J Am Oil Chem Soc (2011) 88:1397–1407
DOI 10.1007/s11746-011-1806-z

ORIGINAL PAPER

Micellization of Beta-Carotene from Soy-Protein Stabilized Oil-in-Water Emulsions under In Vitro Conditions of Lipolysis

Amir Malaki Nik · Amanda J. Wright · Milena Corredig

Received: 22 October 2010 / Revised: 21 February 2011 / Accepted: 9 March 2011 / Published online: 2 April 2011

Abstract The details of the interfacial changes occurring during digestion of an oil-in-water emulsion stabilized with soy protein isolate (SPI) and the impact of the changes on lipolysis and subsequent release of β-carotene (BC) were studied using a two-step in vitro digestion model with varying composition containing bile salts (BS), colipase (COL), phospholipids (PL), and phospholipase A_2 (PLA_2). The hydrolysis of the interfacial layer by pepsin resulted in significant changes in the emulsion droplet size distribution. The introduction of bio-surfactants (BS and PL), in combination with the release of lipid digestion products in the duodenal stage, resulted in re-emulsification of the oil droplets. During this stage a significant increase in the negative charge of the oil droplets from −40 to −70 mV, was also observed, indicating significant changes in interfacial composition. The activity of the pancreatic triglyceride lipase (PTL), as monitored by the liberation of free fatty acids, was largely governed by the composition of digestive fluids. In the presence of BS alone, lipid hydrolysis reached 66.1 ± 3.4%, while addition of PL inhibited lipolysis. Inclusion of PLA_2 was found to increase the rate of lipolysis markedly, and the introduction of COL, along with BS, significantly increased the efficiency of the lipolysis such that a maximum lipid hydrolysis of 81.2 ± 1.2% was reached with about 50% transfer of BC to the mixed micelles.

A. Malaki Nik (✉) · M. Corredig
Department of Food Science, University of Guelph, Guelph, ON N1G 2W1, Canada
e-mail: amalakin@uoguelph.ca

A. J. Wright
Department of Human Health and Nutritional Sciences, University of Guelph, Guelph, ON N1G 2W1, Canada

Keywords Soy-protein stabilized emulsions · Lipolysis · In vitro digestion · Droplet size · β-Carotene micellization

Introduction

Oil-in-water emulsions find widespread applications in both the food and pharmaceutical industries. Despite the established knowledge about the relationship between emulsion droplets structure and processing functionality [1], due to the complex nature of the reactions occurring during digestion, much less is understood in terms of the effect of the interfacial properties on the changes occurring during gastro-intestinal (GI) transit; in particular, the influence of interfacial composition and structure on lipolysis. In the last few years, novel applications and specialized designs have been arising for oil-in-water emulsions, aimed at sophisticated delivery of highly lipophilic bioactives into liquid foods and controlled release of bioactive molecules in the GI tract [2]. These efforts require a greater understanding of how to design the emulsions to optimize the interactions with the GI parameters during digestion and release.

Soy protein isolate (SPI) is extensively employed as a functional ingredient in food emulsions. This isolate is composed of two main protein fractions, glycinin and β-conglycinin, which, in combination, represent more than 70% of the total proteins present in soybean seeds. Glycinin is a heterogeneous hexamer with a molecular weight of about 360 kDa [3]. Each monomer consists of one basic and one acidic polypeptide, linked by a single disulfide bond [4]. The other main protein, β-conglycinin, is a trimeric glycoprotein with molecular weight around 180 kDa and consists of three subunits, α', α, and β [5]. Soy proteins adsorb at the oil–water interface as aggregates with a

thickness of 30–40 nm with little change in their conformational structure, and with a surface coverage between 10–20 mg/m^2 [6]. In addition to their excellent amino acid profiles, their consumption has been linked to a number of health benefits and biological activities. Of particular relevance to the use of SPI in nutritional emulsions, these proteins seem to embed bioactive peptides with satiety and hypolipidemic effects [7–9].

Emulsion digestion includes a number of physico-chemical and enzymatic events occurring during transit through the gastrointestinal (GI) tract. Although some changes occur in the mouth [10–12], the major reactions occurring to protein-stabilized emulsions initiate in the stomach with the action of pepsin. The impact of gastric digestion on the physico-chemical properties of the droplets depends largely on interfacial composition, conformation, concentration, and most importantly, the susceptibility of the adsorbed proteins to pepsin [13]. Emulsions stabilized with whey protein isolate (0.5% WPI) or β-lactoglobulin (1% β-LG) are reportedly stable under acidic conditions, in the absence of proteolysis [14, 15]. However, hydrolysis of the interfacial layers by pepsin leads to extensive droplet aggregation of emulsions. Hence, the proteolysis of proteins originally present in emulsion and the stability of the droplets are highly dependent on various factors, including ionic strength and the presence of mucin [15], phospholipids [16], and protein concentration [17].

The majority of lipid digestion occurs in the upper part of the small intestine (duodenum) through the interfacial action of pancreatic triglyceride lipase (PTL). The activity of PTL, in turn, depends on various factors, including the type and concentration of emulsifiers present in a food [13, 18–20], the presence of bile salts (BS), phospholipids (PL), colipase (COL), phospholipase A_2 (PLA_2), and the products of lipolysis [21–23]. The impact of surface-active molecules on PTL binding and activity is dependent on their adsorption at the oil–water interface [14]. In general, the size of oil droplets (i.e. the available surface area), along with the interfacial composition, are fundamental in determining the extent of PTL activity [24]. Most recently, it was hypothesized that lipid hydrolysis can be reduced by engineering multi-component interfaces and it was demonstrated that lipid digestion was reduced in emulsion droplets coated with β-LG-chitosan-pectin [25]. The encapsulation of oil droplets within calcium alginate beads in the form of filled hydrogel particles was also proposed as a strategy to control the rate of lipid digestion [26]. More understanding on the mechanisms occurring during digestion as a function of interfacial composition would enable the food industry to design oil-in-water emulsion systems better tailored for reduced or enhanced lipolysis and for the delivery of bioactives.

Oil-in-water emulsions can act as carrier vehicles for lipophilic bioactives. In the current study, β-carotene (BC) was used as a model of highly lipophilic molecules to be encapsulated. BC belongs to the carotenoids, a class of plant pigments with a complex adsorption pathway [27]. Due to its hydrophobic nature, BC exhibits poor bioavailability and optimization of its delivery is a challenge for the functional food and nutraceutical industry [27, 28]. BC bioavailability depends on the release from the food matrix, its incorporation into mixed BS micelles, followed by transit across the unstirred water layer and finally uptake by the intestinal cells [29]. However, the details related to release and micellization are not completely understood.

The impact of various emulsifiers and processing conditions on the overall behavior of food emulsions in the GI tract have been extensively studied [13, 18, 30]. However, the results are limited by the simplified in vitro digestion models employed. Indeed, the models do not reflect physiological conditions, but rather focus on particular stages of the process. The overall goal of this study was to understand the main factors governing the changes occurring to SPI-stabilized emulsions during in vitro digestion, focusing on the changes in interfacial composition, and the effect of these changes on lipid hydrolysis and the release and micellization of bioactive molecules in the GI tract (using BC as a model compound). The study was aimed at determining the underlying physical principles controlling GI lipolysis of food emulsions. Ultimately, by modifying interfaces, and with physiologically relevant in vitro digestion models, it should be possible to derive principles for the interfacial design of more efficient delivery systems and to be able to control and optimize lipolysis and bioavailability of lipid-based molecules.

