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Combining 2-DE immunoblots and mass spectrometry to identify putative soybean (*Glycine max*) allergens

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ABSTRACT

Soybean is recognized as a commonly allergenic food, but the identity of important allergens is not well studied. Recently, some global regulatory agencies started requiring quantitative analysis of individual allergens, including unproven allergens, as part of the risk assessment for genetically engineered (GE) soybeans. We sought to identify soybean proteins that bind IgE from any of 10 individual soybean-sensitized subjects. Soybean IgE binding proteins were identified by 2-DE immunoblots using sera from four soy-allergic and plasma from six soy-sensitized human subjects. Corresponding spots were excised from stained gels, digested, and analyzed using a quadrupole TOF Synapt G2-S tandem mass spectrometer. Results showed the major IgE binding proteins were subunits of either β-conglycinin (Gly m 5) or glycinin (Gly m 6). Soybean Kunitz trypsin inhibitor (SKTI) was a significant IgE binding protein for four subjects. Soybean agglutinin, seed biotinylated protein (SBP) of 65 kDa, late embryogenesis protein (LEP), and sucrose-binding protein were identified as IgE binding only for soy-sensitized subjects. We conclude that the major soybean allergens are isoforms of Gly m 5, Gly m 6, and possibly SKTI and that requirements for quantitative measurement of proteins that are not clear allergens is not relevant to safety.

1. Introduction

Soybean (*Glycine max* [L.] Merr.) is a nutritionally important source of edible oil and protein for human food and animal feed. However, soybean is recognized as one of the common allergenic food sources requiring food allergen labeling ("Big Eight" in the U.S. and among the top 14 in the European Union). Eight foods and food groups are thought to account for up to 90% of IgE mediated food allergic reactions in the U.S. (Taylor and Hefle, 2001). Soybean food allergy is relatively common in infants and young children in the U.S. due to the exposure to soy-based formula for those with cow's milk allergy (Zeiger et al., 1999). However, most children outgrow their soybean food allergy by the age of 10 years (Savage et al., 2010), although a few individuals become sensitized and/or allergic as adults.

The precise prevalence of soybean food allergy in the general population is unknown. An accurate estimate of soybean allergy would require studies of general populations in different countries and evaluation of various age groups using clinical demonstration of adverse reactions to soybean products. Preferably the studies would be double-blind, placebo-controlled food challenges (DBPCFC) to rule out false-positive reactions. Other clinical tests that could be useful to demonstrate an IgE-mediated mechanism for the adverse reactions can be performed either by skin prick tests (SPT) or by determination of soybean protein specific IgE levels in serum (Taylor et al., 2015). A number of publications appear to over-estimate the prevalence of soybean allergy. Most were conducted on referred allergic populations or atopic populations without confirmation of clinical reactions. A study in Italy estimated that 6.1% (8/131) of atopic children have soybean allergy (Magnolfi et al., 1996). A second study in Italy evaluated 505 allergic children with symptoms of atopic dermatitis, cow's milk allergy, urticaria, asthma, rhinitis following reported consumption of soybean and found that 6% (31/505) had positive by SPT to soybean, but only 6 of those 31 children were positive when tested by DBPCFC. Thus, a more accurate estimate of allergic-children in Italy having true soybean food

Abbreviations: CHAPS, 3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate; DBPCFC, double-blind placebo-control food challenge; GE, genetically engineered; LEP, late embryogenesis protein; MOWSE, molecular weight search; MW, molecular weight; NCBI, National Center for Biotechnology; OECD, Organization of Economic Co-operation and Development; SBP, seed biotinylated protein; SKTI, soybean Kunitz trypsin inhibitor; SPT, skin prick test; vdc, volts of direct current; WHO/IUIS, World Health Organization and International Union of Immunological Societies

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allergy is 1.2% (6/505) (Bruno et al., 1997). Additional studies have been conducted by questionnaire, relying on subjects' self-reported allergy-like symptoms related to soybean consumption, which likely leads to an overestimation of prevalence (Gupta et al., 2011; McGowan and Keet, 2013). A recent systematic review summarizing 40 studies on the worldwide prevalence of soybean allergy and concluded that 0.27% of the general population likely have soybean allergy (Katz et al., 2014).

Over 90% of soybeans grown in the U.S. are genetically engineered (GE) varieties that are bred into many genetic lines (Fernandez-Cornejo et al., 2014). A number of independent scientific studies have reviewed the evidence regarding the risks of GE crops. These studies conclude that based on scientific evidence, commercialized GE crops are safe for human consumption and the environment (Delaney et al., 2018; Domingo, 2007; Goodman et al., 2013; Hoff et al., 2007; Kuiper et al., 2001; Sten et al., 2004). Despite extensive research, some scientists and a few non-governmental organizations (NGOs) debate the safety of GE crops, particularly in countries of the European Union. The majority of GE crops are grown in the U.S., Brazil, Argentina and Canada (http://www.isaaa.org/gmapprovaldatabase/default.asp). Some governments that collectively represent over 50% of the global population (e.g., India, China, Bangladesh, Indonesia, Pakistan, the Philippines, Ethiopia and Egypt) have approved only a few GE crops for cultivation such as cotton, even though many studies have demonstrated the GE crops are not substantially different from commonly cultivated non-GE varieties (Delaney et al., 2018). Most countries allow importation of commodities from specific GE varieties for use in food, feed or industrial products. Use and cultivation in almost all countries require safety evaluations with most countries following guidelines of the CODEX Alimentarius Commission (Codex, 2009).

