



MALDI based identification of whey protein derived tryptic marker peptides that resist protein glycation

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ABSTRACT

Cow's milk and dairy products are a common cause of food allergy especially for children. The extensive use of milk proteins, especially whey, in food products poses a serious threat to allergic consumers, therefore reliable detection methods are needed in order to ensure meticulous labeling and to protect allergic consumers. The aim of the study was to do a screening of the tryptic peptides derived from whey proteins which were still identifiable using MALDI-TOF/MS and MS/MS analysis despite severe modification through food processing simulating reactions. A bottom-up approach was therefore used. Whey protein mixtures were subjected to the Maillard reaction with glucose, both in the absence or presence of soluble wheat proteins. Despite severe changes on protein level as evidenced by a time dependent increase of protein bound carbonyls, fluorescent compounds, and aggregation as well as a parallel decrease of available lysine residues, several peptides were proven to remain stable. Using MALDI-TOF/MS and MS/MS analysis of tryptic digests of the glycosylated proteins, two unmodified peptides derived from β -lactoglobulin were identified (¹⁵Val–Arg⁴⁰ and ⁴¹Val–Lys⁶⁰). These stable peptides could serve as analytical targets for the development of new robust analytical approaches for detection of undeclared whey protein residues in foods.

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1. Introduction

Cow's milk and dairy products are major nutrients in the human diet, especially during infancy (Mouecoucou, Fremont, Villaume, Sanchez, & Mejean, 2007). Though at one time whey proteins were considered as by-product of the cheese making process, nowadays, due to their wide ranging nutritional, biological, and functional properties, whey proteins are often used in food formulations as low-cost protein ingredients (Liu, Xiong, & Butterfield, 2000). However, whey-protein fractions represented by β -lactoglobulin (β -LG) and α -lactalbumin (α -LA) are recognized as major allergens (Lam et al., 2008). Therefore, their extensive use in foods might pose a serious threat to the milk allergic consumers.

It is well known that various processing methods may affect the allergenicity of the food products. Changes in allergenic activity may be due to hydrolysis, denaturation or via chemical reactions with other food components, leading to structural modifications of the allergen's epitopes. Maillard reaction which involves the reaction between amino acids and reducing sugars is promoted by thermal processing and long storage (Contreras-Calderon, Guerra-Hernandez, & Garcia-Villanova, 2009).

There is abundant evidence that thermal processing and non-enzymatic browning reactions can have a great impact on the IgE-binding activity of food allergens. However, the effects observed are highly dependent on the type of allergen studied (Gruber, Becker, & Hofmann, 2005; Gruber, Vieths, Wangorsch, Nerkamp, & Hofmann, 2004; Nakamura, Watanabe, Ojima, Ahn, & Saeki, 2005; Nakamura et al., 2008).

Apart from the consequences of this and other reactions occurring in foods on the allergenicity of proteins, they are relevant from an analytical prospective as well. Nowadays, only enzyme-linked immunosorbent assay (ELISA) is used in routine food analysis for the determination of food allergens because it is cheap, easy to perform and relatively sensitive. However, commercially available ELISA kits are mainly developed using antibodies against purified allergens isolated from soluble protein fractions obtained from the natural products. Due to the multitude of chemical and structural changes occurring on the protein level during food processing, these receptor based methods may lack robustness because of the altered affinity between the antibodies and the modified proteins (Cucu et al., 2011; Downs & Taylor, 2010; Platteau et al., 2011; Scaravelli, Brohee, Marchelli, & van Hengel, 2009). This may lead to erroneous results, which in view of the potential consequences linked to the undeclared presence of hidden allergens can represent a significant health risk.

Mass spectrometry is one of the dominant approaches used for the identification of proteins and peptides. Given that food allergens are mainly proteins, mass spectrometry is gaining more and more

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attention in the context of the food allergens detection. The use of mass spectrometric approaches for the identification of specific highly stable peptides as markers for the presence of peanut allergens was previously described (Shefcheck, Callahan, & Musser, 2006; Shefcheck & Musser, 2004). Weber, Raymond, Ben Rejeb, and Lau (2006) identified two peptides originating from casein as potential milk markers and traces of milk could be detected in spiked cookies. Further, quantification of β -LG in milk products was achieved by Czerwenka, Maier, Potocnik, Pittner, and Lindner (2007). Although they reported 100% recovery for the whole milk, the recovery decreased to 50% in strongly processed products. The aim of this study was to identify segments of whey proteins which were still identifiable using matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF/MS) and MS/MS analysis, even after the proteins were subjected to various changes on the molecular level as a consequence of a progressive Maillard reaction in the presence and absence of soluble wheat proteins. These stable peptides hold potential to be used for the development of new quantitative analytical methods for the detection of whey protein residues in processed foods.

2. Materials and methods

2.1. Reagents and standards

Whey protein isolate (Lacprodan® DI-9224) was kindly provided by Acacris Food Belgium (Londerzeel, Belgium). Soluble wheat proteins (Meripro 711) obtained by a property owned process including partial hydrolysis, were kindly provided by Syral Belgium N.V. (Aalst, Belgium). Chemicals and standards of analytical grade were obtained from Sigma-Aldrich (Bornem, Belgium), VWR (Leuven, Belgium) and Acros Organics (Geel, Belgium). Solvents were of analytical grade from Chem-Lab (Zedelgem, Belgium). Gel and standards for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were from Bio-Rad (Nazareth, Belgium). The Krypton protein stain was from Thermo Scientific (Aalst, Belgium). Milk chocolate (14% dry milk powder) and cookies (2.4% dry milk powder) were purchased in a local store.

