



Comprehensive analysis of the peanut allergome combining 2-DE gel-based and gel-free proteomics

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ABSTRACT

In this work, we explored the “deep” seed peanut proteome by using both two dimensional electrophoresis (2-DE)-based analysis run under reducing and non-reducing condition (protein-centric) and LC-MS/MS gel-free proteomic (peptide-centric). The former approach allowed to identify high molecular weight disulfide-linked Ara h 1 and Ara h 3 heteroligomers and Ara h 1 homoligomers linked through covalent bonds other than disulfides. The occurrence of these protein complexes revealed natural interactions between Ara(s) subunits with a possible involvement in the allergenic potential of peanut.

The second approach, also referred to as shot-gun proteomics, allowed the identification of 149 gene products, including low-abundance proteins escaped the 2-DE detection. Interestingly, we identified 60 proteins never catalogued previously. The complementary exploitation of two proteomic approaches enabled the access to new relevant information about the complexity of the peanut proteome, with special emphasis to the complement of allergens (allergome).

1. Introduction

Peanuts, the seeds of the legume plant *Arachis hypogaea*, are responsible of one among the most severe and life-long persistent food allergies, affecting nearly 1% of children and 0.6% of adults within the general population of Westernized countries (Sicherer & Wood, 2013). Overall peanut proteins account for approximately 24% in mature seed. World Health Organization and International Union of Immunologic Societies Subcommittee (WHO/IUIS) have catalogued seventeen protein allergens in peanut seeds (www.allergen.org), which have been classified into six different allergen superfamilies, including the cupin (Ara h 1 and Ara h 3/4), prolamin (Ara h 2, Ara h 6, Ara h 7, and Ara h 9), profilin (Ara h 5), Bet v 1 (Ara h 8), oleosin (Ara h 10, Ara h 11, Ara h 14, Ara h 15) and defensin (Ara h 12, Ara h 13, Ara h 16, Ara h 17) superfamilies (Bublin & Breiteneder, 2014). Each of these allergens might trigger immune response in susceptible subjects, with a variety of symptoms and prognosis depending on both individuals and offending agent(s). In general, Ara h 1, Ara h 3, Ara h 2 have been associated with primary sensitization, while Ara h 2 and Ara h 6 are commonly considered the most clinically relevant in terms of basophile activation, IgE binding properties and skin prick test (Chassaigne, Trégoat, Nørgaard,

Maleki, & van Hengel, 2009; Mueller, Maleki, & Pedersen, 2014).

Because peanut is largely used as a food ingredient and it is responsible of persistent food allergic reactions, the molecular structure of peanut proteins has been the subject of fervent investigation over the last decades. The knowledge about the composition of peanut allergens has been significantly advanced by the latest progresses of proteomic methods (Mamone, Picariello, Addeo, & Ferranti, 2011). So far, most of the reports have exploited the SDS-PAGE-based proteomic, providing deep insights in the peanut proteome and contributing to determine the serum IgE-binding properties as well as the stability of allergens to proteolytic digestion (Chruszcz et al., 2011; Di Stasio et al., 2017; Koppelman, Hefle, Taylor, & de Jong, 2010). Recently, a more detailed characterization about composition of peanut proteins and their isoforms has been achieved through two dimensional electrophoresis (2-DE) (Grishina, Bardina, & Grishin, 2017). The “classical” 2-DE/mass spectrometry (MS) proteomic approach has been used to determine the protein profile of mature seeds (Schmidt et al., 2009), to monitor protein accumulation and spatial-temporal expression during seed development (Kang, Srivastava, Ozias-Akins, & Gallo, 2007), to associate cultivar to allergenic potential (Chassaigne et al., 2009; Kottapalli et al., 2008; Schmidt et al., 2009) and to evaluate IgE-binding affinity of

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specific allergen types (Guo, Liang, Chung, Holbrook, & Maleki, 2008; Porterfield et al., 2009). Similar to other *Leguminosae* seeds, major storage proteins of peanut tend to form large molecular weight (MW) multimeric complexes, including both homo- and hetero-oligomeric aggregates. For instance, vicilin-like Ara h 1 is reported to form non-covalent protein aggregates up to 600–700 kDa (Shin et al., 1998). Likewise, the two sub-units (acid and basic) of Ara h 3, covalently linked by an intermolecular disulfide bridge, are further associated into non-covalent hexameric aggregates (Jung, Scott, Nam, & Beaman, 1998). To improve the quality of protein separation, 2-DE analysis is commonly performed under denaturing and reducing conditions (Chevalier, Hirtz, Sommerer, & Kelly, 2009). In these conditions the information about the quaternary organization of proteins goes lost, hampering the structural elucidation of protein aggregates via disulfides and the composition of interacting polypeptides. To the best of our knowledge, there is no comprehensive 2-DE investigation on peanut proteins considering the formation of complexes and aggregates, which may provide significant additional insights if compared to the canonical 2-DE-based proteomic exploration.

