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Food Research International

journal homepage: www.elsevier.com/locate/foodres

Hypoallergenic hydrolysates of egg white proteins modulate allergen responses induced *ex vivo* on spleen cells from sensitized mice

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ARTICLE INFO

Article history:

Received 29 July 2016

Received in revised form 19 September 2016

Accepted 20 September 2016

Available online xxxx

Keywords:

Food allergy

Hypoallergenic egg white hydrolysates

Immunomodulatory peptides

BALB/c mouse

Th1 and Th2 cytokines

ABSTRACT

This study describes the *in vivo* allergenicity of enzymatic hydrolysates of egg white proteins (ovalbumin, lysozyme and ovomucoid) and explores the possibility that they could modulate T cell cytokine responses to egg allergens *ex vivo*, using splenocytes from BALB/c mice sensitized to individual egg proteins or to their mixtures in different proportions. The hydrolysate of ovalbumin with pepsin could be regarded as a good candidate for peptide-based immunotherapy on the grounds of its reduced ability to trigger allergic symptoms in a passive cutaneous anaphylaxis assay and its potential to reduce Th2 responses (release of IL-4 and IL-5) induced by egg allergens in the spleen cell cultures, but also to enhance Th1 responses (release of TNF- α and IFN- γ). While it is possible to obtain chromatographic fractions containing peptides with different Th2-inhibiting or promoting properties, as judged by cytokine production, selective peptide enrichment did not lead to an increase in the immunomodulating efficiency as compared with the whole ovalbumin hydrolysate, possibly due to the presence in the latter of a combination of immunogenic peptides with synergistic or adjuvant actions.

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1. Introduction

Egg allergy is common among European children below the age of three, with a prevalence ranging between 0.2 and 2%, as judged by objective appraisals (Nwaru et al., 2014). Despite many children spontaneously outgrow this condition, up to 15–20% remain allergic (Sicherer et al., 2014). Within the population with persistent egg allergy, the severity of the adverse reactions to egg usually increases over the years and the strict adherence to an avoidance diet critically decreases their quality of life (Martorell et al., 2013).

Oral immunotherapy using raw whole egg is one of the most promising treatment options for egg allergy (Vickery, 2012). Egg oral immunotherapy has shown to be efficient in the induction of desensitization, with a rate of success between 50 and 90% (Iacono et al., 2013; Meglio et al., 2013; Perezábad et al., 2015). However, the high number of associated adverse reactions has prompted the investigation of strategies aimed at reducing the allergenicity of the treatment preparations while

maintaining or improving their immunomodulating potential. In this respect, peptides represent a safer alternative to full allergens, as they produce fewer side effects and increase adherence to therapy (Casale & Stokes, 2011; Moldaver & Larché, 2011; Yang, Yang, & Mine, 2010).

T cell epitopes have been identified from several food allergens *in silico* and *ex vivo*, by screening the proliferative responses and cytokines released by peripheral blood mononuclear cells (PBMCs) from allergic patients or by tetramer-guided epitope mapping (Pascal, Konstantinou, Masilamani, Lieberman, & Sampson, 2013; Prickett et al., 2011). In this respect, it should be mentioned that, in the search of T cell-activating peptides for immunotherapy, those which stimulate a predominant Th2 cytokine profile in PBMCs or specific T cells from allergic patients are regarded as the best option (Pascal et al., 2013; Pastorello et al., 2010; Prickett et al., 2011; Tordesillas et al., 2009). However, and as suggested in mice experiments, peptides that do not induce Th2 cytokines can play a role in regulating allergic responses and even provide and extra help, by deviating predominantly Th2 to Th1 responses or down-regulating both Th2 and Th1 responses (Prioult, Pecquet, & Fliss, 2005; Yang & Mine, 2009; Wai, Leung, Leung, & Chu, 2016).

Enzymatically digested allergens could be a more practical therapeutic option than synthetic dominant T cell epitopes, as the former would provide multiple allergen-derived peptides with adequate T cell-stimulating capacities for HLA-diverse patients (Kulis et al., 2012; Yang et al., 2009). Previous work has shown that low IgE-binding hydrolysates of the main egg allergens: ovalbumin (OVA), lysozyme (LYS) and ovomucoid (OM), modify the cellular and humoral immune responses of murine spleen and mesenteric lymph node cells, and

Abbreviations: AE-OP, peptide fractions of the hydrolysate of OVA with pepsin separated by anion exchange FPLC; CT, cholera toxin; EW, egg white; LA, hydrolysate of LYS with alcalase; LP, hydrolysate of LYS with pepsin; LYS, lysozyme; MA, hydrolysate of OM with alcalase; MP, hydrolysate of OM with pepsin; OA, hydrolysate of OVA with alcalase; OM, ovomucoid; OP, hydrolysate of OVA with pepsin; OVA, ovalbumin; PBMCs, peripheral blood mononuclear cells; PCA, passive cutaneous anaphylaxis; RP-OP, peptide fractions of the hydrolysate of OVA with pepsin separated by RP-HPLC; TFA, trifluoroacetic acid.

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Please cite this article as: Lozano-Ojalvo, D., et al., Hypoallergenic hydrolysates of egg white proteins modulate allergen responses induced *ex vivo* on spleen cells from sensitized mice, *Food Research International* (2016), <http://dx.doi.org/10.1016/j.foodres.2016.09.021>

human PBMCs to different stimuli, suggesting potential activities that could be used to polarize Th2 and Th1 immune functions in the control of inflammatory, pro-oxidant or allergic conditions (Lozano-Ojalvo, Molina, & López-Fandiño, 2016a; Lozano-Ojalvo, Molina, & López-Fandiño, 2016b). In this work, we assess their allergenicity *in vivo*, by means of passive cutaneous anaphylaxis tests (PCA), and explore the possibility that they could modulate cytokine responses to egg allergens *ex vivo*, using splenocytes from BALB/c mice sensitized to individual egg proteins or to their mixtures in different proportions. Finally, the hydrolysates were fractionated, in an attempt to enrich the immunostimulating and immunomodulating peptides, and the sequences contained in each fraction were identified by mass spectrometry.

2. Materials and methods

2.1. Enzymatic hydrolysates of egg white proteins

OVA grade VI, LYS, OM and porcine pepsin (EC 3.4.23.1, 3440 U/mg) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and alcalase (EC 3.4.21.62, 2.4 U/g) from Novozymes A/S (Bagsvaerd, Denmark). Crude egg white (EW) was obtained in the laboratory from chicken fresh shell eggs and, following lyophilization, its protein content was determined by the Kjeldahl method. The lipopolysaccharide level of proteins and enzymes was quantified by the Pierce LAL Chromogenic Endotoxin Quantitation Kit (Thermo Scientific, Waltham, MA, USA) and, when required, reduced to <3 EU/mg by size exclusion chromatography (Pablos-Tanarro, López-Expósito, Lozano-Ojalvo, López-Fandiño, & Molina, 2016).

Hydrolyses were carried out as explained by Lozano-Ojalvo et al. (2016a). Briefly, proteins were dissolved (5 mg/mL) in Milli-Q water (adjusted to pH 1.5 with HCl) or in phosphate buffer (pH 7.0) and treated, respectively, with 172 U/mg of pepsin at 37 °C for 24 h, followed by neutralization to pH 7.0, or with 0.005 U/mg of alcalase at 50 °C for 60 min. Enzymes were inactivated by heating at 95 °C for 15 min and samples were centrifuged at 5000 g for 10 min. The concentration of the hydrolysates and their fractions (obtained as indicated below) was adjusted according to their protein content, as determined by the bicinchoninic acid assay (Pierce BCA Protein Assay Kit, Thermo Scientific).

2.2. Isolation of peptide fractions

Semi-preparative RP-HPLC was performed on a Waters Series 600 HPLC equipped with Millennium 3.2 Software for data acquisition (Waters Corporation, Milford, MA, USA), using a Prep Nova Pak® HR C18 column (300 × 7.8 mm i.d., 6 µm particle size; Waters). The operating conditions were: column temperature, 30 °C; flow rate, 4 mL/min; injection volume, 400 µL (4 mg of protein); solvent A, 1 mL/L trifluoroacetic acid (TFA) in Milli-Q water and solvent B, 0.8 mL/L TFA in HPLC grade acetonitrile. Elution was conducted with a linear gradient of solvent B in A from 0 to 60% B in 100 min, followed by washing with 100% B and conditioning the column. Five fractions were collected using Waters Fraction Collector II. Acetonitrile was removed by rotary evaporation and aliquots were lyophilized for further use.

Alternatively, fractionation was also conducted by anion exchange chromatography on an ÄKTA Explorer FPLC System (GE Healthcare, Freiburg, Germany) using a Mono Q HR 5/5 column (50 × 5 mm i.d., 10 µm particle size; GE Healthcare). The operating conditions were: flow rate, 1 mL/min; injection volume 1 mL (10 mg of protein); solvent A, 20 mM Tris pH 9.0 and solvent B, 20 mM Tris pH 9.0 containing 1 M NaCl. Elution was conducted with a linear gradient of solvent B in A from 0 to 100% B in 15 min, followed by washing with 100% B and conditioning the column. Two fractions were collected using Fraction Collector Frac-950 (GE Healthcare). These were purified by solid phase

extraction on OASIS HLB Plus-short-cartridges (225 mg, 60 µm; Waters) and lyophilized for further use.

2.3. Peptide identification

RP-HPLC with UV detection (214 nm), connected on-line to an electrospray ion source and a quadrupole ion trap mass analyser (ESI-MS/MS), was performed using an Agilent 1100 Series HPLC equipment (Agilent Technologies, Waldbronn, Germany) with a Hi-Pore® Reversed Phase RP-318 Column (250 × 4.6 mm i.d.; Bio-Rad Laboratories, CA, USA) and an Esquire 3000 mass spectrometer (Bruker Daltonik, Bremen, Germany). The operating conditions were: flow rate, 0.8 mL/min; injection volume, 50 µL; solvent A, 0.37 mL/L TFA in Milli-Q water and solvent B, 0.27 mL/L TFA in HPLC grade acetonitrile. Elution was conducted with a linear gradient of solvent B in A from 0 to 70% in 75 min, followed by 100% B for 30 min. Ion source parameters were: nebulizer pressure, 60 psi; dry gas, 8 L/min and dry temperature, 350 °C. Using Data Analyses TM (version 3.0; Bruker Daltonik), the *m/z* spectral data were processed and transformed to spectra representing mass values. Biotools (version 2.1; Bruker Daltonik) was used to process the MS(n) spectra and Mascot software (version 2.3.3, Matrix Science, London, UK) to perform peptide sequencing. For each sample, a minimum Mascot score corresponding to *P* < 0.05 was considered as a prerequisite for validation of peptide identification.

2.4. Mice sensitization and spleen stimulation

Six-week-old female specific-pathogen-free BALB/c mice, purchased from Charles River Laboratories (Saint Germain sur l'Arbresle, France), were sensitized with 1 mg of either OVA, OM or LYS, plus 10 µg of cholera toxin (CT, List Biologicals, Campbell, USA), or poly-sensitized with a mixture of 1 mg of each of the three proteins plus 10 µg of CT, on days 0, 1, 2, 7, 14, 21, 28, 35 and 42 by oral gavage. Another mouse group was similarly sensitized with 5 mg of EW plus 10 µg of CT. Naïve mice just received sterile sodium phosphate buffer. Each group consisted of 5 mice. On day 49, mice were euthanized by CO₂ inhalation. Sera were collected by centrifugation of the blood at 1500 ×g for 15 min and kept at −20 °C until used. The concentration of OVA-, LYS- and OM-specific antibodies was determined as explained in Pablos-Tanarro et al. (2016). All protocols involving animals followed the European legislation (Directive 2010/63/UE) and were approved by the CSIC Bioethics Committee and the Comunidad de Madrid (Ref PROEX 089/15).