2.2. Preparation of the model systems

The model systems were prepared in 0.1 M phosphate buffer pH 7.4 by mixing 1% (w/v) whey proteins with 6% (w/v) glucose (Cucu et al., 2011). Two types of model systems with wheat proteins were also considered. One model system was obtained by mixing 0.25% (w/v) of whey proteins with 0.75% (w/v) of wheat proteins and 6% (w/v) of glucose. Another model system was obtained by mixing 0.5% (w/v) whey proteins with 0.5% (w/v) wheat proteins and 6% (w/v) glucose. Respective model systems without glucose were prepared as control.

2.3. Protein bound carbonyls

Protein bound carbonyls were measured after converting them to the corresponding 2,4-dinitrophenylhydrazones, according to the method of Levine et al. (1990) with slight modifications (Cucu et al., 2011). The carbonyl content was calculated using a molar absorption coefficient of $22,000 \text{ M}^{-1} \text{ cm}^{-1}$.

2.4. Reactive lysine residues

The loss of reactive lysine residues was monitored using derivatization with *ortho*-phthalaldehyde (OPA) in the presence of 2-mercaptoethanol as described previously (Cucu et al., 2011).

2.5. Fluorescent products

Samples were diluted to 1 mg/mL protein in phosphate buffer saline pH 7.4 (135 mM NaCl, 1.5 mM KH_2PO_4 , 8 mM Na_2HPO_4 , 2.7 mM KCl) and the relative fluorescence intensity (RFI) was measured after excitation at 340 nm and emission at 415 nm as previously reported (Corzo-Martinez, Moreno, Olano, & Villamiel, 2008) using a Cary 50 UV-vis Spectrophotometer from Varian (Sint-Katelijne-Waver, Belgium).

2.6. Protein determination

Protein was assessed by determining the nitrogen content according to the Kjeldahl procedure. A factor of 6.38 was used to convert nitrogen to whey protein and 5.70 to convert to wheat proteins.

2.7. SDS-PAGE

A 4% acrylamide stacking and a 15% acrylamide resolving gel were used for protein separation. Aliquots of 10 μL sample containing 10 μg of protein were added to 10 μL Laemmli sample buffer containing 2-mercaptoethanol and heated at 95 °C for 5 min. Precision Plus Protein unstained (Bio-Rad) was used as molecular weight marker. Krypton fluorescence staining was used for bands visualization. The gels were further scanned using the Molecular Imager PharoFX Plus from Bio Rad.

2.8. MALDI-TOF/MS and MS/MS

Native and glycosylated whey proteins solution (in the presence or absence of soluble wheat proteins) were digested with trypsin and the resulting peptide mixtures were separated on a RP column as follows. An aliquot of 60 μg of total protein were digested overnight with trypsin at 37 °C in 50 mM ammonium bicarbonate pH 7.8 at a protease to protein ratio of 1:48 (w/w). Peptides were concentrated by vacuum centrifugation and finally redissolved in 100 μL of 0.1% formic acid. Digested peptides were first separated using the Ettan liquid chromatography (LC) system equipped with an UV detector from GE Healthcare (Brussels, Belgium). The RP column was a $220 \times 2.1 \text{ mm}$ Spheri-5 PTC column with particle size of 5 μm from Higgins Analytical Inc. (Mountain View, California, USA). The column was equilibrated with 100% H_2O with 0.1% trifluoroacetic acid (TFA) and elution was achieved by the following stepwise gradient with acetonitrile 100% with 0.1% TFA (mobile phase B): 0 min to 18 min—0% B; 18 min to 58 min—70% B; 58 min to 68 min—100% B; 68 min to 73 min—100% B; 73 min to 110 min—0% B. The flow rate was 0.1 mL/min and fractions of 0.2 mL were collected (the first 40 fractions). The eluted peptides were monitored at 220 and 280 nm. A total of 40 fractions were collected and each was analyzed by MALDI-TOF in the MS mode to detect the peptide present in the digest. The collected fractions were dried and redissolved in 10 μL of 0.1% formic acid. Finally, 1 μL of these peptide fractions was co-crystallized with 1 μL of α -cyano-4-hydroxycinnamic acid (α -CHCA) as matrix (5 mg of α -CHCA in 1 mL of 50% acetonitrile in 0.2% TFA). The Applied Biosystems MDS Sciex 4800 Plus MALDI TOF/TOF (Foster City, California, USA) operating in positive-ion reflectron mode was used for the peptide detection. External calibration was performed with a mix of des-Arg¹-Bradykinin, Angiotensin I, Glu¹-Fibrinopeptide B, ACTH (1–17 clips), ACTH (18–39 clips), ACTH (7–38 clips) (Applied Biosystems 4700 proteomic analyzer Mass Standards Kit). Totals of 2000 shots were accumulated for each MS spectrum. For selected peptides, MS/MS spectra were collected. Protein identification was performed by searching in the protein sequence database (Swiss-Prot) using the Mascot search engine (<http://www.matrixscience.com>). The following search parameters were used: taxonomy—mammalian; precursor mass tolerance = 100 ppm; fragment mass tolerance = 0.5 Da. The enzyme entry was set for trypsin

