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In vitro digestibility and allergenicity of emulsified hen egg

Rodrigo Jiménez-Saiz, Víctor M.P. Ruiz-Henestrosa [1], Rosina López-Fandiño, Elena Molina *

Institute of Food Science Research (CIAL) CSIC-UAM, C/Nicolás Cabrera, 9, 28049, Madrid, Spain

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ABSTRACT

Whole hen egg produced a fine stable O/W emulsion. The presence of egg proteins as part of the emulsion did not change their IgE binding, but it slightly increased the digestibility of the main allergens present in the egg-white. The observation that egg white proteins, forming part of an emulsion system did not become a much more effective substrate for pepsin indicates that, in the case of egg white proteins, there were not adsorption-induced changes that would considerably increase their flexibility and proteinase susceptibility. The increased digestibility of the emulsion resulted in a slightly lower IgE-binding capacity of the *in vitro* gastric and duodenal digests compared to those obtained from the egg in solution.

1. Introduction

Hen's egg, either as a whole or its constituents (egg yolk and white), is a key ingredient in many food products. Egg proteins are extensively utilized in many foods by virtue of their nutritional value and important functional properties for industrial applications. These include gel formation, foaming capacity and emulsifying ability, among others, that are useful in the preparation of bakery foods, bakery mixes, mayonnaises, salad dressings, and many convenience foods (Campbell, Raikos, & Euston, 2003). Nevertheless, egg is also known because of its allergenic potential and, in fact, it is regarded as one of the most allergenic foods. Egg allergens have been studied in depth. The major ones: ovalbumin (OVA), ovomucoid (OM), lysozyme (LYS) and ovotransferrin (OVT) are found in the egg white, but the egg yolk also contains allergenic proteins such as α-livetin and apovitellenins I and VI (Mine & Yang, 2008).

While the oral mucosa is, in some cases, the first place of antigen uptake, the ability of food proteins to sensitize and/or elicit allergic reactions is linked to their resistance to gastroduodenal digestion, which ultimately lets them interact with the intestinal mucosa where absorption occurs (Untersmayr & Jensen-Jarolim, 2006). Therefore, any factor that affects protein digestibility, whether increasing, decreasing it, or inducing a different proteolysis pattern, might change their capacity to induce or trigger an allergic reaction. In this respect, the food matrix and the processing practices greatly influence the allergic potential of food proteins (Foegeding & Davis, 2011).

Interactions of proteins with lipids to form emulsions and other structures are deliberately introduced during the preparation of foods or may occur in the gastrointestinal tract as a consequence of the digestive process. Proteins, due to their amphipathic nature, adsorb efficiently at the oil/water interface, lowering the surface tension and stabilizing the system and, as a consequence, they may undergo conformational changes. Thus, Agboola and Dalgleish (1996) reported that adsorbed β-lactoglobulin (β-Lg) is more easily digested by trypsin than soluble β-Lg, an observation that they attributed to a greater flexibility and accessibility of susceptible bonds on the interface. Similarly, the rate of pepsin digestion of β-Lg and β-casein is increased when they are presented in emulsions (Macierzanka, Sancho, Mills, Rigby, & Mackie, 2009; Sarkar, Goh, Singh, & Singh, 2009). On the other hand, recent work has highlighted the importance of physiological surfactants, such as phospholipids, bile salts and lipolysis products, not only in the gastrointestinal processing of proteins adsorbed to emulsions, but also in solution (Mackie & Macierzanka, 2010; Maldonado-Valderrama, Wilde, Macierzanka, & Mackie, 2011). However, despite the increasing scientific interest on the behavior of food–protein stabilized emulsions during simulated gastrointestinal digestion, to the best of our knowledge, there are no studies on the effect of emulsification on egg proteins, in terms of digestibility and allergenicity.

The long-term physicochemical stability of egg-stabilized emulsions largely depends on the presence of egg yolk in the system. This implies that the more flexible and surface-active yolk lipoproteins

Abbreviations: EM, egg emulsion; ES, egg solution; OVA, ovalbumin; OM, ovomucoid; LYS, lysozyme; OVT, ovotransferrin; GD, gastric digestion; DD, duodenal digestion; β-Lg, β-lactoglobulin; PC, phosphatidylcholine; CEM, cream phase of the egg proteins emulsion.

