

Sensitizing and Eliciting Capacity of Egg White Proteins in BALB/c Mice As Affected by Processing

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Supporting Information

ABSTRACT: This study assesses to what extent technological processes that lead to different degrees of denaturation of egg white proteins affect their allergenicity. We focused on heat (80 °C, 10 min) and high-pressure (400 MPa and 37 °C, 10 min) treatments and used a BALB/c mouse model of food allergy. Oral sensitization to egg white using cholera toxin as adjuvant induced the production of IgE and IgG1 isotypes and led to severe clinical signs following challenge with the allergen. Extensive protein denaturation caused by heat treatment increased its ability to induce Th1 responses and reduced both its sensitizing and eliciting capacity. Heated egg white stimulated the production of IgE over IgG1 antibodies directed, at least in part, toward new epitopes exposed as a result of heat treatment. Conversely, partial denaturation caused by high-pressure treatment increased the ability of egg white to stimulate Th2 responses and its allergenic potential.

KEYWORDS: egg allergy, heat treatment, high pressure, BALB/c, sensitization, anaphylaxis, IgE, IgG1

■ INTRODUCTION

Food allergy is a major health problem in Western countries, affecting, approximately, 5% of adults and 8% of children, and egg proteins are the second leading cause of allergy during infancy and early childhood.¹ Although it is a complex scenario, it is broadly accepted that the prevalence and severity of food allergies are increasing alarmingly and the economic and social impacts are growing. In addition, so far, there is no approved treatment for food allergy and sensitized individuals need to avoid ingestion of food to which they are allergic.²

Thermal processing techniques, commonly used during food production, have the potential to affect food allergens by inducing structural alterations, such as unfolding and aggregation. The effects of temperature on different food allergens have been extensively investigated, and there is a general agreement that it is very important to understand how heat treatments alter the structure of food proteins and the subsequent gastrointestinal digestibility, both of which influence their allergenicity.^{3–5} Other processing techniques, such as high hydrostatic pressure, can also lead to denaturation of proteins depending on the pressure level, temperature, and chemical conditions. Johnson et al.⁶ did not observe substantial changes in the secondary structure and aggregation state of a selection of purified food allergens after high-pressure application, although, depending on the protein, pressurization may induce sufficient structural modifications to affect susceptibility to digestion and immunoreactive properties, lowering or enhancing the binding to IgE and the capacity to trigger mast cell activation and produce clinical reactions.⁴

Physicochemical changes caused by heat treatment on egg proteins are often associated with a decrease in their allergenicity.⁷ Heating of egg proteins increases the digestibility of ovalbumin (OVA), the most abundant egg allergen, and lowers the binding of egg allergens to IgE from patient sera,^{8–10} although the reactivity of IgE from egg allergic patients toward

the native or heated forms varies depending not only on whether egg has been extensively or partially cooked, but also on their individual susceptibility.¹¹ However, whereas oral exposure to cooked eggs is likely to be the most frequent source of immunization, the sensitizing potential of heated proteins administered by the oral route has not been studied in depth. With regard to high-pressure treatments, Hildebrandt et al.¹² reported that the IgE binding of egg white allergens in meat products decreases with increasing pressure, from 400 to 700 MPa. It is also known that simultaneous enzymatic treatment and pressurization increase the susceptibility of OVA to hydrolysis by pepsin, trypsin, and chymotrypsin.¹³ Nevertheless, the information available does not allow drawing a clear picture of the effect of mild denaturation treatments on the allergenicity of egg and, in fact, there are no *in vivo* data on the allergenic potential of pressurized egg proteins.

The aim of this study was to investigate to what extent technological processes that lead to different degrees of denaturation of egg proteins affect their sensitization and elicitation ability in a mouse model. We have focused on moderate heat and high-pressure treatments applied, respectively, at 80 °C and 400 MPa for 10 min, which are usual operating conditions in food processing for which effects on the allergenic potential of egg white have not been previously addressed. Raw, heated, and pressurized egg white (EW, HEW, and PEW, respectively) were orally administered to BALB/c mice. Serum antibody levels were recorded, and the severity of anaphylaxis was evaluated following allergen challenge. Special attention was paid to the specificity of the generated antibodies and the systemic cytokine profiles.

Received: March 9, 2017

Revised: May 3, 2017

Accepted: May 3, 2017

Published: May 3, 2017

MATERIALS AND METHODS

Proteins, chemicals, and enzymes for *in vitro* digestion were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified. Antibodies were from BD Biosciences (San Diego, CA, USA). Eggs came from organic-crop fed poultry and were purchased from a local supermarket.

Egg White Proteins. Egg white (EW), separated from fresh eggs and commercial egg proteins—ovalbumin grade V (OVA), ovomucoid type III-O (OM), and lysozyme (LYS)—were used. Heated egg white (HEW) was obtained by heating at 80 °C for 10 min and pressurized egg white (PEW) by high hydrostatic pressure treatment (high-pressure equipment, Stansted ISO-LAB system, Essex, UK) at 400 MPa for 10 min at 37 °C. Samples were freeze-dried, analyzed for protein content by the Kjeldahl method (85.12, 84.70, and 84.23% in EW, HEW, and PEW, respectively), and structurally characterized by SDS-PAGE and circular dichroism (CD). The lipopolysaccharide content (analyzed by the Pierce LAL Chromogenic Endotoxin Quantitation Kit, Thermo Scientific, Waltham, MA, USA) was below 1 UE/mg in all proteins and egg white samples except for OVA, which was purified by size exclusion chromatography.¹⁴

Structural Analysis by SDS-PAGE and CD. Samples for SDS-PAGE were dissolved at 2 mg of protein/mL in sample buffer that contained 50 mM Tris-HCl (pH 6.8), 10% v/v glycerol, 2% w/v SDS, and 0.002% w/v bromophenol blue, in the absence or presence of 5% β -mercaptoethanol, and heated for 10 min at 95 °C. Electrophoretic separations were carried out at 120 V on Precast Criterion XT 12% Bis-Tris gels (Bio-Rad, Hercules, CA, USA) using XT MES as running buffer (Bio-Rad). Precision Plus Protein Unstained Standard was used as molecular weight marker. Gels were stained with Coomassie Blue, and images were taken with a Molecular Imager Versadoc MP 5000 system and processed using Quantity One 1-D analysis software (all from Bio-Rad).

