Title: Identification of Peanut Allergens Extracted Using Protea® Cell Lysis Kits

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Introduction

Protea acid labile surfactant cell lysis kits are used to extract proteins from peanut samples which are further identified by MALDI-TOF mass spectrometry. Peanut proteins are classified into two main groups, albumins or globulins. Globulins are subdivided into arachin and conarachin proteins and the albumins are comprised of agglutinins, lectin-reactive glycoproteins, protease inhibitors, α-amylase inhibitors and phospholipases (1). Some peanut proteins are known food allergens, capable of triggering allergic reactions. Several peanut proteins have been identified as peanut allergens, namely Arah1 to Arah8. Of these Arah proteins, peanut allergies are mainly caused by Arah1, Arah2, and Arah3/4 (1). These allergens are homologous to the seed storage protein families of vivilin, conglutin, and glycinin (2). Arah1 is known to be the most abundant allergen followed by Arah2, and then Arah3/4. Peanut proteins are classified as either albumins or globulins.

This work demonstrates the ability of Protea cell lysis kits based on acid labile surfactant chemistries to effectively extract proteins, with very high yield, from peanut samples. A proper assessment of individual peanut allergens in these samples is carried out using 1D and 2D gel electrophoresis followed by MALDI-TOF mass spectrometry. Protea's anionic surfactant based cell lysis kit is used to extract samples for 1-D gel electrophoresis, whereas the non-ionic based cell lysis kit is used to extract proteins for 2-D gel electrophoresis, after which gel spot were excised, digested with trypsin and subjected to mass spectrometry analysis. After digestion, the samples did not require sample clean-up for MALDI-TOF-MS analysis confirming the compatibility of the lysis kits with mass spectrometry

Experimental

Protein Extraction

Raw peanuts (*Arachis hypogaea*) were ground using mortar and pestle into a fine and homogeneous powder. Hexane was used to remove lipids from the peanut flour. This process was done by adding enough hexane to fully wet all flour then sonicating for 15 minutes. The supernatant was then removed, and the delipidation step was repeated twice. The flour was left in a fume hood to fully evaporate any liquids. Peanut flour was rinsed twice at 4°C using TBS buffer (25 mM tris with 150 mM NaCl), pH 7.5. The proteins from peanut flour were extracted using both the ProteaPrep Anionic and Non-ionic Cell Lysis Kits (Protea Biosciences Group, Morgantown, WV, USA). Protein extraction was carried out for 30 minutes with sonication under ambient conditions. The supernatant was collected after centrifuging at 12000 x g for 10 minutes. Peanut extracts were cleaned with an Amicon Ultra 3 membrane kit from Millipore (Billerica, Massachusetts, USA) prior to the determination of their protein content.

1D electrophoresis

For 1D gel electrophoresis analysis, the lysate was combined 1:1 with Laemmli plus β-mercaptoethanol. 1D separation was performed using ProteaGel 10% Solution with a ProteaGel 5% Solution stacking gel (Protea Biosciences Group) with a Mini-PROTEAN® system (BioRad Laboratories, Hercules, CA, USA). Separation was achieved by applying constant 180 V for a run time of 40 minutes. Gels were removed and placed immediately in a fixation solution (50% methanol, 10% acetic acid) for 1 hour. After rehydrating in ddH_2O for 10 minutes, gels were stained with Coomassie stain (20% methanol, 10% ammonium sulfate, 10% phosphoric acid, 0.1 g/L CB G-250).

2D electrophoresis

First dimension IEF was carried out on a PROTEAN® system (BioRad). Protein estimation was performed using ProteaPrep Coomassie Protein Quantitation Kit (Protea Biosciences Group). Five hundred micrograms of protein sample were diluted with IPG rehydration/sample buffer to a volume of 185 µL. The sample was loaded on non-linear 11 cm Immobiline DryStrip (GE Healthcare, Piscataway, NJ, USA). IPG strips were hydrated for 16 hours at room temperature. Separation in the first dimension was performed with an initial voltage of 250 V for 15 minutes, then a gradient up to 8000 V over 2.5 hours, and finally maintenance of 8000 V for 35000 VHours.

The gel strips were equilibrated with 2% SDS in a urea based solution (6 M urea/ 0.375 M tris-HCl/ 20% glycerol) with 2% DTT for 10 minutes and again with 2.5% iodoacetamide for 10 minutes. The gel strips were embedded on top of the SDS-PAGE gel in an agarose overlay (0.5% LM agarose/ 25 mM tris/ 192 mM glycine/ 0.1% SDS/ bromophenol blue)

Second dimension separation was carried out on a Mini-PROTEAN® system (BioRad). A 10% ProteaGel with 5% ProteaGel stacking gel was run at a constant 200 V for 55 minutes. The gel was removed and

immediately placed in fixation solution followed by rehydration and Coomassie G-250 staining.

