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Article

Identification and Protein Characterization of Peanut Lines with Relatively Lower Levels of Major Allergens

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Abstract: Despite the allergen labeling of peanuts, reducing peanut allergenicity through material selection and breeding may provide an avenue to reduce the severity of allergy due to consumption of foods containing peanuts. This study's objective is to select peanut lines (cultivar/variety) with lower potent allergenic peptides. One hundred and twenty-two peanut lines (harvested in 2015, New Mexico State, USA.), including 97 lines from the Core USDA- Griffin collections were used for SDS-PAGE analysis in order to find low allergen peanut lines. Eleven peanut lines (Coded MS-1~MS-11) were identified as having lower allergen (Ara h 1, Ara h 2 and Ara h 3) levels and were re-planted in 2016 for further analysis. One line named MS-7 was selected for lower Ara h 1 (8.5-9.5% of total protein) and Ara h 2 (4.2-6.6% of total protein) content in 2015 and 2016. Two-dimensional (2-D) gel electrophoresis (isoelectric focusing plus SDS-PAGE) was conducted to compare the protein (allergens) distributions of MS-7 and check line (MS-9). The 2-D results supported the SDS-PAGE analysis that MS-7 had lower quantities of Ara h 1 and Ara h 2, the two major peanut allergens. The identified peanut line (MS-7) has potential for processing into lower allergenic peanut food products.

Keywords: peanut cultivars; proteins; allergen composition; SDS-PAGE; 2D-gel electrophoresis

1. Introduction

Peanut allergy is a world-wide problem, especially in the United States, where peanuts and peanut products are commonly consumed [1]. Currently, the only method to prevent allergy is through complete avoidance. However, avoiding peanuts is difficult since it is widely used as a food ingredient in many food products. Consequently, it would be meaningful to search for peanut lines with significantly lower amount of allergen (major allergens such as Ara h 1, Ara h 2) to reduce food allergy intensities [2].

Ara h 1 is a 63.5-65 kDa protein, which shows one protein band in reducing SDS-PAGE with an estimated molecular size of 63 kDa in the report of Kang et al. [3]. Ara h 1 (vicilin, 7S globulin) shares the same overall structural conformation with Ara h 3 (legumin, 11S globulin) and belongs to the cupin superfamily [4]. These types of proteins are considered as storage proteins and energy sources for plants during germination [5]. Approximately 12 to 16% of the peanut total protein is Ara h 1 [6].

Ara h 2 is the most potent allergen in peanuts and shows two protein bands in reducing SDS-PAGE with estimated molecular weights of 20 and 17 kDa [3]. It is a conglutin seed storage protein and a trypsin inhibitor [7]. It belongs to the 2S albumin superfamily [8]. Ara h 2 has 5.9 to 9.3% of the peanut total protein [6].

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Ara h 3 is a 60 kDa protein and a major allergen, which shows five protein bands in reducing SDS-PAGE with estimated molecular sizes of 45, 40, 36, 22 and 14 kDa [3]. It contributes to 50-57% of the total protein content of the peanut [6].

It has been reported that the major allergen (Ara h 1, Ara h 2 and Ara h 3) levels varied among peanut cultivars [3,9]. The "core of core collection" (mini core) including 112 accessions, which was developed by Holbrook and Dong [10], contains the genetic diversity of the entire 831 core accessions of U.S. germplasm collection. Previous study has been conducted on 99 of the 112 accessions for the quantification of allergen composition by Kang and coworkers [3] and in that study 60 accessions in the U.S. Peanut germplasm collection (all belonging to a mini-core collection), along with 88 Florida Peanut breeding program lines were analyzed for major peanut allergen levels. Their study reveals that an accession from India has the lowest level of Ara h 1 (7.0%). An accession from Nigeria has the highest level of Ara h 1 (18.5%), but has the lowest level of Ara h 2 (6.2%). An accession from Zambia has the highest level of Ara h 2 (13.2%), but the lowest level of Ara h 3 (21.8%). Two accessions, 20 breeding lines, and 2 peanut cultivars (Florunner and Georgia Red) contain little or no Ara h 3 isoforms (36 KDa) [3]. In a recent study by Pandey et al. [11] in India, 184 accessions of peanut mini core collection are replanted in two seasons, and analyzed for major allergen content using ELISA. The analysis revealed that the major allergen content of the 184 accessions of peanut are Ara h 1 (4–36,833µg/g), Ara h 2 (41–77,041 µg/g), Ara h 3 (22–106,765 µg/g), Ara h 6 (829–103,892 µg/g), and Ara h 8 (0.01–70.12 µg/g) [11]. Unfortunately, Pandey et al. [11] has not reported the protein extraction recovery of peanut protein. Due to the inconsistent analysis methods between the previous two studies, it was difficult to compare them directly.

Even though previous studies have screened peanut lines from the mini-core collection and for allergen content [3,11], until now, no safe hypoallergenic peanut lines have been found or created. Peanut lines with low allergens still need to be continuously searched for, by looking into available cultivars in different years and planting locations, and improving testing method. In this study, in order to analyze peanut allergen content in a more convincing way, SDS-PAGE was conducted since the band intensity would represent the allergen content distributions more accurately. Furthermore, major allergen levels in peanuts cultivated from two consecutive years could be analyzed. Therefore, 97 of the mini-core peanut accessions and 25 accessions from other resources (harvested in Clovis, NM, USA, 2015) were collected and analyzed, relatively low major allergen level peanuts were pre-screened and replanted (in Clovis, NM, USA, 2016) to select peanut lines with relative low allergen-levels for food processing use or further breeding test. It also would be meaningful to compare the selected low major allergen containing lines with a commercial check line by means of 2D gel electrophoresis (isoelectric focusing followed by SDS-PAGE), which could better reflect the micro-heterogeneity of protein subunit distributions.

Therefore, the objectives of this study were to 1) analyze and select low-allergen peanut lines from 122 peanut lines harvested in Clovis, NM (2015) by using SDS-PAGE; 2) further to re-plant 11 peanut lines in Clovis, NM (2016) and analyzed to confirm the expressions of lower allergen content by means of SDS-PAGE; and 3) to test and compare the major allergen compositions between the one selected with low allergen line and a check (commercial) peanut line by means of 2-dimensional gel electrophoresis.

