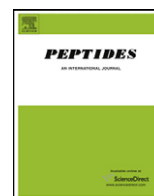




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MALDI based identification of soybean protein markers – Possible analytical targets for allergen detection in processed foods

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ABSTRACT

Soybean (*Glycine max*) is extensively used all over the world due to its nutritional qualities. However, soybean is included in the “big eight” list of food allergens. According to the EU directive 2007/68/EC, food products containing soybeans have to be labeled in order to protect the allergic consumers. Nevertheless, soybeans can still inadvertently be present in food products. The development of analytical methods for the detection of traces of allergens is important for the protection of allergic consumers. Mass spectrometry of marker proteolytical fragments of protein allergens is growingly recognized as a detection method in food control. However, quantification of soybean at the peptide level is hindered due to limited information regarding specific stable markers derived after proteolytic digestion. The aim of this study was to use MALDI-TOF/MS and MS/MS as a fast screening tool for the identification of stable soybean derived tryptic markers which were still identifiable even if the proteins were subjected to various changes at the molecular level through a number of reactions typically occurring during food processing (denaturation, the Maillard reaction and oxidation). The peptides ⁴⁰¹Val-Arg⁴¹⁰ from the G1 glycinin (*Gly m 6*) and the ⁵¹⁸Gln-Arg⁵²⁸ from the α' chain of the β -conglycinin (*Gly m 5*) proved to be the most stable. These peptides hold potential to be used as targets for the development of new analytical methods for the detection of soybean protein traces in processed foods.

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1. Introduction

Over the past decades, food allergies have been recognized as a significant food safety issue. Food allergies involve abnormal responses to specific foods which are otherwise harmless (mostly proteins) and are mediated by the immune system. They are estimated to affect about 2% of the adult population and its prevalence is higher in infants and children (6–8%) [20].

Soybean proteins are extensively used all over the world due to their nutritional qualities, functional properties (e.g. texture, water binding and fat binding), abundance, low cost and because of the technological developments enabling to include soybean proteins in a wide variety of food products as part of a healthy life style. Therefore, soybean proteins and their hydrolysates play an important role in human food habits [21]. However, soybean is included in the “big eight” list together with egg, milk, fish, crustaceans, peanut, wheat and tree nuts, that are responsible of over 90% of food allergies. At least 21 of the proteins present in soybean were reported to be allergenic [27]. Therefore, according to the EU

directive 2007/68/EC, food products containing soybean proteins have to be labeled in order to protect the allergic consumers [7]. Despite this legislation, however, soybean can still inadvertently be present in food products through a number of routes. Food production is increasingly complex with several different products being made in the same facility. Therefore, food allergens can be present due to incorrect labeling, improper handling of re-work, in-process and post-process cross-contamination and insufficient or ineffective equipment cleaning/sanitation procedures when there is a change from one product to the next [11].

The development of analytical methods for the detection of residues of allergenic foods is therefore important for the protection of food-allergic consumers. Moreover, robust analytical methods would allow to verify the effectiveness of the overall allergen-control programs within the food producing plants [25]. However, the detection of allergens in foods is rather difficult due to the fact that they are present in trace amounts and they might be masked by the matrix. Moreover, allergenic foods contain hundreds of proteins of only a few are allergenic. There are, therefore, a number of requirements toward the analytical methods to be used for allergen detection, such as high sensitivity, specificity and a sufficient robustness in order to enable use in complex food matrices.

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Table 1
Identified peptides derived from the Gly m 5 allergen and the trypsin inhibitors from native soybean extract as determined by MALDI-TOF MS and/or MS/MS analysis.

Expected [M+H] ⁺	Measured [M+H] ⁺ (score)	Δmass (Da)	Sequence	Position	Missed cleavages
<i>Gly m 5.0101: α-chain (UNIPROT ID: P13916)</i>					
955.53	955.53	0.00	(R)SPQLQNL(R)	224–231	0
1051.53	1051.51	0.02	(K)NPFLFGSNR(F)	194–202	0
<u>1079.55</u>	1079.40	0.15	(R)SRDPIYSNK(L)	410–418	1
1084.46	1084.34 (13)	0.12	(R)QEEHEQR(E)	113–120	0
1188.52	1188.50	0.02	(R)EEGQQQGEQR(L)	361–370	0
1378.74	1378.72	0.02	(R)QFPFPPPHQK(E)	152–162	0
1391.87	1391.85	0.02	(R)LITLAIIPVKNPGR(F)	306–318	0
1406.73	1406.71 (14)	0.02	(K)TISSEDKPFNL(R)	398–409	0
<u>1465.64</u>	1456.64	0.00	(R)ESEESESELRR(H)	178–189	1
<u>1534.82</u>	1534.80 (34)	0.02	(R)KTISSEDKPFNL(R)	397–409	1
<u>1618.86</u>	1618.84 (37)	0.02	(K)FFEITPEKNPQLR(D)	422–434	1
<u>1770.99</u>	1770.97	0.02	(R)LQESVIVEISKEQR(A)	371–385	1
<u>1792.92</u>	1792.90	0.02	(R)QFPFPPPHQKEER(N)	152–165	1
2025.95	2025.95 (11)	0.00	(K)EQQEQEQEQEQLPVR(K)	479–494	0
<u>2038.88</u>	2038.61	0.27	(R)QEEHEQREEQEWPR(K)	113–127	1
2152.03	2151.94 (127)	0.09	(R)VPSTGTTYYVNPNNENLR(L)	287–305	0
2459.15	2459.12 (56)	0.03	(R)FESFSLSTEAQQSYLQGFNR(N)	319–339	0
<i>Gly m 5.0201 (α'-chain) (UNIPROT ID: P11827)</i>					
986.54	986.54	0.00	(R)SQQLQNL(R)	240–247	0
1078.43	1078.36	0.07	(K)GSEEEQDER(E)	136–144	0
1189.51	1189.49	0.02	(R)EEGQQQGEER(L)	396–405	0
1383.69	1383.60 (50)	0.09	(R)QQQEEQLPVR(K)	518–528	0
1390.71	1390.69	0.02	(R)EHPRPHQPHQK(E)	145–155	0
<u>1511.78</u>	1495.64 (38)	0.14	(R)QQQEEQLPVR(K)	518–529	1
<i>Gly m 5.0301 (β-chain) (UNIPROT ID: P25974)</i>					
956.52	956.50	0.02	(R)SPQLENLR(D)	63–70	0
1235.59	1235.58	0.01	(R)VLFGEEEEEQR(Q)	195–204	0
1256.61	1256.62	0.01	(K)QEEEPLEVR(Y)	320–329	0
<u>1361.72</u>	1361.69	0.03	(R)NFLAGKDNVVR(Q)	371–382	1
1407.68	1407.67	0.01	(K)TISSEDEPFNL(R)	232–243	0
<u>1512.77</u>	1495.64 (50) ^a	0.13	(K)QKQEEPLEVR(Y)	318–329	1
<u>1618.86</u>	1618.83	0.03	(K)FFEITPEKNPQLR(D)	256–268	1
1773.88	1773.87	0.01	(R)QVQELAFPGSAQDVR(L)	387–402	0
<i>Trypsin inhibitor A (UNIPROT ID: P01070)</i>					
997.42	997.44	0.02	(K)DAMDGWFR(L)	136–143	0
1163.64	1163.59	0.05	(K)GIGTISSPYR(I)	77–87	0
1200.71	1201.62 (10) ^b	0.09	(K)NKPLVVQFQK(L)	190–199	0
<i>Trypsin inhibitor B (UNIPROT ID: P01071)</i>					
1200.71	1201.62 (10) ^b	0.09	(K)NKPLVVQFQK(V)	166–175	0
<i>Kunitz trypsin inhibitor (UNIPROT ID: P25272)</i>					
1200.71	1201.62 (10) ^b	0.09	(K)NKPLVVQFQK(F)	188–197	0

The assigned score: >13 = significant homology; >26 = identity or extensive homology ($p < 0.05$); underlined – peptides with missed cleavages. The lack of parenthesis after the peptide mass indicated that no MS/MS was performed due to lower abundance.