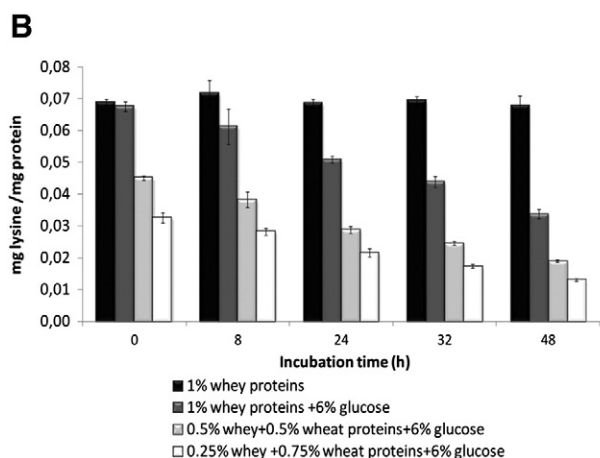
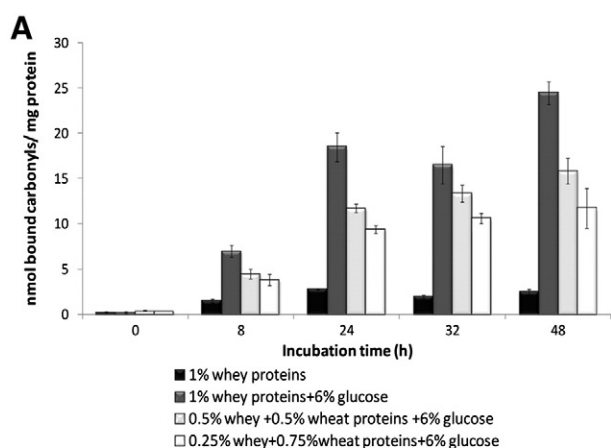


Fig. 1. Formation of protein bound carbonyls (A) and loss of available lysine residues (B) in whey protein due to incubation with glucose in the presence or absence of wheat proteins; data points represent mean values $\pm$ SD of four independent determinations.

or no enzyme, the maximum number of missed cleavages was set to two and oxidized methionine was considered as peptide modification. For the direct MALDI-TOF/MS and MS/MS, 6 μ g of the protein mixture were digested with trypsin (1:48 enzyme to protein ratio). The obtained peptides, vacuum dried, were redissolved in 10 μ L HCOOH 0.1% and 1 μ L of it was directly spotted on the MALDI plate together with 1 μ L of α -CHCA matrix.

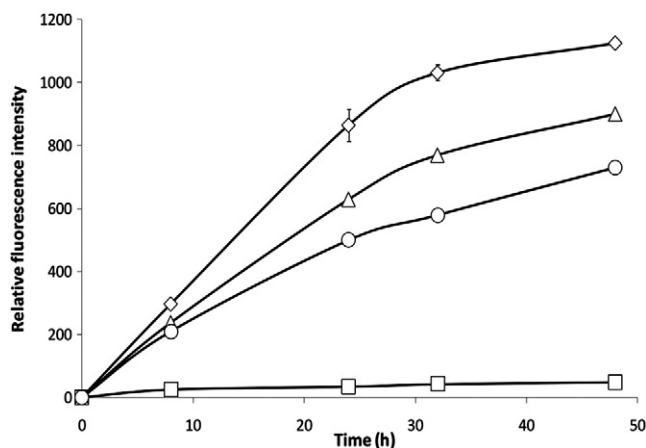


Fig. 2. Formation of fluorescent compounds in the whey protein incubated in the presence or absence of wheat proteins with glucose at 70 °C; data points represent mean values $\pm$ SD of two independent determinations. $\square$ —1% whey proteins, $\diamond$ —1% whey proteins with 6% glucose, Δ —0.5% whey proteins with 0.5% wheat proteins and 6% glucose, $\circ$ —0.25% whey proteins with 0.75% wheat proteins and 6% glucose.

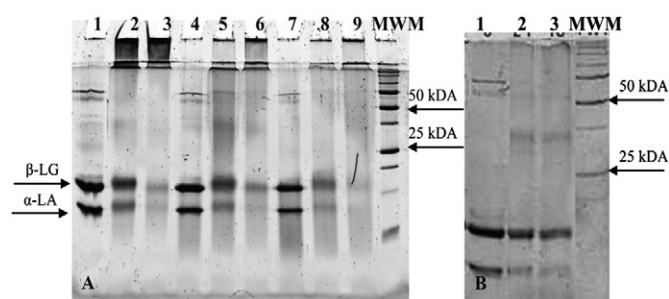


Fig. 3. A—SDS-PAGE pattern of whey proteins incubated in the presence or absence of wheat proteins with glucose: lane 1 to 3—1% whey protein native, 24 and 48 h incubated; lane 4 to 6—0.5% whey and 0.5% wheat proteins native, 24 and 48 h incubated; lane 7 to 9—0.25% whey and 0.75% wheat proteins native, 24 and 48 h incubated; lane 10—marker, B—1% whey proteins incubated alone: lane 1—native, lanes 2–3 incubated for 24 and 48 h respectively, lane 4—marker.

Melted chocolate in a water bath (1 g) was mixed with 10 mL of hexane to remove the lipids. The defatting was repeated 3 times with subsequent centrifugations. The defatted pellet was further mixed with 10 mL of 8 M urea for 1 h at room temperature. The mixture was centrifuged and the clear supernatant was diluted 1:10 with 50 mM ammonium bicarbonate pH 7.8 to lower the urea concentration before the digestion step. Digestion was carried out with 60 μ L of the diluted sample and 1.25 μ g of trypsin. The resultant peptides were separated on a RP column as mentioned above. For cookies, 10 g of sample were mixed with 15 mL of 2.9 M NaCl, 3 mM CaCl buffer and 2 μ L of α -amylase. The mixture was incubated for 4 h at room temperature. After centrifugation, 10 mL of urea (8 M) were added and samples were treated as previously.