Results and Discussion

To identify the position of major peanut allergens, bands from a 1D gel were excised and taken through tryptic digestion. All visible bands were processed for identification. The bands that were chosen are indicated on the gel image in Figure 1. The digests were then analyzed with an AB Sciex MALDI-TOF/TOF mass spectrometer. Results for the highest scoring proteins for each band are summarized in Tables 1 and 2.

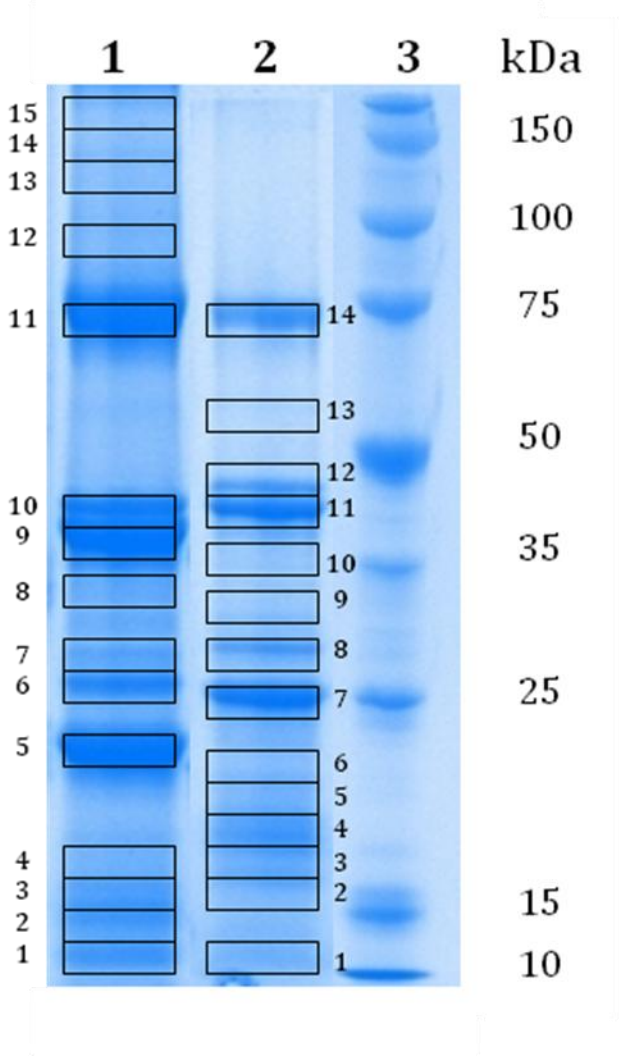

Figure 1. 1D gel separation of peanut lysate extracted using ProteaPrep Cell Lysis Kits, Mass Spec Grade. Lane 1, 20 µL of peanut lysate extracted with ProteaPrep Anionic Cell Lysis Kit, Mass Spec Grade; Lane 2, 20 µL of peanut lysate extracted with ProteaPrep Non-ionic Cell Lysis Kit, Mass Spec Grade; Lane 3, protein molecular weight markers.

Table 1: Peanut lysate sample identification from Protea® ProteaPrep Anionic Cell Lysis Kit, Mass Spec Grade.

Spot ID	Protein Accession	Protein Description	Protein Score
Peanut 1	gi|22135348	trypsin inhibitor	285
Peanut 2	gi|52001219	arachin Ahy-1	177
Peanut 3	gi|33416100	photosystem II subunit H	58
Peanut 4	gi|118776570	arachin 6	201
Peanut 5	gi|118776570	arachin 6	121
Peanut 6	gi|118776570	arachin 6	368
Peanut 7	gi|52001219	arachin Ahy-1	292
Peanut 8	gi|22135348	trypsin inhibitor	190
Peanut 9	gi|9864777	Gly1	338
Peanut 10	gi|52001219	arachin Ahy-1	223
Peanut 11	gi|1168391	Allergen Ara h 1, clone P41B precursor (Ara h I)	83
Peanut 12	gi|1168391	Allergen Ara h 1, clone P41B precursor (Ara h I)	141
Peanut 13	gi|1168391	Allergen Ara h 1, clone P41B precursor (Ara h I)	166
Peanut 14	gi|1168391	Allergen Ara h 1, clone P41B precursor (Ara h I)	209
Peanut 15	gi|46560478	conarachin	226

Table 2: Peanut lysate sample identification from Protea® ProteaPrep Non-ionic Cell Lysis Kit, Mass Spec Grade.