2. Materials and Methods

2.1. Materials

Peanuts (shell-removed) with 122 lines harvested in Clovis, NM (2015) were provided by New Mexico State University. Ninety-seven lines of these 122 germplasms came from the US mini-core collection with Plant Introduction (PI) number located in USDA-Griffin (University of Georgia). Twenty-five lines of them belonged to other resources, including experimental lines, a check line (Valencia, code MS-9 in this study) and private company lines. Peanut lines with low levels of major allergen (Ara h 1, Ara h 2, or Ara h 3) were selected based on SDS-PAGE-band intensity analysis. The screened peanuts (coded MS-1 ~ MS-11, including check line MS-9) were replanted and harvested in

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Clovis, NM (2016) to increase the seed quantity for a food processing study, which we had published [12]. Human plasma from six individuals only with peanut allergy (IgE levels CAP-FEIA > 100 kU/I; contained anti-Ara h 1, Ara h 2 and Ara h 3 antibodies) were purchased from PlasmaLab International (Everett, WA, USA). All the other chemicals of the analytical grade were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. The Protein Extraction Method for Raw Peanut

The protein extraction from raw peanut powder was according to an earlier study [19] with some modifications. Briefly, approximately 5 g of the pre-frozen peanut kernel with skin (-80 ℃) were milled with a Magic bullet blender (Model No. MBR-1101, Capbran Holdings, LLC, Los Angeles, CA) into coarse powder. About 4 g were ground with mortar and pestle in liquid nitrogen to fine powder (could pass through 0.5 mm sieve). Five hundred milligrams of peanut powder in a 15 mL centrifuge tube were defatted using 10 mL acetone with shaking for 2 hr in an orbital shaker. The suspension was centrifuged (2795 g for 15 min) and the pellet was dried under an exhaust hood overnight at room temperature. The defatted flours were shaken in a 15 mL centrifuge tube with 3 mL of 0.02 M sodium phosphate, pH 8.5, plus 10 mM EGTA at room temperature for 2 hr. After centrifugation at 2800 g for 15 min at room temperature, the supernatant was collected and diluted 30-fold. The protein content of the obtained supernatant was determined using the Bradford's method [13].

2.3. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was conducted according to our previous study [14] using a Mini Protein Tetra System (BioRad, Hercules, CA). Soluble protein extracts (adjusted to 2 mg/mL) were mixed with an equal volume of sample buffer, containing 5% β-mercaptoethanol. The mixture was boiled for 5 min to denature the proteins. Electrophoresis was performed on 13.5% (non-gradient) acrylamide gels for 1 hr at 50 V followed by 1.5 hr at 100 V. Protein markers (ranging from 10-250 kDa, Product No.1610373, Bio-Rad, Hercules, CA) was used to identify and estimate the major allergens and their molecular mass. For the quantification of peanut major allergens (Ara h 1, Ara h 2 and Ara h 3), gels were scanned and analyzed by a Molecular Imager (Bio-Rad Chemidoc™ XRS+, Hercules, CA) equipped with Image Lab™ Analysis Software (version 5.2). The major allergens and their relative compositions were calculated based on the band intensity and total area of their subunits. Allergen protein levels were determined and expressed as the percentage of total detectable protein.

2.4. Two-Dimensional Gel Electrophoresis of Selected Peanut Protein Extracts

Two-dimensional electrophoresis was performed according to a previous study [15] with modifications. Peanut powder (0.5 g, without defatting procedure) was extracted by 3 mL 20 mM TBS buffer, pH 7.4, plus 150 mM NaCl for 2 hr. The protein extracts (aqueous fractions) were collected by centrifugation (2800 g) for 15 min at room temperature. Cleanup Kit (Catalog #163-2130, BioRad, Hercules, CA) was used to purify and precipitate the protein extractions for isoelectric focusing (IEF). Prepared proteins (150 micrograms) were loaded onto each precast immobilized pH gradient (IPG) strip (pH 3–10, BioRad, Hercules, CA) and focused using a Protean IEF System (BioRad) overnight. After focusing, the proteins were reduced by immersing the IPG strips in an equilibration buffer (50 mM Tris-HCl pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 1% dithiothreitol) for 15 min, and alkylated with 2.5% w/v iodoacetamide in the equilibration buffer for 15 min. The IPG strips were then placed directly onto 13.5% polyacrylamide-SDS Mini gels (8.6 × 6.8 cm) for the second-dimensional electrophoresis. After electrophoresis, the proteins in the gels were stained with Coomassie blue R-250 and then destained with a destaining solution (40% methanol and 10% acetic acid). After destaining, gels were scanned and analyzed by a Molecular Imager (Bio-Rad Chemidoc™ XRS+, Hercules, CA) equipped with Image Lab™ Analysis Software (version5.2). Each identical spot (based on the results of western-blot and the previous literature) was quantified with Image Lab™ Analysis Software and expressed as percentage of the total allergen content.

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2.5. Western-Blot Analysis

Two-dimensional gels of two peanut lines (MS-7 and MS-9, raw peanut) were analyzed by western-blot analysis, using plasma containing IgE antibodies from peanut allergic individuals as described in our study [14]. In brief, protein extracts were transferred from the SDS–PAGE gel to an Immobilon-P membrane. The membrane then was blocked with a SuperBlock solution (contains a proprietary protein in pH 7.4 PBS, Cat No. 37515, ThermoFisher, Waltham, MA) to provide low background interference. After blocking, the membrane was incubated for 1 hr with a pooled plasma diluted 1:20 (v/v) in Superblock/TBS-Tween 20 (1:1, v/v). The membrane was washed with TBS-Tween 20 and incubated with a rabbit anti-human IgE-peroxidase (1:250) for 1 hr, and then washed with TBS-Tween 20 for 3 times (10 min each time). After washing, the membrane was then incubated in the SuperSignal™ West Pico Chemiluminescent Substrate (Fisher Scientific, Pittsburgh, PA) for 1 min to develop reaction for the detection of horseradish peroxidase (HRP) activity from the secondary antibodies. The incubated membrane was scanned and analyzed by a Molecular Imager (Bio-Rad Chemidoc™ XRS+, Hercules, CA) equipped with Image Lab™ Analysis Software (version 5.2).