Spleen cells were collected and processed under sterile conditions. Pooled splenocytes from each group (*n* = 5) were cultured in 48-well plates (4 × 10⁶ cells/mL) and stimulated in triplicate with concanavalin A (2.5 µg/mL), as positive control; RPMI-1640 medium, as negative control; and the intact proteins, their hydrolysates or peptide fractions at different concentrations (from 10 to 200 µg/mL). Inactivated enzymes were also tested at a concentration equivalent to that present in 200 µg/mL of the hydrolysates. The immunomodulating effect of the hydrolysates and their selected fractions, at a concentration of 200 µg/mL, was also tested concomitantly with the intact allergens (200 µg/mL).

Supernatants were collected after 72 h at 37 °C in 5% CO₂, and stored at −80 °C until analysis of IL-4, IL-5 and TNF-α by ELISA using commercial kits (eBioscience). In case of the supernatants of cell cultures incubated with the peptide fractions, the levels of IL-4, IL-5, IL12p70 and IFN-γ were determined by Luminex (eBioscience).

2.5. Passive cutaneous anaphylaxis (PCA)

PCA tests were performed as described by López-Expósito, Chicón, Belloque, López-Fandiño, and Berin (2012). Briefly, naïve BALB/c mice were injected intradermally in the right ear pinna with 20 µL of pooled sera (*n* = 5) from OVA-, LYS- or OM-sensitized mice and with pooled sera from naïve mice in the left ear pinna. Twenty four hours later, mice (2 per test) were injected intravenously with 200 µg of the

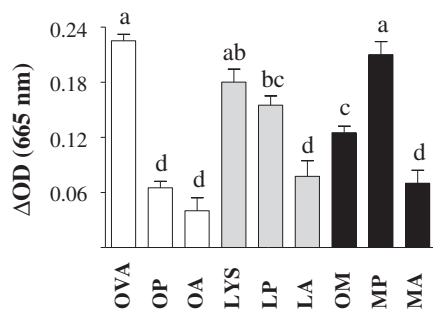


Fig. 1. Allergenicity of the egg proteins ovalbumin (OVA), lysozyme (LYS), ovomucoid (OM) and their hydrolysates with pepsin (OP, LP and MP, respectively) and alcalase (OA, LA and MA, respectively) assessed by passive cutaneous anaphylaxis tests. Allergen-specific dye extravasation was calculated by correcting the allergen or hydrolysate induced absorbance with the absorbance of the supernatant of the ear injected with pooled naïve sera. Data are expressed as mean $\pm$ SEM. Different letters indicate statistically significant differences ($P < 0.05$).

respective intact protein (OVA, OM or LYS) or the corresponding hydrolysates in 100 μ L of 1% Evans blue dye (Sigma-Aldrich). One hour after protein administration, mice were euthanized by CO₂ inhalation and ears were individually collected and incubated overnight in *N,N*-

dimethylformamide (Sigma-Aldrich). The absorbance of the supernatant was measured at 655 nm in order to quantify the extravasation of the dye.

2.6. Statistical analysis

All data were presented as means $\pm$ standard error of the mean (SEM) and subjected to ANOVA followed by Tukey *post hoc* multiple-comparison test using GraphPad Prismv5 (GraphPad Software Inc., San Diego, USA). P values < 0.05 were considered statistically significant.

3. Results

3.1. Capacity to elicit allergic responses in vivo

The ability of the egg white protein hydrolysates to trigger cutaneous allergic reactions was assessed *in vivo* using PCA tests with sera of mice mono-sensitized to OVA, LYS or OM (Fig. 1). The concentration of specific antibodies of the sera used for passive sensitization were: i) in OVA-sensitized mice: OVA-specific IgE, 1020.2 ± 192.9 ng/mL and OVA-specific IgG1, $10,667.9 \pm 2517.5$ ng/mL; ii) in LYS-sensitized mice: LYS-specific IgE 54.0 ± 12.3 ng/mL and LYS-specific IgG1, $36,566.8 \pm 5567.8$ ng/mL; iii) in OM-sensitized mice: OM-specific IgE, 869.4 ± 1146.3 ng/mL and OM-specific IgG1, $29,006.3 \pm 1036.8$ ng/

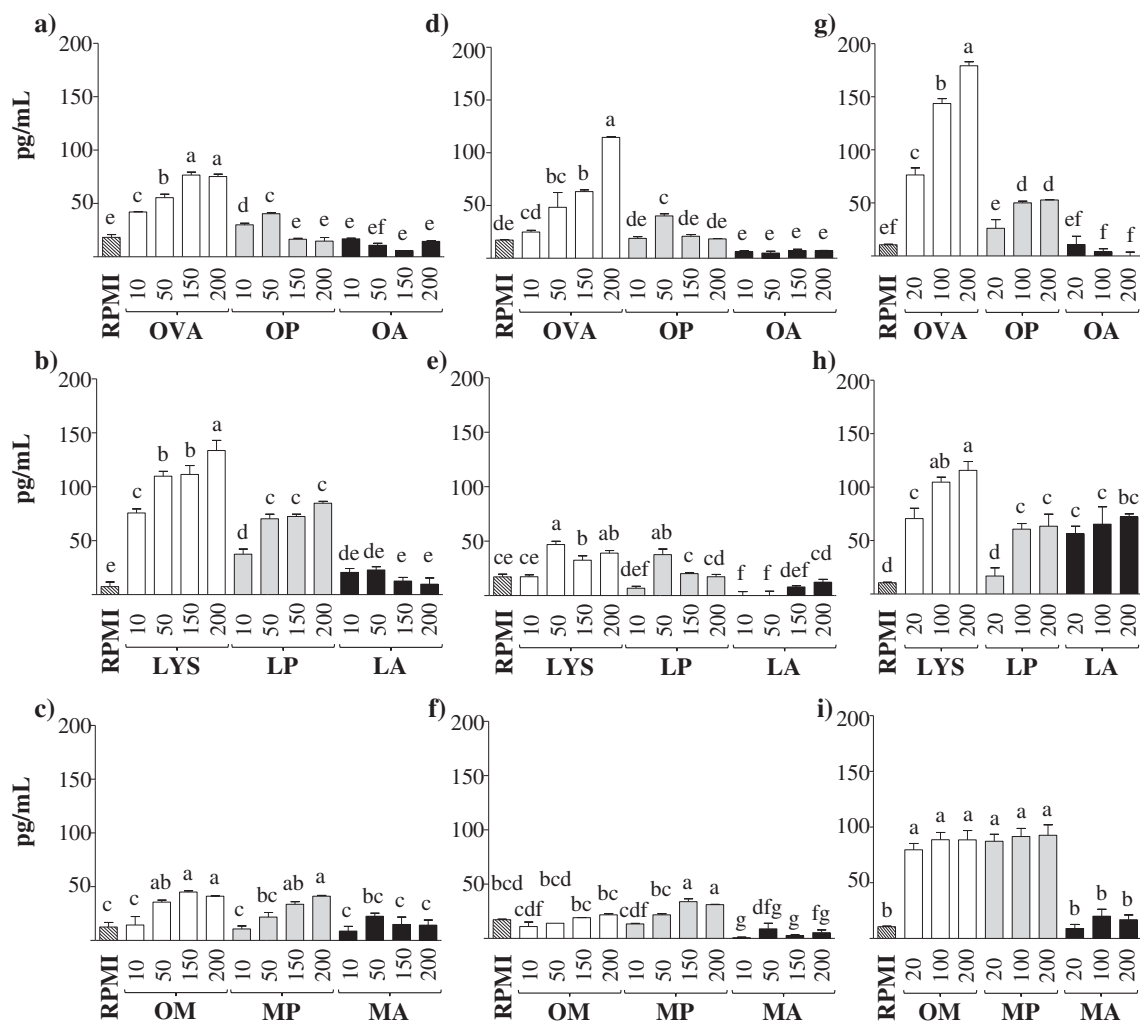


Fig. 2. Immunostimulating effects of different concentrations (from 10 to 200 μ g/mL) of ovalbumin (OVA), lysozyme (LYS), ovomucoid (OM) and their hydrolysates with pepsin (OP, LP and MP, respectively) and alcalase (OA, LA and MA, respectively) on the secretion of IL-4 by splenocytes from mono-sensitized (a, b and c), poly-sensitized (d, e and f) and egg white-sensitized (g, h and i) mice. Data are expressed as mean $\pm$ SEM. Different letters indicate statistically significant differences ($P < 0.05$).

mL. In sera from naïve mice, no IgE or IgG1 antibodies specific for egg proteins were detected. Intravenous challenge of mice with the respective allergens caused a significant extravasation, as measured by the localized release of Evans blue into the tissues, in the ears injected with sera from sensitized mice compared with the control ears, injected with sera from naïve mice.

In contrast with the corresponding intact protein, dye extravasation was significantly lower in mice challenged with the hydrolysate of OVA with pepsin and with the hydrolysates produced with alcalase, which indicates a diminished eliciting capacity (Fig. 1). However, the hydrolysates of LYS and OM with pepsin caused, respectively, a local reaction similar or even higher than that of the allergenic protein from which they derive.

3.2. Immunostimulating activity in spleen cells from sensitized mice

Spleen cells from mice sensitized to OVA, LYS or OM were stimulated with the protein used in each case for sensitization and its hydrolysates with pepsin and alcalase at various concentrations. As expected, incubation with the sensitizing protein induced a significant dose-dependent release of the Th2 cytokines IL-4 (Fig. 2a–c) and IL-5 (Supporting Information Fig. S1a–c). OVA and LYS, unlike OM, also increased the production of the Th1 cytokine TNF- α , although no clear dose-effect relationships were apparent (Fig. 3a–c).

In general terms, hydrolysis of OVA and LYS with pepsin reduced their Th2-stimulating capacity, while pepsin treatment did not affect the OM-induced secretion of IL-4 and IL-5. Hydrolysis of OVA, LYS and OM with alcalase abrogated their ability to stimulate Th2 responses in spleen cells, yielding Th2 cytokine levels equivalent to those of the negative control (RPMI) (Fig. 2a–c and Supporting Information Fig. S1a–c). The hydrolysates of OVA with pepsin and alcalase, at the highest concentrations (150 and 200 $\mu\text{g/mL}$), enhanced the production of TNF- α by the splenocytes compared with the intact protein, whereas no major differences were found between LYS, OM and their hydrolysates regarding TNF- α secretion (Fig. 3a–c).

Very similar results, concerning Th2 responses, were found in the splenocytes from mice poly-sensitized to the same amount of the three proteins, as well as in those from mice sensitized to EW (Fig. 2d–i and Supporting information Fig. S1d–i). With respect to Th1 cytokines, the hydrolysates of OVA, LYS and OM induced a lower production of TNF- α by the splenocytes from EW-sensitized mice than the proteins from which they derive (Fig. 3d–i). To discard an immunostimulating influence of the inactivated enzymes, their effect on splenocytes from EW-sensitized mice was evaluated at the maximum concentration used in the hydrolysates and no significant differences were observed in the production of the Th2 and Th1 cytokines studied as compared with the negative control (Supporting information Table S1).

