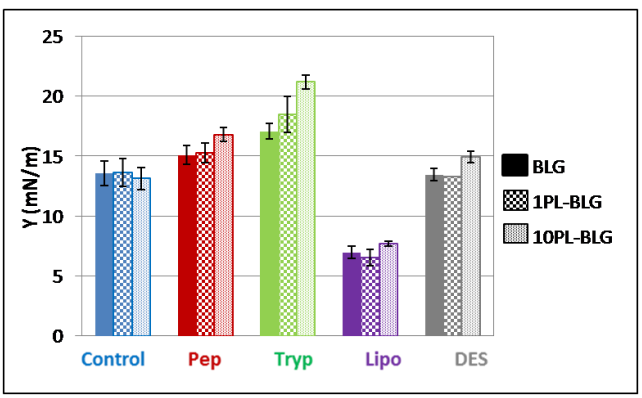


Figure 4: Interfacial tension of BLG (0.1 g/l), 1PL-BLG (0.1 g/l) and 10PL-BLG (0.1 g/l) proteins adsorbed into olive oil-water interface were measured *in vitro* digestion. Conditions of each digestion step are met through subphase exchange of solutions shown in Table 1.Values are obtained as mean of at least three replicate measurements.

Once established the properties of the control layers we can evaluate the impact of PL-treatment on the *in-vitro* digestibility of the adsorbed layers. The experimental design used to investigate the effects of *in-vitro* digestion on interfacial layers formed by BLG and PL-BLG is similar to that used in previous studies[19]. Real food products are usually pre-formed under ambient conditions and then subjected to physiological conditions on consumption. Hence, an interfacial protein layer was first pre-

formed under control conditions and the subsequent gastrointestinal media conforming the digestion model (Table 1) applied to this control interface. In order to characterize the interfacial layers, we report the interfacial tension, the dilatational and the shear response of BLG and PL-BLG layers subjected to the *in-vitro* digestion model shown in Table 1. These magnitudes are discussed in a combined way so that we can analyze the interfacial layers at the molecular level. This combined analysis of dilatational and shear parameters along with interfacial tension (coverage) offers new generic aspects of the digestion profile.

1. In-vitro digestion: Interfacial tension

Figure 4 shows the final interfacial tension of BLG, 1PL-BLG and 10PL-BLG adsorbed layers onto the olive oil-water interface following simulated *in-vitro* digestion consisting of the sequential exposure to the physiological media shown in Table 1. For each protein (BLG, 1PL-BLG and 10PL-BLG) we would obtain a curve similar to that shown in Figure 1. Figure 4 plots the interfacial tension values obtained at the end of digestion step, i.e. after 1 hour adsorption under the new physiological conditions. The values plotted in Figure 4 are obtained as mean of at least three replicate measurements of the whole digestion.

Firstly, Figure 4 shows how the control layers provided similar values of interfacial tension for BLG and PL-BLG as discussed in the previous section (aprox 13.5 mN/m). Secondly, the effect of pepsin (Table 1) provides an increase in the interfacial tension value, i. e. dilution of the interfacial layer, which appears fairly similar for BLG (15.1 mN/m) and for 1PL-BLG (15.3 mN/m) but higher for 10PL-BLG (16.8 mN/m). The fact that pepsin hydrolysis dilutes the interfacial layer has been discussed in detail previous works [1, 19, 26, 52]. This is due to exposure of pepsin susceptible sites owing to the adsorption process and the interfacial unfolding undergone by BLG at the olive oil-water interface. The fact that this happens similarly for PL-BLG, where is even more noticeable, corroborates increase of surface hydrophobicity the laxer structure of BLG induced by the PL-treatment which seems to promote pepsin hydrolysis. This also agrees with the results reported in Figure 2 in solution.

After passage through the gastric phase (Table 1), the interfacial layer enters into the duodenal phase which we have divided in three sequential steps: trypsinolysis, lipolysis and desorption of soluble digestion products. Again, Figure 1 displays an example of the whole *in-vitro* digestion process undergone by the interface[19] and Figure 4, the final interfacial tensions recorded at the end of each digestion step.

