



Immunoreactivity of hen egg allergens: Influence on *in vitro* gastrointestinal digestion of the presence of other egg white proteins and of egg yolk

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ABSTRACT

Hen egg white comprises of a complex mixture of proteins, which greatly differ in their physicochemical characteristics and relative abundance. We aimed to identify potential undiscovered egg allergens within the egg white proteome and investigated the existence of matrix effects on the proteolytic stability and resultant IgE-binding of the allergenic proteins. In addition to the main egg allergens: ovalbumin (OVA), ovomucoid (OM) and lysozyme (LYS), two minor egg white proteins, tentatively identified as ovoinhibitor and clusterin, were found to react with serum IgE from egg-allergic patients. Egg white exhibited residual immunoreactivity after gastrointestinal digestion due to the presence of intact OVA and LYS, as well as of several IgE-binding peptides derived from OVA. The presence of egg yolk slightly increased the susceptibility to hydrolysis of egg white proteins and abrogated bile salt-induced precipitation of LYS in the duodenal medium. However, the resultant immunoreactivity against IgE of egg white proteins after *in vitro* digestion was not significantly modified by the presence of yolk components.

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1. Introduction

Hen eggs are an important and inexpensive supply of high quality proteins in the human diet, but also the second most frequent source of allergic reactions, particularly in children (Rona et al., 2007). The main allergens: ovomucoid (OM), ovalbumin (OVA), ovotransferrin (OVT) and lysozyme (LYS), named Gal d 1–4, respectively, are contained in the egg white, while two yolk-specific allergens have been reported so far: α -livetin (Gal d 5) (Schade & Chacana, 2007) and protein YGP42 (Gal d 6) (Amo, Rodríguez-Pérez, Blanco, Villota, & Juste, 2010). Recent works have considerably widened our knowledge of the egg white proteome, characterising several minor proteins (Guerin-Dubiard, Pasco, Molle, Desert, & Nau, 2006; Mann & Mann, 2011; D'Alessandro, Righetti, Fasoli, & Zolla, 2010; D'Ambrosio et al., 2008), but their potential contribution to egg allergy has not been explored.

In order to elicit an IgE response, an allergen must be able to cross the intestinal barrier, retaining sufficient structural integrity to interact with the immune system. Therefore, a major characteristic of many food allergens is their resistance to gastric digestion (Moreno, 2007). However, several food allergens have been identified that are sensitive to these processes; while many non-allergenic proteins are as resistant to degradation as allergenic proteins (Fu, 2002). These observations underline the importance of taking into account the complex factors that play a role in physiological diges-

tion and of evaluating the ability of the fragments generated upon digestion to retain biologically relevant IgE epitopes.

An aspect of food allergens that remains to be elucidated is the influence of the food matrix on the immune responses (Nowak-Węgrzyn & Fiocchi, 2009). The food matrix has been suggested to affect the allergenic properties of proteins by providing adjuvant stimuli to the specialised gut mucosal immune system or by protecting them from digestion (Teuber, 2002). In fact, the stability of proteins to digestion may be altered in the presence of various components that form part of the food matrix, such as soluble polysaccharides (Polovic et al., 2007), lipids (Macierzanka, Sancho, Mills, Rigby & Mackie, 2009) or protease inhibitors (Maleki et al., 2003). We have previously assessed the immunoreactivity of the major egg allergens, OVA, OM and LYS before and after simulated digestion (Jiménez-Saiz, Belloque, Molina, & López-Fandiño, 2011a; Martos, Contreras, Molina, & López-Fandiño, 2010; Jiménez-Saiz, Martos, Carrillo, López-Fandiño, & Molina, 2011b). However, the evaluation of basic matrix effects on the stability of these allergens might contribute to a better understanding of the allergenic properties of proteins that are not completely resistant to digestion as well as those of the stable proteins.

In this paper we studied the IgE reactivity of egg white proteins with the double objective to find potential undiscovered allergens among the minor proteins and to evaluate some general matrix effects on their proteolytic stability and resultant IgE-binding. Susceptibility to *in vitro* digestion assays was examined in the context of mixtures of compounds (whole egg white or egg white and yolk) and compared with previous results on isolated proteins. We

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also assessed the immunoreactivity of egg white proteins after *in vitro* digestion with a view to find resistant peptides that preserved IgE epitopes.

2. Materials and methods

2.1. Reagents and materials

All reagents and materials were purchased from Bio-Rad (Richmond, CA, USA) unless otherwise specified. For *in vitro* digestion, chemicals and enzymes were purchased from Sigma–Aldrich (St. Louis, MO, USA) unless otherwise specified. Eggs came from organic-crop fed poultry and were purchased from a local supermarket.