Materials and Methods

Materials

Soy protein isolate (SPI) with 86.5 ± 0.2% purity was supplied by the Solae Company (Solae, St. Louis, MO, USA). Pepsin (from porcine stomach mucosa with an activity of 1,020 U mg^{-1} protein), porcine pancreatin (4 × USP, contains amylase, lipase, trypsin, chymotrypsin, and ribonuclease), phospholipase A_2 (from porcine pancreas), porcine bile extract (containing ~49 wt% BS consisting of hyodeoxycholic acid (1–5%), deoxycholic acid (0.5–7%), cholic acid (0.5–2%), glycodeoxycholic acid (10–15%) and taurodeoxycholic acid (3–9%), according to the supplier), porcine colipase (lyophilized, essentially salt free), and pyrogallol (99%, A.C.S reagent) were purchased from Sigma–Aldrich (St. Louis, MO, USA).

Soybean lecithin (containing 70% phosphatidylcholine and 10% phosphatidylethanolamine) was provided by Lipoid (Lipoid GmbH, Ludwigshafen, Germany). β-carotene (all-*trans*, Type 1 synthetic, >95% purity) and bleached and refined soybean oil were purchased from Sigma.

The non-esterified fatty acid kit (NEFA-HR2) was purchased from Wako Pure Chemical Industries (Wako diagnostics, VA, USA). All other chemicals and reagents were of analytical grade and obtained from Sigma. Ultrapure water was used to prepare all the reagents and buffers.

Emulsion Preparation

A stock solution of β-carotene (BC) in soybean oil (SO) was prepared as previously described [31]. SPI (1.5 wt%) was dispersed in water and stirred for 5 h at room temperature to ensure protein hydration and solubilization. The emulsion was prepared by homogenizing 10 wt% BC–SO stock oil solution in the protein solution with a handheld mixer (Ultra-Turrax, IKA T18 Basic, Germany) followed by four passes at 40 MPa through a microfluidizer (110S Microfluidizer Processor, Microfluidics, MA, USA).

In Vitro Gastro-Duodenal Digestion

The details of the composition of the different in vitro digestion model fluids used in this study were reported elsewhere [14]. In brief, the gastric phase was carried out by treating the SPI-stabilized emulsions with simulated gastric fluids (SGF) containing 3.2 mg pepsin and 12.6 mg of pyrogallol (as an antioxidant) per mL of the mixture (emulsion-SGF). The mixture was incubated at 37 °C for 1 h using a temperature-controlled shaking water bath (New Brunswick Scientific Co., Inc., NJ) at 250 rpm and final pH of 2 (emulsion-SGF mixture). The pH of the mixture was monitored during 1 h gastric digestion and no significant change was observed. After the gastric phase, the mixture was incubated with simulated duodenal fluids (SDF) containing 5 mg mL^{-1} pancreatin with or without colipase (1:1 molar ratio with lipase) and simulated bile fluids (SBF) containing 8 mM bile salts (BS) with or without 5 mM phospholipids (PL). In the case of phospholipase A_2 addition, 450 µL of (PLA_2) was added into 5 mL of the digestive mixture. The final mixtures were incubated at pH 6.5 and at 37 °C for 2 h (shaking water bath, New Brunswick Scientific Co., Inc., NJ). Therefore, the five different in vitro digestion models used in this study consist of (1) BS, (2) BS–PL, (3) BS–COL, (4) BS–PL–COL, and (5) BS–PL–COL–PLA_2.

Isolation of Micellar Phase

After the in vitro digestion, the micellar fraction was separated from the undigested oil droplets and particulate materials by centrifuging at 144,000 *g* at 7 °C for 1 h using a Sorvall WX Ultra 80 ultracentrifuge (Mandel Scientific, ON, Canada). The collected aqueous fraction was filtered (0.22 µm nylon filters, Fisher Scientific) to remove any crystalline BC. The aqueous phase was used to quantify the amount of BC transfer, as described below.

Particle Size and ζ-Potential Measurements

The particle size distribution and average particle diameters ($D_{3,2} = (\Sigma\ n_i d_i^3/\Sigma\ n_i d_i^2)$) before and during in vitro digestion were determined using a Mastersizer 2000S (Malvern Instruments Inc., Southborough, MA). Refractive indices of 1.46 and 1.33 were used for the emulsion droplets and dispersant (i.e. water), respectively. For the gastric stage, water at pH 2 was used for size measurements. The average diameters reported were calculated for three batches of each sample. ζ-potential measurements were conducted using a Nano Series Zetasizer (Malvern Instruments lnc., Southborough, MA). All samples, including initial emulsions and digestive mixtures, were diluted 500-fold with 5 mM phosphate buffer at similar pH as performed during the digestion (pH 6.5) to minimize multiple scattering effects. The ζ-potential measurements are reported as the average and standard deviation of three measurements.

Confocal Laser Scanning Microscopy (CLSM)

The microscopic images were recorded using an upright Leica DM 6000B microscope connected to a Leica TCS SP5 system (Heidelberg, Germany) coupled with the Leica LAS AF Imaging software. 0.1 wt% Nile Red was added to the oil phase and stirred at room temperature for 1 h. The oil phase was then filtered using 0.45 µm nylon filters (Fisher Scientific) and used to prepare the emulsions. Before and during digestion, samples were transferred onto a concave microscopy slide and covered with a cover slip. Images were taken using a 63× magnification oil immersion objective at 543 nm.

Free Fatty Acid Determination

The extent of lipid hydrolysis was monitored by quantification of liberated fatty acids (i.e. free fatty acids, FFA) during the digestion, as described in detail by Malaki Nik et al. [14]. Following acidic extraction, the amount of FFA at certain time intervals was determined using a non-esterified fatty acid (NEFA) kit (Wako Diagnostics, VA, USA) and UV–vis micro-plate spectrophotometer (Spectramax plus, Molecular Devices Corporation, CA, USA) by measuring the absorbance at λ_{max} of 550 nm. The quantification was carried out using a standard curve prepared

with oleic acid ranging from 0.1–2 mM and the percent hydrolysis was calculated with respect to the total moles of fatty acid present initially. The kinetic parameters, including maximum product conversion (P_{max}) and initial reaction rate (υ_0) were determined by plotting $[P]/t$ against $[P]$, where $[P]$ is the concentration of liberated FFA at time t. P_{max} and υ_0 were obtained by extrapolation to $[P]/t = 0$ and $[P] = 0$, respectively. Curve fitting was carried out using Sigma Plot, Enzyme Kinetics Module version 1.3 (Systat Software Inc., CA, USA).

β-Carotene Extraction and Measurement

BC was extracted from the dispersed (micellar) phase using solvent extraction as described elsewhere [31]. The amount of BC was quantified by measuring the absorbance at 450 nm using Hewlett Packard 8451A Diode Array Spectrophotometer. The proportion of BC transferred to the micellar phase was calculated based on the initial amount of BC present in each emulsion. In this paper, "bioaccessibility" refers to the proportion of BC originally present in the emulsion, which was incorporated into the aqueous micellar phase of the digestate.

Statistical Analysis

All results were analyzed statistically using ANOVA and Duncan multiple comparisons testing with SAS version 9.1 (SAS Institute Inc., NC, USA). Data shown are the average of at least three independent experiments and significance was considered at $p < 0.05$.