3. Results and discussion

3.1. Assessment of the Maillard reaction

First of all, the impact of incubation of whey proteins with glucose was assessed using a number of general parameters in order to have an idea about the degree of induced modifications on protein level. It is well known that the interaction of reducing sugars or their polycarbonyl derivatives might lead to the formation of protein bound carbonyls (Stadtman & Levine, 2000). Indeed, their reaction with the side chain of proteins, mainly with lysine residues, gives rise to the advanced glycation end products (AGEs). Therefore, the amount of protein bound carbonyls was used as an indicator of the Maillard reaction degree (Liggins & Furth, 1997). As expected, an increase in the amount of protein bound carbonyls due to incubation of whey proteins with glucose was observed (Fig. 1A). After 24 h incubation at 70 °C an almost 100 fold increase in protein bound carbonyls amount was observed while after 48 h a 130 fold increase as compared to the initial amount present. Incubation of whey protein alone led to a slight increase in protein bound carbonyls amount probably due to the presence of residual lactose in the whey protein isolate (<0.2%). This proves that the formation of protein carbonyls in whey proteins was primarily due to interaction between the glucose and/or its break down products with the side chain of the whey proteins. Similarly, increases of the amount of protein bound carbonyls have been previously observed after incubation of BSA with glucose and fructose (Liggins & Furth, 1997). Further, Maillard reaction was induced in the presence of wheat proteins. Wheat proteins were chosen for the model system to simulate the presence of bulk proteins in food products. Similarly, an increase in protein bound carbonyls was observed for the model systems containing 0.5% and 0.75% wheat proteins. However, the increase observed was less prominent as compared to the model system containing no wheat proteins. After 48 h of incubation with glucose an approximately 38 fold

Table 1
Sequence of the 2 major whey allergens (the signal peptide was excluded), underlined –residues representing the IgE binding epitopes (Jarvinen et al., 2001).

α-LA—primary accession number P00711		
1	ELTKCEVFRE LKDLKGYGGV SLPEWVCTTFTHTSGYDTQAI VQNNSTHEY	50
51	G LFOQNNKIWC KDDQNPSSN ICNISCDFL DDDLTDDIMCVKILDKVG	100
101	NYWLAHKALC SEKLDQWLCE KL	123
β-LG—primary accession number P02754		
1	LIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVYVEELKPTP	50
51	EGDLEILLQKWENGECAQKKIAEKTIPAVFKIDALNENKVLVDLDYK	100
101	KYLLFCMENSAPQSLACQCLVRTPEVDDEALEKFDKALKALPMHIRLS	150
151	FNPTQLLEEQCHI	162

increase in the protein bound carbonyls was registered for the model system containing 0.5% wheat proteins and a 30 fold increase was observed in the case of the model system containing 0.75% wheat protein.

As previously mentioned, reducing sugars and their degradation products can interact with basic amino acids, more specifically with the lysine residues. The loss of lysine residues has been for a long time, used as an indicator for both the early and advanced stages of the Maillard reaction (Contreras-Calderon et al., 2009). Reactive lysine residues were therefore determined before and during incubation with glucose. As can be seen from Fig. 1B, an almost 50% decrease of reactive lysine residues was registered during incubation of 1% whey proteins with 6% glucose for 48 h. On the other hand, no decrease of reactive lysine residues was recorded in the case of incubation of protein alone even after 48 h. The reaction systems containing 0.5% and 0.75% wheat proteins contained approximately 50% and 75% less reactive lysine residues as compared with the model system containing no wheat proteins due to the known lysine deficiency of wheat proteins. Nevertheless, a similar trend in losses of reactive lysine residues was observed during incubation of these mixtures with glucose.

It has been previously shown that the incubation of proteins with reducing sugars leads to the development of fluorescent compounds at a greater extent than in their absence. These compounds are mainly formed in the advanced stages of the Maillard reaction (Rufian-Henares, Guerra-Hernandez, & Garcia-Villanova, 2002). In our case, a constant increase in the relative fluorescence intensity for the whey protein incubated with glucose in the presence or absence of wheat proteins was observed during the 48 h of incubation (Fig. 2). Upon introduction of soluble wheat proteins in the reaction systems accumulation of fluorescent compounds over time was less intense. This was in correspondence with the lower amount of reactive sites in the whey/soluble wheat protein mixtures as evidenced by their lower reactive

lysine residues content (Fig. 1B). In the absence of glucose, formation of fluorescent compounds was negligible.

The electrophoretic pattern of the whey protein under reducing conditions was characterized by 2 major bands representing α-LA with a molecular weight of 14 kDa and β-LG with a molecular weight of 18 kDa (Fig. 3A lane 1, 4 and 7, Fig. 3B lane 1). Additionally, 2 minor bands in the region of 70 kDa representing the heavy chains of the immunoglobulins could be also observed. Considering the above observed modification in whey proteins, modification in the electrophoretic pattern was also expected. Fig. 3A and B shows the SDS-PAGE pattern of the reactions systems incubated with and without glucose for 48 h. Severe changes in the SDS-PAGE pattern due to incubation with glucose were observed. The intensity of the bands representing β-LG and α-LA decreased with the incubation time and smearing progressed. Incubation for 48 h led to aggregates formation with even much bigger masses: clearly, their migration into the running gel was hindered. Since SDS-PAGE experiments were performed under reducing conditions, the observed protein aggregation cannot be due to disulfide formation. This was most probably due to the covalent bonds formed between the formed carbonyls and the available α- or ε-amino groups. Incubation of the whey proteins alone led to slight decreases of the intensity of the band representing the α-LA with formation of a new band with a molecular weight of about 36 kDa representing probably a dimer of the β-LG. This slight aggregation might be either due to the trace amounts of lactose still present in the whey protein isolate or due to the heat treatment alone.

