Reduction of whey protein concentrate antigenicity by using a combined enzymatic digestion and ultrafiltration approach

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Abstract The global interest in saving food resources is leading to recycle wasted-food materials to extract useful nutrients. In dairy industry, the recycling of whey proteins determines their utilization in the healthy-addressed foods, which, however, can cause immunological responses in allergic subjects. In this work, a whey protein concentrate (WPC) was alternatively hydrolyzed with pepsin, papain, trypsin and rennin in order to attenuate or abolish the β -lactoglobulin (BLG) antigenicity. The electrophoretic profiles of both pepsin and papain WPC hydrolysates proved the disappearance of the BLG band, even though a slight antigenicity was still found by ELISA. Pepsin hydrolysates, filtered through a 10-kDa cut-off membrane, did not produce immunological response. A deeper investigation carried out on pepsin digested and ultrafiltered samples by LC–MS/MS showed the disappearance of the immunoreactive BLG-fragment IVTQMKGLDIQK-VAGTW. The remaining peptides, partially overlapped to major IgE binding epitopes, were not able to give immunoreactivity response. The combined WPC pepsin digestion with ultrafiltration confirmed to be a user-friendly strategy to reduce markedly the WPC antigenicity. The improvement of this two-steps process could be used to produce novel hypoallergenic infant food formulas.

Keywords β -Lactoglobulin · Antigenicity · Enzymatic digestion · ELISA · LC–MS

Introduction

Food allergies are one of the major public health concerns worldwide. Most individuals with a cow's milk protein allergy and/or intolerance show clinical symptoms at the ages of 6–12 months, when their mother's milk may be replaced or supplemented with milk of animal origin and integrated with complementary foods (Sicherer and Scott 2011). Recently, whey proteins (WP), well known for their high nutritional value and functional properties, have gained increased interest for their applications as functional ingredients in foods (Birsén and Akin 2012). Peptides from WP have been demonstrated to exert antimicrobial (Quintieri et al. 2012; Baruzzi et al. 2015; Caputo et al. 2015), antioxidant (Mann et al. 2015), antihypertensive and anti-cancer properties (Morris and Fitzgerald 2008).

The latest efforts directed to save food resources are mainly based on the exploitation of large volumes of whey wastes arising from cheese production, that represent a good source of proteins to be utilized for the production of new added-value food products. Notably, whey protein concentrates (WPC) and whey protein isolates (WPI), thanks to high nutritional value of their proteins, have become important ingredients in the manufacturing of added-value products including dairy, infant formulas, sport nutrition foods, meats, bakery, confections, snack foods, and beverages (Solak and Akin 2012).

However, the major constituent of WP is the β -lactoglobulin (BLG; 55–60%), which also causes the main allergic reaction in children (Chen and Gao 2012; Breiteneder and Mills 2013). In clinical studies, BLG showed

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the highest rate of oral food challenges in children with milk allergies (Schutte and Paschke 2007).

New research over the last ten years has reported that there has been a reduction of immunoreactivity by enzymatic hydrolysis, although some discrepancies can be found in the reported data. On the one hand, proteolysis using various enzymes or microbial proteinases, also in combination with heat denaturation, has been widely applied (Nakamura et al. 1993; Kim et al. 2007; El Mecherfi et al. 2011); the reduction in BLG IgE immunoreactivity in the hydrolysates by in vitro test was also demonstrated (Izquierdo et al. 2008; Shi et al. 2014). Many hydrolytic processes were carried out using mixed protein substrates, which made the process kinetics and peptide source identification rather complicated (Lakshman et al. 2011). In order to contain allergen risk for whey protein based foods, several analytical methods and laboratory immune assays have been proposed for the identification of allergenic proteins and their peptides (Monaci and Visconti 2010; Monaci and Visconti 2012) resulting in an ongoing update of allergen databases (Sircar et al. 2014). Even though, extensively hydrolyzed protein formulas (EHF) have been developed so far to manage the allergies of cow's milk and digestive disorders (Hays and Wood 2005), little is known on the allergenic whey protein epitopes and/or their disappearance following this strategy (Peñas et al. 2006a; 2006b; Benedé et al. 2014).