In spite of the ability of 2-DE to preserve the information about the intact proteins and occurrence of possible isoforms, shotgun proteomics has emerged as a powerful technique overcoming many of the 2-DE pitfalls (Rogowska-Wrzęsinska, Le Bihan, Thaysen-Andersen, & Roepstorff, 2013). According to the shotgun proteomic workflow, the tryptic digests of an unfractionated protein extract is resolved by liquid chromatography-high resolution tandem mass spectrometry (LC-MS/MS). Proteolysis dramatically increases the complexity of the analytical system. However, the method takes advantage from the much higher sensitivity in the analysis of peptides compared to proteins and from the possibility of sequencing at least one peptide arising from each parent protein. A software-assisted inference process allows recovering back the identity of the proteome components, but the information about the original intact polypeptides and possible isoforms is lost at the digestion stage (Nesvizhskii & Aebersold, 2005).

Although preliminary attempts to characterize the peanut proteome using the shotgun “peptide-centric” workflow have been reported (Kottapalli et al., 2013; White et al., 2013), they lack a direct correlation with the 2-DE-based analysis.

Herein, the peanut proteome was investigated by using complementary 2DE-based and shotgun proteomic approaches. In particular, the former was applied under both reducing and nonreducing conditions, in order to investigate the covalent protein complexes of peanut, while the latter had the specific purpose to enhance the proteome coverage, expanding the dynamic range of the electrophoresis-based methods.

2. Material and methods

2.1. Chemicals

Raw peanuts (Virginia, Zambia and China variety) were provided by Besana (Milano, Italy). Dithiothreitol (DTT), iodoacetamide (IAA), Tris-HCl, urea, ammonium bicarbonate (AMBIC), acetonitrile (ACN) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Calcium chloride (CaCl_2) was provided from J.T. Baker (Rutland, Austin, USA). Trifluoroacetic acid (TFA) was from Fluka. Proteomic grade modified trypsin Gold® was provided by Promega (Madison, WI, USA). Reagents for electrophoresis analysis were from Bio-Rad (Milan, Italy).

2.2. Preparation of peanut protein for electrophoresis analysis

Raw peanut flour from individual varieties (100 mg) was defatted by stirring thrice for 10 min in five volumes (w/v) of diethyl ether. Proteins were extracted from the flour pellet in 1 mL of urea 7 M, TBS buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl) with 20 mM DTT (reduced sample) or without DTT (unreduced sample). After 1 h stirring at

room temperature, the suspension was centrifuged at 10,000g for 30 min and supernatant was collected. Proteins were alkylated by incubation with IAA (55 mM final concentration) at room temperature for 30 min. Protein concentration was determined with the Bradford assay (Kit from Bio-Rad). Finally, samples were desalted against 25 mM AMBIC using Econo-Pac 10 DG columns (Bio-Rad), lyophilized and stored at -20°C .

2.3. Sample preparation for shotgun proteomic analysis

Peanut flour (100 mg) was extracted with 1 mL of urea 7 M, TBS containing 20 mM DTT for 1 h at 50°C . In this case, the defatting step was omitted in order to minimize the possible loss of hydrophobic proteins. Sample was centrifuged at 5000g for 30 min and 55 mM IAA was incorporated into the supernatant, incubating for 30 min in the dark. To quench the alkylation reaction, DTT was added up 30 mM final concentration and incubated for additional 15 min at room temperature. Protein concentration was determined with the modified Lowry assay (kit from Sigma-Aldrich). The peanut protein extract (100 μg) was diluted with ten volumes of 50 mM AMBIC, pH 8.0, containing 1 mM CaCl_2 and incubated overnight at 37°C with modified proteomic grade trypsin (Promega, Madison, WI, USA) using a 1:20 (w/w) trypsin-to-protein ratio. Resulting peptides were desalted using Sep-Pak® C18 cartridges (Waters, Milford, MA, USA) and finally dried in a “speed-vac” centrifuge, prior to LC-MS/MS analysis.

2.4. SDS – PAGE analysis

Peanut proteins were analysed by monodimensional (1-DE) SDS – PAGE (Bio-Rad, Mini-Protean) on precast 12% acrylamide gel. Protein aliquots were dissolved in Laemli buffer (0.125 M Tris-HCl pH 6.8, 5% SDS, 20% glycerol, 5% (w/v) β -mercaptoethanol (βMe), 0.02% bromophenol blue) at a $2.0\text{ }\mu\text{g}/\mu\text{L}$ concentration and denatured in a boiling water bath for 5 min. A final volume of $10\text{ }\mu\text{L}$ was loaded into the SDS-PAGE wells. Analysis under nonreducing condition was carried out in the same way of reducing SDS-PAGE, but omitting βMe . Electrophoresis was performed at constant voltage (100 V) at room temperature. After migration, the gels were stained with Coomassie Brilliant Blue G-250 and finally imaged with a LABScan scanner (Amersham Bioscience/GE Healthcare, Uppsala, Sweden).