In the case of the model systems containing also 0.5% and 0.75% wheat proteins the intensity of the bands representing β-LG and α-LA decreased. Moreover, in the non-treated samples smearing could be observed especially in the region below 20 kDa. This is due to the fact that the wheat proteins used in the model systems were partially hydrolyzed. Extensive aggregation could be seen in the case of incubation of whey proteins in the presence of wheat proteins as well. However compared to the whey proteins incubated in the absence of soluble wheat proteins, it seems that the aggregation took place in a lesser extent. These results are in line with all the other parameters assessed to monitor the progress of the Maillard reaction upon incubating the protein mixtures in the presence of glucose.

3.2. Detection of stable peptides derived from glycated α-LA and β-LG

MALDI-TOF/MS is a widely used analytical technique due to its excellent sensitivity, relatively high speed, and simplicity. As a consequence of producing mostly singly charged ions, MALDI-TOF is especially suitable for analysis of protein mixtures with or without

Table 2
Identified peptides derived from the native and modified whey proteins. Whey proteins were digested and the obtained peptides were separated on a RP column prior the MALDI-TOF/MS analysis.

Expected [M + H] ⁺	Peptide	Measured mass [M + H] ⁺			
		Native	1% whey ^a	0.5% whey 0.5% wheat ^a	0.25% whey 0.75% wheat ^a
1034.50	¹¹⁵ Leu–Lys ¹²²	1034.45	α-LA 1034.38	n/d	n/d
1200.65	⁹⁹ Val–Lys ¹⁰⁸	1200.61 (46)	1200.51 (42)	1200.64	1200.56
1642.73	⁸⁰ Phe–Lys ⁹³	1642.68	n/d	n/d	n/d
1064.44	⁶¹ Trp–Lys ⁶⁹	1064.39 (24)	β-LG n/d	n/d	n/d
1065.58	⁹² Val–Lys ¹⁰⁰	1065.53 (64)	1065.46 (32)	1065.58	1065.51
1245.58	¹²⁵ Thr–Lys ¹³⁵	1245.52 (93)	1245.42 (21)	1245.59 (86)	1245.48 (87)
1658.78	¹⁴⁹ Leu–Ile ¹⁶²	1658.72 (40)	1658.63	n/d	n/d
2313.25	⁴¹ Val–Lys ⁶⁰	2313.19 (105)	2313.03 (52)	2313.00 (7)	2313.15 (115)
2707.37	¹⁵ Val–Arg ⁴⁰	2707.30 (132)	2707.12 (68)	2707.10 (50)	2707.20 (88)

^a – 48 h incubated with 6% glucose at 70 °C, in parenthesis assigned score: > 21 = significant homology; > 33 = identity or extensive homology (p<0.05); masses without parenthesis—present with lower intensity without MS/MS analysis; n/d—not detected.