Recently, European legislation (Commission Delegated Regulation, EU 2016/127) has allowed the production of new infant formula manufactured from protein hydrolysates, in addition to tryptic digest, previously regulated, paying attention to requested nutritional composition. In

these cases, in addition to preliminary evaluation, the new products will be defined as hypoallergenic only after a case-by-case clinical evaluation of their safety.

In this work we focus on the characterization of peptide profile in extensively hydrolysed WPC samples endowed with a reduced BLG antigenicity. To this aim WPC was alternatively hydrolysed with several enzymes (pepsin, papain, trypsin and rennin) and ultrafiltered. The hydrolysate with the lowest residual antigenicity was further analysed by LC–MS/MS to identify the sequences of released peptides.

Materials and methods

Experimental design, chemicals and reagents

Experimental activities described in the following paragraphs have been summarized in a graphical scheme in the Fig. 1. Whey protein concentrate (WPC) was purchased from Armor (Protarmor 80; Armor Protéines, Le Pont, France) and was composed (as dry matter) by β -lactoglobulin (60%; BLG), α -lactalbumin (20%, ALA), bovine serum albumin (8%; BSA) and immunoglobulins (10%) as reported in the technical data sheet. Porcine pepsin (EC 3.4.23.1; 250 units/mg solid), porcine trypsin (EC 3.4.21.4; $\geq 10,000$ BAEE units/mg), papain (E.C. 3.4.22.2; 3 units/mg), and rennin from calf stomach (E.C. 3.4.23.4; ≥ 20 units/mg) were obtained from Sigma (Sigma-Aldrich Chemicals, St. Louis, MO). All chemicals and reagents for the electrophoresis analysis were obtained from Bio-Rad (Bio-Rad Laboratories srl, Milan, Italy).

Fig. 1 Graphical scheme of the experimental plan carried out in the present work

Enzymatic hydrolysis of WPC

WPC powder (5%, w/v) was dissolved in distilled water; temperature and pH were adjusted with respect to the enzyme used before starting each hydrolysis reaction. Briefly, pepsin and rennin (3%, w/w of substrate) digestions were carried out at pH 3.0 and 37 °C, whereas trypsin (0.375%, w/w of substrate) and papain (5%, w/w of substrate) reactions were performed at pH 8.0 and 37 °C, or pH 6.2 and 55 °C, respectively. WPC powder, dissolved in hydrolysis buffers without enzymes were also included. After 4 h of incubation, each reaction was stopped by heating samples at 90 °C in a water bath for 15 min. Except for the WPC tripsyn digest, the pH of the remaining hydrolysates was re-adjusted to 7.0 by using 1 M NaOH. Insoluble solids were removed in all samples by centrifugation at 10,000×g for 20 min, at 4 °C. Each supernatant was collected and quantified for residual protein content by the Bradford's method against a calibration curve of BLG (ranging from 0.1 to 1.0 mg/mL).

SDS–PAGE and ultrafiltration

The electrophoretic analysis of WPC hydrolysates was performed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE), using the Criterion™ Cell. Samples (10 µl) were mixed with sample buffer (1:1, v/v) consisting of 4% SDS, 3% β-mercaptoethanol, 10% glycerol, 50 mM Tris–HCl pH 6.8 and applied to 4–20% Criterion™ TGX precast gels. Electrophoresis was carried out initially at 100 V for 15 min and then at 150 V for 60 min. After run, gel was stained with Bio-safe Coomassie stain (Bio-Rad Laboratories srl), following the manufacturer's instructions. Destained gels, digitally acquired by the Image Scanner III (GE Healthcare, USA), were processed using the ImageMaster 2D Platinum 7.0 (GE Healthcare). Protein bands on SDS–PAGE were visually recognized and matched. The densitometric analysis of the gels was performed by subtracting the lane-based background and band intensity was measured by the area under its intensity profile curve.