* Corresponding author. Tel.: +34 91 0017 938; fax: +34 91 0017 905.
E-mail address: e.molina@csic.es (E. Molina).
[1] Present address: Departamento de Industrias, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria (1428), Buenos Aires, Argentina.

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are preferred on the interfaces to the globular egg white proteins. While egg-white proteins adsorb to the interfaces covered with yolk lipoproteins only to a limited extent (Drakos & Kiosseoglou, 2008), it cannot be excluded that the adsorbed proteins as well as those present in the serum phase of the emulsion behave towards digestion differently than the native proteins in solution (Sarkar et al., 2009). From this background, this work studies the *in vitro* digestibility and human IgE-binding capacity of egg proteins that form part of food emulsions under the conditions that these products are usually consumed, with the aim to achieve a better understanding of the gastrointestinal processing of egg emulsions and the way the emulsion system affects the allergenic potential of the egg.

2. Materials and methods

2.1. Materials and emulsions processing

Fresh hen eggs (category A) and extra virgin olive oil were bought at a local store. The eggs were cracked, pooled and gently homogenized, avoiding foam formation, by using an Ultra-Turrax (T-25 basic, IKA, Germany) and the mixture was freeze dried and stored at −20 °C. The pH of the pool was 7.46 and the protein content 53.4%, as estimated by the Kjeldahl method. The oil was purified by mixing with 10% (w/v) Florisil (Sigma-Aldrich, MO, USA; Bahtz et al., 2009) with vigorous stirring for 48 h at room temperature (RT). After another 48 h, the oil was centrifuged (5000 *g*) and the supernatant was removed and stored at RT avoiding light exposure until used.

Egg solutions (ES) containing 8% (w/v) freeze dried whole egg (4.27% of egg protein) were prepared in 0.15 M NaCl, adjusted with citric acid to pH 4.2, with gentle stirring for 30 min. Egg emulsions (EM) contained 8% (w/v) freeze dried whole egg in 0.15 M NaCl, pH 4.2 and 25% of olive oil (v/v) and were homogenized by using an Ultra-Turrax (T-25 basic) at 11,000 rpm. A total volume of 50 mL of emulsion was prepared each time. The cream phases of the EM were obtained by centrifugation (10,000 g) and careful removal of the subnatants.

2.2. Stability of the EM

2.2.1. Conductivity measurements

The specific electrical conductivity of the EM was checked, at least in triplicate, by using a conductivity meter (Konductometer CG 855, Schott, Mainz, Germany). The conductivity measurements are directly related to the creaming of the droplets (Kato, Fujishige, Matsudomi, & Kobayashi, 1985). The conductivity was checked in the ES before adding the oil and in the EM at different times, up to 240 h at RT.

2.2.2. Phase separation

Digital pictures of the EM (fresh and stored for 4 h) placed into a 15 mL graduated tube were taken at different times in order to corroborate the absence of phase separation. The tubes were kept at RT and at least duplicates of each sample were prepared. A digital camera (IXUS 100IS, Canon) was used.

2.2.3. Differential interference contrast microscopy (DIC)

The microstructure of the EM (fresh emulsions and emulsions after 4 h) was studied using a DIC microscope Leica model AF6000 LX (Wetzlar, Germany). A drop of the EM was placed on the slide, covered by a cover slip, and observed under a magnification of ×20. At least 2 replications were prepared for each sample and they were examined without any previous dilution. Images of the EM were taken using a digital camera (Andor 885, Belfast, Ireland) from different fields on each slide.