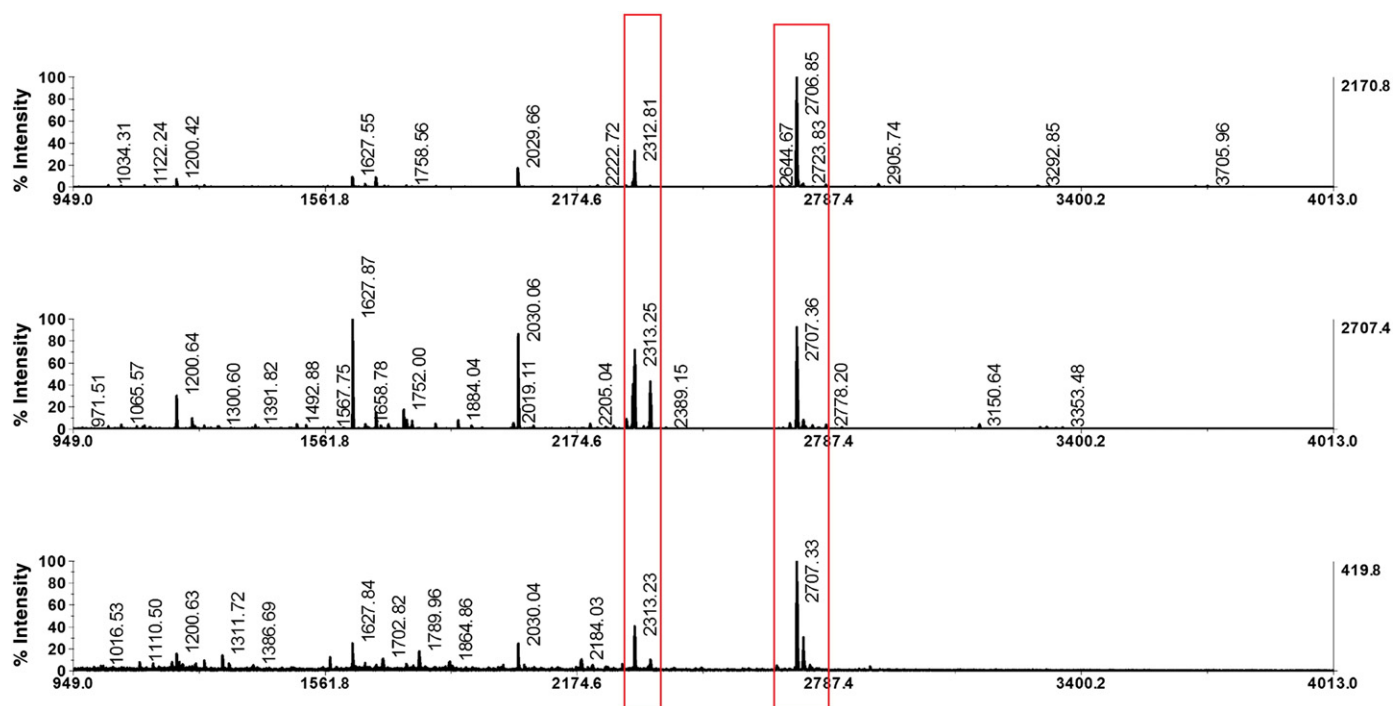


Fig. 4. MALDI-TOF/MS spectra of native and glycated whey proteins: upper—native whey proteins, middle—1% whey proteins incubated with 6% glucose for 48 h at 70 °C, lower—0.25% whey proteins with 0.75% wheat proteins incubated with 6% glucose for 48 h at 70 °C. After treatment proteins were digested with trypsin and directly analyzed via MALDI-TOF/MS. Stable peptides are marked in open boxes.

pre-fractionation. The only disadvantage of the MALDI-TOF/MS is lower reproducibility (mostly shot to shot) which makes the quantitative measurements more difficult. In this study, the MALDI-TOF was mainly used for the identification of peptides which remained stable even after the induced food processing simulating reactions. As previously reported, mass spectrometric based methods represent a valuable tool in food allergen analysis. However, reliable markers have been found for only few food allergens (peanut, casein). Therefore, efforts should be devoted to the discovery of such markers and development of mass spectrometric methods for the allergen detection (Careri et al., 2007; Monaci & Visconti, 2009). Screening of especially stable allergen derived peptides is very important when complex matrixes are analyzed via mass spectrometric techniques so the selection of good peptide markers is of great importance.

Table 1 shows the sequence of the β -LG and α -LA obtained from Swiss-Prot. Trypsin cleaved products of the α -LA may vary from 1 to 42 amino acids while for the β -LG from 1 to 26 amino acids if no missed cleavages are to be expected. Peptides with masses below

950 Da can be hardly identified using the MALDI-TOF/MS due to interferences caused by the matrix. Therefore the peptides were identified within a mass range of 950 to 4000 Da.

The predicted number of peptides with masses higher than 950 Da obtained after tryptic digestion was five for the α -LA while for the β -LG this was seven. Three out of the five peptides derived from α -LA could be identified after digestion of native whey and subsequent separation on the RP column before MALDI-TOF/MS and MS/MS analysis. For β -LG six peptides could be identified (Table 2). This represents about 22.5% and 50% protein coverage, respectively. The protein sequence coverage was calculated as the percentage of the amino acids residues in the protein sequence that has been identified. The lower protein coverage obtained for the α -LA might be due its lower concentrations present in the protein isolate. When the peptides were spotted directly without previous separation on the RP column, the same peptides corresponding to the α -LA and β -LG were identified in the native whey protein solution (Table 2). However, the identity of the peptides could not be confirmed by MS/MS for all peptides since they were present with lower intensity.

Table 3

Identified peptides derived from the native whey protein with their corresponding assigned scores as determined by direct MALDI-TOF/MS and MS/MS analysis.

Expected [M + H] ⁺	Peptide	Measured mass [M + H] ⁺			
		Native	1% whey ^a	0.5% whey 0.5% wheat ^a	0.25% whey 0.75% wheat ^a
1034.50	¹¹⁵ Leu–Lys ¹²²	1034.31	α -LA 1034.49	1034.50	n/d
1200.65	⁹⁹ Val–Lys ¹⁰⁸	1200.64 (42)	1200.64 (40)	1200.65	1200.63
1642.73	⁸⁰ Phe–Lys ⁹³	1642.43	n/d	1642.76	n/d
1064.44	⁶¹ Trp–Lys ⁶⁹	1064.24	β -LG n/d	1064.45	n/d
1065.58	⁹² Val–Lys ¹⁰⁰	1065.37	1065.57	1065.53	n/d
1245.58	¹²⁵ Thr–Lys ¹³⁵	1245.35	1245.58	1245.64	1245.61
1658.78	¹⁴⁹ Leu–Ile ¹⁶²	1658.47	1658.78	1658.79 (62)	1658.75
2313.25	⁴¹ Val–Lys ⁶⁰	2312.85 (59)	2313.25 (55)	2313.29 (34)	2313.23 (56)
2707.37	¹⁵ Val–Arg ⁴⁰	2706.90 (190)	2707.36 (123)	2707.35 (109)	2707.33 (123)

^a – 48 h incubated with 6% glucose at 70 °C, in parenthesis assigned score: > 21 = significant homology; > 33 = identity or extensive homology ($p < 0.05$); masses without parenthesis—present with lower intensity without MS/MS analysis; n/d—not detected.