Results and Discussion

Proteolysis of Interfacial Layer

It has been previously demonstrated using SDS–PAGE electrophoresis [32] that, upon incubation of SPI-stabilized emulsions with SGF containing pepsin, rapid degradation of almost all soy protein subunits present in both the cream and serum phases occurred within the first 2 min. The proteolysis of soy protein subunits continued for the entire length of the in vitro gastric phase (1 h) with the presence of polypeptides <14 kDa observed. Subsequent incubation with SDF containing trypsin and chymotrypsin led to further degradation of the soy polypeptides with no visible bands in the SDS–PAGE gels [32].

The Impact of In Vitro Digestion Conditions on Stability of SPI-Stabilized Emulsions

The changes in droplet size of SPI emulsions during in vitro conditions were followed by measuring the mean droplet diameter ($D_{3,2}$) (Fig. 1) and particle size distribution of selected emulsions (Fig. 2). The oil-in-water emulsions prepared with 1.5 wt % SPI showed a monomodal size distribution (Fig. 2) with a $D_{3,2}$ value of 0.23 ± 0.02 μm (Fig. 1). There was no change in mean droplet diameter of the emulsion-SGF mixtures in the absence of pepsin (control sample) with a $D_{3,2}$ value of 0.24 ± 0.01 μm at the end of the in vitro gastric phase (Fig. 1). Although a similar mean droplet diameter was

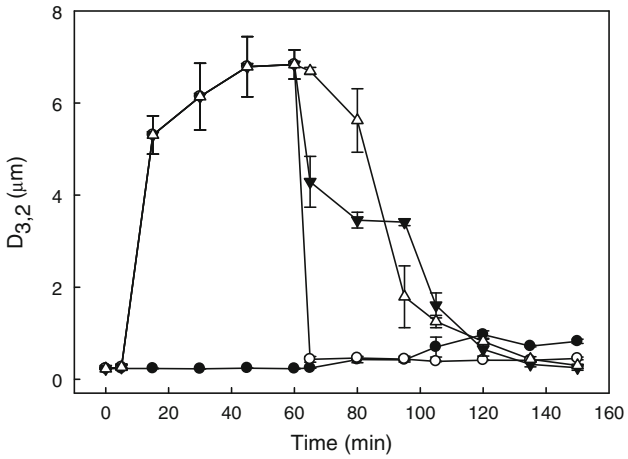

Fig. 1 Changes in average droplet diameter ($D_{3,2}$) of emulsion incubated with SGF (without pepsin), followed by SDF (without enzymes) and SBF (without BS and PL) (*filled circle*); emulsion incubated with SGF (with pepsin) followed by SDF (without enzymes) and SBF (without BS and PL) (*open circle*); and emulsion incubated with SGF (with pepsin) (*open circles*) and then SDF-SBF with PL (*open upward triangles*) or without PL (*filled downward triangles*). Data represent the average of three independent experiments with standard deviation

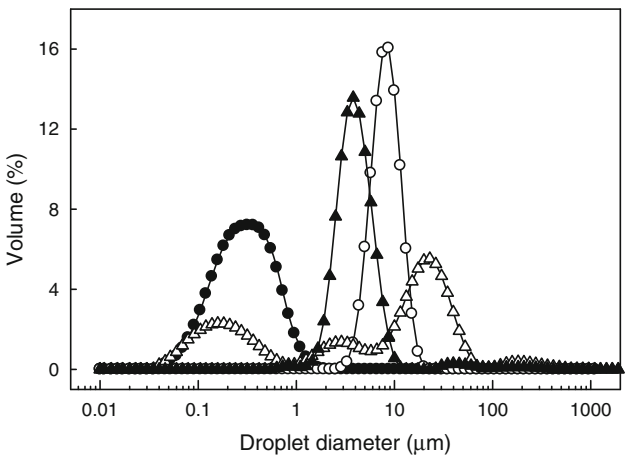

Fig. 2 Particle size distribution of original emulsion droplet (*filled circles*), emulsion after incubation with SGF (*open circles*), incubation with SGF-SDF-SBF (*filled upward triangles*), and SGF-SDF-SBF with PL (*open upward triangles*). The particle size distribution was measured after 30 min of duodenal incubation. Graph is representative of three independent experiments

observed, the particle size distribution showed a small population of droplets >1 μm (data not shown). These results suggested that, in spite of the general stability of the emulsion, some droplet aggregation might occur under the gastric conditions (very low pH and high ionic strength, around 72 mM). Incubation of the control sample with SDF and SBF containing inorganic salts, but no enzymes, resulted in a significant increase in mean droplet diameter, which reached a plateau value of $D_{3,2}$ around 0.8 μm (Fig. 1). This increase in size was most likely caused by the further increase in ionic strength of the mixture to around 200 mM.

Incubation of emulsions with SGF containing pepsin resulted in a substantial increase in the $D_{3,2}$ of the emulsion droplets. The mean droplet diameter increased significantly and gradually from 0.23 ± 0.02 to 6.68 ± 0.65 μm (Fig. 1, $p < 0.05$), while still maintaining a monomodal distribution (Fig. 2). This indicated that pepsinolysis of the interfacial layer would significantly reduce the stability of oil-in-water emulsions. Interestingly, stability of the emulsion droplet against gastric digestion has been shown that would affect the rate of gastric emptying with subsequent impact of lipolysis during the duodenal transit [33].

When the pepsin-digested emulsion droplets were then dispersed in SDF (no enzyme) and SBF (without BS and PL), a significant decrease in the $D_{3,2}$ value was measured (Fig. 1). Under these conditions, the droplet diameter was reduced from around 7 μm at the end of the gastric phase to around 0.4 μm and remained unchanged until the end of the duodenal phase (Fig. 1), indicating that droplet flocculation was the main mechanism involved in destabilization of the emulsion droplets by pepsin in the SGF. The change in pH (from 2 to 6.5 in the SDF), in combination with the dilution and continuous agitation, caused disruption of the emulsion flocs.

Figures 1 and 2 also illustrate the changes in the average and droplet size distribution of the emulsion-SGF mixture when BS was added to the simulated fluids. With the addition of BS, there was a decrease in the droplet size during incubation with SDF and SBF, but at different rates than when only ions were present in the system (compare filled inverted triangles with empty circles in Fig. 1). It is known that BS cause profound changes at an oil–water interface by adsorbing on the surface and causing displacement of other emulsifiers [34]. In the presence of BS, a monomodal size distribution was obtained within the first 30 min of the duodenal stage (Fig. 2). It is important to note that most of the lipid hydrolysis occurred within first 30 min of reaction. Therefore, oil droplet size is an important variable within this period, as it implies differences in the surface area available for lipase adsorption. The slower rate of decrease in the mean droplet diameter in the samples containing BS compared to the control reflects the complex exchanges occurring at the interface. The competition of BS with SPI peptides for the interface showed a slower re-emulsification than in the presence of SPI peptides alone. In addition, it is also important to note in the presence of BS alone and in the absence of lipase, that the mean emulsion droplet diameter remained constant over the 2 h of duodenal digestion with a value of around 4 μm (data not shown). Hence, the products of lipid hydrolysis also played a major role in decreasing the size distribution of the emulsion, most likely causing displacement of BS from the interface. Indeed, in the presence of lipid hydrolysis, the emulsion droplets showed a plateau after the in vitro digestion comparable to that of the control emulsions.