2.6. Statistical Analyses

Experiments were performed in duplicate for enzymatic processing and triplicate for analysis. Data were subject to the analysis of variance (ANOVA) using SAS (Version 9.4, SAS Inst. Inc., Cary, NC, U.S.A.). Significant differences among means were determined by the Duncan multiple range test procedure for independent samples at $P < 0.05$.

3. Results

3.1. Peanut Protein/Peptide Analysis and Screening

3.1.1. Peanut Lines Harvested in 2015

The allergen content (% of total protein) was calculated based on the band intensities of the SDS-PAGE, which was the same calculating method as a previous study [3]. Among 97 lines of peanuts from the mini-core collections (harvested in 2015), Ara h 1 varied from 6.33 to 17.02%; Ara h 2 varied from 3.66 to 9.29%; Ara h 3 varied from 46.62 to 61.97% (Table 1A).

Table 1. A. The major allergen content of 97 USDA mini core peanut cultivars harvested in year 2015 (Clovis, NM).

No.	PI number	Ara h 1	Ara h 2	Ara h 3	Total
1	152146	13.93±1.14	8.31±0.24	53.69±2.32	75.92±3.30
2	155107	13.19±1.10	8.29±0.66	54.89±2.76	76.36±3.72
3	157542	14.74±1.19	7.36±0.51	50.82±2.30	72.91±3.21
4	158854	13.84±0.82	8.65±0.71	50.81±1.93	73.30±2.66
5	159786	14.27±1.05	6.91±0.12	50.28±2.30	71.46±3.47
6	162655	14.24±1.14	8.24±0.76	54.09±1.78	76.56±2.28
7	162857	13.83±1.27	7.25±0.24	51.30±0.95	72.38±1.47
8	196622	14.75±0.21	7.34±0.10	49.34±0.29	71.44±0.40
9	196635	14.44±0.18	7.43±0.19	49.56±2.77	71.43±3.06
10	200441	14.10±0.20	8.36±0.48	52.19±1.32	74.64±2.00
11	240560	13.90±0.24	8.89±0.18	52.40±1.46	75.19±1.89
12	259617	14.22±0.08	9.11±0.21	52.85±0.69	76.17±2.98
13	259658	15.24±0.02	8.09±0.02	49.03±0.72	72.36±2.76
14	259851	14.64±0.18	7.73±0.08	50.24±1.64	72.62±1.91
15	262038	14.49±0.01	8.43±0.43	51.82±0.84	74.74±3.28
16	268586	10.18±0.71	9.29±0.28	54.78±1.15	74.24±2.14

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17	268696	13.59±0.48	7.64±0.27	54.34±1.21	75.57±3.96
18	268755	10.82±0.36	8.36±0.18	56.10±0.89	75.28±2.42
19	268806	14.31±0.03	7.88±0.20	53.13±0.77	75.32±3.00
20	268868	14.87±0.20	6.55±0.35	50.81±0.66	72.23±1.21
21	268996	14.54±0.35	7.70±0.33	49.06±0.67	71.29±2.10
22	270786	15.36±0.56	6.78±0.29	52.70±1.77	74.84±2.62
23	270905	14.10±0.15	8.23±0.29	49.97±1.47	72.31±1.91
24	270907	14.75±0.19	7.11±0.18	49.63±2.61	71.49±2.98
25	270998	13.28±0.49	6.49±0.34	50.06±1.09	69.82±1.92
26	271019	13.14±0.08	4.33±0.36	54.73±1.45	72.20±1.89
27	274193	12.31±0.57	6.51±0.26	49.78±2.79	68.60±3.62
28	288146	12.68±1.20	5.02±0.42	53.76±0.41	71.46±2.03
29 (MS-1)	288210	6.33±0.22[1]	6.55±0.58	57.19±2.95	70.05±3.75
30	290536	13.02±0.90	5.45±0.59	50.76±0.57	69.22±2.06
31	290560	12.71±0.34	5.81±0.39	53.31±1.85	71.83±2.58
32	290594	14.40±1.64	4.06±0.04	52.53±3.49	70.99±5.17
33	290620	11.39±0.23	5.14±0.66	53.53±2.07	70.06±2.95

Note: The allergen content was expressed by the % of total soluble protein. [1]Represent significant lower level of that major allergen ($P < 0.05$).

Table 1. A. (continued).

No.	PI number	Ara h 1	Ara h 2	Ara h 3	Total
34 (MS-2)	292950	13.36±0.50	7.27±0.71	47.27±2.72[1]	68.90±3.93
35	295250	14.29±1.16	6.40±0.36	52.27±3.01	72.96±4.52
36	295309	14.77±0.33	5.27±0.19	51.37±1.57	71.42±2.09
37	295730	12.71±0.88	4.63±0.22	54.39±1.05	71.73±2.15
38	296550	9.75±1.16	4.06±0.15	57.45±1.96	71.26±3.27
39 (MS-3)	296558	12.53±1.02	6.85±0.52	48.67±2.40[1]	68.05±3.94
40	298854	10.99±0.11	7.14±0.21	53.30±2.70	71.43±3.02
41	313129	12.64±0.86	6.46±0.13	53.14±3.01	72.24±4.00
42	319768	10.33±1.24	6.91±0.13	54.32±1.46	71.56±2.82
43	319770	13.04±0.46	4.60±0.18	53.59±3.32	71.24±3.96
44	323268	14.41±2.00	6.24±0.91	54.38±1.71	72.96±4.15
45	325943	10.96±2.05	5.12±0.65	55.92±4.03	72.31±6.53
46	331297	13.03±1.68	4.83±0.16	53.10±1.14	70.94±3.14
47	337293	10.82±0.30	5.17±0.44	52.77±4.90	70.82±6.10
48	337399	12.04±1.14	5.43±0.45	54.94±4.12	72.10±5.91
49	337406	14.21±0.09	4.81±0.32	56.07±2.25	75.10±2.50
50	338338	13.49±0.37	4.94±0.31	54.84±0.88	73.27±1.56
51	339960	13.57±0.33	5.89±0.43	54.18±0.74	73.64±1.50
52 (MS-4)	343384	17.02±0.16	6.95±0.65	46.62±1.25[1]	70.59±3.76
53 (MS-5)	343398	14.60±0.59	4.07±0.29[1]	52.88±3.46	69.55±4.35
54	355268	14.94±0.37	4.89±0.59	52.76±1.00	72.59±1.97
55	355271	16.97±0.18	4.76±0.53	50.34±0.71	72.07±1.42
56	356004	12.81±0.74	5.96±0.60	52.80±0.83	71.58±2.17
57	370331	14.60±0.17	6.01±0.11	52.21±0.29	72.82±0.56
58	371521	14.73±0.05	5.20±0.24	52.08±0.59	72.00±0.88
59	372271	14.22±0.42	4.18±0.17	52.42±1.58	70.82±2.18
60	372305	12.11±1.24	4.57±0.13	55.85±2.72	73.03±4.09
61	399581	15.48±0.07	5.91±0.19	50.94±1.44	72.34±1.70
62	403813	12.60±0.27	6.19±0.55	58.11±1.60	76.89±2.42
63	407667	9.30±0.15	4.81±0.29	58.26±3.12	72.37±3.55

