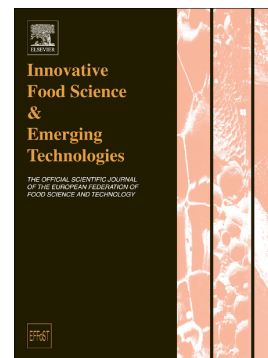


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PEPSIN TREATMENT OF WHEY PROTEINS UNDER HIGH PRESSURE PRODUCES HYPOALLERGENIC HYDROLYSATES

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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); **mMCP-1:** mouse mast cell protease-1; **PCA:** passive cutaneous
anaphylaxis; **WP:** whey protein isolate

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 starting 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.

Key words: cow's milk allergy; whey proteins; enzymatic hydrolysis; high-pressure; BALB/c mice; anaphylaxis; antibody production; T-cell activation

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 (Muraro et al., 2014; de Silva 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 96/4/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 (López-Fandiño, 2005; Bu, Luo, Chen, Liu, & Zhu, 2013; Cheison & Kulozik, 2015). Enzymatic treatments under high hydrostatic pressure considerably enhance proteolysis of β -Lg and reduce its IgE-

binding (Peñas, Préstamo, Baeza, Martínez-Molero, & Gómez, 2006; Peñas, Snel, Floris, Préstamo, & Gómez 2006; Chicón, Belloque, Alonso, Martín-Alvarez, & López-Fandiño 2008 and Chicón, López-Fandiño, Alonso, & Belloque, 2008). 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 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 (Liu, Jiang & Peterson, 2014; Abd El-Salam & El-Shibiny, 2017). 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 et al., 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 $\pm$ 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 less than 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 et al., 2008b). Chromatographic separations were performed with a RP318 column (250 x 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. Biotoools (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 less than 10 times in 15 min; 2: puffiness around eyes and

mouth, scratching nose and mouth more than 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 CO₂ inhalation.

2.6. Determination of specific immunoglobulins and mouse mast cell protease-1

Blood and feces were collected after each sensitization dose and after the i.p. challenge. Sera were obtained by centrifugation at 2500 x g for 10 min and kept at -20°C until analysis, and feces were lyophilized. Serum levels of total, WP-specific, and HWP-specific IgE and IgG1 were quantified by ELISA as previously described by Pablos-Tanarro, López-Expósito, Lozano-Ojalvo, López-Fandiño & Molina (2016). Briefly, 96-well plates were coated with 0.2 µg/well of anti-mouse IgE and IgG1 (BD Biosciences, San Diego, CA, USA) for total IgE and IgG1 quantification and for building reference curves or with 0.5 µg/well of either WP or HWP for measurement of specific-antibodies. After an overnight incubation at 4°C, plates were blocked and incubated with diluted serum samples (1:100 for total IgE, 1:10000 for total IgG1, 1:30 for WP-specific IgE, 1:5 for HWP-specific IgE, 1:1000 for WP-specific IgG1 and 1:100 for HWP-specific IgG1) or serial dilutions of mouse IgE and IgG1 isotypes (BD Biosciences). Detection was carried out by adding biotinylated anti-mouse IgE and IgG1, followed with an avidin-horseradish peroxidase incubation step (BD Biosciences). Reactions were developed with ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonate) as substrate (Roche, Mannheim, Germany) and read at 405 nm.

Determination of total, WP-specific and HWP-specific IgA was carried out in the supernatants of lyophilized fecal samples homogenized in PBS during 3 h and centrifuged at 4000 x g for 10 min. Quantification of IgA levels was performed as described for IgE and IgG1, except for the use of anti-mouse IgA antibodies and IgA isotype control for the reference curves (BD Biosciences). Supernatants were diluted 1:100 for total IgA, 1:30 for WP-specific IgA and 1:5 for HWP-specific IgA.

Mast cell degranulation was evaluated in serum samples collected after i.p. challenge by determining the concentration of mouse mast cell protease-1 (mMCP-1) using a commercial ELISA kit (eBioscience, San Diego, CA, USA).

2.7. *Passive cutaneous anaphylaxis assay*

Passive cutaneous anaphylaxis (PCA) assays were performed as described by López-Expósito, Chicón, Belloque, López-Fandiño, & Berin, (2012). Naïve BALB/c mice (2 per test) were injected intradermically in the right ear pinna with 20 µL of either unheated or heat-inactivated (56°C for 3 h; Li et al., 2000) pooled sera from WP- or HWP-sensitized mice (n=6), and with pooled serum from control mice (n=6) in the left ear pinna. Twenty-four hours later, mice were injected intravenously with 200 µg of the respective protein, either WP or HWP, in 100 µL of 1% Evans blue dye (Sigma-Aldrich). One hour after protein inoculation, mice were euthanized 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 by extrapolation from a reference curve with known amounts of Evans blue, and normalized to the weight of each ear.

2.8. *Splenocyte culture, cell proliferation and cytokine quantitation*

Spleens were individually collected and processed under sterile conditions. Splenocytes were cultured in 48-well plates (4×10^6 cells/mL) and stimulated, in duplicate, with concanavalin A ($2.5 \mu\text{g/mL}$), as positive control, RPMI-1640 medium, as negative control, WP, HWP, α -La or β -Lg ($200 \mu\text{g/mL}$). CFSE-based proliferation assays were carried out as previously described by Lozano-Ojalvo, Molina, & López-Fandiño, (2016). After 72 h of incubation at 37°C in 5% CO_2 , cells were harvested for analytical flow cytometry with a Gallios instrument (Beckman Coulter, Krefeld, Germany) and supernatants were collected and stored at -80°C until analysis of cytokines (IL-5, IL-13, TNF- α and IFN- γ) by ELISA, using commercial kits (eBioscience).