Spot ID	Protein Accession	Protein Description	Protein Score
Peanut 1	gi|5542503	Chain A, Trypsin Inhibitors With Rigid Tripeptidyl Aldehydes	169
Peanut 2	gi|21314465	allergen Arah3/Arah4	431
Peanut 3	gi|21314465	allergen Arah3/Arah4	345
Peanut 4	gi|52001221	arachin Ahy-2	145
Peanut 5	gi|23965775	methionine aminopeptidase	42
Peanut 6	gi|230338	Chain E, Structure Of The Trypsin Binding Domain Of Bowman-Birk	58
Peanut 7	gi|21314465	allergen Arah3/Arah4	263
Peanut 8	gi|118776570	arachin 6	363
Peanut 9	gi|21314465	allergen Arah3/Arah4	239
Peanut 10	gi|9864777	Gly1	72
Peanut 11	gi|21314465	allergen Arah3/Arah4	336
Peanut 12	gi|21314465	allergen Arah3/Arah4	163
Peanut 13	gi|52001221	arachin Ahy-2	99
Peanut 14	gi|46560478	conarachin	144

To identify the isoforms of peanut allergens from the 2D gel, spots were excised and taken through tryptic digestion. The gel spots were chosen using the above data to guide the selection. The samples are indicated below on the gel image in Figure 2. The digests were analyzed using an AB Sciex MALDI-TOF/TOF mass spectrometer. Results are summarized in Table 3.

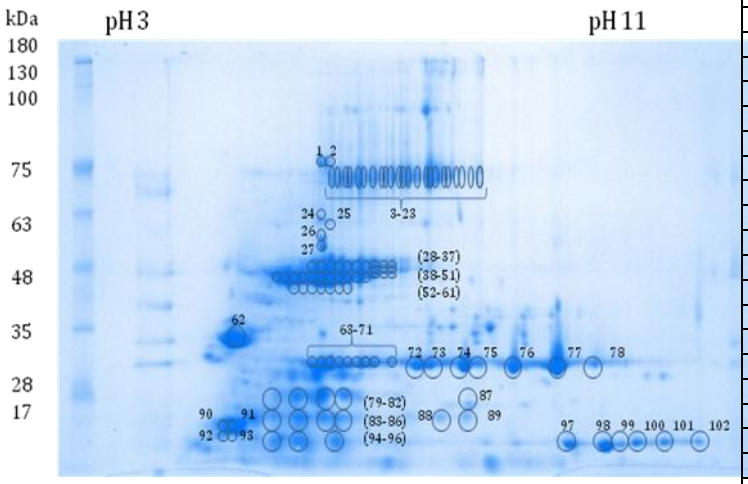

Figure 2. A 2D gel image of 500 µg of peanut lysate extracted using ProteaPrep Non-ionic Cell Lysis Kit, Mass Spec Grade. Spots of interest were excised, submitted to tryptic digestion and analyzed by MALDI-TOF/TOF.

Table 3: Peanut lysate sample identification from Protea® ProteaPrep Non-ionic Cell Lysis Kit, Mass Spec Grade.