^a Gln → pyro Glu on the N-terminus.

^b Deamidation (Asn or Gln).

The immunoassays as well as the DNA-based methods, although routinely used for the allergen detection in foods, are criticized regarding the fact that they lack accuracy and robustness especially for processed foodstuffs [4,6,19,22]. Mass spectrometry (MS) is growingly used in clinical biomarker analysis and is gaining attention in the context of food allergen detection as well. Quantification of food allergens at protein level has been previously used, especially in the case of milk allergens [5,17]. Quantification of food allergens at the peptide level has been also used. This approach was mostly based on the finding of specific markers resulting after the proteolytic digestion of native and/or modified proteins. The use of specific markers from peanut proteins for confirmation and quantitative determination of hidden allergenic peanut proteins in different food matrices has been described [2]. A mass spectrometric method was developed for the detection of peanut proteins in ice cream and chocolate based on the identification of peptide markers derived from *Ara h 1* using strategies reflecting proteomic approaches [23,24]. In another work, potential peptide biomarkers for peanut proteins using mass spectrometry were characterized and showed to be stable after peanut processing [3]. The same technique was used for the development of a method able to detect milk allergens in different food matrices based on two markers derived from casein [26]. The mass spectrometric methods represent

therefore a valuable tool for identification of food allergens in different food matrices. However, reliable peptide markers have been found for only a few allergens so far and there is an urgent need to add a number of other food allergens markers, among which soybean as well [18].

The aim of this study was to identify soybean derived tryptic markers which were still identifiable using matrix-assisted laser desorption/ionization mass spectrometry (MALDI-TOF/MS and MS/MS) even after the proteins were subjected to various changes on the molecular level through reactions typically occurring during food processing (denaturation, the Maillard reaction and protein oxidation). These stable peptides hold potential to be used as targets for the development of new quantitative analytical methods for the detection of soybean protein residues in processed foods.

2. Materials and methods

2.1. Reagents and standards

Soluble wheat proteins (Meripro 711) obtained by a property owned process, were kindly provided by Syral Belgium N.V. (Aalst, Belgium). Chemicals and standards of analytical grade

Table 2

Identified peptides derived from the Gly m 6 allergens from native soybean extract as determined by MALDI-TOF MS and/or MS/MS analysis.

Expected [M+H] ⁺	Measured [M+H] ⁺ (score)	Δmass (Da)	Sequence	Position	Missed cleavages
<i>Gly m 6–G1 (UNIPROT ID: P04776)</i>					
979.52	979.50	0.02	(R)LSAEFGSLR(K)	357–365	0
1030.50	1030.47	0.03	(R)GQSSRPQDR(H)	121–129	0
1040.58	1040.58 (7)	0.00	(K)LNALKPDNR(I)	36–44	0
1149.55	1149.53 (70)	0.02	(R)VFDGELQEGR(V)	401–410	0
1172.60	1172.60 (27)	0.00	(K)FLVPPQESQK(R)	482–491	0
<u>1328.68</u>	1328.68	0.00	(K)FLVPPQESQK(R)	482–492	1
1417.65	1417.60	0.05	(K)YQQEQGGHQSQK(G)	195–206	0
1425.86	1425.84 (72)	0.04	(R)VLIVPQNFVVAAR(S)	411–423	0
1978.99	1978.92 (32)	0.07	(R)RPSYTNGPQEYIQGK(G)	81–97	0
<i>Gly m 6–G2 (UNIPROT ID: P04405)</i>					
1040.58	1040.58 (7)	0.00	(K)LNALKPDNR(I)	36–41	0
1263.65	1263.57	0.08	(K)EAFGVNMQIVR(N)	228–238	0
1494.69	1494.64	0.05	(K)YQQQQQGGSSQK(G)	192–204	0
1873.89	1873.89 (90)	0.00	(K)NNNPFSFLVPPQESQR(R)	466–481	0
<u>2229.06</u>	2229.06 (37)	0.00	(R)QVKNNNPFSFLVPPQESQR(R)	463–481	1
<u>2385.03</u>	2385.16	0.13	(R)QVKNNNPFSFLVPPQESQRR(A)	463–482	2
2401.28	2401.24 (37)	0.04	(R)VFDGELQEGGVLPQNFVAAK(S)	391–413	0
<i>Gly m 6–G3 (UNIPROT ID: P11828)</i>					
978.54	978.51	0.03	(K)LSAQFGSLR(K)	343–351	0
1030.50	1030.47	0.03	(K)GQSSRPQDR(H)	118–126	0
1040.58	1040.58 (7)	0.00	(R)LNALKPDNR(I)	36–44	0
<u>1873.89</u>	1873.89 (88)	0.00	(K)NNNPFSFLVPPKESQR(R)	462–477	1
<u>2229.06</u>	2229.06 (37)	0.00	(R)QVKNNNPFSFLVPPKESQR(R)	459–477	2
<i>Gly m 6–G4 (UNIPROT ID: P02858)</i>					
1192.65	1192.65	0.00	(K)LHENIARPSR(A)	388–397	0
<u>1352.71</u>	1352.67 (21)	0.04	(R)RPRQEEPFRER(G)	362–371	2
<u>1398.83</u>	1398.82 (51)	0.01	(R)ISTLNSLTLPALR(Q)	408–420	0
1569.84	1569.77	0.07	(R)AIPSEVLHASYNL(R)	518–531	0
<i>Gly m 6–G5 (UNIPROT ID: P04347)</i>					
1398.83	1398.82 (51)	0.01	(R)ISTLNSLTLPALR(Q)	375–387	0
1543.83	1543.78	0.05	(R)QFGLSAQYVVLRYR(N)	388–400	0

The assigned scores: >13 = significant homology; >26 = identity or extensive homology ($p < 0.05$); underlined – peptides with missed cleavages. The lack of parenthesis after the peptide mass indicated that no MS/MS was performed due to lower abundance.

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 SDS–PAGE were from Bio-Rad (Nazareth, Belgium).

2.2. Soybean protein extraction

Soybeans were provided by Alpro (Wevelgem, Belgium) and Cargill (Mechelen, Belgium). A mixture of the soybeans was made by taking equal amounts of each kind. Beans were frozen with liquid nitrogen and ground in a two-step process, first with a blender (Moulinex, France) and secondly with an Ultra Turrax T25 (IKA, Wilmington, USA). The in-house prepared soybean protein extract was obtained following the protocol described earlier with slight modifications [19]. The protein extract was lyophilized and stored at -20°C until further use.