CD was performed as previously described by Benedé et al.¹⁵ Far (200–250 nm) CD spectra of EW, HEW, and PEW, dissolved at 0.2 mg of protein/mL in 50 mM phosphate buffer, pH 7.0, were recorded at 20 °C using cells with 0.1 cm pathlengths in a Jasco J-810 spectropolarimeter (Jasco Corp., Tokyo, Japan). Spectra represent the average of three accumulations collected at 20 nm/min, with a 2 s time constant, a 0.2 nm resolution, and a sensitivity of 100 mdeg. Buffer blanks were subtracted from each CD spectrum, which were represented as mean specific ellipticity (deg·cm²·cg⁻¹). CDNN secondary structure analysis software (Applied Photophysics Ltd., Surrey, UK) was used.

In Vitro Gastroduodenal Digestion. *In vitro* digestions were carried out as previously described.¹⁶ Briefly, gastric digestions were performed at a protein concentration of 6.4 mg/mL in simulated gastric fluid (35 mM NaCl, pH 2.0) for 60 min at 37 °C, with 182 units/mg protein of porcine pepsin (EC 3.4.23.1, 4220 U/mg protein). Aliquots were withdrawn at 20 and 60 min and reactions stopped by raising the pH to 7.0–7.5 with NaHCO₃. For intestinal digestions, the gastric digests were mixed with 0.25 M Bis-Tris, pH 6.5, 1 M CaCl₂, and a 0.125 M bile salt mixture, containing equimolar quantities of sodium taurocholate and glycodeoxycholic acid. Pancreatic bovine trypsin (EC 232-650-8, type I), α -chymotrypsin (EC 232-671-2; type I-S), and pancreatic porcine lipase (EC 232-619-9; type VI-S) were added to the mixture at enzyme/protein ratios of 34.5, 0.4, and 24.8 units per mg of protein, respectively. Pancreatic porcine colipase (EC 259-490-1) was added at an enzyme:protein ratio of 1/895 (w/w). Duodenal digestions were carried out for 20 and 60 min at 37 °C and stopped by adding trypsin–chymotrypsin inhibitor, at a concentration calculated to inhibit twice the amount of trypsin and chymotrypsin present in the digestion mix. The digests were immediately mixed with SDS-PAGE sample buffer and heated as indicated above.

Animals. Six-week-old female BALB/c mice (Charles River Laboratories, Saint Germain sur l'Abresale, France) were kept under specific-pathogen-free conditions and fed an animal protein-free diet (SAFE, Route de Saint Bris, France) and water *ad libitum*. All protocols involving animals were approved by the CSIC Bioethics

Committee and the Comunidad de Madrid (ref PROEX 089/15) and followed the European legislation (Directive 2010/63/UE).

Sensitization and Challenge Protocols. Mice (distributed in eight groups of five animals) were orally administered the amount of EW (three groups), HEW (two groups), or PEW (two groups) equivalent to 5 mg of protein plus 10 μ g of CT (List Biologicals, Campbell, CA, USA), or just PBS (naïve group). Sensitization was performed during three consecutive days on the first week and once a week during the following 6 weeks. At week 8, mice were orally (50 mg of protein) and intraperitoneally (ip, 100 μ g of protein) challenged, 40 min apart. EW-sensitized mice were challenged with EW, HEW, and PEW; HEW-sensitized mice with EW and HEW; and PEW-sensitized mice with EW and PEW. Naïve mice were challenged just with PBS. Anaphylactic signs and body temperature drops were evaluated 30 min after the oral and ip challenges.¹⁷ All mice were euthanized at the end of the challenge protocol by CO₂ inhalation.

At different experimental points, blood samples were collected by cheek puncture and centrifuged at 2000g for 15 min. Serum levels of mouse mast cell protease-1 (MCP-1) were quantified post-mortem with a commercial ELISA kit (eBioscience, San Diego, CA, USA), as outlined by the manufacturer.

Passive Cutaneous Anaphylaxis (PCA). Following Li et al.,¹⁸ 20 μ L of pooled samples of sera collected post-mortem from five HEW-sensitized mice, either unheated or heat-inactivated (56 °C for 3 h), were intradermally inoculated in the right ear pinna of BALB/c naïve mice (two mice per treatment). Pooled serum from naïve mice was inoculated in the left ear pinna. Twenty-four hours later, mice were intravenously injected with 200 μ g on a protein basis of HEW in 100 μ L of 0.5% Evans blue dye (Sigma-Aldrich). Sixty minutes apart, mice were euthanized by CO₂ inhalation, and ears were individually collected, weighed, and incubated overnight at 55 °C in *N,N*-dimethylformamide (Sigma-Aldrich). The absorbance of the supernatant was measured at 655 nm, corrected with the absorbance of the supernatant from the ear injected with pooled naïve sera, and compared with a standard curve of serial dilutions of Evans blue to quantify dye extravasation per gram of mouse ear.

