



Digestibility and immunoreactivity of soybean β -conglycinin and its deglycosylated form

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ABSTRACT

IgE-mediated allergy to soybean may be connected to an incomplete protein digestion causing an inappropriate immune response in the gut. The aim of this study was to gain insight on the digestibility and immunoreactivity of β -conglycinin and its deglycosylated form. To achieve this goal both proteins were digested mimicking gastrointestinal physiological conditions. Digests were characterised by SDS–PAGE, MALDI–TOF–MS and immunological assays. Data on IgG binding western blot provided key information related to the composition of the digests indicating that whereas deglycosylated β -conglycinin was completely removed by gastrointestinal digestion, α and β subunits of β -conglycinin glycoprotein partially survive the process of digestion. Such differential digestive behaviour might be influenced by glycan moieties, at least to some extent, and should be an additional factor to be considered on its potential allergenicity. Results also support the existence of linear epitopes in the three subunits (α , α' , and β) of both protein forms.

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1. Introduction

Soybean (*Glycine max*) β -conglycinin has been reported as a major food allergen (Ogawa, Bando, Tsuji, Nishikawa, & Kitamura, 1995; Ogawa, Samoto, & Takahashi, 2000). Soybean β -conglycinin is a trimeric glycoprotein composed of very heterogeneous and differently processed polypeptides (Thanh & Shibasaki, 1977). The occurrence of extensive post-translational modifications in the cotyledonary tissues, including proteolysis and glycosylation, increases significantly the polymorphism of this protein class (Casey, Domoney, & Ellis, 1986). Variation in glycosylation pattern of 7S globulins has been reported; α and α' subunits contain two N-linked high mannose glycans, whereas β -subunits generally contain only a single one (Doyle et al., 1986; Thanh & Shibasaki, 1977). Recent studies have demonstrated that IgE reactivity of soybean β -conglycinin may be significantly reduced when it is compared with its glycosylated form (Amigo-Benavent,

Athanasopoulos, Ferranti, Villamiel, & del Castillo, 2009) although is not completely abolished by using glycosidases or are expressed in *Escherichia coli* (Krishnan, Kim, Jang, & Kerley, 2009).

Studies regarding epitope characterisation of β -conglycinin are based on mapping of linear epitopes, being those of conformational nature unexplored yet (Fu, Jez, Kerley, Allee, & Krishnan, 2007; Ogawa et al., 1995). It has been recently demonstrated that all three subunits of soybean β -conglycinin (α , α' and β) share high amino acid sequence homology and are recognised by IgE antibodies present in soybean-allergic patients (Krishnan et al., 2009). The β -conglycinin polypeptides resist and survive, at least to some extent, the transit through the stomach and small intestine (Adachi et al., 2009; Lålles et al., 1999; Wang, Qin, Zhao, & Sun, 2009; Zhao, Qin, Sun, Zhang, & Wang, 2010). Subsequently, they may be absorbed in immunological active form by the gut cells, facilitating the exposition of these allergens to the immune system in order to sensitise a naïve individual and/or elicit an allergic response in a sensitised individual (Astwood, Leach, & Fuchs, 1996; Moreno & Clemente, 2008). Despite the clinical relevance of soybean β -conglycinin in allergenicity, the role of its glycan moieties on its susceptibility to proteolysis by digestive proteases has not been previously reported.

Due to the relevance of the digestibility on the immunoreactivity of the food proteins simulated gastrointestinal digestion

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together with bioinformatic searches and the development of suitable IgE-binding methods are the allergy assessment testing strategies proposed by US Food Drug Administration (FDA), Food and Agricultural Organization of the United Nations (FAO) and the US Codex Office (Mc Clain, Vieths, & Bannon, 2008). In agreement, the aim of this study was to go deeply into a better understanding on the digestibility and immunoreactivity of soybean β -conglycinin and its deglycosylated form. We demonstrate that whereas deglycosylated β -conglycinin is completely hydrolysed by gastrointestinal digestion, α and β subunits of β -conglycinin glycoprotein partially survived the hydrolysis process. Such differential behaviour might indicate that glycan moieties are involved, at least in some extent, in proteolysis resistance and should be taken into account as an additional factor to consider in its potential allergenicity.

2. Materials and methods

2.1. Chemicals and enzymes

Commercial soy protein isolate was purchased from Manuel Riesgo Company (Madrid, Spain). PNGase F (Peptide N-Glycosidase F EC 3.5.1.52, P7367), octyl- β -D-glucopyranoside, pepsin (P-7000) pancreatin (P-3292), 3,3',5,5'-tetramethylbenzidine (TMB) for western and for ELISA were supplied by Sigma-Aldrich (Sigma-Aldrich, Saint Louis, MO, USA). 2-mercaptoethanol and α -ciano-4-hydroxycinnamic acid were from Merck (Hohenbrunn, Germany) and Fluka (Steinheim, Germany), respectively. All other chemicals were of analytical grade.

2.2. Sample preparation

2.2.1. Protein isolation and sample preparation

β -Conglycinin was isolated from commercial soy protein isolate following the method of Nagano, Hirotsuka, Mori, Kohyama, and Nishinari (1992). The obtained protein extract enriched in β -conglycinin was washed twice with distilled water, freeze-dried and stored in dryness at -20°C until use.

The glycoprotein fraction (5.5 mg/ml) was denatured in 20 mM ammonium carbonate buffer (pH 7.4) with 0.11% (w/w) octyl- β -D-glucopyranoside and 5.6 mM 2-mercaptoethanol by heating at 100°C for 10 min. The denatured protein solution was diluted to a final concentration of 5 mg/ml with 20 mM ammonium carbonate buffer (pH 7.4) and divided into two aliquots. Subsequently, 40 U of PNGase F was added to one aliquot that was named as deglycosylated β -conglycinin (5 ml) whereas 80 μl of distilled water were added to the other one that was called as β -conglycinin glycoprotein (5 ml). All samples were incubated in a Thermomixer (Eppendorf, Hamburg, Germany) at 37°C for 24 h with constant stirring at 300 rpm and subsequently heated at 100°C for 10 min. β -Conglycinin deglycosylation was confirmed by differential electrophoretic behaviour employing Periodic Acid Schiff (PAS) staining SDS-PAGE protocol (Amigo-Benavent et al., 2009). All samples were lyophilised and stored at -20°C until use.