Different serum pools from children with clinical allergic symptoms to egg were used at a dilution 1:200 in pH 7.6 Tris buffered saline (TBS) containing 0.05% Tween 20 (TBST). The sera were collected from the Maternal and Child Gregorio Marañón Hospital (Madrid, Spain). The patients' specific serum IgE levels were determined by CAP (GE Healthcare).

2.2. *In vitro* gastric and duodenal digestion

Egg whites and yolks were either separated or mixed with a magnetic stirrer at the same weight ratio found in the egg and their protein concentration was measured by Bradford method (Bradford 1976). An *in vitro* digestion model that takes into account the physiological concentrations of enzymes and surfactants present in the stomach and intestine was used (Moreno, Mackie, & Mills, 2005). Solutions in simulated gastric fluid (SGF, 35 mM NaCl at pH 2) were prepared at a concentration of 6 mg protein/ml. The corresponding substrates in SGF were preheated for 15 min at 37 °C before incubation with porcine pepsin (EC 3.4.23.1, 3440 units/mg) at an enzyme:protein ratio of 1:20, w/w (172 units/mg). The reactions were stopped after 60 min by raising the pH to 7.0 with NaHCO₃.

Duodenal digestions were performed by using, as the starting substrate, the 60 min gastric digests adjusted to pH 7 with the addition of: 1 M CaCl₂, 0.25 M Bis-Tris pH 6.5 and a 0.125 M bile salts mixture containing equimolar quantities of sodium taurocholate and glycodeoxycholic acid. After preheating at 37 °C for 15 min, aqueous solutions of trypsin (EC 232–650–8, 10100 BAEE units/mg protein), α -chymotrypsin (EC 232–671–2; 55 units/mg protein), porcine pancreatic lipase (EC 232–619–9) and colipase (EC 259–490–1) were added to the duodenal mix. The final composition of the mixture was: 6.15 mM of each bile salt, 20.3 mM Bis-Tris, 7.6 mM CaCl₂; and the enzymes referred to the quantity of protein were: 40 units/mg trypsin, 0.5 units/mg α -chymotrypsin, 28.9 units/mg lipase and colipase (enzyme:protein ratio 1:895 w/w). The reactions were stopped after 30 min with trypsin-chymotrypsin inhibitor as per the manufacturer's instructions. The final protein concentration of all samples was adjusted to 4 mg/ml.

Aliquots of egg gastric and gastroduodenal digests were lyophilised for their analysis by 2DE, SDS–PAGE and Western blotting.

2.3. Two-dimensional electrophoresis (2DE)

To improve the relative amount of minor proteins, the egg white (1:1 in PBS) was first treated with the ProteoMiner™ enrichment kit as per the instructions of the manufacturer. Excess of salts and other interferents were removed from the eluates using the ReadyPrep™ 2-D Cleanup kit. Afterwards, 140 μ l of the eluate were mixed with 60 μ l of rehydration buffer (thiourea was added to the buffer to make it 2 M). In the case of lyophilised gastroduodenal digests, enrichment and cleaning steps were omitted. The

powder (62 mg protein) was resuspended in 3 ml of water and 10 μ l volumes were diluted 1/20 in rehydration buffer.

Two hundred micro-litres of samples were loaded on an immobilised pH gradient (IPG) strip pH 4–7 and a rehydration step was carried out in a Protean® IEF Cell at 50 V overnight. Isoelectric focusing was performed as follows: a lineal ramp to 250 V in 15 min, then up to 8000 V in 2 h 30 min; this voltage was maintained until accumulating 20000 Vh and then a rapid ramp down to 500 V, before the voltage was held until reaching 30000 Vh. The total separation time was about 5 h 30 min. The IPG strip was reduced and alkylated for 10 and 15 min with equilibration buffers I and II (Bio-Rad) containing DTT and iodoacetamide, respectively. Then it was washed in XT-MES running buffer and placed in the slot for IPG strip of a Bis-Tris 12% polyacrylamide gel. Agarose was used to link both dimensions. The second dimension was performed at 150 V in a Criterion™ cell using the XT-MES running buffer. SYPRO® Ruby fluorescent dye was used following the manufacturer's instructions for protein detection in the undigested egg white sample. Egg white gastroduodenal digest sample was detected by silver staining following Mortz, Krogh, Vorum, and Gorg (2001).

2.4. SDS–PAGE

Samples were diluted 1:4 (final protein concentration 1 mg/ml) 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. Then, they were heated for 5 min at 95 °C and 40 μ l were loaded on a 12% Bis-Tris polyacrylamide gel (Criterion™ XT). Electrophoretic separation was carried out at 150 V in a criterion cell using a XT-MES running buffer. Gels were stained with Coomassie Blue G-250.