Indirect ELISA for the Detection of Specific IgE and IgG1

Indirect ELISA was performed by coating 96-well plates with 5 μ g/mL (for IgE) or 2 μ g/mL (for IgG1) of protein (OVA, OM, LYS, EW, HEW, and PEW).¹⁷ To build reference curves, plates were coated with rat anti-mouse IgE and IgG1 (BD Biosciences). Blocked plates were incubated overnight at 4 °C with serum samples (1/25 diluted for IgE and 1/1000 or 1/5000 diluted for IgG1). Serial dilutions of mouse IgE and IgG1 were used for the reference curves (BD Biosciences). Subsequently, plates were incubated with biotin rat anti-mouse IgE and IgG1 and streptavidin–HRP (BD Biosciences). Colorimetric reactions were read at 405 nm in a plate reader (Multiskan FC, Thermo Scientific) after the addition of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) as substrate (Roche, Mannheim, Germany).

Antibody-Capture ELISA for the Study of the IgE Binding Capacity

EW, HEW, and PEW were labeled with EZ-link sulphonyl-NHS-LC biotin (Pierce, Rockford, IL, USA) using a 5/1 molecular ratio of biotin/protein. Pooled sera from five mice sensitized to EW, HEW, or PEW (1/100 diluted) were added to plates coated with rat anti-mouse IgE (2 μ g/mL, clone MCA 419, Bio-Rad) and incubated overnight at 4 °C. For direct ELISA, 50 μ L of biotin-labeled antigen (500 ng/mL) was added and incubated for 3 h at room temperature. In the case of the competition assays, mixtures of 25 μ L of biotinylated antigen and 25 μ L of diluted nonlabeled antigen (from 1 ng/mL to 0.5 mg/mL) were added to the plates. After incubation with streptavidin–HRP (BD Biosciences), 3,3',5,5'-tetramethylbenzidine was added as substrate (eBioscience). The colorimetric reaction was stopped with H₂SO₄ and read at 450 nm in a plate reader (Multiskan FC, Thermo Scientific). Results were expressed as B/B_0 , where B_0 and B represent, respectively, the amount of labeled EW, HEW, or PEW bound to the immobilized IgE antibodies in the absence or presence of a known concentration of nonlabeled EW, HEW, or PEW.

Cytokines Released by Spleen Cells. Spleens from individual mice were collected and processed as previously described.¹⁹ Isolated

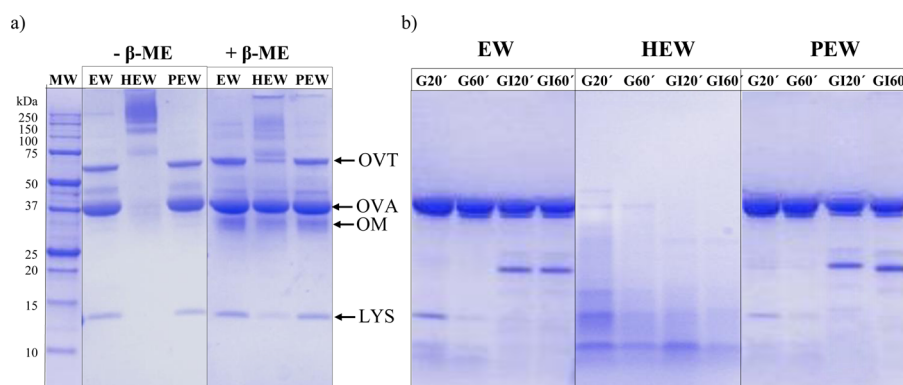


Figure 1. SDS-PAGE patterns of EW, HEW, and PEW, without and with β -mercaptoethanol ($-\beta$ -ME and $+\beta$ -ME, respectively) (a); in vitro gastric (G, 20 and 60 min) and gastrointestinal (GI, 20 and 60 min) digests of EW, HEW, and PEW with β -mercaptoethanol (b). MW, molecular weight marker; OVT, ovotransferrin; OVA, ovalbumin; OM, ovomucoid; LYS, lysozyme.

splenocytes were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 U/mL penicillin, and 50 μ g/mL streptomycin (all from Biowest SAS, Nuaillé, France) at a cellular density of 4×10^6 cells/mL in 48-well plates (nonstimulated control) and incubated with 200 μ g/mL (on a protein basis) of OVA, OM, LYS, EW, HEW, or PEW. Supernatants were collected after 72 h of culture in 5% CO₂ at 37 °C and stored at -80 °C until ELISA analyses (eBioscience) to quantify cytokine production (IFN- γ , IL-4, IL-5, and IL-10).

Statistical Analyses. Experimental results are expressed as means $\pm$ standard error of the mean (SEM). Statistical analyses were performed by one-way analysis of variance (ANOVA), followed by Tukey's test for comparing all groups. Clinical scores are expressed as medians and, in this case, significant differences were determined using the unpaired nonparametric Mann–Whitney test. The t test was used to test differences between unheated and heat-inactivated serum values in the PCA test. The statistical software package GraphPad Prism version 6.0 (GraphPad Software, Inc., La Jolla, CA, USA) was used for the analyses. $P < 0.05$ was considered statistically significant.

RESULTS

Structural Changes and Digestibility of Egg White Proteins as a Result of Heat and High-Pressure Treatments. SDS-PAGE analysis under nonreducing conditions revealed similar patterns for EW and PEW, although HEW showed a completely different profile characterized by the presence of high molecular mass aggregates and the absence of bands corresponding to the main egg white proteins (Figure 1a). At least some of these aggregates were reduced following the addition of β -mercaptoethanol, with the appearance of bands corresponding to ovotransferrin (OVT), OVA, OM, and LYS, which indicates that they were stabilized by disulfide bonds induced by exchanges between free sulfhydryl and disulfide groups of these proteins, whereas other aggregates, possibly stabilized by nonreducible covalent bonds, remained undissociated (Figure 1a).