Concerning trypsinolysis, Figure 4 shows a significant increase in the interfacial tension after trypsinolysis of the pepsin digested BLG and PL-BLG interfaces. This indicates a further dilution of the interfacial layer owing to trypsinolysis of the adsorbed protein. The increase in interfacial tension (decrease in interfacial coverage) is more significant than that recorded due to pepsin hydrolysis (Figure 4). Moreover, the impact of PL-treatment is now more noticeable. Namely, BLG layer increased until 17.1 mN/m, 1PL-BLG layer increased to 18.5 mN/m and increased to 10PL-BLG was 21.2 mN/m.

Accordingly, Figure 4 demonstrates that trypsin cleavage produces soluble products which desorb from the interface hence, diluting the interfacial layer which results in an increase of its interfacial tension. The reason for this is twofold. On one hand, trypsinolysis could be enhanced naturally owing to the physiological conditions of the duodenum. BLG at pH below 3.0 has a rigid structure whereas at pH 7.0 has a more flexible structure[53], which could well allow exposure of more trypsin susceptible sites. On the other hand, the protein entering the trypsin phase has already been partially hydrolyzed by pepsin which again could promote exposure of trypsin susceptible sites. This would explain the further impact of trypsinolysis as compared to pepsinolysis affecting equally BLG and PL-BLG. However, apart from this, the trend in Figure 4 clearly demonstrates that PL-BLG is more susceptible to trypsin hydrolysis than BLG resulting in a lower interfacial coverage of PL-BLG after the trypsin phase (higher interfacial tension). This again originates in the loss of secondary and tertiary structure induced by PL-treatment which again seems to expose more trypsin susceptible sites.

Next, in Figure 4, following trypsinolysis, the interfacial layer enters the lipolysis step, consisting in exposure to duodenal fluid with a mixture of bile salts and lipase (Table 1). No colipase was included in the digestion model since previous works carried out in our lab confirmed no effect in the interfacial behaviour[54, 55], in agreement with Mun and coworkers[56]. Figure 4 shows that the interfacial tension values decreases drastically to 7.0, 6.5 and 7.7 mN/m for BLG, 1PL-BLG and 10PL-BLG respectively upond addition of the duodenal fluid. These values are intermediate between those corresponding to bile salts only and lipase only as studied in detail in previous works [19, 54] and allow evaluation of lipolysis. Due to the presence of bile salts, the interfacial tension cannot be now univocally related to interfacial coverage as previously. The subphase during lipolysis is a complex fluid composed of many bio-surfactants with different interfacial affinities and morphologies[6, 19, 52, 54, 57]. Bile salts are negatively charged amphiphilic surface active molecules with high affinity for the olive oil-water interface[19, 25, 52, 58]. Simultaneously, lipase also adsorbs onto the interface and hydrolyzes olive oil in glycerol and fatty acids[59-61]. Hence, all these species form a very complex interface characterized by very low values of the interfacial tension as recorded in Figure 4. Also, BS micelles could solubilise digestion products developing a similarly complex subphase. The lipolysis step provides similar values for BLG and PL-BLG suggesting that the PL-treatment is not having an impact on lipolysis. However, the dilatational and shear moduli will provide more information on the nature of the interface.

Finally, the last step in the *in-vitro* digestion model consists in depleting the subphase of soluble products; hence promoting the desorption of any reversibly adsorbed material[19, 52, 54]. In this desorption phase, the interfacial tension increased to 13.5, 13.3 and 15.0 mN/m for BLG, 1PL-BLG and 10PL-BLG, respectively. The slightly higher value recorded for 10PL-BLG could imply the desorption of more soluble lipolysis products hence suggesting that 10PL-BLG comprises a weaker barrier to lipolysis. However, this conclusion cannot be solely extracted from the interfacial tension values and we need to consider other variables.

2. In-vitro digestion: Interfacial dilatational rheology

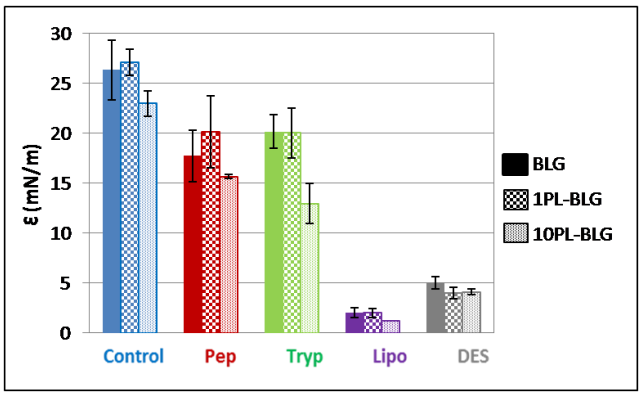


Figure 5: Dilatational elasticity modulus of BLG, 1PL-BLG and 1PL-BLG proteins adsorbed into olive oil-water interface were measured *in vitro* digestion at 0.1 Hz frequency. Conditions of each digestion step are met through subphase exchange of solutions shown in Table 1. Values are obtained as mean of at least three replicate measurements.