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64	429420	13.32±0.00	5.22±0.07	52.23±0.77	70.77±0.85
65	442768	14.43±0.65	4.55±0.23	52.35±0.81	71.32±1.69
66	461427	13.76±0.25	5.20±0.19	52.52±0.40	71.47±0.84

Note: The allergen content was expressed by the % of total soluble protein. [1]represent significant lower level of that major allergen ($P < 0.05$). .

Table 1. A. (continued).

No.	PI number	Ara h 1	Ara h 2	Ara h 3	Total
67	461434	13.15±0.07	5.55±0.14	56.09±0.35	74.79±0.57
68	468271	12.22±0.29	5.92±0.15	56.89±1.18	75.03±1.62
69	471952	12.93±0.13	5.87±0.28	53.98±0.42	72.79±0.83
70	475863	16.44±0.12	4.25±0.14	52.13±1.07	71.82±1.33
71 (MS-6)	475918	13.58±0.40	3.66±0.27[1]	59.33±5.43	76.58±6.00
72	476025	12.95±0.46	4.74±0.10	56.23±2.87	73.92±3.44
73	476432	14.19±0.08	4.21±0.11	55.29±0.79	73.68±0.98
74	476596	13.00±0.02	5.98±0.16	56.18±1.52	75.16±1.70
75	476636	15.83±0.18	5.45±0.23	51.73±0.33	73.01±0.74
76	478819	14.22±0.80	4.63±0.54	51.22±1.87	70.07±3.21
77 (MS-7)	481795	8.41±0.10[1]	4.16±0.45[1]	61.97±2.32	74.54±3.87
78	482120	9.35±0.04	6.19±0.09	60.05±1.06	75.59±1.19
79	482189	13.01±0.07	6.72±0.16	55.15±2.17	74.87±2.40
80	493329	12.75±0.15	6.38±0.54	56.17±2.24	75.30±2.93
81	493356	13.77±0.27	6.73±0.23	50.89±0.64	71.39±1.14
82	493547	13.51±0.27	6.93±0.14	53.59±0.97	74.03±1.38
83	493581	13.87±0.37	6.51±0.28	54.96±4.11	75.34±4.75
84	493631	14.81±0.41	6.61±0.26	53.39±3.22	73.81±3.89
85	493693	14.81±0.07	6.57±0.66	54.18±1.27	75.57±2.00
86	493717	14.34±0.07	7.15±0.92	53.75±2.69	75.25±3.69
87	493729	16.58±0.48	5.67±0.47	52.90±1.38	75.15±2.33
88	493880	14.91±0.31	6.81±0.47	52.43±1.20	74.15±1.98
89	493938	15.88±0.16	7.53±0.04	55.81±3.11	75.22±3.30
90	494018	15.64±0.56	6.52±0.02	52.56±2.35	75.72±2.92
91	494034	7.94±0.01	8.52±0.83	55.56±2.97	72.02±3.81
92	494795	13.53±0.13	7.44±0.43	52.54±2.23	73.51±2.79
93	496401	12.61±0.23	5.77±0.40	52.54±2.39	70.92±3.01
94	496448	12.79±0.39	5.93±0.91	53.15±1.86	71.87±3.15
95	497318	14.84±0.45	5.66±0.07	52.74±1.02	73.24±1.54
96 (MS-8)	497395	8.62±0.11[1]	4.26±0.32	60.03±0.82	72.91±1.25
97	497517	13.09±1.18	7.32±0.51	53.28±5.37	73.69±7.05
Ranges		6.33-17.02	3.66-9.29	46.62-61.97	68.05-76.58

Note: The allergen content was expressed by the % of total soluble protein. [1]represents significant lower level of that major allergen ($P < 0.05$).

Table 1. B. The major allergen content of 25 peanut cultivars from other resources harvested in year 2015 (Clovis, NM).