2.9. Statistical analyses

Data are presented as means $\pm$ SEM, excluding clinical signs, which are expressed as medians. Differences were determined by applying one-way ANOVA followed by Tukey post-hoc test, except for the clinical sign scores, which were evaluated by Mann-Whitney U test. P values < 0.05 were considered statistically significant. Statistical analyses were performed using GraphPad Prism v5 (GraphPad Software Inc., San Diego, CA, USA).

3. Results

3.1. Characterization of the whey protein hydrolysate obtained under high pressure

The extent of hydrolysis of WP with pepsin at atmospheric and high pressure was evaluated by RP-HPLC (Fig. 1). β -Lg resisted pepsin digestion at atmospheric pressure and it did not disappear until 24 h of hydrolysis (Fig. 1b-d). In contrast, high pressure promoted a very rapid disappearance of β -Lg, leaving no traces of intact proteins after 30 min of treatment with pepsin at 400 MPa (Fig. 1e).

The peptide mass distribution of HWP was compared with that of the hydrolysate produced at atmospheric pressure under conditions leading to the complete removal of the main whey proteins (24 h). Molecular masses, as estimated by MALDI-TOF, were lower than 10 kDa in both cases (Fig. 2). However, almost 50% of the peptides contained in HWP had molecular masses above of 3 kDa, whereas hydrolysis at atmospheric pressure led to smaller fragments, with approximately 90% below 3 kDa, which matches the presence of more early-eluting peaks in the RP-HPLC chromatogram of the later (Fig. 1).

RP-HPLC-MS/MS was used to label the peptides with molecular mass lower than 3 kDa in HWP. Eighty fragments corresponding to β -Lg and 22 corresponding to α -La were identified (supplementary Table S1). Peptides from β -Lg covered most of its sequence, with several of them included in regions of the protein previously identified as major human IgE-binding epitopes, such as β -Lg (43-62), β -Lg (86-100) and β -Lg (127-147) (Sélo et al., 1999, Järvinen, Chatchatee, Bardina, Beyer, & Sampson, 2001; Cerecedo et al., 2008; Wang et al., 2010, Benedé et al., 2014). On the other hand, it is likely that peptides, such as those including Cys₆₆ [β -Lg (58-74) and β -Lg (59-73)] are linked to those containing Cys₁₆₀ [β -Lg (156-162)] through a disulphide bond. Fragments from α -La related to the IgE-binding epitopes α -La (1-26) and α -La (93-102) were also found, although no peptides corresponding to the area between amino acids 50 and 83, which comprises the epitope α -La (47-58) (Järvinen et al., 2001), were identified.

3.2. Eliciting capacity

Body temperature and clinical signs were recorded after oral and i.p. challenges with WP and HWP in mice administered WP or HWP plus CT, and compared with

control mice. No anaphylactic symptoms were observed after oral challenge with either WP or HWP in any experimental group (data not shown). However, 30 min after i.p. challenge, mice fed with WP showed a pronounced temperature drop and severe anaphylactic symptoms when challenged with WP, while only one out of six WP-administered mice showed very mild symptoms when challenged with HWP (score 1) (Fig. 3 a and b). No anaphylactic symptoms were observed in mice from the HWP group after i.p challenge with either WP or HWP. In agreement with these results, elevated serum levels of *mMCP-1*, denoting activation and degranulation of mast cell levels, were only significantly increased in WP-administered mice challenged with WP (Fig. 3c).

3.3. Sensitizing capacity: antibody responses

Total and antigen-specific antibody responses were monitored weekly throughout the sensitization period and after challenge (Fig. 4). Total IgE increased in the WP and HWP groups up to the 4th week, with no significant differences between them. The highest levels of IgG1 were reached on the 5th week and corresponded to the WP group. While the concentrations of total IgE and IgG1 were higher in the WP and HWP groups than in the control group by end of the experimental period, no significant differences were found among the 3 groups in total fecal IgA (Fig. 4a, c and e).

Sensitization with WP induced significant levels of serum WP-specific IgE and IgG1 and fecal WP-specific IgA. However, HWP failed to generate WP-specific antibody responses (Fig. 4b, d and f). Furthermore, no HWP-specific IgE or IgA antibodies could be detected in any group (not shown), although HWP-specific IgG1 was present in both, WP and HWP sera, with a significantly higher level in the HWP group (Fig. 4g).

In order to estimate the functionality of the specific IgE and IgG1 antibodies generated, PCA assays were performed. Inoculation of pooled serum from the WP group, but not of that from naïve mice, induced positive reactions following the intravenous administration of WP. In addition, when the pooled serum samples were heated at 56°C during 3 h to inactivate IgE, dye extravasation significantly decreased (Fig. 5). Passive immunization of mice with sera from the HWP group, devoid of detectable specific-IgE (Fig. 4b), caused a lower, but significant, dye extravasation following HWP injection, which was not modified upon heating. These results indicated the generation, in mice from the HWP group, of biologically active HWP-specific IgG1, able to mediate allergic reactions.

3.4. Immunogenic capacity: cellular responses

Spleen cells were cultured and stimulated with WP and HWP, and T cell proliferative responses and Th1/Th2 cytokine production were evaluated. Concanavalin A, a mitogen able to stimulate T lymphocytes, induced a vigorous cell proliferation in all groups (Table 1). Spleen cells from WP- and HWP-administered mice exhibited significant proliferative responses to both WP and HWP, as compared with non-stimulated cells (RPMI), with no differences in the outcomes of WP or HWP stimulation within each group or between both groups.

As expected, splenocytes from mice from the WP group produced allergy-associated Th2 cytokines (IL-13 and IL-5) following stimulation with WP, whereas incubation with HWP induced a lower, albeit significant, response (Fig. 6a and b). Spleen cells of mice from the HWP group released comparably less IL-13 and IL-5 upon WP stimulation and similar levels upon HWP stimulation. With respect to Th1 cytokines, spleen cells from WP and HWP groups produced similar levels of IFN- γ and

TNF- α as a result of incubation with WP, while HWP did not induce the release of these cytokines in any of these groups (Fig. 6c and d).