Addition of PL to the BS mixture also had a marked influence on the rate of mean droplet diameter decrease. Compared to the fluids without PL, the decrease occurred at a slower rate (Fig. 1). The particle size distribution shown in Fig. 2 for this system indicates that the presence of PL caused coalescence of the oil droplets (see also discussion below on change in microstructure). These results clearly demonstrate that PL also adsorbed at the interface, competing with the BS. At the end of duodenal digestion and in the presence of BS–PL, the multimodal sized distribution was found indicating the presence of some large undigested oil droplets (Fig. 2).

Change in Emulsion Microstructure During In Vitro Digestion

The microstructures of the SPI-stabilized emulsions before and during gastro-duodenal digestion are shown in Fig. 3. The original emulsions had a uniform structure with no obvious aggregation or coalescence (Fig. 3a), confirming the particle size analysis data. Incubation of the SPI-stabilized emulsion with SGF without pepsin led to minimal droplet aggregation, as some large droplets are now visible (Fig. 3b). When the emulsion was treated with SGF containing pepsin, large aggregated droplets were observed after 60 min of incubation (Fig. 3c), also in agreement with the particle size measurements. These observations indicate that the newly formed soy peptides were less efficient at stabilizing the emulsion droplets under the in vitro gastric conditions. Figure 3d and h show the microstructure of the digestive mixture in the presence of BS after 15 and 60 min of duodenal incubation, respectively. Aggregated droplets were present and, even after 60 min of incubation, some of the undigested oil droplets remained in the form of aggregated particles, although the $D_{3,2}$ data revealed a decrease in the overall average size (Fig. 1). It is not possible to determine the exact mechanism of flocculation in these systems containing BS. It is hypothesized that depletion flocculation occurred, because of the presence of

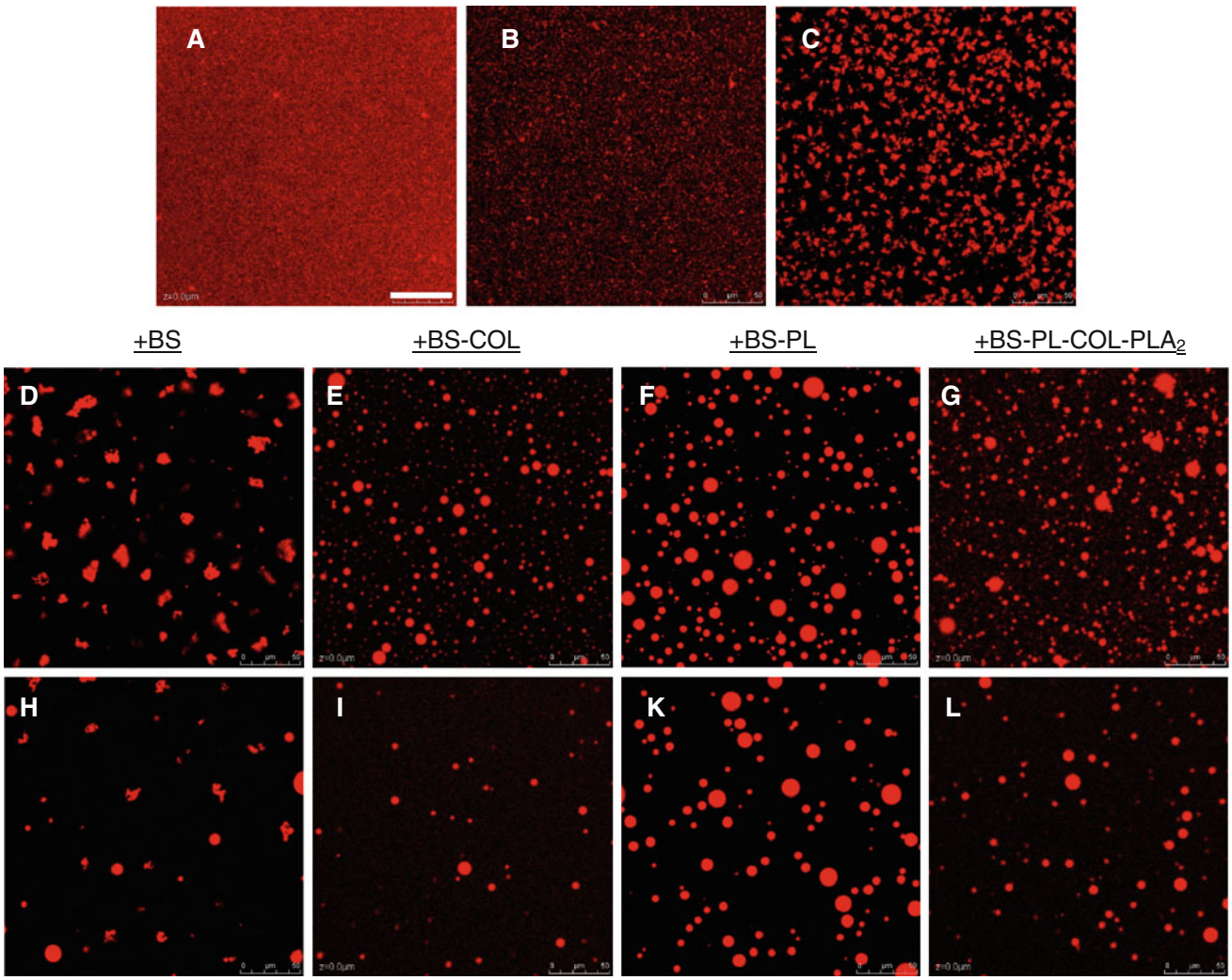

Fig. 3 Confocal micrographs of SPI-stabilized emulsions during various in vitro models: **a** original emulsion, **b** emulsion treated with SGF without pepsin, **c** emulsion treated with SGF with pepsin, **d–l** emulsions treated with SGF, including pepsin and then SDF, containing the following: BS only (**d**, **h**), BS–COL (**e**, **i**), BS–PL (**f**, **k**), BS–PL–COL–PLA_2 (**g**, **l**) after 15 (**d**, **e**, **f**, **g**) and 60 (**h**, **i**, **k**, **l**) min. *Magnification bar* represents 50 μm

BS micelles. It has been proposed previously that high concentration of BS resulted in depletion flocculation of the oil-in-water emulsions [35]. In addition, high ionic strengths cause charge neutralization [36], causing destabilization of the droplets. Finally, bridging of pancreatic lipase between oil droplets [37] or milk fat globules [38] has been proposed recently as a mechanism of droplet aggregation under duodenal condition. All of the above mechanisms could occur in the duodenal in vitro stage in the presence of BS and PTL.

Inclusion of COL with BS significantly altered the microstructure of the emulsions during digestion (Fig. 3e and i). In this case, some coalescence was observed, compared with the emulsions with only BS. Figure 3 also compares the microstructure of emulsions digested with duodenal fluids containing BS or BS with PL (Fig. 3d and h, f and k, respectively). In contrast with the flocculation observed with BS alone, with PL, the oil droplets underwent significant coalescence. A number of large droplets were visible in this sample even after 60 min of duodenal incubation. These results were consistent with the particle size distributions. The mean droplet size values after 15 min of incubation in the presence of BS–PL were similar to those of the gastric stage. However, the confocal microscopy images clearly showed microstructural differences between them (Fig. 3c compared to f). At the end of the gastric phase, the emulsion droplets were extensively flocculated, while after the in vitro duodenal stage, the droplets were coalesced. These results suggest that weaker interfacial films were formed with BS–PL compared to with BS alone. The BS–PL–COL–PLA_2 system was the most physiologically relevant in vitro duodenal digestion

model employed. In this case, even after 60 min (Fig. 3l), some undigested small oil droplets were observed, together with a few coalesced droplets. In all samples, fewer oil droplets were observed after 60 min, showing the lipolysis of the oil droplets. However, the difference was more pronounced for the system containing BS–COL (Fig. 3i).