No.	Line name	Ara h 1	Ara h 2	Ara h 3	Total
1	M1	11.60±0.33	6.96±0.23	52.37±0.46	70.94±1.02
2	M2	12.74±0.58	6.14±0.08	50.43±0.74	69.30±1.40
3	M3	12.49±0.13	6.55±0.08	51.46±0.26	70.50±0.47
4	M4	12.01±0.37	7.12±0.08	51.73±3.16	70.87±3.61
5	M5	12.63±0.18	6.70±0.03	50.59±1.07	74.92±1.29
6	M6	12.44±0.43	6.75±0.02	51.90±0.92	71.09±1.37

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7	M7	12.38±0.05	6.67±0.06	52.57±0.52	71.62±0.63
8	M8	12.10±0.33	7.05±0.04	51.12±0.93	70.27±1.31
9	M9	13.34±0.90	6.46±0.28	51.19±4.08	70.98±5.27
10	M10	12.74±0.17	6.54±0.16	51.11±1.01	70.39±1.34
11 (MS-9)	Valencia-A	13.11±0.13	6.46±0.21	51.13±2.40	70.70±3.74
12	Valencia-C	13.63±0.33	6.21±0.32	51.69±0.56	71.53±1.22
13	GT-101	13.08±0.46	8.58±0.57	53.08±3.96	74.73±4.99
14	GT-102	13.25±0.65	11.50±0.50	50.69±0.72	75.44±1.87
15	GT-136	12.51±0.09	8.03±0.15	52.51±1.59	73.05±1.84
16	GT-112	13.32±0.06	6.84±0.22	50.63±0.99	70.79±1.27
17	GT-118	12.99±0.03	6.59±0.26	50.44±1.44	70.02±1.72
18	Kennedy	12.11±0.47	7.70±0.30	51.36±1.13	71.16±1.91
19	NM02322	12.59±0.52	8.35±0.12	50.41±0.71	71.35±1.35
20 (MS-10)	NM02565	16.03±0.33	8.65±0.04	46.70±1.29[1]	71.38±4.67
21	G-Red	13.17±0.57	8.07±0.44	53.35±2.88	74.59±3.90
22	G-Valencia	12.12±0.12	7.26±0.11	52.74±0.28	72.12±0.51
23	SunMex	13.62±0.87	7.55±0.61	54.01±3.93	75.18±5.41
24 (MS-11)	NM-308	18.10±2.22	9.75±0.38	43.98±4.66[1]	71.83±3.26
25	NM-309	14.63±0.84	7.97±0.61	52.56±4.04	75.17±5.48
Ranges		11.60-18.10	6.14-9.75	43.98-53.08	69.30-75.44

Note: The allergen content was expressed by the % of total soluble protein. *represent significant lower level of that major allergen ($P < 0.05$).

Fifty accessions of peanuts in our study were the same as the previous study [3], in which 99 accessions from a mini-core collection were used for peanut allergen quantification by means of SDS-PAGE. It was important to note that the peanut lines with the lowest level of Ara h 1 (PI 288210), Ara h 2 (PI 372305) and Ara h 3 (PI 494795) were all included in our study. In the Kang's study [3], low Ara h 1 line (PI 288210), low Ara h 2 line (PI 372305) and low Ara h 3 line (PI 494795) had 7%, 6.2%, and 21.8% of Ara h 1, Ara h 2, and Ara h 3, respectively. In our study, PI 288210 (MS-1) had also contained the lowest amount of Ara h 1 (6.33%) (Table 1A). For Ara h 2 content, PI 372305 contained 4.57% of the Ara h 2, which was not the lowest among all screened in the mini core collections planted in 2015. The relatively low Ara h 2 content peanut lines in our study were PI 343398 (MS-5), PI 475918 (MS-6), and PI 481795 (MS-7) (Table 1A), which had not been studied in the Kang's study [3]. Regarding Ara h 3 content, PI 494795 had 52.54% of the Ara h 3, which was not the lowest as compared with PI 292950 (MS-2), PI 296558 (MS-3) and PI 343384 (MS-4). Since the inconsistent results of the peanut allergen content existed between our study and Kang's study, a correlation study was conducted to better understand the peanut allergen consistency between two batches of peanuts (with same line numbers) from different production locations and years studied in our research. A correlation of major peanut allergens (Ara h 1, Ara h 2) was analyzed and the correlation was not significant ($R2 < 0.2$, $P > 0.05$), indicating that both year and location could affect the level of allergen. Therefore, it would be meaningful to screen the peanut in our study in order to obtain relatively lower allergen peanut lines for replanting in the following year (2016). Among 25 lines from other resources (Table 1B), Ara h 1 varied from 11.60% to 18.10%; Ara h 2 varied from 6.14 to 9.75%, Ara h 3 varied from 43.98% to 53.08% (Table 1B). Among 122 peanut lines in total, two lines (MS-1 and MS-8) were selected with lower Ara h 1; two lines (MS-5 and MS-6) were selected with lower Ara h 2; five lines (MS-2, MS-3, MS-4, MS-10, MS-11) were selected with lower Ara h 3; one line (MS-7) was selected with lower Ara h 1 & Ara h 2 (Tables 1A & 1B).

3.1.2. 2015. Selected Peanut Lines Harvested in 2016

From the results of the analyses of 2015 peanuts, eleven peanut lines were selected, re-planted and harvested in 2016. Ara h 1 varied from 7.84 to 15.52%; Ara h 2 varied from 6.65 to 8.95%; Ara h 3 varied from 46.05 to 57.85% (Table 2). MS-1 and MS-8 had relatively lower Ara h 1 (6-10%) in 2015

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and 2016. MS- 5 and MS-6 had relatively lower Ara h 2 (3.6-7.5%) in both years. MS-2 and MS-3 had relatively lower Ara h 3 (46-48%) in both years. MS-7 had relatively lower Ara h 1 (8.4-9.5%) & Ara h 2 (4.2-6.7%) in both years. However, the total percentage of these three major allergens were not significantly different among all accessions within the same year (Table 2).

Table 2. The major allergen content of 11 selected peanuts harvested in year 2015 and 2016.