2.3. In vitro gastro duodenal digestions

In vitro digestions were performed following Moreno, Mellon, Wickham, Bottrill, and Mills (2005) and Martos, Contreras, Molina, and Lopez-Fandiño (2010). ES and EM were subjected to gastric digestion in simulated gastric fluid (SGF, 35 mM NaCl) at pH 2.0, containing porcine pepsin (EC 3.4.23.1, 3440 units/mg, Sigma) at an enzyme/substrate ratio of 1:20 w/w (172 units/mg) and phosphatidylcholine (PC, P3841, Sigma) at a concentration of 6.64 mM. Aliquots were taken at 10, 20, 30 and 60 min. 60 min-gastric digests were readjusted at pH 6.5 and subjected to duodenal digestions with the addition of 1 M $CaCl_2$, 0.25 M Bis–Tris pH 6.5, and a 0.125 M bile salt mixture containing equimolar quantities of sodium taurocholate (Sigma) and glycodeoxycholic acid (Sigma). Trypsin (EC 232-650-8, 10100 BAEE units/mg protein, Sigma), α-chymotrypsin (EC 232-671-2; 55 units/mg protein, Sigma), porcine pancreatic lipase (EC 232-619-9, Sigma) and colipase (EC 259-490-1, Sigma), prepared in 35 mM NaCl adjusted to pH 7.0, were added to the duodenal mix. The final composition of the mixture was 4.15 mg/mL of protein, 6.15 mM of each bile salt, 20.3 mM Bis–Tris, 7.6 mM $CaCl_2$ and the enzymes referred to the quantity of protein were: 40 units/mg trypsin, 0.5 units/mg α-chymotrypsin, and 28.9 units/mg lipase and colipase (enzyme/substrate ratio 1:895 w/w). Duodenal digestions were stopped after 30 min with Orlistat (O4139, Sigma-Aldrich, enzyme/substrate ratio 1:70 w/w) and Bowman-Birk trypsin–chymotrypsin inhibitor from soybean (T9777, Sigma-Aldrich). Aliquots were kept at −20 °C until use for SDS-PAGE, RP-HPLC and ELISA analysis.

2.4. SDS-PAGE

SDS-PAGE was performed using Criterion XT Precast Gels with 4–12% Bis–Tris (Bio-Rad Laboratories, Hercules, CA). Samples were diluted in a buffer containing 62.5 mM Tris–HCl pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 5% (v/v) β-mercaptoethanol and 0.0025% (w/v) bromophenol blue and heated at 95 °C for 10 min. Electrophoresis was carried out at 90–100 V for 2 h and at RT, in XT MES running buffer (Bio-Rad). Gels were fixed in a 40% methanol and 10% (wt/v) acetic acid solution, followed by staining with Coomassie Blue R-250 (Bio-Rad). Precision Plus Protein Unstained molecular mass standards (Bio-Rad) were used. Images were taken with a Versa Doc imaging system (Bio-Rad) and the software Quantity One (Bio-Rad) was used for band analyses.

2.5. RP-HPLC

Analyses were conducted in a Hi-Pore RP-318 (250×4.6 mm internal diameter) column (Waters, Milford, MA) in a Waters 600 HPLC system. Solvent A was 0.37% (v/v) trifluoroacetic acid (Scharlau Chemie, Barcelona, Spain) in double-distilled water and solvent B was 0.27% (v/v) trifluoroacetic acid in HPLC-grade acetonitrile (Lab-Scan, Gliwice, Poland). The chromatographic conditions were as in Quiros, Chichon, Recio, and Lopez-Fandino (2007). Detection was at 220 nm and data were processed by using the Empower 2 Software (Waters).

2.6. Human IgE binding by inhibition ELISA

Human-IgE binding was evaluated by inhibition ELISA following Jimenez-Saiz, Martos, Carrillo, Lopez-Fandino, and Molina (2011) with some variations: freeze dried egg was used as a coating antigen diluted in 0.1 M bicarbonate buffer, pH 9.6 to 200 μg/mL; a total of 9 sera from egg allergic patients were mixed in three different pools (Table 1). Polyclonal rabbit anti-human IgE (A0094, Dako, Glostrup, Denmark) and polyclonal swine anti-rabbit immunoglobulins labeled with horseradish peroxidase (P0399, Dako) were used diluted 1:1000 and 1:2000 (v/v) respectively, in phosphate buffer, pH 7.4, containing 0.05% Tween 20.

Table 1
Specific IgE levels (kU/L) towards egg white, yolk, ovalbumin (OVA) and ovomucoid (OM) of the sera used in the study and age of the patients.