The recently revised European Commission regulation 503/2013 requires mandatory quantitative measurement of endogenous allergens as part of the compositional analysis in the risk assessment for GE soybean, taking into account recommendations of OECD (EU, 2013). The OECD document on soybean allergens lists 15 proteins designated as “allergens” as shown in Table 20 of the OECD recommendations for soybean (OECD, 2012), which was derived primarily from one literature review by L'Hocine and Boye (L'Hocine and Boye, 2007). This requirement has been questioned and subjected to criticism because several of the listed soybean proteins are only putative allergens (e.g., Gly m Bd 30 K, Gly m Bd 28 K, lectin, lipoxygenase) or are poorly characterized proteins (e.g., no amino acid sequences for the 39 kDa and 50 kDa and P22-25 proteins) (Ladics et al., 2014). A review by Selb et al., in 2017 discusses the evidence and the lack of evidence for the relevance of some proteins in the OECD list (Selb et al., 2017). There are no studies demonstrating that individual proteins cause objective reactions based on blinded challenges in allergic consumers. In two studies, IgE reactivity of soybean allergic individual's sera with the single soybean allergens Gly m 5 and Gly m 6 was found to be associated with clinical reactions caused by soybean (Holzhauser et al., 2009; Ito et al., 2011). In addition, these are very abundant proteins. In populations of northern Europe, Gly m 4 is the most commonly recognized proteins using serum IgE from clinically allergic adult subjects, while more proteins are often recognized in extracts of soybean (Mittag et al., 2004). We recently showed that individual soybean allergic or soybean sensitized subjects show individual IgE binding patterns using reducing and non-reducing one-dimensional gel chromatography and immunoblotting and individual patterns in total IgE binding to proteins in non-denaturing extracts using extracts of multiple soybean lines grown in two or three locations (Lu et al., 2018). Those results led us to investigate the individual proteins bound by IgE using multiple subjects.

In recent years, the combination of 2-DE immunoblots coupled with mass spectrometry of isolated protein spots has become a powerful tool to identify potential allergens from various sources (Batista et al., 2007; Choopong et al., 2016; Chuang et al., 2010; Nakamura and Teshima, 2013). The aim of this study was to identify the IgE binding proteins from soybean using sera from soy-allergic subjects and plasma from soy-sensitized subjects. The identification of IgE binding proteins is important to achieve a better understanding of soybean allergens for soybean allergy diagnosis, identification of possible immunotherapy reagents and for risk assessment and risk management of foods. A second potential outcome of this study was to verify the relevance of proteins listed by the OECD as being soybean allergens and to question the relevance of quantitation for the specified 15 “allergens” to the risk assessment of GE soybean.

2. Material and methods

2.1. Soybean protein extraction

Raw, full-fat flour samples of a non-GE soybean line (*Glycine max*) were produced from soybeans grown in multiple locations by Pioneer Hi-Bred International, Inc. Protein extraction was performed by the trichloroacetic acid/acetone method described by Natarajan et al., in 2005 (Natarajan et al., 2005), and modified as described previously (Panda et al., 2013) using 8 M urea and 2% 3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate (CHAPS) to extract the protein. The protein content of clarified extracts was determined by Bradford assay (Bio-Rad) (Ramagli, 1999).

2.2. Soy-allergic human sera and soy-sensitized human plasma

Samples from ten adult human subjects were provided from two sources: the University Hospital Zürick, Switzerland provided sera from four soy-allergic patients and PlasmaLab International, Everett, WA, U.S. (www.plasmalab.com) provided plasma samples from six soy-sensitized subjects. The four soy-allergic patients had a history of allergic reactions to consuming soybean and their soybean allergies were confirmed by DBPCFC. The six soy-sensitized patients had only self-reported allergies to soybean and displayed detectable ImmunoCAP® scores to soybean. Clinical symptoms of allergy and sensitivity to soybean and ImmunoCAP® scores are listed in Table 1. Sera from five individuals without reported allergy to soybean who did not experience symptoms in an open food challenge with soy milk and with very low soybean-specific IgE (< 0.35 kU/L ImmunoCAP® value) were used as negative controls. It is important to note that soybean allergy occurs mostly in young children who become tolerant by 10 years of age and

Table 1
Soy-allergic human sera and soy-sensitized plasma. Human subject selection was based on clinical history or self-reported history related to consumption of soybean. ImmunoCAP® results are shown, and n/a means not available, OAS is oral allergy syndrome.

Subject ID	ImmunoCAP® (kU/l)			Symptoms as reported by history
	Soy	Gly m 4	Total IgE	
Soy-allergic human sera				
Neb-4	2.06	n/a	n/a	OAS, nausea, emesis
Neb-5	9.23	n/a	n/a	OAS, dyspnea, hypotension
Neb-7	< 0.35	26.2	n/a	OAS
Neb-8	6.42	1.58	n/a	OAS, nausea, emesis, diarrhea, gastrointestinal pain
Soy-sensitized human plasma				
9735-RE	5.00	n/a	> 5000	Anaphylaxis to peanut. Soybean causes sore throat, itchy mouth, and queasy stomach.
19392-CS	71.60	n/a	896	Angioedema, vomiting
20197-BH	3.00	n/a	n/a	Itchy throat with nuts and raw vegetables
23508-JK	6.7	n/a	n/a	Multiple allergies, not well-defined
24924-LH	13	n/a	n/a	Multiple allergies, not well-defined
26730-AB	5	n/a	n/a	Anaphylaxis to peanuts, dyspnea, angioedema, hives

only rarely in adults. Thus, it is difficult to obtain samples from a large number of soybean allergic subjects. Individual sera and plasma were collected previously from subjects who provided voluntary consent for studies with specific institutional review board approval (Zürich) or by a U.S. Food and Drug Administration licensed facility (PlasmaLab).