Changes in ζ-Potential Values of the Oil Droplets During In Vitro Digestion

The changes in the ζ-potential values during the in vitro digestion of the various models are summarized in Table 1. The emulsion droplets prepared with SPI had a ζ-potential value of −38.9 ± 0.7 mV at pH 6.5. Treating the emulsion with SGF with and without pepsin at pH 2 resulted in a change to approximately +10 mV, which remained constant throughout the in vitro gastric stage (data not shown). The absolute value of ζ-potential depends not only on pH but also on ionic strength. As reported recently [15], a higher ionic strength significantly reduces the net positive charge of emulsions, as the ions shield the charges around the droplets. In addition to the extensive hydrolysis of the interfacial layer during gastric digestion, emulsion droplet flocculation can be attributed to the low net charge of the oil droplets under gastric conditions. A ζ-potential value of 44.9 ± 1.1 mV was obtained for the emulsion-SGF mixture after pH adjustment to 6.5, indicating that the liberation of (poly) peptides upon pepsinolysis caused an increase in the magnitude of the charge at the interface.

A ζ-potential value of about −50 mV was recorded for the control samples treated with SDF (without enzyme) and SBF (without BS and PL). This value remained unchanged throughout the duodenal phase (Table 1). Compared to the control sample, significant changes in the absolute value of ζ-potential were observed during the duodenal phase of in vitro digestion in the presence of different bio-surfactants. In the presence of BS, the negative ζ-potential gradually increased, reaching a plateau within the 60 min of digestion (Table 1). As previously discussed for the particle size data (Fig. 1), the adsorption of BS on the SPI-peptide covered interface occurred at a slow rate. In contrast, recent work [14] demonstrated that BS adsorb quickly onto whey protein stabilized interfaces (i.e. within 10 min of duodenal stage). The results seem to demonstrate that the displacement of interfacial film by BS is highly dependent on the original protein used to stabilize the emulsion. A similar trend was also found for the SPI-emulsion incubated with SDF containing BS–COL (Table 1).

Incorporation of PL in the in vitro SDF containing BS resulted in a rapid and significant change in ζ-potential within the first 2 min of the duodenal phase. The value of ζ-potential reached −62.3 ± 1.4 mV after 2 min, with a further increase in negative charge to 72.9 ± 1.1 mV at the end of digestion (Table 1). The results suggest that PL readily adsorbed at the oil–water interface along with the BS. Comparing this value with the oil-in-water emulsions stabilized with PL (−52.5 ± 0.6 mV, data not shown), it can be concluded that the oil–water interface was covered by a mixed BS–PL interfacial film with more BS present at the interface. The PL molecules most likely aided in the displacement of the soy proteins and peptides from the interface.

Addition of COL or COL–PLA_2 to the BS–PL system also resulted in a rapid increase in the negative ζ-potential and the value reached a plateau within 5 min of the duodenal stage (Table 1), confirming that, in all the in vitro digestion models containing PL, the change in ζ-potential values occurred significantly faster compared to those without PL (Table 1).

Table 1 ζ-potential (mV) values after the gastric phase and with exposure to duodenal fluids of variable composition

Time (min)	Control[A]	BS	BS–COL	BS–PL	BS–PL–COL	BS–PL–COL–PLA_2
0[B]	-44.9 ± 1.1^{b}	-44.9 ± 1.1^{a}	-44.9 ± 1.1^{a}	-44.9 ± 1.1^{a}	-44.9 ± 1.1^{b}	-44.9 ± 1.1^{a}
2	$-50.1 \pm 1.6^{a,x}$	$-45.4 \pm 2.4^{a,y}$	$-41.5 \pm 5.2^{a,y}$	$-62.3 \pm 1.4^{b,z}$	$-67.7 \pm 2.5^{a,z}$	$-65.9 \pm 1.4^{b,z}$
5	$-49.9 \pm 1.6^{a,x}$	$-54.9 \pm 2.9^{b,x}$	$-55.0 \pm 2.6^{b,x}$	$-61.6 \pm 2.5^{b,y}$	$-68.8 \pm 5.3^{a,z}$	$-67.7 \pm 3.1^{b,z}$
10	$-49.9 \pm 0.7^{a,x}$	$-60.1 \pm 2.8^{c,y}$	$-59.9 \pm 3.9^{b,y}$	$-65.6 \pm 3.7^{c,z}$	$-68.1 \pm 6.1^{a,z}$	$-71.3 \pm 1.5^{b,z}$
15	$-51.4 \pm 0.8^{a,x}$	$-58.2 \pm 2.5^{c,y}$	$-65.7 \pm 4.7^{c,z}$	$-64.8 \pm 1.2^{c,z}$	$-67.6 \pm 3.4^{a,z}$	$-71.3 \pm 0.8^{b,z}$
30	$-50.6 \pm 1.3^{a,x}$	$-64.6 \pm 3.1^{d,y}$	$-68.0 \pm 4.2^{c,y}$	$-66.6 \pm 0.7^{c,y}$	$-68.1 \pm 3.0^{a,y}$	$-71.5 \pm 0.9^{b,y}$
60	$-51.5 \pm 0.6^{a,x}$	$-71.9 \pm 3.0^{e,y}$	$-76.9 \pm 1.3^{d,y}$	$-71.1 \pm 2.5^{d,y}$	$-69.7 \pm 6.7^{a,y}$	$-71.5 \pm 3.4^{b,y}$
90	$-50.8 \pm 0.9^{a,x}$	$-74.2 \pm 1.8^{e,y}$	$-76.7 \pm 1.6^{d,y}$	$-70.8 \pm 1.1^{d,y}$	$-68.3 \pm 5.4^{a,y}$	$-71.1 \pm 2.1^{b,y}$
120	$-50.6 \pm 1.3^{a,x}$	$-74.8 \pm 1.0^{e,y}$	$-76.9 \pm 3.5^{d,y}$	$-72.9 \pm 1.1^{d,y}$	$-69.4 \pm 3.8^{a,y}$	$-68.5 \pm 1.3^{b,y}$

Data are the averages of at least three independent experiments ± standard deviation

Different superscripts indicate significant difference within a column (a to e) and within a row (x–z) at $p < 0.05$

[A] Contained SDF (without pancreatin) and SBF (without BS or PL)

[B] Determined after adjustment to pH 6.5, but prior to SDF and SBF addition

The Rate of Lipid Hydrolysis

To analyze the extent of triglyceride (TG) digestion by pancreatic triglyceride lipase (PTL), the emulsion-SGF was subjected to the various in vitro digestion models. Hence, the lipolysis experiments were carried out at constant substrate and PTL concentrations. Fig. 4a and b show the plot of product formation as a function of time, for the different digestion models. Moreover, all the kinetic parameters including maximal product formation (P_{max}) and initial rates (υ_0) represented in Table 2 were obtained by plotting $[P]/t$ against $[P]$ (Fig. 4c and d).