		Ara h 1		Ara h 2		Ara h 3		Total	
Low in (allergen)	**Code No.**	**2015**	**2016**	**2015**	**2016**	**2015**	**2016**	**2015**	**2016**
Ara h1	MS-1	**6.33±0.22 g**	**10.33±0.52 cd**[1]	6.55±0.58 d	8.95±0.90 a[1]	57.17±2.95 a	51.70±3.65 abc[1]	70.05±3.75 a	70.98±5.07 a
Ara h3	MS-2	14.36±0.80 cd	12.90±0.77 b	7.27±0.71 c	7.11±0.59 bc	47.27±2.72 c	52.06±3.29 abc	68.90±3.93 a	72.07±4.66 a
Ara h3	MS-3	12.53±1.02 e	15.52±1.05 a[1]	6.85±0.52 d	7.22±0.48 bc	**48.67±2.40 bc**	**46.05±4.10 c**	68.05±3.94 a	68.78±5.62 a
Ara h3	MS-4	17.02±0.16 ab	15.26±1.69 a[1]	6.95±0.65 d	8.07±0.59 ab	**46.62±1.25 c**	**46.82±2.38 c**	70.59±3.76 a	70.15±4.66 a
Ara h2	MS-5	14.60±0.59 c	13.57±1.32 ab	**4.07±0.29 ef**	**7.11±0.67 bc**[1]	52.88±3.46 b	50.83±4.05 bc	69.55±4.35 a	71.51±6.04 a
Ara h2	MS-6	13.58±0.90 de	12.19±1.32 bc	**3.66±0.27 f**	**7.47±0.90 bc**[1]	59.33±5.43 ab	53.78±3.33 ab	76.58±6.00 a	73.44±5.55 a
Ara h1/Ara h2	MS-7	**8.41±0.10 f**	**9.49±1.31 de**	**4.16±0.45 ef**	**6.65±0.51 c**[1]	61.97±2.32 a	56.45±5.05 ab	74.54±3.87 a	72.59±6.88 a
Ara h1	MS-8	**8.62±0.11 f**	**7.84±1.22 e**	4.26±0.32 e	8.43±1.02 ab	60.14±3.85 a	57.85±4.91 a	73.03±3.28 a	74.12±7.15 a
Check	MS-9	13.11±0.13 d	12.29±1.12 bc	6.46±0.21 d	7.93±1.00 abc	51.13±2.40 b	52.23±3.38 abc	70.70±3.74 a	72.45±5.51 a
Ara h3	MS-10	16.03±0.33 b	12.59±1.47 b[1]	8.65±0.04 b	8.04±0.93 ab	46.70±1.29 c	51.78±4.12 abc	71.38±4.67 a	72.42±6.52 a
Ara h3	MS-11	18.10±2.22 a	12.83±1.12 b[1]	9.75±0.38 a	7.29±0.99 bc	43.98±4.66 c	55.05±5.61 ab[1]	71.83±3.26 a	75.18±7.73 a
	Range	6.33-18.10	7.84-15.52	3.66-9.75	6.65-8.95	43.98-61.97	46.05-57.85	68.05-76.58	68.78-75.18

Note: The results are the means of 3 determinations ± SD within a row followed by different letters are significantly different ($P < 0.05$). The allergen content was expressed by the % of total soluble protein. Data in **bold** represents the content were lower in both 2015 and 2016. Check cultivar was Valencia variety for comparison. [1]represent significant different between two year's data ($P < 0.05$).

In the study of Kang et al. [3], 60 accessions in the U.S. Peanut collection (all belonged to the min-core collection), along with the 88 Florida Peanut breeding program lines were analyzed for major peanut allergen levels. An accession from Nigeria had the highest level of Ara h 1 (18.5%), but the lowest level of Ara h 2 (6.2%). An accession from Zambia had the highest level of Ara h 2 (13.2%), but with the lowest level of Ara h 3 (21.8%). Our results were consistent with this report, which cited major allergen distribution varied among all lines but with similar total content. In Kang et al. [3] study, 20 lines, and two peanut cultivars (Florunner and Georgia Red) contained no or little of a 36 kDa Ara h 3 isoform, Ara h 3-im. In our study, MS-4 was found with little Ara h 3-im, and with very clear band on the other major allergens such as Ara h 1 and Ara h 2 (Figure 1).

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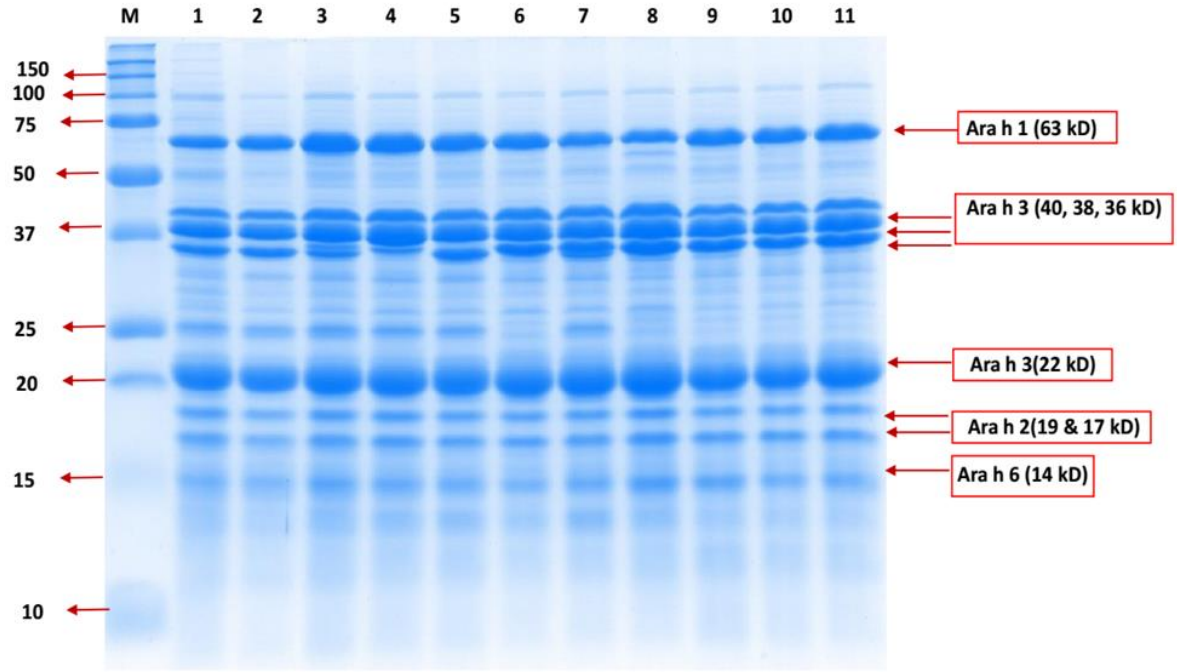

Figure 1. SDS-PAGE analysis of the eleven selected peanuts with lower allergens (harvested in 2016). M represents the molecular mass marker (molecular masses are shown beside the markers). Ara h 1 (63 kD), Ara h 2 (17, 19kD), Ara h 3 (22, 36, 38 & 40 kD) and Ara h 6 (14 kD) represents the major allergen of the peanuts. Lane 1-11 represents screened peanuts (MS-1– MS-11) polypeptides. MS-9 (Valencia) represents the check.