2.5. 2-DE analysis

Peanut proteins were dissolved in immobilized pH gradient (IPG) strip rehydration buffer containing 8 M urea, 2% (w/v) CHAPS, 2% (v/v) Pharmalytes pH 4.0–7.0 (namely, Pharmalytes 4–6.5 and 5–7 in the 1/1 ratio), traces of bromophenol blue, with 20 mM DTT (reduced sample) or without DTT (unreduced sample). Notably, the 2DE gels were slightly overloaded ($1.0\text{ mg}/0.500\text{ mL}$), aiming to improve the visualization of the low-abundance proteins, especially the high molecular weight aggregates. Immobiline Dry Strips (pH 4–7 linear gradient, 11 cm, Bio-Rad Laboratories, Inc., Hercules, CA) were rehydrated overnight in the Immobiline Dry-Strip Reswelling Tray (Amersham Pharmacia). Isoelectrofocusing (IEF) was carried out using the Multiphor II system (Pharmacia Biotech, Uppsala, Sweden). The proteins were focused up to 15,000 Vh at a maximum voltage of 6000 V at 20°C . After focusing, proteins were reduced for 15 min in equilibration buffer (6 M urea, 30% glycerol, 2% SDS, 2% DTT), and alkylated for 15 min with 2.5% IAA. In the case of the nonreducing conditions, the equilibration buffer was deprived of DTT or IAA. SDS-PAGE in the second dimension was performed on a 12% polyacrylamide gel using a Protean II system (BioRad Laboratories, California). The run was performed at 220 V constant voltage, and each gel was stained in a water solution containing 0.05% (w/v) Coomassie Brilliant Blue R-250, 50% (v/v) methanol, 0.7% (v/v) acetic acid, and destained in water solution containing 10% (v/v) acetic acid and 40% (v/v) methanol.

2.6. Protein in-gel digestion

Protein bands/spots (1-DE/2-DE, respectively) were repeatedly washed with 25 mM AMBIC/acetonitrile (1/1, v/v) up to complete destaining. The proteins were reduced for 1 h at 56 °C with 10 mM DTT in 25 mM AMBIC and alkylated for 1 h at room temperature with 55 mM IAA in 25 mM AMBIC. Proteins were in-gel digested overnight at 37 °C using proteomic grade trypsin (12.5 ng/μL) in 25 mM AMBIC. Peptides were extracted twice in 5% formic acid/acetonitrile (1/1, v/v), dried in a “speed-vac” centrifuge and finally reconstituted in 50 μL of 0.1% TFA for MS analysis.

2.7. LC-high resolution (HR)-MS/MS analysis

Tryptic digests were separated using an Ultimate 3000 ultra-high performance liquid chromatography instrument (Dionex/Thermo Scientific, San Jose, CA, USA), coupled to a Q-Exactive Orbitrap mass spectrometer (Thermo Scientific). Peptide mixtures, re-suspended in 0.1% (v/v) TFA solution, were loaded through a 5 mm long, 300 μm i.d. pre-column (LC Packings, USA) using a Famos autosampler (Thermo) and separated with an EASY-Spray™ PepMap C18 column (2 μm, 15 cm × 75 μm) 3 μm particles, 100 Å pore size (Thermo Scientific). Eluent A was 0.1% formic acid (v/v) in Milli-Q water, eluent B was 0.1% formic acid (v/v) in acetonitrile. The separation was carried out at a flow rate of 300 nL/min with a linear gradient from 4% to 40% of solution B over 60 min for 1-DE/2-DE in-gel tryptic digests or over 240 min for the gel-free shotgun analysis. Mass spectra, generated by Xcalibur Software 3.1 version (Thermo Scientific), were elaborated using the Proteome Discoverer 2.1 software (Thermo Scientific). Matching entries from both 2-DE protein spots and shotgun analysis were filtered according to the following parameters: two tryptic missed cleavage, 5 ppm tolerance for precursor ions and 0.01 Da for MS/MS fragments, carbamidomethylation of cysteines as a fixed modification, oxidized Met and N-terminus Gln as pyroglutamic acid as variable peptide modifications. The identification of protein entries was validated using the Target Decoy PSM Validator (Proteome Discoverer software), restricting the search at 0.01 False Discovery Rate (FDR).

3. Results and discussion

3.1. Electrophoresis of peanut extract of varieties

Proteins from three commercial peanut cultivars (Virginia, China and Zambia), extracted by an optimized urea-based protein recovery method, were analysed by SDS-PAGE (12% gradient) under both reduction and nonreducing conditions (Fig. 1). Preliminarily, the major allergens Ara h 1, Ara h 3, Ara h 2 and Ara h 6 h were assigned according to the electrophoretic mobility as suggested by previous investigations (Apostolovic et al., 2013; Di Stasio et al., 2017; Koppelman et al., 2010; Koppelman et al., 2016). Among the cultivars, the electrophoretic profiles were similar except for Ara h 3 isoforms which appeared as a triplet band in Virginia and China samples and doublet band in Zambia (Koppelman et al., 2016). Interestingly, SDS-PAGE revealed that under nonreducing conditions all the cultivars contained significant amounts of high MW (97 up to > 250 kDa) aggregates, which for the most disappeared after reduction of disulfide bonds (dashed boxes in Fig. 1). When analysed by LC-MS/MS, these protein bands contained signals of tryptic peptides from both Ara h 1 and Ara h 3. At the high MW protein range a single band at ca. 110 kDa was not affected by disulfide reduction. By LC-MS/MS analysis of in-gel tryptic digests, this protein band was identified as Ara h 1 (not shown), in line with Maleki et al., (Maleki et al., 2000), suggesting that intermolecular cross-links can be established through covalent bonds other than disulfide linkages.