2.2.2. In vitro digestion of β -conglycinin and its deglycosylated form

β -Conglycinin and its deglycosylated form were digested, as previously described by Moreno, Rubio, Olano, and Clemente (2006), mimicking adult human gastrointestinal tract (GIT) conditions (Daugherty & Mrsny, 1999). A 10 mg amount of glycoprotein or its deglycosylated form were suspended in 8 ml of 120 mM NaCl, mixed and allowed to stand at room temperature for 15 min. The pH of the samples was adjusted to 2.0 with 5 M HCl, the volume brought to 10 ml with 120 mM NaCl solution and 0.1 ml of a pepsin solution (5 mg in 2.5 ml of 0.1 M HCl; enzyme-substrate ratio [E/S] = 0.02 [w/w]) added to each sample.

The flasks were placed in a shaker (100 oscillations/min) at 37°C for 1 h that corresponded to a simulated gastric digestion. For the intestinal digestion step, the pH was raised to 6.0 with 1 M NaHCO_3 dropwise, and 2.5 ml of pancreatin-bile salts mixture (2.5 mg of pancreatin and 15 mg of bile extract [B-8756, Sigma-Aldrich] in 25 ml of 100 mM NaHCO_3 ; [E/S] = 0.025 [w/w]) were added. The pH was adjusted to pH 7.5 with 1 M NaOH and the volume brought to 15 ml with 120 mM NaCl. Pancreatin digestion was carried out in a shaker (100 oscillations/min) at 37°C for 2 h. After protein hydrolysis, digestive enzymes were inactivated by heat treatment at 85°C for 5 min in a water bath. Control samples having only the digestive enzymes in buffered solutions were also obtained. Digestions were performed in triplicate. All protein samples were collected and stored at -20°C until use.

2.3. SDS-PAGE analysis

SDS-PAGE analysis was carried out to monitor the electrophoretic pattern of β -conglycinin and its deglycosylated form before and after simulated gastrointestinal digestion. Samples containing 1 mg protein/ml (32.5 μl) or its equivalent (digested products) were mixed with 5 μl of DTT (0.5 M) and 12.5 μl of NuPAGE[®] LDS sample buffer (4X) (Invitrogen, Barcelona, Spain). Twenty microliters were loaded onto the gel. Separation was performed on a 4% to 12% linear gradient polyacrylamide NuPAGE[®] Novex Bis-Tris pre-cast gels employing a continuous buffer system (NuPAGE[®] MES SDS running buffer, Invitrogen) for 50 min and constant voltage of 200 V. Unstained protein molecular markers Mark 12[™] (Invitrogen) were applied to the gel. Electrophoretic bands were stained with Coomassie blue staining Kit (Invitrogen), and the electrophoretic pattern was analysed by the software Quantity One (BioRad).

2.4. Immunoassays

Polyclonal rabbit *anti*-soybean sera (IgG) (Sigma-Aldrich) were used to study the antigenicity of proteins and digests. A pooled sample of equal volumes of plasma from five patients suffering a history of adverse reaction to soy (PlasmaLab International, Everett, WA, USA) was used to monitor the allergenicity of undigested and digested proteins.

2.4.1. Western blot

SDS-PAGE was carried out as it has been described above. Pre-stained protein molecular markers ranging from 10 to 250 kDa (BioRad, California, USA) were applied to the gel. The electrophoretic bands were transferred onto a 0.45 μm PVDF membrane (Bio-Rad) using the XCell II Blot module (Invitrogen) at 30 V for 2.5 h. Blots were blocked with TBST [0.05% (v/v) Tween-20 in Tris-buffered saline (TBS)] containing 10% (w/v) milk powder for 1 h at room temperature, washed with TBST and incubated overnight at 4°C with a polyclonal rabbit *anti*-soybean sera or plasma from soybean allergic people diluted 1:3000 or 1:25 (v/v), respectively. After that, the membranes were washed and incubated with a 1:4000 (v/v) horseradish peroxidase labeled goat *anti*-rabbit IgG (Sigma) or 1:1000 (v/v) polyclonal rabbit *anti*-human IgE (DakoCytomation, Glostrup, Denmark) in TBST containing 10% (w/v) milk powder for 2 h at room temperature. Blots were washed five times with TBST and the immunoreactive bands detected by means of peroxidase reaction with TMB.

2.4.2. Competitive inhibition ELISA

Competitive inhibition ELISA was carried out as previously described (Amigo-Benavent et al., 2009) with slight modifications. Polystyrene high-bind microtitration plates (Costar 3590, New York, USA) were coated with β -conglycinin glycoprotein (150 ng/well) dissolved in 50 mM carbonate buffer pH 9.5. After overnight

incubation at 4 °C, plates were washed with PBS (phosphate saline buffer [50 mM NaCl, 0.15 mM H₂KPO₄, 0.2 mM HNa₂PO₄ and 0.3 mM KCl] pH 7.2), and the remaining protein-binding sites in coated wells were blocked by adding 300 µl of 2% (w/v) BSA in 50 mM sodium carbonate buffer pH 9.5 and incubated at 37 °C for 1 h. Plates were washed three times with PBS containing 0.05% (v/v) Tween 20 buffer (PBST) to remove the excess of BSA. β-Conglycinin, its deglycosylated form or their corresponding gastric and gastrointestinal digests (100 µl/well) in the range of concentrations 0.25 to 2500 µg/ml, together with 100 µl/well of anti-soybean sera diluted 1:3000 in PBST, were added to the plates

and incubated for 1 h at room temperature. Subsequently, plates were washed five times with PBST to remove the excess of primary antibody. One hundred microliters of horseradish peroxidase labeled goat anti-rabbit IgG diluted 5000 fold in PBS were added to wells as conjugated secondary antibody and incubated at 37 °C for 1 h. Plates were washed five times with PBST to remove the excess of secondary antibody. Detection of the antigen was performed adding 100 µl of the chromogenic TMB solution for ELISA to each well and incubation in darkness at room temperature for 10 min. The colorimetric reaction was stopped by adding 100 µl 2 M HCl. The intensity of the resulting colour was read in a microplate reader at 450 nm (Biotek Power Wave XS, Izasa, Madrid, Spain). The percentage of inhibition was calculated by using the formula: $100 - 100(A_2 - A_1)/(A_0 - A_1)$, where A_0 is the absorbance in the absence of inhibitor (maximum absorbance); A_2 is the absorbance at any given concentration and A_1 is the average absorbance of the control wells that do not contain any primary antibody (minimal signal). All measurements were carried out in triplicate. IC₅₀ values were calculated interpolated as the amount of inhibitor that produces 50% of inhibition. High IC₅₀ values denoted low immunoreactivity response whilst low levels of this parameter indicated a powerful immunological response.

