JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2008,** *56,* 11809–11816 **11809**

Changes in the Ovalbumin Proteolysis Profile by High Pressure and Its Effect on IgG and IgE Binding

IVAN LÓPEZ-EXPÓSITO,[†] ROSA CHICÓN,[†] JOSEFINA BELLOQUE,[†] ISIDRA RECIO,[†] ELENA ALONSO,[‡] AND ROSINA LÓPEZ-FANDIÑO*,[†]

Instituto de Fermentaciones Industriales, Consejo Superior de Investigaciones Científicas (CSIC), Juan de la Cierva 3, Madrid 28006, Spain, and Hospital Infantil Gregorio Marañón, Doctor Esquerdo 46, Madrid 28007, Spain

Egg proteins are responsible for one of the most common forms of food allergy, especially in children, and one of the major allergens is ovalbumin (OVA). With the aim to examine the potential of high pressure to enhance the enzymatic hydrolysis of OVA and modify its immunoreactivity, the protein was proteolyzed with pepsin under high-pressure conditions (400 MPa). Characterization of the hydrolysates and peptide identification was performed by reversed-phase high-performance liquid chromatography−tandem mass spectrometry (RP-HPLC−MS/MS). The antigenicity (binding to IgG) and binding to IgE, using the sera of patients with specific IgE to OVA, were also assessed. The results showed that, upon treatment with pepsin at 400 MPa, all of the intact protein was removed in minutes, leading to the production of hydrolysates with lower antigenicity than those produced in hours at atmospheric pressure. However, the exposure of new target residues only partially facilitated the removal of allergenic epitopes, because the hydrolysates retained residual IgG- and IgE-binding properties as a result of the accumulation of large and hydrophobic peptides during the initial stages of hydrolysis. These peptides disappeared at later stages of proteolysis, although reactivity toward IgG and IgE was not completely abolished. Some fragments identified in the hydrolysates (such as Leu_{124}−Phe_{134}, Ile_{178}−Ala_{187}, Leu_{242}−Leu_{252}, Gly_{251}−Ile_{259}, Lys_{322}−Gly_{343}, Phe_{358}−Phe_{366}, and Phe_{378}−Pro_{385}) carried previously identified IgE-binding epitopes. Because some of the peptides found, such as Phe_{358}−Phe_{366}, probably contain only one binding site for IgE, the possibility to use high pressure to tailor hydrolysates that contain mostly peptides with only one IgE-binding site, which may help the immune system to tolerate egg proteins, is suggested.

KEYWORDS: Ovalbumin; high hydrostatic pressure; pepsin; proteolysis; allergenicity

INTRODUCTION

IgE-mediated allergic reactions to food affect approximately 6% of children and 3−4% of adults in the U.S. (*1*). It is well-known that certain food proteins are involved in the process of sensitization and recognition by IgE; however, not much is known about the structural or functional properties that are responsible for their capacity to modulate the immune response (*2*). Egg represents, together with cow's milk, the most common cause of allergic reactions in children, and ovalbumin (OVA), the major protein in egg white (58%, w/w), is considered a dominant allergen (*3*).

OVA is a glycoprotein with a molecular mass of 45 000 Da. Its sequence comprises 385 amino acids and includes 6 cysteines, with a single disulfide bond between Cys 73 and Cys 120. It has two potential phosphorylation sites, at Ser 68 and Ser 344, and a single carbohydrate chain, which accounts for 3% in weight, covalently linked to the amide nitrogen of Asn 292 (*4*). OVA partially resists hydrolysis with pepsin (>60 min at pH 1.2) (*5*). Thermal denaturation of OVA can decrease its IgE binding capacity and the proliferative response of peripheral blood mononuclear cells, in a way that depends upon individual variations in susceptibility (*6, 7*). These characteristics are also shared by many allergenic proteins, which withstand processing and digestion in the gastrointestinal tract and, thus, keep a certain degree of three-dimensional structural integrity to trigger the immune reaction (*8*).

Because food allergens are generally resistant to heat and proteases, most processing practices applied in food manufacturing are not effective in eliminating their allergenic potential, with an exception being the extensive enzymatic hydrolysis, as used on milk proteins for infant formula, which has led to marketed hypoallergenic products (*9*). In this respect, it should be mentioned that, when using hydrolysis to destroy epitope structures, the main challenge is to maintain the palatability and nutritional and functional properties of the original protein (*10*).

* To whom correspondence should be addressed. Telephone: 34-91-5622900. Fax: 34-91-5644853. E-mail: rosina@ifi.csic.es.

[†] CSIC.

[‡] Hospital Infantil Gregorio Marañón.

10.1021/jf8023613 CCC: $40.75
Published on Web 11/18/2008

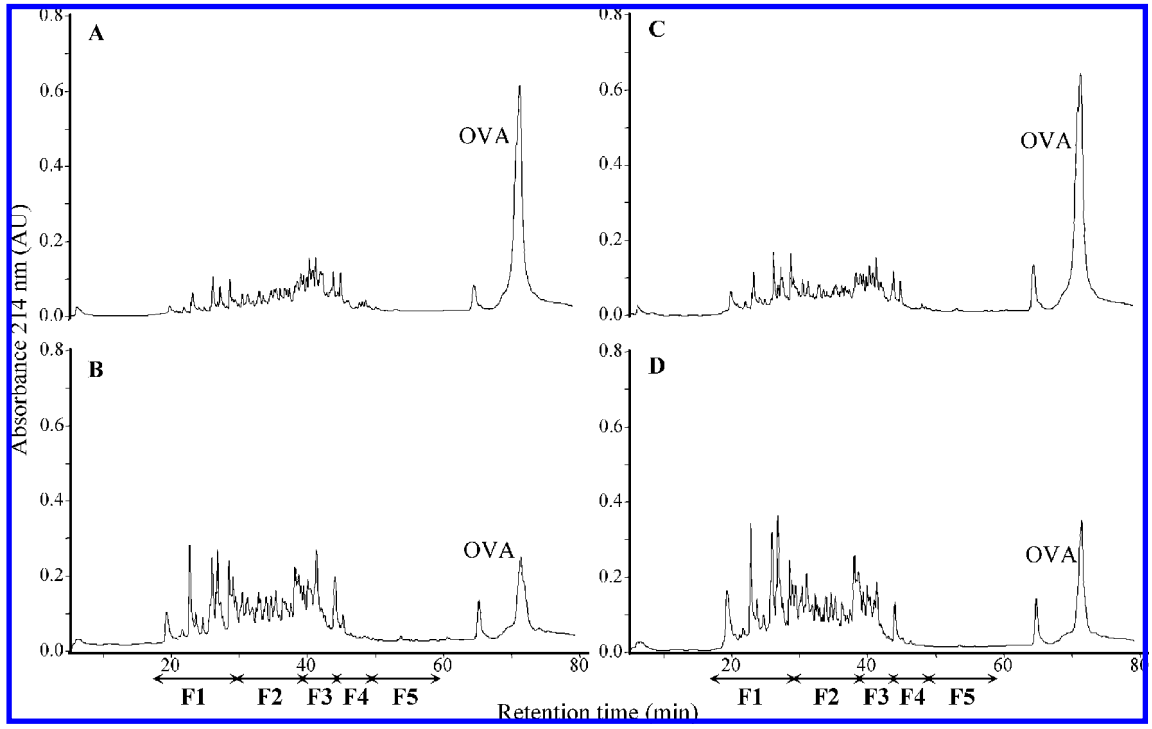

Figure 1. RP-HPLC patterns of OVA hydrolyzed with pepsin at atmospheric pressure at an enzyme/substrate ratio of 1:20 for (A) 2 h and (B) 24 h and at an enzyme/substrate ratio of 1:5 for (C) 2 h and (D) 24 h.