Overall, our results indicate that the hydrolysates of OVA and LYS with pepsin and alcalase promoted a less pronounced *ex vivo* Th2

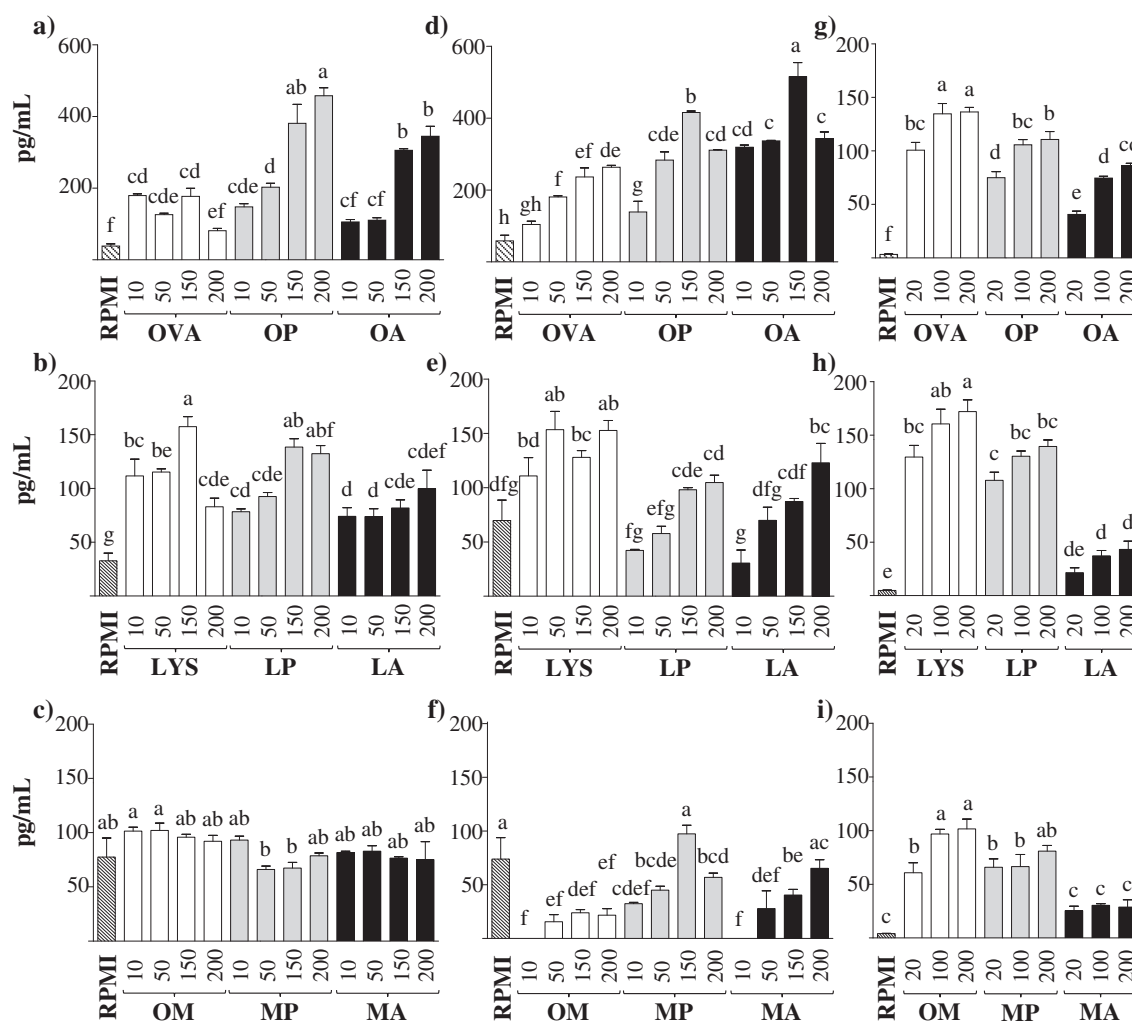


Fig. 3. Immunostimulating effects of different concentrations (from 10 to 200 $\mu\text{g/mL}$) of ovalbumin (OVA), lysozyme (LYS), ovomucoid (OM) and their hydrolysates with pepsin (OP, LP and MP, respectively) and alcalase (OA, LA and MA, respectively) on the secretion of TNF- α by splenocytes from mono-sensitized (a, b and c), poly-sensitized (d, e and f) and egg white-sensitized (g, h and i) mice. Data are expressed as mean $\pm$ SEM. Different letters indicate statistically significant differences ($P < 0.05$).

Table 1

Immunomodulating effects of the hydrolysates of ovalbumin (OVA), lysozyme (LYS) and ovomucoid (OM) with pepsin (OP, LP and MP, respectively) and alcalase (OA, LA and MA, respectively). Splenocytes from mono- and poly-sensitized mice were stimulated with the allergen (200 µg/mL) in the absence or presence of the hydrolysates (200 µg/mL), and the secretion of IL-4, IL-5 and TNF-α was evaluated. Data are expressed as mean ± SEM. Different letters indicate statistically significant differences ($P < 0.05$) among each allergen and its hydrolysates for each cytokine and type of sensitization.

		Mono-sensitized mice			Poly-sensitized mice (OVA + LYS + OM)		
		IL-4	IL-5	TNF-α	IL-4	IL-5	TNF-α
OVA	OVA	75.3 ± 2.2 ^a	478.1 ± 10.9 ^a	81.3 ± 6.6 ^c	114.6 ± 0.6 ^a	142.0 ± 5.8 ^a	219.6 ± 4.1 ^a
OVA + OP		28.9 ± 2.1 ^c	183.3 ± 9.5 ^b	451.9 ± 11.6 ^a	24.8 ± 0.6 ^b	65.6 ± 3.4 ^b	243.7 ± 16.2 ^a
OVA + OA		49.1 ± 2.4 ^b	113.5 ± 1.4 ^c	124.3 ± 3.4 ^b	18.9 ± 3.5 ^b	24.0 ± 1.8 ^c	208.1 ± 19.0 ^a
LYS	LYS	133.6 ± 9.5 ^a	493.3 ± 15.3 ^a	83.0 ± 8.1 ^b	39 ± 2.4 ^b	92.3 ± 5.0 ^b	106.9 ± 26.4 ^a
LYS + LP		165.3 ± 7.4 ^a	389.9 ± 18.4 ^b	162.7 ± 8.6 ^a	54.5 ± 2.8 ^a	121.1 ± 6.5 ^a	120.4 ± 12.3 ^a
LYS + LA		161.5 ± 11.1 ^a	352.7 ± 15.1 ^b	151.0 ± 6.9 ^a	35.9 ± 2.4 ^b	109.1 ± 2.5 ^{ab}	114.2 ± 7.9 ^a
OM	OM	41.2 ± 0.3 ^a	59.0 ± 6.7 ^a	92.1 ± 5.5 ^a	23.2 ± 0.85 ^b	37.8 ± 1.9 ^b	14.3 ± 4.0 ^b
OM + MP		69.0 ± 12.1 ^a	83.9 ± 2.3 ^a	91.2 ± 8.2 ^a	62.6 ± 11.3 ^a	131.0 ± 8.8 ^a	92.6 ± 7.5 ^a
OM + MA		54.5 ± 11.5 ^a	83.7 ± 8.1 ^a	79.8 ± 2.5 ^a	16.0 ± 1.2 ^b	33.7 ± 2.3 ^b	15.8 ± 0.8 ^b

activation of spleen cells from sensitized mice than the respective intact proteins, with comparable outcomes in simple (mono-sensitized) and complex (poly-sensitized or EW-sensitized) models of egg allergy. This prompted us to investigate whether they could down-regulate the allergic responses induced by the allergens from which they derive.

3.3. Immunomodulating activity in spleen cells from sensitized mice

In order to test whether the allergen-induced responses of immune cells from sensitized mice could be modulated by the hydrolysates, splenocytes were stimulated with the allergens used for sensitization in the presence of their corresponding hydrolysates at a concentration of 200 µg/mL of each substance, and the impact on the secretion of Th2 and Th1 cytokines was determined. OVA-induced production of IL-4 and IL-5 was inhibited by co-incubation of spleen cells from both OVA-sensitized and poly-sensitized mice with the hydrolysates of OVA with pepsin and alcalase ($P < 0.05$, Table 1). However, the hydrolysates of LYS only modestly reduced the secretion of IL-5 stimulated by LYS in splenocytes from mice mono-sensitized to this protein. The hydrolysates of OM failed to impair the production of these cytokines, which even increased when the hydrolysate of OM with pepsin was present in the culture ($P < 0.05$), in the case of the poly-sensitized animals. On the other hand, the hydrolysates of OVA and LYS with both enzymes tended to promote the release of the Th1 cytokine TNF-α, when used together with the intact proteins for stimulation of the spleen cells from mono-sensitized mice (Table 1).

In spleen cells from mice sensitized to EW, the hydrolysates of OVA, unlike those of LYS and OM, significantly reduced the EW-induced secretion of both IL-4 and IL-5 (the hydrolysate of LYS with pepsin also significantly decreased the release of IL-4). In addition, the hydrolysates of OVA and LYS with pepsin increased the production of TNF-α (Table 2). These results thus indicate that the hydrolysate of OVA with pepsin

was able to bias allergen-specific immune responses, helping to restore the Th1/Th2 balance.

3.4. Identification of peptides in the most active fractions

With the aim to enrich and identify bioactive peptide sequences, the hydrolysate of OVA with pepsin, which had shown the most promising properties in terms of reduction of Th2 immunity and enhancement of Th1 responses, was separated by RP-HPLC at a semi preparative scale into 5 fractions (labelled RP-OP1 to RP-OP5) and by anion exchange FPLC in 2 fractions (AE-OP1 and AE-OP2). These were collected after about 30 runs to obtain enough material to determine protein concentration and immunostimulating and immunomodulating activities on splenocytes from EW-sensitized mice, before being analysed by RP-HPLC-MS/MS.

In the hydrolysate of OVA with pepsin, RP-HPLC fractions RP-OP1 and RP-OP2 stimulated the lowest production of the Th2 cytokines IL-4 and IL-5, and RP-OP3, RP-OP4 and RP-OP5, the highest. Fraction RP-OP2 considerably reduced the production of both IL-4 and IL-5 induced by EW in the splenocytes, while RP-OP4 enhanced the production of IL-4 and did not modify that of IL-5 (Fig. 4a and c). Spleen cells from EW-sensitized mice responded to stimulation with fractions RP-OP3, RP-OP4 and RP-OP5 releasing IL-12p70. IL-12 favours dendritic cell-driven differentiation of CD4+ T cells to IFN-γ-producing Th1 cells when it is present at an early stage of T cell activation (Macatonia et al., 1995). Furthermore, when added together with EW, RP-OP4 and RP-OP5, as well as OP, enhanced the release of IL-12p70 (Fig. 4b). However, the whole hydrolysate promoted IFN-γ secretion induced by EW to a much higher extent than any of the individual RP-HPLC fractions (Fig. 4d). On the other hand, the first fraction obtained by anion exchange chromatography (AE-OP1) induced weaker Th2 (IL-4 and IL-5) and Th1 responses (IFN-γ), compared with the second fraction (AE-OP2), although it significantly enhanced IFN-γ production by EW (Fig. 4a-d).

The peptides present in the hydrolysate of OVA with pepsin and its isolated fractions were identified by mass spectrometry (Supporting information Tables S2–S9). In the RP-HPLC fractions, peptides ranging from 5 to 22 amino acids were found, with some coincident sequences, mainly between contiguous fractions. In turn, in AE-OP1 and AE-OP2, we identified, respectively, 67 and 64 peptides distributed along the whole OVA molecule, with 16 identical sequences, although they shared many analogous peptides with amino- and carboxy-terminal truncations. Fig. 5 shows the fragments identified in the most potent immunomodulatory fractions: RP-OP2, RP-OP4, AE-OP1 and AE-OP2 (the respective RP-HPLC chromatograms are shown in Supporting Information Figs. S2 and S3). The comparison between RP-OP2 and RP-OP4, which exerted distinct Th2 and Th1 cytokine-producing effects in combination with EW, showed a different occurrence of sequences within wide parts of the protein. In particular, RP-OP4 lacked peptides in the areas 98–227, 248–275, 285–360 and 366–386. Interestingly, fractions

Table 2

Immunomodulating effects of the hydrolysates of ovalbumin, lysozyme and ovomucoid with pepsin (OP, LP and MP, respectively) and alcalase (OA, LA and MA, respectively). Splenocytes from egg white (EW)-sensitized mice were stimulated with EW (200 µg/mL) in the absence or presence of the hydrolysates (200 µg/mL), and the secretion of IL-4, IL-5, and TNF-α was evaluated. Data are expressed as mean ± SEM. Different letters indicate statistically significant differences ($P < 0.05$) for each cytokine.