In the study of Pandey et al. [11], the low levels of major allergens Ara h 1, Ara h 2 and Ara h 3 peanut lines could reach to 4 μg/g, 41 μg/g, 22 μg/g from a 184 mini-core accessions, respectively. The major allergen content (Ara h 1, Ara h 2 and Ara h 3) of Pandey's study showed extremely low level, as compared with our study and Kang's study. The reason for the extremely significant differences may be largely due to the different analytical methods. The study of Pandey et al. [11] used ELISA method for quantification. It would be meaningful and interesting to analyze the same peanut used in the Pandey's study for allergen content by means of SDS-PAGE, to double check if there were some natural peanut lines containing such low amount of specific major allergens. Furthermore, the total major allergen content of each peanut lines had not been measured in the study of Pandey et al. [11], which made it difficult to understand their 'hypoallergenic lines' in a quantitative manner.

3.1. Two-Dimensional (2D) SDS-PAGE and Western-Blot Analyses of MS-7 and MS-9 Protein Extracts

The major protein (allergen) distribution of MS-7 and MS-9 were further compared using two-dimensional SDS-PAGE and western-blot techniques (Figures 2 and 3). According to the results from western-blot by using a commercial human plasma product (collected from a pool of people who are allergic to peanuts), containing anti-peanut IgE (Figures 2B and 3B), and comparisons with previous literatures [15], 19 distinct IgE-binding spots were labeled in the corresponded SDS-PAGE (Figures 2A & 3A) with number 1 to 19. The sensitivity of the antibodies in the human plasma was dependent upon the composition of the people (patients) with different sensitivities to various allergens. However, the results from the second dimension (SDS-PAGE) should be valid for relative comparisons of the quantities of allergens between two different varieties of peanuts. Spot 1 [pI (isoelectric point): 6.0-7.5] belonged to ten isoallergens of Ara h 1; spot 2 to 5 (pI: 5.0-5.4) belonged to isoallergens of Ara h 2; and spot 6 to 19 (pI: 4.2-8.2) belonged to isoallergens of Ara h 3 [15]. The quantification of each spot was conducted and results are shown in Table 2, which was expressed as the percentage of the total allergen content. The contents of Ara h 1 isoallergens (pI: 5.0-5.4) and Total

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Ara h 2 isoallergens (pI: 5.0-5.4) were significantly higher in MS-9 (Table 3). On the contrary, the contents of Total Ara h 3 isoallergens (pI: 4.2-8.2) were significantly higher in MS-7 (Table 3).

Figure 2. Two-dimensional (2D) SDS-PAGE (**A**) and Western-blot (**B**) of MS-7 (selected) peanut (harvested in 2016) protein. Human plasma (containing anti-peanut IgE antibody) was used for western-blot analysis. Major allergenic spots (1-8) were labeled in the correlated location in the SDS-PAGE. Spot 1: Ara h 1; Spot 2-5: Ara h 2; spot 6-19: Ara h 3, which were identified according to published works of [15,20].

Figure 3. Two-dimensional (2D) SDS-PAGE (**A**) and Western-blot (**B**) of MS-9 (check) peanut (harvested in 2016) protein. Human plasma (containing anti-peanut IgE antibody) was used for western-blot analysis. Major allergenic spots (1-8) were labeled in the correlated location in the SDS-PAGE. Spot 1: Ara h 1; Spot 2-5: Ara h 2; spot 6-19: Ara h 3, which were identified according to the published works [15,20].

Table 3. Quantification of major allergen spots between two peanut cultivars based on two-dimensional gels.

Spot No.[1]	Allergen	Estimate pI[2]	Percentage of total allergen	
			MS-7	MS-9
1	Ara h 1	6.0-7.5	28.02±2.22 b	37.23±2.92 a
2	Ara h 2	5.0	0.44±0.05 b	1.05±0.06 a
3	Ara h 2	5.1	0.86±0.06 b	1.26±0.11 a
4	Ara h 2	5.3	1.43±0.13 b	3.02±0.22 a
5	Ara h 2	5.4	1.68±0.14 b	3.02±0.23 a
2-5	Total Ara h 2	5.0-5.4	4.41±0.31 b	6.45±0.45 a
6	Ara h 3	4.2	8.08±0.57 a	4.12±0.29 b
7	Ara h 3	4.5	1.91±0.13 a	1.72±0.12 a
8	Ara h 3	4.6	1.00±0.07 a	1.03±0.07 a
9	Ara h 3	5.1	1.77±0.12 a	1.92±0.13 a
10	Ara h 3	6.8	0.91±0.06 a	0.25±0.02 b
11	Ara h 3	7.1	2.23±0.16 a	1.48±0.10 b
12	Ara h 3	8.2	1.41±0.10 a	1.12±0.08 b
13	Ara h 3	5.1	3.48±0.24 a	1.15±0.08 b
14	Ara h 3	6.0	3.43±0.24 a	1.17±0.08 b
15	Ara h 3	6.5	1.94±0.14 a	1.49±0.10 b
16	Ara h 3	6.8	0.82±0.13 a	0.57±0.04 b
17	Ara h 3	6.0	2.53±0.09 a	2.65±0.19 a
18	Ara h 3	6.4	3.71±0.27 a	1.74±0.12 b
19	Ara h 3	8.1	2.34±0.16 a	1.89±0.13 b
6-19	Total Ara h 3	4.2-8.2	35.57±2.49 a	22.29±1.56 b
1-19	Total allergens	-	67.00±4.69 a	66.97±4.01 a