Splenocyte incubation with the major whey proteins, α -La and β -Lg, revealed that β -Lg induced the strongest cytokine responses; in most cases, equivalent to those induced by WP, whereas α -La did not significantly increase cytokine production (Fig. 6).

4. Discussion

Treatment of WP with pepsin under high hydrostatic pressure (400 MPa) produced in 30 min a hydrolysate devoid of intact allergens, including β -Lg, a protein very resistant to proteolysis at acidic pH (Fig. 1). Approximately, 50% of the peptides contained in HWP had molecular masses between 10 and 3 kDa, while most of the fragments resulting from the complete hydrolysis of β -Lg at atmospheric pressure (90%) were below 3 kDa (Fig. 2). Hydrolysis of β -Lg with pepsin under high pressure follows a progressive proteolysis mechanism that involves a very rapid disappearance of the protein into large fragments, which are then degraded into smaller peptides. In contrast, at atmospheric pressure, the persistence of the intact protein and the comparatively lower amount of medium-size fragments indicate that proteolysis is more difficult, but once intermediate peptides are released, there are no restrictions on their hydrolysis to smaller fragments (Belloque et al., 2007, Chicón et al., 2008b).

Previous work showed that the large, and presumably hydrophobic, fragments contained in HWP contribute to enhance heat stability and maintain the capacity to efficiently form emulsions, while retaining a reduced, but noticeable, residual binding to IgE from egg allergic patients (Chicón et al., 2009). In fact, RP-HPLC-MS/MS revealed the presence of allergenic epitopes from both β -Lg and α -La (supplementary table S1).

Challenge with HWP, however, did not cause body temperature changes, anaphylaxis signs or significant release of *mMCP-1* in BALB/c mice sensitized to WP (Fig. 3), what allows inferring that it is not likely to induce clinical symptoms in patients diagnosed with allergy to whey milk proteins. This observation is in line with other studies that reported that partially hydrolyzed whey-based formulas do not to elicit anaphylactic reactions in C3H/HeOuJ mice (Knipping et al., 2012; van Esch et al., 2010, 2011a) or Brown Norway rats (Bøgh et al., 2015) sensitized to whey proteins. In fact, the degree of hydrolysis β -Lg, the dominant whey protein causing allergic reactions in mice sensitized to whey, correlates with its allergenic reactivity and hence, the complete removal of intact β -Lg abolishes its ability to activate mast cells and to trigger anaphylaxis *in vivo* (Bossios et al., 2011; López-Expósito et al., 2012).

Less is known on the capacity to induce sensitization of hydrolyzed versus intact allergens, including β -Lg. Hydrolysis has been reported to reduce the sensitizing potency of cow's milk formulas (Niggemann, Nies, Renz, Herz, & Wahn, 2001). However, depending on the hydrolysis conditions, the sensitizing potential of hydrolyzed food products can be maintained, or even enhanced, if new epitopes are formed (Krogsho, Andersen, Rasmussen, Jacobsen, & Madsen, 2014). Thus, hydrolysis fragments of Ara h1 retain IgE-binding and T-stimulatory properties (Eiwegger et al., 2006), being able to sensitize Brown Norway rats by the i.p. route, as long as there is the right combination of peptides with an adjuvant effect or the peptides are in an aggregated state (Bøgh et al., 2009; Bøgh, Barkholt, Rigby, Mills, & Madsen, 2012). At this respect, it was found that the hydrolysis products of β -Lg with masses lower than 4.5 kDa fail to induce IgE, IgG1 and IgA antibodies in this rat model, despite they form aggregated complexes (Bøgh, Barkholt, & Madsen, 2013), but this may not hold true for bigger fragments.

As judged by the antibody responses they generated, HWP exhibited a much less sensitizing capacity than WP. Administration of HWP plus CT to mice did not lead to the production of WP-specific antibodies nor to allergic signs upon WP challenge (Fig. 3 and 4), and thus, complies with the European Guidelines which preclude that hypoallergenic hydrolyzed formula induce sensitization, in animals, to the intact proteins from which they are manufactured. Nevertheless, HWP increased the levels of non-specific IgE and IgG1, as compared with the control group, and even if it did not produce detectable specific IgE antibodies, it was able to generate HWP-specific IgG1, indicating that the peptides contained in the hydrolysate maintained residual immunogenicity (Fig. 4). Furthermore, the PCA assays indicated that the IgG1 antibodies generated were functional, that is, potentially able to trigger local allergic responses (Fig. 5). The development of food allergy in mouse models has been described to occur through two independent pathways, comprising either IgE and mast cells or IgG1 and macrophages (Finkelman, 2005). Although, in contrast to systemic anaphylaxis in the mouse, PCA is entirely mast cell-dependent, in addition to IgE, it can also be mediated by IgG1 antibodies whose binding to mast cells is promoted by the Th2 cytokine IL-4 (Finkelman, 2007). In any case, the low levels of serum HWP-specific IgG1 generated may explain why neither oral nor i.p challenge with HWP induced anaphylaxis in HWP-administered mice. On the other hand, there is no evidence for the clinical relevance of IgG-mediated food allergy in humans (Finkelman Khodoun, & Strait, 2016).

IgG1 from sera of mice sensitized to WP were also reactive towards plate-bound HWP (Fig. 4). Despite the resistance of β -Lg to pepsin digestion, it is likely that mice fed orally with WP and HWP become sensitized to similar fragments, although there

was a higher level of HWP-specific IgG1 in the HWP group, as compared to the WP group.