To unveil further structural differences after processing, far-UV CD spectra were collected and compared (Figure 2). The spectra of HEW confirmed secondary structure changes as a result of heat treatment. CD also showed that PEW was structurally similar to EW with respect to the secondary structure content.

Following simulated gastrointestinal digestion, there was an incomplete degradation of EW and PEW proteins (Figure 1b). In particular, OVA partially resisted successive gastric and duodenal in vitro hydrolyses. However, HEW was more susceptible to proteolysis, and neither aggregates nor individual

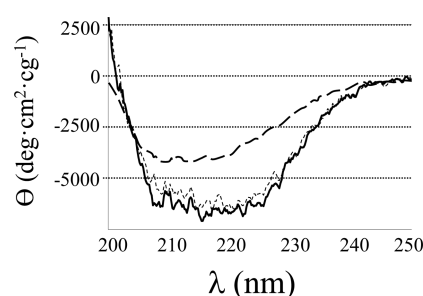


Figure 2. Far-UV circular dichroism spectra of EW (solid line), HEW (long-dash line), and PEW (short-dash line) at pH 7.0 and 20 °C.

native proteins were detected in the SDS-PAGE gels at the end of the digestion process.

Sensitizing and Eliciting Potential of Processed Egg White Proteins. Serum concentrations of IgE and IgG1 specific to EW and to the individual proteins, OVA, OM, and LYS, were determined during the sensitization period by indirect ELISA (Supplementary Figure 1). Table 1, which compares the antibody levels at the eighth week just before challenge, indicates that HEW-sensitized mice presented a higher amount of EW-specific IgE than mice sensitized to EW. In particular, the OVA-specific IgE response was greater in HEW-sensitized than in EW-sensitized mice, although the LYS-specific IgE response was lower. In addition, HEW-specific IgE was also detected in serum from HEW-sensitized mice at a higher concentration than that of EW-specific IgE. Although the difference did not reach statistical significance, this suggests the formation of IgE antibodies directed toward different antigenic determinants exposed by heat treatment. There were no significant differences in the levels of EW-, OVA-, and OM-specific IgE between EW- and PEW-sensitized mice, although LYS-specific IgE was the highest in mice sensitized to PEW. Sensitization to PEW generated comparable amounts of EW- and PEW-specific IgE (Table 1).

Sensitization to HEW did not lead to a significant EW-specific IgG1 response as compared with naïve mice. Furthermore, the concentration of HEW-specific IgG1 in the serum of HEW-sensitized mice was also low (Table 1). However, oral administration of EW and PEW plus CT significantly increased the levels of EW-specific IgG1, with PEW-sensitized animals showing equivalent amounts of EW- and PEW-specific IgG1. We detected similar concentrations of

Table 1. Protein-Specific IgE and IgG1 Levels in the Sera of Mice Sensitized to EW, HEW, or PEW Measured at the Eighth Week (before Challenge) by Indirect ELISA^a

sensitivity group	protein-specific immunoglobulin					
	EW	OVA	OM	LYS	HEW	PEW
IgE (ng/mL)						
EW	1248.5 ± 216.7ABb	1407.7 ± 272.3ABb	2121.9 ± 362.9Aa	517.4 ± 62.9Bb		
HEW	2835.6 ± 316.0Aa	3031.6 ± 309.7Aa	2839.5 ± 588.3Aa	38.0 ± 33.8Bc	3674.8 ± 558.9Aa	
PEW	1910.6 ± 275.9ABab	1903.9 ± 318.8ABb	2906.2 ± 439.1Aa	958.0 ± 191.4Ba		1901.0 ± 298.2ABa
naïve	0.0 ± 0.0c	0.0 ± 0.0c	0.0 ± 0.0b	0.0 ± 0.0c	0.0 ± 0.0b	0.0 ± 0.0b
IgG1 (μg/mL)						
EW	554.1 ± 45.4Aa	276.0 ± 37.4Ba	191.8 ± 34.7Ba	314.6 ± 18.1Ba		
HEW	69.1 ± 9.5Ab	76.3 ± 8.2Ab	36.9 ± 10.2Bb	1.8 ± 0.1Cb	77.5 ± 16.1Aa	
PEW	643.9 ± 50.8Aa	371.9 ± 53.5Ba	221.5 ± 48.1Ba	364.9 ± 29.5Ba		694.5 ± 75.5Aa
naïve	2.3 ± 0.6b	2.5 ± 0.2b	1.8 ± 0.1b	1.7 ± 0.2b	1.3 ± 0.1b	1.7 ± 0.2b

^aNaïve mice were used as controls. Data are expressed as means ± SEM ($n = 5$). Different upper case letters (A–C) indicate significant differences ($P < 0.05$) within rows. Different lower case letters (a–c) indicate significant differences ($P < 0.05$) within columns for each antibody (IgE, IgG1).

OVA-, OM-, and LYS-specific IgG1 within each mouse group, and no differences between both groups.

The ability to trigger anaphylactic reactions of HEW and PEW, as compared to EW, was assessed in mice sensitized to EW (Figure 3). Oral challenge with HEW did not cause a significant drop in temperature as compared with naïve mice administered PBS, nor did challenge with EW, whereas PEW significantly decreased body temperature in mice (Figure 3a). Systemic anaphylactic symptoms were evident in all cases, but there were no significant differences among EW-sensitized mice challenged with the protein preparations submitted to processing under different conditions (Figure 3b). Similar results were obtained after subsequent ip challenges, although, in this case, HEW produced the least severe clinical signs. Challenge with HEW also led to the lowest release of MCP-1, indicative of mast cell degranulation (Figure 3c). Overall, these results imply that HEW and PEW induced, respectively, weaker and stronger anaphylactic responses than EW in EW-sensitized mice.