All samples showing the disappearance of BLG band on SDS–PAGE and their related controls were selected and filtered on Amicon® Ultra-4 Centrifugal Filter membranes with 10-kDa molecular-weight cut-off membrane (Merck Millipore Ltd, Tullagreen, Ireland) for 40 min at 7500×g.

Enzyme-linked immunosorbent assay (ELISA)

Digested ultrafiltered WPC samples, as well as not ultrafiltered digests and controls, were assayed for the immunoreactivity by using the RIDASCREEN® β-Lactoglobulin ELISA kit (R4901, R-Biopharm, Darmstadt,

Germany), according to the manufacturer's instructions. This competitive enzyme immunoassay is generally used for the quantitative analysis of native and processed BLG in hydrolyzed milk products containing the related peptides.

Liquid chromatography and mass spectrometry analysis

Samples showing the lowest BLG content by ELISA analysis were analysed by LC–MS/MS in order to identify peptides released after enzymatic digestion. For MS analysis, a Linear IonTrap Mass Spectrometer Velos Pro™ (Thermo Fisher Scientific, San José, CA, USA) equipped with a UHPLC pump and an ESI interface was used. Analyses were performed in data-dependent acquisition (DDA) mode enabling a global proteomics analysis. In this mode the mass analyser performs two scan events: a full MS scan event (first event) and then 20 most abundant ions are selected for the subsequent MS/MS analysis (second event). In this frame ion charge screening is activated and only doubly and triply charged ions are typically selected for the subsequent CID activation.

Separation of peptides was accomplished on the Acclaim™ PepMap100 analytical column (internal diameter 1 mm, length 150 mm, 3 µm particle size) as previously reported by Monaci et al. (2014). Samples were four-fold diluted (v/v) in mobile phase before the injection. Raw data were processed via commercial software Proteome Discoverer™ version 1.3 (Thermo Fisher Scientific) and identification was achieved by Sequest search algorithm by interrogating the Uniprot database (DB). Software results were filtered by peptide mass deviation (300 ppm), and peptide confidence (high = 0.01).

Statistical analysis

Residual protein concentrations and BLG derived peptides from digested whey samples were analysed by using one-way ANOVA with SPSS Statistics 21.0 (SPSS, IBM corp. Armonk, NY). Homogeneity of variances was assessed with Levene's test and significantly different means were separated by Duncan's post hoc test ($p < 0.05$).

Results and discussion

Enzymatic hydrolysis of WPC

Aiming at reducing the allergenic potency of whey proteins and enhancing their exploitation in food preparation, the WPC was extensively digested with four different enzyme in order to inactivate BLG antigenicity.

A significant reduction in the protein content was recorded after subjecting WPC to 4 h of hydrolysis with the enzymes pepsin, papain and rennin ($F(6,14) = 2329,90$, $p = 3.61 \times 10^{-20}$). In particular, protein concentration in the pepsin digested WPC was undetectable, whilst the residual protein contents in the papain and rennin hydrolysates (0.246 and 0.791 mg/mL, on average) were significantly ($p < 0.05$) lower than those found in the related undigested controls (2.82 and 4.18 mg/mL, on average). In contrast, a moderate but significant ($p < 0.05$) reduction in protein content was calculated for WPC hydrolyzed with trypsin (1.93 ± 0.05 mg/mL calculated in digested sample and 4.48 ± 0.12 in the undigested sample).