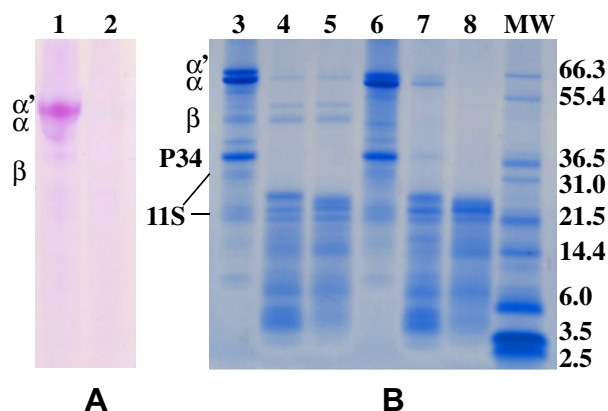


Fig. 1. Periodic acid Schiff stained (A) and Coomassie blue stained (B) SDS-PAGE. Lane 1: undigested β-conglycinin glycoprotein; lane 2: undigested deglycosylated β-conglycinin; lane 3: undigested β-conglycinin glycoprotein; lane 4: gastric digested β-conglycinin; lane 5: gastrointestinal digested β-conglycinin; lane 6: undigested deglycosylated β-conglycinin; lane 7: gastric digested deglycosylated β-conglycinin; lane 8: gastrointestinal digested deglycosylated β-conglycinin; and MW: molecular weight markers.

Table 1

IC₅₀ values of the gastric and gastrointestinal digests from β-conglycinin and its deglycosylated form by competitive inhibition ELISA using polyclonal anti-soybean sera (IgG). Values are expressed as µg of inhibitor/ml and represent the means of triplicate analyses ± SD.

Sample	Glycoprotein	Deglycosylated
Undigested protein	9.5 ± 0.9 ^a	11 ± 1.3 ^a
Gastric digests	45 ± 5.7 ^b	50 ± 5.6 ^b
Gastrointestinal digests	60 ± 5.4 ^c	65 ± 7.0 ^c

Different letters denote statistically significant differences ($p < 0.01$) amongst samples.

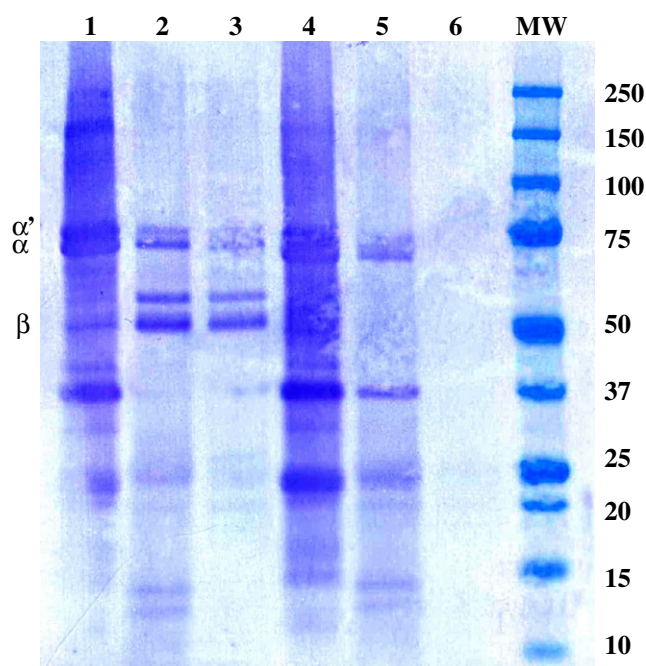


Fig. 2. Western blotting of undigested β-conglycinin, its deglycosylated form and their digests detected by anti-total soy protein antibodies from rabbits (IgG). MW: molecular weight markers; lane 1: undigested β-conglycinin; lane 2: gastric digested β-conglycinin; lane 3: gastrointestinal digested β-conglycinin; lane 4: undigested deglycosylated β-conglycinin; lane 5: gastric digested deglycosylated β-conglycinin; and lane 6: gastrointestinal digested deglycosylated β-conglycinin.

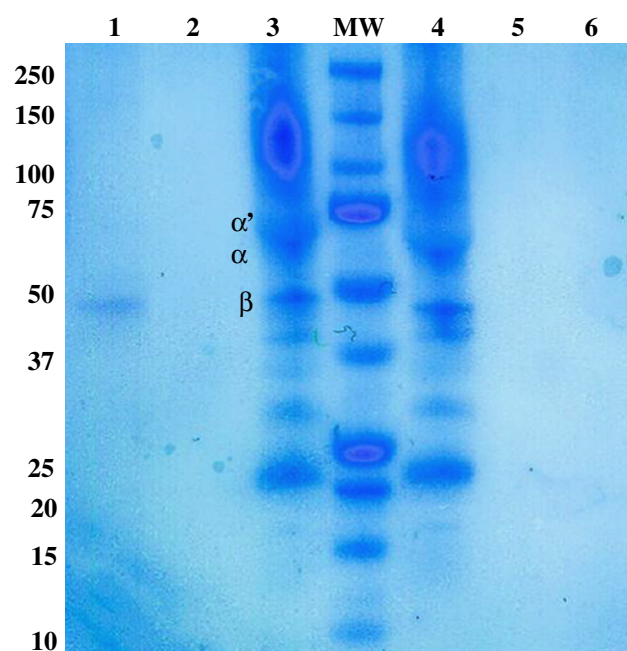


Fig. 3. Western blotting of undigested β-conglycinin, its deglycosylated form and their digests detected by sera from soybean allergic patients (IgE). Lane 1: gastric digested β-conglycinin; lane 2: gastrointestinal digested β-conglycinin; lane 3: undigested β-conglycinin; MW: molecular weight markers; lane 4: undigested deglycosylated β-conglycinin; lane 5: gastric digested deglycosylated β-conglycinin; and lane 6: gastrointestinal digested deglycosylated β-conglycinin.

2.5. MALDI-TOF-MS analysis

MALDI-TOF-MS spectra of peptides were recorded on a Voyager DE-PRO mass spectrometer (PerSeptive Biosystems, Framingham, MA) equipped with an N₂ laser (337 nm, 3 ns pulse width) operating both in the linear and reflector positive ion modes. Prior to mass spectrometry analysis, either β -conglycinin or its deglycosylated form digested by pepsin or sequentially by pepsin and pancreatin were desalted using Zip-Tip C₁₈ (Millipore, Billerica, MA, USA). Peptide samples (1 μ l) were loaded on a stainless steel plate using α -ciano-4-hydroxycinnamic acid as matrix (1 μ l prepared dissolving 0.5 mg in 0.5 ml 0.1% TFA and 50% ACN solution) and dried at room conditions. External mass calibration was carried out by using peptide standards (Sigma). Mass spectrum acquisition was performed by accumulating 200 laser pulses. MALDI mass spectra were scanned from 500 to 6000 m/z. The accelerating voltage was 20 kV.