The lower capacity of HEW, as compared with EW, to elicit systemic reactions was also evident in the sign score of mice sensitized to HEW and challenged either orally or ip with both preparations. In fact, mice sensitized to HEW did not suffer temperature changes nor anaphylactic signs following challenge with HEW (Figure 3a,b). However, both PEW and EW caused similar temperature drops, allergic symptoms, and MCP-1 release in PEW-sensitized mice. It is noteworthy that significant temperature drops and MCP-1 levels were detected only in mice sensitized to PEW (and challenged with either EW or PEW), as well as in mice sensitized to EW and challenged with PEW, which suggests that, besides a superior ability to trigger allergic reactions, PEW held an enhanced sensitization potential.

Specificity of the Generated Antibodies. As mentioned, serum levels of MCP-1 were significantly increased in mice sensitized to EW and PEW (and challenged, respectively, with PEW and either EW or PEW), but not in mice sensitized to HEW (Figure 3c), despite the latter exhibiting the highest specific IgE levels (Table 1). This observation prompted us to investigate the ability of IgE generated in HEW-sensitized mice to develop allergic responses. To this aim, we conducted PCA assays with pooled serum heated at 56 °C to inactivate mouse IgE antibodies.¹⁸ Passive immunization of naïve mice with heated serum from HEW-sensitized mice led to a significantly reduced extravasation of Evan's blue dye to the surrounding tissues upon intravenous challenge with HEW, as compared with the immunization using nonheated serum, denoting that IgE was biologically functional and able to mediate local anaphylaxis reactions (Supplementary Figure 2).

To go deeper into this point, we assessed the binding capacity of IgE present in sera from EW-, HEW-, and PEW-sensitized mice to egg white proteins subjected to different processing methods using a reverse ELISA based on the capture of mouse IgE antibodies. Initially, we compared the binding capacity of the immobilized IgE molecules toward the antigen used for sensitization in each case by a direct test, which confirmed a higher response in HEW-sensitized mice (Figure 4a). Competitive ELISA tests showed that IgE from sera of mice sensitized to HEW bound HEW with a much higher strength than EW and PEW, underscoring that sensitization to HEW generated IgE antibodies specific to different or new epitopes (Figure 4c). As mentioned above, the observation that, despite allergy in HEW-sensitized mice was mainly IgE-

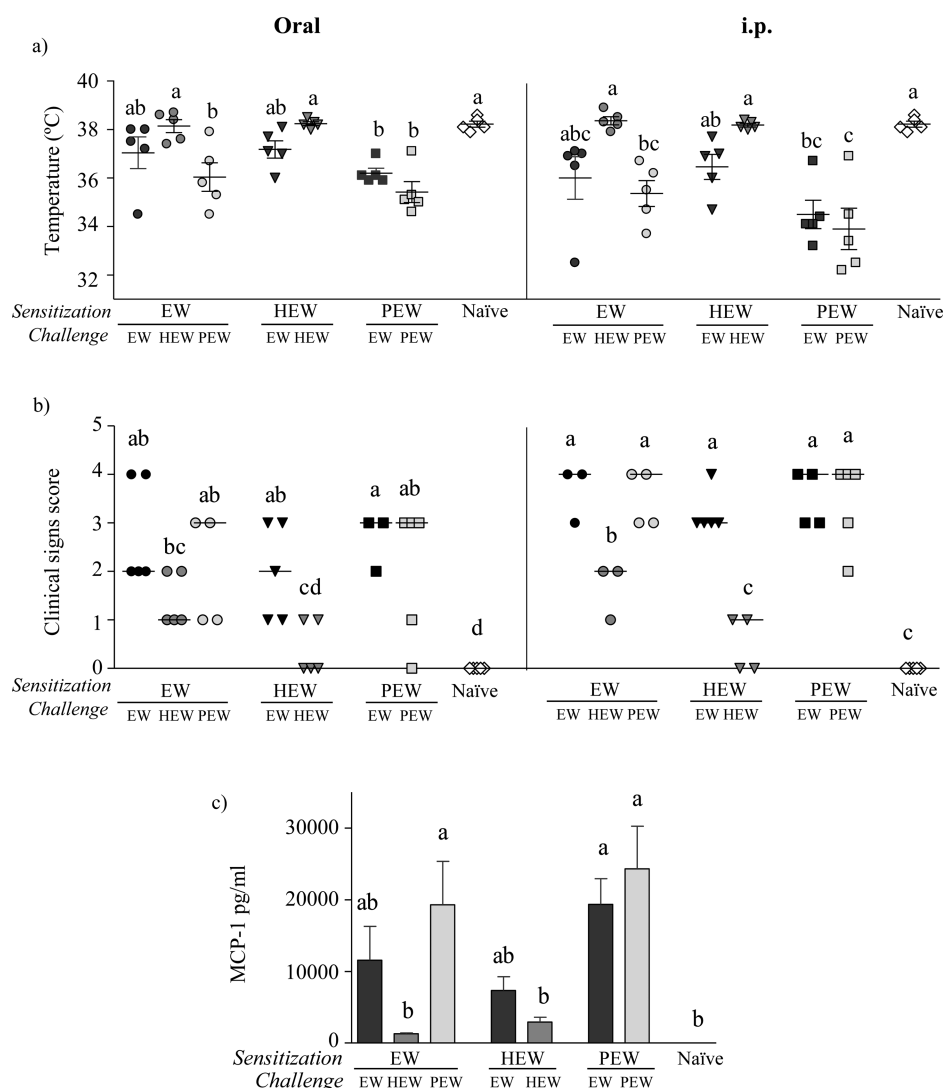


Figure 3. Body temperature (a), clinical sign scores (b), and serum concentrations of MCP-1 (c) in BALB/c mice sensitized to EW, HEW, or PEW and challenged with these protein preparations. Oral challenges were followed by ip challenges 40 min apart. Values are expressed as means $\pm$ SEM (a, c) or medians (b). Different letters indicate statistically significant differences ($P < 0.05$) within orally or ip challenged animals ($n = 5$).