2.3. Two dimensional gel electrophoresis (2-DE) and immunoblotting

Soybean extracts representing 30 μg of protein were diluted to a final volume of 125 μl of rehydration buffer (8 M urea, 2% CHAPS, 50 mM dithiothreitol (DTT) and 0.5% ampholyte pH 3–10) for each 2-DE separation. The 125 μl sample was applied to a non-linear 7 cm immobilized pH gradient strip pH 3–10. Active rehydration was performed at 50 V of direct current (vdc), 20 °C for 12 h, followed by separation with rapid ramping up to 250 vdc for 15 min, gradual ramping up to 4000 vdc for 2 h, then a limit of 4000 vdc which was continued until 37,500 integrated volt x hours was obtained (Panda et al., 2013). The strips were reduced with DTT and alkylated with iodoacetamide equilibration buffer (Bio-Rad). The second dimension gel electrophoresis separation was completed using NuPAGE® Novex 4–12% Bis-Tris ZOOM® gels (Invitrogen). A representative gel was fixed and stained with EZBlue™ Gel stain (Sigma).

Proteins from identical gels were transferred to polyvinylidene difluoride membranes for immunoblots. The membranes were dried completely, fixed and stained with SYPRO Ruby protein blot stain (Bio-Rad) before blocking. The images of stained membranes were taken with a Gel Logic 440 image station (Kodak), then membranes were rewet with methanol and blocked with 5% non-fat dry milk. Individual soybean allergic or non-allergic sera were diluted based on prior knowledge of soybean-specific IgE (1: 5, 1:10 or 1:20, v:v) and incubated with the membranes overnight. Unbound IgE was removed by four washes with 0.5% Tween in PBS. Bound IgE was detected using monoclonal anti-human IgE (Southern Biotech) diluted 1:1,000, then washed to remove unbound detection antibody. Bound anti-IgE was detected with SuperSignal West Dura chemiluminescent substrate (Thermo Fisher Scientific). Immunoblot images were captured in the Kodak Gel Logic 440, without light and without a filter at the same condition focal length as the images of SYPRO stained membranes.

2.4. Protein spots sampling and trypsin digestion

To locate the position of IgE binding proteins, the SYPRO stained membranes and the immunoblot images were overlapped and compared. After the IgE binding spots were identified for all 10 human samples, corresponding to EZBlue stained protein spots, the spots were excised from the 2-DE gels based on apparent MW and pI, placed in individual microfuge tubes and then digested (Natarajan et al., 2006; Shevchenko et al., 1996). Briefly, the protein samples were washed with 100 mM ammonium bicarbonate, reduced with 10 mM DTT, alkylated with 55 mM iodoacetamide, washed twice with 100 mM ammonium bicarbonate, and digested *in situ* with 10 ng/μl trypsin (Promega). Peptides were extracted with two 60 μl aliquots of 1:1 acetonitrile:water containing 1% formic acid. These extracts were dried using a SpeedVac and then reconstituted in 12 μl of 0.1% formic acid for application in the liquid chromatography-tandem mass spectrometry (LC-MS/MS) system.

2.5. LC-MS/MS

Formic-acid reconstituted extract solutions (4 μl) were injected onto a trapping column (300 μm x 1 mm) in line with a 75 μm x 150 mm 1.7 μm BEH130 C18 reversed phase LC column (Waters). Peptides of each sample were eluted from the column using 0.1% formic acid (A)/95% acetonitrile: 0.1% formic acid (B) gradient at a flow rate of 270 nl/min. Gradients were developed with the following time profile: 0 min 0% B, 5 min 5% B, 35 min 35% B, 40 min 45% B, 42 min 60% B, 45 min 90% B, 48 min 95% B, and 50 min 5% B. Eluted peptides were analyzed using a quadrupole TOF Synapt G2-S MS/MS spectrometer (Micromass Waters) with electrospray ionization. Analyses were performed using data-dependent acquisition with lock mass correction. The MS/MS data were processed with Distiller software (Matrix Science) to produce peak lists for database searching using MASCOT (Matrix Science). Data were searched against the National Center for Biotechnology (NCBI) non-redundant database in 2014, with the search restricted to proteins in green plants (*Viridiplantae*). The following search parameters were used: mass accuracy 0.1 Da, enzyme specificity trypsin, fixed carbamidomethylated modification, and variable modification oxidized methionine. Protein identifications were based on random probability scores with a minimum value of 25.

2.6. Identification of putative allergenic proteins using MASCOT data

The scoring of MASCOT incorporated a probability-based implementation of the molecular weight search (MOWSE) algorithm with the formula: MOWSE score = $-10 \times \text{Log}(P)$, where P is the probability that the observed match is a random event. A random match will have a high probability (P), therefore indicated by a low MOWSE score, whereas a valid match will have a low probability as indicated by a high MOWSE score (Weber et al., 2006). MASCOT ranks the quality of the peptide matches and sums the scores of the detected peptides to calculate a total protein MOWSE score with the highest scoring proteins representing the most likely protein detected in the gel spot. The top matches of proteins were selected based on the highest ranking of the protein MOWSE score.

3. Results

3.1. 2-DE gel patterns and immunoblots

The stained 2-DE gel image of the soybean extract is shown in Fig. 1 (A). As is typical for a soybean seed extract, most of the proteins localize in the acidic pI range of between 3 and 7, and individual proteins were between 10 and 90 kDa mass. Some of the proteins were identified only in single spots, and others occurred in series of spots of approximately the same MW but at different pI values.