Spleen cells from mice sensitized to WP exhibited similar proliferative responses to stimulation with WP and HWP (Table 1). Furthermore, HWP induced a significant production of IL-13 and IL-5 from the splenocytes of WP-sensitized mice, which was comparable to that induced by intact β -Lg (Fig. 6a and b). These results show that HWP contained immunodominant T cell epitopes able to mount Th2 responses. These, in mice, are responsible for the maturation of B cells into plasma cells that produce IgE and IgG1, and for the generation of inflammatory responses that mediate anaphylaxis to a great extent (Oyoshi, Oettgen, Chatila, Geha, & Bryce, 2014). Furthermore, the splenocytes of HWP-administered mice responded to stimulation with HWP with a significantly increased secretion of IL-13 and IL-5, which was similar to that induced by WP. These observations, together with the absence of a clear Th1-stimulating effect (IFN- γ and TNF- α ; Fig. 6c and d), indicate that HWP kept to some extent the immunogenicity of the intact allergen, but lacked its sensitizing and eliciting potential.

Hydrolysates that show reduced IgE reactivity and maintain T cell-stimulating abilities have proved, in mice, to be a safe treatment for food allergy, working through the same mechanisms as whole protein immunotherapy (Kulis et al., 2012). In addition, partially hydrolyzed β -Lg, as well as intact β -Lg, but not short tryptic peptides, can prevent further sensitization to this allergen (Adel-Patient et al., 2011; 2012). Similarly, partial, but not extensively hydrolyzed whey, with limited sensitizing properties, retains the putative capacity to prevent allergic symptoms when given orally to mice before sensitization (van Esch et al., 2011 b). The immunostimulating potential of hydrolysates seems to be mainly supported by the combination of the generated peptides and, particularly, by large ones resulting from partial hydrolysis (Pecquet, Bovetto, Maynard,

& Fritsché, 2000). In this respect, we have shown that high hydrostatic pressure produced, in minutes, a hypoallergenic hydrolysate of whey proteins, containing 50% of peptides with molecular masses between 10 and 3 kDa, which retained sufficiently immunogenicity to stimulate Th2 responses, but not enough to generate specific IgE or IgG1 antibodies that could mediate systemic anaphylaxis. In view of these characteristics, this hydrolysate could be used in the management of milk allergy in diagnosed patients or to induce tolerance to milk whey proteins in therapeutic or preventive approaches.

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Figure captions

Fig. 1. RP-HPLC patterns of a whey protein isolate (a) and its hydrolysates with pepsin at atmospheric pressure during 4 (b), 8 (c) and 24 h (d), and under high pressure (400 MPa) during 30 min (e). α -La: α -lactalbumin, β -Lg: β -lactoglobulin.

Fig. 2. Peptide mass distribution in the whey protein hydrolysates obtained by pepsin treatment at atmospheric pressure during 24 h (black bars), and under high pressure (400 MPa) during 30 min (white bars). Bars represent the masses included in each range as percentage of all the masses detected by MALDI-TOF (means $\pm$ SEM, n=3).

Fig. 3. Anaphylaxis in control mice (CT) and mice administered whey protein isolate plus CT (WP+CT) or WP hydrolyzed with pepsin at 400 MPa during 30 min plus CT (HWP+CT), intraperitoneally (i.p.) challenged with WP or HWP. (a) Body temperature, (b) clinical symptom score, and (c) serum concentration of mouse mast cell protease 1 (*mMCP-1*). Data are expressed means (a and c) $\pm$ SEM or medians (b) (n=6). * $P < 0.05$.

Fig. 4. Serum levels of total and specific IgE, IgG1 and IgA at different time points in control mice (CT), and mice administered whey protein isolate plus CT (WP+CT) or WP hydrolyzed with pepsin at 400 MPa during 30 min plus CT (HWP+CT). (a) Total IgE, (b) WP-specific IgE, (c) total IgG1, (d) WP-specific IgG1, (e) total IgA, (f) WP-specific IgA and (g) HWP-specific IgG1. Data are means $\pm$ SEM (n=6). Different lowercase letters indicate significant differences ($P < 0.05$) among different weeks within mouse groups and different uppercase letters indicate significant differences ($P < 0.05$) among different mouse groups within weeks.

Fig. 5. Passive cutaneous anaphylaxis assay with unheated and heated (56°C for 3 h) pooled serum samples from mice administered whey protein isolate plus cholera toxin (WP+CT) or WP hydrolyzed with pepsin at 400 MPa during 30 min plus cholera toxin (HWP+CT). Allergen-specific dye extravasation was calculated by correcting the WP or HWP induced absorbance with the absorbance of the supernatant of the ear passively sensitized with pooled control serum. Data are means $\pm$ SEM (n= 2). Different letters indicate statistically significant differences ($P < 0.05$).

Fig. 6. Effects of whey protein isolate (WP), α -lactalbumin (α -La), β -lactoglobulin (β -Lg), and WP hydrolyzed with pepsin at 400 MPa during 30 min (HWP) on the secretion of (a) IL-13, (b) IL-5, (c), IFN- γ and (d) TNF- α by splenocytes from control mice (CT) and mice administered WP plus CT (WP+CT) or HWP plus CT (HWP+CT). Data are means $\pm$ SEM (n=6). Different lowercase letters indicate significant differences ($P < 0.05$) among stimuli within each mouse group and different uppercase letters indicate significant differences ($P < 0.05$) among mouse groups within each stimulus.

Table 1. Effects of a whey protein isolate (WP) and WP hydrolyzed with pepsin at 400 MPa during 30 min (HWP) on the proliferation index, assessed by CFSE labelling, of splenocytes from control mice (CT) and mice administered WP or HWP plus cholera toxin. Cells stimulated with 2.5 µg/mL of concanavalin A (ConA) were used as positive control and RPMI-treated cells as negative control. Data are means ± SEM (n= 6).

