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Pepsin treatment of whey proteins under high pressure produces hypoallergenic hydrolysates

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ABSTRACT

BALB/c mice were used to assess the ability of a whey protein hydrolysate obtained by pepsin treatment under high pressure (400 MPa, 37 °C, 30 min, HWP), to induce anaphylaxis, antibody production and cytokine responses in comparison with the whey protein isolate (WP) from which it is derived. HWP did not contain intact allergens and 50% of its peptides ranged between 10 and 3 kDa. Challenge with HWP did not induce clinical signs, body temperature changes or release of mast cell proteinase-1 in mice sensitized to WP. Immunization of mice with HWP did not produce WP-specific antibodies or allergic reactions upon HWP or WP challenge and thus, it can be considered hypoallergenic. However, HWP stimulated Th2 responses in splenocytes from sensitized mice. These characteristics make HWP a good candidate to be used in the management of milk allergy in diagnosed patients or to induce tolerance to whey proteins.

Industrial relevance: Hydrolysis with pepsin under high pressure produces in minutes a whey protein hydrolysate that complies with the health claims of the European guidelines on infant and follow-on formulas related to the reduction of risk to allergy to milk proteins. This process constitutes an alternative to the exhaustive enzymatic hydrolysis treatments used in the processing of hypoallergenic formulas that release short peptides and free amino acids to adversely affect organoleptic properties and technological value.

1. Introduction

Allergy to bovine milk is one of the most common types of food allergy during early childhood. In infants diagnosed with cow's milk allergy or at high risk of allergy development, the use of hypoallergenic formulas is frequently recommended (de Silva et al., 2014; Muraro et al., 2014). Hypoallergenic formulas are based on hydrolyzed cow's milk proteins which show reduced allergenicity with reduction in the size of peptides. Accordingly, they are classically categorized as extensively (< 3 kDa) or partially (3–10 kDa) hydrolyzed, on the basis of the molecular weight distribution of their peptide fragments, and designed, respectively, to avoid clinical symptoms and to prevent potential sensitization (Bøgh, Barkholt, & Madsen, 2015). European guidelines on infant and follow-on formulas (those intended up to 24-month-old infants) establish that hypoallergenicity needs to be assessed by showing that they are not able to orally sensitize animals to the intact protein from which they are manufactured (Commission Directive 2006/141/EC). This has prompted the validation of animal models of cow's milk allergy with the purpose to provide scientific data to support hypoallergenicity claims (van Esch et al., 2013).

Hypoallergenic formulas are frequently manufactured from the milk whey protein fraction, which contains a high amount of β-lactoglobulin (β-Lg) (about half of the protein content of whey), a major allergen absent from human milk (Roth-Walter et al., 2014). Susceptibility of β-Lg to proteolysis is relatively low and various chemical and physical treatments have been used with the purpose to promote its hydrolysis, such as the use of denaturing agents, heating or high pressure (Bu, Luo, Chen, Liu, & Zhu, 2013; Cheison & Kulozik, 2015; López-Fandiño, 2005). Enzymatic treatments under high hydrostatic pressure considerable enhance proteolysis of β-Lg and reduce its IgE-binding (Chicón, Belloque, Alonso, Martín-Alvarez, & López-Fandiño, 2008; Chicón, López-Fandiño, Alonso, & Belloque, 2008; Peñas, Préstamo, Baeza, Martínez-Molero, & Gómez, 2006 and Peñas, Snel, Floris, Préstamo, & Gómez, 2006). The effect of pressure has been traced to protein unfolding that exposes to the enzymatic action regions not easily accessible in the native structure. Thus, proteolysis under high

Abbreviations: CT, cholera toxin; α-La, α-lactalbumin; β-Lg, β-lactoglobulin; HWP, whey protein hydrolysate obtained by pepsin treatment under high pressure at 400 MPa (37 °C, 30 min); *m*MCP-1, mouse mast cell protease-1; PCA, passive cutaneous anaphylaxis; WP, whey protein isolate

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pressure, in contrast to atmospheric pressure, leads to a rapid breakdown of the intact protein and to the accumulation of large and intermediate size peptides, which are then further degraded to smaller fragments (Belloque, Chicón, & López-Fandiño, 2007).

High pressure might, therefore, constitute an alternative to the exhaustive enzymatic hydrolysis treatments used in the processing of hypoallergenic formulas that release short peptides and free amino acids to adversely affect organoleptic properties and technological value (Abd El-Salam & El-Shibiny, 2017; Liu, Jiang, & Peterson, 2014). From the point of view of the manufacturing industry, optimization of the proteolysis process is essential, not only to eliminate the allergenic potential, but also to maintain or enhance sensory and functional properties, such as emulsification, which are important for preparing stable formulations.

Previous results have shown that pepsin treatment under high hydrostatic pressure produces a whey protein hydrolysate that exhibits reduced IgE-binding and improved heat stability and emulsion activity (Chicón, Belloque, Alonso, & López-Fandiño, 2009). The combination of low antigenicity with the capacity to form emulsions may allow the production of milk-based ingredients for hypoallergenic preparations. The aim of this work was to investigate the eliciting, sensitizing and immunogenic properties of this hydrolysate. To this end, a BALB/c mouse model of cow's milk allergy was used for the evaluation of the ability of the hydrolysate to induce anaphylactic reactions, antibody production and cytokine responses in comparison with intact whey proteins.