The IgE binding from four soy-allergic and six soy-sensitized subjects showed clearly distinct patterns to soybean proteins, indicating the diversity of individual responses to soybean proteins (Fig. 2). However, some common immunoreactive proteins were detected. The numbering of the IgE binding spots in Fig. 2 corresponds to the spot numbers in Fig. 1 (B). Collectively, 36 IgE binding spots that were the primary targets of IgE from the 10 human subjects' sera and plasma samples were labeled on the stained gel image in Fig. 1 (B). The plasma from non-soybean allergic individuals used as negative controls, showed no IgE binding to any protein in the soybean extracts (data not shown).

3.2. Protein identities analyzed by LC-MS/MS

The IgE binding spots labeled on the gel in Fig. 1 (B) were analyzed by mass spectrometry. The MS/MS data of the IgE binding proteins is shown in Table 2. Only the identities of the highest protein MOWSE score hits are listed. Because LC-MS/MS almost never detects full-length coverage of any protein, there is some uncertainty of the complete sequences of the proteins. In addition, often peptides from more than one protein can be identified for an individual spot. The results are discussed in the context of individual matches and potential biological meaning. The numbers of peptides matched and percentage of coverage also indicate a high degree of confidence that the proteins identities are accurate.

As shown in Table 2, the isoforms of the two abundant seed storage proteins β-conglycinin (Gly m 5, three subunits) and glycinin (Gly m 6, five subunits) were located at several different positions (different MW

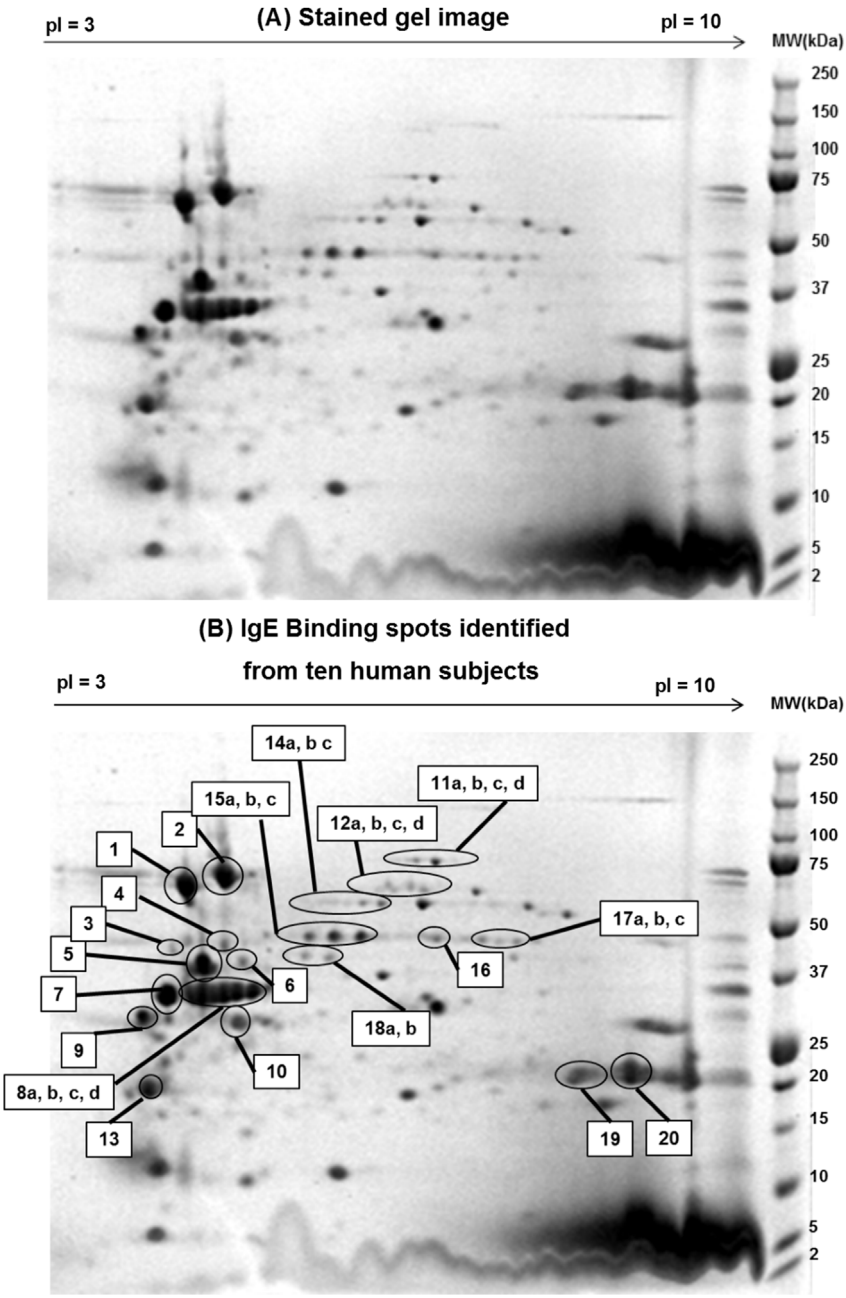

Fig. 1. Separation of soybean proteins by 2-DE shown by Coomassie staining (A) and spot designation where of stained spots that correspond to IgE binding for at least one of the ten subjects (B). Each spot was excised and placed in a separate microfuge tube for digestion by trypsin and injection in LC-MS/MS.