2.3. Preparation of the model systems

2.3.1. Protein glycation in the presence or absence of other proteins

The model systems were prepared in 0.1 M phosphate buffer pH 7.4 by mixing 1% (w/v) soybean proteins with 6% (w/v) glucose. Two types of model systems containing wheat proteins were also considered. One model system was obtained by mixing 0.25% (w/v) of soybean 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) soybean proteins with 0.5% (w/v) wheat proteins and 6% (w/v) glucose. Respective model systems without glucose were prepared as control. The mixtures were loaded in closed falcon tubes that were incubated in a water bath at 70°C for up to 48 h [4].

2.3.2. Protein oxidation in the presence or absence of lipids

The reaction systems were prepared in 50 mM 3-morpholinopropanesulfonic acid (MOPS) pH 7.4 by mixing 1% (w/v) oil with 2% (w/v) protein isolate by means of a vortex for 2 min. The reaction systems in sealed falcon tubes were incubated at 70°C in the presence of 10 μM copper sulfate solution to initiate the oxidation and 0.2 g/L sodium azide to prevent microbial growth. After incubation, samples were treated with 15% TCA (final concentration) and incubated for 10 min on ice. After centrifugation for 10 min at $10,000 \times g$ the pellet was redissolved in 2% SDS and the pH was adjusted to approximately 10 with 10 M NaOH to facilitate the solubilization of the pellet. Any undissolved particles were further removed by centrifugation at $10,000 \times g$ for 10 min. The clear supernatant was used for analysis.

A 200 μL protein solution (10 mg/mL) in 100 mM phosphate buffer pH 8 or 5.8 was oxidized with 50 μL hypochlorous acid (0.5 mmol/g protein) at 30°C for 10 min. The initial concentration of the hypochlorous acid was determined by iodometric titration. After treatment, the samples were immediately immersed in ice.

2.4. Protein determination

Protein content was assessed by determining the nitrogen content according to the Kjeldahl procedure [1]. A factor of 5.71 was used to convert nitrogen to soybean protein.

2.5. MALDI-TOF/MS and MS/MS

An aliquot of 60 μg of total protein were digested 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%

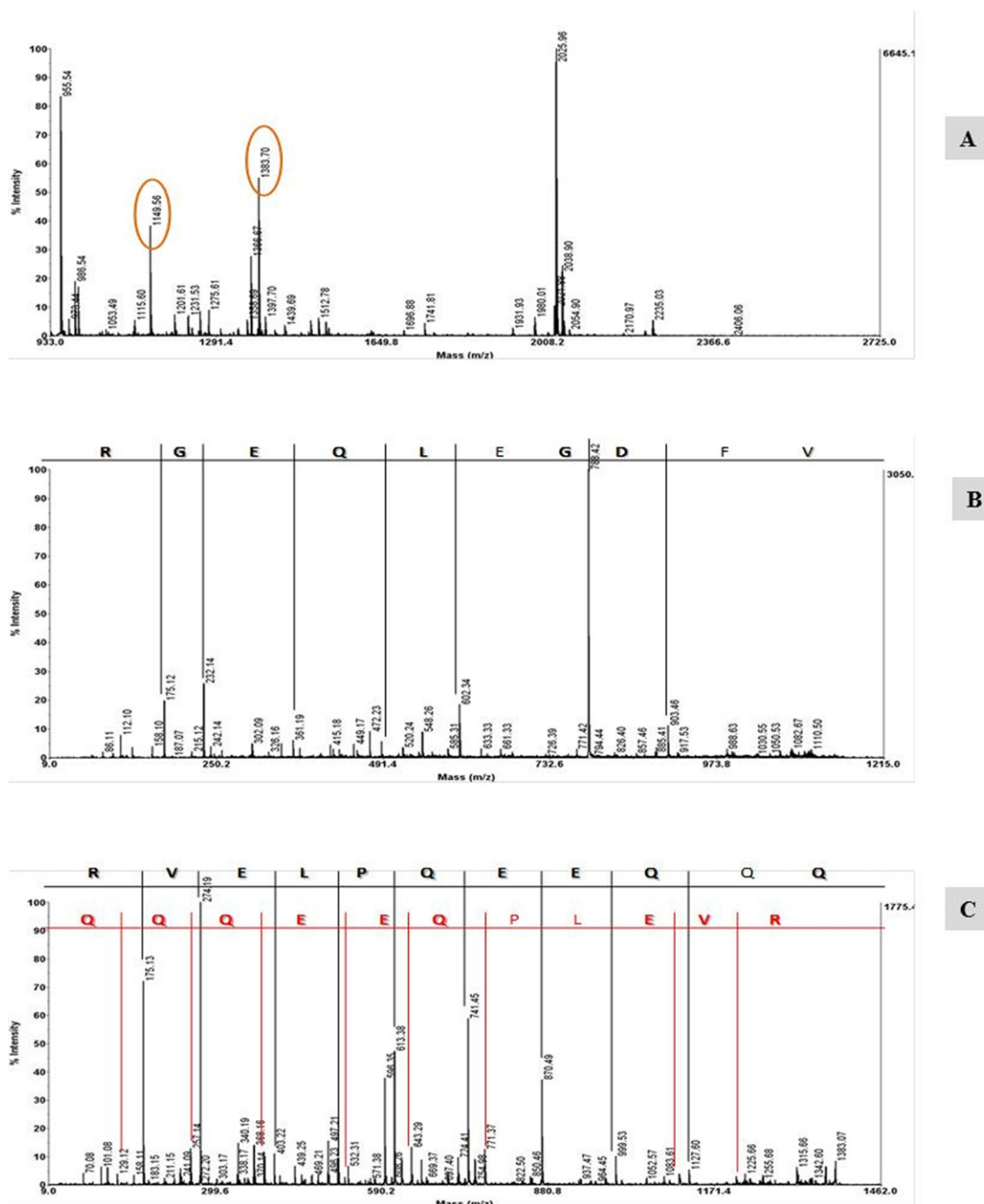


Fig. 1. (A) MS spectrum of the soybean proteins 48 h incubated with glucose. The stable peptides $^{401}\text{Val-Arg}^{410}$ and the $^{518}\text{Gln-Arg}^{528}$ are encircled. (B and C) MS/MS spectra of the $^{401}\text{Val-Arg}^{410}$ and $^{518}\text{Gln-Arg}^{528}$. Matched Y- and b-ions are indicated in bold black and red respectively. The amino acid sequence is indicated in the one letter code. (For interpretation of the references to color in figure caption, the reader is referred to the web version of the article.)

formic acid. Digested peptides were first separated using the Ettan LC system equipped with an UV detector from GE Healthcare (Brussels, Belgium). The RP column was a 220 mm $\times$ 2.1 mm Spheri-5 PTC column with particle size of 5 μm from Higgins Analytical Inc. (Mountain View, CA, USA) on which the peptides were loaded. The column was equilibrated with H_2O 100% with 0.1% trifluoroacetic acid (TFA) and elution was achieved by the following step gradient with acetonitrile 100% with 0.1% TFA. The flow rate was 0.1 mL/min and fractions of 0.2 mL were collected. The eluted peptides were monitored at 220 and 280 nm. The collected fractions were dried and redissolved in 10 μL of 0.1% formic acid. Finally, 1 μL of this peptide fraction 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, CA, USA) operating in reflectron positive ion 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 clip), ACTH (18-39 clip), ACTH (7-38 clip) (Applied Biosystems 4700 proteomic analyzer Mass Standards Kit). A total of 2000 shots were accumulated for each MS and MS/MS spectrum respectively. Protein identification was performed by searching in the protein sequence database (SwissProt, Swiss Institute of Bioinformatics) using the Mascot

Table 3

Identified peptides derived from the modified soybean Gly m 5 allergen with their corresponding assigned scores as determined by MALDI-TOF MS and MS/MS analysis.