	Mouse group		
	CT	WP+CT	HWP+CT
ConA	2.5 ± 0.2 ^{aA}	2.63 ± 0.3 ^{aA}	2.1 ± 0.5 ^{aA}
RPMI	0.1 ± 0.1 ^{bA}	0.1 ± 0.0 ^{cA}	0.1 ± 0.0 ^{cA}
WP	0.1 ± 0.1 ^{bB}	0.6 ± 0.1 ^{bA}	0.7 ± 0.1 ^{bA}
HWP	0.2 ± 0.1 ^{bB}	0.6 ± 0.1 ^{bA}	0.7 ± 0.04 ^{bA}

^{a-c} Different lowercase letters indicate significant differences (P<0.05) within columns

^{A-B} Different uppercase letters indicate significant differences (P<0.05) within rows

Figure 1

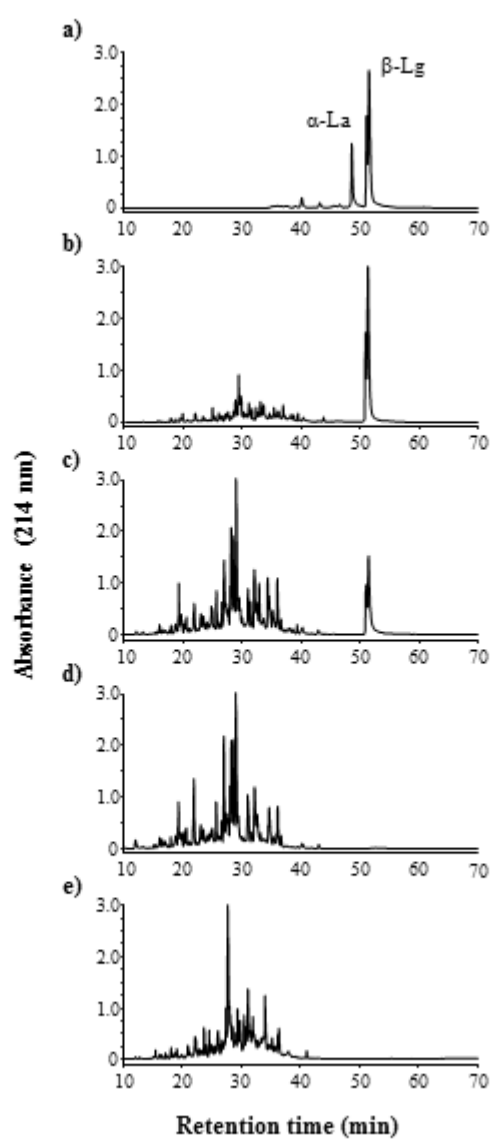


Figure 2

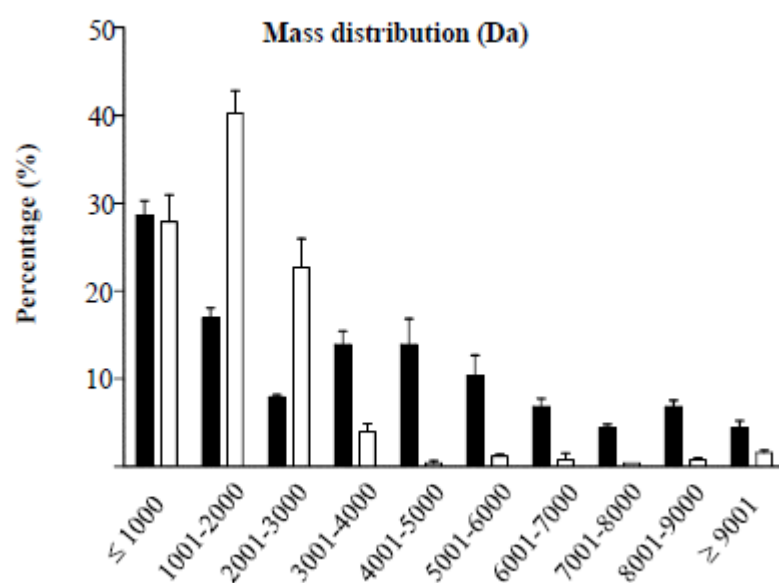


Figure 3

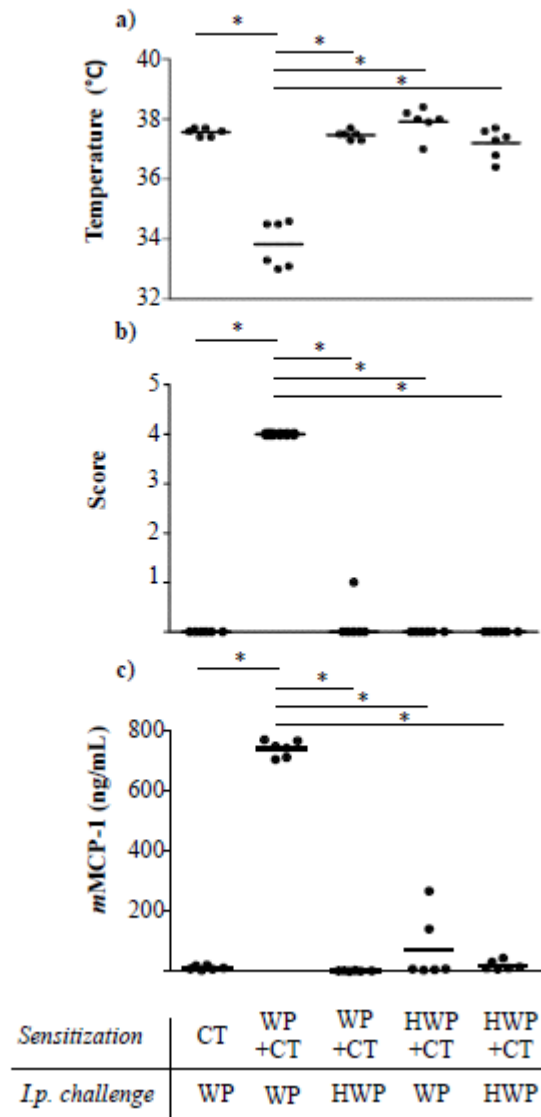


Figure 4

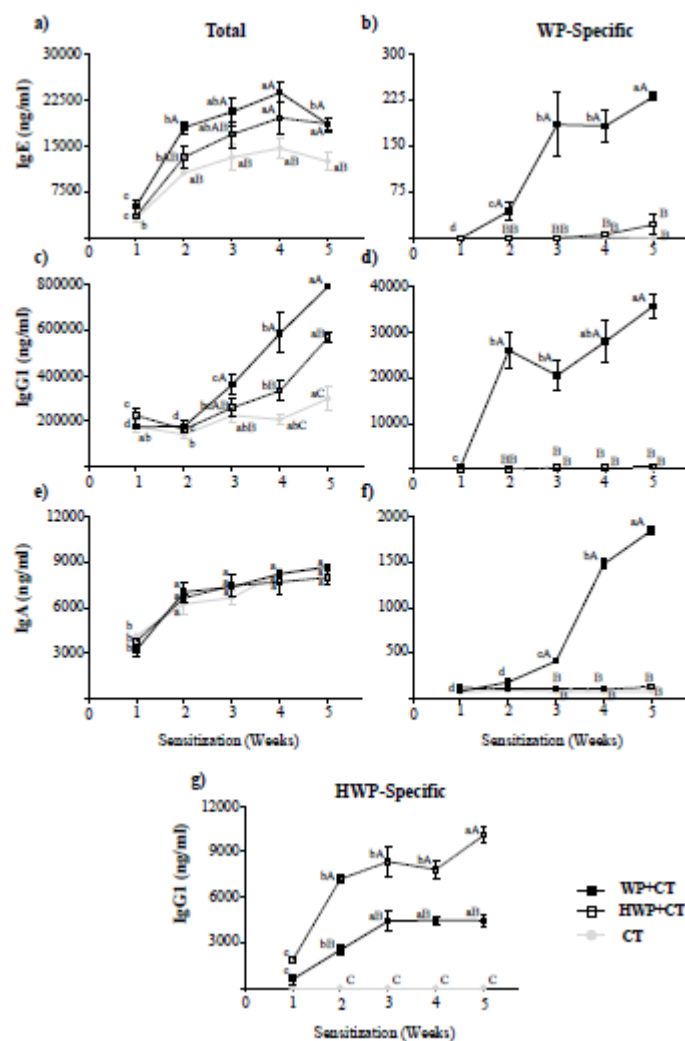


Figure 5

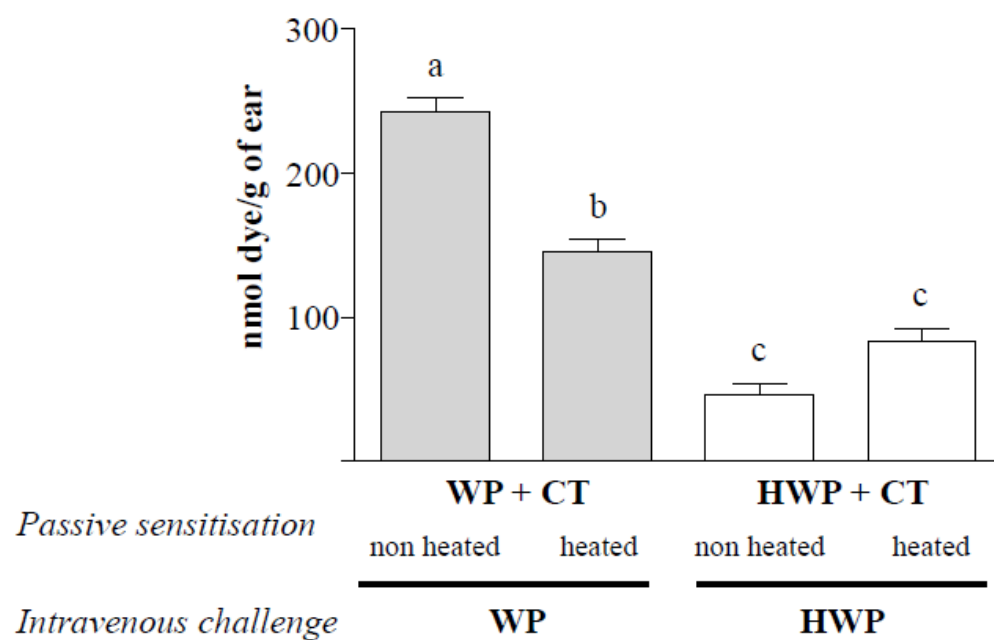
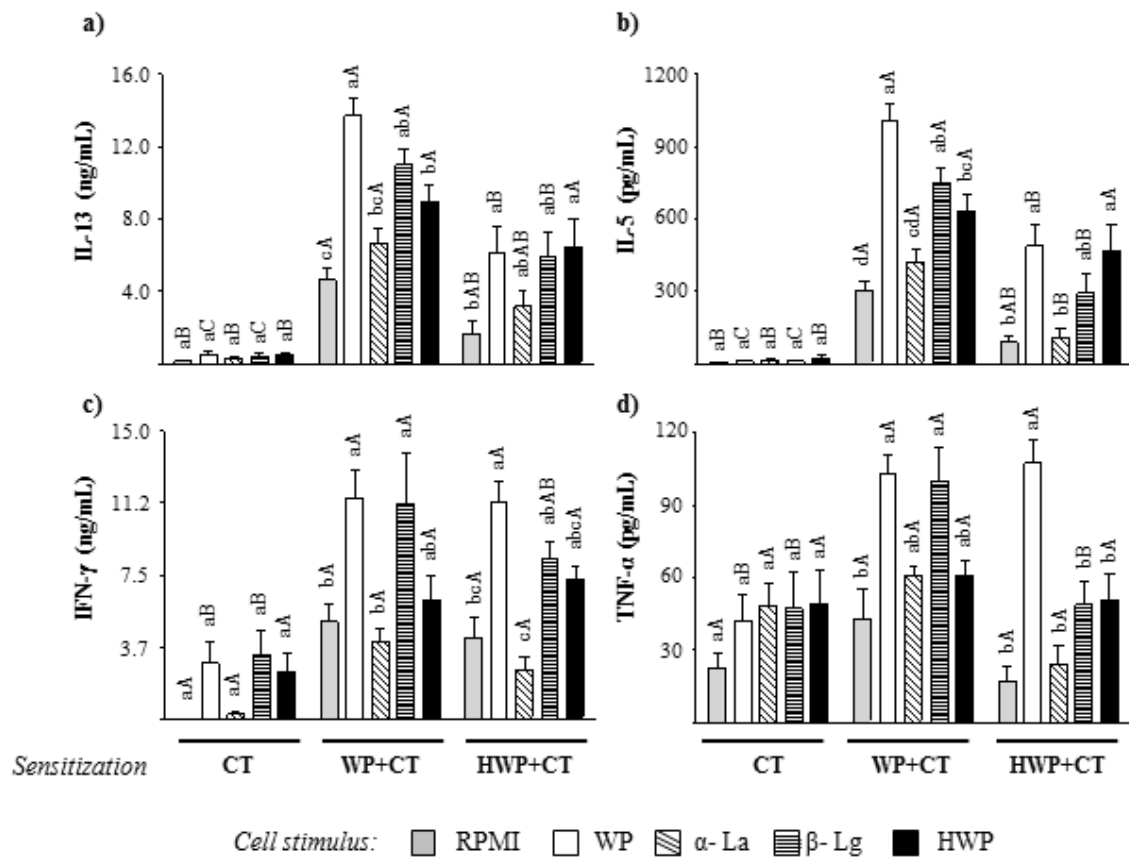