Thus, extensive enzymatic hydrolysis may not represent a viable alternative for egg proteins, because they are used as ingredients in food products for their unique functional properties, such as foaming, emulsifying, and gelling (*10, 11*).

A novel approach is to use physical treatments, such as heat or high hydrostatic pressure under certain conditions, to unfold proteins and expose new targets to proteolysis (*12*−*17*). In fact, treatment of OVA under high pressures up to 400 MPa, with chymotrypsin, trypsin, and pepsin, promotes its hydrolysis and changes the proteolytic pattern (*18*). Furthermore, in the case of other food proteins, such as β-lactoglobulin (β-Lg), it has been demonstrated that enzyme treatments under high pressure rapidly remove the intact protein and lead to an important reduction in the IgG- and IgE-binding properties of the hydrolysates, with no need for extensive hydrolysis (*19, 20*). The objective of this study was to examine the potential of proteolysis under high pressure to modify the immunoreactivity of OVA, with the aim of obtaining hydrolysates that could be used as hypoallergenic ingredients.

MATERIALS AND METHODS

Enzyme Treatments at High and Atmospheric Pressure. OVA (grade VI, 99% purity, Sigma Chemicals Co., St. Louis, MO) was subjected to enzymatic hydrolysis with porcine pepsin A (EC 3.4.23.1, 570 units/mg, Sigma) at atmospheric and high pressure. Briefly, pepsin was added to OVA (2.5 mg/mL) in 50 mM citrate buffer at pH 2.5 at two different enzyme/substrate ratios (1:5 and 1:20 E/S), immediately poured into Eppendorf vials (1700 μL), avoiding headspace, and pressurized at 400 MPa, for 10, 60, and 120 min at 37 °C using a 900 HP apparatus (Eurotherm Automation, Lyon, France). The pressure was raised at a rate of 2.5 MPa/s and released at the same rate. The temperature of the hydrostatic fluid medium was controlled by water circulating through a jacket surrounding the pressure vessel, and before pressure processing, it was set to 37 °C. The temperature increase as a result of the pressure treatments was approximately 2 °C/100 MPa. This temperature increase might transitory alter pepsin activity. However, all samples were processed in the same manner, thus allowing for a comparison among them. Immediately after removal from the high-pressure unit, the enzyme reactions were stopped by the addition of 2 N NaOH to pH 6.5−7.

Controls were obtained by conducting the hydrolysis at atmospheric pressure (0.1 MPa) on native OVA at 37 °C. Samples were immediately freeze-dried and reconstituted as needed for subsequent analyses. All reactions were performed in triplicate.

Preparation of Peptide Fractions. Semi-preparative reversed-phase high-performance liquid chromatography (RP-HPLC) was performed on a Waters Series 600 HPLC, equipped with Millennium 3.2 Software for data acquisition (Waters Corporation, Milford, MA). A Prep Nova Pak HR C_{18} column (300 × 7.8 mm i.d., 6 μm particle size, and 60 Å pore size) (Waters) was used. Solvent A was a mixture of water/trifluoroacetic acid (1000:1), and solvent B was a mixture of acetonitrile/trifluoroacetic acid (1000:0.8). Operating conditions were column temperature, 30 °C; flow rate, 4 mL/min; injection volume, 300 μL (protein concentration of approximately 2.5 mg/mL). The elution was performed with a linear gradient of solvent B in A from 0 to 50% B in 60 min, followed by washing at 70% B and conditioning of the column. Absorbance was recorded at 214 nm. Five fractions were collected using a Waters Fraction Collector II. The fractions were lyophilized and dissolved in water, and their protein concentrations was determined by the bicinchoninic acid (BCA) assay (Pierce, Rockford, IL), using bovine serum albumin as the standard. Then, each fraction was further diluted with water to a protein concentration of 2.5 mg/mL or 2.5 μg/mL, for RP-HPLC−tandem mass spectrometry (MS/MS) or enzyme-linked immunosorbent assay (ELISA) analyses, respectively.

RP-HPLC−MS/MS. RP-HPLC with UV detection, online with electrospray ionization, and quadrupole ion trap instrument (ESI−MS/MS) analyses were performed as previously described by Chicón et al. (*17*), using an Agilent 1100 series HPLC equipment (Agilent Technologies, Waldbronn, Germany), with a Hi-Pore reversed-phase RP-318 column (250 × 4 mm i.d.) (Bio-Rad Laboratories, Hercules, CA) and an Esquire 3000 mass spectrometer (Bruker Daltonik, Bremen, Germany). The identification approach involved the search for the masses in a database of OVA, including sequence modifications (phosphorylation and glycosylation) and the matching of partial sequences (sequence tags) identified by tandem MS with the peptides selected by mass. Sequence data were obtained from UniProtKB/Swiss-Prot (accession number P01012).