Expected [M+H] ⁺	Peptide	Measured mass [M+H] ⁺ (score)					
		1% soybean ^a	0.5% soybean 0.5% wheat ^a	0.25% soybean 0.75% wheat ^a	2.5 mmol HOCl/g	5 mmol HOCl/g	Oxidized soybean oil ^b
Gly m 5.0101: α-chain (UNIPROT ID: P13916)							
955.53	²²⁴ Ser-Arg ²³¹	955.54 (61)	955.42 (51)	955.54 (47)	955.48 (57)	956.49 (14) ^d	955.51
1051.53	¹⁹⁴ Asn-Arg ²⁰²	1051.53	1051.46 (61)	1051.55	1051.49	1051.52	1051.44
1079.55	⁴¹⁰ Ser-Lys ⁴¹⁸	1079.55 (25)	1079.54 (27)	1079.57 (30)	1079.47	1079.51	n/d
1084.46	¹¹³ Gln-Arg ¹²⁰	1084.49 (52)	1084.48 (63)	1084.50 (47)	1084.38	1084.41	n/d
1188.52	³⁶¹ Glu-Arg ³⁷⁰	1188.55	1188.54	1188.54	1188.42 (48)	1188.46 (36)	1188.44
1378.74	¹⁵² Gln-Lys ¹⁶²	1378.74 (55)	1378.61 (48)	1378.74 (54)	1378.68	1378.70	1378.61
1391.87	³⁰⁶ Leu-Arg ³¹⁸	1391.91 (66)	1391.76 (43)	1391.74 (35)	1391.83	1391.85	1391.52
1406.73	³⁹⁸ Thr-Arg ⁴⁰⁹	1406.73 (52)	1406.60 (59)	1406.73 (60)	1406.70	1406.70 (40)	n/d
1465.64	¹⁷⁸ Glu-Arg ¹⁸⁹	1465.67	1465.65	1465.67	1465.67 (10)	1465.71	1465.62
1534.82	³⁹⁷ Lys-Arg ⁴⁰⁹	1534.82 (28)	1534.67	1534.79	1534.76 (92)	1534.79 (67)	1534.67
1618.86	⁴²² Phe-Arg ⁴³⁴	1618.90 (90)	1618.73	n/d	1618.79 (57)	1618.83 (82)	n/d
1770.99	³⁷¹ Leu-Arg ³⁸⁵	n/d	1770.86	1770.86	1770.94 (18)	1770.83 (11)	n/d
1792.92	¹⁵² Gln-Arg ¹⁶⁵	1792.73	1792.92	n/d	1792.85 (13)	n/d	n/d
1939.78	¹³⁶ Gly-Arg ¹⁵¹	1936.69 (122)	1939.69 (154)	1939.74 (128)	1939.55 (106)	1939.64	n/d
2025.95	⁴⁷⁹ Glu-Arg ⁴⁹⁴	2025.96 (148)	2025.74 (158)	2025.97 (138)	2025.85 (139)	2025.91 (123)	2025.81
2038.88	¹¹³ Gln-Arg ¹²⁷	2038.90 (68)	2038.70 (77)	2038.90 (42)	n/d	n/d	n/d
2152.03	²⁸⁷ Val-Arg ³⁰⁵	2152.04 (150)	2151.83 (147)	2152.04 (114)	2151.94 (169)	2151.99 (80)	n/d
2169.03	³²² Met-Arg ³⁴⁰	n/d	n/d	n/d	n/d	2170.95 (23) ^d	n/d
2253.84	¹³³ Gly-Arg ¹⁵¹	n/d	2253.88	n/d	2253.68 (109)	2253.79 (100)	n/d
2459.15	³¹⁹ Phe-Arg ³³⁹	2458.94 (142)	2459.01 (190)	2459.15	2459.07 (123)	2459.09	n/d
Gly m 5.0201 (α'-chain) (UNIPROT ID: P11827)							
986.54	²⁴⁰ Ser-Arg ²⁴⁷	986.54 (49)	986.53 (40)	986.55 (38)	986.48 (47)	986.51	986.57
1078.43	¹³⁶ Gly-Arg ¹⁴⁴	1078.43 (37)	1078.45 (51)	1078.46 (42)	1078.35	1078.40	n/d
1189.51	³⁹⁶ Glu-Arg ⁴⁰⁵	1189.52 (76)	1189.53 (63)	1189.54 (39)	n/d	1189.48	n/d
1383.69	⁵¹⁸ Gln-Arg ⁵²⁸	1383.70 (77)	1366.52 (62) ^c	1383.70 (71)	1383.61 (36)	1383.66	1383.59 (91)
1390.71	¹⁴⁵ Glu-Lys ¹⁵⁵	1390.73 (21)	1390.71 (37)	1390.74 (24)	1390.80	1390.77	1390.44
1511.78	⁵¹⁸ Gln-Lys ⁵²⁹	n/d	n/d	n/d	1511.67	1511.70 (14)	1512.63 (74) ^d
2422.06	¹¹⁰ Gln-Arg ¹²⁷	2422.07	2422.04 (63)	n/d	n/d	n/d	n/d
Gly m 5.0301 (β-chain) (UNIPROT ID: P25974)							
956.52	⁶³ Ser-Arg ⁷⁰	956.54	956.55	956.53	956.48	956.49 (15)	956.41
1235.59	¹⁹⁵ Val-Arg ²⁰⁴	1235.60	1235.48 (60)	1235.59	n/d	1235.57 (17)	n/d
1256.61	³²⁰ Gln-Arg ³²⁹	1256.63	1256.48	1256.63	1256.55 (29)	1256.58 (34)	1256.52
1361.72	³⁷¹ Asn-Arg ³⁸²	1361.73 (54)	1361.62	1361.71	1361.67	n/d	n/d
1372.62	¹¹⁴ Asp-Arg ¹²⁵	1372.64	1372.62 (58)	1372.65 (67)	n/d	n/d	n/d
1407.68	²³² Thr-Arg ²⁴³	1497.72	1407.56	1407.55	1407.62	1407.66 (34)	1407.56
1512.77	³¹⁸ Gln-Arg ³²⁹	1512.76 (46)	1512.76	1512.80 (63)	1512.68	n/d	1512.63 (92)
1618.86	²⁵⁶ Phe-Arg ²⁶⁸	1618.90 (90)	1618.73	n/d	1618.79 (57)	1618.83 (82)	n/d
1773.88	³⁸⁷ Gln-Arg ⁴⁰²	1773.92 (115)	1773.73 (100)	1773.88	1773.81 (21)	1773.72 (31)	n/d

The assigned score: >13 = significant homology; >26 = identity or extensive homology ($p < 0.05$); the lack of parenthesis after the peptide mass indicated that now MS/MS was performed due to lower abundance; n/d – not detected; underlined peptide masses – peptides with a missed cleavage; shaded with bold – stable peptides.

^a 48 h incubated with 6% glucose at 70 °C.

^b 48 h incubated with highly oxidized soybean oil at 70 °C.

^c Gln → pyro Glu on the N terminus.

^d Deamidation (Asn and Gln).

search engine (<http://www.matrixscience.com>). The following search parameters were used: taxonomy–*viridiplantae*; precursor mass tolerance = 100 ppm; fragment mass tolerance = 0.5 Da. The enzyme entry was set for trypsin, the maximum number of missed cleavages was set to 2 and oxidized methionine, tryptophan and histidine, deamidation (Gln and Asn) and Gln → pyro-Glu modification were considered as peptide modifications.