		EW-sensitized mice		
		IL-4	IL-5	TNF-α
EW		127.9 ± 13.0 ^a	161.7 ± 6.0 ^a	118.1 ± 10.3 ^{bcd}
EW + OP		59.1 ± 11.5 ^{bc}	67.6 ± 13.8 ^c	157.7 ± 6.9 ^a
EW + OA		35.6 ± 11.0 ^c	18.1 ± 3.4 ^d	107.9 ± 15.8 ^{cd}
EW + LP		104.7 ± 8.3 ^a	123.2 ± 7.8 ^b	148.6 ± 8.9 ^{ab}
EW + LA		94.8 ± 6.8 ^{ab}	162.6 ± 2.6 ^a	124.7 ± 8.3 ^a
EW + MP		115.9 ± 10.3 ^a	130.6 ± 12.3 ^{ab}	96.4 ± 2.2 ^{dc}
EW + LA		109.3 ± 4.2 ^a	135.5 ± 1.5 ^{ab}	83.4 ± 6.6 ^d

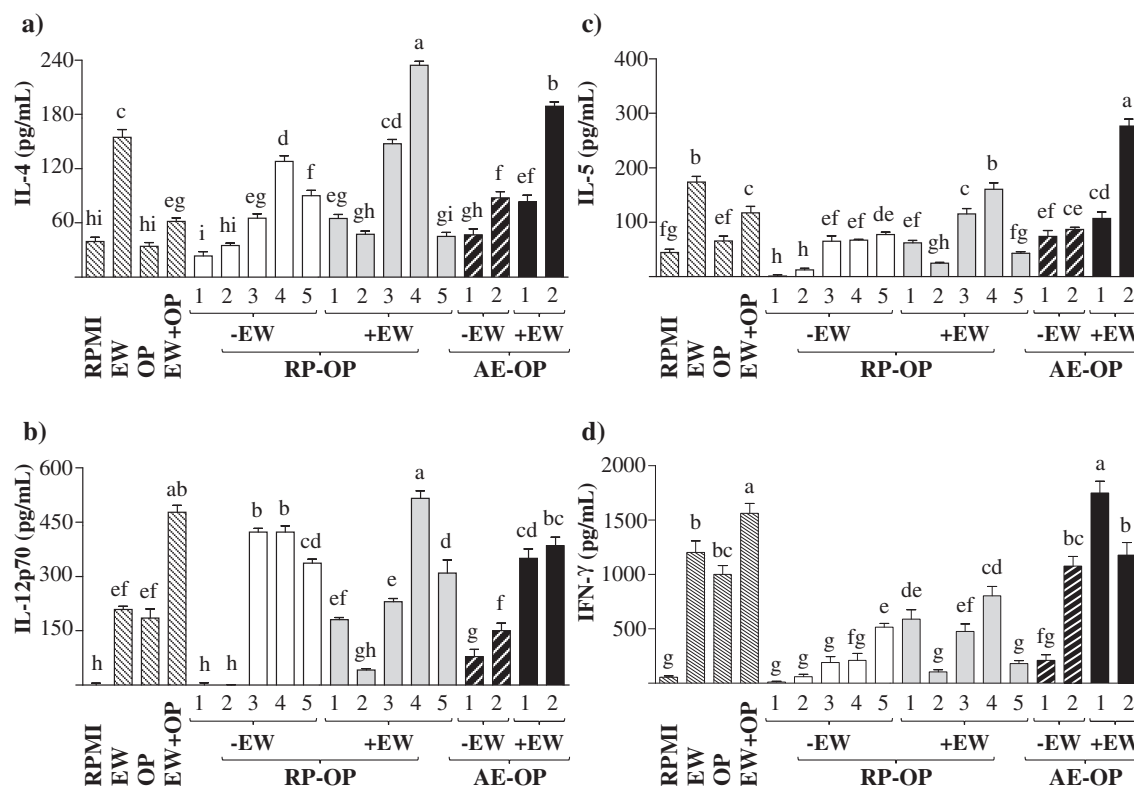


Fig. 4. Immunostimulating and immunomodulating effects of the fractions obtained from the hydrolysate of ovalbumin with pepsin (OP) by semi-preparative RP-HPLC (RP-OP1 to RP-OP5) and anion exchange chromatography (AE-OP1 and AE-OP2). Splenocytes from egg white (EW)-sensitized mice were stimulated with OP and its fractions (200 μ g/mL) in the absence or presence of EW (200 μ g/mL), and the secretion of IL-4 (a), IL-5 (b), IL-12p70 (c) and IFN- γ (d) was evaluated. Data are expressed as mean $\pm$ SEM. Different letters indicate statistically significant differences ($P < 0.05$).

RP-OP2 and AE-OP1, that down-regulated Th2 responses, shared 26 peptides in common, some of which, such as those within the areas 205–211 and 369–376, were absent from both RP-OP4 and AE-OP2, which exerted the opposite effect. However, the complex peptide composition of these fractions hindered the identification of sequences that could play a role to diminish Th2 responses and, in general, the correlation of peptide structure and immunoregulatory function.

4. Discussion

This study evidences the immunoregulatory role of OVA hydrolysed with pepsin and alcalase on splenocytes from mice orally sensitized to OVA, as well as on splenocytes from mice orally sensitized to identical amounts of OVA, OM and LYS. In both models, OVA hydrolysates induced a low Th2 activation and significantly suppressed the release of IL-4 and IL-5 promoted by intact OVA, stimulating the secretion of the Th1 cytokine TNF- α . Furthermore, OVA hydrolysates combined the capacity to modulate the immune response *ex vivo* with a reduced ability to trigger cutaneous allergic symptoms *in vivo* in a PCA assay.

Interestingly, the hydrolysates of OVA, also significantly inhibited the EW-induced secretion of IL-4 and IL-5 in mice sensitized to EW, which consists of 54% OVA, 11% OM and 3.4% w/w LYS. This observation suggests that the sole administration of OVA hydrolysates could result in significant protection against bystander allergens. In fact, immunotherapy with OM-depleted heated EW effectively protects from allergic reactions to BALB/c mice sensitized to whole EW, despite OM is being considered an immunodominant allergen in this mouse model (Jiménez-Saiz, Rupa, & Mine, 2011).

Previous results had shown that hydrolysates of egg white proteins with alcalase down-regulate the production of Th2 cytokines and the secretion of IgE by human PBMCs from non-allergic donors artificially

Th2-skewed by culture with IL-4 and anti-CD40; while, in the same cell model, hydrolysates with pepsin stimulated the restoration of the Th1/Th2 balance by the simultaneous decrease in IL-5 and IL-13 and increase in IFN- γ (Lozano-Ojalvo et al., 2016b). The present results basically confirm those findings, in accordance with Holvoet, Zuercher, Julien-Javaux, Perrot, and Mercenier (2013), who reported parallel outcomes with the non-antigen-specific model of Th2-skewed human PBMCs and allergen-primed murine splenocytes. However, the assay using spleen cells from egg white protein-sensitized mice provided a more refined characterization of the cytokine responses produced by the hydrolysates from OVA, LYS and OM, in line with the proven suitability of the BALB/c mouse model to establish mechanisms and preventive or therapeutic strategies against food allergy and the fact that the spleen is a source of both antigen-presenting cells and allergen-specific T cells (Pablos-Tanarro et al., 2016; Yang & Mine, 2009). Moreover, the use of splenocytes from sensitized BALB/c mice enabled the assessment of the ability of the hydrolysates to modulate the specific T cell responses induced by the sensitizing allergens, which allowed discriminating OVA hydrolysates as the most promising therapeutic compounds.

The observation that complete hydrolysis of OVA and LYS with pepsin reduced their ability to promote Th2 effector pathways highlights the importance that resistance to pepsin digestion plays in the allergenicity of these proteins (Benedé, López-Expósito, Molina, & López-Fandiño, 2015). In contrast, both intact OM and its hydrolysates with pepsin induced very similar cytokine profiles. OM (whose structure is stabilized by 9 disulphide bridges) is proteolysed by pepsin in minutes, but the resulting peptides form big fragments linked by disulphide bonds (Benedé, López-Fandiño, Reche, Molina, & López-Expósito, 2013). OM, therefore, behaves as certain food allergens that, despite being digested, retain the T cell stimulatory properties of the intact protein, such as Ara h 1 from peanut (Eiwegger et al., 2006).

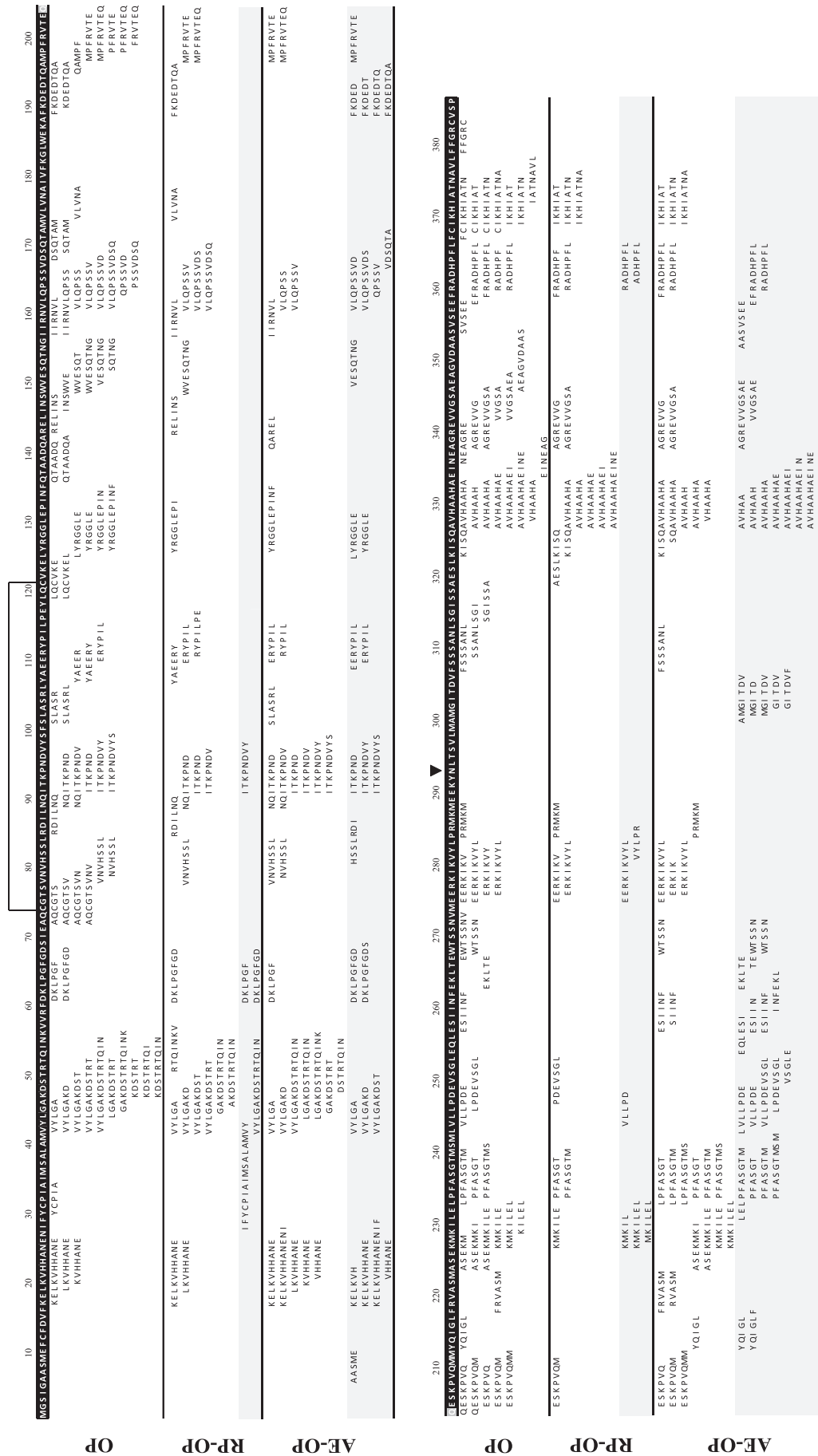


Fig. 5. Peptide sequences, identified by RP-HPLC-MS/MS (ESI-MS/MS), in the hydrolysate of ovalbumin with pepsin (OP), and in the fractions separated by semi-preparative RP-HPLC: RP-OP2 (white □) and RP-OP4 (light grey □) and anion exchange chromatography: AE-OP1 (white □) and AE-OP2 (light grey □). ▼: Carbohydrate chains. □: Disulphide bonds.