Serum	Patient	Age	IgE (kU/L)			
			Egg white	Egg yolk	OVA	OM
Pool 1	1	3	10.2	1.71	11.1	2.94
	2	12	>100	85.7	7.9	69.2
	3	–	–	–	15	16
Pool 2	4	2	7	2.2	7.84	1.4
	5	–	Egg > 100			
	6	–	Egg > 100			
Pool 3	7	1	7.44	0.89	3.03	7.51
	8	–	Egg > 100			
	9	–	–	–	62	80

2.7. Statistical analyses

Density analyses of the SDS-PAGE bands, HPLC peak areas and IgE-binding results were statistically processed. Density analyses of the SDS-PAGE bands and HPLC peak areas were expressed as relative percentages while for IgE-binding, a non-linear adjustment of the data obtained for each dilution was applied for each serum and sample. The adjustment model was a sigmoid curve of inhibition dose–response with variable slope, from which the IC_{50} (the protein concentration that binds 50% of seric IgE) was obtained with the program GraphPad Prism 5 for Windows (GraphPad software, San Diego, CA). Both relative percentages and IC_{50} of each sample, were expressed as a mean ± standard error for n = 2. Significant differences ($p<0.05$) were evaluated by one-way analysis of variance followed by post hoc multiple-comparison using Tukey's test.

3. Results and discussion

3.1. Characterization of EM stability

Conductivity (mS/cm) is commonly used as a measurement of the stability of emulsions (Azzam & Omari, 2002; Kato et al., 1985). As shown in Fig. 1, the conductivity of EM was lower than the conductivity of ES and it remained stable during the first 48 h, showing that the oil droplets were homogeneously distributed in the entire sample volume (Gundersen, Saether, & Sjoblom, 2001). After 48 h, the values of conductivity started to increase, probably because the dispersed oil droplets began to rise and coalesce to form floating layer of oil (Gundersen et al., 2001).

Fig. 2 shows the visual appearance and the microstructure of EM, freshly prepared and after 4 h. EM was very stable, without signs of phase separation for the first 4 h. Furthermore, the images taken

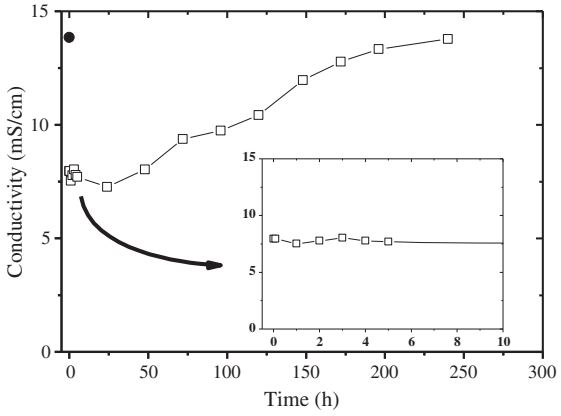

Fig. 1. Changes in the conductivity of the egg emulsion (EM, open squares) with time. The closed circle represents the conductivity of egg proteins in solution (ES). The inset represents the conductivity changes within the first 10 h.

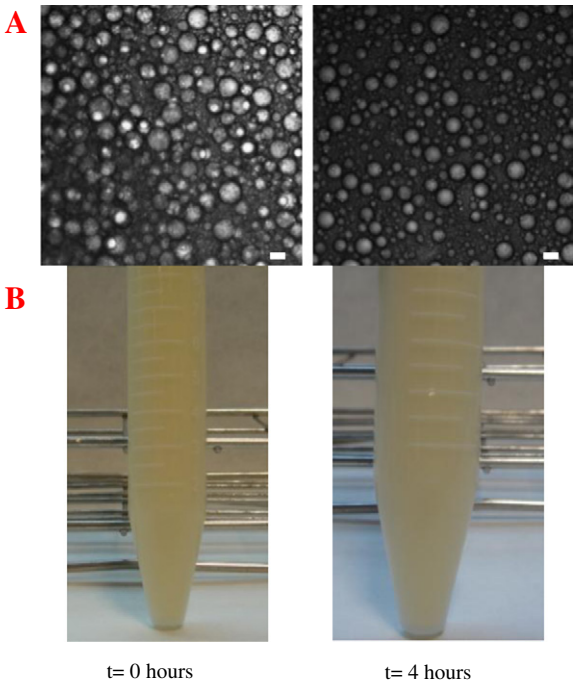

Fig. 2. Microstructure A) and visual appearance B) of the egg emulsion (EM) freshly made and after 4 h. The scale bar represents 20 μm.

from the optical microscope were typical of a homogeneous emulsion with small oil drops, surrounded by a well-defined interfacial film, dispersed in a continuous phase. During 4 h, there were no relevant changes in the microstructure of the EM, which showed a low tendency to flocculate (Camino & Pilosof, 2011).