3. Results

The native and modified soybean proteins were subjected to tryptic digestion prior to the MALDI-TOF/MS and MS/MS analysis.

Initially, the native soybean extracts were analyzed for the peptide pattern. Soybean proteins contain a number of trypsin inhibitors which in the native proteins might affect the tryptic digestion typically applied for MALDI-TOF analysis. Therefore, for the native proteins, the samples were incubated in an ultrasound bath at 30 °C for 10 min before the trypsin was added [10]. This ensured therefore partial inactivation of the trypsin inhibitors and

an effective cleavage of the peptides. Although soybeans contain a larger number of proteins [14], peptides from mainly β-conglycinin (Gly m 5) the α, β and α' subunits, glycinins (Gly m 6) from G1 to G5 and trypsin inhibitors were identified in the digest of the native soybean extract (Tables 1 and 2). The identity of some of the identified peptides was moreover confirmed by MALDI-TOF MS/MS with high scores. There are 24 possible peptides to be detected from the α-chain of the Gly m 5 (primary accession number – P13916) within the mass range of 950–4000 Da, which represent 60% sequence coverage. Out of the 24 expected peptides, 10 were identified in the native soybean protein extract together with another 7 peptides which contained one missed cleavage (Table 1). Similarly as for the α-chain, the α'-chain of the Gly m 5 (primary accession number – P11827), 24 possible peptides can be formed, which represents 58.6% sequence coverage. From these, 5 peptides were identified together with one peptide with a missed cleavage. Finally, for the β-chain of the Gly m 5 (primary accession number – P25974), 19 possible peptides can be formed during tryptic digestion, which represents 62.3% sequence coverage. Of these, 5 were identified in the native soybean proteins together with another 3 peptides each with one missed cleavage.

Table 4
Identified peptides derived from the modified soybean proteins (*Gly m 6*) with their corresponding assigned scores as determined by MALDI-TOF MS and MS/MS analysis.

Expected [M+H] ⁺	Peptide	Measured mass [M+H] ⁺ (score)					
		1% soybean ^a	0.5% soybean 0.5% wheat ^a	0.25% soybean 0.75% wheat ^a	2.5 mmol HOCl/g	5 mmol HOCl/g	Oxidized soybean oil ^b
<i>Gly m 6 – G1 (UNIPROT ID: P04776)</i>							
979.52	³⁵⁷ Leu-Arg ³⁶⁵	979.52	979.42 (39)	979.52	979.48 (36)	979.50 (56)	n/d
1030.50	¹²¹ Gly-Arg ¹²⁹	1030.51 (22)	1030.50	1030.53	1030.41 (16)	1030.46	1030.42 (16)
1040.58	³⁶ Leu-Arg ⁴⁴	1040.57	1040.57	1040.57	1040.51 (36)	1040.55 (40)	n/d
1149.55	⁴⁰¹Val-Arg⁴¹⁰	1149.56 (53)	1149.43 (59)	1149.56 (54)	1149.50 (60)	1149.53 (66)	1149.46 (61)
1172.60	⁴⁸² Phe-Lys ⁴⁹¹	1172.63	1172.51	1172.62	1172.57 (30)	1172.60 (39)	n/d
<u>1328.68</u>	⁴⁸² Phe-Arg ⁴⁹²	1328.66	n/d	n/d	1328.68 (45)	1328.60 (26)	n/d
1417.65	¹⁹⁵ Tyr-Lys ²⁰⁶	1417.67 (65)	1417.65 (73)	1417.68 (74)	1417.55	1417.51	n/d
1425.86	⁴¹¹ Val-Arg ⁴²³	1425.73 (73)	1425.76 (93)	1425.74 (72)	1425.82 (86)	1425.83 (82)	n/d
1742.89	¹⁸¹ Arg-Lys ¹⁹⁴	1742.93 (53)	1742.74 (53)	1742.72 (29)	1742.81 (8)	n/d	n/d
1978.99	⁸¹ Arg-Lys ⁹⁷	1979.00 (32)	1978.82 (30)	1979.00 (44)	1978.92 (96)	n/d	n/d
<i>Gly m 6 – G2 (UNIPROT ID: P04405)</i>							
1040.58	³³ Leu-Arg ⁴¹	1040.57	1040.57	1040.57	1040.51 (36)	1040.55 (40)	n/d
1263.65	²²⁸ Glu-Arg ²³⁸	1263.68 (63)	1263.55 (61)	1263.53 (66)	n/d	n/d	n/d
1494.69	¹⁹² Tyr-Lys ²⁰⁴	1494.72 (114)	1494.70 (102)	1494.74 (103)	1494.60 (97)	n/d	1494.58
1873.89	⁴⁶⁶ Asn-Arg ⁴⁸¹	1873.75 (124)	1873.82 (130)	1873.78 (116)	1873.78 (104)	1873.88 (132)	n/d
<u>2229.06</u>	⁴⁶³ Gln-Arg ⁴⁸¹	n/d	n/d	n/d	2029.96 (48)	n/d	n/d
<u>2385.03</u>	⁴⁶³ Gln-Arg ⁴⁸²	n/d	n/d	n/d	n/d	n/d	2385.02 (14)
2401.28	³⁹¹ Val-Lys ⁴¹³	n/d	n/d	n/d	n/d	2401.22 (127)	n/d
<i>Gly m 6 – G3 (UNIPROT ID: P11828)</i>							
978.54	³⁴³ Leu-Arg ³⁵¹	n/d	979.42 (39) [§]	n/d	979.48 (40) [§]	979.50 (56) [§]	n/d
1030.50	¹¹⁸ Gly-Arg ¹²⁶	1030.51 (22)	1030.50	1030.53	1030.41 (16)	1030.46	1030.42 (16)
1040.58	³⁶ Leu-Arg ⁴⁴	1040.57	1040.57	1040.57	1040.51 (36)	1040.55 (40)	n/d
1873.89	⁴⁶² Asn-Arg ⁴⁷⁷	1873.75 (124)	1873.82 (130)	1873.78 (116)	1873.78 (102)	1873.88 (130)	n/d
2229.06	⁴⁵⁹ Gln-Arg ⁴⁷⁷	n/d	n/d	n/d	2229.96 (48)	n/d	n/d
<i>Gly m 6 – G4 (UNIPROT ID: P02858)</i>							
1192.65	³⁸⁸ Leu-Arg ³⁹⁷	1192.66 (26)	1192.65	1192.68	1192.58	n/d	n/d
1398.83	⁴⁰⁸ Ile-Arg ⁴²⁰	1398.70 (61)	1398.74 (64)	1398.71 (54)	1398.79 (57)	1398.80 (30)	n/d
1569.84	⁵¹⁸ Ala-Arg ⁵³¹	1569.88 (92)	1569.71 (58)	1569.68 (68)	1569.78	n/d	n/d
<i>Gly m 6 – G5 (UNIPROT ID: P04347)</i>							
1398.83	³⁷⁵ Ile-Arg ³⁸⁷	1398.68	1398.74	1398.72	1398.79 (60)	1398.80 (32)	n/d
1543.83	³⁸⁸ Gln-Arg ⁴⁰⁰	1543.66 (55)	1543.81	1543.82	1543.75	1543.74	n/d

Parenthesis assigned score: >13 = significant homology; >26 = identity or extensive homology ($p < 0.05$); the lack of parenthesis after the peptide mass indicated that now MS/MS was performed due to lower abundance; n/d – not detected; underlined peptide masses – peptides with a missed cleavage; shaded with bold – stable peptides.