mediated and HEW-specific IgE displayed higher affinity for HEW than for EW (Supplementary Figure 2 and Figure 4c), HEW triggered less severe allergic responses than EW in these animals following both oral and systemic challenges (Figure 3) shows that heating impaired its ability for mast cell cross-linking and activation, given that challenge with EW was able to trigger anaphylactic reactions in HEW-sensitized mice.

On the other hand, as illustrated in Figure 4b,d, both EW and PEW inhibited with similar affinity the binding of IgE from sera of EW or PEW-sensitized mice to labeled EW and PEW, respectively, whereas HEW was a much weaker inhibitor, particularly of the binding of specific IgE to biotinylated EW. This reveals that heat treatment destroyed IgE-binding epitopes and that these were maintained when egg white was submitted to less severe denaturation conditions, such as 400 MPa applied for 10 min. Additionally, it denotes that sensitization to both EW and PEW induced the production of IgE antibodies with similar specificities.

T Cell Responses in Spleen Cell Cultures. Splenocytes from EW-, HEW-, and PEW-sensitized mice were stimulated with the three protein preparations, as well as with the main egg allergens, OVA, OM, and LYS (Figure 5). Cytokines produced

in cultures from naïve mice were undetectable (results not shown). In general terms, spleen cells from sensitized mice within each group released equivalent amounts of IFN- γ , IL-4, IL-5, and IL-10 in response to either the individual proteins or the raw and processed egg white forms, with the latter stimulating the highest production. However, splenocytes from HEW-sensitized mice were more prone to Th1 responses, as judged by the release of IFN- γ to the culture media, and those from PEW-sensitized mice released more Th2 (IL-4 and IL-5) cytokines and IL-10, following incubation with the stimuli.

DISCUSSION

In this study, the ability of egg white subjected to denaturation by means of heat treatment (80 °C, 10 min) and high hydrostatic pressure (400 MPa, 10 min) to sensitize and trigger allergic responses was compared in BALB/c mice. Thermal treatment affected egg protein secondary structure and caused polymerization, as determined by CD and SDS-PAGE, in agreement with Mine et al.,²⁰ who described a marked increase in β -sheet and a concomitant decrease in α -helix structure, exposure of hydrophobic residues, and aggregation by disulfide

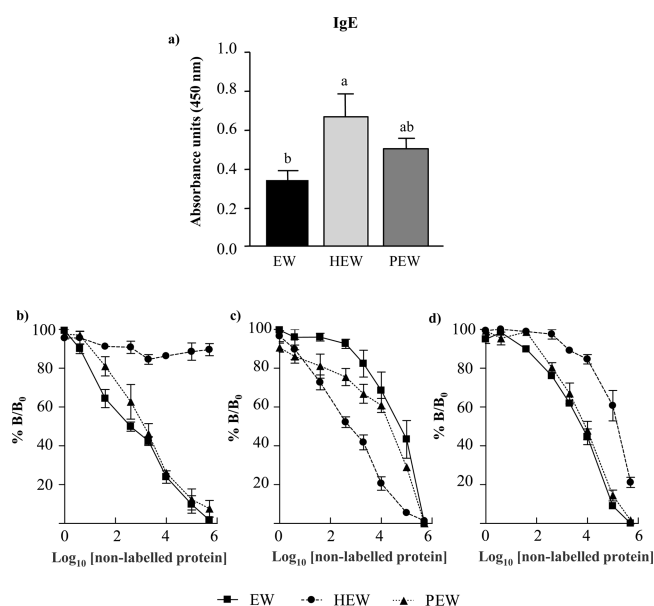


Figure 4. Binding capacity of IgE from pooled serum samples of mice ($n = 5$) sensitized to EW, HEW, and PEW to each of these protein preparations determined by direct antibody capture ELISA (a); competition of EW, HEW, and PEW for the binding of IgE antibodies present in serum of EW- (b), HEW- (c), and PEW-sensitized mice (d) to biotin-labeled EW, HEW, and PEW, respectively. Results are presented as means $\pm$ SEM, and different letters indicate statistically significant differences ($P < 0.05$) (technical triplicates).

bridges with increasing temperature from 60 to 90 °C. On the other hand, and also in accordance with our results, few changes in the secondary structure of OVA and LYS have been reported between the native proteins and those pressurized up to 600 MPa.^{21,22} High-pressure treatments can result in a pressure-dependent exposure of buried sulfhydryl groups leading to EW protein aggregation, although at 40 °C this occurs only above 600 MPa.²³ Therefore, the heat and high-pressure processing conditions applied led, respectively, to extensive and limited denaturation of egg white proteins. Assessment of resistance to simulated gastrointestinal digestion, which is another indicator of structural stability, revealed that, as expected, HEW was far more prone to hydrolysis than EW, which partially withstood digestion, as previously reported for heated and native OVA,^{9,24,25} whereas EW and PEW exhibited similar susceptibility to proteolysis.