3.2. In vitro digestibility of the EM

SDS-PAGE and RP-HPLC analyses were conducted on ES, EM and their respective digests (Figs. 3 and 4 and Table 2) to compare their *in vitro* digestibility. Both ES and EM showed a similar *in vitro* digestion pattern by SDS-PAGE, although slight differences were found. Basically, the digestion of egg proteins was somewhat favored when they formed part of the emulsions. The most abundant allergen, the egg white, OVA, that was clearly seen in the gel as large band of

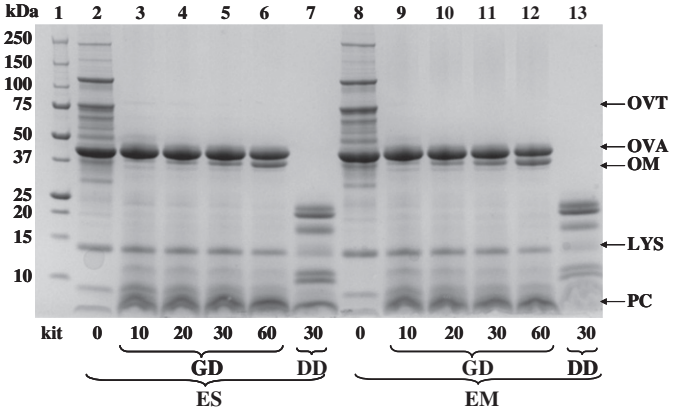

Fig. 3. SDS-PAGE analysis of egg proteins in solution (ES) and in emulsion (EM) and their gastric (GD) and duodenal digests (DD). Lane 2: ES; lanes 3–6: ES subjected to gastric digestion for 10, 20, 30 and 60 min; lane 7: ES subjected to gastric digestion for 60 min followed by duodenal digestion for 30 min; lane 8: EM; lanes 9–12: EM subjected to gastric digestion for 10, 20, 30 and 60 min; lane 13: EM subjected to gastric digestion for 60 min followed by duodenal digestion for 30 min Lane 1: kit of protein standards with different molecular masses. OVA: ovalbumin; OVT: ovotransferrin; OM: ovomucoid; LYS: lysozyme; PC: phosphatidylcholine.

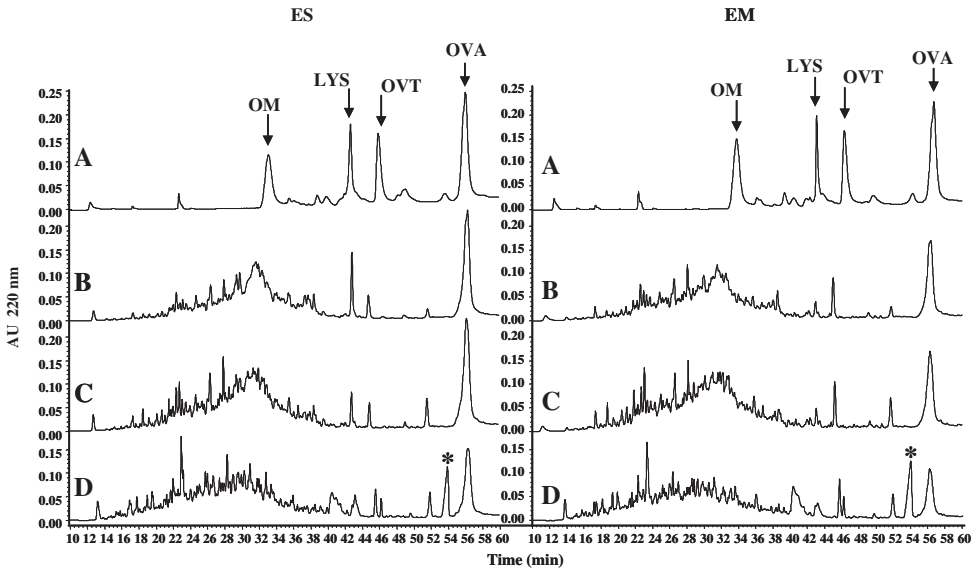

Fig. 4. RP-HPLC analysis of egg proteins in solution (ES) and in emulsion (EM) (A) and their gastric (GD) (B, C) and duodenal (DD) (D) digests. (B) Egg proteins subjected to gastric digestion for 20 min; (C) egg proteins subjected to gastric digestion for 60 min; (D) egg proteins subjected to gastric digestion for 60 min followed by duodenal digestion for 30 min; OVA: ovalbumin; OVT: ovotransferrin; OM: ovomucoid; LYS: lysozyme; bile salts are represented as *.