The dilatational behavior of protein adsorbed layers is a complex magnitude which offers interesting new information about the stability of emulsions, which are formed during the digestion as the food bolus goes through the digestive tract[1, 19, 21, 25, 26, 52, 62]. The interfacial dilatational modulus is defined by the change in surface tension caused by a small deformation of the interface and provides information about inter and intra-molecular protein cross-linking[19, 20]. Hence, dilatational measurements are in response to a compression expansion stress, and tend to be more sensitive to the composition and structure of the surface film. The dilatational moduli were measured at the end of each digestion step (Table 1) for BLG, 1PL-BLG and 10PL-BLG. Figures 5 and 6 show the results of dilatational elasticity and dilatational viscosity recorded at 0.1 Hz of BLG, 1PL-BLG and 10PL-BLG following the sequential *in-vitro* digestion model consisting of 5 steps displayed in Table 1.

In the control phase, the dilatational elasticity and viscosity are similar for native and 1PL-BLG proteins and slightly lower for 10PL-BLG protein as discussed in detail in Table 2.

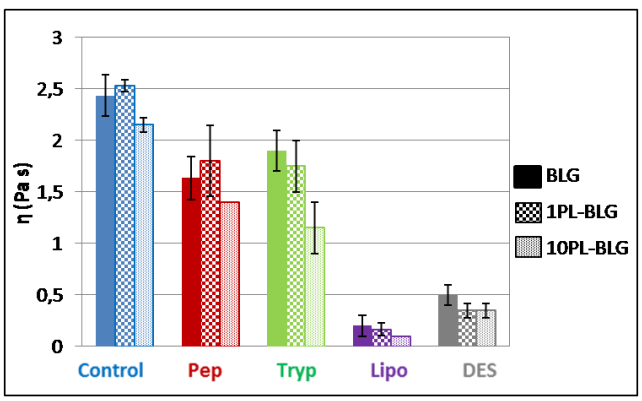


Figure 6: Viscosity of BLG, 1PL-BLG and 10PL-BLG proteins adsorbed into olive oil-water interface were measured *in vitro* digestion at 0.1 Hz frequency. Conditions of each digestion step are met through subphase exchange of solutions shown in Table 1. Values are obtained as mean of at least three replicate measurements.

The next step was the passage through the stomach where pepsinolysis dilutes the interfacial layer due to hydrolysis of the protein as seen in Figure 4. Interestingly, the elasticity and viscosity of adsorbed BLG and PL-BLG decreased also due to the action of pepsin (Figures 5 and 6). This decreased elasticity owing to pepsinolysis has been discussed in detail for BLG in previous works[19, 21, 26, 52, 62] and ascribed to the cleavage of BLG which disrupts the cohesive BLG network. Figure 5 shows that this reduction in dilatational elasticity happens similarly for PL-BLG hence indicating that pepsin cleaves similarly adsorbed BLG and PL-BLG layers.

After the passage through the stomach we simulated *in-vitro* the passage through the duodenum in three sequential steps: trypsinolysis, lipolysis and desorption of soluble products.

In the trypsin phase, the duodenal media contains trypsin and chimotrypsin in a buffer at pH 7 and in the presence of Ca^{+2} ions. Trypsinolysis was found to dilute further the interfacial layer in Figure 4 in all cases owing to hydrolysis of adsorbed protein network. Conversely, Figure 5 shows a more complex response of the dilatational elasticity to trypsin action. The dilatational elasticity of native BLG increases, remains for 1PL-BLG and decreases for 10PL-BLG with respect to the previous digestion step (pepsin). This is a fascinating finding which could imply important differences in digestibility between BLG and PL-BLG. The trypsin cleavage occurs very differently for BLG and for PL-BLG. A previous work already linked decreasing elasticity values with improved digestibility of emulsified BLG and increasing dilatational elasticity values with less digestibility of emulsified β-casein (BCS)[19]. Accordingly, this result could well point in the same direction. The PL-treatment improves the trypsinolysis of adsorbed BLG layer possibly by exposing more susceptible sites. 1PL-BLG shows already some reduction in elasticity but 10PL-BLG clearly diminishes the elasticity of the interface owing to the cleavage on network forming sites. Also, the PL-treatment produced aggregates[14] which could well contribute lower elasticity and viscosity values.