2.6. Peptide structural characterisation by LC-ESI-Q/TOF-MS/MS analysis

Peptides derived from *in vitro* gastrointestinal digestion of β -conglycinin and its deglycosylated form were identified by

LC-ESI-Q/TOF-MS/MS. Tandem MS spectra were obtained by using a QTOF instrument model Q-STAR PULSAR (Applied Biosystems, CA, USA) equipped with a nanospray interface (Protana, Denmark). Dried samples were resuspended in 0.1% TFA, desalted using ZipTip C₁₈ microcolumns (Millipore) and sprayed from gold-coated “medium-length” borosilicate capillaries (Protana). The capillary voltage used was 800 V. Double-charged ion isotopic clusters were selected by using the quadrupole mass filter and then induced to fragment by collision. The collision energy was 20–40 eV, depending on the size of the peptide. The collision-induced dissociation was processed by using Analyst software (Applied Biosystems). The deconvoluted MS/MS spectra were manually interpreted with the help of Peptools (Agilent Technologies, Santa Clara, CA, USA) software taking into account the known amino acid sequences of the α , α' and β subunits of soybean β -conglycinin described in Swiss-Prot protein database (their corresponding accession numbers are P13916, P11827 and P25974, respectively).

Identified peptides were submitted to <http://www.immuneepitope.org> (National Institute of Allergy and Infectious Diseases, Bethesda, MD, USA) database in order to look for sequences previously described as epitopes. The sequences found were submitted with the following search fields: linear sequence, peptide from protein and substring.

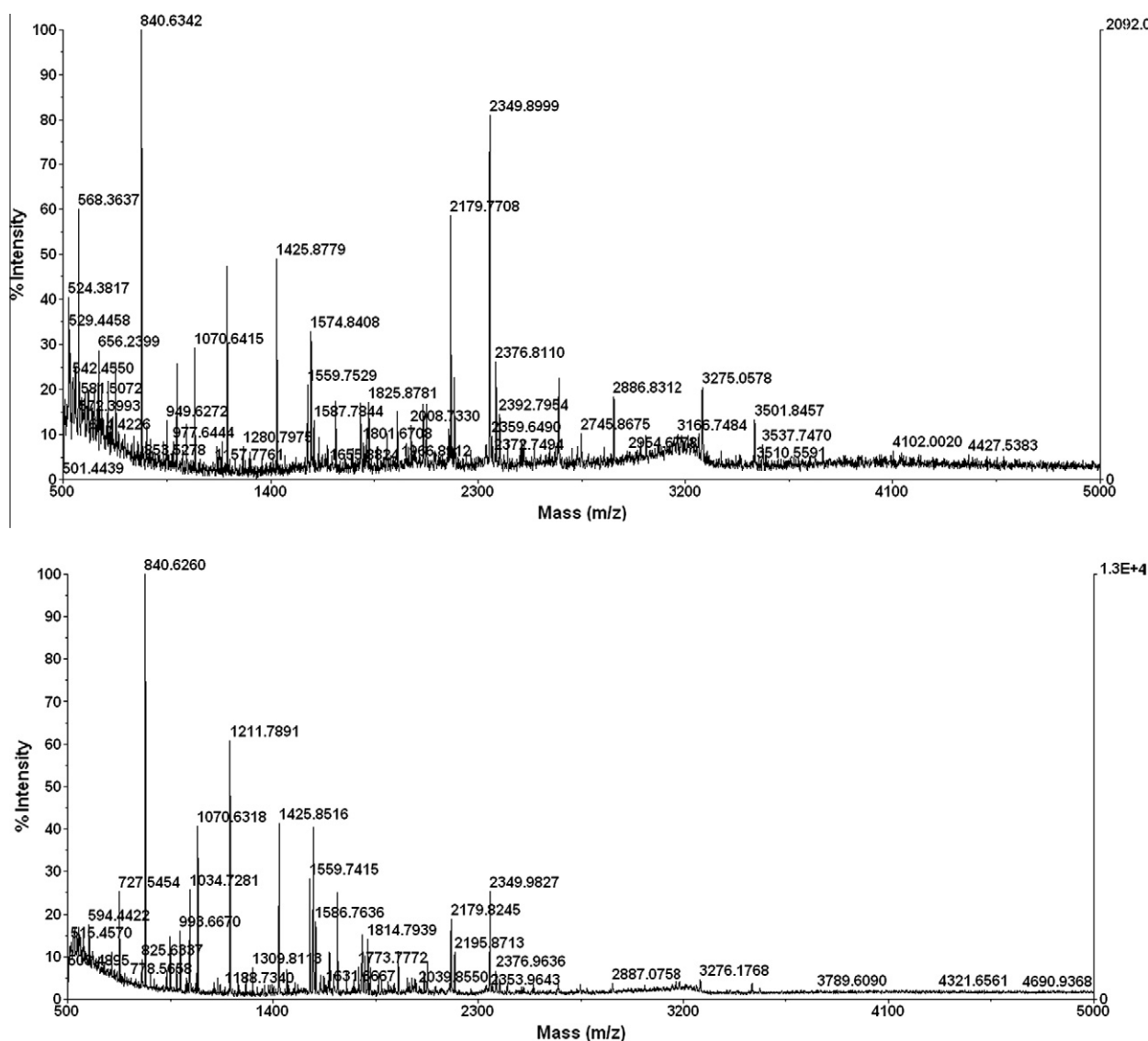


Fig. 4. MALDI-TOF mass spectra of gastric digested β -conglycinin (upper panel) and its deglycosylated form (lower panel).

2.7. Statistical analysis

SPSS Statistics Program for Windows version 15.0 software package (Inc. Chicago, IL, USA) was employed for statistical analysis of the data with the level of significance set at 99%. One way analysis of variance (ANOVA) was used to look at differences in antigenicity (IgG immunoreactivity) amongst all samples followed by DMS test.

3. Results

The resistance to gastrointestinal digestion of β -conglycinin and its deglycosylated form was investigated. The removal of glycan moieties from β -conglycinin primary structure by PNGase F activity was confirmed by differences in electrophoretic mobility and specific glycan's staining (PAS staining). No carbohydrate bound to polypeptide bands were detected after deglycosylation (Fig. 1A) indicating that the glycan moieties were effectively removed by PNGase F activity under our experimental conditions.