^a 48 h incubated with 6% glucose at 70 °C.

^b 48 h incubated with highly oxidized soybean oil at 70 °C.

[§] deamidation (Asn and Gln).

For the trypsin inhibitor A and B (primary accession numbers – P01070 and P01071), 10 possible peptides can be formed, which represent 81% sequence coverage. However, only 3 peptides could be identified for trypsin inhibitor A and 1 for the trypsin inhibitor B. For the Kunitz trypsin inhibitor (primary accession number – P25272), 9 possible peptides can be formed upon tryptic digestion, which represents 77.5% sequence coverage and

just one could be identified in the digested native soybean proteins.

For the G1 unit of the *Gly m 6* (primary accession number – P04776), there are 11 predicted peptides for the acid subunit and 10 for the basic subunit, which represents 67.6 and 90.6% sequence coverage respectively for each subunit. Out of these, 4 peptides from the acid and 4 from the basic subunits could be detected

Table 5
Identified peptides derived from the modified soybean proteins (trypsin inhibitors) with their corresponding assigned scores as determined by MALDI-TOF MS and MS/MS analysis.

Expected [M+H] ⁺	Peptide	Measured mass [M+H] ⁺ (score)							
		1% soybean ^a	0.5% soybean	0.5% wheat ^a	0.25% soybean	0.75% wheat ^a	2.5 mmol HOCl/g	5 mmol HOCl/g	Oxidized soybean oil ^b
Trypsin inhibitor A (UNIPROT ID: P01070)									
997.42	¹³⁶ Asp-Arg ¹⁴³	997.31		997.35 (57)		997.35	n/d	n/d	n/d
1163.64	⁷⁷ Gly-Arg ⁸⁷	1163.67 (56)		1163.54 (39)		1163.53	n/d	n/d	n/d
1200.71	¹⁹⁰ Asn-Lys ¹⁹⁹	1200.62		1200.60		1200.63	1200.65 (35)	1201.59 (18) ^c	n/d
1230.53	¹⁴⁷ Val-Lys ¹⁵⁶	1230.52 (45)		1230.40		1230.54 (38)	1230.46 (66)	1230.50	n/d
Trypsin inhibitor B (UNIPROT ID: P01071)									
1200.71	¹⁶⁶ Asn-Lys ¹⁷⁵	1200.62		1200.60		1200.63	1200.65 (35)	1201.59 (18) ^c	n/d
1230.53	¹²³ Val-Lys ¹³²	1230.52 (45)		1230.40		1230.54 (38)	1230.46 (66)	1230.50	n/d
Kunitz trypsin inhibitor (UNIPROT ID: P25272)									
1200.71	¹⁸⁸ Asn-Lys ¹⁹⁷	1200.62		1200.60		1200.63	1200.65 (35)	1201.59 (18) ^c	n/d
1351.63	¹³⁶ Asp-Arg ¹⁴⁶	1351.49 (85)		1351.54 (91)		1351.52	n/d	n/d	n/d

Parenthesis assigned score: >13 = significant homology; >26 = identity or extensive homology ($p < 0.05$); the lack of parenthesis after the peptide mass indicated that now MS/MS was performed due to lower abundance; n/d – not detected.

^a 48 h incubated with 6% glucose at 70 °C.

^b 48 h incubated with highly oxidized soybean oil at 70 °C.

^c Deamidation (Asn and Gln).

(Table 2). Additionally, a peptide with a missed cleavage from the basic subunit could be also detected. For the G2 and G3 subunits of the *Gly m 6* (primary accession numbers – P04405 and P11828, respectively), 9 predicted peptides can be formed after tryptic digestion of both the acid and basic subunits, which represents 52.5 and 93.9% sequence coverage respectively. However, only three peptides derived from the acid subunit and 4 peptides from the basic subunits could be detected. One of the peptides derived from the basic subunit of the G2 glycinin, however, was present with 2 missed cleavages. For the G3 glycinin, only two peptides from the acid and three from the basic subunits of which one with missed cleavage were identified. For the G4 subunit of the *Gly m 6* (primary accession number – P02858), contains two acid subunits and one basic subunit. Four peptides from the first acid subunit, 9 from the second acid subunit and 11 peptides from the basic subunits can be expected after the tryptic digestion, which represents 73.2, 61.9 and 86.5 sequence recovery, respectively. However, only 3 peptides from the basic subunit were identified along with a peptide from the second acid subunit which, however, contained a missed cleavage. Finally, for the G5 subunit of the *Gly m 6* (primary accession number – P04347), 11 predicted peptides can be expected from the acid subunit while from the basic subunit 10, which represents 67.8 and 86.0% respectively. None of the peptides derived from the acid subunit could be identified in the digest of the native soybean proteins extract while two could be identified from the basic subunit.

Further, a special focus on the identification of stable soybean derived tryptic markers even if the proteins were subjected to various changes on the molecular level through the Maillard reaction and protein oxidation was made. Two peptides, ⁴⁰¹Val-Arg⁴¹⁰ from

Table 6

Stable peptide derived from soybean and their specificity: shaded – unmatched residues, underlined – predicted linear epitopes.

⁵¹⁸ Gln-Arg ⁵²⁸	<u>QQQEEQPLEVR</u> <u>484</u> QQQEEQPLEVR ⁴⁹⁴	<i>Gly m 5.0201</i> <i>Gly m 5.0101</i>
⁴⁰¹ Val-Arg ⁴¹⁰	VFDGEIQEGR <u>402</u> VFDGELEAGR ⁴¹¹ <u>403</u> VFDGELEAGR ⁴¹²	<i>Gly m 6</i> <i>Pis v 5</i> <i>11S globulin from chickpea</i>

the G1 glycinin (*Gly m 6*) and the ⁵¹⁸Gln-Arg⁵²⁸ from the α' chain of the *Gly m 5* proved to be the most stable and could be detected and confirmed with high scores in all modified samples (Fig. 1, Tables 3 and 4). However, besides being stable, these peptides have to be specific to soybean in order to avoid false positive results and ideally they should cover known IgE binding epitopes [3]. For the assessment of the specificity of the identified peptides, the BLAST tool (Basic Local Alignment Search Tool, SwissProt database) was used (Table 5). The ⁵¹⁸Gln-Arg⁵²⁸ from the α'-subunit of the *Gly m 5* is specific for the soybean although there is 100% homology with a peptide in its α-subunit. It was, however, not possible to state whether this peptide is part of an IgE binding epitope because the IgE binding epitopes of the α'-subunit of the *Gly m 5* are not known yet. Nevertheless, using the immunoinformatic tool BepiPred 1.0 (<http://www.cbs.dtu.dk/services/BepiPred/>), it was seen that this peptide is part of a predicted linear IgE binding epitope which ranges from ⁵¹³Glu-Asp⁵²⁶ [15]. The peptide ⁴⁰¹Val-Arg⁴¹⁰ from the G1 glycinin (*Gly m 6*) has limited homology with the 11S globulin from chickpea and pea (Table 6). Using the BepiPred 1.0 software it was observed that the peptide is part of a linear IgE binding epitope ranging from ⁴⁰⁵Glu-Gln⁴⁰⁷.