Sensitization to HEW induced IgE antibodies specific to native and denatured egg white proteins. In fact, the serum concentration of EW-specific IgE, as determined by indirect ELISA, was significantly higher in HEW-sensitized mice than in EW-sensitized mice, and that of HEW-specific IgE was even greater. Because indirect ELISA does not avoid interactions of plate-bound antigens with specific IgG1, which may impair the binding and detection of IgE antibodies, a selective IgE capture ELISA was conducted.²⁶ Even though biotin labeling may vary depending on protein structure, hindering comparison, this method also showed that severe denaturation of egg white proteins caused by heat treatment stimulated the production of IgE antibodies, which were, at least in part, different in their specificity from those produced upon sensitization with native allergens. In addition, PCA assays revealed that such IgE antibodies were biologically functional in the development of allergic reactions. However, even if HEW was able to generate

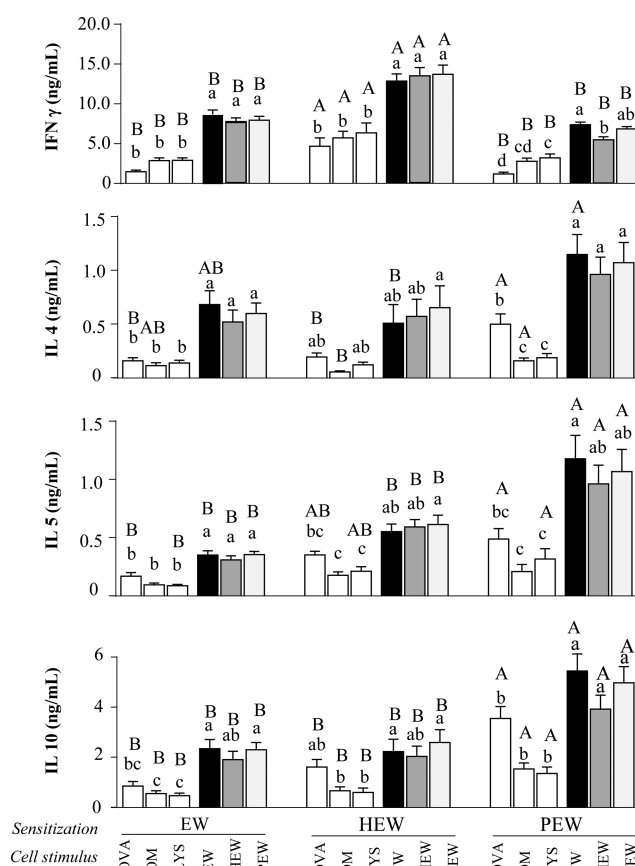


Figure 5. Production of IFN γ , IL-4, IL-5, and IL-10 by splenocytes of mice sensitized to EW, HEW, or PEW and stimulated with individual egg proteins (OVA, OM, and LYS), as well as with these protein preparations. Data are expressed as means $\pm$ SEM ($n = 5$). Different lower case letters indicate statistically significant differences ($P < 0.05$) within each sensitized group, and different upper case letters indicate statistically significant differences ($P < 0.05$) among different sensitization groups for the same stimulus.

the most vigorous IgE response, its eliciting capacity, both in EW- and HEW-sensitized mice, was very low.

It is well documented that heat treatment of egg proteins decreases their allergenic potential. Approximately 70% of egg-allergic children tolerate extensively heated eggs and, in these cases, the inclusion of baked egg in the diet accelerates the development of oral tolerance.^{27–30} The mechanisms responsible for the reduced allergenicity of heat-treated (100 °C, 5–60 min) EW, OVA, and OM were investigated in BALB/c and C3H/HeJ mice. Results pointed at the enhanced digestibility of heated OVA, which reduces its basophil activation capacity, and the impaired absorption of immunologically active forms of the allergens through the intestinal epithelium.^{24,31,32} Similarly, in the case of milk whey proteins, heat treatment and subsequent aggregation were reported to promote their uptake from Peyer patches rather than from intestinal epithelium cells, which reduces their capacity to elicit anaphylactic reactions, but enhances their immunogenicity.³³ In this respect, although there seems to be a general agreement that severe heating reduces the capacity of egg proteins to trigger allergic reactions, much less is known of the effect of processing on their sensitizing potential.

It was reported that mice sensitized by ip administration to OVA heated at 70 °C for 10 min develop lower levels of OVA-

specific IgE than mice sensitized to native OVA.³⁴ However, in that study, the administration route may have masked the impact of digestion and absorption in the gastrointestinal tract on the immunogenicity of the protein, because both the native and denatured forms were equally recognized by the generated IgE antibodies. Likewise, native and aggregated OVA (80 °C, 6 h) ip administered to mice generated similar levels of IgE antibodies specific to either OVA form.³⁵ Following oral administration, the physicochemical changes caused by heat treatment on egg white protein structure, and consequent decreased stability toward proteolysis, possibly altered antigen processing and presentation by dendritic cells and T cell priming, ultimately leading to the induction, in HEW-sensitized mice, of IgE over IgG1 antibodies with different specificities compared with those produced in EW-sensitized mice. In addition, both structural changes brought about by heat treatment and increased degradation during *in vivo* digestion could have impaired the ability of HEW for mast cell cross-linking and activation, despite the IgE antibodies generated showing high affinity toward HEW epitopes. Our results indicate that mice sensitized to HEW by oral administration were more susceptible to Th1 responses than those sensitized to EW, which points at a more equilibrated Th1/Th2 balance indicative of a lower sensitization status, an aspect also observed when heated OVA was used for mice immunization through the ip route.^{34,35} It has been described that different forms of the same antigen can activate distinct patterns of T cell commitment in mice with consequences in the generated antibodies. Thus, OVA chemically modified to give rise to high molecular weight polymers shifts the activation of the OVA-reactive T cell repertoire toward a Th1 phenotype *in vivo*, which correlates with the antibody responses.³⁶ It should be noted that, in our work, spleen cells from HEW-sensitized mice produced the highest level of IFN- γ *ex vivo* regardless of the stimulus. In fact, EW, HEW, and PEW induced the same cytokine responses on cells from naïve or sensitized mice, which does not support heat-denatured egg proteins intrinsically stimulating a Th1 profile over a Th2 one.³⁷