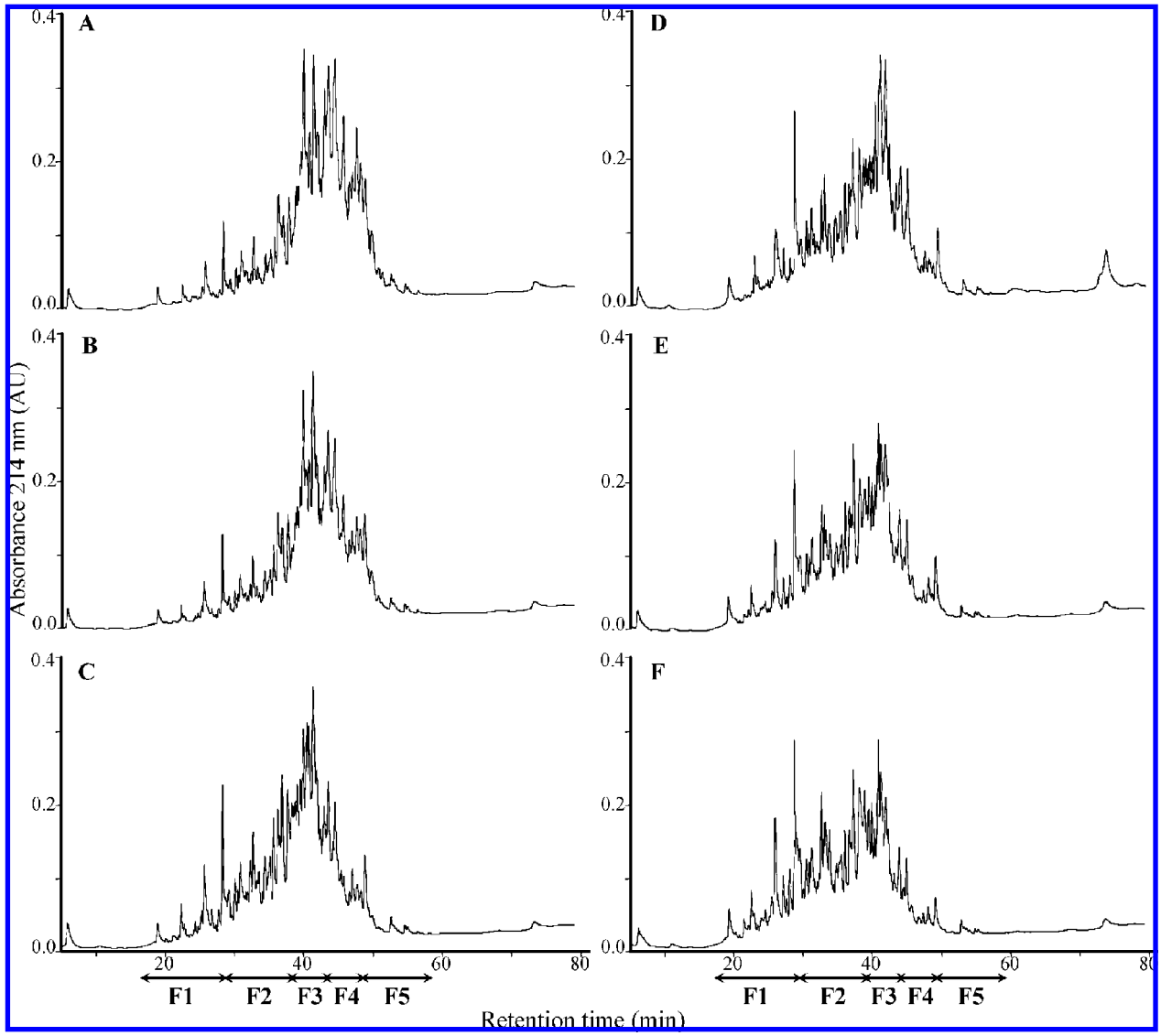

Figure 2. RP-HPLC patterns of OVA hydrolyzed with pepsin at 400 MPa at an enzyme/substrate ratio of 1:20 for (A) 10 min, (B) 60 min, and (C) 120 min and at an enzyme/substrate ratio of 1:5 for (D) 10 min, (E) 60 min, and (F) 120 min.

ELISA Experiments. ELISA assays were conducted as previously described by Chicón et al. (*19*). Individual serum samples from children allergic to OVA were collected in the hospital Gregorio Marañón (Madrid, Spain). All patients showed specific immunoglobulin E (IgE) antibodies toward egg proteins determined by the FEIA-CAP System (Pharmacia Diagnostics, Uppsala, Sweden). Individual sera were always used for IgE-binding experiments, while the pooled serum of five healthy non-allergic people was used as a negative control (NCS).

For the estimation of the IgE-binding, high binding polystyrene microtiter plates (Corning, Cambridge, MA) were used as a solid support. Single wells were coated with 50 μL of antigen (2.5 μg/mL of OVA or OVA hydrolysates) in 0.01 M phosphate-buffered saline (PBS) solution at pH 7.4 and incubated overnight at 6 °C. Plates were washed 3 times with PBS containing 0.05% Tween 20 (PBST), using a Microplate Washer (Nunc, Roskilde, Denmark). This washing system was used after each incubation step. Residual free binding sites were blocked with PBS containing 2.5% Tween 20 for 1 h at room temperature. Then, plates were incubated with 50 μL/well of serum diluted in PBST overnight at 6 °C. The same dilution of NCS was used as a negative control. Rabbit anti-human IgE conjugated with horseradish peroxidase (HRP) (DakoCytomation, Glostrup, Denmark) was added (50 μL/well, diluted 1:1000 in PBST) and incubated for 1 h at room temperature. Before the addition of the enzyme substrate, a signal amplification system based on the subsequent addition of biotinyl- tyramide and streptavidin-HRP was used following the instructions of the manufacturer (ELAST ELISA amplification system, PerkinElmer Life Sciences, Waltham, MA). A solution of freshly prepared *o*-phenylene-diamine dihydrochloride (OPD, from DakoCytomation) containing H_2O_2 was added. Plates were incubated for 30 min at room temperature in the dark, and the reaction was stopped by adding 50 μL/well of 0.5 M H_2SO_4. Optical densities were read at 492 nm on an automated ELISA plate reader Multiskan Ascent (Labsystems, Helsinki, Finland). ELISA determinations were carried out in triplicate, and measurements were averaged. A blank without antigen (PBS), the negative control (NCS), and positive controls (different concentrations of OVA) were included in each plate.

IgG binding of the samples was evaluated against commercial HRP-conjugated anti-chicken OVA IgG raised in rabbit, following the ELISA described above, with slight modifications. Briefly, wells were coated with 50 μL of antigen as described above. After the blocking step, plates were incubated for 1 h at room temperature with 50 μL/well of HRP-conjugated rabbit anti-chicken OVA (from Gene Tex, Inc.), diluted 1:3000 in PBS. Finally, 50 μL/well of the OPD solution were added and incubated for 30 min.

RESULTS AND DISCUSSION

Hydrolysis of OVA by Pepsin under High Pressure. Pepsin hydrolyzed OVA slowly at atmospheric pressure. As illustrated in **Figure 1**, there was intact protein left after 24 h of hydrolysis at the highest enzyme/substrate ratio. OVA was reported to be