The hydrolysate of OVA with alcalase, unlike that of OVA with pepsin, exerted on spleen cells from sensitized mice a negligible Th2-stimulating role. A likely explanation is that all the hydrolysates with alcalase contains short peptides, with molecular masses lower than 1500 Da, that provide a poor sequence coverage (Lozano-Ojalvo, 2016a; 2016b). This possibly correlates with a low proportion of full length T epitopes with the minimum core sequence of 9 amino acids required for recognition by reactive T cells (Prickett et al., 2011). For its part, the hydrolysate of OVA with pepsin could be regarded as a good candidate for peptide-based immunotherapy on the grounds of its potential to reduce Th2 responses induced by egg allergens, but also to enhance Th1 responses. In fact, the peptides contained in this hydrolysate increased the production of dendritic cell-derived cytokines, such as IL-12 which, in turn, translated into an enhanced capacity to induce Th1 cell activation, as evidenced from the IFN- γ secretion in the spleen cell culture.

Semi-preparative chromatographic separation of OVA hydrolysed with pepsin yielded peptide fractions with different immunostimulating and immunomodulating properties. In particular, hydrophilic peptides (RP-OP2) and peptides with low affinity for the anion exchange column (AE-OP1) hindered the secretion of Th2 cytokines stimulated by EW in the spleen cells from EW-sensitized mice (in contrast to RP-OP4 and AE-OP2, respectively). Cationic host-defense peptides are known to exert immunomodulating properties beyond their direct antimicrobial action through the stimulation of innate immune responses and the modification of T and B cell responses (Hilchie, Wuerth, & Hancock, 2013). Similarly, cationic peptides, from bovine β -lactoglobulin, unlike anionic ones, are tolerogenic and prevent β -lactoglobulin sensitization in mice (Pecquet, Bovetto, Maynard, & Fritsché, 2000). However, despite there were similarities in the peptides contained in RP-OP2 and AE-OP1, with common sequences absent from other fractions, it was not possible to trace peptide composition to Th2 down-regulating activities. Furthermore, the observation that peptide enrichment by reverse-phase and anion-exchange fractionation of the hydrolysate did not lead to an increase in its immunomodulating efficiency suggests that factors other than peptide hydrophilicity or charge influence the ability to bias an allergic immune response. In fact, so far, very little is known regarding the structural and sequence requirements underlying the immunoregulatory properties of peptides (Haney & Hancock, 2013).

It is noteworthy that the whole hydrolysate of OVA with pepsin, as well as all its derived fractions, except for RP-OP4, contained several peptides included in the immunodominant sequence OVA (323–339), which comprises multiple T cell epitopes restricted by the MHC class I-A^d molecule of BALB/c mice, such as OVA (324–332) (SQAVHAAHA), OVA (327–335) (VHAAHAEIN) and OVA (329–337) (AAHAEINEA) (Robertson, Jensen, & Evavold, 2000). Furthermore, the hydrolysate and certain fractions contained peptides related to OVA (39–50), OVA (147–158) and OVA (263–274), which, as well as OVA (329–340), induce proliferation and IFN- γ secretion in spleen cell of BALB/c mice subcutaneously sensitized to OVA, without stimulating the production of IL-4 (Yang & Mine, 2009). The occurrence in the whole OVA hydrolysate of a combination of immunogenic structures with synergistic or adjuvant actions could explain its stronger immunomodulating potential as compared with its separated peptide fractions (Bøgh, Barkholt, Rigby, Mills, & Madsen, 2012; Yang et al., 2009 and 2010).

5. Conclusions

The assessment of the immunostimulating and immunomodulating capacity of hydrolysates of egg white proteins on splenocytes from sensitized BALB/c mice showed that the hydrolysate of OVA with pepsin stimulated low Th2 responses and also down-regulated Th2 responses induced by intact egg allergens *ex vivo*, while contributing to increase Th1 cytokine secretion. Hydrolysis of OVA with pepsin appears as a cheap, simple and safe approach for a reproducible production of

immunomodulating peptides with a reduced ability to trigger allergic symptoms. These results, if confirmed *in vivo*, could have implications in future treatment strategies of egg allergy.

Acknowledgments

Financial support was received from the project AGL2014-59771-R from MINECO (Spain). The authors are participants in the COST-Action ImpARAS FA1402. D. L-O is recipient of an FPU fellowship from MECO (Spain).

Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.foodres.2016.09.021>.

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Table S1. Effects of inactivated pepsin and alcalase, at a concentration equivalent to that present in 200 µg/mL of the hydrolysates, on the secretion of IL-4, IL-5, TNF-α and IFN-γ by splenocytes from egg white (EW)-sensitized mice. The secretion induced by EW is also shown for comparison. Data are expressed as mean ± SEM. Different letters indicate statistically significant differences (P< 0.05).

	RPMI	Pepsin	Alcalase	EW
IL-4	10.6 ± 0.5 ^b	0.8 ± 1.3 ^b	0.4 ± 0.7 ^b	127.9 ± 13.0 ^a
IL-5	18.9 ± 2.7 ^b	14.3 ± 2.3 ^b	15.1 ± 3.3 ^b	161.7 ± 6.0 ^a
TNF-~	3.4 ± 0.3 ^b	7.1 ± 3.3 ^b	4.3 ± 2.3 ^b	118.1 ± 10.3 ^a
IFN-¥	17.2 ± 0.7 ^b	17.6 ± 25.8 ^b	31.6 ± 54.2 ^b	1777.4 ± 65.0 ^a

Table S2. Peptide sequences, identified by RP-HPLC-MS/MS (ESI-MS/MS), in the hydrolysate of ovalbumin with pepsin.

Measured M/z	Calculated MH+	z	Mascot Score	Rt(min)	Range	Sequence
1204.6	1204.6	1+	23	20.8	17 - 26	KELKVHHANE
947.5	947.5	1+	22	20.5	19 - 26	LKVHHANE
834.4	834.4	1+	16	16.0	20 - 26	KVHHANE
566.2	566.3	1+	13	31.5	30 - 34	YCPIA
522.2	522.3	1+	19	30.4	42 - 46	VYLGA
765.4	765.4	1+	8	26.5	42 - 48	VYLGAKD
953.5	953.5	1+	45	26.3	42 - 50	VYLGAKDST
1210.6	1210.6	1+	15	26.0	42 - 52	VYLGAKDSTRT
783.4	1565.8	2+	11	29.2	42 - 55	VYLGAKDSTRTQIN
948.5	948.5	1+	9	19.7	44 - 52	LGAKDSTRT
660.0	1318.7	2+	16	19.9	45 - 56	GAKDSTRTQINK
707.3	707.4	1+	8	13.9	47 - 52	KDSTRT
948.4	948.5	1+	12	23.4	47 - 54	KDSTRTQI
1062.6	1062.6	1+	7	20.2	47 - 55	KDSTRTQIN
676.4	676.4	1+	10	37.5	61 - 66	DKLPGF
848.4	848.4	1+	25	34.8	61 - 68	DKLPGFGD
566.1	566.2	1+	15	13.9	72 - 77	AQCGTS
665.3	665.3	1+	10	21.8	72 - 78	AQCGTSV
779.3	779.3	1+	6	19.0	72 - 79	AQCGTSVN
878.4	878.4	1+	7	27.3	72 - 80	AQCGTSVNV
755.4	755.4	1+	21	28.6	78 - 84	VNVHSSL
656.3	656.3	1+	21	25.0	79 - 84	NVHSSL
758.4	758.4	1+	6	28.3	85 - 90	RDILNQ
929.5	929.5	1+	13	20.9	89 - 96	NQITKPND
1028.5	1028.5	1+	12	25.6	89 - 97	NQITKPNDV
687.3	687.4	1+	17	19.7	91 - 96	ITKPND
949.5	949.5	1+	18	28.1	91 - 98	ITKPNDVY
1036.5	1036.5	1+	12	26.1	91 - 99	ITKPNDVYS
533.1	533.3	1+	16	22.8	101 - 105	SLASR
646.4	646.4	1+	5	32.9	101 - 106	SLASRL
667.2	667.3	1+	11	21.1	107 - 111	YAEER
830.4	830.4	1+	21	24.4	107 - 112	YAEERY
790.4	790.4	1+	19	38.9	110 - 115	ERYPIL
719.4	719.4	1+	20	24.7	119 - 124	LQCVKE
832.4	832.5	1+	43	34.8	119 - 125	LQCVKEL
807.4	807.4	1+	6	29.2	125 - 131	LYRGGLE
694.4	694.4	1+	7	24.8	126 - 131	YRGGLE
1018.5	1018.5	1+	5	32.0	126 - 134	YRGGLEPIN
1165.6	1165.6	1+	12	42.3	126 - 135	YRGGLEPINF
633.2	633.3	1+	18	13.8	136 - 141	QTAADQ
704.2	704.3	1+	10	16.8	136 - 142	QTAADQA
731.4	731.4	1+	6	26.7	143 - 148	RELINS
747.3	747.4	1+	9	29.8	146 - 151	INSWVE
749.3	749.3	1+	11	25.2	149 - 154	WVESQT
920.4	920.4	1+	5	24.3	149 - 156	WVESQTNG
734.3	734.3	1+	12	14.9	150 - 156	VESQTNG
506.0	506.2	1+	17	7.5	152 - 156	SQTNG
727.5	727.5	1+	17	36.0	157 - 162	IIRNVL
1126.6	1126.7	1+	9	33.6	157 - 166	IIRNVLQPSS
630.3	630.3	1+	28	21.5	161 - 166	VLQPSS
729.4	729.4	1+	16	28.8	161 - 167	VLQPSSV