Fig. 1B shows the Coomassie blue staining SDS-PAGE profiles of the glycoprotein, its deglycosylated form and their corresponding digests. As can be observed, electrophoretic bands corresponding to α' ($M_r \sim 68$ kDa), α ($M_r \sim 64$ kDa) and β ($M_r \sim 50$ kDa) subunits of β -conglycinin were detected (Fig. 1B, lane 3) (Amigo-Benavent, Silván, Moreno, Villamiel, & del Castillo, 2008). The three electrophoretic bands forming the deglycosylated protein were also detected by applying this technical approach (Fig. 1B, lane 6). Soybean P34 ($M_r \sim 37$ kDa), usually co-purified with β -conglycinin (Wilson, Martínez-Villaluenga, & de Mejia, 2008), and faint bands of 11S globulin were observed as contaminants in the protein preparations.

The electrophoretic bands corresponding to α and β subunits of β -conglycinin were observed whereas the α' subunit was not detected in the corresponding gastric digest (Fig. 1B, lane 4). On the other hand after pepsinolysis, only the electrophoretic band corresponding to deglycosylated α subunit was observed (Fig. 1B, lane 7). In addition, new electrophoretic bands with apparent molecular masses in the range from 5 to 27 kDa were also found (Fig. 1B,

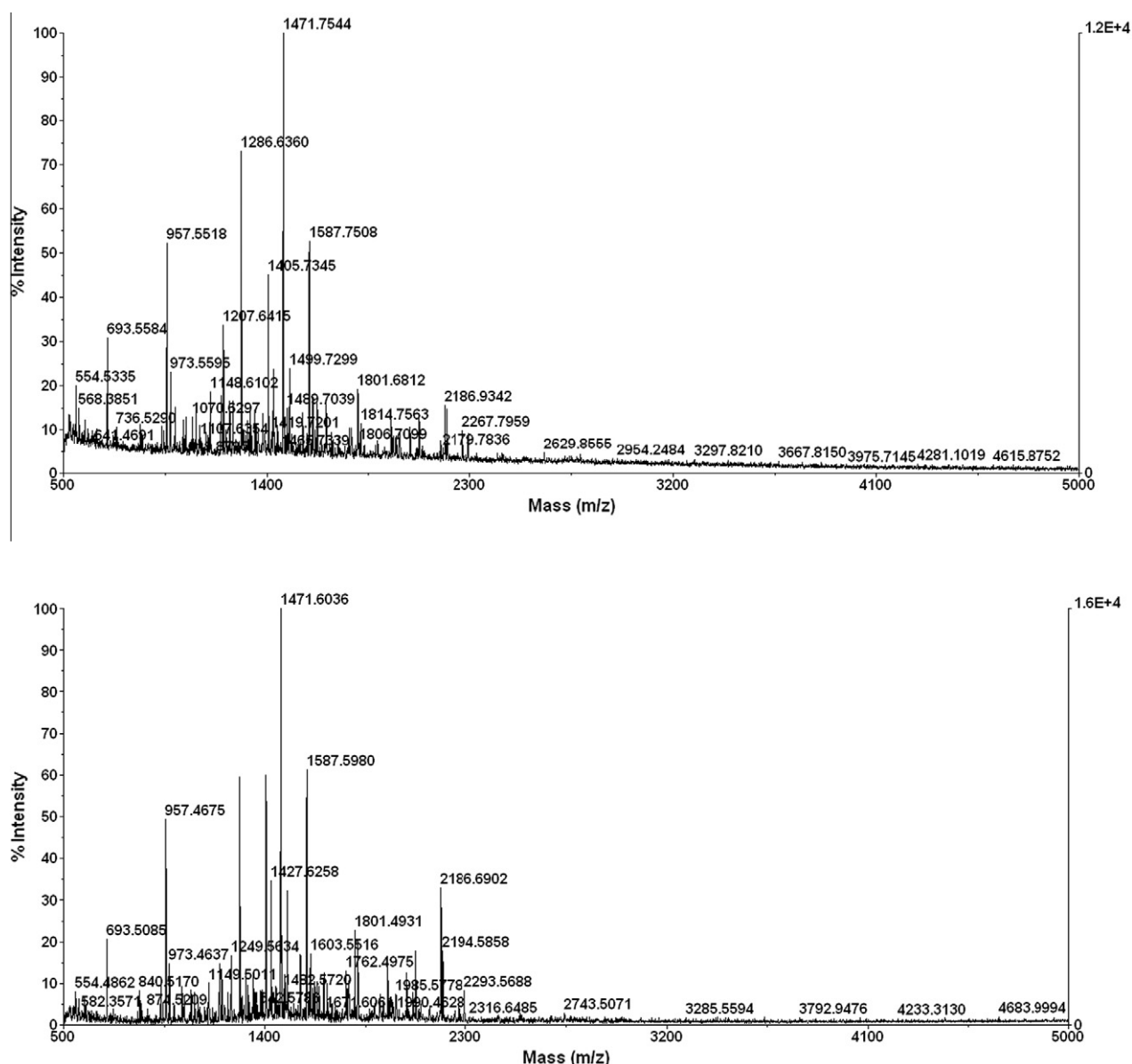


Fig. 5. MALDI-TOF mass spectra of gastrointestinal digested β -conglycinin (upper panel) and its deglycosylated form (lower panel).

lanes 4 and 7). After gastrointestinal digestion of β -conglycinin, α and β subunits were observed (Fig. 1B, lane 5), whilst no protein bands were detected in the sample corresponding to deglycosylated form (Fig. 1B, lane 8). Peptide profiles from both proteins obtained by gastric (Fig. 1B, lanes 4 and 7) and gastrointestinal (Fig. 1B, lanes 5 and 8) digestions differed.

The IgG immunoreactivity of proteins and their corresponding digestion products are shown in Fig. 2. The different subunits of β -conglycinin and those derived from its deglycosylated form reacted with the polyclonal IgG antibodies (Fig. 2, lanes 1 and 4). After gastric digestion, IgG binding polypeptides with molecular masses corresponding to α and α' subunits were observed in both glycoprotein (Fig. 2, lane 2) and its deglycosylated form (Fig. 2, lane 5). For the glycoprotein, an IgG binding polypeptide with molecular mass corresponding to the β subunit was also detected after gastric digestion (Fig. 2, lane 2). However, in the case of the deglycosylated form such immunoreactive band (β subunit) was not detected (Fig. 2, lane 5). In addition, IgG binding peptides with $M_r \sim 15$, 25 and 37 kDa were detected (Fig. 2, lanes 2 and 5). After gastrointestinal digestion, the IgG binding ability was only associated to the β -conglycinin' subunits (Fig. 2, lane 3) whereas no immunoreactive bands were found in the gastrointestinal digests of the deglycosylated form (Fig. 2, lane 6).