Figure 6



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.

Highlights

- Pepsin under high pressure produces a hypoallergenic hydrolysate of whey proteins.
- Fifty percent of the peptides have between 10 and 3 kDa and contain IgE-binding epitopes.
- Challenge with the hydrolysate does not induce anaphylaxis.
- Oral administration of the hydrolysate does not generate specific IgE, IgG1 or IgA.
- The hydrolysate retains sufficient immunogenicity to stimulate Th2 responses.

Supplementary information

Table S1. Peptide sequences, identified by RP-HPLC-MS/MS, in the hydrolysate of whey proteins with pepsin obtained at 400MPa during 30 minutes.

Measurement M/z	Calculated MH ⁺	z	Mascot Score	Retention time (min)	Range	Sequence	Protein
674.4	674.4	1+	15	28.2	1-6	LIVTQT	β-Lg
805.4	805.4	1+	10	35.0	1-7	LIVTQTM	β-Lg
990.5	990.6	1+	14	32.0	1-9	LIVTQTMKG	β-Lg
1218.7	1218.7	1+	23	38.3	1-11	LIVTQTMKGLD	β-Lg
893.4	893.4	1+	17	26.6	4-11	TQTMKGLD	β-Lg
792.4	792.4	1+	10	25.8	5-11	QTMKGLD	β-Lg
1130.6	1130.6	1+	10	41.3	10-19	LDIQKVAGTW	β-Lg
716.4	716.4	1+	33	21.6	12-18	IQKVAGT	β-Lg
902.5	902.5	1+	31	34.1	12-19	IQKVAGTW	β-Lg
661.4	661.4	1+	12	30.5	14-19	KVAGTW	β-Lg
565.2	565.2	1+	39	17.9	23-28	AMAASD	β-Lg
494.1	494.2	1+	22	16.8	24-28	MAASD	β-Lg
956.5	956.5	1+	37	31.6	33-41	DAQSAPLRV	β-Lg
652.3	652.3	1+	16	32.7	42-46	YVEEL	β-Lg
1489.7	1489.7	1+	38	36.4	42-54	YVEELKPTPEGDL	β-Lg
809.8	1618.8	2+	24	34.5	42-55	YVEELKPTPEGDL	β-Lg
1326.7	1326.7	1+	25	34.3	43-54	VEELKPTPEGDL	β-Lg
1098.5	1098.6	1+	18	32.1	45-54	ELKPTPEGDL	β-Lg
856.3	856.4	1+	9	21.4	46-53	LKPTPEGD	β-Lg
969.5	969.5	1+	9	30.8	46-54	LKPTPEGDL	β-Lg
1098.5	1098.6	1+	20	28.8	46-55	LKPTPEGDL	β-Lg
743.3	743.4	1+	28	17.4	47-53	KPTPEGD	β-Lg
856.4	856.4	1+	16	27.4	47-54	KPTPEGDL	β-Lg
985.4	985.5	1+	13	25.3	47-55	KPTPEGDL	β-Lg
1023.5	2046.0	2+	17	33.1	58-74	LQKWENDECAQKKIIAE	β-Lg
902.3	1803.9	2+	14	31.7	59-73	QKWENDECAQKKIIA	β-Lg
674.4	674.4	1+	24	28.2	71-76	IIAEKT	β-Lg
885.6	885.5	1+	13	29.7	74-81	EKTKIPAV	β-Lg
1032.6	1032.6	1+	19	38.7	74-82	EKTKIPAVF	β-Lg
756.4	756.5	1+	9	29.5	75-81	KTKIPAV	β-Lg
903.6	903.6	1+	21	38.3	75-82	KTKIPAVF	β-Lg
706.4	706.4	1+	25	40.7	82-87	FKIDAL	β-Lg
949.4	949.5	1+	11	37.5	82-89	FKIDALNE	β-Lg
1612.8	1613.1	1+	10	45.3	82-95	FKIDALNENKVLVL	β-Lg
559.3	559.3	1+	11	30.6	83-87	KIDAL	β-Lg
802.4	802.4	1+	15	27.7	83-89	KIDALNE	β-Lg
916.4	916.5	1+	15	26.3	83-90	KIDALNEN	β-Lg
1143.6	1143.6	1+	36	29.7	83-92	KIDALNENKV	β-Lg
1355.7	1355.8	1+	54	41.6	83-95	KIDALNENKVLVL	β-Lg
716.4	716.4	1+	15	22.1	87-92	LNENKV	β-Lg
603.3	603.3	1+	15	17.8	88-92	NENKV	β-Lg
716.3	716.4	1+	10	26.1	88-93	NENKVL	β-Lg
928.5	928.5	1+	30	39.0	88-95	NENKVLVL	β-Lg
685.4	685.5	1+	19	38.5	90-95	NKVLVL	β-Lg
571.4	571.4	1+	9	38.1	91-95	KVLVL	β-Lg
932.4	932.4	1+	20	22.6	96-102	DTDYKKY	β-Lg
1158.6	1158.6	1+	28	38.8	96-104	DTDYKKYLL	β-Lg
1305.7	1305.7	1+	13	44.7	96-105	DTDYKKYLLF	β-Lg
827.5	827.5	1+	24	36.9	99-104	YKKYLL	β-Lg
974.6	974.6	1+	16	43.9	99-105	YKKYLLF	β-Lg
664.4	664.4	1+	17	34.7	100-104	KKYLL	β-Lg
811.5	811.5	1+	13	42.7	100-105	KKYLLF	β-Lg
643.2	643.2	1+	10	29.0	105-109	FCMEN	β-Lg
1371.4	1371.5	1+	15	29.8	105-116	FCMENSAEPEQS	β-Lg
783.2	783.3	1+	12	19.2	106-112	CMENSAE	β-Lg
1224.4	1224.5	1+	21	21.2	106-116	CMENSAEPEQS	β-Lg
990.3	990.4	1+	13	17.5	108-116	ENSAEPEQS	β-Lg
861.3	861.4	1+	15	17.0	109-116	NSAEPEQS	β-Lg
747.3	747.3	1+	25	16.6	110-116	SAEPEQS	β-Lg