844.4	844.4	1+	11	26.7	161 - 168	VLQPSSVD
1059.5	1059.5	1+	11	25.0	161 - 170	VLQPSSVDSQ
632.3	632.3	1+	7	20.4	163 - 168	QPSSVD
719.2	719.3	1+	17	17.0	164 - 170	PSSVDSQ
652.2	652.3	1+	10	20.1	168 - 173	DSQTAM
537.2	537.2	1+	9	20.0	169 - 173	SQTAM
515.2	515.3	1+	11	27.6	174 - 178	VLVNA
953.4	953.4	1+	13	21.8	189 - 196	FKDEDTQA
806.3	806.4	1+	18	16.0	190 - 196	KDEDTQA
593.3	593.3	1+	14	42.5	195 - 199	QAMPF
879.4	879.4	1+	13	35.6	197 - 203	MPFRVTE
1007.5	1007.5	1+	7	34.1	197 - 204	MPFRVTEQ
748.4	748.4	1+	19	30.1	198 - 203	PFRVTE
876.4	876.5	1+	8	28.6	198 - 204	PFRVTEQ
779.4	779.4	1+	13	26.6	199 - 204	FRVTEQ
815.4	815.4	1+	10	17.8	204 - 210	QESKPVQ
946.5	946.5	1+	5	25.5	204 - 211	QESKPVQM
687.3	687.4	1+	16	17.4	205 - 210	ESKPVQ
818.4	818.4	1+	15	25.3	205 - 211	ESKPVQM
949.4	949.4	1+	19	30.6	205 - 212	ESKPVQMM
593.3	593.3	1+	22	38.6	213 - 217	YQIGL
710.3	710.4	1+	5	34.5	218 - 223	FRVASM
565.2	565.3	1+	9	19.5	224 - 228	ASEKM
806.4	806.4	1+	19	28.6	224 - 230	ASEKMKI
1048.6	1048.6	1+	13	33.2	224 - 232	ASEKMKILE
761.5	761.5	1+	7	32.3	227 - 232	KMKILE
874.6	874.5	1+	14	41.9	227 - 233	KMKILEL
615.4	615.4	1+	16	39.4	229 - 233	KILEL
823.4	823.4	1+	14	39.6	233 - 240	LPFASGTM
579.3	579.3	1+	20	23.5	234 - 239	PFASGT
797.3	797.3	1+	20	28.4	234 - 241	PFASGTMS
685.3	685.4	1+	19	34.1	244 - 249	VLLPDE
829.3	829.4	1+	13	37.3	246 - 253	LPDEVSGL
722.3	722.4	1+	13	42.6	257 - 262	ESIINF
619.3	619.3	1+	18	21.5	263 - 267	EKLTE
822.4	822.4	1+	5	41.3	267 - 273	EWTSNNV
594.2	594.3	1+	8	19.2	268 - 272	WTSSN
901.5	901.5	1+	16	25.9	275 - 281	EERKIKV
1177.7	1177.7	1+	11	36.8	275 - 283	EERKIKVYL
935.6	935.6	1+	11	27.7	276 - 282	ERKIKVY
1048.6	1048.7	1+	7	36.4	276 - 283	ERKIKVYL
662.3	662.3	1+	13	26.3	284 - 288	PRMKM
725.3	725.3	1+	6	29.8	307 - 313	FSSSANL
374.9	748.4	2+	17	26.5	309 - 316	SSANLSGI
521.1	521.3	1+	12	20.7	314 - 319	SGISSA
1132.6	1132.6	1+	16	23.8	323 - 333	KISQAVHAAHA
605.4	605.3	1+	6	16.2	327 - 332	AVHAAH
676.3	676.4	1+	26	18.6	327 - 333	AVHAAHA
805.4	805.4	1+	20	18.4	327 - 334	AVHAAHAE
918.5	918.5	1+	46	27.2	327 - 335	AVHAAHAEI
1161.6	1161.6	1+	23	24.2	327 - 337	AVHAAHAEINE
605.3	605.3	1+	11	17.6	328 - 333	VHAAHA
632.3	632.3	1+	11	16.5	334 - 339	EINEAG
675.3	675.3	1+	11	15.3	336 - 341	NEAGRE
687.4	687.4	1+	7	21.9	338 - 344	AGREVVG
845.4	845.4	1+	22	22.6	338 - 346	AGREVVGSA
432.1	432.2	1+	19	18.1	342 - 346	VVGSA
632.3	632.3	1+	6	20.3	342 - 348	VVGSAEA
790.4	790.4	1+	14	25.1	346 - 354	AEAGVDAAS
550.2	550.2	1+	17	17.0	354 - 358	SVSEE

1131.6	1131.6	1+	7	40.5	358 - 366	EFRADHPFL
1002.5	1002.5	1+	11	39.9	359 - 366	FRADHPFL
742.4	742.4	1+	10	27.9	360 - 365	RADHPF
855.5	855.4	1+	18	36.4	360 - 366	RADHPFL
1046.5	1046.5	1+	21	32.6	367 - 375	FCIKHIATN
785.4	785.4	1+	8	24.5	368 - 374	CIKHIAT
899.5	899.5	1+	13	22.7	368 - 375	CIKHIATN
970.5	970.5	1+	12	24.5	368 - 376	CIKHIATNA
682.4	682.4	1+	12	23.4	369 - 374	IKHIAT
796.4	796.5	1+	16	21.3	369 - 375	IKHIATN
701.4	701.4	1+	9	42.2	372 - 378	IATNAVL
629.3	629.3	1+	5	33.6	379 - 383	FFGRC

Table S3. Peptide sequences, identified by RP-HPLC-MS/MS (ESI-MS/MS), in the Fraction 1 of the hydrolysate of ovalbumin with pepsin separated by semi-preparative RP-HPLC (RP-OP1).

Measured M/z	Calculated MH+	z	Mascot Score	Rt(min)	Range	Sequence
835.5	835.4	1+	15	15.6	45 - 52	GAKDSTRT
734.4	734.3	1+	13	15.0	150 - 156	VESQTNG
806.4	806.4	1+	33	15.9	190 - 196	KDED TQA
687.5	687.4	1+	16	17.3	205 - 210	ESKPVQ
619.4	619.3	1+	15	21.3	263 - 267	EKLTE
594.3	594.3	1+	16	19.1	268 - 272	WTSSN
676.4	676.4	1+	16	18.3	327 - 333	AVHAAHA
805.4	805.4	1+	25	18.1	327 - 334	AVHAAHAE
692.3	692.3	1+	23	18.9	352 - 358	AASVSEE

Table S4. Peptide sequences, identified by RP-HPLC-MS/MS (ESI-MS/MS), in the Fraction 2 of the hydrolysate of ovalbumin with pepsin separated by semi-preparative RP-HPLC (RP-OP2).

Measured M/z	Calculated MH+	z	Mascot Score	Rt(min)	Range	Sequence
1204.8	1204.6	1+	37	20.4	17 - 26	KELKVHHANE
947.6	947.5	1+	38	20.3	19 - 26	LKVHHANE
522.3	522.3	1+	17	30.0	42 - 46	VYLGA
765.5	765.4	1+	16	26.1	42 - 48	VYLGAKD
953.6	953.5	1+	35	25.9	42 - 50	VYLGAKDST
605.9	1210.6	2+	8	25.6	42 - 52	VYLGAKDSTR
1190.7	1190.6	1+	7	20.2	45 - 55	GAKDSTRTQIN
1133.7	1133.6	1+	11	20.0	46 - 55	AKDSTRTQIN
858.6	858.5	1+	21	23.3	51 - 57	RTQINKV
848.6	848.4	1+	19	34.5	61 - 68	DKLPGFGD
755.5	755.4	1+	18	28.2	78 - 84	VNVHSSL
656.4	656.3	1+	23	24.6	79 - 84	NVHSSL
758.5	758.4	1+	13	27.9	85 - 90	RDILNQ
929.6	929.5	1+	28	20.6	89 - 96	NQITKPND
687.4	687.4	1+	19	19.4	91 - 96	ITKPND
786.5	786.4	1+	18	24.1	91 - 97	ITKPNDV
830.5	830.4	1+	21	24.0	107 - 112	YAEERY
790.5	790.4	1+	14	38.8	110 - 115	ERYPIL
887.6	887.5	1+	27	37.0	111 - 117	RYPILPE
904.6	904.5	1+	15	35.9	126 - 133	YRGGLEPI
731.4	731.4	1+	8	26.3	143 - 148	RELINS
920.5	920.4	1+	22	23.8	149 - 156	WVESQTNG
727.5	727.5	1+	7	35.8	157 - 162	IIRNVL
729.5	729.4	1+	14	28.4	161 - 167	VLQPSSV
931.6	931.5	1+	14	25.7	161 - 169	VLQPSSVDS
1059.6	1059.5	1+	28	24.7	161 - 170	VLQPSSVDSQ
515.2	515.3	1+	18	27.3	174 - 178	VLVNA
953.5	953.4	1+	18	21.5	189 - 196	FKDEDTQA
879.5	879.4	1+	9	35.2	197 - 203	MPFRVTE
1007.6	1007.5	1+	11	33.7	197 - 204	MPFRVTEQ
818.5	818.4	1+	21	25.0	205 - 211	ESKPVQM
761.5	761.5	1+	21	31.9	227 - 232	KMKILE
579.4	579.3	1+	16	23.1	234 - 239	PFASGT
710.4	710.3	1+	11	30.8	234 - 240	PFASGTM
716.4	716.3	1+	24	31.3	247 - 253	PDEVSGL
722.5	722.4	1+	10	42.5	257 - 262	ESIINF
901.7	901.5	1+	18	25.4	275 - 281	EERKIKV
1048.7	1048.7	1+	11	36.2	276 - 283	ERKIKVYL
662.5	662.3	1+	8	25.9	284 - 288	PRMKM
875.5	875.5	1+	8	27.1	319 - 326	AESLKISQ
1132.7	1132.6	1+	11	23.4	323 - 333	KISQAVHAAHA
676.4	676.4	1+	41	18.1	327 - 333	AVHAAHA
805.6	805.4	1+	11	18.1	327 - 334	AVHAAHAE
918.5	918.5	1+	20	26.8	327 - 335	AVHAAHAEI
1161.7	1161.6	1+	21	23.8	327 - 337	AVHAAHAEINE
687.3	687.4	1+	24	21.5	338 - 344	AGREVVVG
845.5	845.4	1+	17	22.3	338 - 346	AGREVVGSA
889.5	889.4	1+	11	33.8	359 - 365	FRADHPF
855.5	855.4	1+	16	36.2	360 - 366	RADHPFL

682.5	682.4	1+	14	22.9	369 - 374	IKHIAT
796.5	796.5	1+	17	21.0	369 - 375	IKHIATN
867.6	867.5	1+	19	22.8	369 - 376	IKHIATNA

Table S5. Peptide sequences, identified by RP-HPLC-MS/MS (ESI-MS/MS), in the Fraction 3 of the hydrolysate of ovalbumin with pepsin separated by semi-preparative RP-HPLC (RP-OP3).

Measured M/z	Calculated MH+	z	Mascot Score	Rt(min)	Range	Sequence
783.4	1565.8	2+	9	28.6	42 - 55	VYLGAKDSTRTQIN
847.5	1693.9	2+	12	27.1	42 - 56	VYLGAKDSTRTQINK
676.4	676.4	1+	28	37.2	61 - 66	DKLPGF
935.5	935.4	1+	11	34.1	61 - 69	DKLPGFGDS
1028.4	1028.5	1+	7	25.3	89 - 97	NQITKPNDV
949.5	949.5	1+	26	27.8	91 - 98	ITKPNDVY
1036.6	1036.5	1+	13	25.9	91 - 99	ITKPNDVYS
646.4	646.4	1+	8	32.5	101 - 106	SLASRL
790.5	790.4	1+	19	38.7	110 - 115	ERYPIL
807.4	807.4	1+	6	38.1	125 - 131	LYRGGLE
1150.1	2299.2	2+	6	31.4	142 - 161	ARELINSWVESQTNGIIRNV
1225.8	1225.7	1+	8	36.9	157 - 167	IIRNVLPQSSV
879.5	879.4	1+	15	35.2	197 - 203	MPFRVTE
1007.6	1007.5	1+	23	33.7	197 - 204	MPFRVTEQ
748.4	748.4	1+	12	29.8	198 - 203	PFRVTE
876.5	876.5	1+	8	33.6	198 - 204	PFRVTEQ
761.5	761.5	1+	15	31.9	227 - 232	KMKILE
928.5	928.4	1+	18	36.6	234 - 242	PFASGTMSM
829.2	829.4	1+	14	37.0	246 - 253	LPDEVSGL
722.4	722.4	1+	18	42.4	257 - 262	ESIINF
1048.8	1048.7	1+	7	36.1	276 - 283	ERKIKVYL
919.7	919.6	1+	11	35.7	277 - 283	RKIKVYL
1132.8	1132.6	1+	20	23.6	323 - 333	KISQAVHAAHA
1002.6	1002.5	1+	7	39.6	359 - 366	FRADHPFL
855.5	855.4	1+	25	36.2	360 - 366	RADHPFL

Table S6. Peptide sequences, identified by RP-HPLC-MS/MS (ESI-MS/MS), in the Fraction 4 of the hydrolysate of ovalbumin with pepsin separated by semi-preparative RP-HPLC (RP-OP4).