and pI) on the 2-DE gels. For example, the α subunit of β-conglycinin was identified at spots 1, 3, 6, and 16; the α' subunit was identified at spots 2 and 4; and the β subunit of β-conglycinin was identified at spots 14a, 14b, 15a, 15b, 15c, 17c, and 18b. Glycinin G1 subunit was identified at spots 8c, 8d, 19, and 20; glycinin G2 subunit was identified at spots 8a, 8b; and glycinin G4 subunit was identified at spots 7 and 9. The occurrence of multiple positions might be due to post-translational modifications of protein or peptide cross-linking to other proteins or peptides (Green-Church et al., 2008). In certain, the protein spots resolve as a series of spots that are likely to be minor variants of the same protein, i.e., either minor isoform differences or differences created by post-translational modification or post-extraction chemical changes (Kischkel et al., 2000; Machamer and Cresswell, 1982). For example, spots 11a, 11b, 11c are seed biotinylated protein (SBP) 65 kDa; spots 12a, 12b are sucrose-binding protein; spots 12c, 12d are late embryogenesis protein (LEP); and spots 14a, 14b and 15a, 15b, 15c represent β-conglycinin, β subunits.

It was determined that multiple significant MOWSE hits (different proteins) commonly occurred for one spot. Those are proteins that have similar MW and pI values and thus co-migrate to the same position on the 2-DE gel. For instance, for spot 1, the highest MOWSE scoring hit shows 56% sequence coverage of a β-conglycinin, α subunit (precursor) sequence composed of 605 amino acids; the second MOWSE scoring hit shows 40% sequence coverage of a β-conglycinin, α subunit sequence composed of 623 amino acids; the third scoring hit shows 44% sequence coverage of a β-conglycinin, α' subunit sequence composed of 559 amino acids. Yet, only the top significant hits are reported in Table 2. It must be noted that the identification of multiple proteins in a single spot correlating to an IgE binding spot does not necessarily mean they all bind IgE or are putative allergens. Serum IgE binding tests with individual purified proteins would be required to determine the specificity of binding with certainty. Clinical tests with individual pure

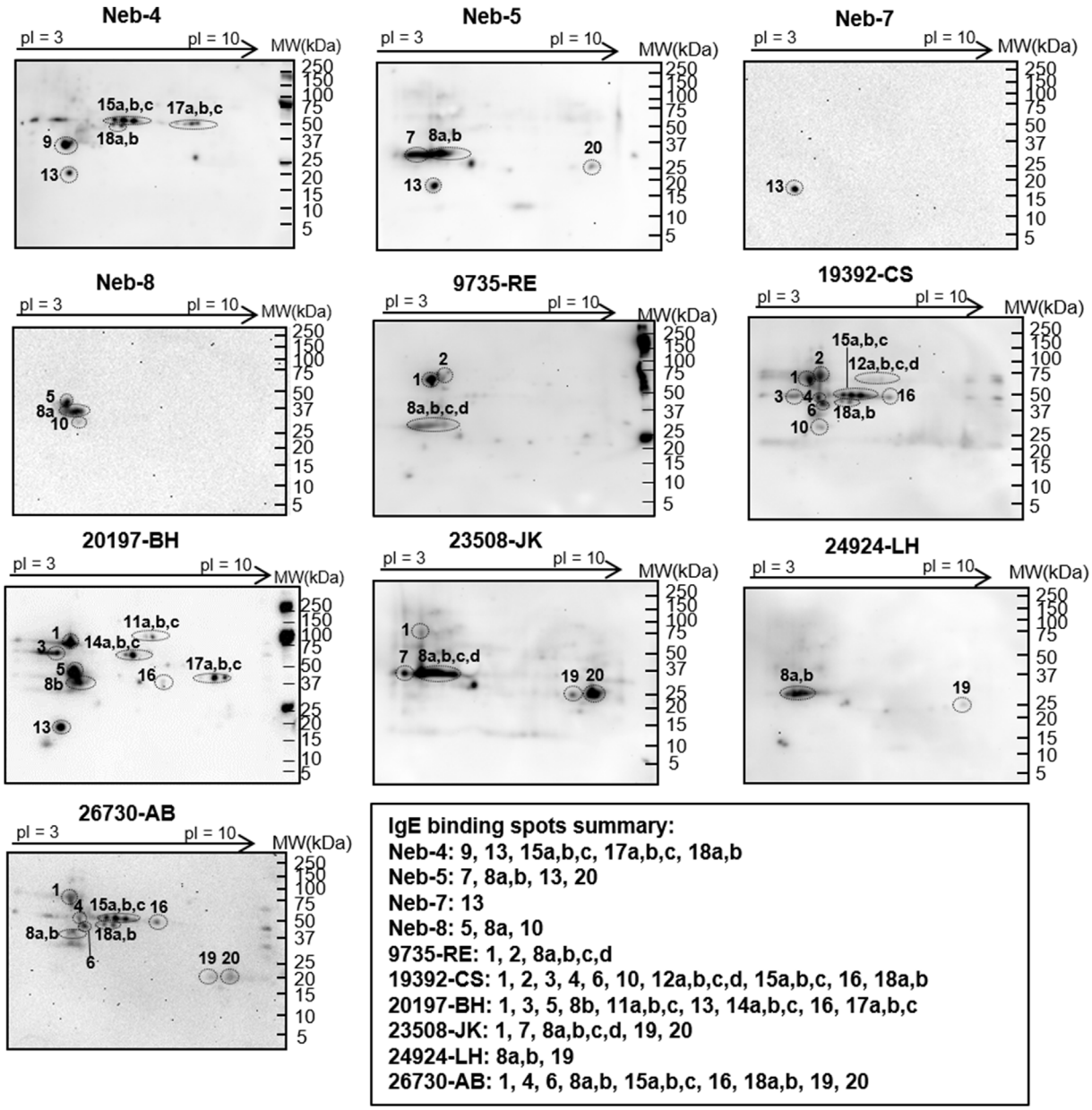

Fig. 2. Identification of soybean IgE binding proteins by 2-DE immunoblots for all ten subjects. Four subjects (Neb-4, Neb-5, Neb-7 and Neb-8) were clinically diagnosed with allergy to soybean, in Switzerland. The last six subjects were self-reported as allergic to soybean and their plasma tested positive with IgE binding to soybean by ImmunoCAP® by PlasmaLab in Everette, WA. Spot numbers correspond to stained proteins identified by LC-MS/MS.

proteins would be needed to demonstrate clinically relevant immunoreactivity by SPT, or by oral food challenges.