Quantitative results based on the IgG immunoreactivity of the samples obtained by competitive inhibition ELISA using commercial polyclonal anti-total soybean protein antibodies from rabbit are summarised in Table 1. The digests of both proteins were

significantly less immunoreactive than the parental proteins ($p < 0.01$). The immunoreactivity decreased as a function of the progress of digestion being less antigenic the gastrointestinal digests than the gastric digests. The total IgG immunoreactivity of the digests derived from both proteins were not statistically different ($p > 0.01$) by this approach.

The data on IgE immunoreactivity of both proteins and their digests are shown in Fig. 3. The three subunits of β -conglycinin and those forming its deglycosylated form reacted with IgE antibodies from a pooled sample of sera of people allergic to soybean (Fig. 3, lanes 3 and 4) suggesting the presence of linear epitopes. Only a faint electrophoretic band corresponding to the β -subunit was observed for the gastric glycoprotein digests (Fig. 3, lane 1) whereas no immunoreactive bands were detected in the digestion products of the deglycosylated form (Fig. 3, lane 5). After gastrointestinal digestion, no IgE binding epitopes were detected in the digests of both protein forms.

The mass spectra obtained by MALDI-TOF-MS analysis of the peptides forming the gastric and gastrointestinal hydrolysates of both proteins are shown in Figs. 4 and 5, respectively. Fig. 4 shows little differences on the peptide composition of gastric digests corresponding to glycoprotein and deglycosylated protein. However, the mass spectra (500–6000 m/z) shown in Fig. 5 were very similar indicating the major presence of common peptides in gastrointestinal digests of both β -conglycinin and its deglycosylated form. Table 2 shows the amino acid sequences of those common peptides identified in the gastrointestinal digests of both proteins

Table 2

Identified peptides by LC-ESI-QTOF-MS/MS of the gastrointestinal hydrolysates from β -conglycinin and its deglycosylated form. Peptide numbers refer to the N-terminal and to the C-terminal of each peptide.

Measured mass [M]	Expected mass [M + H ⁺]	Peptide identity	Theoretical mass	Subunit position
692.95	693.95	YRILE	692.39	α' (187–191) α (171–175)
840.19	841.19	YRILEF	839.45	α' (187–192) α (171–176)
934.21	935.21	VNDDRRDS	933.38	α' (223–230) α (207–214) β (83–90)
987.32	494.66	LLPHHADAD	987.48	α' (200–208) β (60–68)
1069.34	535.67	SPQLQNLRD	1069.55	α (162–170)
1083.48	542.74	NVISQIPSQV	1083.59	α' (517–526) α (483–492)
1134.30	1134.30	SLVNDDRRDS	1133.49	α (205–214)
1134.36	568.18			
1147.42	574.71	TLVNDDRRDS	1147.51	α' (221–230)
1177.36	589.68	YVNPNDNEN	1177.49	α' (248–257) α' (267–276) α (232–241)
1341.46	671.73	KPFNLRSDPI	1341.75	α (342–352)
1342.42	672.21	EPFNLRSDNPI	1341.72	α (213–223)
1426.56	714.28	LAIPVKNKGRFES	1426.79	α' (282–294) α (247–259) β (35–46)
1467.63	490.21	NKRSPQLENLRD	1468.80	
1468.50	735.25			
1471.52	736.76	FEITPEKNPQLR	1470.78	α (361–372) β (232–243)
1488.48	745.24	FVDAQPKKKEEGN	1488.76	α (516–528)
1488.52	745.26	FVDAQPQQKEEGN	1488.68	α' (550–562)
1489.40	745.70			
1510.52	756.26	NEGALLPHFNSKA	1509.79	α (384–397)
1510.60	756.30			β (255–268)
1586.61	529.87	FEITPEKNPQLRD	1585.10	α (361–373) β (232–244)
1773.72	887.86	YVNPNDNENLRIT	1772.91	α (232–246)
1917.66	959.83	NFLAGSKDNVISQIPSQV	1917.00	α' (509–526)
2186.31	729.77	LAIPGSAQDVERLLKKQRE	2185.20	β (366–384)
2186.88	729.96	LAIPGSAQAVEKLLKNQRES	2186.19	α (495–514)
2186.90	1094.45			
2188.02	1095.06			

Table 3

Match peptides found in this study that correspond with immunoreactive peptides of β -conglycinin reported in <http://www.immuneepitope.org> database. Amino acids are in single-letter code.

Identified peptides	Epitope code	Assay antigen	Assay description
YRILE	49,028, 75,618	PQLQNLRDYRILEFN YRILEFNSKPNTLL	Western negative ^a
YRILEF	49,028, 75,618	PQLQNLRDYRILEFN YRILEFNSKPNTLL	Western negative ^a
VNDDRDS	27,337	ILSLVNDDRDSYRL	Western negative ^a
NVISQIPSVQ	46,401	NVISQIPSVQELAF	Western negative ^a
SLVNDDRDS	27,337	ILSLVNDDRDSYRL	Western negative ^a
KPFNLSRDPI	57,370	SEDKPFNLSRDPIY	Western negative ^a
FVDAQPKKKEGN	73,927	YFVDAQPKKKEGNK	Western negative ^a

^a Tested with sera (IgG) from pigs by Fu, Jez, Kerley, Allee, and Krishnan (2007).

by LC-ESI-Q/TOF-MS/MS. It is the first time that such high number of peptides derived from mimicked gastrointestinal digestion of β -conglycinin and deglycosylated protein is sequenced. Moreover, the data also supported the hypothesis of a little influence of the glycans on the rate of protein digestibility. The amino acid sequences obtained by proteomic analysis were submitted to bioinformatic searches in allergen database (Table 3). The peptide sequences herein described have been reported by others as non-immunoreactive. The proteomic results agreed with those obtained by immunoassays indicating a reduction of the immunoreactivity by proteolysis and the existence of linear polypeptide epitopes in β -conglycinin.