Measured M/z	Calculated MH+	z	Mascot Score	Rt(min)	Range	Sequence
903.5	1805.9	2+	9	35.6	28 - 43	IFYCPIAIMSALAMVY
783.4	1565.8	2+	21	28.6	42 - 55	VYLGAKDSTRQTIN
676.4	676.4	1+	15	37.1	61 - 66	DKLPGF
848.5	848.4	1+	16	34.5	61 - 68	DKLPGFGD
949.6	949.5	1+	11	27.8	91 - 98	ITKPNDVY
632.5	632.4	1+	8	34.2	227 - 231	KMKIL
874.6	874.5	1+	21	41.5	227 - 233	KMKILEL
746.6	746.4	1+	23	43.3	228 - 233	MKILEL
556.3	556.3	1+	16	34.1	244 - 248	VLLPD
1177.9	1177.7	1+	24	36.3	275 - 283	EERKIKVYL
647.5	647.4	1+	9	29.9	281 - 285	VYLPR
855.6	855.4	1+	9	36.1	360 - 366	RADHPFL
699.5	699.3	1+	8	37.3	361 - 366	ADHPFL

Table S7. Peptide sequences, identified by RP-HPLC-MS/MS (ESI-MS/MS), in the Fraction 5 of the hydrolysate of ovalbumin with pepsin separated by semi-preparative RP-HPLC (RP-OP5).

Measured M/z	Calculated MH+	z	Mascot Score	Rt(min)	Range	Sequence
1210.8	1210.6	1+	8	25.7	42 - 52	VYLGAKDSTRT
783.5	1565.8	2+	15	28.6	42 - 55	VYLGAKDSTRTQIN
676.4	676.4	1+	31	37.1	61 - 66	DKLPGF
848.4	848.4	1+	18	34.4	61 - 68	DKLPGFGD
755.6	755.4	1+	23	28.3	78 - 84	VNVHSSL
949.6	949.5	1+	20	27.8	91 - 98	ITKPNDVY
1036.6	1036.5	1+	17	25.8	91 - 99	ITKPNDVYS
790.5	790.4	1+	17	38.6	110 - 115	ERYPIL
791.5	2372.2	3+	7	78.0	150 - 171	VESQTNGIIRNVLPSSVDSQT
727.6	727.5	1+	16	35.8	157 - 162	IIRNVL
1225.8	1225.7	1+	11	36.8	157 - 167	IIRNVLPSSV
879.6	879.4	1+	9	35.2	197 - 203	MPFRVTE
710.4	710.4	1+	8	34.1	218 - 223	FRVASM
761.5	761.5	1+	24	31.9	227 - 232	KMKILE
615.5	615.4	1+	11	39.2	229 - 233	KILEL
823.4	823.4	1+	14	39.3	233 - 240	LPFASGTM
710.3	710.3	1+	22	30.7	234 - 240	PFASGTM
829.5	829.4	1+	8	36.9	246 - 253	LPDEVSGL
722.5	722.4	1+	25	42.4	257 - 262	ESIINF
919.8	919.6	1+	16	35.5	277 - 283	RKIKVYL
1132.8	1132.6	1+	33	23.5	323 - 333	KISQAVHAAHA
1161.7	1161.6	1+	11	23.9	327 - 337	AVHAAHAEINE
1002.7	1002.5	1+	11	39.5	359 - 366	FRADHPFL
855.6	855.4	1+	16	36.0	360 - 366	RADHPFL

Table S8. Peptide sequences, identified by RP-HPLC-MS/MS (ESI-MS/MS), in the Fraction 1 of the hydrolysate of ovalbumin with pepsin separated by anion exchange chromatography (AE-OP1).

Measured M/z	Calculated MH+	z	Mascot Score	Rt(min)	Range	Sequence
1204.8	1204.6	1+	26	19.9	17 - 26	KELKVHHANE
716.4	1431.8	2+	24	24.9	17 - 28	KELKVHHANENI
947.7	947.5	1+	13	19.6	19 - 26	LKVHHANE
834.5	834.4	1+	21	15.6	20 - 26	KVHHANE
706.4	706.3	1+	24	15.0	21 - 26	VHHANE
522.3	522.3	1+	20	29.4	42 - 46	VYLGA
765.4	765.4	1+	17	25.3	42 - 48	VYLGAKD
783.4	1565.8	2+	22	27.8	42 - 55	VYLGAKDSTRQTIN
652.4	1303.7	2+	9	22.6	44 - 55	LGAKDSTRQTIN
716.5	1431.8	2+	7	21.4	44 - 56	LGAKDSTRQTINK
835.4	835.4	1+	17	14.7	45 - 52	GAKDSTRT
934.5	934.5	1+	13	19.4	48 - 55	DSTRQTIN
676.5	676.4	1+	29	36.4	61 - 66	DKLPGF
755.5	755.4	1+	26	27.4	78 - 84	VNVHSSL
656.4	656.3	1+	22	23.8	79 - 84	NVHSSL
929.5	929.5	1+	22	20.2	89 - 96	NQITKPND
1028.6	1028.5	1+	19	24.7	89 - 97	NQITKPNDV
687.4	687.4	1+	23	19.0	91 - 96	ITKPND
786.5	786.4	1+	14	23.6	91 - 97	ITKPNDV
949.5	949.5	1+	29	27.0	91 - 98	ITKPNDVY
1036.6	1036.5	1+	16	25.1	91 - 99	ITKPNDVYS
646.5	646.4	1+	7	31.7	101 - 106	SLASRL
790.5	790.4	1+	15	37.8	110 - 115	ERYPIL
661.5	661.4	1+	8	37.6	111 - 115	RYPIL
1165.7	1165.6	1+	7	41.9	126 - 135	YRGGLEPINF
616.5	616.3	1+	9	43.6	141 - 145	QAREL
727.5	727.5	1+	10	34.6	157 - 162	IIRNVL
630.4	630.3	1+	21	21.0	161 - 166	VLQPSS
729.5	729.4	1+	23	28.0	161 - 167	VLQPSSV
879.5	879.4	1+	15	34.3	197 - 203	MPFRVTE
1007.6	1007.5	1+	12	32.7	197 - 204	MPFRVTEQ
687.4	687.4	1+	22	17.0	205 - 210	ESKPVQ
818.5	818.4	1+	11	24.1	205 - 211	ESKPVQM
949.5	949.4	1+	24	29.6	205 - 212	ESKPVQMM
593.3	593.3	1+	24	38.0	213 - 217	YQIGL
710.4	710.4	1+	8	32.9	218 - 223	FRVASM
563.3	563.3	1+	15	22.3	219 - 223	RVASM
806.5	806.4	1+	13	26.7	224 - 230	ASEKMKI
1048.7	1048.6	1+	21	31.9	224 - 232	ASEKMKILE
761.5	761.5	1+	22	30.8	227 - 232	KMKILE
874.6	874.5	1+	15	41.4	227 - 233	KMKILEL
692.4	692.4	1+	8	33.4	233 - 239	LPFASGT
823.5	823.4	1+	12	39.2	233 - 240	LPFASGTM
910.5	910.4	1+	24	37.0	233 - 241	LPFASGTMS
579.3	579.3	1+	8	22.7	234 - 239	PFASGT
710.4	710.3	1+	22	30.1	234 - 240	PFASGTM
797.4	797.3	1+	19	27.4	234 - 241	PFASGTMS
722.4	722.4	1+	11	42.4	257 - 262	ESIINF
593.3	593.3	1+	12	42.1	258 - 262	SIINF

594.3	594.3	1+	17	18.7	268 - 272	WTSSN
1177.8	1177.7	1+	8	35.4	275 - 283	EERKIKVYL
673.5	673.4	1+	16	17.2	276 - 280	ERKIK
1048.8	1048.7	1+	9	34.8	276 - 283	ERKIKVYL
662.4	662.3	1+	10	24.7	284 - 288	PRMKM
725.4	725.3	1+	9	28.9	307 - 313	FSSSANL
1132.8	1132.6	1+	22	22.9	323 - 333	KISQAVHAAHA
891.5	891.4	1+	16	22.8	325 - 333	SQAVHAAHA
605.4	605.3	1+	17	15.7	327 - 332	AVHAAH
676.4	676.4	1+	42	17.7	327 - 333	AVHAAHA
605.4	605.3	1+	21	17.2	328 - 333	VHAAHA
687.4	687.4	1+	22	21.0	338 - 344	AGREVVVG
845.5	845.4	1+	22	21.9	338 - 346	AGREVVGSA
1002.6	1002.5	1+	24	38.7	359 - 366	FRADHPFL
855.5	855.4	1+	11	34.1	360 - 366	RADHPFL
682.5	682.4	1+	21	22.4	369 - 374	IKHIAT
796.5	796.5	1+	16	20.5	369 - 375	IKHIATN
867.6	867.5	1+	13	22.5	369 - 376	IKHIATNA

Table S9. Peptide sequences, identified by RP-HPLC-MS/MS (ESI-MS/MS), in the Fraction 2 of the hydrolysate of ovalbumin with pepsin separated by anion exchange chromatography (AE-OP2).

Measured M/z	Calculated MH+	z	Mascot Score	Rt(min)	Range	Sequence
508.2	508.2	1+	15	17.7	6, - 10	AASME
753.5	753.5	1+	18	20.5	17 - 22	KELKVH
1204.7	1204.6	1+	34	20.3	17 - 26	KELKVHHANE
789.9	1578.8	2+	8	26.5	17 - 29	KELKVHHANENIF
706.4	706.3	1+	13	15.1	21 - 26	VHHANE
522.2	522.3	1+	9	29.7	42 - 46	VYLGA
765.5	765.4	1+	15	25.8	42 - 48	VYLGAKD
953.6	953.5	1+	30	25.7	42 - 50	VYLGAKDST
848.5	848.4	1+	24	34.0	61 - 68	DKLPGFGD
935.6	935.4	1+	14	33.7	61 - 69	DKLPGFGDS
827.5	827.4	1+	8	23.5	81 - 87	HSSLRDI
687.4	687.4	1+	15	19.3	91 - 96	ITKPND
949.6	949.5	1+	9	27.4	91 - 98	ITKPNDVY
1036.6	1036.5	1+	6	25.5	91 - 99	ITKPNDVYS
919.6	919.5	1+	15	38.8	109 - 115	EERYPIL
790.5	790.4	1+	14	38.2	110 - 115	ERYPIL
807.5	807.4	1+	13	28.3	125 - 131	LYRGGLE
694.4	694.4	1+	12	24.3	126 - 131	YRGGLE
734.4	734.3	1+	17	13.6	150 - 156	VESQTNG
844.5	844.4	1+	19	26.2	161 - 168	VLQPSSVD
931.6	931.5	1+	18	25.7	161 - 169	VLQPSSVDS
517.4	517.3	1+	10	16.6	163 - 167	QPSSV
620.3	620.3	1+	11	15.5	167 - 172	VDSQTA
653.4	653.3	1+	18	19.1	189 - 193	FKDED
754.4	754.3	1+	14	19.9	189 - 194	FKDEDT
882.5	882.4	1+	10	19.6	189 - 195	FKDEDTQ
953.5	953.4	1+	18	21.2	189 - 196	FKDEDTQA
879.5	879.4	1+	8	34.7	197 - 203	MPFRVTE
593.4	593.3	1+	26	38.1	213 - 217	YQIGL
740.5	740.4	1+	13	45.6	213 - 218	YQIGLF
1065.6	1065.5	1+	13	45.4	231 - 240	LELPFASGTM
579.3	579.3	1+	29	23.0	234 - 239	PFASGT
710.3	710.3	1+	23	30.4	234 - 240	PFASGTM
928.5	928.4	1+	8	36.3	234 - 242	PFASGTMSM
798.4	798.5	1+	12	40.7	243 - 249	LVLLPDE
685.4	685.4	1+	40	33.5	244 - 249	VLLPDE
1041.7	1041.6	1+	15	41.8	244 - 253	VLLPDEVSGL
829.5	829.4	1+	17	36.8	246 - 253	LPDEVSG
504.1	504.3	1+	7	16.0	250 - 254	VSGLE
718.5	718.4	1+	6	28.7	254 - 259	EQLESI
575.3	575.3	1+	13	26.8	257 - 261	ESIIN
722.4	722.4	1+	19	42.3	257 - 262	ESIINF
763.5	763.4	1+	9	37.0	260 - 265	INFEKL
619.4	619.3	1+	10	20.7	263 - 267	EKLTE
824.4	824.3	1+	9	23.3	266 - 272	TEWTSSN
594.3	594.3	1+	23	18.9	268 - 272	WTSSN
706.4	706.3	1+	15	34.9	300 - 306	AMGITDV
536.1	536.2	1+	20	25.9	301 - 305	MGITD
635.4	635.3	1+	21	34.3	301 - 306	MGITDV