approximately 45 kDa, is known to be very stable to pepsin and its digestion leads to a SDS-PAGE pattern with two hydrolysis fragments of ~40 and <10 kDa (Takagi, Teshima, Okunuki, & Sawada, 2003). The band corresponding to OVA underwent a faster decrease in intensity with the advance of gastric digestion in EM than in ES (Fig. 3, lanes 3–6, 9–12). Accordingly, the appearance of the hydrolysis fragment of ~40 kDa was more rapid during the hydrolysis of EM, but the differences were not significant (Table 2). The RP-HPLC analyses (Fig. 4) corroborated this observation, showing that OVA, which elutes with a retention time of 56 min, was digested faster as part of the EM, an effect that was still noticeable at the end of duodenal digestion (Table 2). Similarly, LYS, also recognized by its high stability to gastric digestion (Jimenez-Saiz, Martos, Carrillo, Lopez-Fandino, & Molina, 2011), was more resistant to hydrolysis by pepsin in ES than in EM (Figs. 3 and 4). On the contrary, OM is very prone to pepsin digestion, disappearing in less than 10 min, with the formation of degradation products of ~18 kDa, ~13 kDa and <3 kDa (Jimenez-Saiz, Belloque, Molina, & Lopez-Fandino, 2011). Under our conditions, we could not detect differences in the hydrolysis of OM between ES and EM.

Due to its compact globular structure, OVA exhibits poor emulsifying properties, although the egg is rich in other surface-active compounds, such as the phospholipids of the egg-yolk, which further promote the interfacial adsorption of OVA (Mine, Kobayashi, Chiba, & Tada, 1992). Similarly, LYS does not exhibit good emulsifying properties, which are improved by chemical and enzymatic modifications aimed to increase the amphipathicity of the protein (Shu, Sahara, Nakamura, & Kato, 1996). In order to confirm the presence of the egg white proteins on the interface, the emulsion was centrifuged and the cream, separated from the liquid subnatant, was analyzed by SDS-PAGE, as illustrated in Fig. 5, showing the presence of the main egg white and yolk proteins surrounding fat globules.

Table 2
Density analyses of the SDS-PAGE bands corresponding to OVA and its 40 kDa digestion fragment and HPLC peak areas corresponding to OVA following *in vitro* gastric (GD) and duodenal digestions (DD), in solution (ES) and in emulsion (EM), expressed as relative percentages. Different letters indicate significant differences ($P<0.05$) within each group.

Sample	SDS-PAGE				RP-HPLC	
	OVA		OVA fragment		OVA	
	ES	EM	ES	EM	ES	EM
Undigested	100^{a}	100^{a}	0^{a}	0^{a}	100^{a}	100^{a}
10 min GD	100^{a}	82.9^{b}	0^{a}	17.1^{b}	–	–
20 min GD	$81.1^{b,c}$	$76.6^{b,c}$	$18.6^{b,c}$	$23.4^{b,c}$	74.7^{b}	$60.5^{b,c,d}$
30 min GD	$76.5^{b,c}$	$70.6^{c,d}$	$23.5^{b,c}$	$29.4^{c,d}$	–	–
60 min GD	$71.5^{c,d}$	62.0^{d}	$28.5^{c,d}$	38.0^{d}	$74.2^{b,c}$	$57.7^{c,d}$
30 min DD	–	–	–	–	$65.7^{b,d}$	56.2^{d}