3.2. 2-DE analysis

Proteins from peanut varieties were separated by 2-DE under both reducing and nonreducing conditions (Fig. 2). In all cases, the 2-DE maps evidenced a significant heterogeneity within the protein families due to the presence of multiple proteoforms, also including components differing by post-translational modifications. In agreement with the SDS-PAGE analysis, three main spot groups at high MW (estimated 240, 210, 180 and kDa) occurred in the 2-DE map of nonreduced protein extracts, which for the most disappeared upon disulfide reduction, confirming their nature of disulfide-linked protein aggregates (Fig. 2).

A detailed 2-DE proteomic investigation was carried out on Virginia peanut, chosen as a model cultivar (Koppelman et al., 2010; Kottapalli et al., 2008; Schmidt et al., 2009) (Fig. 3). The 2-DE isolated spots were characterized by LC-MS/MS analysis of tryptic peptides. Proteins are inventoried in Table S1 and Table S2, relevant to the 2-DE maps generated in the absence (Fig. 3A) or in the presence (Fig. 3B) of DTT, respectively, as the reducing agent. The main allergen families are individually examined in the following sections.

3.2.1. Ara h 1

The protein spots of Ara h 1 (7S vicilin-like) belonged to two co-migrating isoforms, namely P14B and clone-P17, which differ each other by a series of punctiform substitutions and share 94.6% homology. Under nonreducing conditions (Fig. 3A; Table S1) the expected full-length Ara h 1 occurred in the poorly resolved spots 21–25 (ca. 68 kDa, pI range 6–6.5). Because of N-glycosylation micro-heterogeneity, the Ara h 1 spots partially overlapped those of Ara h 3 isoforms, so that the two protein families co-migrated in spots 24 and 25. In contrast, after reduction (Fig. 3B; Table S2), as a consequence of a splitting of the Ara h 3 subunits, Ara h 1 was clearly resolved into multiple spots, namely spots no. 12–19 and 37, at estimated MW 68 kDa, covering a relatively large pI range (pI 6.0–6.5), due to N-glycosylations (Chassaigne et al., 2009).

Additional shorter Ara h 1 forms, with experimental MW lower than theoretical one, were detected in the 2-DE maps of both nonreduced (Spot 48, 47; Fig. 3A) (Table S1) and reduced (spots 21–24, 34–36, 40, 47, 48, 66, 67; Fig. 3B; Table S2) protein extracts. Low MW isoforms of Ara h 1 probably were proteolytic fragments produced by endogenous proteases (Chassaigne et al., 2009).

Covalent polymeric aggregates of Ara h 1 were identified in the high MW region of nonreduced 2-DE gel (Fig. 3A; Table S1). Almost all the high MW spots disappeared under reducing conditions (Fig. 3B; Table S2) due to the disulfide nature of the inter-chain linkages. The LC-MS/MS analysis of high MW aggregates at 240 kDa (spot 1), 210 kDa (spots 2–3), 180 kDa (spots 4–7) and 90–110 kDa (spots 8–11 and 14–20) contained tryptic peptides matching both Ara h 1 and Ara h 3 (Fig. 3A). This finding suggested that Ara h 1 and Ara h 3 might co-migrate even as aggregates due to the poor resolution of nonreducing 2-DE or, most likely, it is possible that additional disulfide bonds are established between Ara h 1 and Ara h 3 chains, producing heteropolymeric aggregates, which naturally occur into mature peanut seeds. The capability of Ara h 1 to establish disulfide bonds, generating both hetero- and homo-polymeric aggregates, contrasts with previous observations which excluded the involvement of Ara h 1 in disulfide cross-links (Shewry, Napier, & Tatham, 1995).

Actually, the mature protein chains of both the Ara h 1 isoforms contain seven Cys residues, at least one of which is expected to be susceptible of inter-molecular pairing, supporting on a structural basis the possibility it plays a role in the formation of disulfide-mediated aggregates (Khan, Di, Patel, & Nanda, 2013). Interestingly, the Ara h 1 tryptic peptide containing the consensus triplet for the N-glycosylation escaped the MS detection in all the Ara h 1-containing spots, confirming its effective glycosylation. The specific monitoring of the glycopeptide (s) and the characterization of the glycoforms would require a dedicated analysis.