4. Discussion

Several studies have shown the potential clinical relevance of some legume seed proteins as emerging allergens, including β -conglycinin, a major storage protein from soybean. This dietary protein has demonstrated its ability to bind IgE from allergic patient sera (Holzhauser et al., 2009; Krishnan et al., 2009), being recently considered as a potential diagnostic marker for severe allergic

reactions to soybean (Holzhauser et al., 2009). It has been hypothesised that IgE-mediated adverse reaction to soybean may be connected to an incomplete protein digestion; such resistance to the proteolytic activity of digestive enzymes could allow small amounts of protein to be absorbed in immunologically active forms by the gut, facilitating the exposition of these allergens to the immune system in order to sensitise a naïve individual and/or elicit an allergic response in a sensitised individual (Adachi et al., 2009; Moreno, 2007; Moreno and Clemente, 2008). Consequently, we have evaluated the stability of β -conglycinin to simulated gastrointestinal digestion and analysed the role of its glycan moieties on protein digestibility. Such stability is thought to be an important attribute in the assessment of potential allergenicity (Astwood et al., 1996; Thomas et al., 2007). Regarding this, Adachi et al. (2009) have shown differences in resistance to digestion of two soybean based foods and suggested that the presence of undigested allergens in the digestive tract is a prerequisite for the development of food-dependent exercise induced anaphylaxis.

As recently reported by Holzhauser et al. (2009) and Krishnan et al. (2009), we have confirmed the presence of linear epitopes in the three subunits (α , α' and β) of β -conglycinin. In addition, we report for the first time that under extreme conditions (denaturation, see materials and methods section for specific details) used prior deglycosylation, the IgG (Fig. 2, lanes 1 and 4) and IgE (Fig. 3, lanes 3 and 4) binding properties of β -conglycinin remained. Interestingly, the electrophoretic and immunological data suggest that the rate of proteolysis of β -conglycinin, at least to little extent, may be influenced by its structural glycans (Fig. 1B, lanes 5 and 8; Fig. 2, lanes 2–3 and 5–6; and Fig. 3, lanes 1 and 5). The IgG binding studies performed by western-blot technique revealed that whereas deglycosylated β -conglycinin was completely removed by gastrointestinal digestion, α and β subunits of β -conglycinin glycoprotein survived to some extent the digestive process, being the α' subunit more susceptible to proteolytic attack (Fig. 1B, lane 4). As occurred with other glycoproteins (Wilson, Wu, Peluso, & Nock, 2002), β -conglycinin glycans might limit the accessibility of pepsin to cleavage sites (Fig. 6) because the sequence of glycosylation are close to some of them.

A number of studies have evaluated the effects of enzymatic hydrolysis on digestibility, immunoreactivity and/or allergenicity of legume proteins (Clemente, 2000), included those from soybean (Amigo-Benavent et al., 2008; Song, Martinez-Villaluenga, & de

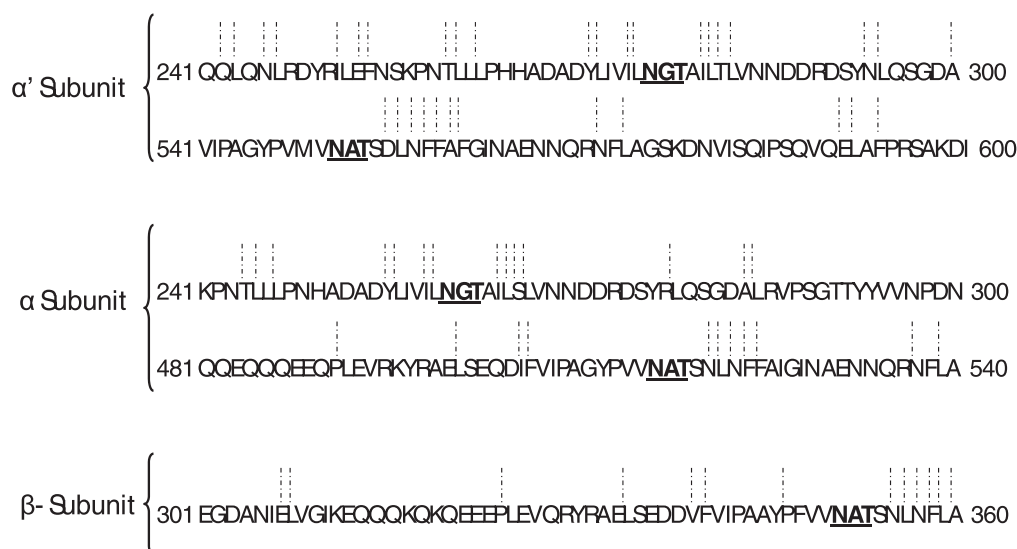


Fig. 6. Primary sequence corresponding to N-glycosylated regions of β -conglycinin subunits. The numbers indicated the amino acid sequence position. The discontinuous line means the peptide cleavage site of pepsin under the conditions studied here (pH >2). Bold underlined amino acids denote glycosylation sites.

Mejia, 2008), lentil (Cabanillas et al., 2010) and chickpea (Clemente, Vioque, Sanchez-Vioque, Pedroche, & Millán, 1999). Table 3 shows β -conglycinin matched the peptides and the amino acid sequences of the peptides identified in the gastrointestinal hydrolysates (Table 2). In all cases, the gastrointestinal peptides hereby found were fragments of sequences already described as non-immunoreactive against IgG antibodies produced in pigs in the database used as reference (Table 3).

In conclusion, our study supports a relationship between digestibility and immunoreactivity of β -conglycinin. The present investigation also suggests that α , α' and β subunits of the β -conglycinin contain linear IgE binding polypeptides although conformational epitopes may not be discarded. Western-blot analysis showed that β -conglycinin glycoprotein may survive, to some extent, the gastrointestinal digestion. Its *in vitro* pepsin digestibility may be enhanced by deglycosylation with PNGase F. As a consequence, the structural glycans seem to affect the rate of proteolysis and the amount of intact protein (epitopes) available to reach up the intestinal tract that will develop an allergenic response. Further research is necessary in order to determine the minimum amount of glycoprotein able to cause an allergenic response. Our results pinpoint the importance of using different analytical approaches for ensuring the hypoallergenicity of digestion products.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.foodchem.2011.06.015](https://doi.org/10.1016/j.foodchem.2011.06.015).