Note: The results are the means of 3 determinations ± SD within a row followed by different letters are significantly different ($P < 0.05$). All spots are demonstrated with IgE-binding properties by western-blot and previous literature. [1]Spot 1: isoallergens of Ara h 1; spot 2-5: isoallergens of Ara h 2; spot 6-19: isoallergens of Ara h 3, which were identified according to the published works of [15,20]. [2]pI: Isoelectric Points

4. Discussions

Among the major allergen molecules of Ara h 1, Ara h 2 and Ara h 3, it had been reported their allergenicities (potencies) ranked in the order from high to low : Ara h 2 > Ara h 1 > Ara h 3 [16,17]. Therefore, the peanut cultivars with a lower content of Ara h 2 and Ara h 1 are preferred for human consumption. As shown in the study of Krause et al. [18], peanut lines with reduced Ara h 1 had not shown reduced allergenicity. It was meaningful to search for some low major allergen lines for future's cross-breeding or transgenic preparation for consuming after food processing. Therefore,

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MS-7 was chosen for the 2D-gel electrophoresis study for its relatively lower allergen levels in both years with Ara h 1 & Ara 2. MS-9 (Valencia, a popular commercial variety in the USA) was chosen as the check (control) for comparison. Koppelman and co-workers [6] had tested four major varieties of peanuts, including Valencia, Runner, Virginia and Spanish, to compare for their allergen contents. The results showed no significant differences between the tested samples by means of SDS-PAGE analyses, and minor differences by means of ELISA. Therefore, these market varieties of peanuts may contain relatively similar quantity of allergens. Based on the literature studies, it made sense to screen for low allergen peanuts from a broader source, including mini-core and experimental lines than merely analyzing traditional market-type peanuts.

The second-dimensional SDS-PAGE results were mutual supported with one-dimensional SDS-PAGE (Figure 1, Table 2), and consistent with the study of Kang et al., [3] who found that the major allergens in peanut seed would compensate for each other with the similar total allergen content (not reactivity). In the study of Schmidt et al. [19], they had compared an Indonesian peanut line (Kacang Asin) with a common peanut variety Virginia, and their results showed virtually nearly no Ara h 1 band/spot in the 1D/2D SDS-PAGE of Kacang Asin, and the Ara h 2 allergen level was also apparently lower than the Virginia variety in this Indonesian peanut line. Meanwhile, the Ara h 3 level was high in this Indonesian variety. Comparing with this previous study [20], the Ara h 1 level (9.49%) of MS-7 in the current study was relatively high. However, both two varieties (Kacang Asin and MS-7) had relatively lower Ara h 1 and Ara h 2, which would be preferred for the food industry. The Ara h 2 spots (spots 2-5) had less intensities as compared to Ara h 1 isoallergen spots (spot 1), the same situation had been reported in an earlier study of Chassaigne et al. [15] by using the same protein extraction method (TBS buffer, pH 7.4). The relatively mild extraction method in their studies might have resulted in a low extraction rate, which was reflected by lower band intensities. The relative mild treatment (neutral pH extraction) would have less influence on the protein denaturation during extraction and resulted in a better resolution in the 2D-gel. It is interesting to note that the extraction ratio of Ara h 3 was relatively lower by using the mild extraction method, the reason might be due to some basic subunit of Ara h 3 cannot be thoroughly extracted. However, one-dimensional (1D) SDS-PAGE (protein extracted with a slightly alkaline pH of 8.5) may reflect the extract ratio of the peanut allergen better. In the literature 2D-gel electrophoresis was more used for protein isoform qualification determinations than quantification. While, isoforms of Ara h 1-Ara h 3 in both varieties of peanuts were observed in our analyses (Figures 2 & 3), the main objective of our 2D-gel study was to provide a supporting information for our 1D gel and to show the potential differences of allergen isoform distributions (micro-heterogeneity of proteins) in a visible manner. In the future step, the spots labeled on the 2D-gel for both MS-7 and MS-9 should be taken for quantitative proteomic study.

In the study of Dodo et al. [21], peanuts with reduced (silenced) Ara h 2 by means of genetic engineering was found to have less allergenicity, which was analyzed by a combination of SDS-PAGE, western-blot and ELISA assays. Chu et al. [22] had the same results by silencing Ara h 2 and Ara h 6. However, it still has not been fully accepted by all scholars that peanut variety with reduced Ara h 2 would possess low allergenicity [23], because it needs to be taken into consideration the sensitivity of the population to different allergens that is dependent upon ethnicity and the geographic distribution. In USA, large amount of people had allergen sensitivity to Ara h 1, Ara h 2, and Ara h 3. While in the Mediterranean area, Ara h 9 is the dominant allergen for the peanut allergy than any others as revealed by two studies [24,25]. Therefore, it should be noted that genetic method or screening method alone would be not enough to reduce the peanut allergen into a safe level for all population.

It is commonly believed that a combination of several processing methods, such as HPP and heat; ultrasound and enzymatic hydrolysis or germination; and enzymatic hydrolysis and roasting, may have the potential to reduce the allergenicity in a significant level [2]. In our published study, the selected peanut (MS-7) also showed lower IgE-binding properties after roasting and hydrolysis as compared with the control peanut (MS-9) treated by the same processing methods [12]. Nonetheless, our current study provided a very meaningful reference to the geneticist, breeders and food industry for future improvement of peanuts for human consumption.

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5. Conclusions

Since Ara h 1 and Ara h 2 could produce allergic reactions in 90% of the patients, who had allergy to peanuts, whereas Ara h 3 only contributed to 50% of the IgE reaction to peanut allergy patients [16]. It would be meaningful to screen the peanut lines with both lower Ara h 1 & Ara h 2. By using the rapid electrophoresis method, peanut MS-7 was identified to have a lower levels of the allergens (Ara h 1 & Ara h 2) compare to other lines. If we could use MS-7 to cross-breed with itself (back-crossing) or with other low major allergen lines, some lower allergen lines may be obtained. Furthermore, using this rapid SDS-PAGE method, more lines from USA and from the whole world (as many as 14,000 lines) can be analyzed; and a better line may be identified. The selected line could be used for further food processing treatments and could contribute to achieve ideal hypoallergenic foods in the future.

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