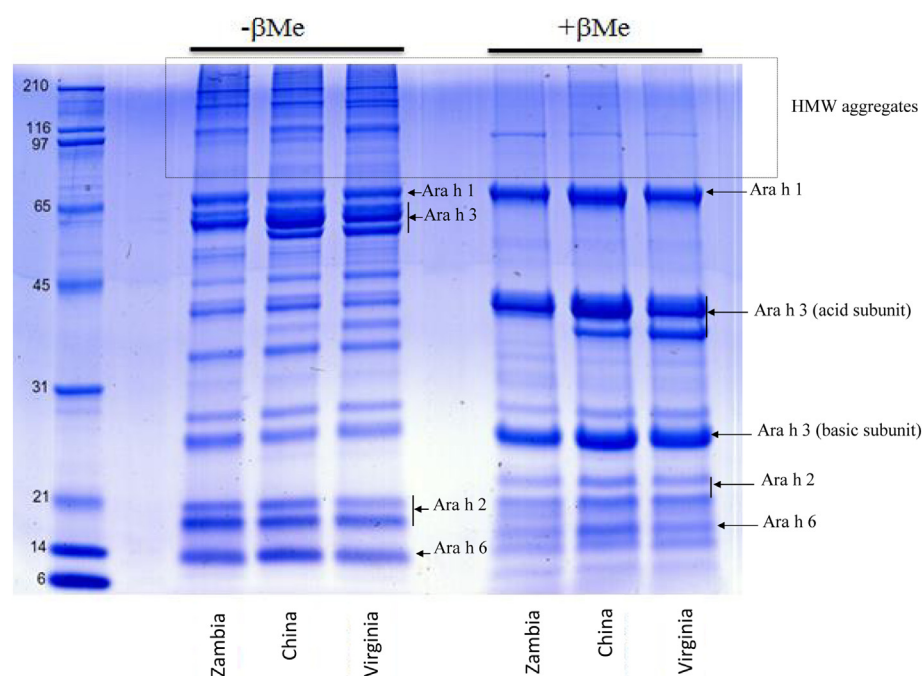


Fig. 1. SDS-PAGE comparison of Virginia, Zambia and China peanut proteins under nonreducing ($-\beta\text{Me}$) and reducing ($+\beta\text{Me}$) conditions. The major allergens were assigned in line with previous investigations (Di Stasio et al., 2017; Koppelman et al., 2010; Koppelman et al., 2016). Inserts delimited by dashed lines indicate the HMW aggregate.

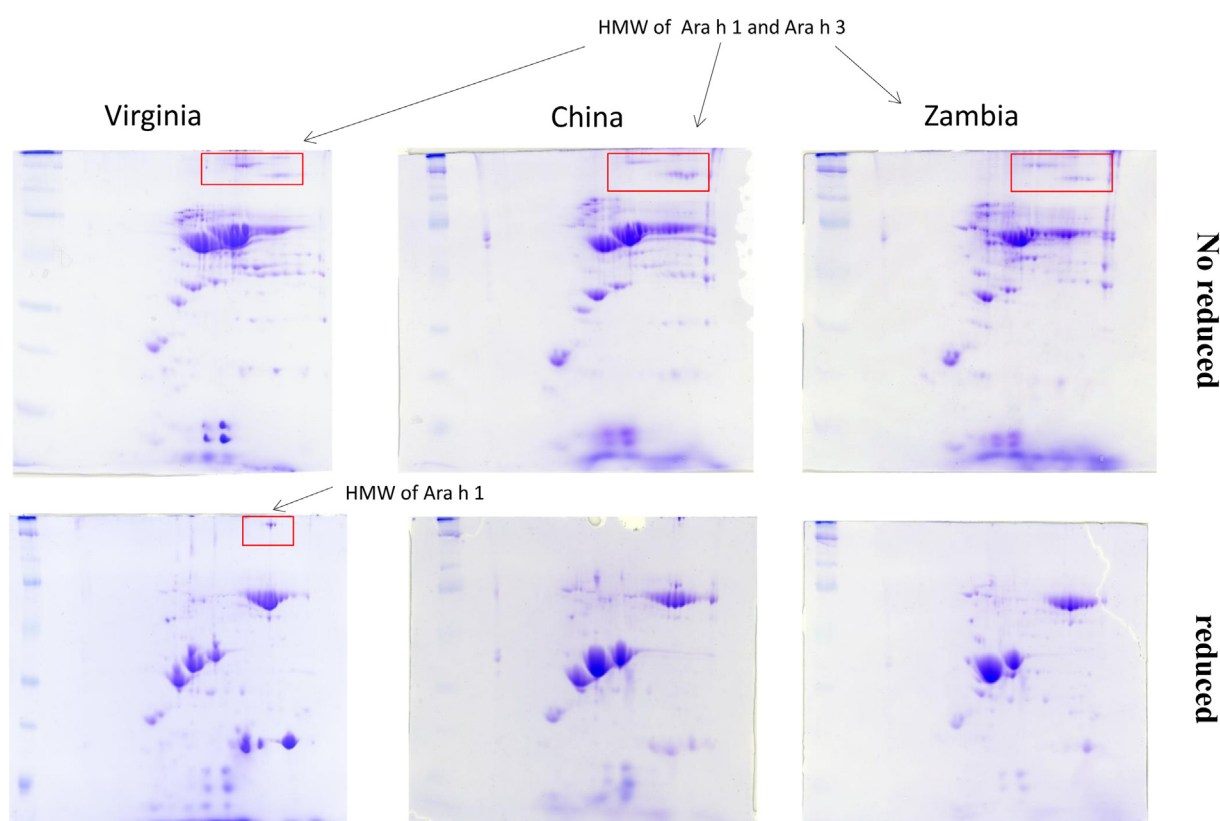


Fig. 2. 2-DE comparison of peanut proteins of Zambia, China and Virginia cultivars, under reducing and nonreducing conditions. HMW regions are delimited by lines.