678.3	678.3	1+	20	39.3	117-122	LVCQCL	β-Lg
815.4	815.4	1+	20	24.8	123-129	VRTPEVD	β-Lg
930.5	930.5	1+	13	24.6	123-130	VRTPEVDD	β-Lg
1059.5	1059.5	1+	20	25.5	123-131	VRTPEVDDE	β-Lg
1130.5	1130.5	1+	21	26.4	123-132	VRTPEVDDEA	β-Lg
574.1	574.2	1+	30	19.3	126-130	PEVDD	β-Lg
703.2	703.3	1+	28	20.1	126-131	PEVDDE	β-Lg
780.2	2338.3	3+	18	47.2	130-149	DEALEKFDKALKALPMHIRL	β-Lg
856.4	1712.0	2+	22	39.3	132-146	ALEKFDKALKALPMH	β-Lg
1047.5	2094.2	2+	24	45.0	132-149	ALEKFDKALKALPMHIRL	β-Lg
820.9	1640.9	2+	24	37.9	133-146	LEKFDKALKALPMH	β-Lg
1012.1	2023.2	2+	40	43.4	133-149	LEKFDKALKALPMHIRL	β-Lg
666.4	666.3	1+	21	19.9	134-138	EKFDK	β-Lg
978.6	978.6	1+	13	25.6	134-141	EKFDKALK	β-Lg
1049.6	1049.6	1+	18	27.9	134-142	EKFDKALKA	β-Lg
764.4	1527.8	2+	55	35.7	134-146	EKFDKALKALPMH	β-Lg
955.5	1910.1	2+	29	42.7	134-149	EKFDKALKALPMHIRL	β-Lg
950.5	950.6	1+	9	41.7	142-149	ALPMHIRL	β-Lg
879.5	879.5	1+	9	41.0	143-149	LPMHIRL	β-Lg
806.4	806.4	1+	11	37.8	150-156	SFNPTQL	β-Lg
871.4	871.4	1+	15	31.3	156-162	LEEQCHI	β-Lg
914.9	1829.0	2+	12	39.9	4-18	TKCEVFRELKDLKGY	α-La
1121.6	2242.2	2+	12	42.8	4-23	TKCEVFRELKDLKGYGGVSL	α-La
660.3	660.4	1+	6	19.4	10-14	RELKD	α-La
836.4	836.5	1+	22	28.6	12-18	LKDLKGY	α-La
982.4	982.4	1+	12	44.7	24-31	PEWVCTTF	α-La
756.3	756.3	1+	17	42.5	26-31	WVCTTF	α-La
564.2	564.2	1+	11	19.2	32-36	HTSGY	α-La
908.4	908.4	1+	25	18.9	32-39	HTSGYDTQ	α-La
979.4	979.4	1+	7	20.7	32-40	HTSGYDTQA	α-La
597.3	597.3	1+	11	19.5	36-40	YDTQA	α-La
1090.5	1090.5	1+	12	21.4	40-49	AIVQNNDSTE	α-La
1019.5	1019.5	1+	10	18.6	41-49	IVQNNDSTE	α-La
578.3	578.2	1+	18	20.4	84-88	DLTDD	α-La
721.4	721.4	1+	11	24.5	89-94	IMCVKK	α-La
926.5	1852.0	2+	7	46.3	88-103	DIMCVKKILDKVGINY	α-La
1062.6	1062.6	1+	25	37.5	89-97	IMCVKKILD	α-La
868.9	1737.0	2+	51	44.1	89-103	IMCVKKILDKVGINY	α-La
808.4	808.4	1+	11	30.4	97-103	DKVGINY	α-La
693.4	693.4	1+	9	30.1	98-103	KVGINY	α-La
821.4	1641.8	2+	28	35.3	104-117	WLAHKALCSEKLDQ	α-La
728.3	1455.8	2+	33	28.6	105-117	LAHKALCSEKLDQ	α-La
791.4	791.4	1+	20	41.5	118-123	WLCEKL	α-La