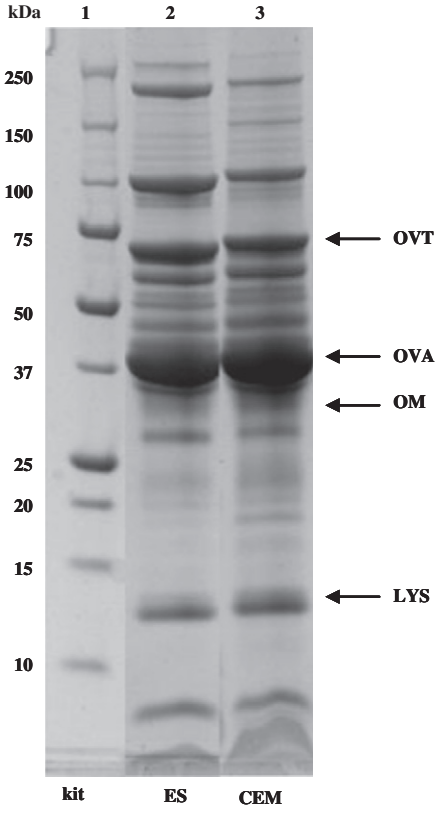

Fig. 5. SDS-PAGE analysis of egg proteins in solution (ES) and of the cream phase of the egg proteins emulsion (CEM). Lane 1: kit of protein standards with different molecular mass; lane 2: ES; lane 3: CEM.

Overall, based on the RP-HPLC and SDS-PAGE analyses, the *in vitro* digestibility of egg proteins in EM was only slightly favored compared to that of the egg proteins in ES. It should be noted that, in the case of other proteins, such as β-casein and β-Lg, the molecules adsorbed on oil/water interfaces are digested by pepsin at a much faster rate than the proteins in solution (Macierzanka et al., 2009). Furthermore, a significantly higher extent of hydrolysis has also been reported for unadsorbed milk whey proteins in emulsions, as compared with the proteins in solution (Nik, Wright, & Corredig, 2010; Sarkar et al., 2009). This is because the emulsifying activity of these proteins involves the unfolding and spreading of the molecules at the oil water/interface mainly through hydrophobic interactions and this leads to changes in the secondary and tertiary structures and to the exposure of buried residues with the subsequent augment of hydrolysis (Bergenstahl & Claesson, 1997; Beverung, Radke, & Blanch, 1999; Nilsson, Osmark, Fernandez, & Bergenstahl, 2007). In our study, the observation that egg white proteins, forming part of an emulsion system did not become a much more effective substrate for pepsin, indicates that there were no adsorption-induced changes that would considerably increase their flexibility and the accessibility of their enzyme cleavage sites. In fact, the egg proteins that are most resistant to pepsin action, OVA and LYS, exhibit a very high conformational stability at pH 2.0 (de Laureto, Frare, Gottardo, van Dael, & Fontana, 2002; Tatsumi, Yoshimatsu, & Hirose, 1999).

On the other hand, the simulated gastric digestion of proteins in emulsions in the presence of PC has been reported to occur mainly in solution, because PC causes displacement of the adsorbed proteins from the interfaces (Macierzanka et al., 2009). In our study, the inclusion of vesicular PC in the simulated gastric fluid at a final concentration of 6.64 mM to mimic physiological conditions could have caused the displacement of the egg white proteins adsorbed with other more surface active components of egg yolk, causing their digestion to take place mainly in solution and minimizing the differences in digestibility between EM and ES. Furthermore, it is known that PC itself affects the kinetics of pepsinolysis of certain proteins in solution such as α-lactalbumin and β-Lg (Moreno, Mackie, & Mills, 2005). However, the presence of PC does not affect the secondary or tertiary structure of OVA and LYS in solution and it does not change the susceptibility of OVA to pepsin action, while it slightly increases LYS resistance to digestion (Jimenez-Saiz, Martos, Carrillo, Lopez-Fandino, & Molina, 2011b; Martos et al., 2010).

In the duodenal phase of the *in vitro* digestion a destabilization of the pepsin-digested EM was visually observed (results not shown). Bile acids present in the duodenal medium easily displace proteins adsorbed at the interfaces forming mixed micelles with fatty acids, and phospholipids (Macierzanka et al., 2009; Sarkar, Horne, & Singh, 2010). Therefore, the hydrolysis would mainly occur in solution, where bile acids are found to promote digestion of several dietary proteins, as it is the case of OVA or myoglobin, an effect that can be enhanced by the presence of PC (Gass, Vora, Hofmann, Gray, & Khosla, 2007; Martos et al., 2010).