In spite of the DTT-induced disappearance of aggregates with estimated MW 210 and 180 kDa, the reduction did not affected the spot train at mass line 240 kDa (spots no. 1–3)- detected only as a faint band in the 1D SDS-PAGE (Fig. 1) - and the isolated spots at ~ 110 kDa (spots no. 4 and 5) (Fig. 3B), which were univocally identified by LC-MS/MS as homopolymers of Ara h 1 cross-linked via covalent bonds other than disulfides (Table S2). This finding confirmed those obtained by 1-D

SDS-PAGE-MS analysis by other authors (Koppelman et al., 2016; Maleki et al., 2000) and by us in this work. However, the additional aggregates at 240 kDa are compatible with the occurrence of stable tetrameric Ara h 1 homopolymers with non-disulfide covalent cross-linkages, which have been never reported previously. Several kinds of covalent bonds could be involved in the spontaneous formation of protein aggregates (Gerrard, 2002). In the case of Ara h 1, glycans might

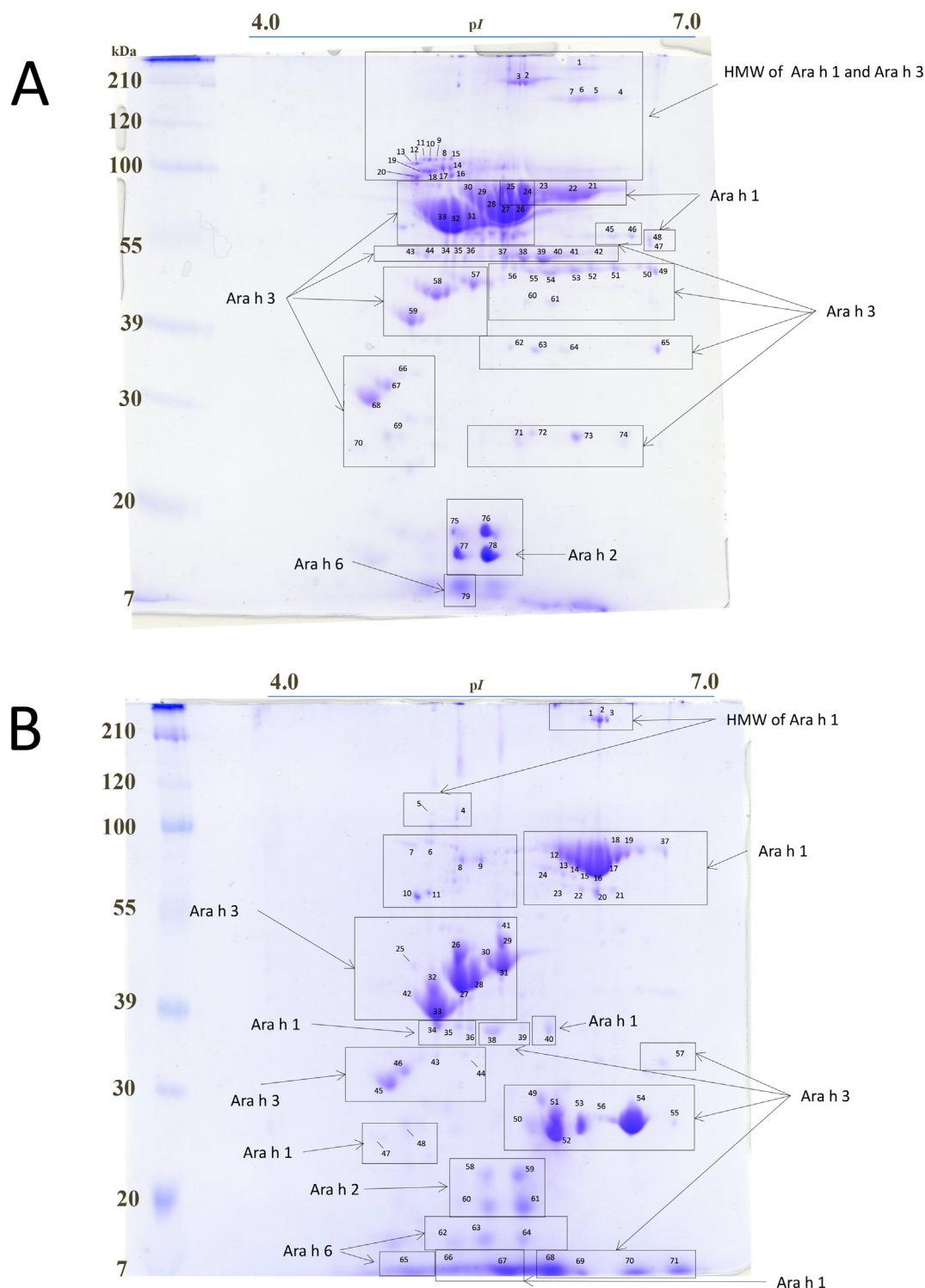


Fig. 3. 2-DE of peanut proteins (Virginia cultivar) separated under unreducing (panel A) and reducing (panel B) conditions. Spots as indicated by numbers were submitted to LC-MS/MS base analysis (Table S1 and Table SII). HMW regions and individual protein isoforms are annotated and delimited by dashed lines.

be responsible of the polypeptide inter-linkage as well. However, the specific nature of the covalent bonds and their potential involvement in the intrinsic allergenicity of peanut proteins should be elucidated with dedicate investigations.

3.2.2. Ara h 3

Ara h 3 dominated the 2-DE maps of peanut proteins and demonstrated a significant heterogeneity due to several simultaneous factors, including: i) occurrence of multiple isoforms, ii) post-synthesis

processing, which involves the splitting in an acidic and a basic subunits held together by a disulfide, analogously to the 11S globulins from other *Leguminosae* (e.g. soybean) or non-*Leguminosae* tree nuts (e.g. walnut and hazelnut), iii) progressive N-terminal trimming of the basic subunit, iv) possible glycosylation (Piersma, Gaspari, Hefle, & Koppelman, 2005). Prior to reduction, Ara h 3 migrated as three full-length polypeptides at estimated mass lines 60 (spot 24, 25, 29–30), 58 (spot 26–28) and 56 kDa (spot 31–33), covering the 5.5–6 pI range. Peptides arising from Ara h 3 were also found in a series of minor spots