504.2	504.3	1+	23	27.7	302 - 306	GITDV
651.3	651.3	1+	15	40.6	302 - 307	GITDVF
468.2	468.3	1+	15	17.0	327 - 331	AVHAA
605.4	605.3	1+	20	15.7	327 - 332	AVHAAH
676.4	676.4	1+	18	18.1	327 - 333	AVHAAHA
805.5	805.4	1+	35	17.9	327 - 334	AVHAAHAE
918.6	918.5	1+	26	26.4	327 - 335	AVHAAHAEI
1032.6	1032.5	1+	27	22.7	327 - 336	AVHAAHAEIN
1161.7	1161.6	1+	31	23.6	327 - 337	AVHAAHAEINE
854.4	854.4	1+	14	21.7	330 - 337	AAHAEINE
974.6	974.5	1+	7	22.1	338 - 347	AGREVVGSAE
561.3	561.3	1+	9	18.1	342 - 347	VVGSAE
692.3	692.3	1+	9	18.4	352 - 358	AASVSEE
1131.6	1131.6	1+	11	39.8	358 - 366	EFRADHPFL
855.5	855.4	1+	25	35.4	360 - 366	RADHPFL

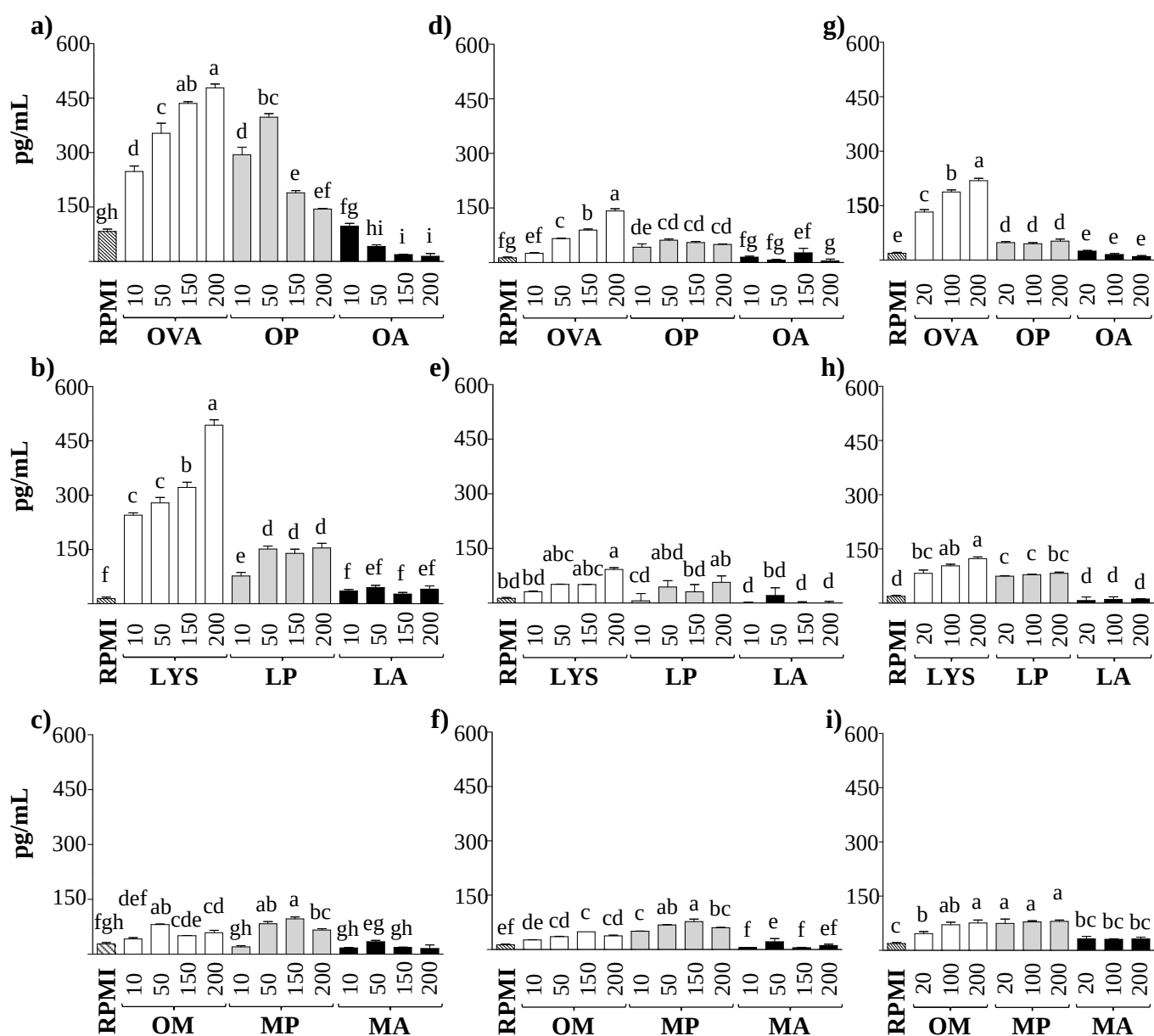


Figure S1. Immunostimulating effects of different concentrations (from 10 to 200 µg/ml) of ovalbumin (OVA), lysozyme (LYS), ovomucoid (OM) and their hydrolysates with pepsin (OP, LP and MP, respectively) and alcalase (OA, LA and MA, respectively) on the secretion of IL-5 by splenocytes from mono-sensitized (a, b and c), poly-sensitized (d, e and f) and egg white-sensitized (g, h and i) mice. Data are expressed as mean ± SEM. Different letters indicate statistically significant differences (P < 0.05).

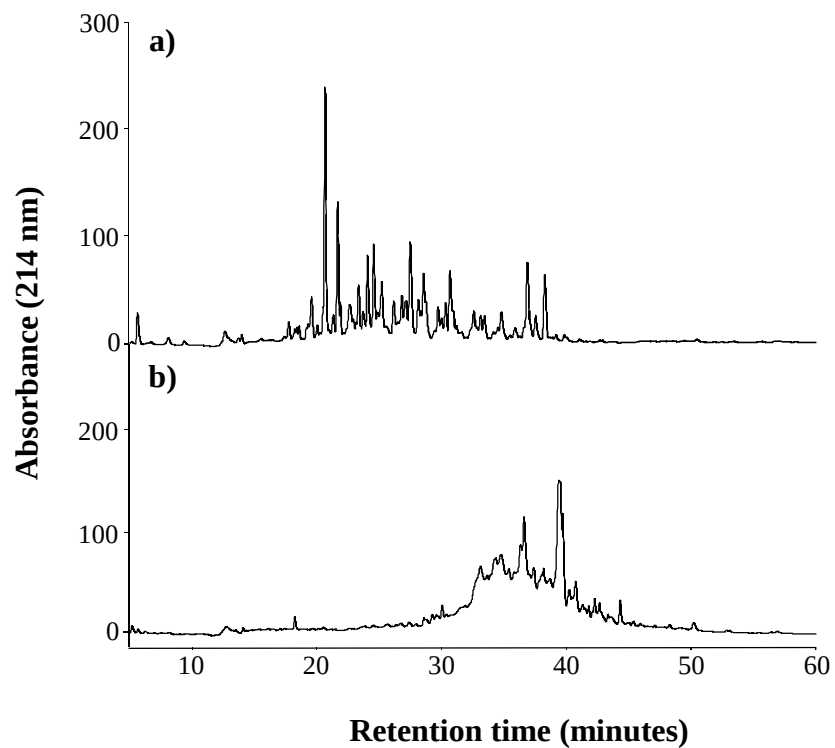


Figure S2. RP-HPLC patterns of the peptide fractions obtained from the hydrolysate of ovalbumin with pepsin (OP) by semi-preparative RP-HPLC: RP-OP2 (a) and RP-OP4 (b).

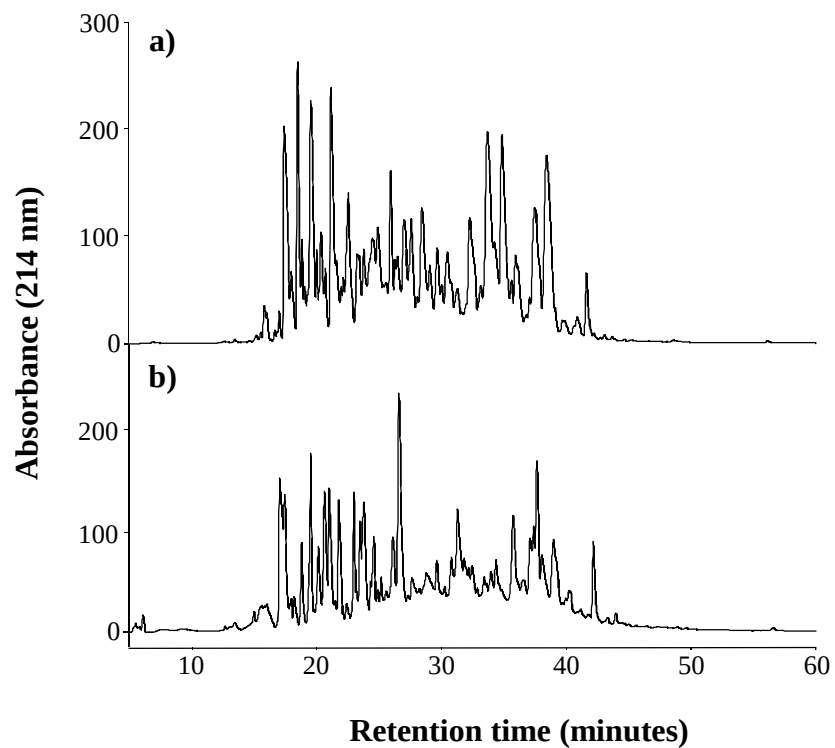


Figure S3. RP-HPLC patterns of the peptide fractions obtained from the hydrolysate of ovalbumin with pepsin (OP) by anion exchange chromatography: AE-OP1 (a) and AE-OP2 (b).