The enzymatic activity produced hydrolysis levels of 61.4%, 84.18%, 95.08 and 100% for trypsin- rennin- papain- and pepsin digested WPC. SDS–PAGE profiles of the hydrolysates and undigested WPC samples are shown in Fig. 2. At the end of the enzymatic reaction, ALA and BLG were almost completely digested by pepsin and papain (Fig. 2, lanes 3 and 5). This result was in contrast with that reported by other authors showing a resistance of BLG to pepsin digestion (Mandalari et al. 2009), and therefore combined pepsin hydrolysis with heat or

microwave treatments (Bertrand-Harb et al. 2002; El Mecherfi et al. 2011). In the present work, the more extended digestion time (up to 4 h) and the slightly higher pH value of the pepsin buffer (pH 3.0) than those generally applied for the optimal pepsin activity (pH 1.5–2.5), probably favored the susceptibility of BLG to proteolysis.

Papain caused the disappearance of BSA and BLG (Fig. 2 lane 3). These results were partially consistent with those of Peñas et al. (2006a) who found an elevated hydrolytic activity of papain towards BLG and other whey proteins at pH 7.0 and 70 °C. Conversely, Izquierdo et al. (2008) reported the hydrolysis with papain only under microwave irradiation. SDS–PAGE electrophoresis showed also the appearance of aggregates with a molecular weight higher than 29 kDa, probably attributed to hydrophobic BLG fragments as reported by Lieske and Konrad (1996).

Even though rennin- and trypsin digested WPC showed protein content of 0.79 ± 0.08 and 1.93 ± 0.05 mg/ml respectively, their protein patterns on SDS–PAGE were similar and characterized principally by BLG and ALA bands (Fig. 2, lanes 4 and 9). Tryptic resistance of ALA has been reported by Nakamura et al. (1993). On the contrary, although BLG becomes less stable under alkaline conditions facilitating the release of amino acids from the partially hydrolyzed protein (Lakshman et al. 2011), no significant change in the BLG band intensity was found after trypsin digestion.

Enzyme-linked immunosorbent assay (ELISA)

WPC hydrolysates digested with pepsin and papain, showing the disappearance of BLG band on SDS–PAGE, were further assayed for their immunoreactivity by an ELISA commercial kit able to detect reactive peptides in highly hydrolyzed milk products and then commonly used by food manufacturers to check cross-contamination by whey proteins in all food processing steps (HaiYing et al. 2012).

After 4 h of incubation a significant reduction (by 85.3%, on average; $p < 0.05$) in BLG content was observed in WPC digested with pepsin (Table 1). As discussed above, our data disagreed with the information reported by other Authors instead reporting BLG resistance to pepsin hydrolysis for a shorter time (ca. 60 min) (Mandalari et al. 2009; Benedé et al. 2014). The extended WPC incubation with pepsin showed similar results to those found by Kilara and Panyam (2003) that demonstrated as combination of pepsin and chymotrypsin was found the best treatment to reduce both ALA and BLG allergenicity.

A residual BLG content was instead found in the papain hydrolysed sample. This result apparently contrasts with the disappearance of the BLG band in SDS–PAGE.

Fig. 2 SDS–PAGE of whey protein hydrolysates after 4 h of reaction with each enzyme. Lane 1: molecular weight marker; lane 2: standard of whey protein (BLG β -Lactoglobulin, ALA α -lactalbumin, BSA bovine serum albumin, BLF Lactoferrin); lane 3, papain WPC hydrolysate; lane 4, trypsin-WPC hydrolysate; lane 5, pepsin WPC hydrolysate; lane 6, undigested WPC dissolved in hydrolysis buffer pH 6.2; lane 7, undigested WPC dissolved in hydrolysis buffer pH 3; lane 8, rennin-WPC hydrolysate; lane 9, undigested WPC dissolved in hydrolysis buffer pH 8; lane 10, undigested WPC dissolved in water