spread out at ca. 52–56 kDa (spot 42–46) at the acidic and basic side, as well as in low abundance spots at ca. 34–43 kDa (spot 49–65) and 24–25 kDa (spot 69–74), at the acidic and basic side of the gel, respectively (Fig. 2, Table S1). These latter spots of Ara h 3 are probably due to a partial proteolysis affecting the full length polypeptides. Additional Ara h 3 forms, with experimental MW higher than theoretical one, were detected in spot 12 and 13 in the 2-DE maps of nonreduced gel (Fig. 3A, Table S1). Following reduction, the expected acidic (pI 5–6) and basic (pI 6–8) Ara h 3 subunits were detected in spots 25–33, 41–42 and 49–56, respectively (Fig. 3B; Table S2) (Piersma et al., 2005). Ara h 3 fragments were also found in spot 38–39, 43–46, and 57. Several additional low MW Ara h 3 fragments (ca. 7 kDa, spot no. 68–71) were generated by the DTT-mediated release of proteolyzed subunits.

Interestingly, low-abundance isoforms of Ara h 3 did not completely disappear under reducing conditions and were still detected in the 56–60 kDa mass range (spots no. 6–11; Fig. 3B). Since LC-MS/MS analysis matched the sequences in both acidic and basic subunits, we deduce that these spots corresponded to proteolytic isoforms of Ara h 3 of different origin than post-translational processing (Dam et al., 2009).

Notably, whereas Ara h 3 was engaged with Ara h 1 in the formation of disulfide-linked heteropolymeric aggregates, no evidence of Ara h 3-containing aggregates persisted under reducing conditions.

3.2.3. Ara h 2 and Ara h 6

Ara h 2 and Ara h 6, both members of the 2S albumin family, were the only proteins detected in the 2-DE maps in addition to Ara h 1 and Ara h 3. In line with previous characterizations (Chatel, Bernard, & Orson, 2003), Ara h 2 occurred as two isoforms (2.01 and 2.02), which included multiple spots each, differing by pI. They were spots no. 75–78 in nonreduced (Fig. 3A; Table S1) and spots no. 58–61 in reduced 2-DE gels (Fig. 3B; Table S2).

Under nonreducing conditions, Ara h 6 (expected 14.6 kDa) was detected as a single polypeptide chain (spot no. 79; Fig. 3A; Table S1), which did not evidence the presence of multiple proteoforms (Bernard et al., 2007). Analogously to Ara h 3, Ara h 6 occurs as a heterodimer produced by *endo*-proteolytic cleavage of a single polypeptide precursor, whose subunits remain linked via a disulfide bond. The disulfide reduction split Ara h 6 into multiple spots, namely 62–64 at ~9 kDa and 65 at ~7 kDa (Fig. 3B; Table S2), probably arising from different post-translational proteolytic processing of the 9 kDa subunit, in agreement with the typical electrophoretic pattern of Ara h 6 previously observed (Suhr, Wicklein, Lepp, & Becker, 2004).