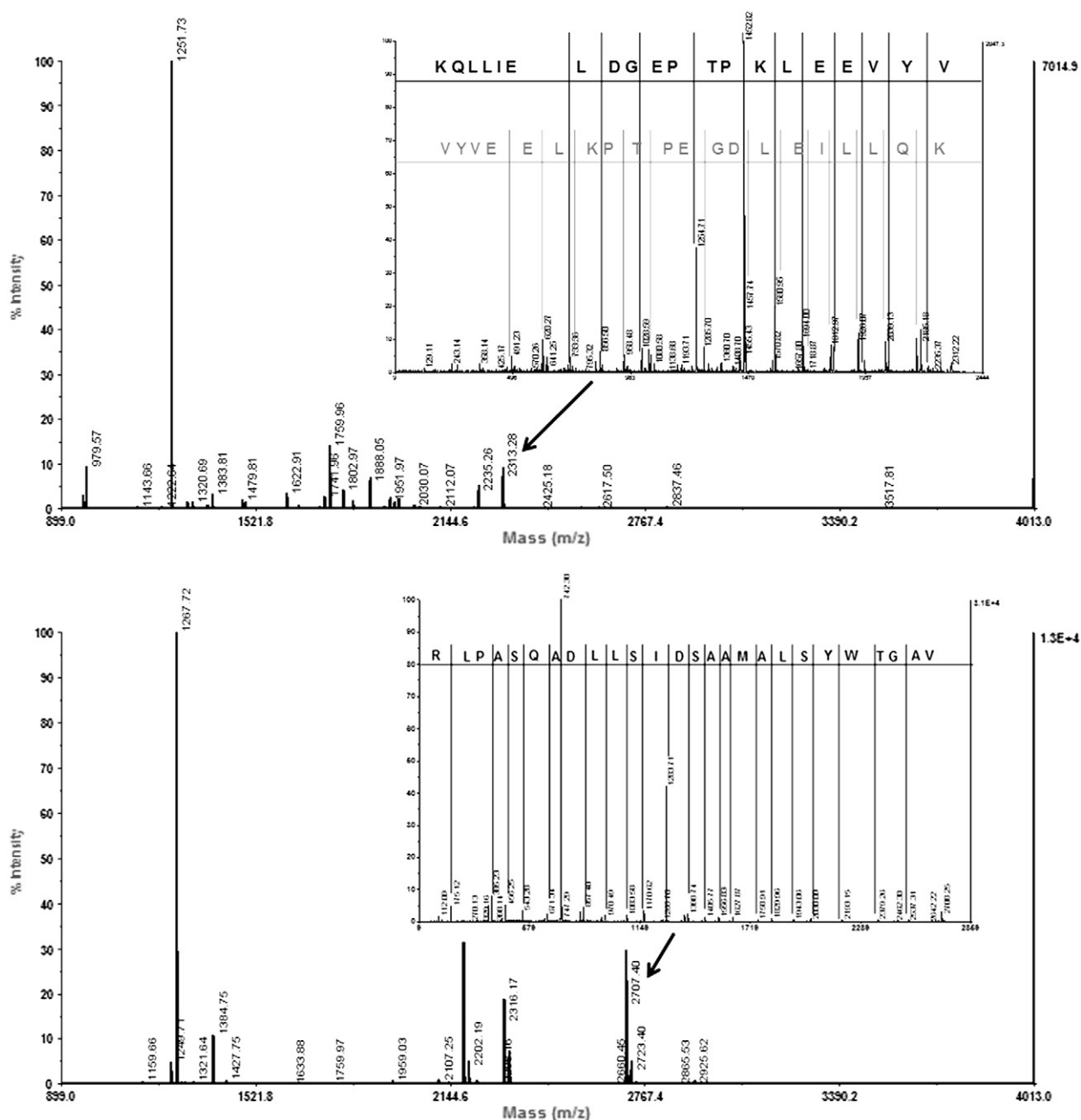


Fig. 5. MALDI-TOF/MS and MS/MS spectra of the chocolate sample. The stable peptides $^{41}\text{Val-Lys}^{60}$ and $^{15}\text{Val-Arg}^{40}$ are marked with arrows. Matched Y- and b-ions are indicated in black and gray respectively. The amino acid sequence is indicated in the one letter code.

This is not unusual, given the ion suppression effect typically observed when analyzing highly complex peptide samples.

As a result of the complex chemical modifications occurring during the Maillard reaction as also evidenced above, a number of peptides present in the tryptic digest of the native whey proteins could not be observed in the digest of the glycated proteins. This might be due to the modification of the lysine and arginine residues (as mentioned above) which moreover represent the cleavage sites of the trypsin. Therefore this led to a less effective cleavage of the peptides. Given the complexity of the peptide mixtures of the glycated proteins obtained after the digestion, it was not possible to attribute the merely general changes monitored in the first part of the study to specific molecular changes on peptide or amino acid level by using the applied proteomic approach even if no soluble whey proteins were present in the model systems. However, several peptides previously observed by MALDI-TOF/MS analysis of the native whey protein isolate could still

be observed after incubation of a 1% whey protein solution for 48 h at 70 °C in the presence of 6% glucose. The peptides $^{99}\text{Val-Lys}^{108}$ and $^{115}\text{Leu-Lys}^{122}$ from α -LA and the peptides $^{41}\text{Val-Lys}^{60}$, $^{15}\text{Val-Arg}^{40}$, $^{149}\text{Leu-Ile}^{162}$, $^{92}\text{Val-Lys}^{100}$ and $^{125}\text{Thr-Lys}^{135}$ from β -LG could be still identified. Interestingly, as can be seen from Table 1, several of these peptides were reported previously to be part of IgE binding epitopes of α -LA and β -LG respectively (Jarvinen, Chatchatee, Bardina, Beyer, & Sampson, 2001). As a further elaboration of this study, systems in which apart from the whey proteins, also other proteins were present, were investigated. It should be noted that, whey proteins are typically used with other food proteins or as in the case of accidental allergen contamination, are even present as a minor component. In these cases, it should be considered that whey proteins might form cross-links with other (bulk) proteins present, further affecting their structure and potentially their detectability such as seen before (Cucu et al., 2011; Platteau et al., 2011). Therefore, the effect of the presence

Table 4

Identified stable peptides derived from β -LG in the chocolate and cookie sample. Extracted proteins were digested and the obtained peptides were separated on a RP column prior the MALDI-TOF/MS analysis.