2.5. Western blotting

Mono- and bidimensional unstained gels were soaked in transfer buffer (48 mM Tris, 39 mM glycine, 20% methanol, pH 9.2) for 20 min. Proteins were electroblotted into a nitrocellulose membrane using a Trans-Blot® SD apparatus for 30 min at 18 V.

For detection with commercial polyclonal antibodies, the nitrocellulose membranes were incubated in TBST containing 1% bovine serum albumin (TBST–1% BSA) overnight at 4 °C. Next day, OVA antibody (GeneTex, Irvine, CA, USA), rabbit anti chicken OM antibody (Immune Systems Ltd., Devon, United Kingdom) and LYS antibody (Abcam, Cambridge, UK), 1/30000, 1/10000 and 1/20000 diluted in TBST–0.1% BSA, respectively, were incubated with the membrane for 1 h at room temperature. All incubations were followed by 5 washing steps with TBST of 5 min each. Finally, the membrane was developed with the chemiluminescent substrate (Novagen, Darmstadt, Germany). Image acquisition (exposure time 10 min) was performed using the VersaDoc Imaging System.

For detection with human sera, the membranes were incubated in TBST–1% BSA for 3 h and dipped into a pool of sera from children with clinical allergic symptoms to egg at (1/40 diluted in TBST–0.1% BSA) for 24 h at 4 °C. Next, the membrane was incubated with biotin-conjugated anti-human-IgE antibody (Southern Biotech, Birmingham, AL, USA) 1/500 diluted in TBST–0.1% BSA, overnight at 4 °C. Finally, Horseradish peroxidase (HRP)–Streptavidin (DakoCytomation, Glostrup, Denmark) diluted 1:3000 in TBST–0.1% BSA was added and incubated for 1 h. All incubations were followed by 5 washing steps with TBST for 5 min each. The chemiluminescent substrate Amersham™ ECL™ Prime (GE Healthcare, Uppsala, Sweden) was used and light emission detected for 5 min in the VersaDoc™ imaging system.

2.6. Mass spectrometry analyses of peptide bands and spots

Bands and spots of interest were manually excised from the corresponding gels using biopsy punches. Bands and spots selected for analysis were digested with trypsin according to Shevchenko, Tomas, Havlis, Olsen, and Mann (2006). Peptide bands from the monodimensional gel were in-gel reduced and alkylated prior to trypsin digestion. After digestion, the supernatant was collected and spotted onto a MALDI target plate with α -cyano-4-hydroxycinnamic acid matrix for protein identification by peptide mass fingerprint. Analyses were performed in a 4700 Proteomics MALDI-TOF/TOF Analyzer (Perseptive Biosystems, Framingham, MA). SwissProt DB v. 57.15 with taxonomy restriction to Metazoa (Animals) was searched using MASCOT 2.2 (matrixscience.com) through the Global Protein Server v3.6 from ABSciex. Search parameters were carbamidomethyl cysteine as fixed modification and oxidised methionine as variable modification; peptide mass tolerance 50 ppm and allowance for 1 missed trypsin cleavage site. Probability scores were greater than the score fixed by MASCOT as significant with P -value < 0.05.

2.7. RP-HPLC

Samples were diluted to a protein concentration of 2 mg/ml, filtered through a 0.2 μ m pore size and then separated in a Hi-Pore RP-318 (250 $\times$ 4.6 mm internal diameter) column (Bio-Rad) in a Waters 600 HPLC (Waters Corporation, Milford, MA, USA) equipped with a 717 plus autosampler and UV detector. The digests were eluted by using 0.037% (v/v) trifluoroacetic acid in double-distilled water as solvent A and 0.027% (v/v) trifluoroacetic acid in acetonitrile as solvent B, at 1 ml/min, and detection was at 220 nm. The chromatographic method was that described by Quirós, Chicón, Recio, and López-Fandiño (2007). Data were processed by using Empower 2 Software (Waters Corporation).

2.8. Inhibition ELISA

Immunoreactivity of egg white and yolk digests was quantified by inhibition ELISA as follows. Single wells of polystyrene microtiter plates were coated with undigested egg white and yolk samples at a protein concentration of 50 μ g/ml in PBS, and incubated overnight at 4 °C. Plates were washed with PBST after each subsequent incubation steps. Blocking was performed with PBS containing 2.5% Tween 20 for 3 h at room temperature. Then, serial dilutions of each sample (not less than seven) were incubated for 2 h with serum pools (1:1, v:v), and 50 μ l volumes were added to each well. After a further 2 h of incubation, 50 μ l of polyclonal rabbit anti-human IgE (Dako Denmark A/S, Glostrup, Denmark) 1:1000 diluted in PBST, were added per well and incubated for 1 h. Next, 50 μ l of polyclonal swine anti-rabbit immunoglobulins/HRP (Dako Denmark A/S) were added and incubated for another hour. The tyramide-biotin and streptavidin-HRP amplification system was used following the instructions of the manufacturer (ELAST ELISA amplification system, Perkin-Elmer Life Sciences, Waltham, MA, USA). Finally, tetramethyl benzidine was used as a substrate. The reaction was stopped with 0.5 M sulphuric acid. Absorbance was measured at 450 nm. A negative control without serum (undigested egg white and yolk sample at 1 mg/ml in PBST) and positive controls (serum pools diluted in PBS) were included in each plate.