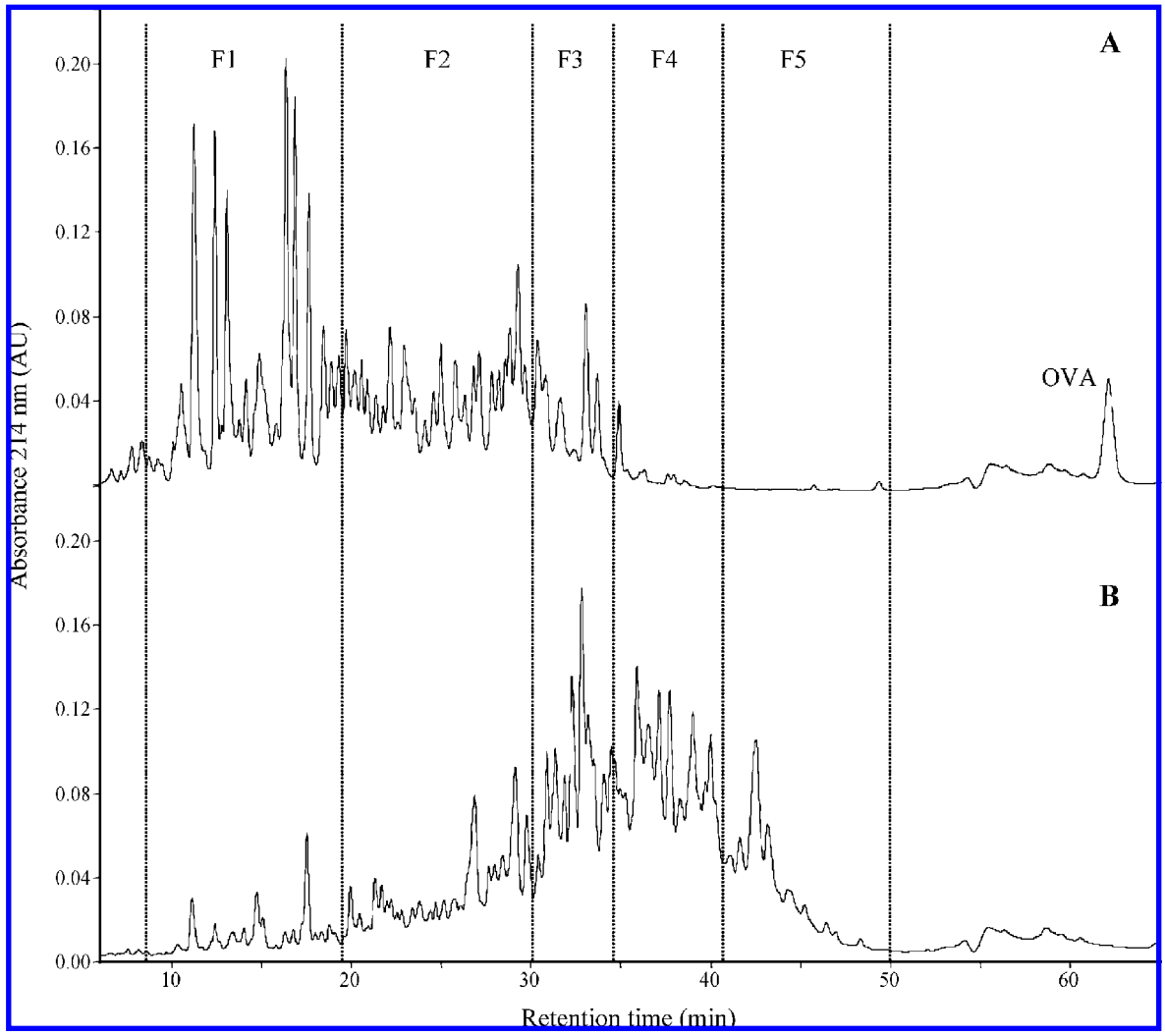

Figure 3. Semi-preparative RP-HPLC chromatograms of OVA hydrolyzed with pepsin (A) at an enzyme/substrate ratio of 1:5 at 0.1 MPa for 24 h and (B) at an enzyme/substrate ratio of 1:20 at 400 MPa for 10 min. Five fractions (F1−F5) were automatically collected.

very stable to pepsin at atmospheric pressure and enzyme/protein ratios similar to those of the present study, such as 1:10, used by Astwood et al. (*5*). This stability decreases at higher ratios, such as 13:1, used by Fu et al. (*21*), stressing the importance of the assay conditions when trying to correlate pepsin resistance with the allergenic potential of proteins (*22*).

The susceptibility of OVA to proteolysis by pepsin was considerably enhanced by the simultaneous application of high pressure and enzyme treatment, with no remaining protein even after 10 min at 400 MPa (**Figure 2**). The comparison between the RP-HPLC chromatograms of OVA treated with pepsin at atmospheric and high pressure revealed quantitative differences, because there was an increased level of all proteolysis products when the hydrolyses were conducted under high pressure, but also qualitative differences in the peptide pattern.

We quantified the peak areas of five peptide groups corresponding to different regions of the chromatogram, F1 (18−30 min), F2 (30−40 min), F3 (40−44 min), F4 (44−48 min), and F5 (48−58), relative to the total peptide area (PF) that included all peptide groups (**Figures 1** and **2**). As illustrated in **Table 1**, hydrophobic, late eluting peptides (F4 and F5), were predominant in the hydrolysates produced under high-pressure conditions at the initial stages of hydrolysis (**Figure 2**). Furthermore, many of these peptides were absent from the hydrolysates produced at atmospheric pressure (**Figure 1**), which strongly suggests that high-pressure treatment of OVA at acidic pH exposed new cleavage sites to enzymatic hydrolysis. Peptides present in fractions F4 and F5 were further cleaved as the hydrolysis proceeded, leading to an increase in fractions F1 and F2. In addition, the higher the concentration of enzyme, the faster the disappearance of the intermediate peptides in the late-eluting fractions. Thus, treatment under high pressure for 120 min at an enzyme/substrate ratio of 1:20 produced a hydrolysate with a peptide pattern very similar to that produced at an enzyme/substrate ratio of 1:5 for only 10 min (**Table 1** and **Figure 2**).

These observations are consistent with previous results, showing that pressurization (300−400 MPa) of OVA during the enzyme treatments accelerated its hydrolysis by trypsin, chymotrypsin, and pepsin. It was observed that hydrolysis under high pressure produced differences in the peptide pattern, particularly with pepsin at acidic pH, that were consistent with a higher accessibility of the enzyme to hydrophobic regions of the substrate exposed during pressurization (*18*). OVA was reported to be less stable to high pressure when treated at pH 3.0 than at neutral pH. At acidic pH, the exposure of the hydrophobic core is observed at much lower pressures (*23, 24*).

IgG- and IgE-Binding Properties of the Hydrolysates. The IgG-binding properties of the hydrolysates were estimated by indirect ELISA using IgG raised against OVA (**Table 1**). The hydrolysates produced at atmospheric pressure, which contained a considerable amount of intact OVA, exhibited a substantial residual antigenicity. The more extensive pro-

Table 1. Relative Area of RP-HPLC Peptide Fractions (Shown in **Figures 1** and **2**) and Antigenicity (ELISA Response against Anti-OVA IgG Raised in Rabbit) of Pepsin Hydrolysates of OVA Obtained under Atmospheric (0.1 MPa) and High (400 MPa) Pressure

pepsin hydrolysis			fraction relative area[a]					antigenicity[b]
pressure (MPa)	time (min)	E/S	F1/PF	F2/PF	F3/PF	F4/PF	F5/PF	IgG binding
0.1	0							3.93 ± 0.06
0.1	10	1:20	0.14	0.18	0.29	0.25	0.14	3.10 ± 0.04
0.1	60	1:20	0.21	0.23	0.27	0.18	0.11	2.75 ± 0.10
0.1	120	1:20	0.25	0.35	0.21	0.12	0.07	2.71 ± 0.03
0.1	480 (8 h)	1:20	0.35	0.28	0.21	0.12	0.04	2.57 ± 0.04
0.1	1440 (24 h)	1:20	0.40	0.34	0.13	0.10	0.03	1.99 ± 0.08
0.1	10	1:5	0.27	0.22	0.26	0.15	0.10	2.69 ± 0.01
0.1	60	1:5	0.29	0.37	0.16	0.12	0.06	2.62 ± 0.05
0.1	120	1:5	0.29	0.46	0.13	0.08	0.04	2.72 ± 0.04
0.1	480 (8 h)	1:5	0.36	0.43	0.11	0.07	0.02	2.36 ± 0.11
0.1	1440 (24 h)	1:5	0.46	0.40	0.08	0.05	0.02	1.91 ± 0.09
400	10	1:20	0.07	0.18	0.20	0.26	0.29	1.87 ± 0.01
400	60	1:20	0.11	0.23	0.25	0.15	0.26	1.61 ± 0.03
400	120	1:20	0.17	0.28	0.29	0.11	0.15	1.37 ± 0.01
400	10	1:5	0.20	0.26	0.31	0.12	0.12	1.35 ± 0.11
400	60	1:5	0.23	0.28	0.30	0.09	0.10	1.20 ± 0.13
400	120	1:5	0.30	0.33	0.24	0.06	0.07	0.71 ± 0.03

[a] Fractions F1 (18−30 min), F2 (30−40 min), F3 (40−44 min), F4 (44−48 min), and F5 (48−58 min) are shown in **Figures 1** and **2**. PF = F1 + F2 + F3 + F4 + F5. [b] ELISA response in absorbance (492 nm) units. Values are means ± standard deviations ($n = 3$).