Table 7

Mass increase of the modified peptide fragments derived from the *Gly m 5* and suggested interpretations.

Expected [M+H] ⁺	Measured [M+H] ⁺	Δmass (Da)	Sequence	Modification	Type of reaction
<i>Gly m 5.0101: α-chain (UNIPROT ID: P13916)</i>					
1079.55	1111.45	+31.90	(R)SRDPIYSNK(L)	Oxidation (Y → Topa)	Incubation with 2.5 mmol HOCl/g
	1095.59	+16.04		Oxidation (Y → Dopa)	Incubation with oxidized oil
<u>1534.82</u>	1696.88	+162.06	(R)KTISSEDKPFNL(R)	Amadori product	Incubation with glucose
<u>1618.86</u>	1780.95	+162.09	(K)FFEITPEKNPQL(R)	Amadori product	Incubation with glucose
<u>1770.99</u>	1828.80	+57.81	(R)LQESVIVEISKEQ(R)	Carboxymethyl residue	Incubation with glucose
	1954.98	+162.06		Amadori product	Incubation with glucose
<u>1792.92</u>	1850.85	+57.93	(R)QFPFPRPPHQKEER(N)	Carboxymethyl residue	Incubation with glucose
	1808.84	+15.92		Oxidation (H → 2-oxo-histidine)	Incubation with 2.5 mmol HOCl/g
<u>2038.88</u>	2054.97	+16.09	(R)QEEHEQREEQEWPR(K)	Oxidation (H → 2-oxo-histidine or W → hydroxytryptophan)	Incubation with 2.5 mmol HOCl/g
	2070.73	+31.85		Oxidation (H → 2-oxo-histidine and W → hydroxytryptophan)	
<i>Gly m 5.0201 (α'-chain) (UNIPROT ID: P11827)</i>					
1390.71	1422.51	+31.80	(R)EHPRPHQPHQK(E)	Oxidation (HH)	Incubation with 2.5 mmol HOCl/g
<i>Gly m 5.0301 (β-chain) (UNIPROT ID: P25974)</i>					
<u>1361.72</u>	1523.77	+162.05	(R)NFLAGEKDNVVR(Q)	Amadori product	Incubation with glucose
	1419.66	+57.94		Carboxymethyl residue	
<u>1512.77</u>	1674.83	+162.06	(K)QKQEEEPLEVR(Y)	Amadori product	Incubation with glucose
	1570.81	+58.04		Carboxymethyl residue	
<u>1618.86</u>	1780.95	+162.09	(K)FFEITPEKNPQL(R)	Amadori product	Incubation with glucose

The amino acid sequence of the peptides is indicated in the one letter code, the residues in bold are the amino acids residues where modification due to oxidation or glycation takes place; underlined – peptides with missed cleavages.

Table 8
Mass increase of the modified peptide fragments derived from Gly m 6 and trypsin inhibitor A and suggested interpretations.

Expected [M+H] ⁺	Measured [M+H] ⁺	Δmass (Da)	Sequence	Modification	Type of reaction
<i>Gly m 6 – G1 (UNIPROT ID: P04776)</i>					
<u>1328.68</u>	1490.61	+161.93	(K)FLVPPQESQ K R(A)	Amadori product	Incubation with glucose
1417.65	1386.58	+57.90	(K)YQQEQGGHQS K (G)	Carboxymethyl residue	Incubation with 5 mmol HOCl/g
1978.99	1433.71	+16.06	(R)RPSYTNGPQE I YIQQ G K(G)	Oxidation (Y → Dopa or H → 2-oxo-histidine)	Incubation with 5 mmol HOCl/g
	2026.75	+47.85	(R)RPSYTNGPQE I YIQQ G K(G)	Oxidation (Y → Dopa and Y → Topa)	Incubation with oxidized oil
<i>Gly m 6 – G2 (UNIPROT ID: P04405)</i>					
1040.58	1202.61	+162.03	(K)LNAL K PDNR(I)	Amadori product	Incubation with glucose
1263.65	1295.55	+31.90	(K)EAFGVNM Q IVR(N)	Oxidation (M → methionine sulfone)	Incubation with 2.5 mmol HOCl/g
1494.69	1510.66	+15.97	(K)YQQQQQGG S Q S K(G)	Oxidation (Y → Dopa)	Incubation with 5 mmol HOCl/g
	1526.75	+32.06	(K)YQQQQQGG S Q S K(G)	Oxidation (Y → Topa)	Incubation with 5 mmol HOCl/g
<i>Gly m 6 – G3 (UNIPROT ID: P11828)</i>					
<u>1873.89</u>	1931.93	+58.04	(K)NNNPFSFLVPP K ESQR(R)	Carboxymethyl residue	Incubation with glucose
<i>Gly m 6 – G4 (UNIPROT ID: P02858)</i>					
<u>1569.84</u>	1617.76	+47.92	(R)AIPSEVLA H SYNLR(Q)	Oxidation (H → 2-oxo-histidine and Y → Topa)	Incubation with 5 mmol HOCl/g
<i>Gly m 6 – G5 (UNIPROT ID: P04347)</i>					
1543.83	1559.57	+15.74	(R)QFGLSAQY V VL R (N)	Oxidation (Y → Dopa)	Incubation with oxidized oil
<i>Trypsin inhibitor A (UNIPROT ID: P01070)</i>					
997.42	1029.40	+31.98	(K)DAMDGW F R(L)	Oxidation (M → methionine sulfoxide and W → hydroxytryptophan)	Incubation with 2.5 mmol HOCl/g
	1045.52	+48.10	(K)DAMDGW F R(L)	Oxidation (M → methionine sulfoxide and W → N-formylkynurenine or M → methionine sulfone and W → hydroxytryptophan or W → Hydroxyformylkynurenine)	Incubation with 5 mmol HOCl/g and incubation with oxidized oil
	1061.35	+63.93	(K)DAMDGW F R(L)	Oxidation (M → methionine sulfone and W → N-formylkynurenine or M → methionine sulfoxide and W → Hydroxyformylkynurenine)	Incubation with 2.5 mmol HOCl/g

The amino acid sequence of the peptides is indicated in the one letter code, the residues in bold are the amino acids residues where modification due to oxidation or glycation take place; underlined – peptides with missed cleavages.

4. Discussion

In this study a special focus on the identification of stable soybean derived tryptic markers even if the proteins were subjected to various changes on the molecular level through the Maillard reaction and protein oxidation was made. These stable peptides can be further used for the development of quantitative analytical methods for the detection of soybean protein residues in processed foods. However, besides being stable, the peptides used for quantitative analytical methods should meet other criteria such as specificity for soybean and lack of missed cleavages in order to avoid erroneous results as well as good detectability in mass spectrometric set-ups. For the latter reason, we only considered peptides with masses ranging between 950 and 4000 Da. MALDI-TOF/MS was chosen because it is a robust and fast method extensively used for peptide identification while requiring very low amounts of sample. The actual sample analysis after sample preparation is often taking less than one minute which allows a fast screening of stable peptides derived from modified soybean proteins.

Considering that the number of peptides formed upon tryptic digestion was rather high, and that using direct MALDI-TOF/MS analysis ion suppression effects may occur, the peptide mixture was initially separated in 40 fractions on a RP column before the MALDI-TOF/MS analysis. The MS mode was used to characterize the peptide pattern of the soybean proteins. To unambiguously identify the peptides, MS/MS analysis for selected peptides was performed. The MS/MS spectra were further subjected to a search in the SwissProt data base.