Expected [M + H] ⁺	Peptide	Chocolate	Cookie
		β -LG	
1245.58	¹²⁵ Thr–Lys ¹³⁵	1245.59 (78)	n/d
2313.25	⁴¹ Val–Lys ⁶⁰	2313.28 (126)	+
2707.37	¹⁵ Val–Arg ⁴⁰	2707.40 (223)	2707.34 (41)

In parenthesis assigned score: >33 = identity or extensive homology, + present with lower intensity without MS/MS analysis, n/d—not determined; chocolate contained 7.5 mg whey proteins/g and cookies contain 1.3 mg whey proteins/g sample as theoretically calculated from the ingredient list.

of wheat proteins on the stability of the peptides derived from whey proteins during Maillard reactions was investigated as well. Upon glycation of a 0.5% whey and 0.5% wheat protein mixture in the presence of 6% glucose for 48 h at 70 °C, only one peptide from α -LA (⁹⁹Val–Lys¹⁰⁸) and four unmodified peptides from β -LG could still be detected: ⁹²Val–Lys¹⁰⁰, ¹⁵Val–Arg⁴⁰, ⁴¹Val–Lys⁶⁰ and ¹²⁵Thr–Lys¹³⁵. These peptides were also identified in the case where 0.25% whey and 0.75% wheat protein mixtures were glycated with 6% glucose for 48 h at 70 °C. Upon the introduction of other protein, detection of stable peptides derived from whey proteins became more difficult due to the complexity of the resulting peptide mixture. Interestingly however, from the Maillard reaction parameters monitored it could be concluded that the protein mixtures containing both whey and wheat proteins showed less intense changes upon incubation with glucose compared to the whey protein mixtures alone (Figs. 1–3). This discrepancy clearly demonstrates the potential effect of protein cross-linking on the stability of allergenic proteins and the peptides thereof. As the target proteins became partially cross-linked with other proteins, trypsin digestibility could be altered, but obviously the produced peptides could contain segments of the other proteins as well. Nevertheless, it was shown that these four β -LG derived peptides and one α -LA derived peptide remained unaltered.

In order to avoid tedious chromatographic separation, direct MALDI-TOF/MS and MS/MS analysis of the tryptic digest was performed as well. As expected, the number of peptides detected and confirmed by MS/MS was lower due to earlier mentioned suppression effect. When 1% whey proteins were incubated with 6% glucose the peptide ⁹⁹Val–Lys¹⁰⁸ and ¹¹⁵Leu–Lys¹²² from α -LA and the peptides ⁴¹Val–Lys⁶⁰, ¹⁵Val–Arg⁴⁰, ¹⁴⁹Leu–Ile¹⁶², ⁹²Val–Lys¹⁰⁰ and ¹²⁵Thr–Lys¹³⁵ from β -LG were detected. However, only the peptides ⁴¹Val–Lys⁶⁰ and ¹⁵Val–Arg⁴⁰ could be identified and also confirmed by MS/MS with high scores. These two peptides are specific for β -LG from cow milk although to some other mammal species from the *Bos* genus as well. Nevertheless, when 0.25% and 0.5% wheat proteins were added to the model systems, peptide ⁴¹Val–Lys⁶⁰ and peptide ¹⁵Val–Arg⁴⁰ from β -LG could still be detected by direct MALDI TOF/MS and MS/MS (Table 3). This was possible regardless of the fact that only 150 ng of whey proteins were present per spot in the model system containing 0.25% whey. It should be noted however that the signal intensity of the detected peptides was severely decreased probably due to this lower amounts present (Fig. 4). These stable peptides can, therefore, be used as analytical target to detect the presence of whey proteins in foods using quantitative mass spectrometric techniques (such as LC-MS methods especially the ones making use of heavy peptides for quantitative purposes). Since synthesis of heavy peptides is rather costly, a screening of such stable peptides might help avoid unnecessary and costly synthesis of the marker peptides for the LC-MS methods.

As a preliminary elaboration of the applied proteomic approach, real food samples such as milk chocolate and cookie were tested for the presence of the stable peptides. As can be seen from Table 4 and Fig. 5 peptides ⁴¹Val–Lys⁶⁰ and ¹⁵Val–Arg⁴⁰ were found in chocolate

as well as cookie samples (data not shown). Moreover, their identity was confirmed by MS/MS analysis. The peptides ¹²⁵Thr–Lys¹³⁵ was also found in the chocolate samples but not in the cookies sample. The presence of these peptides, even if derived from samples initially having milk as an ingredient and being subjected to the processing, indicates that they are stable not only under the studied food processing simulating conditions, but also in real food matrices.

4. Conclusions

This study showed that despite severe protein modifications induced during heating of whey proteins with glucose both in the absence and in the presence of soluble wheat proteins, ⁴¹Val–Lys⁶⁰ and ¹⁵Val–Arg⁴⁰ peptides derived from β -LG remained stable. These peptides can serve as analytical targets for the development of robust quantitative methods. MALDI-TOF/MS represents an excellent technique for fast and efficient screening of such stable peptides during food processing simulating reactions.

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