3.3. Human IgE-binding of the gastroduodenal digests

Human IgE-binding of the ES and EM gastric (60 min) and duodenal digests (30 min) was estimated by inhibition ELISA as illustrated in Fig. 6. The presence of egg proteins as part of the emulsion did not change their IgE binding, with both EM and ES showing similar IC_{50}. The progress of digestion led to the breakdown of allergenic determinants as reflected by the higher IC_{50} of the hydrolysates. The fact that egg protein digestibility was slightly increased in the EM was also reflected in the IgE-binding, leading to a lower IgE-binding ability in the EM digests as compared to the ES digests, although the results varied depending on the serum pool used, as a result of different sensitivities among individuals.

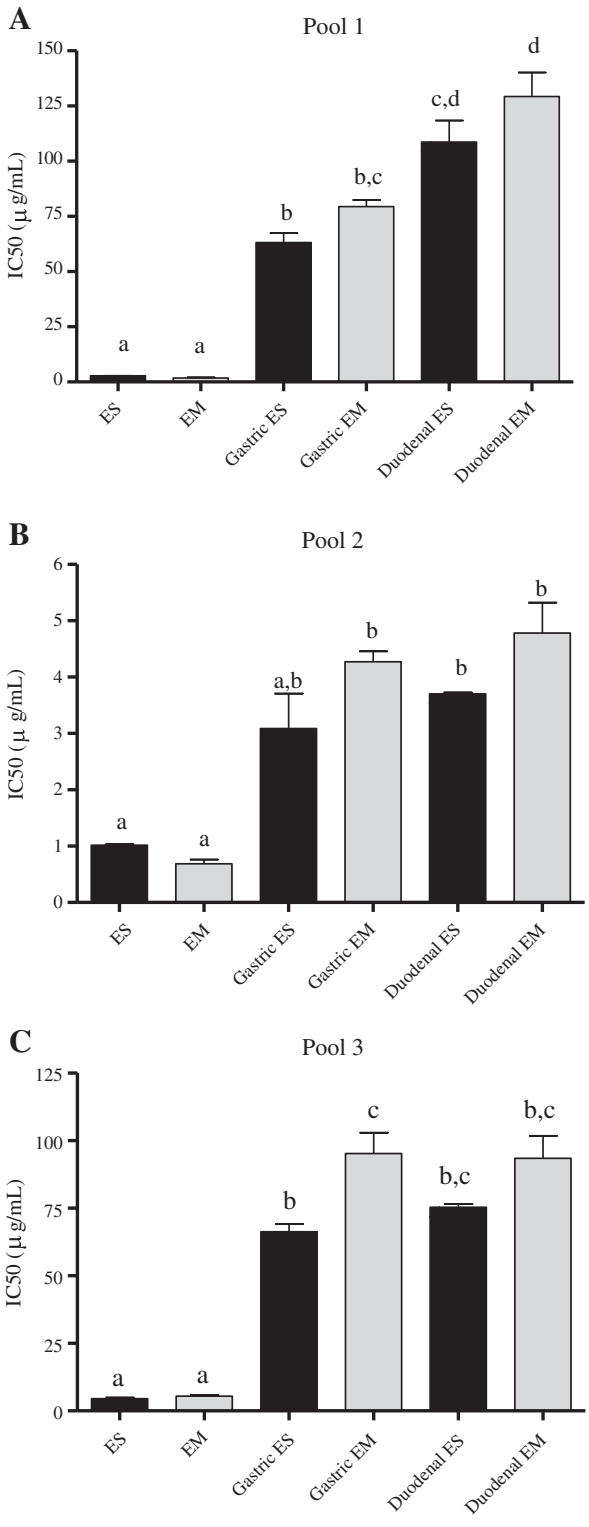

Fig. 6. Human IgE-binding by inhibition ELISA of egg proteins in solution (ES), in emulsion (EM,) and their respective gastric (60 min) and duodenal (30 min) digests. The IgE levels of the individual sera used in the pools are shown in Table 1.

As far as we know, there are no previous reports on the reactivity against IgE of emulsified proteins or their digestion products. The present results suggest that the incorporation of whole egg into an emulsion may not vary substantially the potential of egg allergens to induce adverse reactions in sensitized individuals, except for a reduced immnunoreactivity that could derive from a slightly increased susceptibility to digestion.

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