In the lipolysis phase, the elastic moduli decreased similarly for the native and the PL-BLG in a similar way. This low elastic moduli correlates with the formation of a fluid layer [63], composed of bile salts as indicated in Figure 4 and in agreement with previous findings [19, 52, 54].

In the final desorption phase, the soluble product of the digestion are eliminated from the bulk of the drop and only the amphipatic product adsorbed into olive oil water interface were remain[19, 52, 54]. The interfacial tension was found to increase due to this desorption in all cases (Figure 4), slightly more for 10PL-BLG. The values of the dilatational elasticity and viscosity in the desorption phase increase with respect to the lipolysis step but remain low. This indicates that the protein network does not recover and did not resist lipolysis in any case. Insoluble products of lipolysis (fatty acids) could also be present at the interface preventing the formation of a cohesive interfacial network. 10PL-BLG protein showed a slightly lower dilatational elastic modulus that BLG and 1PL-BLG after the desorption phase (Figure 5) which correlates with the slightly higher interfacial tension value in Figure 4. This finding could imply that the 10PL-BLG layer offered less resistance to lipolysis. More soluble products were formed (increased interfacial tension) and more insoluble products at the interface preventing the formation of a cohesive interfacial network (lower dilatational elasticity). However, this reduction, although significant is very small and the most noticeable effect appears in the trypsin digestion.

3. *In-vitro* digestion: Interfacial shear rheology

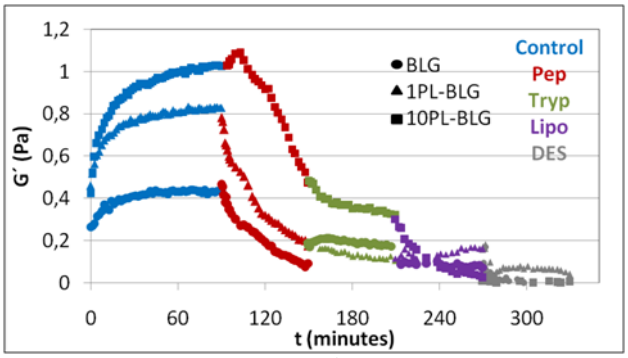


Figure 7: Shear elasticity modulus of BLG, 1PL-BLG and 10PL-BLG proteins adsorbed onto the olive oil-water interface following *in vitro* digestion (Table 1). Values are obtained as mean of at least three replicate measurements.

The impact of PL-treatment on the *in-vitro* digestibility of the adsorbed layers was also followed by interfacial shear rheology. Figure 7 shows the interfacial behavior of BLG, 1PL-BLG and 10 PL-BLG adsorbed layers onto the olive oil-water interface following simulated *in-vitro* digestion consisting of the sequential exposure to the physiological media shown in Table 1.

Treated BLG (1PL and 10PL) significantly enhanced the shear response of the adsorbed layers comparing to the native BLG, at least up to the trypsinolysis phase. Although interfacial tension showed similar values between treated and non-treated samples and dilatational modulus showed weaker adsorbed layers in the treated samples, shear elasticity of 1PL-BLG and 10PL-BLG appeared to be significantly higher than BLG shear elasticity throughout the adsorption process of the control phase. Although 10PL sample had a much higher starting point (0.2 Pa from native protein against 0.4 Pa from 10PL-BLG), the adsorption process is significantly more extensive in the treated protein. The slope of the adsorption at earlier stages is much higher for treated samples as well as the duration of the slope before the surface space is covered. Therefore, the elasticity values for the treated samples are clearly much higher than the non-treated samples even if the starting point was the same.

As mentioned above, PL-treatment induced drastic structural changes in the secondary and tertiary structures[14]. Those conformational changes and the increase in surface hydrophobicity and molecular flexibility are closely related to the improvement in the viscoelasticity of the interface by favoring intermolecular interactions. In fact, the rapid increase of the shear elastic constant indicates faster and probably different intermolecular associations between proteins.