A nonlinear adjustment of the data obtained for each dilution was applied for each serum and sample. The adjustment model was a sigmoidal curve of inhibition dose–response with a variable slope, from which the IC₅₀ (the concentration that binds 50% of seric IgE) was obtained with the program GraphPad PRISM 4 (www.graphpad.com).

3. Results and discussion

3.1. Immunoreactivity against IgE of egg white proteins

With the aim to identify potential allergens among the minor proteins present in egg white, the samples subjected to an enrichment protocol, as explained in the Materials and Methods section, to increase the relative amount of minor proteins before the separation by 2DE. Fig. 1A shows the tentative identification of the proteins in the resultant 2DE gel, following Guerin-Dubiard et al. (2006). The most abundant proteins, such as OVA, OM and LYS appeared as well defined spots corresponding to their different isoforms. However, OVT was poorly detected in the 2DE gel, probably as a result of the enrichment process, based on bead-coupled peptide libraries, which would reduce the most abundant proteins rather than equalising the concentration of minor and major components (Keidel, Ribitsch, & Lottspeich, 2010).

Egg white proteins in the gel were electroblotted onto a nitrocellulose membrane and incubated with a pool of sera from egg-allergic subjects (Fig. 1B). The proteins that presented the highest binding to IgE were OVA, OVA-Y and LYS isoforms. OVA, a glycoprotein with a molecular mass of 45 kDa that exists in three levels of phosphorylation and constitutes the most abundant protein in egg white (54%), is regarded as a major allergen (Mine & Yang, 2008); while OVA-Y, with a molecular mass of about 53 kDa (Nau, Pasco, Désert, Mollé, & Guérin-Dubiard, 2005), presents 57% identity with OVA and bears OM-like carbohydrate chains (Hirose, Doi, Kitabatake, & Narita, 2006). The immunoreactivity of LYS, which is also regarded as an important egg allergen (Mine & Yang, 2008), against IgE was outstanding. Guerin-Dubiard et al. (2006) reported the 2D separation of LYS spots corresponding to monomer and dimer forms of the protein. We also found a third spot, which also presented a high IgE binding, with the same apparent isoelectric point, but with a molecular mass that could correspond to the trimer. Finally, OM, which is considered the

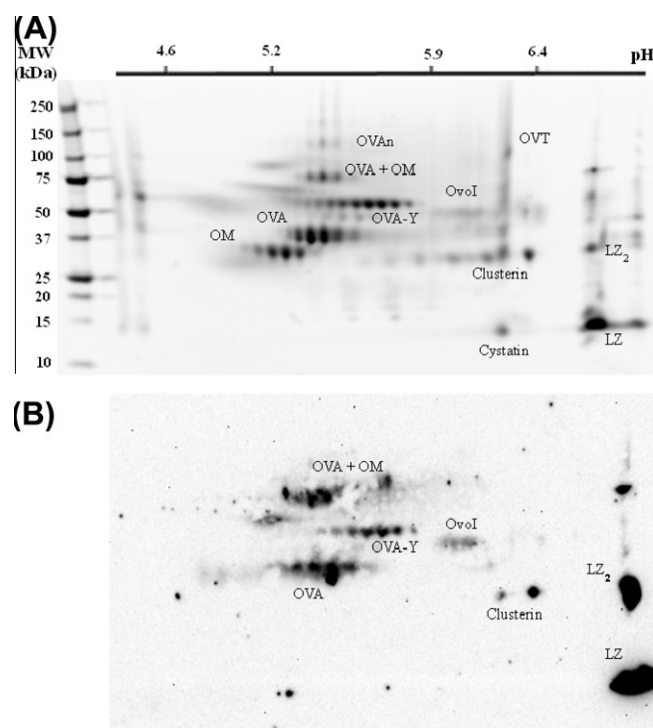


Fig. 1. 2DE of egg white proteins with Coomassie staining (A) and Western blotting of egg white proteins using a serum pool of egg allergic patients (mean specific IgE level to egg white: 13.2 kU/L) (B).

immunodominant egg protein (Mine & Yang, 2008), is also probably bound IgE, but OM isoforms, which appeared as diffuse bands with a higher apparent molecular mass than its real mass as a result of its high degree of glycosylation, were probably masked by OVA spots.