Table 2. ELISA Response (Absorbance at 492 nm) against IgE from Individual Sera of Allergic Patients (S1−S5) of Pepsin Hydrolysates of OVA Obtained under High Pressure (400 MPa) (Values Are Means ± Standard Deviations, $n = 3$)

serum IgE binding						
			pepsin hydrolysis at 400 MPa			
serum[a]	OVA control	E/S	10 min	30 min	60 min	120 min
S1	0.97 ± 0.11	1:20	0.65 ± 0.11	0.92 ± 0.06	0.87 ± 0.14	0.74 ± 0.09
		1:5	0.88 ± 0.11	0.84 ± 0.19	0.62 ± 0.03	0.71 ± 0.09
S2	0.78 ± 0.23	1:20	0.43 ± 0.08	0.33 ± 0.01	0.43 ± 0.25	0.26 ± 0.10
		1:5	0.43 ± 0.13	0.57 ± 0.02	0.44 ± 0.09	0.17 ± 0.07
S3	1.19 ± 0.04	1:20	0.82 ± 0.06	0.85 ± 0.06	0.89 ± 0.08	0.93 ± 0.03
		1:5	0.95 ± 0.18	0.90 ± 0.05	0.79 ± 0.07	0.66 ± 0.01
S4	2.5 ± 0.14	1:20	1.59 ± 0.26	1.92 ± 0.18	1.44 ± 0.19	1.74 ± 0.06
		1:5	1.32 ± 0.16	1.85 ± 0.08	1.24 ± 0.13	0.91 ± 0.15
S5	2.05 ± 0.20	1:20	1.35 ± 0.31	1.07 ± 0.26	1.52 ± 0.38	1.30 ± 0.34
		1:5	1.45 ± 0.16	0.92 ± 0.54	1.09 ± 0.23	0.74 ± 0.31

[a] Specific IgE antibody titters to OVA in the sera used were S1, 53.6 kU/L; S2, 57.1 kU/L; S3, 31.8 kU/L; S4, 36.3 kU/L; and S5, 32.1 kU/L. S1 and S2 were diluted 1:50, while S3, S4, and S5 were diluted 1:20.

teolysis under high pressure, as compared to atmospheric pressure, was reflected in a lower antigenicity of the hydrolysates. However, the hydrolysates obtained under high pressure still exhibited a clear response against IgG, even if there was not intact OVA left, indicating that some of the proteolysis products retained antigenic epitopes. The antigenicity decreased as fractions F4 and F5 decreased, which suggests that peptides present in these fractions were involved in the immune reaction.

With regard to the IgE-binding properties of the hydrolysates, it should be mentioned that, even if no remaining OVA was left after enzymatic treatments at 400 MPa, these retained a noticeable reactivity against IgE (**Table 2**). IgE antibodies to OVA from sera of patients with allergy to egg white are known to differ from IgG or IgA, in that they are more

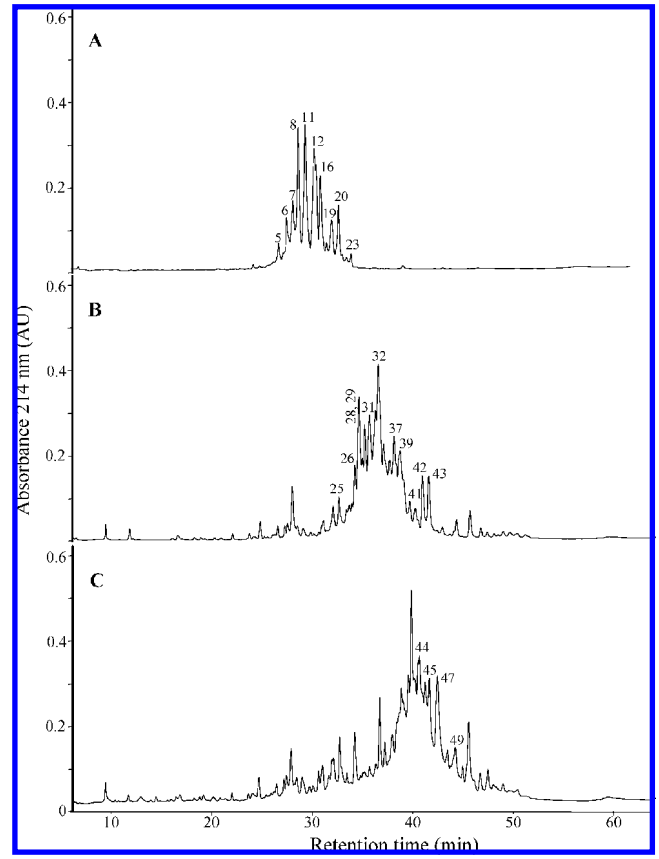

Figure 4. RP-HPLC pattern of the fractions (A) F3, (B) F4, and (C) F5, isolated from the hydrolysate of OVA treated with pepsin at an enzyme/substrate ratio of 1:20 at 400 MPa for 10 min as shown in **Figure 3B**. Peak numbers refer to the peptide sequences indicated in **Table 4**.

reactive against the denatured or fragmented protein (*6*). This is probably because anti-OVA IgE antibodies preferentially recognize sequential epitopes (*25*). Furthermore, the responses to native, denatured, or pepsin-treated OVA may vary depending upon the individual susceptibility of the patient (*7*) or between patients with persistent egg allergy and those who have outgrown egg allergy (*26*). In general terms, little reduction in the response was found in the hydrolysates obtained at 400 MPa for 60 min as compared to 10 min. The lowest IgE binding was found in the hydrolysates obtained at the longest hydrolysis time (120 min) with the highest enzyme/substrate ratio (1:5 E/S) that showed the lowest proportion of peptide products in F4 and F5 (**Table 1**). This suggests that IgE immunoreactive fragments were present in F4 and F5.

IgG and IgE Binding Properties of the Peptides Released by Pepsin on OVA under Pressure. We then tried to confirm the contribution of the RP-HPLC late-eluting fractions from the OVA hydrolysates to the IgE binding, as well as to investigate the peptide sequences potentially responsible for this reactivity. For this purpose, we selected two hydrolysates: one obtained under atmospheric pressure, 1:5 E/S, and 24 h and one produced under 400 MPa, 1:20 E/S, and 10 min, which were representative, respectively, of a low and high content of intermediate peptides. These were separated by RP-HPLC at a semi-preparative scale into five different fractions as shown in **Figure 3**. These fractions are similar to those shown in **Figures 1** and **2** and **Table 1**, although the retention time intervals differ because a semi-preparative HPLC piece of equipment was used in this case. The fractions

Table 3. ELISA Response (Absorbance at 492) against IgG (Using Anti-OVA Raised in Rabbit) and IgE (Using Individual Sera of Patients Allergic to OVA, S1−S5) of the Peptide Fractions F1−F5 Obtained by Semi-preparative RP-HPLC from Pepsin Hydrolysates of OVA as Shown in **Figure 3**[a]

pepsin treatment				serum[b] IgE binding[c]			
pressure/time	E/S ratio	fraction	IgG binding[c]	S1	S2	S5	S6
0.1 MPa/24 h	1:5	F1	0.10 ± 0.01	0.06 ± 0.05	0.02 ± 0.02	0.02 ± 0.01	0.04 ± 0.09
		F2	0.18 ± 0.02	0.23 ± 0.04	0.14 ± 0.01	0.12 ± 0.08	0.60 ± 0.03
		F3	0.27 ± 0.02	1.96 ± 0.05	0.27 ± 0.08	0.24 ± 0.01	1.29 ± 0.05
		F4	0.25 ± 0.01	1.79 ± 0.26	0.26 ± 0.07	0.24 ± 0.09	1.91 ± 0.01
400 MPa/10 min	1:20	F1	0.09 ± 0.01	−0.04 ± 0.02	0.01 ± 0.01	−0.02 ± 0.01	0.09 ± 0.01
		F2	0.13 ± 0.02	−0.04 ± 0.04	0.01 ± 0.01	−0.04 ± 0.01	0.03 ± 0.06
		F3	0.31 ± 0.01	0.66 ± 0.09	0.37 ± 0.09	0.13 ± 0.02	0.27 ± 0.01
		F4	0.54 ± 0.02	1.97 ± 0.51	0.45 ± 0.10	0.40 ± 0.08	1.83 ± 0.18
		F5	0.56 ± 0.01	1.99 ± 0.06	0.15 ± 0.10	0.37 ± 0.02	1.68 ± 0.06
OVA control			1.54 ± 0.05	1.93 ± 0.02	0.25 ± 0.01	0.51 ± 0.02	2.15 ± 0.04

[a] Antibody binding to nonhydrolysed OVA is also shown. [b] Specific IgE antibody titters to OVA in the sera used were S1, 53.6 kU/L; S2, 57.1 kU/L; S5, 32.1 kU/L; and S6, 65.6 kU/L. S1 and S6 were diluted 1:40; S2 was diluted 1:50; and S5 was diluted 1:20. [c] Values are means ± standard deviations ($n = 3$).

were diluted to the same protein concentration and tested individually for IgG and serum IgE binding.