3.3. Frequency of IgE positive sera/plasma to individual soybean allergens

The identities of the IgE binding proteins in serum and plasma samples for each soybean allergic subject are summarized in Table 3. Six out of the ten soy-allergic or soy-sensitized subjects had IgE binding to one or more of the β-conglycinin subunits (Gly m 5). Eight out of the ten subjects had IgE binding to one or more of the glycinin subunits (Gly m 6). Only subject Neb-7 did not show IgE binding to either one or more subunits of Gly m 5 or Gly m 6. That subject's IgE only bound to SKTI. Three of the four clinically confirmed soy-allergic subjects' sera showed IgE binding to SKTI, and one of the soybean sensitized subjects displayed similar binding response in plasma to SKTI. Two out of the ten subjects showed IgE binding to soybean agglutinin (spot 10). Three other proteins were only bound by IgE from the soy-sensitized subjects and those included SBP 65 kDa (spots 11a, 11b and 11c), LEP (spots 12c, 12d), and sucrose-binding protein (spots 12a, 12b).

4. Discussion

Most of the IgE binding proteins identified by the ten soy-allergic or soy-sensitized subjects were subunits of either β-conglycinin (Gly m 5) or glycinin (Gly m 6), which are the abundant seed storage proteins that have been identified by others as major soybean allergens (Holzhauser et al., 2009). Beta-conglycinin and glycinin subunits make up 30% and 40% to the total seed protein, respectively. The β-conglycinin is a trimeric protein composed of three subunits in various mixtures of α (~72 kDa, pI of 5.18), α' (~76 kDa, pI of 4.90), and β (~53 kDa, pI of 5.66–6.00) subunits (Krishnan et al., 2009). The α subunit of β-conglycinin was called Gly m Bd 60 K in earlier references (Ogawa et al., 2000). Glycinin is a hexameric protein that is an assembly by five different subunits: G1 (A1aB1b, 53.6 kDa), G2 (A2B1a, 52.4 kDa), G3

Table 2
Identification of IgE binding proteins by LC-MS/MS. Spot numbers correspond to Fig. 1B. No significant identity matches were found for spots 14c and 18a. Theoretical mass of the individual proteins and the expected pI values are shown along with the MOWSE score, number of peptide matches to the identified protein accession and the percent coverage of identified protein sequences is shown for each spot.

Spot*	NCBI Accession GI#	Protein Type	Theoretical Mass (Da)/pI	Protein MOWSE Score	No. Peptides Matched	Sequence Coverage (%)
1	P13916.2	β-conglycinin, α	70535/5.07	3173	107	56
2	BAA74452.2	β-conglycinin, α′	65160/5.23	3127	116	62
3	P13916.2	β-conglycinin, α	70535/5.07	1320	33	32
4	BAA74452.2	β-conglycinin, α′	65160/5.23	2170	68	51
5	CAA55977.1	Glycinin G5	58608/5.52	1555	48	40
6	P13916.2	β-conglycinin, α	70535/5.07	1656	49	38
7	CAA37044.1	Glycinin G4	64351/5.21	1559	36	28
8a	P04405.2	Glycinin G2	54927/5.46	4416	76	44
8b	P04405.2	Glycinin G2	56299/5.46	2180	37	42
8c	P04776.2	Glycinin G1	56299/5.89	2075	40	50
8d	1FXZ_A	Glycinin G1	54047/5.78	1254	42	47
9	CAA37044.1	Glycinin G4	64351/5.78	1371	29	27
10	2SBA_A	Soybean agglutinin	27555/5.21	1200	41	49
11a	Q39846.1	SBP of 65 kDa	67894/6.1	587	10	22
11b	Q39846.1	SBP of 65 kDa	67894/6.1	2281	47	53
11c	Q39846.1	SBP of 65 kDa	67894/6.1	2919	62	65
12a	Q04672.1	Sucrose-binding protein	60884/6.42	833	14	24
12b	Q04672.1	Sucrose-binding protein	60884/6.42	877	15	29
12c	AAA33985.1	LEP	50613/6.33	1436	30	49
12d	AAA33985.1	LEP	50613/6.33	189	3	11
13	1BA7_A	SKTI	20310/4.61	974	45	88
14a	1IPJ_A	β-conglycinin, β	47879/5.67	1228	23	45
14b	1IPJ_A	β-conglycinin, β	47879/5.67	485	10	31
15a	1IPJ_A	β-conglycinin, β	47879/5.67	1906	42	56
15b	BAD98463.1	β-conglycinin, β	48358/5.67	1980	51	56
15c	1IPJ_A	β-conglycinin, β	47879/5.67	2055	54	56
16	P13916.2	β-conglycinin, α	70535/5.07	1723	42	37
17a	ACU18647.1	Unknown protein	43082/6.28	1723	45	81
17b	ACU18647.1	Unknown protein	43082/6.28	912	19	61
17c	1IPK_A	β-conglycinin, β	47947/5.67	759	14	37
18b	1IPJ_A	β-conglycinin, β	47879/5.67	1490	35	50
19	P04776.2	Glycinin G1	56299/5.89	980	40	33
20	P04776.2	Glycinin G1	56299/5.89	1035	65	33