Table 1 BLG and BLG-derived peptides (ppm) detected using the RIDASCREEN® β -Lactoglobulin kit in selected digested samples; WPC papain hydrolysate (papHWPC), pepsin WPC hydrolysate (pHWPC) and their undigested controls (WPC: WPC dissolved in water, WPC pH 3: WPC dissolved in the reaction buffer of pepsin, WPC pH 6: WPC dissolved in the reaction buffer of papain); P10 kDa: permeate from filtration of each sample by 10-kDa molecular-weight cut-off

Samples	BLG and BLG-derived peptides (ppm)*
WPC	524.57 $\pm$ 0.78 ^h
WPC_P10 kDa	7.13 $\pm$ 0.20 ^b
papHWPC	249.23 $\pm$ 0.48 ^f
papHWPC_P10 kDa	39.93 $\pm$ 0.71 ^d
pHWPC	77.47 $\pm$ 0.37 ^e
pHWPC_P10 kDa	— ^a
WPC pH 6	433.96 $\pm$ 3.71 ^g
WPC pH6_P10 kDa	— ^a
WPC pH 3	579.61 $\pm$ 0.36 ⁱ
WPC pH 3_P10 kDa	24.67 $\pm$ 0.63 ^c

* Values represent mean $\pm$ SD (N = 3). Same superscript letters represent similar mean values ($p > 0.05$) as established by Duncan's post hoc test. One-way ANOVA $F(9,20) = 104,837$, $p = 1 \times 10^{-44}$

—: Not detected; limit of detection 0.115 ppm β -lactoglobulin

However, Wróblewska et al. (2004) showed that the immunoblotting of WPC hydrolysates, obtained after 2 h of reaction with papain registered a reaction occurring between BLG and anti-BLG antibodies. Traces of proteins with higher molecular masses (*ca.* 33 kDa) were also immunostained, indicating that agglomerates of BLG reacted with the specific antibody.

The immunoreactivity of BLG digests was found to be consistent with the levels of intact BLG present showing almost negligible IgE binding after gastrointestinal digestion (Benedé et al. 2014). In fact, in order to remove large allergenic peptides or undigested proteins, the proteolytic process is usually combined with the ultrafiltration process (Restani et al. 2009).

In this work the samples were fractioned with a 10 kDa cut-off membrane and analysed by the enzyme-linked immunosorbent assay, able to detect also the protein fragments. The results showed that residual peptides in the ultrafiltrate samples of papain digested WPC retained a significantly ($p < 0.05$) lower BLG immunoreactivity than the related controls. However, as the pepsin digested and ultrafiltered WPC samples did not show any residual content of reactive BLG or derivative peptides (Table 1), they were selected to be further analysed by LC–MS/MS.

Liquid chromatography and mass spectrometry analysis

In the light of these results, the pool of peptides generated by enzymatic hydrolysis followed by ultrafiltration was analysed and identified by LC–MS/MS analysis. Among hundreds of peptides identified in the digested or/and ultrafiltered samples we addressed our attention to those potentially involved in the immunoreactivity recorded by the ELISA assay. Table 2 shows the aminoacid sequence of residual peptides released from BLG, contained in the WPC pepsin digest, before and after ultrafiltration. It is worth mentioning that the peptides in the digested and

Table 2 Peptides sequences belonging to BLG (Accession N° P02754; Score 21.86; Coverage 29.78) and identified by LC–MS/MS analysis in the pepsin digested WPC, before and after ultrafiltration (10 kDa) process