Two minor proteins tentatively identified as ovoinhibitor (Ovol) and clusterin, following Guerin-Dubiard et al. (2006), also reacted against the pool of human sera. Ovol accounts for 1.5% of total egg white protein and presents 22.5% of sequence identity with OM. Cross-reactivity between these proteins was demonstrated using a rabbit anti-OM antibody (Matsuda, Watanabe, & Nakamura, 1983), which suggests that antigenic determinants may be located in homologous regions. Indeed, several OM IgE epitopes (Mine & Zhang, 2002) are found within regions that present sequence homology with Ovol kazal-like domains 1, 2 and 7, and one study has shown that Ovol strongly reacts with serum from hen egg allergic patients in immunodot assays (Peng, 2002). Clusterin is a molecular chaperone, which is a protein that stabilises partially folded proteins to prevent their aggregation and precipitation (Mann et al., 2003). Hitherto, its IgE reactivity has not been reported.

3.2. Residual immunoreactivity of the main egg allergens following *in vitro* gastroduodenal digestion of egg white

The susceptibility of egg white proteins to *in vitro* gastroduodenal digestion and their residual immunoreactivity were investigated by SDS–PAGE/2DE and Western blotting (Figs. 2 and 3). As part of egg white, intact OVA and LYS remained after the duodenal phase, with the appearance, at this stage, of a number of resistant fragments within a wide range of molecular masses (Fig. 2A, lanes 1–3). Western blotting with polyclonal OVA (Fig. 2B) and OM (Fig. 2C)-specific antibodies allowed the detection of their degradation products. It should be mentioned that, as compared to the hydrolysis of isolated OVA (Martos et al., 2010), a higher content of intact OVA was found following duodenal hydrolysis of egg white, which could be attributed to the presence of OM, an important trypsin inhibitor that partially retains its inhibitory activity

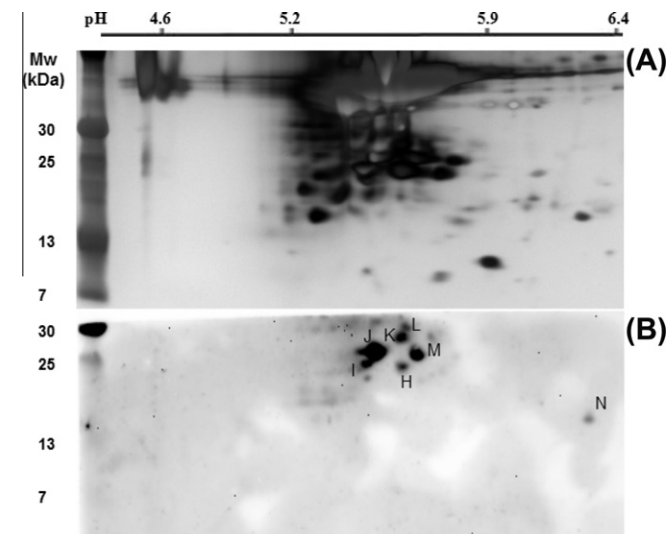


Fig. 3. 2DE of egg white gastroduodenal digest with Coomassie staining (A) and Western blotting of fragments lower than 30 kDa from egg white gastroduodenal digest using a serum pool of egg-allergic patients (mean specific IgE level to egg white: 70.5 kU/l) (B). MW: molecular weight marker. IgE-binding spots are indicated by letters H–N.

after pepsin digestion (Kovacs-Nolan, Zhang, Hayakawa, & Mine, 2000; Takagi et al., 2005). Furthermore, Western blotting evidenced polyclonal OM-specific antibody binding to intact OM in the gastric and duodenal digests (Fig. 2C), indicating that this generally pepsin-labile protein could also be protected from the enzymatic action in the egg white matrix. In fact, OM disappears after only 10 min of gastric digestion when the protein is tested alone (Jiménez-Saiz et al., 2011a; Kovacs-Nolan et al., 2000).

The IgE-binding capacity of the egg white gastroduodenal digests is shown in the immunoblot of Fig. 2D. The remaining OVA and the fragments named a–f strongly reacted against serum IgE, demonstrating the important residual immunoreactivity of egg white allergens after digestion. These peptides were cut off from