Unlike in the case of sensitization to HEW, sensitization to EW and PEW gave rise to an allergic response likely mediated by both IgE and IgG1. Both isotypes can participate in anaphylaxis in mice, although the IgG1 pathway differs from the classical IgE pathway in that it is mainly triggered by the binding of circulating antigen–IgG1 complexes to Fc γ RIII receptors on macrophages, instead of the cross-linking of IgE bound to FC ϵ RI on basophils or mast cells.³⁸ IgG1-dependent anaphylaxis in EW- and PEW-sensitized mice could have been facilitated by the large excess of specific antibodies from the IgG1 isotype with respect to the IgE isotype, particularly after the entry of the allergen into systemic circulation. Indeed, despite the fact that the clinical relevance of non-IgE-mediated anaphylaxis in humans is controversial, IgG-mediated anaphylaxis can occur in the presence of large concentrations of antigens and of specific IgG antibodies.³⁹ Thus, even if these results obtained in mice cannot be directly translated to a human situation, the fact that, in mice, IgE and IgG1 are Th2-driven isotypes induced by IL-4⁴⁰ argues for a superior sensitization capacity of EW and PEW over HEW.

EW and PEW induced equivalent IgE and IgG1 titers, and the specificity of the generated antibodies was also very similar. However, spleen cells of PEW-sensitized mice responded with a higher secretion of IL-4, IL-5, and IL-10 to stimulation with either EW, HEW, and PEW or to the individual EW proteins,

which denotes a Th2 bias typical of the allergic status and suggests that high-pressure processing increased the sensitizing potential of egg white proteins. Furthermore, unlike challenge with EW, challenge of mice sensitized to EW with PEW provoked significant temperature drops and mast cell degranulation as compared to naïve mice, suggesting that limited denaturation caused by high hydrostatic pressure increased the eliciting potential. There is some information on the impact of high hydrostatic pressure processing on the IgG- and IgE-binding properties of food proteins⁴ but, to the best of our knowledge, there are no published data on its effects on the induction of antibody responses and the acquisition of allergic sensitization or the elicitation of allergic reactions *in vivo*.

It is worth mentioning that, in EW-sensitized mice, the levels specific IgE were in the order OM $\geq$ OVA $\geq$ LYS, and there were similar concentrations of OVA-, OM-, and LYS-specific IgG1, despite these proteins being present in very different proportions (OVA, 54%; OM, 11%; and LYS, 3.5% w/w, respectively). It is assumed that OM plays a predominant role in egg allergy, but, in general terms, the contribution of the individual protein components and, in particular, the influence of LYS, is insufficiently known.⁷ As compared with EW, LYS in HEW generated a significantly lower IgE response, whereas LYS-specific IgE response in PEW was significantly higher. LYS is regarded as a thermolabile allergen.⁴¹ Unfolding of LYS becomes irreversible when the temperature is >80 °C, with α -helices being thermodynamically and kinetically more stable than β -structures.⁴² However, under high hydrostatic pressure conditions (600 MPa, 30 min, 40 °C), partial and reversible unfolding of LYS occurs.²² Our results suggest that heat-induced aggregation and enhanced flexibility brought about by high pressure decreased and enhanced, respectively, LYS sensitizing potential, in support of previous findings showing that LYS structure plays an important role in its immunogenicity. In fact, immunization of mice with LYS derivatives of different conformational stability revealed that the least stable derivative leads to the most potent Th2 response and IgE production, which was associated with a higher susceptibility of the unfolded form to be processed by antigen-presenting cells.^{43,44}

In conclusion, extensive protein denaturation caused by heat treatment of egg white not only reduced its eliciting capacity but also its sensitizing capacity in a BALB/c model of egg allergy. HEW stimulated IgE responses over IgG1 responses; however, given the involvement of both isotypes in anaphylaxis in this animal model, the overall result is likely a lower degree of sensitization, reinforced by the observation that HEW-sensitized mice were more prone to Th1 responses than EW-sensitized mice. Furthermore, HEW induced the production of antibodies directed toward new epitopes exposed by heat treatment or released as a consequence of the enhanced digestibility of the heated allergen. Conversely, partial denaturation caused by high-pressure treatment increased the ability of EW to stimulate Th2-biased responses and its allergenic potential.

■ ASSOCIATED CONTENT

📄 Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.7b00953.

Supplementary Figure 1: Levels of IgE and IgG1 specific for EW, OVA, OM, and LYS in sera from mice sensitized to EW, HEW, and PEW at different days throughout the sensitization period determined by indirect ELISA. Supplementary Figure 2: Passive cutaneous anaphylaxis assay with unheated and heated pooled sera ($n = 5$) from HEW-sensitized mice. ($P < 0.05$) (PDF)

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Funding

This work was supported by MINECO (Project AGL2014-59771R and contract of A.P.-T.) and MECD (through D.L.-O. contract).

Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

CD, circular dichroism; CT, cholera toxin; EW, raw egg white; HEW, heated egg white; ip, intraperitoneal; LYS, lysozyme; MCP-1, mast cell protease-1; OVA, ovalbumin; OM, ovomucoid; PCA, passive cutaneous anaphylaxis; PEW, pressurized egg white

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