As shown in **Table 3**, fractions F1, F2, and F3 showed a similar reactivity toward IgG in the two hydrolysates. Fractions F1 and F2 presented a low reactivity against IgG in both hydrolysates. In contrast, the highest antigenicity corresponded to fractions F4 and F5 of the hydrolysate produced under high pressure. The binding of the fractions to IgE from sera of patients allergic to OVA followed a similar trend, although the reactivity toward IgE was comparatively higher than toward IgG, taking as a reference the binding responses of the intact protein (**Table 2**). Disregarding the variations among the individual sera, which showed a slightly different specificity toward the fractions, the highest IgE response corresponded to fractions F4 and F5. Therefore, this strongly supports that these fractions, which were important in the hydrolysates obtained under high pressure, were responsible for much of the IgE binding of the unfractionated hydrolysates. It is worth noting that F3 also exhibited a high reactivity toward IgE in the hydrolysates produced under atmospheric pressure and, to a lesser extent, in the hydrolysates obtained under high pressure.

The identification of the peptides present in F3, F4, and F5 from the hydrolysate obtained under high pressure was undertaken by RP-HPLC−MS/MS (**Figure 4**). **Table 4** lists the peptides identified in these fractions, showing that some of them included or overlapped previously reported IgE and T epitopes recognized by allergic subjects or model animals. Thus, many fragments found in F3 and F4 were contained within the large CNBr fragments 41−172 and 301−385 that, according to Kahlert et al. (*27*), react with IgE of patients. On the other hand, Mine and Rupa (*26*) determined the entire mapping of the IgE-binding epitopes in the primary sequence of OVA, finding five distinct regions mainly composed of hydrophobic residues (38−49, 95−102, 191−200, 243−248, and 251−260). The peptides (Asn_{88}−Phe_{99}, Phe_{99}−Leu_{105}, Leu_{242}−Leu_{252}, Val_{243}−Leu_{252}, Gly_{251}−Ile_{259}, and Glu_{256}−Phe_{261}) that include or partially overlap with those fragments were found in the present study. Furthermore, the fragment Leu_{124}−Phe_{134} contained the epitope region 127−136 that was shown to be reactive against antisera from orally challenged mice (*28*).

In addition, Honma et al. (*29*) identified an allergenic epitope of OVA in the C-terminal region (residues 347−385), which was very accessibly located at the surface of the protein. Five peptides found in this study (Ala_{351}−Leu_{365}, Phe_{358}−Leu_{365}, Phe_{358}−Phe_{366}, Arg_{359}−Phe_{366}, and Phe_{378}−Pro_{385}) are related to that region. The latter is also similar to the sequence 375−384, reported as an IgE epitope in sensitized mice (*28*). However, while Ala_{351}−Leu_{365} resembles the synthetic peptide 347−366, which is thought to contain two epitopes that are recognized by IgE, and thus, is able to release histamine from basophils or mast cells (*29*), Phe_{358}−Leu_{365}, Phe_{358}−Phe_{366}, and Arg_{359}−Phe_{366} are very close to the haptenic peptide 357−366, which most likely contains only one IgE epitope and inhibits histamine release by human basophils by blocking the reaction between IgE molecules and multivalent peptides (which contain two or more allergenic determinants located at the right distance) (*29*). Interestingly, the peptide sequences Phe_{358}−Leu_{365}, Phe_{358}−Phe_{366}, and Arg_{359}−Phe_{366} either correspond or include the antihypertensive peptides FRADHPFL and RADHPFL (*30*−*32*). In fact, several previous studies have reported that the hydrolysis of egg white with pepsin releases antihypertensive peptides and that the simultaneous application of high pressure can be used to promote the quick production of specific active sequences (*18*). This is the case of certain peptides found in the present study that could exert their antihypertensive effect through antioxidant and/or angiotensin-I-converting enzyme inhibitory mechanisms (such as YAEERYPIL, peak 11; *31*−*33*) or through a vascular-relaxing mechanism (such as ESIINF, peak 19 or YRGGL-EPINF, peak 20; *34*).

Similarly, several fragments were found that corresponded to Val_{175}−Phe_{180}, Val_{175}−Leu_{183}, Ala_{177}−Trp_{184}, Ala_{177}−Ala_{187}, Ile_{178}−Trp_{184}, and Ile_{178}−Ala_{187}, all related to the fragment 173−196 that, according to Ben Nasser et al. (*35*), contains a T cell determinant of OVA and promotes oral tolerance to this protein. This fragment stimulates proliferation and cytokine [interkeukin (IL)-2] production by OVA-specific T helper (Th)1 cells, in turn suppressing the IgE response to OVA in rats. Furthermore, the peptides Lys_{322}−Gly_{343} and Ala_{329}−Gly_{348} are related to the immunodominant B and T cell epitope 323−339 (*36*). This peptide was tested for its ability to decrease the risk of anaphylaxis inherent in conventional-specific immunotherapy, by combining the loss of IgE bridging with the ability to restore the balance between Th1 and Th2 activity. However, it failed to induce T-cell-specific tolerance, characterized by a diminished Th2 response and a reduced IL-4 and IL-5 production. In contrast, it was found that the fragment 323−339 enhanced Th2 responses (*37, 38*).

The results obtained support the hypothesis that OVA is hydrolyzed by pepsin under pressure following a "progressive proteolysis" mechanism. This implies that high pressure leads to a rapid removal of the intact protein, while intermediate fragments are proteolyzed at a slower rate. The accelerated proteolysis of the OVA substrate under pressure leads to the production in 10 min of hydrolysates with lower antigenicity than those produced in