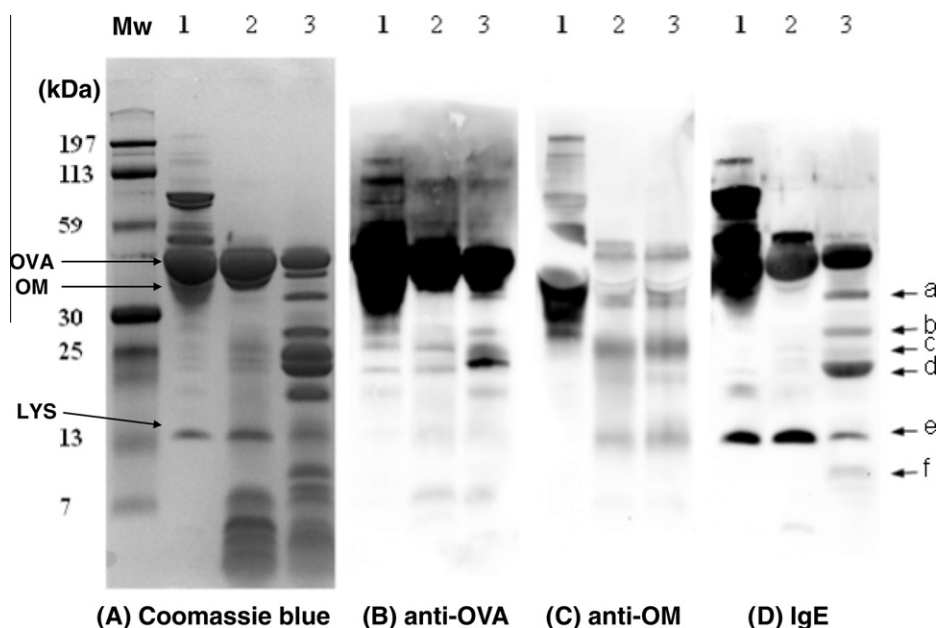


Fig. 2. SDS–PAGE with Coomassie staining (A) and Western blotting (B, C and D) of egg white proteins: egg white (1), gastric digest (2) and gastroduodenal digest (3). MW: molecular weight marker. Membranes were probed against ovalbumin antibody (B, anti-OVA), ovomucoid antibody (C, anti-OM) antibodies, and a serum pool (D, IgE) of egg-allergic patients (mean specific IgE level to egg white: 70.5 kU/L). IgE-binding fragments are indicated by letters a–f.

Table 1Protein identification of immunoreactive bands and spots by peptide mass fingerprinting. All scores were significant according to Mascot Search Engine ($p < 0.05$).

Bands/spots	Identification	Coverage (%)	Peptides matched	Score
a	Ovalbumin	24	6	63
b	Ovalbumin	34	11	99
c	Ovalbumin	39	13	85
d	Ovalbumin	32	10	72
e	Lysozyme C	63	12	122
f	Ovalbumin	20	7	212
	Lysozyme C	13	2	81
H	Ovalbumin	33	16	631
I	Ovalbumin	35	12	85
J	Ovalbumin	45	17	127
K	Ovalbumin	35	14	85
L	Ovalbumin	29	11	253
M	Ovalbumin	35	12	83
N	Chymotrypsin bovine	15	4	143