(A1bB2, 52.2 kDa), G4 (A5A4B3, 61.2 kDa), and G5 (A3B4, 55.4 kDa). Each individual glycinin protein is translated as a single protein that is cleaved into an acidic and a basic peptide chain, linked by a disulfide bond (Prak et al., 2005). Our results are consistent with previous findings that all subunits of β-conglycinin and glycinin bound IgE in sera from some DBPCFC positive soybean allergic subjects (Holzhauser et al., 2009). In addition, the levels of IgE responses to β-conglycinin and glycinin were associated with severe clinical reactions caused by soybean (Ito et al., 2011). The results of this study confirmed that β-conglycinin and glycinin are the two major clinically important soybean allergens in the soy-allergic and soy-sensitive population from the U.S. and Switzerland tested in this study.

Soybean Kunitz trypsin inhibitor was bound by IgE from three of the four clinically confirmed soy-allergic subjects, which indicates SKTI

Table 3
Summary of the identities of IgE binding soybean proteins with all ten subjects. Numbers represent spots of the stained gel as shown in Fig. 1B. Only subject Neb-7 did not show IgE binding to either one or more β-conglycinin subunits (Gly m 5) or glycinin subunit (Gly m 6). That subject's IgE only bound to SKTI.

Subjects										
Proteins	Spot Numbers[1]									
	Neb-4	Neb-5	Neb-7	Neb-8	9735-RE	19392-CS	20197-BH	23508-JK	24924-LH	26730-AB
Gly m 5 (β-conglycinin)										
β-conglycinin, α					1	1, 3, 6, 16	1, 3, 16	1		1, 6, 16
β-conglycinin, α′					2	2, 4				4
β-conglycinin, β	15a,b,c, 18b, 17c					15a,b,c, 18b	14a,b, 17c			15a,b,c, 18b
Gly m 6 (Glycinin)										
Glycinin G1		20			8c,d			8c,d, 19, 20	19	19, 20
Glycinin G2		8a,b		8a	8a,b		8b	8a,b	8a,b	8a,b
Glycinin G4	9	7						7		
Glycinin G5				5			5			
Other possible soybean allergens										
SKTI	13	13	13				13			
Soybean agglutinin				10		10				
SBP of 65 kDa							11a,b,c			
LEP						12c,d				
Sucrose-binding protein						12a,b				

might be an important food allergen for soy-allergic patients. SKTI accounts for 4–7% of the total extractable protein in soybean with a MW of ~22 kDa and pI of 4.5 (Taylor et al., 2015). The SKTI protein has previously been reported to be an inhalation allergen among 14 bakers suffering from respiratory symptoms to soybean (Baur et al., 1996). In one study, inhalation of purified SKTI caused positive clinical reactivity in two sensitized bakers (Quirce et al., 2002). Allergic reactions caused by ingestion of SKTI have only been confirmed in one individual, who was exposed to SKTI occupationally (Moroz and Yang, 1980). SKTI is not included in the World Health Organization and International Union of Immunological Society (WHO/IUIS) database, and the food allergenicity of SKTI needs to be investigated further.

Soybean agglutinin was identified as an IgE binding protein with sera from one soy-allergic sera and from plasma of one soy-sensitized subject in this study. Soybean agglutinin is also known as a soy lectin that is an anti-nutritional factor in soybean. It accounts for approximately 10% of the total protein (Maria John et al., 2017; Natarajan et al., 2009). Soybean agglutinin has a MW of approximately 120 kDa and pI of 5.8. It is composed of four identical subunits of 30 kDa. There is a carbohydrate moiety bound to the agglutinin by an *N*-acetylglucosaminyl linkage to asparagine (Lotan et al., 1974). Lectins are glycoproteins found in plant seeds, which have been shown to have non-specific IgE binding to carbohydrate moieties inducing allergy-like symptoms (Shibasaki et al., 1992). It is not clear whether the agglutinins can elicit allergic reactions or can stimulate basophils to release histamine. However, peanut agglutinin was identified as a specific IgE binding protein from peanut allergic patients (Burks et al., 1994). A few review papers list soybean agglutinin as a soybean allergen based on IgE binding (L'Hocine and Boye, 2007; Wang et al., 2014; Wilson et al., 2005), but little is known about the allergenicity of soybean agglutinin. Batista et al. (2007) and our study identified soybean agglutinin as a potential soybean allergen using the combination of 2-DE immunoblot and LC-MS/MS techniques. However, further studies would be needed to verify the allergenicity of soybean agglutinin.

Three other proteins, including SBP, LEP, and sucrose-binding protein, were identified as IgE binding proteins only among soy-sensitized subjects. These IgE binding proteins are potential allergens, but more work is required for verification. Batista et al. identified LEP as a potential allergen for soybean using similar techniques as those reported here (Batista et al., 2007). Recently, SBP (Gly m 7) was purified and confirmed as a soybean allergen using peanut and soybean-allergic human subjects (Riascos et al., 2016). The Gly m 7 protein description has fulfilled the primary criteria of WHO/IUIS Allergen Nomenclature Subcommittee and is included in the database. Sucrose binding protein has also been identified as an IgE binding soy protein in 53% of thirty-two North American soybean-allergic subjects in one study (Gagnon et al., 2010).