Spot ID	Protein Accession	Protein Description	Protein Score
1	gi|9864777	Gly1	243
2	gi|22135348	trypsin inhibitor	61
3	gi|46560478	conarachin	229
4	gi|46560478	conarachin	189
5	gi|46560478	conarachin	443
6	gi|46560478	conarachin	310
7	gi|46560478	conarachin	170
8	gi|68552534	Ribosomal protein L7/L12	66
9	gi|46560478	conarachin	82
10	gi|46560478	conarachin	268
11	gi|46560478	conarachin	198
12	gi|46560478	conarachin	263
13	gi|46560478	conarachin	232
14	gi|46560478	conarachin	168
15	gi|1168391	Allergen Ara h 1, clone P41B precursor (Ara h l)	456
16	gi|46560478	conarachin	434
17	gi|46560478	conarachin	143
18	gi|46560478	conarachin	208
19	gi|32491072	hypothetical protein WGLp323	56
20	gi|145237828	hypothetical protein An07g04780	52
21	gi|22135348	trypsin inhibitor	74
22	gi|73980575	PREDICTED: similar to SMC6 protein	58
23	gi|68552534	Ribosomal protein L7/L12	64
24	gi|9864777	Gly1	363
25	gi|115464543	Os05g0484800	58
26	gi|1669765	iron superoxide dismutase	46
27	gi|22135348	arachin Ahy-4	164
28	gi|22135348	trypsin inhibitor	72
29	gi|22135348	allergen Arah3/Arah4	339
30	gi|22135348	allergen Arah3/Arah4	150
31	gi|22135348	trypsin inhibitor	79
32	gi|22135348	allergen Arah3/Arah4	150
33	gi|116253518	putative LysR family transcriptional regulator	55
34	gi|22135348	allergen Arah3/Arah4	173
35	gi|15229438	60S ribosomal protein L36a/L44 (RPL36aA)	46
36	gi|230338	allergen Arah3/Arah4	83
37	gi|22135348	trypsin inhibitor	56
38	gi|9864777	Gly1	63
39	gi|22135348	arachin Ahy-4	135
40	gi|22135348	allergen Arah3/Arah4	231
41	gi|22135348	Gly1	401
42	gi|57669861	arachin Ahy-4	230
43	gi|22135348	allergen Arah3/Arah4	368
44	gi|22135348	allergen Arah3/Arah4	381
45	gi|22135348	allergen Arah3/Arah4	386
46	gi|22135348	allergen Arah3/Arah4	483
47	gi|22135348	allergen Arah3/Arah4	270
48	gi|22135348	arachin Ahy-4	236
49	gi|22135348	allergen Arah3/Arah4	195
50	gi|22135348	trypsin inhibitor	83
51	gi|22135348	arachin Ahy-4	203
52	gi|9864777	Gly1	570
53	gi|9864777	Gly1	615
54	gi|57669861	arachin Ahy-4	469
55	gi|22135348	arachin Ahy-4	588
56	gi|9864777	Gly1	515
57	gi|9864777	Gly1	484
58	gi|9864777	Gly1	694
59	gi|9864777	Gly1	403
60	gi|9864777	Gly1	450
61	gi|22135348	arachin Ahy-2	497
62	gi|118776570	arachin 6	235
63	gi|9864777	Gly1	319
64	gi|9864777	Gly1	382
65	gi|9864777	Gly1	366
66	gi|9864777	Gly1	360
67	gi|9864777	Gly1	61
68	gi|9864777	Gly1	254
69	gi|9864777	Gly1	302
70	gi|57669861	arachin Ahy-4	329
71	gi|118776570	arachin 6	301
72	gi|118776570	arachin 6	492
73	gi|118776570	arachin 6	407
74	gi|37789212	allergen Arah3/Arah4	233
75	gi|118776570	arachin 6	337
76	gi|126457770	hypothetical protein BURPS1106A_A1463	53
77	gi|9864777	Gly1	297
78	gi|9864777	Gly1	305
79	gi|147639154	allergen II	192
80	gi|148613175	Ara d 2.01	277
81	gi|158344609	conglutin	406
82	gi|158344609	conglutin	311
83	gi|150005204	putative EPS related membrane protein	54
84	gi|75114094	Conglutin precursor (Allergen Ara h 6)	254
85	gi|158344609	Conglutin	243
86	gi|89099005	propionyl-CoA carboxylase	58
87	gi|118776570	arachin 6	308
88	gi|230338	2S protein 2	252
89	gi|230338	2S protein 2	164
90	gi|230338	allergen Arah3/Arah4	157
91	gi|118776570	arachin 6	158
92	gi|9864777	Gly1	111
93	gi|118776570	arachin 6	320
94	gi|17225991	conglutin	304
95	gi|75114094	Conglutin precursor (Allergen Ara h 6)	282
96	gi|118776570	arachin 6	244
97	gi|52001219	arachin Ahy-1	430
98	gi|22135348	allergen Arah3/Arah4	392
99	gi|21314465	allergen Arah3/Arah4	487
100	gi|5712199	glycinin	540
101	gi|5712199	glycinin	603
102	gi|118776570	arachin 6	593

Identifications of various isoforms of peanut allergens were obtained in the mass ranges 65-68 kDa, 45-50 kDa, and 15-18 kDa. The isoforms found in the mass range of 65-68 kDa gave an identification of Arah1. The allergen found at 45-50 kDa was identified as Arah3/4. Arah2 was identified in the mass range of 15-18 kDa. Multiple storage proteins were also identified along with other known allergens.

Conclusion

The combination of ProteaPrep Cell Lysis Kits, 1D and 2D PAGE, along with MALDI-TOF/TOF allows for specific detection and identification of multiple allergens and peanut proteins from the 2-D gel. Within this gel, the allergen Ara h 1 was resolved and detected in 15 protein spots. Allergen Ara h 2 was identified in 7 protein spots in the mass range of 15-25 kDa. Ara h 3/4 had multiple mass ranges with positive identification: 14 kDa, 30 kDa, and 45-55 kDa. Several storage proteins along with other allergens were identified. The complexity of peanut allergens is further supported by the multitude of identifications from allergens and protein sub-units. The ability of various cell lysis buffers to extract different proteins and allergens at a multitude of mass ranges adds an insightful view of naturally occurring allergen modification.

References:

(1) Loza C, Brostoff J Peanut allergy. Clin Exp Allergy 1995; 25(6): 493-502

(2) Bohle B, Swoboda I, Spitzauer S, et al. Food Antigens: structure and function. Food Allergy: Adverse reactions to Foods and Food Additives. Blackwell Scientific Publications 2003: 38-50