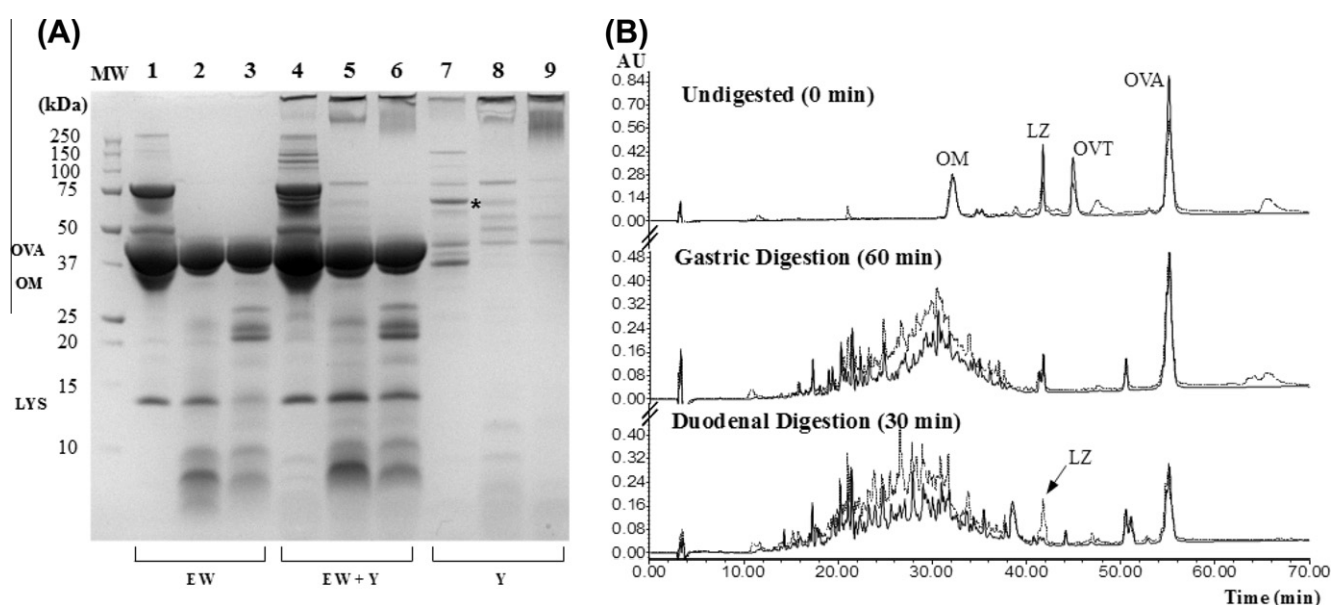


Fig. 4. (A) SDS-PAGE of undigested (1, 4, 7), gastric digests (2, 5, 8) and gastroduodenal digests (3, 6, 9) of egg white (EW, lanes 1–3), the mixture of egg white and yolk (EW + Y, lanes 4–6) and egg yolk (Y, lanes 7–9). MW: molecular weight marker. * indicates α -livetins. (B) RP-HPLC chromatograms of gastric and duodenal digests of egg white (solid line) and a mixture of egg white and yolk (dotted line).

the gel and subjected to tryptic digestion and MS analyses. As shown in Table 1, almost all IgE-reactive bands were found to derive from OVA. Intact LYS and a LYS-derived peptide produced after digestion (bands e and f) were also identified as responsible for the IgE-binding. To our surprise, no OM-derived peptides were identified in the IgE-reactive bands. Other authors (Lee & Kim, 2010) have also failed to identify OM by MALDI-TOF mass spectrometry, ascribing this difficulty to the protein stability against denaturation and trypsinization. However, it cannot be discarded that bands a, c, d and e, that reacted against the OM-specific antibody (Fig. 2C, lane 3), would also contain IgE-reactive OM-derived fragments.

2DE (Fig. 3A), followed by Western blotting (Fig. 3B) was also used to examine the IgE-reactive peptides present in the egg white gastroduodenal digest. As a means to avoid predominant IgE-binding to OVA, the nitrocellulose membrane was cut and only the transferred proteins below 30 kDa were probed against a pool of sera from egg-allergic patients. Several spots (highlighted in Fig. 3B), of around 25 kDa and within an apparent isoelectric point range of 5–6, produced the strongest binding signals. These spots were cut off from the 2D gel and trypsinized for MS identification

(Table 1, spots H–N). The most reactive fragments were found to derive from OVA in agreement with the fragments identified following SDS-PAGE (Table 1, bands a–d and f). Neither Ovot, nor clusterin, or IgE-reactive fragments deriving from these two proteins were detected in the immunoblot, which points at their degradation and loss of immunoreactivity during the enzymatic digestion.

3.3. Residual immunoreactivity following *in vitro* gastroduodenal digestion of egg white and yolk

We next assessed the effect of the egg yolk matrix on the susceptibility of egg allergens to digestion. Fig. 4A compares the *in vitro* hydrolysis of egg white, egg yolk, and their mixture in the proportion found in whole egg, regarding egg yolk (Fig. 4A, lanes 7–9), stacked proteins at the top of the gel probably corresponded to yolk granular proteins, such as the major complex lipovitellin-fosvitin (Wallace, 1978) and bands of molecular weight > 50 kDa to soluble fragments of the highly abundant LDL apoprotein ApoB, as deduced from their apparent molecular masses. In fact, Jolivet, Boulard, Beaumont, Chardot, and Anton