In a previous study, a number of soybean specific tryptic peptides that could be used for absolute quantitation of soybean

proteins in soybeans were reported [9]. The authors have implied that the identified peptides could be used for absolute quantification of soybean allergens. However, they did not investigate the impact of food processing on these specific peptides. Moreover, up until now the stability of these peptides, which moreover contain amino acids susceptible to modifications through oxidation or glycation, was not reported. The lack of guarantee that these peptides can be detected in both raw and processed products containing soybean can undermine their suitability to be used as markers for the detection of soybean in especially processed foods.

After treatment under the food processing simulating reactions, the proteins are subjected to a number of molecular changes characterized by modification of amino acids residues, formation of protein bound carbonyls and changes in the electrophoretic pattern. During the Maillard reaction, mainly modifications of the basic amino acids (lysine and arginine) via interaction with glucose took place [19]. During protein oxidation however, tryptophan, methionine, tyrosine and histidine residues in soybean proteins were mainly modified (unpublished data). This indicates that mass shifts of the peptides due to oxidation and glycation cannot be excluded [8,12,16]. Moreover, it was expected that the peptide stability will highly depend on the induced reaction. Indeed, for example peptide ²²⁸Glu-Arg²³⁸ of the glycinin G2 was observed in its original state even after 48 h of incubation with glucose in the presence or absence of wheat proteins. The same peptide however, could not be detected anymore when the proteins were oxidized either via hypochlorous acid or lipid induced oxidation (Table 4). The reason for the inability to detect this peptide in the oxidized proteins is obviously due to the presence of the methionine residues (Met²³⁴) which was probably modified to methionine sulfone through

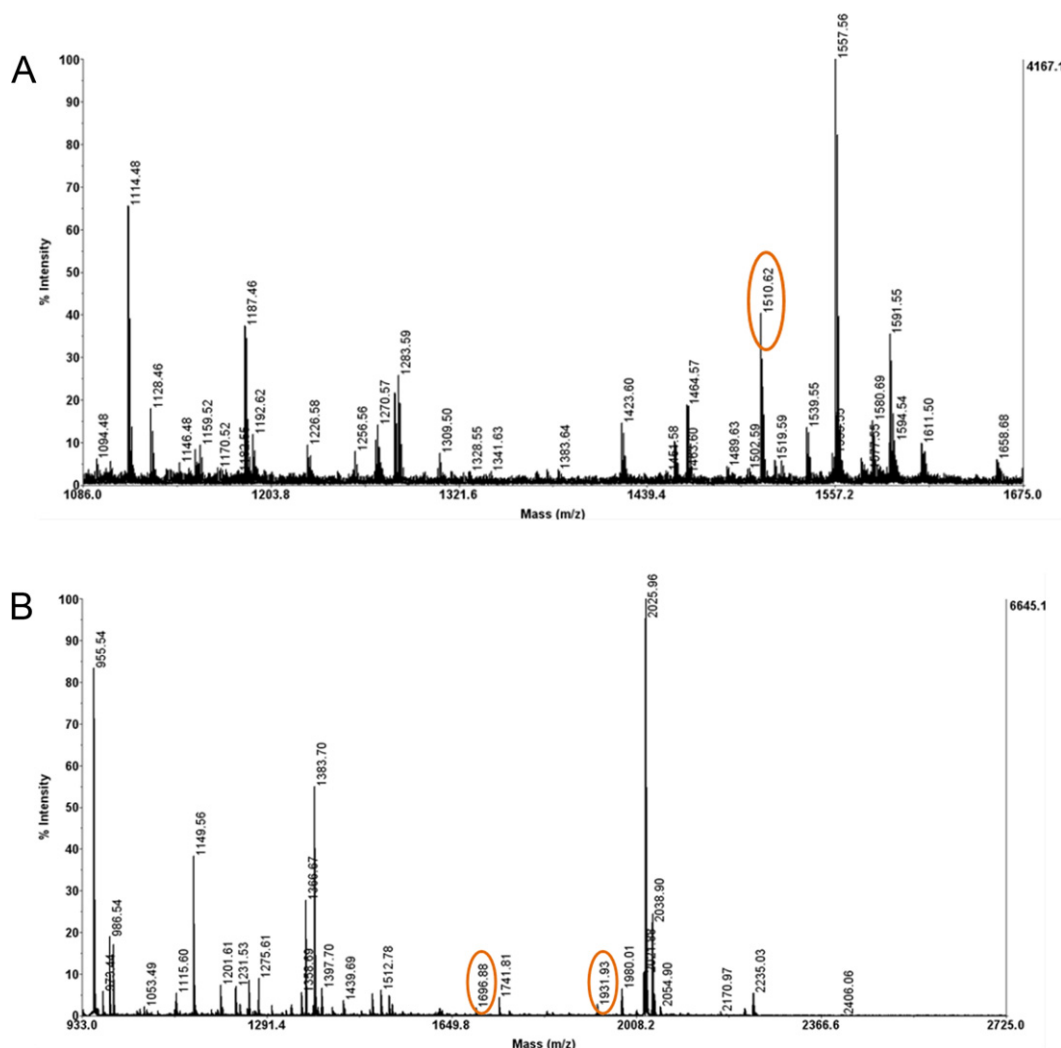


Fig. 2. (A) MS spectrum of soybean proteins incubated with 5 mmol HOCl/g protein. The peptide with the mass 1494.69 with a mass shift of +16 Da due to tyrosine residue oxidation is encircled. (B) MS spectrum of soybean proteins 48 h incubated with glucose. The peptide 1534.82 with a mass shift of +162 Da due to Amadori product formation and the peptide 1873.89 with a mass shift of +58 Da due to carboxymethyl residue formation are encircled.

oxidation of its side chain [16]. The oxidation of the methionine residue would imply that a peptide with a Δ mass of 32 Da would be formed as also evidenced from the pick table list (Table 7). On the contrary, the peptide $^{482}\text{Phe-Arg}^{492}$ of the G1 glycinin was apparently modified during incubation with glucose but was stable during oxidation. The instability during the Maillard reaction is obviously due to the presence of the lysine residues (Lys^{491}) which could interact with either glucose or other degradation products therefore, leading to its modification through formation of an Amadori product or a carboxymethyl residue with Δ mass of 162 Da or 58 Da respectively [13]. Besides these 2 peptides, a number of other peptides with mass shifts were detected in the peptide pool obtained from the modified soybean proteins (Tables 7 and 8). These peptides could be identified using the pick list table and the mass spectra (Fig. 2). For each mass shift, interpretations of the possible modifications were moreover given.

However, in food products a combination of glycation and oxidation are taking place therefore, a focus was made only on the peptides which could be detected with high scores regardless of the type of modification induced. In our case peptides $^{401}\text{Val-Arg}^{410}$ from the G1 glycinin (*Gly m 6*) and the $^{518}\text{Gln-Arg}^{528}$ from the α' chain of the *Gly m 5* proved to be the most stable and could be detected and confirmed with high scores in all modified samples (Fig. 1, Tables 3 and 4). Moreover, these peptides were

specific to soybean and part of predicted linear IgE binding epitopes. Therefore, these two peptides are possible candidates for the development of new robust quantitative methods for allergen detection in especially processed foods. MALDI-TOF/MS proved to be an excellent technique for fast and efficient screening of such stable peptides during food processing simulating reactions.

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