Table 4. Peptide Sequences Arising from the Hydrolysis of OVA with Pepsin under High-Pressure Conditions (400 MPa, 1:20 E/S, and 10 min), as Determined by RP-HPLC−MS/MS

peak[a]	calculated mass[b]	protein fragment	sequence	OVA epitopes reported in humans	OVA epitopes reported in model animals
1	918.6	276−282	RKIVYL		275−280[c]
2	1047.6	275−282	ERKIVYL		275−280[c]
3	1176.7	274−282	EERKIVYL		275−280[c]
4	1424.7	88−99	NQITKPNDVYSF	95−102[d], 41−172[e]	
5	2285.2	322−343	KISQAVHAAHAEINEAGREVVG	323−339[f], 301−385[e]	323−332[c], 323−339[g,h]
6	911.4	378−385	FFGRCVSP	347−385[i], 301−385[e]	375−384[c]
7	1774.9	84−98	RDILNQITKPNDVYS	41−172[e]	
8	1001.5	358−365	FRADHPFL	347−385[i], 301−385[e]	
9	822.4	216−222	LFRVASM		
10	1496.8	217−229	FRVASMASEKMKI		
11	1152.6	106−114	YAEERYPIL	41−172[e]	
12	792.5	99−105	FSLASRL	95−102[d], 41−172[e]	
13	675.4	178−183	IVFKGL		
14	722.4	211−216	MYQIGL		
15	1032.5	282−289	LPRMKMEEK		
16	1579.8	102−114	ASRLYAEERYPIL	41−172[e]	103−108[c]
17	1160.6	223−232	ASEKMKILEL		
18	746.5	177−183	AIVFKGL		
19	721.4	256−261	ESIINF	251−260[d]	
20	1164.6	125−134	YRGGLEPINF		127−136[c]
21	854.4	210−216	MMYQIGL		
22	933.5	230−238	LELPFASGT		
23	1040.6	243−252	VLLPDEVSGL	243−248[d]	
24	808.4	291−297	YNLTSVL		
25	1000.5	251−259	GLEQLESII	251−260[d]	
26	661.4	175−180	VNAIVF		173−196[j]
27	1939.0	13−28	DVFKELKVHHANENIF	11−19[k]	
28	1674.8	351−365	AASVSEEFRADHPFL	347−385[i], 301−385[e]	
29	1189.7	178−187	IVFKGLWEKA		173−196[j]
30	1001.5	359−366	RADHPFLF	347−385[i], 301−385[e]	
31	959.6	175−183	VNAIVFKGL		173−196[j]
32	1277.7	124−134	LYRGGLEPINF	41−172[e]	127−136[g]
33	852.4	299−306	AMGITDVF		
34	1270.7	177−187	AIVFKGLWEKA		173−196[j]
35	2628.3	148−171	WVESQTNGIIRNVLQPSSVDSQTA	41−172[e]	
36	739.4	212−217	YQIGLF		
37	1148.6	358−366	FRADHPFLF	347−385[i], 301−385[e]	
38	1852.0	217−232	FRVASMASEKMKILEL		
39	1936.9	329−348	AAHAEINEAGREVVGSAEAG	323−339[f], 301−385[e]	323−332[c], 323−339[g,h]
40	983.5	298−306	MAMGITDVF		
41	861.5	178−184	IVFKGLW		173−196[j]
42	1153.7	242−252	LVLLPDEVSGL	243−248[d]	
43	1282.6	230−241	LELPFASGTMSM		
44	932.5	177−184	AIVFKGLW		173−196[j]
45	1523.8	228−241	KILELPFASGTMSM		
46	2070.0	223−241	ASEKMKILELPFASGTMSM		
47	2614.3	218−241	RVASMASEKMKILELPFASGTMSM		
48	1395.7	229−241	ILELPFASGTMSM		
49	2761.4	217−241	FRVASMASEKMKILELPFASGTMSM		

[a] Peak numbers refer to **Figure 4**. [b] Monoisotopic mass for the neutral molecule, calculated from the amino acid sequence. [c] From ref *38*. [d] From ref *26*. [e] From ref *27*. [f] From ref *36*. [g] From ref *37*. [h] From ref *38*. [i] From ref *29*. [j] From ref *35*. [k] From ref *39*.

hours at atmospheric pressure. However, those hydrolysates retain residual IgG- and IgE-binding properties because of the accumulation of long and hydrophobic peptides during the initial stages of hydrolysis. These fragments are formed to a much greater extent than during proteolysis at atmospheric pressure, being reduced as proteolysis evolves.

It seems that, under the present conditions, the exposure of new peptide targets because of conformational changes of OVA under pressure only partially facilitated the removal of allergenic epitopes. However, it remains to be demonstrated whether most of the peptides formed retain just one or more relevant IgE-binding epitopes. Taking this into account and considering that the peptide pattern and the immunoreactivity of hydrolysates obtained under high pressure can be modified by the selection of the appropriate enzyme, pressure, and hydrolysis time, it could be possible to design hydrolysates with reduced allergenicity for not sensitizing patients at risk but capable of helping the immune system to develop a tolerance to egg proteins.

LITERATURE CITED

(1) Mine, Y.; Yang, M. Concepts of hypoallergenicity. In *Bioactive Egg Compounds*; Huopalahti, R., López-Fandiño, R., Anton, M., Schade, R., Eds.; Springer-Verlag: Heidelberg, Germany, 2007; Vol. 14, pp 5−158.

(2) Bufe, A. The biological function of allergens: Relevant for the induction of allergic diseases. *Int. Arch. Allergy Immunol.* **1988**, *117*, 215–219.

(3) Walsch, B. J.; Hill, D. J.; Macoun, P.; Cairns, D.; Howden, M. E. Detection of four distinct groups of hen egg allergens binding IgE in the sera of children with egg allergy. *Allergol. Immunopathol.* **2005**, *33*, 183–191.