References

- Adachi, A., Horikawa, T., Shimizu, H., Sarayama, Y., Ogawa, T., Sjolander, S., et al. (2009). Soybean β -conglycinin as the main allergen in a patient with food-dependent exercise-induced anaphylaxis by tofu: Food processing alters pepsin resistance. *Clinical and Experimental Allergy*, 39, 167–173.
- Amigo-Benavent, M., Athanasopoulos, V., Ferranti, P., Villamiel, M., & del Castillo, M. D. (2009). Role of carbohydrate moieties on the *in vitro* immunoreactivity of soy (*Glycine max*) β -conglycinin. *Food Research International*, 42, 819–825.
- Amigo-Benavent, M., Silván, J. M., Moreno, F. J., Villamiel, M., & del Castillo, M. D. (2008). Protein quality, antigenicity, and antioxidant activity of soy-based foodstuffs. *Journal of Agricultural and Food Chemistry*, 56, 6498–6505.
- Astwood, J. D., Leach, J. N., & Fuchs, R. L. (1996). Stability of food allergens to digestion *in vitro*. *Nature Biotechnology*, 14(10), 1269–1273.
- Cabanillas, B., Pedrosa, M. M., Rodriguez, J., Gonzalez, A., Muzquiz, M., Cuadrado, C., et al. (2010). Effects of enzymatic hydrolysis on lentil allergenicity. *Molecular Nutrition and Food Research*, 54, 1266–1272.
- Casey, R., Domoney, C., & Ellis, N. (1986). Legume storage protein and their genes (vol 3, pp. 1–95). In B. J. Miflin (Ed.), *Oxford Surveys of Plant Molecular and Cell Biology*. Oxford, United Kingdom: Oxford University Press.
- Clemente, A. (2000). Enzymatic protein hydrolysates in human nutrition. *Trends in Food Science and Technology*, 11, 254–262.
- Clemente, A., Vioque, J., Sanchez-Vioque, R., Pedroche, J., & Millán, F. (1999). Production of extensive chickpea (*Cicer arietinum* L.) protein hydrolysates with reduced antigenic activity. *Journal of Agricultural and Food Chemistry*, 47, 3776–3781.
- Daugherty, A. L., & Mersny, R. J. (1999). Transcellular uptake mechanisms of the intestinal epithelial barrier - Part one. *Pharmaceutical Science and Technology Today*, 2, 144–151.
- Doyle, J. J., Schuler, M. A., Godette, W. D., Zenger, V., Beachy, R. N., & Slightom, J. L. (1986). The glycosylated seed storage proteins of *Glycine max* and *Phaseolus vulgaris* - structural homologies of genes and proteins. *The Journal of Biological Chemistry*, 261, 9228–9238.
- Fu, C. J., Jez, J. M., Kerley, M. S., Allee, G. L., & Krishnan, H. B. (2007). Identification, characterization, epitope mapping, and three-dimensional modeling of the α -subunit of α -conglycinin of soybean, a potential allergen for young pigs. *Journal of Agricultural and Food Chemistry*, 55, 4014–4020.
- Holzhauser, T., Wackermann, O., Ballmer-Weber, B. K., Bindeslev-Jensen, C., Scibilia, J., Perono-Garoffo, L., et al. (2009). Soybean (*Glycine max*) allergy in Europe: Gly m 5 (β -conglycinin) and Gly m 6 (glycinin) are potential diagnostic markers for severe allergic reactions to soy. *The Journal of Allergy and Clinical Immunology*, 123, 452–458.
- Krishnan, H. B., Kim, W.-S., Jang, S., & Kerley, M. S. (2009). All three subunits of soybean β -conglycinin are potential food allergens. *Journal of Agricultural and Food Chemistry*, 57, 938–943.
- Lalles, J.-P., Tukur, H. M., Salgado, P., Mills, C. E. N., Morgan, M. R. A., Quillien, L., et al. (1999). Immunochemical studies on gastric and intestinal digestion of soybean glycinin and β -conglycinin *in vivo*. *Journal of Agricultural and Food Chemistry*, 47, 2797–2806.
- Mc Clain, S., Vieths, S., & Bannon, G. A. (2008). Assessment of food proteins for allergenic potential. In B. G. Hammond (Ed.), *Food safety of proteins in agricultural biotechnology* (pp. 209–222). Boca Raton: CRC Press Taylor & Francis Group.
- Moreno, F. J. (2007). Gastrointestinal digestion of food allergens: Effect on their allergenicity. *Biomedicine and Pharmacotherapy*, 61, 50–60.
- Moreno, F. J., & Clemente, A. (2008). 2S albumin storage proteins: What makes them food allergens? *The Open Biochemistry Journal*, 2, 16–28.
- Moreno, F. J., Rubio, L. A., Olano, A., & Clemente, A. (2006). Uptake of 2S albumin allergens, Ber e 1 and Ses i 1, across human intestinal epithelial Caco-2 cell monolayers. *Journal of Agricultural and Food Chemistry*, 54, 8631–8639.
- Nagano, T., Hirotsuka, M., Mori, H., Kohyama, K., & Nishinari, K. (1992). Dynamic viscoelastic study on the gelation of 7S globulin from soybeans. *Journal of Agricultural and Food Chemistry*, 40, 941–944.
- Ogawa, T., Bando, N., Tsuji, H., Nishikawa, K., & Kitamura, K. (1995). Alpha-subunit of β -Conglycinin, an allergenic protein recognized by IgE antibodies of soybean-sensitive patients with atopic-dermatitis. *Bioscience, Biotechnology and Biochemistry*, 59, 831–833.
- Ogawa, T., Samoto, M., & Takahashi, K. (2000). Soybean allergens and hypoallergenic soybean products. *Journal of Nutritional Science and Vitaminology*, 46, 271–279.
- Song, Y. S., Martinez-Villaluenga, C., & de Mejia, E. G. (2008). Quantification of human IgE immunoreactive soybean proteins in commercial soy ingredients and products. *Journal of Food Science*, 73, T90–T99.
- Thanh, V. H., & Shibasaki, K. (1977). β -Conglycinin from soybean proteins isolation and immunological and physicochemical properties of the monomeric forms. *Biochimica et Biophysica Acta*, 490, 370–384.
- Thomas, K., Herouet-Guicheney, C., Ladics, G., Bannon, G., Cockburn, A., Crevel, R., et al. (2007). Evaluating the effect of food processing on the potential human allergenicity of novel proteins: International workshop report. *Food and Chemical Toxicology*, 45, 1116–1122.
- Wang, T., Qin, G. X., Zhao, Y., & Sun, Z. W. (2009). Comparative study on the stability of soybean (*Glycine max*)-conglycinin *in vivo*. *Food and Agricultural Immunology*, 20, 295–304.
- Wilson, S., Martinez-Villaluenga, C., & de Mejia, E. G. (2008). Purification, thermal stability, and antigenicity of the immunodominant soybean allergen P34 in soy cultivars, ingredients, and products. *Journal of Food Science*, 73(6), T106–T114.
- Wilson, D. S., Wu, J., Peluso, P., & Nock, S. (2002). Improved method for pepsinolysis of mouse IgG₁ molecules to F(ab)₂ fragments. *Journal of Immunological Methods*, 260, 29–36.
- Zhao, Y., Qin, G. X., Sun, Z. W., Zhang, B., & Wang, T. (2010). Stability and immunoreactivity of glycinin and β -conglycinin to hydrolysis *in vitro*. *Food Agricultural Immunology*, 21, 253–263.