2. Materials and methods

2.1. Proteins and mice

Whey protein isolate (WP) (LACPRODAN DI-9224K, 84.5% protein content as determined by the Kjeldahl method) was from Arla Foods Ingredients (Sønderhøj, Denmark). Porcine pepsin (EC 3.4.23.1, 3440 U/mg), α-lactalbumin (α-La) and β-Lg were purchased from Sigma-Aldrich (St. Louis, MO, USA). The lipopolysaccharide levels of the milk proteins and pepsin were quantified by the Pierce LAL Chromogenic Endotoxin Quantitation Kit (Thermo Scientific, Waltham, MA, USA) and, after purification of α-La by size exclusion chromatography (Pablos-Tanarro, López-Expósito, Lozano-Ojalvo, López-Fandiño, & Molina, 2016), all levels were < 1 EU/mg.

Six-week-old female specific-pathogen-free BALB/c mice were purchased from Charles River Laboratories (Saint Germain sur l'Arbresle, France), housed under standard conditions and fed an animal protein-free diet (SAFE, Route de Saint Bris, France). 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).

2.2. Hydrolysis of the whey protein isolate under high hydrostatic pressure

WP, dissolved in Milli-Q water (5 mg/ml) and adjusted to pH 1.5, was pre-incubated at 37 °C for 10 min prior to the addition of 172 U/mg protein of porcine pepsin. The mixture was immediately vacuum-sealed in polyethylene bags, avoiding headspace, and pressurized at 400 MPa and 37 ± 2 °C using an Iso-lab 900 High Pressure Food Processor (Mod FPG7 100:9/2C, Stansted Fluid Power Ltd. Essex, UK) with water as pressure-transmitting fluid. The pressure was raised at a rate of 600 MPa/min, maintained for 30 min, and released in < 4 s. The conditions to produce WP hydrolyzed with pepsin under high pressure (HWP) were chosen on the basis of previous experiments, which showed complete elimination of the intact allergenic proteins, reduced IgE-binding and improved functional properties (Chicón et al., 2009). As a control experiment, hydrolysis was also conducted at atmospheric pressure (0.1 MPa) at 37 °C, under continuous shaking, up to 24 h. After removal from the high pressure unit or the water bath, pepsin reaction was stopped by raising the pH to 7.0 with 2 N NaOH. Samples were lyophilized, their protein content determined by the Kjeldahl method, and stored at − 20 °C until used.

2.3. MALDI-TOF mass spectrometry

Peptide mass distribution of the hydrolysates was analyzed by MALDI-TOF using a Bruker AUTOflex Speed spectrometer (Bruker Daltonik GmbH, Bremen, Germany). The hydrolysates (0.5 µl dissolved at a concentration of 5 µg/ml) were loaded on a dry 2.5-dihydroxybenzoic acid (DHB) matrix spot (0.5 µl of 20 mg/ml DHB in acetonitrile/methanol, 70/30%, containing 1% trifluoroacetic acid) onto a Bruker Anchorchip target. All mass spectra were initially calibrated with Peptide Calibration Standard and Protein Calibration Standard I (Bruker Daltonik). Mass spectra were acquired in positive reflection, by summing 50 laser pulses at a fixed slide target position, using a 337 nm nitrogen laser and an acceleration voltage of 20 kV.

2.4. RP-HPLC-MS/MS

For RP-HPLC analyses with UV detection (214 nm) and on-line electrospray ionization (ESI-MS/MS), an Agilent 1100 Series HPLC (Agilent Technologies, Waldbronn, Germany) and an Esquire 3000 mass spectrometer (Bruker Daltonik) were used. HWP was analyzed after a reducing step using dithiothreitol, at a final concentration of 70 mM and pH 7.0, for 1 h at 37 °C (Chicón, López-Fandiño, et al., 2008). Chromatographic separations were performed with a RP318 column (250 × 4.6 mm, Bio-Rad). The operating conditions were: flow rate, 0.8 ml/min; injection volume, 50 µl; solvent A, 0.37 ml/l trifluoroacetic acid in Milli-Q water; and solvent B, 0.27 ml/l trifluoroacetic acid 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, 12 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.

2.5. Sensitization and challenge of mice

Twenty-four mice (4 groups) were fed by oral gavage, during three consecutive days on the first week and once per week during the following 4 weeks, with the equivalent of 5 mg of protein per mouse of WP (two groups, $n = 6$/group) or HWP (two groups, $n = 6$/group) in PBS, plus 10 µg of cholera toxin (CT) (List Biologicals, Campbell, CA, USA). Control mice ($n = 6$) just received 10 µg of CT in PBS. One week after the last sensitization dose, one of the WP- or HWP-sensitized mice groups was challenged with WP and the other groups were challenged with HWP. Control mice were challenged with WP. Oral challenge (20 mg protein per mouse) was followed by an intraperitoneal (i.p.) challenge (100 µg protein per mouse) 40 min apart. Anaphylactic responses were evaluated by scoring clinical signs (0: no signs; 1: scratching nose and mouth < 10 times in 15 min; 2: puffiness around eyes and mouth, scratching nose and mouth > 10 times in 15 min; 3: wheezing and labored respiration, cyanosis around the mouth and tail, diarrhea and difficulty in walking normally; 4: no activity after prodding; and 5: death) and rectal temperature 30 min after each challenge, as described by Pablos-Tanarro et al. (2016). Mice were then euthanized by CO_2 inhalation.