During the next stage of pepsinolysis, these more exposed proteins (BLG, 1PL-BLG and 10PL-BLG) are more sensitive to be hydrolyzed. In fact, the pepsinolysis step showed a more rapid decrease in shear elasticity for both 1PL-BLG and 10PL-BLG comparing to BLG. It clearly seems that this decrease is more pronounced for the shear elasticity than dilatational elasticity. This can be understood since shear measurements are more sensitive to intermolecular interactions[64] and pepsin seems to disrupt the cohesive BLG network.

During the trypsinolysis phase, shear elasticity of native BLG slightly increases, as it also happens for the dilatational elasticity, whereas for 1PL-BLG (it reaches similar values than BLG) and 10PL-BLG the shear elasticity decreases at a lower extent than the pepsinolysis phase. This finding corroborates the idea that PL-treatment improves the trypsinolysis of adsorbed BLG layer by exposing more susceptible sites and therefore digestibility of emulsified BLG could be significantly improved.

When the interfacial layer goes into the lipolysis step, the presence of bile salts and lipase makes the shear elasticity decreased in a similar way from all BLG and PL-BLG samples, showing a much weaker and more fluid interface. Only 1 PL-BLG appears to have a slightly increase through the lipolysis, which can give an idea of a more cohesive interfacial network caused by the mentioned conformational changes in the treated protein. Finally, during the last step, the final desorption phase, the values of the shear elasticity decrease to show a complete fluid layer. In accordance with dilatational measurements, this indicates that the protein network does not recover at all and did not resist lipolysis in any case.

Conclusions

Findings from this study demonstrate that PL-treatment of BLG can have a significant impact on digestibility both in solution and adsorbed at interfaces. Also, according to the shear rheology, PL-treatment can improve the emulsion stability. We have mimicked the passage through the gastrointestinal tract and measured *in-situ* the effects of gastrointestinal processing on the proteins. Moreover, the sequential digestion model allows considering cumulative effects and synergisms. *In-vitro* digestion of BLG and PL-BLG in solution already showed improved digestibility of PL-BLG concerning proteolysis due to enhanced surface hydrophobity of BLG after PL-treatment which exposes pepsin susceptible sites. Then, i*n-vitro* digestion of adsorbed BLG provides further detail of this improved digestibility by combined analyisis of shear and dilatational rheology of adsorbed layers. Pepsin partially hydrolyses interfacially adsorbed BLG and PL-BLG molecules under gastric conditions lowering both the interfacial coverage and the interfacial elasticity of the network. Although PL-BLG shear elasticity values are higher than BLG values, both treated samples suffered a more rapid loss in their elasticity. Trypsinolysis of the interfacial adsorbed layer also lowers the interfacial coverage of BLG and PL-BLG but affects differently the interfacial packing of the resulting network. Trypsin hydrolysis increased the interfacial dilatational and shear modulus of BLG whereas decreases the interfacial dilatational and shear elasticity of PL-BLG. This weakening of the interfacial network importantly correlates with the improved digestibility of PL-BLG found in solution. The observed sensitivity of the hydrolysis profile to the conformation of the protein at the interface suggests a possible route to controlling the digestibility of the proteins through manipulation of the interfacial characteristics. The PL-treatment had also a slight effect facilitating lipid hydrolysis in agreement with the weaker network remaining after the trypsin phase. This again, points out the importance of cumulative enzymatic effects on the digestion of interfacial network. A very important aspect is that despite facilitating digestibility PL-treatment does not affect the functional properties of the protein. These results offer generic conformation possibly applicable to emulsified systems. However, in order to fully understand the whole picture and design emulsified systems, these measurements need to be extended to emulsions. Results so far allow concluding that PL-treatment on proteins is a promising alternative to control both proteolysis and lipolysis, thus contributing to the battle against food allergies and obesity and providing a basis for the rational design of food products.

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Abbrevations

BLG, β-lactoglobulin; PL-BLG, pulsed light treated β-lactoglobulin; BS, bile Salts; PL, pulsed light; SDS, Sodium Dodecyl Sulfate; BCS, β-casein,sodium glycodeoxycholate; NaGDC, sodium taurocholate; NaTC.

Notes and references

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