Despite the large number (up to 21) of reported soybean allergens (Selb et al., 2017; Wilson et al., 2005), their biological significance and immunologic characteristics have not been extensively studied compared to the proteins in other allergenic foods, such as peanut. Many of the reported soybean allergens were identified by serum IgE binding using subjects with atopic dermatitis or sera from subjects whose soybean food allergy was not confirmed by controlled food challenge (Hiemori et al., 2004; Ogawa et al., 1991, 1993; Tsuji et al., 1997). The 15 potential soybean allergens list in the OECD soybean consensus document on compositional considerations for new GE varieties of soybean includes Gly m 1, Gly m 2, Gly m 3, Gly m 4, Gly m 5, Gly m 6, Gly m Bd 30 K, Gly m Bd 28 K, Gly m 8, SKTI, soybean lectin, lipoxygenase, and three uncharacterized proteins (50 kDa, 39 kDa, and P22-25 proteins) (OECD, 2012).

Hydrophobic soybean hull protein Gly m 1 and the soybean hull defensin Gly m 2 were not detected in this study, as expected, because these two soy-hull proteins are airborne allergens (i.e., atmospheric soybean dust) and only relevant to respiratory allergy. The short peptides of Gly m 1 and Gly m 2 were identified as belonging to proteins from studies of the Barcelona, Spain asthma epidemic near the port facility in the 1980s (Hernando et al., 1989; Sunyer et al., 1989). The sequence of Gly m 2 was unknown except that recent cloning studies matched the peptide to a full-length protein sequence. The clinical relevance of these two soy-hull proteins is unknown.

The soybean profilin Gly m 3 and the pathogenesis-related class 10 protein Gly m 4 are important IgE binding (cross-reactive) proteins for some European soybean and birch pollen allergic subjects who typically experience oral allergy syndrome when ingesting soybeans (Jenkins et al., 2005; Mittag et al., 2004). However, the clinical relevance of Gly m 3 and Gly m 4 as important allergens is limited geographically corresponding to areas where birch pollen allergy is common. Little evidence supports their allergenicity in foods. Detecting IgE binding to profilin Gly m 3 depends on the integrity of a conformational epitopes (Rihs et al., 1999). The 2-DE procedure used in this study probably disrupted the integrity of the conformational epitopes of Gly m 3. In addition, since Gly m 3 requires the full-length, correctly folded protein to bind IgE, it is not likely to be a major allergen in cooked or processed soy products. Interestingly Gly m 4, also known as starvation-associated-message 22 (SAM) is > 50% identical to the Bet v 1 family of birch pollen allergens and seems to initially be due to cross-reactivity. The primary reports of clinical food allergy related to Gly m 4 are subjects who were consuming large quantities of a nutritional lightly processed food supplement (Kleine-Tebbe et al., 2002). Gly m 4 was not detected by IgE binding in this study even though the serum Neb-7 has a Gly m 4 specific IgE ImmunoCAP® value of 26.2 kU/l (Table 1). It is possible that the protein is relatively easy to denature, and conformational epitopes of Gly m 4 might not be present in a 2-DE immunoblot. Although the newly described 2S albumin of soybean (Gly m 8) was reported to be an important as a biomarker for clinically severe food to soybean for populations in Japan and Netherlands (Ebisawa et al., 2013; Klemans et al., 2013) we did not detect IgE binding to it. There seems to be little evidence that supports the importance of Gly m 4 or Gly m 8 outside of the subjects from restricted geographies.

The other seven OECD listed putative soybean allergens (Gly m Bd 28 K, Gly m Bd 30 K, lectin, lipoxygenase, P22-25, unknown 39 kDa, and unknown 50 kDa) were poorly characterized in published scientific literature, and are they were not detected in our study. There is insufficient evidence to suggest they cause soybean food allergy. Gly m Bd 28 K and 30 K were reported to be strong and frequent allergens in the 1990s in Japan; however, the IgE binding to the two allergens were identified in the population of atopic dermatitis patients who had detectable IgE to soybean tested by either radioallergosorbent assay or SPT (Bando et al., 1996; Helm et al., 2000; Ogawa et al., 1993; Tsuji et al., 1997). No food challenge or other convincing proof of soybean allergies were reported in those studies. Thus, further evidence is needed to classify Gly m Bd 28 K and Gly m Bd 30 K as confirmed food allergens. Only one peer-reviewed publication indicated that soybean lectin and lipoxygenase are potential allergens for bakers suffering from respiratory symptoms to soybean (Baur et al., 1996), however a recent review suggested that those authors likely used an impure test substance (Ladics et al., 2014). The sequences and characteristics of three proteins; P22-25, 39 kDa and 50 kDa remain unknown and it is not clear why they have been listed by the OECD as allergens of soybean (Ladics et al., 2014).

5. Conclusion

Current evidence suggests that the soybean proteins responsible for food allergy are the major seed storage proteins, β-conglycinin (Gly m 5, with three subunits) and glycinin (Gly m 6, with five subunits), and possibly SKTI. The prevalence and importance of other proteins in soybean food allergy have not been conclusively demonstrated. Carefully designed and performed studies are needed with isolated and purified potential soy allergenic proteins to be tested in individuals with clear evidence of allergic reactions to soy following oral challenge,

especially DBPCFC, to expand the list for food safety concerns. Thus, recommendations by the European Food Safety Authority for quantitative analysis of all the OECD listed soy "allergens" for evaluation of the safety of new GE soybeans is not justified in our opinion.

Conflicts of interest

ML, YJ, RC, BB-W, RG declared no conflict of interest. The sponsor company played no role in designing the experiments or preparing the manuscript. The results and discussions presented in this article do not necessarily represent the views or scientific work of Pioneer Hi-Bred International, Inc.

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