In all models, lipolysis occurred rapidly and without any lag phase. However, the extent of lipolysis depended highly on the composition of the digestive fluids. In the model containing only BS, the reaction reached a plateau within 20 min of about 130 μmol mL^{-1}. In other words, 66.1 ± 3.4% of the original TG were converted into MG and FFA at the end of the duodenal stage (Table 2). The limited hydrolysis of TG in the presence of BS confirmed the inhibitory effect of BS along with lipolytic products on PTL activity that was described in previous work [14].

Addition of COL to the in vitro digestion fluids increased the extent of lipolysis, but not the initial rate (Table 2). The highest value of P_{max} (196.3 ± 2.5 μmol mL^{-1}) was noted for systems containing BS–COL (Fig. 4a), corresponding to 81.2 ± 1.2% of lipid hydrolysis (Table 2). These results are in good agreement with previous published work proposing that COL improves the binding of PTL to its substrate through formation of a PTL–COL complex [39]. In the current study, inclusion of COL led to a 15.1% increase in lipolysis compared to BS alone. Possibly the COL–PTL complex remained at the interface for longer, and had a higher resistance to displacement by the BS and lipolytic products, than PTL alone. Reportedly, under the same experimental conditions, incubation of a whey protein stabilized emulsion (0.5% protein) with SDF and SBF containing COL and BS resulted in a significantly higher value of initial velocity along with higher P_{max} [14]. However, in this case the addition of COL in combination with BS increased only P_{max}. Again, these results stress the importance of COL in terms of lipid hydrolysis, and that its effect is governed by the original and modified composition of the interfacial layer.

The values of PTL initial velocity were considerably higher in all systems containing PL (Table 2). The highest υ_0 was recorded in the presence of BS–PL with a value of

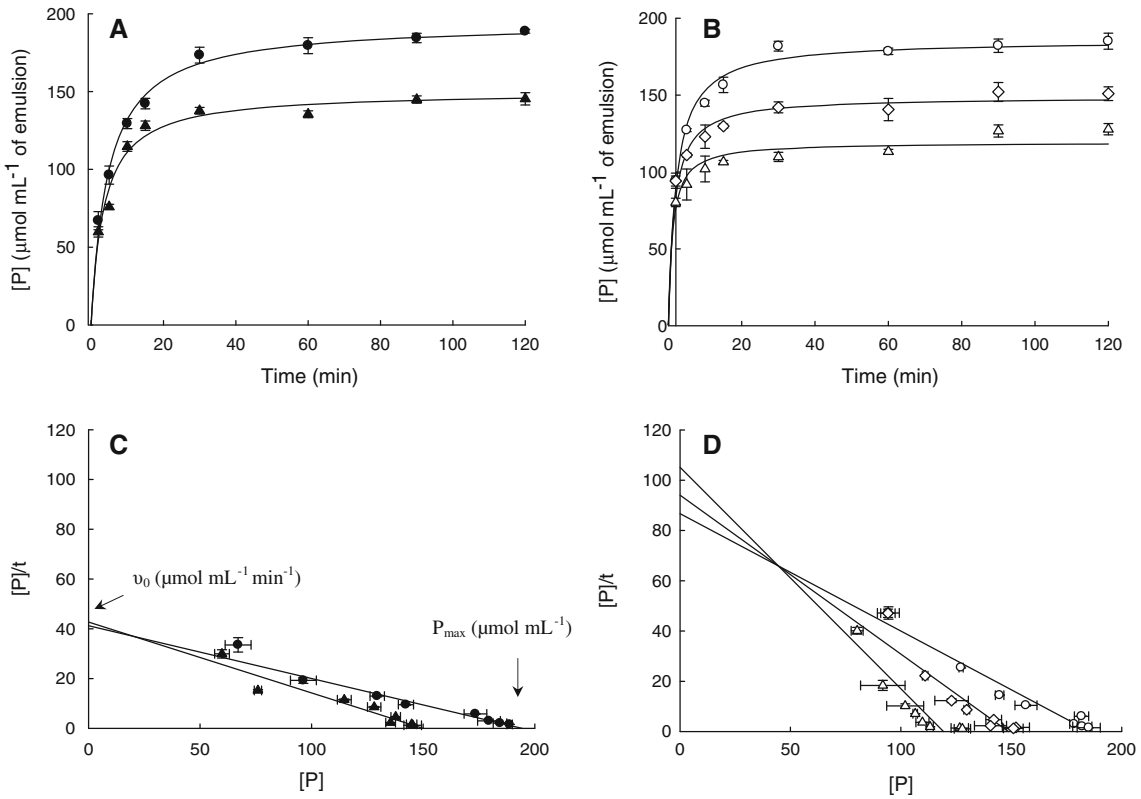

Fig. 4 Product formation with PTL activity (as FFA concentration) (**a**, **b**) and the corresponding linear plot of $[P]/t$ versus $[P]$ for determination of kinetic parameters (**c**, **d**). The concentration of products was measured in the various in vitro models: BS only (*filled upward triangles*); BS–COL (*filled circles*); BS–PL (*open upward triangles*), BS–PL–COL (*open diamonds*), and BS–PL–COL–PLA_2 (*open circles*). Data shown is the averages of three independent experiments with standard deviation

Table 2 The kinetic parameters of lipid hydrolysis by PTL for the different duodenal conditions

	P_{max} (μmol mL^{-1})	υ_0 (μmol mL^{-1} min^{-1})	r^2	Lipid hydrolysis (%)
BS	150.0 ± 2.6^{c}	42.5^{c}	0.95	66.1 ± 3.4^{c}
BS–PL	120.1 ± 2.9^{d}	105.0^{a}	0.87	51.1 ± 5.0^{d}
BS–COL	196.3 ± 3.2^{a}	41.1^{c}	0.97	81.2 ± 1.2^{a}
BS–PL–COL	147.4 ± 2.7^{c}	93.9^{b}	0.82	59.8 ± 5.3^{b}
BS–PL–COL–PLA_2	186.3 ± 2.5^{b}	86.4^{b}	0.94	79.2 ± 2.3^{a}

Different superscripts within a column indicate significant difference between means, $p < 0.05$

105.0 μmol mL^{-1} min^{-1}. Furthermore, a higher υ_0 was found with BS–PL–COL and BS–PL–COL–PLA_2 compared to the systems lacking PL (Table 2). The addition of PL along with BS also led to a rapid increase in the generation of FFA, reaching a plateau within 20 min of duodenal incubation (Fig. 4b). However, the lowest P_{max} values were observed in the systems containing BS–PL (120.1 ± 2.9 μmol mL^{-1}). Together with the particle size and ζ-potential data, it is possible to conclude that the preferential adsorption of PL at the interface enhanced the PTL inhibition. The lowest percentage of lipolysis was recorded for the in vitro digestion containing BS–PL, with a value of 51.0 ± 5.0% (Table 2).

Addition of COL to the BS–PL system slightly minimized the inhibitory effect of BS–PL on PTL a value of 147.4 μmol mL^{-1} or 59.8 ± 5.3% lipid digestion observed (Table 2). These data seem to confirm that the oil–water interface was covered by both BS and PL, and inclusion of COL significantly improved the PTL binding and activity. Lipolysis was 8.8% higher compared to the in vitro digestion system containing only BS–PL (Table 2).