3.3. Shotgun proteomic analysis

> 100 protein spots were resolved by 2-DE gels, providing a clear picture of the major storage protein families and possible isoallergens, with an estimation of their pI/MW and information about the effects of proteolytic events. On the other hand, Ara h 2 and Ara h 6 allergens along Ara h 1 and Ara h 3, which together account for about 87% of storage peanut protein, were clearly individuated in 2-DE maps.

The pitfalls of 2-DE to detect low-abundance proteins as well as to separate hydrophobic and extremely acidic or basic proteins have been largely reported (Rogowska-Wrzesinska et al., 2013). Overall, these limitations hampered the detection of many among the expected gene products occurring in peanut seeds and already catalogued among the peanut allergens, such as oleosins (Ara h 10/11), defensins (Ara h 12/13), lipid transfer protein (LTP, Ara h 9), besides enzymes involved in metabolic processes, not described as allergens yet. The shotgun proteomics enabled accessing to groups of low-abundance proteins (Zhang, Fonslow, Shan, Baek, & Yates, 2013). Using the shotgun proteomic approach, 149 gene product entries were identified with high confidence (Table S3), including the main storage proteins Ara h 3 (ten identified isoforms), Ara h 1 (isoform P17 and P418), as well as Ara h 6 and two isoforms of Ara h 2 (Ara h 2.0.1 and 2.0.2), in line with the 2-

DE based proteomic analysis.

Differently from the 2-DE, shotgun proteomics allowed the identification of minor allergens and metabolic enzymes in addition to the dominant storage protein components. Two isoallergens of the Ara h 9 protein, namely LTP isoallergen 1 (ABX56711) and LTP isoallergen 2 (ABX75045) were detected and identified. In analogy to the homologous protein from other plant sources, LTP is considered a clinically relevant peanut food allergen, especially in the Mediterranean area (Krause et al., 2009). Ara h 7, belonging to the conglutinin 2S albumin protein family occurred as two isoforms. This finding was in agreement with previous ones, reporting the missed detection of Ara h 7 by 2-DE and the identification of two isoallergens sharing high sequence homology (Schmidt et al., 2009). Ara h 8, homologous to Bet v 1 allergen, which may justify cross-sensitization of some peanut allergic subjects to birch pollen (Mittag et al., 2004), was identified as well with 64% of the protein sequence coverage.

Among the identified oleosins, which consist of several gene products tightly associated to the seed oil bodies, oleosin Ara h 10 (oleosin 17.8), Ara h 11.0101 (oleosin 1), Ara h 11.0102 (oleosin 2), Ara h 14 (oleosin variant A) and Ara h 15.0101 (oleosin 3) are clinically relevant peanut allergens and most likely associated with severe allergic symptoms (Schwager et al., 2017). Minor oil body proteins such as steroleosin B-type and A-type, whose allergenic properties have not been proven so far, were also identified. In the current investigation, the identification of oleosins was successful because the crude protein extracts were trypsinized before defatting the peanut seeds, according to the alternative protocol of sample preparation described above. Actually, due to their peculiar properties, the purification of peanut oleosins requires specific protocols of extraction making use of detergents or denaturing and delipidating agents (Schwager et al., 2015).

Low-abundance Ara h 12 (defensin 1) and Ara h 13 (defensin 3), with still uncertain allergenic properties (Petersen et al., 2015), were confidently identified. Many additional minor protein entries identified by shotgun proteomics were enzymes involved in metabolism of starch, proteins expressed in response to stress and involved in defence mechanisms. The allergenic potential of these latter proteins still awaits to be investigated. Overall, our analysis allowed to catalogue nearly 60 low abundant peanut seed gene products more than the unique previous attempt carried out with shotgun proteomics, probably because of the better performances of the Q-Exactive Orbitrap instrument (White et al., 2013).

4. Conclusions

The results of this investigation pointed out the complexity of peanuts allergome. 2-DE analysis run under reducing and nonreducing conditions highlighted the natural occurrence of HMW disulfide-linked multimers of Ara h 1 and Ara h 3. Due to poor resolution of 2-DE deprived of DTT, further dedicated studies would be necessary to definitely assess if Ara h 1 and Ara h 3 form exclusively homopolymers or also heteropolymers. Another relevant insight achieved was the identification of HMW aggregates of Ara h 1 stable under reducing condition. The observation of all these complexes may reveal natural interactions between Ara(s) subunits with a potential involvement in their allergenic potential, still unknown. Herein results also confirmed the complementarity of the “protein-centric” and “peptide-centric” proteomic methodologies, when the purpose of the study is a “discovery-driven” characterization of the proteome. In fact, 2-DE analysis practically allowed only the detection of the major storage proteins, including the main four allergen families (Ara h 1, Ara h 3, Ara h 2 and Ara h 6), while it failed to disclose the “deep” peanut proteome. In contrast, the shotgun analysis enabled accessing to a more comprehensive coverage of the peanut allergome, but losing information about the quaternary structure of the proteins and the possible occurrence of isoallergens (Rogowska-Wrzesinska et al., 2013).

Conflict of interest

The authors declare no competing financial interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodres.2018.09.045>.

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