Sequence	BLG fragments	PEP	X _{CORR}	MH ⁺	Δ M (ppm)	m/z	Missed cleavages	Retention time
<i>Pepsin hydrolysis</i>								
IVTQMKGLDIQKVAGTW	2–18	6.8×10^{-6}	6.21	1989.07	−4.35	995.04	1	16.41
LDAQSAPLRVY	32–42	1.46×10^2	4.58	1232.69	21.13	616.84	2	8.95
VEELKPTPEGDL	43–54	0.175	4.66	1326.48	142.95	663.75	1	7.76
KIDALNENKVL	83–94	0.12	3.02	1256.69	−23.73	628.85	1	4.85
KIDALNENKVLVL	83–96	3.03×10^2	4.72	1468.87	−0.64	734.94	2	12.95
VRTPEVDDEAL	123–133	0.18	3.32	1243.52	−74.79	622.26	0	5.79
VRTPEVDDEALEKF	123–136	0.015	4.99	1648.16	208.43	824.58	1	11.34
<i>Pepsin hydrolysis followed by ultrafiltration (10 kDa)</i>								
LDAQSAPLRVY	32–42	0.056	3.47	1232.69	−21.13	616.85	2	9.94
VEELKPTPEGDL	43–54	0.175	4.66	1326.48	142.95	663.75	1	7.76
KIDALNENKVL	83–94	0.069	3.86	1256.69	−23.73	628.85	1	5.67
KIDALNENKVLVL	83–96	0.196	4.97	1468.87	−0.64	734.94	2	13.30
VRTPEVDDEAL	123–133	0.18	3.32	1243.52	−74.79	622.26	0	5.79
VRTPEVDDEALEKF	123–136	0.015	4.99	1648.16	208.43	824.58	1	11.34

unfiltered samples differed only for the peptide IVTQMKGLDIQKVAGTW corresponding to the sequence 2–18 amino acids of BLG. This peptide partially overlapped with BLG (1–14) epitope recognized by sera from milk allergic patients (Järvinen et al. 2001), suggesting a possible explanation of the reduced BLG antigenic response in the ultrafiltered pepsin digested WPC. However, the ultrafiltered pepsin digested WPC still showed BLG (32–42), BLG (43–54), BLG (83–94/96) and BLG (123–133/136) peptides. Among these, BLG (32–42) overlapped with the sequence BLG (29–41) as well as BLG (83–94/96) overlapped with BLG (86–100) that did not react with patient's sera or did not show IgE binding when they were included in a larger fragment (Benedé et al. 2014). At contrary, the remaining peptides belonged to the two immunoreactive BLG regions identified as major IgE epitopes, namely 43–68 and 121–146 (Benedé et al. 2014), although in this study the immunoreactivity of these regions was not evidenced by ELISA assays. As recently published (Villas-Boas et al. 2015) the occurrence of BLG peptides 42–54 and 41–60 was still found after simulated gastrointestinal digestion; however in this study the IgE binding (%) estimated by inhibition ELISA (from six patient's sera) was only the $0.79\% \pm 0.18$ in comparison with native undigested BLG ($115.41\% \pm 16.28$).

In addition, the immunoreactivity of IgE antibodies for BLG epitopes can be affected by peptide length and the presence of critical amino acids (Cocco et al. 2007). Thus, despite the allergenic BLG (43–54) and BLG (123–133/136) peptides occurrence in pepsin-digested WPC as shown by MS–MS analysis, we can argue that their immunoreactivity or amount is too low to be detected by the commercial ELISA kit applied in this study and showing 0.5 ppm BLG quantification limit and 0.115 ppm BLG detection limit.

Conclusion

In this paper WPC hydrolysates obtained with four proteolytic enzymes were evaluated for residual BLG antigenicity, in order to obtain at least one hypoallergenic product with high nutritional value.

Despite extensive digestion of whey proteins, the lowest BLG antigenicity was shown only when pepsin is used. The ultrafiltration of pepsin-digested WPC allowed to completely drop BLG reactivity, even though LC–MS/MS analysis still detected some allergenic epitopes. This is the first work showing a new two-stage approach based on pepsin digestion and ultrafiltration in order to obtain extensively hydrolysed formula. In addition, results of this work show that this method is more effective than trypsin-based process of WPC, actually allowed by European regulation

for infant formulae, in reducing BLG antigenicity. Even though, clinical trials are needed to assess the residual antigenicity of other milk allergens and to prevent cow's milk protein allergy, the promising results of this work pave the way for a safer exploitation of whey proteins.

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