The effect of PL needs to be considered in light of the presence of PLA_2 in the duodenal fluids. This enzyme converts PL into lysophospholipids (Lyso–PL) and FFA. As shown in Table 2, the addition of PLA_2 into the system containing BS–PL–COL did not change the initial velocity of lipolysis compared to when only BS–PL–COL are present; however, significantly higher P_{max} was found (Table 2). This suggests that hydrolysis of adsorbed PL at the oil–water interface by PLA_2 and the generated FFA and Lyso–PL modified the structure of the interfacial layer. It was reported [21] that Lyso–PL has a high affinity for mixed BS micelles and may readily desorb from the oil–water interface. The same positive impact of PLA_2 on the PTL activity has been also recently reported for whey protein stabilized interfaces [14].

β-Carotene Bioaccessibility

The amounts of bioaccessible β-carotene (BC) isolated from the micellar phase (the centrifugal supernatant) are summarized in Fig. 5. In the presence of BS alone, 48.4 ± 2.9% BC transfer was obtained. In contrast,

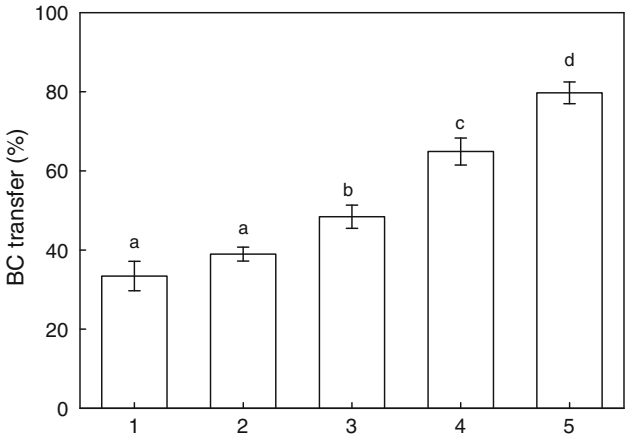

Fig. 5 The total amount of BC transferred to the aqueous phase in the presence of **1** BS–PL, **2** BS–PL–COL, **3** BS, **4** BS–COL, and **5** BS–PL–COL–PLA_2. Means and standard deviations indicated for three independent experiments, letters show statistical differences ($p < 0.05$)

addition of PL along with BS significantly reduced the amount of micellarized BC, as only 33.4 ± 3.7% BC was recovered in the aqueous phase. The combination of BS–COL significantly increased the amount of micellarized BC compared to BS alone. However, addition of COL to the BS–PL had no effect on BC transfer (38.9 ± 1.7%). It seemed that the impact of BS, PL, and COL on BC transfer originated from the impact of these bio-surfactants and cofactor on the extent of lipid hydrolysis (Table 2).

The highest bioaccessibility was obtained in the in vitro digestion model that included PLA_2, in addition to BS–PL–COL. In this case, a high degree of lipid hydrolysis was observed (Table 2) and 79.7 ± 3.4% of the BC was transferred to the aqueous phase. Comparing the BC bioaccessibility for the BS–COL system (treatment 4 in Fig. 5) versus the model containing BS–PL–COL and PLA_2 (treatment 5 in Fig. 5) demonstrates that the presence of Lyso–PL liberated during PL hydrolysis significantly increased BC micellization. Indeed, in both cases, 80% of the TG were hydrolyzed; however, significantly higher BC transfer occurred in the presence of Lyso–PL.

Figure 6 shows that there was a positive correlation between the extent of lipid hydrolysis and BC bioaccessibility (Fig. 6, $r^2 = 0.94$). This is consistent with recent

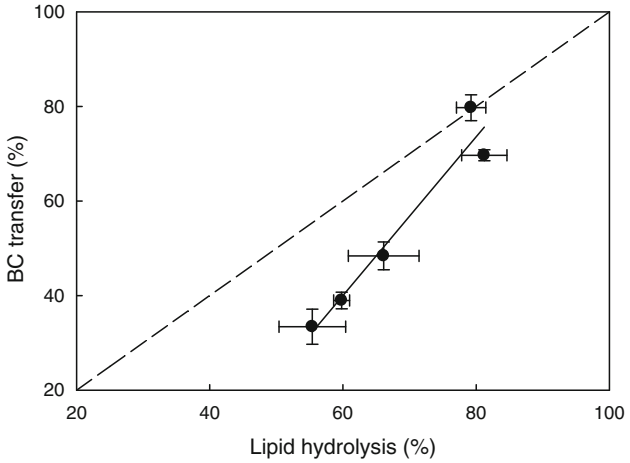

Fig. 6 Correlation between β-carotene (BC) transfer and lipid hydrolysis at the end of the duodenal phase with BS, BS–PL, BS–PL–COL, BS–COL, and BS–PL–COL–PLA_2

reports [14] that a higher extent of lipolysis led to more BC transfer to the aqueous phase. However, prior to the linear region in Fig. 6, a clear lag phase was observed such that approximately 55% lipolysis resulted in approximately 30% BC transfer. In addition, the release behavior of BC was below the theoretical line indicating that BC had a higher tendency towards the oil phase than the micellar phase. This trend might be explained by a preferential partitioning and hence a concentration of BC in the oil phase during the course of digestion. A similar trend was recently reported for the lipophilic drug progesterone, which became more concentrated in the oil phase as the oil was digested [40]. The present work also illustrates that factors other than lipolysis might play a role in the micellization behaviour of BC. In particular, the composition of the mixed micelles is implicated based on the fact that BC bioaccessibility was higher when Lyso–PL were present compared to only BS and PL (Fig. 6).

Conclusions

The gastro-intestinal behavior of SPI-stabilized emulsions has been explored, by considering the influence of the in vitro conditions on lipolysis and micellization of BC. During the gastric stage, the hydrolysis of the interfacial layer led to significant flocculation of the emulsion droplets. The particle size, ζ-potential, and microstructure of the emulsion droplets were markedly affected by the presence of BS and PL in the in vitro duodenal stage. BS and PL were found to play a significant role in PTL activity by affecting PTL access to its substrate. Both COL and PLA_2 enhanced the extent of lipolysis, although through different mechanisms. The amount of TG hydrolyzed also was correlated with the amount of BC transferred to the mixed BS micelles, and Lyso–PL was seen to play an important role in BC micellization.