(4) Huntington, J. A.; Stein, P. E. Structure and properties of ovalbumin. *J. Chromatogr., B* **2001**, *756*, 189–198.
(5) Astwood, J. D.; Leach, J. N.; Fuchs, R. L. Stability of food allergens to digestion in vitro. *Nat. Biotechnol.* **1996**, *14*, 1269–1273.
(6) Honma, K.; Kohno, Y.; Saito, K.; Shimojo, N.; Tsunoo, H.; Niimi, H. Specificities of IgE, IgG and IgA antibodies to ovalbumin. Comparison of binding activities to denatured ovalbumin or ovalbumin fragments of IgE antibodies with those of IgG or IgA antibodies. *Int. Arch. Allergy Immunol.* **1994**, *103*, 28–35.
(7) Nishida, T.; Kondo, N.; Agata, H.; Fukutomi, O.; Shinoda, S.; Suzuki, Y.; Shimozawa, N.; Tomatsu, S.; Orii, T. Proliferative responses towards native, heat-denatured and pepsin treated ovalbumin by peripheal blood mononuclear cells from patients with hen's egg-sensitive atopic dermatitis. *Biotherapy* **1994**, *8*, 33–40.
(8) Taylor, S. L.; Lehrer, S. B. Principles and characteristics of food allergens. *Crit. Rev. Food Sci. Nutr.* **1996**, *36*, S91–S118.
(9) Høst, A.; Halken, S. Hypoallergenic formulas—When, to whom and how long: After more than 15 years we know the right indication. *Allergy* **2004**, *59* (Supplement 78), 45–52.
(10) Van Beresteijn, E. C. H.; Peeters, R. A.; Kaper, J.; Meijer, R. J. G. M.; Robben, A. J. P. M.; Schmidt, D. G. Molecular mass distribution, immunological properties and nutritive value of whey protein hydrolysates. *J. Food Prot.* **1994**, *57*, 619–625.
(11) Lechevalier, V.; Jeantet, R.; Arhaliass, A.; Legrand, J.; Nau, F. Egg white drying: Influence of industrial processing steps on proteins structure and functionalities. *J. Food Eng.* **2007**, *83*, 404–413.
(12) Bonomi, F.; Fiocchi, A.; Frøkiær, H.; Gaiaschi, A.; Iametti, S.; Poiesi, C.; Rasmussen, P.; Restani, P.; Rovere, P. Reduction of immunoreactivity of bovine β-lactoglobulin upon combined physical and proteolytic treatment. *J. Dairy Res.* **2003**, *70*, 51–59.
(13) Dufour, E.; Hervé, G.; Haertle, T. Hydrolysis of β-lactoglobulin by thermolysin and pepsin under high hydrostatic pressure. *Biopolymers* **1995**, *35*, 475–483.
(14) Stapelfeldt, H.; Petersen, P. H.; Kristiansen, K. R.; Qvist, K. B.; Skibsted, L. H. Effect of high hydrostatic pressure on the enzymatic hydrolysis of β-lactoglobulin B by trypsin, thermolysin and pepsin. *J. Dairy Res.* **1996**, *63*, 111–118.
(15) Maynard, F.; Weingand, A.; Hau, J.; Jost, R. Effect of high pressure treatment on the tryptic hydrolysis of bovine β-lactoglobulin AB. *Int. Dairy J.* **1998**, *8*, 125–133.
(16) Knudsen, J. C.; Otte, J.; Olsen, K.; Skibsted, L. H. Effect of high hydrostatic pressure on the conformation of β-lactoglobulin A as assessed by proteolytic peptide profiling. *Int. Dairy J.* **2002**, *12*, 791–803.
(17) Chicón, R.; Belloque, J.; Recio, I.; López-Fandiño, R. Influence of high hydrostatic on the proteolysis of β-lactoglobulin A by trypsin. *J. Dairy Res.* **2006**, *73*, 121–128.
(18) Quirós, A.; Chicón, R.; Recio, I.; López-Fandiño, R. The use of high hydrostatic pressure to promote the proteolysis and release of bioactive peptides from ovalbumin. *Food Chem.* **2007**, *104*, 1734–1739.
(19) Chicón, R.; Belloque, J.; Alonso, E.; Martín-Álvarez, P.; López-Fandiño, R. Hydrolysis under high hydrostatic pressure as a means to reduce the binding of β-lactoglobulin to IgE from human sera. *J. Food Prot.* **2008**, *7*, 1453–1459.
(20) Chicón, R.; López-Fandiño, R.; Alonso, E.; Belloque, J. Proteolytic pattern, antigenicity and serum IgE binding of β-lactoglobulin hydrolysates obtained by pepsin and high pressure treatments. *J. Dairy Sci.* **2008**, *91*, 928–938.
(21) Fu, T.-J.; Abbot, U. R.; Hatzos, C. Digestibility of food allergens and nonallergenic proteins in simulated gastric fluid—A comparative study. *J. Agric. Food Chem.* **2002**, *50*, 7154–7160.
(22) Taylor, S. L. Comment on digestibility of food allergens and nonallergenic proteins in simulated gastric fluid—A comparative study. *J. Agric. Food Chem.* **2003**, *51*, 5183–5184.
(23) Smith, C.; Galazka, V. B.; Wellner, N.; Summer, I. G. High pressure unfolding of ovalbumin. *Int. J. Food Sci. Technol.* **2000**, *35*, 361–370.
(24) Van der Plancken, I.; Van Loey, A.; Hendricks, M. E. G. Combined effect of high pressure and temperature on selected properties of egg white proteins. *Innovative Food Sci. Emerging Technol.* **2005**, *6*, 11–20.
(25) Mine, Y.; Zhang, J. W. Comparative studies on antigenicity and allergenicity of native and denatured egg white proteins. *J. Agric. Food Chem.* **2002**, *50*, 2679–2683.
(26) Mine, Y.; Rupa, P. Fine mapping and structural analysis of immunodominant IgE allergenic epitopes in chicken ovalbumin. *Protein Eng.* **2003**, *16*, 747–752.
(27) Kahlert, H.; Petersen, A.; Becker, W. M.; Schlaak, M. Epitope analysis of the allergen ovalbumin (Gal d II) with monoclonal antibodies and patients' IgE. *Mol. Immunol.* **1992**, *29*, 1191–1201.
(28) Mine, Y.; Yang, M. Epitope characterization of ovalbumin in BALB/c mice using different entry routes. *Biochim. Biophys. Acta, Proteins Proteomics* **2007**, *1774*, 200–212.
(29) Honma, K.; Kohno, Y.; Saito, K.; Shimojo, N.; Horiuchi, T.; Hayashi, H.; Suzuki, N.; Hosoya, T.; Tsunoo, H.; Niimi, H. Allergenic epitopes of ovalbumin (OVA) in patients with hen's egg allergy: Inhibition of basophil histamine release by haptenic ovalbumin peptide. *Clin. Exp. Immunol.* **1996**, *103*, 446–453.
(30) Fujita, H.; Usui, H.; Kurahashi, K.; Yoshikawa, M. Isolation and characterization of ovokinin, a bradykinin B1 agonist peptide derived from ovalbumin. *Peptides* **1995**, *16*, 785–790.
(31) Miguel, M.; Recio, I.; Gómez-Ruiz, J. A.; Ramos, M.; López-Fandiño, R. Angiotensin I-converting enzyme inhibitory activity of peptides derived from egg white proteins by enzymatic hydrolysis. *J. Food Prot.* **2004**, *67*, 1914–1920.
(32) Miguel, M.; López-Fandiño, R.; Ramos, M.; Aleixandre, M. A. Short-term effect of egg white hydrolysate products on the arterial blood pressure of hypertensive rats. *Br. J. Nutr.* **2005**, *94*, 731–737.
(33) Dávalos, A.; Miguel, M.; Bartolomé, B.; López-Fandiño, R. Antioxidant activity of peptides derived from egg white proteins by enzymatic hydrolysis. *J. Food Prot.* **2004**, *67*, 1939–1944.
(34) Miguel, M.; Manso, M.; Aleixandre, M. A.; Alonso, M. J.; Salaices, M.; López-Fandiño, R. Vascular effects, angiotensin I-converting enzyme (ACE)-inhibitory activity, and antihypertensive properties of peptides derived from egg white. *J. Agric. Food Chem.* **2007**, *55*, 10615–10621.
(35) Ben Nasser, I.; Boyoka, P. N.; Ben Aissa, F. F.; Jeddi, M.; Tomé, D. The [173−196] fragment of ovalbumin suppresses ovalbumin-specific rat IgE responses. *Int. Immunopharmacol.* **2003**, *3*, 1569–1579.
(36) Elsayed, S.; Apold, J.; Holen, E.; Vik, H.; Florvaag, E.; Dybendal, T. The structural requirements of epitopes with IgE binding capacity demonstrated by three major allergens from fish, egg and tree pollen. *Scand. J. Clin. Lab. Invest.* **1991**, *Supplement 204*, 17–31.
(37) Janssen, E. M.; Wauben, M. H.; Jonker, E. H.; Hofman, G.; Van Eden, W.; Nijkamp, F. P; Van Oosterhout, A. J. Opposite effects of immunotherapy with ovalbumin and the immundominant T-cell epitope on airway eosinophilia and hyperresponsiveness in a murine model of allergic asthma. *Am. J. Respir. Cell Mol. Biol.* **1999**, *21*, 21–29.
(38) Barbey, C.; Donatelly-Dufour, N.; Batard, P.; Corradin, G.; Spertini, F. Intranasal treatment with ovalbumin but not the major T cell epitope ovalbumin 323−339 generates interleukin-10 secreting T cells and results in the induction of allergen systemic tolerance. *Clin. Exp. Allergy* **2004**, *34*, 654–662.
(39) Elsayed, S.; Stavseng, L. Epitope mapping of region 11−70 of ovalbumin (Gal d I) using five synthetic peptides. *Int. Arch. Allergy Immunol.* **1994**, *104*, 65–71.

Received for review July 30, 2008. Revised manuscript received October 15, 2008. Accepted October 23, 2008. This work was supported by the projects AGL-2005-03384, CSD-2007-00063 (Ministerio de Educación y Ciencia, Spain), and S-0505/AGR/0153 (Comunidad Autónoma de Madrid, Spain).

JF8023613