Food Research International 89 (2016) 661–669

Contents lists available at ScienceDirect

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 21 September 2016

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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http://dx.doi.org/10.1016/j.foodres.2016.09.021
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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 CO_2 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 (4×10^6 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% CO_2, 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 intradermically 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

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