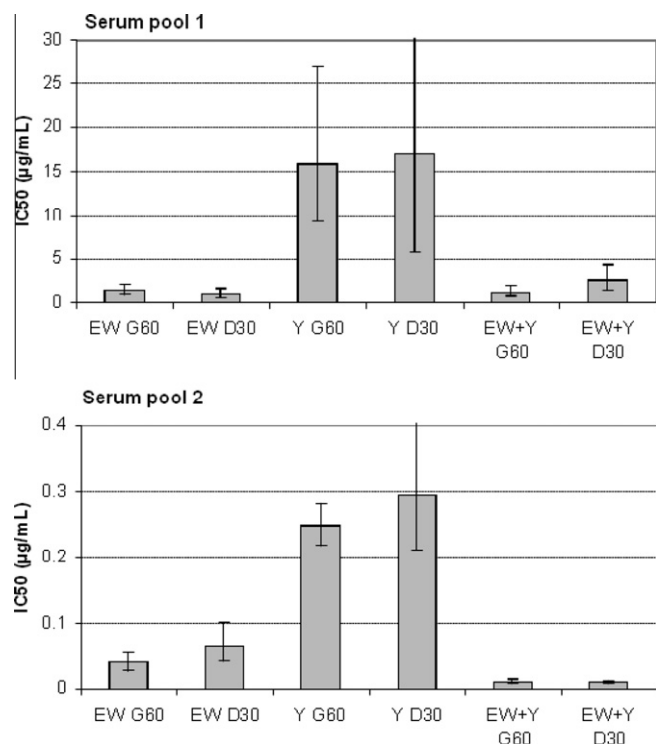


Fig. 5. Inhibition ELISA of gastric (G60) and gastroduodenal digests (D30) of egg white (EW), egg yolk (Y), and a mixture of both (EW + Y). The concentration that binds 50% (IC₅₀) of IgE from two different serum pools is represented. Mean specific IgE levels to egg white were 15.1 kU/L for pool 1 and 13.29 kU/L for pool 2 and IgE levels to egg yolk < 5kU/L. Bars indicate the standard error of the mean.

(2006) reported the existence of Apo B internal and C-terminal fragments of molecular mass 86, 64 and 55 kDa with a migration pattern similar to that shown in Fig. 4A lane 7. α -livetin (Gal d 5), a very abundant yolk plasma protein that is also considered an allergen (Quirce et al., 2000), is probably the prominent band of (approximately) 70 kDa, indicated with an asterisk in Fig. 4A. Pepsin partially cleaved it during gastric digestion and it became completely hydrolysed by duodenal enzymes, either in the absence or presence of egg white (Fig. 4A, lanes 4–6), suggesting that α -livetin is a digestion-labile allergen in whole egg.

In general terms, egg yolk did not exert a major influence on the digestion of egg white proteins, except for a slight increase in their susceptibility to hydrolysis in view of the differences observed in the digestion profiles: mainly, the higher intensity of the bands corresponding to degradation products in SDS–PAGE (Fig. 4A, lanes 2, 3 and 5, 6) or the higher number and concentration of peptides eluting between min 20 and 35 in RP–HPLC (Fig. 4B).

An increased amount of intact LYS after duodenal digestion in the presence of yolk was noteworthy (Fig. 4A, lanes 3 and 6 and 4B). It was reported that LYS precipitates under simulated duodenal conditions, probably because of its electrostatic interaction with bile salts (Jiménez-Saiz et al., 2011b) and thus, the present observation suggests that LYS precipitation due to bile salts could be prevented by yolk components. In fact, low density lipoproteins (that account for 66% of total yolk dry matter) are able to bind bile salts (Ceryak, Bouscarel, & Fromm, 1993), which would leave less surfactant molecules available to induce LYS aggregation. In addition, there is also a high concentration of phosphatidylcholine in egg yolk (approximately 1.7 mmol), which partially avoids LYS precipitation induced by bile salts (Jiménez-Saiz et al., 2011b). It could, therefore, be inferred that LYS in whole egg remains soluble under duodenal conditions and resists proteolysis to a large extent.

On the other hand, bile salts can promote the digestion of several dietary proteins, an effect that is modulated (either enhanced or diminished) by the presence of phosphatidylcholine or lipolysis products, such as fatty acids or monoglycerides, which compete for bile acid incorporation into the mixed micelles, changing the free bile acid concentration available for interaction (Gass, Vora, Hofman, Gray, & Khosla, 2007; Maldonado-Valderrama, Wilde, Macierzanka, & Mackie, 2011). In this respect, our results suggest that neither yolk phospholipids, lipoproteins or the lipids potentially released from them upon digestion, due to the presence of porcine pancreatic lipase and colipase in the duodenal mix, and would considerably affect the cleavage of egg white allergens by pancreatic enzymes.

Whether these differences could imply changes in immunoreactivity was addressed by comparing the IgE-binding of the gastric and duodenal digests of egg white, egg yolk and their mixture by inhibition ELISA using two different serum pools (3 subjects each) (Fig. 5). As expected, the digestion of egg yolk produced the least reactive hydrolysates. However, no significant differences were found between the egg white digests obtained in the absence or presence of egg yolk. Moreover, in both cases, gastric and duodenal digests had comparable IgE-binding capacities despite the higher extent of proteolysis of allergens, especially of OVA, in the latter (Fig. 4), which stressed that the fragments produced during duodenal digestion bore IgE epitopes (Figs. 2D and 3B).

4. Conclusion

Two minor proteins in egg white, clusterin and ovoinhibitor, were found to be able to bind IgE from egg-allergic patients, although their immunoreactivity was lost after simulated gastrointestinal digestion. The presence of egg yolk slightly increased the susceptibility to hydrolysis of egg white proteins and reduced bile salt-induced precipitation of LYS in the duodenal medium, what suggests that LYS, a highly immunoreactive protein, may remain soluble when whole egg is ingested resisting enzymatic proteolysis. However, the resultant immunoreactivity against IgE of egg white proteins after *in vitro* digestion was not significantly modified by the presence of yolk components.

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