References

1. Dalgleish DG (2006) Food emulsions—their structures and structure-forming properties. Food Hydrocoll 20(4):415–422
2. McClements DJ, Decker EA, Weiss J (2007) Emulsion-based delivery systems for lipophilic bioactive components. J Food Sci 72(8):R109–R124
3. Nielsen NC, Dickinson CD, Cho TJ, Thanh VH, Scallon BJ, Fischer RL, Sims TL, Drews GN, Goldberg RB (1989) Characterization of the glycinin gene family in soybean. Plant Cell 1(3):313–328
4. Staswick PE, Hermodson MA, Nielsen NC (1984) Identification of the cystine which links the acidic and basic components of the glycinin subunits. J Biol Chem 259:13431–13435
5. Yamauchi F, Yamagishi T, Iwabuchi S (1991) Molecular understanding of heat induced phenomena of soybean proteins. Food Rev Int 7:283–322
6. Keerati-u-rai M, Corredig M (2010) Heat-induced changes occurring in oil/water emulsions stabilized by soy glycinin and beta-conglycinin. J Agric Food Chem 58(16):9171–9180
7. Aoyama T, Fukui K, Nakamori T, Hashimoto Y, Yamamoto T, Takamatsu K, Sugano M (2000) Effect of soy and milk whey protein isolates and their hydrolysates on weight reduction in genetically obese mice. Biosci Biotechnol Biochem 64(12):2594–2600
8. Martinez-Villaluenga C, Bringe NA, Berhow MA, de Mejia EG (2008) β-Conglycinin embeds active peptides that inhibit lipid accumulation in 3T3-L1 adipocytes in vitro. J Agric Food Chem 56(22):10533–10543
9. Duranti M, Lovati MR, Dani V, Barbiroli A, Scarafoni A, Castiglioni S, Ponzone C, Morazzoni P (2004) The α' subunit from soybean 7S globulin lowers plasma lipids and upregulates liver beta-VLDL receptors in rat feed a hypercholesterolemic diet. J Nutr 134(6):1334–1339
10. Silletti E, Vingerhoeds MH, Norde W, van Aken GA (2007) Complex formation in mixtures of lysozyme-stabilized emulsions and human saliva. J Colloid Interf Sci 313(2):485–493
11. Vingerhoeds MH, Blijdenstein TBJ, Zoet FD, van Aken GA (2005) Emulsion flocculation induced by saliva and mucin. Food Hydrocoll 19(5):915–922
12. van Aken GA, Vingerhoeds MH, de Hoog EHA (2007) Food colloids under oral conditions. Curr Opin Coll Int Sci 12(4–5):251–262
13. McClements DJ, Decker EA, Park Y, Weiss J (2008) Designing food structure to control stability, digestion, release and absorption of lipophilic food components. Food Biophys 3(2):219–228
14. Malaki Nik A, Corredig M, Wright AJ (2010) Changes in WPI-stabilized emulsion interfacial properties in relation to lipolysis and beta-carotene transfer during exposure to simulated gastric-duodenal fluids of variable composition. Food Dig 1:14–27
15. Sarkar A, Goh KKT, Singh H (2010) Properties of oil-in-water emulsions stabilized by beta-lactoglobulin in simulated gastric fluid as influenced by ionic strength and presence of mucin. Food Hydrocoll 24(5):534–541
16. Mandalari G, Mackie AM, Rigby NM, Wickham MSJ, Mills ENC (2009) Physiological phosphatidylcholine protects bovine beta-lactoglobulin from simulated gastrointestinal proteolysis. Mol Nutr Food Res 53:S131–S139

17. Malaki Nik A, Wright AJ, Corredig M (2010) Surface adsorption alters the susceptibility of whey proteins to pepsin-digestion. J Colloid Interf Sci 344:372–381
18. Mun S, Decker EA, McClements DJ (2007) Influence of emulsifier type on in vitro digestibility of lipid droplets by pancreatic lipase. Food Res Int 40(6):770–781
19. Beysseriat M, Decker EA, McClements DJ (2006) Preliminary study of the influence of dietary fiber on the properties of oil-in-water emulsions passing through an in vitro human digestion model. Food Hydrocoll 20(6):800–809
20. Reis PM, Raab TW, Chuat JY, Leser ME, Miller R, Watzke HJ, Holmberg K (2008) Influence of surfactants on lipase fat digestion in a model gastro-intestinal system. Food Biophys 3(4):370–381
21. Borgstrom B (1980) Importance of phospholipids, pancreatic phospholipase A2 and fatty acid for the digestion of dietary fat. Gastroenterology 78:954–962
22. Reis P, Holmberg K, Miller R, Leser ME, Raab T, Watzke HJ (2009) Lipase reaction at interfaces as self-limiting processes. Comptes Rendus Chimie 12(1–2):163–170
23. Reis P, Holmberg K, Miller R, Kragel J, Grigoriev DO, Leser ME, Watzke HJ (2008) Competition between lipases and monoglycerides at interfaces. Langmuir 24(14):7400–7407
24. McClements DJ, Decker EA, Park Y (2009) Controlling lipid bioavailability through physicochemical and structural approaches. Crit Rev Food Sci Nutr 49(1):48–67
25. Li Y, Hu M, Xiao H, Du YM, Decker EA, McClements DJ (2010) Controlling the functional performance of emulsion-based delivery systems using multi-component biopolymer coatings. Eur J Pharm Biopharm 76:38–47
26. Li Y, Hu M, Du YM, Xiao H, McClements DJ (2011) Control of lipase digestibility of emulsified lipids by encapsulation within calcium alginate beads. Food Hydrocoll 25(1):122–130
27. Yonekura L, Nagao A (2007) Intestinal absorption of dietary carotenoids. Mol Nutr Food Res 51:107–115
28. Chu BS, Ichikawa S, Kanafusa S, Nakajima M (2007) Preparation of protein-stabilized beta-carotene nanodispersions by emulsification–evaporation method. J Am Oil Chem Soc 84(11):1053–1062
29. Borel P (2003) Factors affecting intestinal absorption of highly lipophilic food microconstituents (fat-soluble vitamins, carotenoids and phytosterols). Clin Chem Lab Med 41(8):979–994
30. Mun S, Decker EA, Park Y, Weiss J, McClements DJ (2006) Influence of interfacial composition on in vitro digestibility of emulsified lipids: potential mechanism for chitosan's ability to inhibit fat digestion. Food Biophys 1(1):21–29
31. Wright AJ, Pietrangelo C, MacNaughton A (2008) Influence of simulated upper intestinal parameters on the efficiency of beta carotene micellarisation using an in vitro model of digestion. Food Chem 107(3):1253–1260
32. Malaki Nik A, Wright AJ, and Corredig M (2011) Impact of interfacial composition on emulsion digestion and rate of lipid hydrolysis using different in vitro digestion models. Colloids Surf B Biointerfaces 83:321–330
33. Marciani L, Faulks R, Wickham MSJ, Bush D, Pick B, Wright J, Cox EF, Fillery-Travis A, Gowland PA, Spiller RC (2009) Effect of intragastric acid stability of fat emulsions on gastric emptying, plasma lipid profile and postprandial satiety. Br J Nutr 101(6):919–928
34. Maldonado-Valderrama J, Woodward NC, Gunning AP, Ridout MJ, Husband FA, Mackie AR, Morris VJ, Wilde PJ (2008) Interfacial characterization of beta-lactoglobulin networks: displacement by bile salts. Langmuir 24(13):6759–6767
35. Jódar-Reyes AB, Torcello-Gómez A, Wulff-Pérez M, Gálvez-Ruiz MJ, Martín-Rodríguez A (2010) Different stability regimes of oil-in-water emulsions in the presence of bile salts. Food Res Int 43:1634–1641
36. Sandra S, Decker EA, McClements DJ (2008) Effect of interfacial protein cross-linking on the in vitro digestibility of emulsified corn oil by pancreatic lipase. J Agric Food Chem 56(16):7488–7494
37. Reis P, Miller R, Kragel J, Leser M, Fainerman VB, Watzke H, Holmberg K (2008) Lipases at interfaces: unique interfacial properties as globular proteins. Langmuir 24(13):6812–6819
38. Ye A, Cui j, Singh H (2010) Effect of the fat globule membrane on in vitro digestion of milk fat globules with pancreatic lipase. Int Dairy J 20:822–829
39. van Tilbeurgh H, Bezzine S, Cambillau C, Verger R, Carriere F (1999) Colipase: structure and interaction with pancreatic lipase. Biochimica et Biophysica Acta-Molecular and Cell Biology of Lipids 1441(2–3):173–184
40. Day JPR, Rago G, Domke KF, Velikov KP, Bonn M (2010) Label-free imaging of lipophilic bioactive molecules during lipid digestion by multiplex coherent anti-stokes Raman scattering microspectroscopy. J Am Chem Soc 132(24):8433–8439