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An efficient extraction method to enhance analysis of low abundant proteins from soybean seed

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ABSTRACT

Large amounts of the major storage proteins, β-conglycinin and glycinin, in soybean (*Glycine max*) seeds hinder the isolation and characterization of less abundant seed proteins. We investigated whether isopropanol extraction could facilitate resolution of the low abundant proteins, different from the main storage protein fractions, in one-dimensional polyacrylamide gel electrophoresis (1D-PAGE) and two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). 1D-PAGE of proteins extracted by different concentrations (10%, 20%, 30%, 40%, 50%, 60%, 70% and 80%) of isopropanol showed that greater than 30% isopropanol was suitable for preferential enrichment of low abundant proteins. Analysis of 2D-PAGE showed that proteins which were less abundant or absent by the conventional extraction procedure were clearly seen in the 40% isopropanol extracts. Increasing isopropanol concentration above 40% resulted in a decrease in the number of less abundant protein spots. We have identified a total of 107 protein spots using matrix-assisted laser desorption/ionization time of flight mass spectrophotometry (MALDI-TOF-MS) and liquid chromatography–mass spectrometry (LC–MS/MS). Our results suggest that extraction of soybean seed powder with 40% isopropanol enriches lower abundance proteins and is a suitable method for 2D-PAGE separation and identification. This methodology could potentially allow the extraction and characterization of low abundant proteins of other legume seeds containing highly abundant storage proteins.

Proteomic technologies including two-dimensional polyacrylamide gel electrophoresis (2D-PAGE)[1] and mass spectrometry (MS) are powerful and efficient tools that have been successfully used for genetic and proteomic studies of plants, animals, microbes, and humans [1–5]. Recent advances in MS, together with the large number of expressed sequence tag (EST) sequences (http://www.ncbi.nlm.nih.gov/dbEST/) and the availability of several genome sequences substantially increase the accuracy of protein profile characterization from complex protein mixtures.

Recently, proteomic tools have been used to test and detect unintended effects in genetically modified plants [6,7]. 2D-PAGE systems were used to separate various globulin proteins, allergen, and anti-nutritional proteins from soybean seeds including wild and cultivated soybean genotypes [8–12]. Sample extraction and preparation are critical for the analysis of proteins over a wide range of abundance levels and the extraction of protein is highly sample dependent [13]. Analysis of wheat leaf proteins required precipitation of the proteins with cold acetone prior to resuspension in lysis buffer [14]. Extraction methods utilizing phenol followed by precipitation with cold ammonium acetate and methanol, acetone, or ethanol have been used for the seeds, leaves, and woody tissue samples [15]. Recently, Wang and co-workers [16] reported that a TCA method combined with phenol was efficient for extracting leaf proteins from bamboo, lemon, olive, and redwood and from apple, pear, banana, grape, tomato, and orange fruits. Direct precipitation of protein from powdered tissues using TCA/acetone has been used in our laboratory to successfully extract proteins from soybean seeds and leaves [2,9]. Using this extraction method, we resolved both abundant and less abundant proteins. Since soybean seeds contain a large proportion of abundant storage proteins that account for about 70–80% of the total seed protein it remained a challenge to isolate both abundant and less abundant proteins using conventional extraction methods. Low abundance regulatory proteins are mostly out of the scope of standard protein extraction techniques [17]. Aqueous ethanol, isopropanol, and *tert*-

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[1] *Abbreviations used*: CHCA, α-cyanohydroxycinnamic acid; BBI, Bowman–Birk inhibitor; 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis; DTT, dithiothreitol; KTI, Kunitz trypsin inhibitor; LEA, late embryogenesis-abundant proteins; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time of flight mass spectrophotometry; SDS, sodium dodecyl sulfate; SOD, superoxide dismutase.

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butanol are often used to extract prolamins from various cereals. Since our earlier study [18] indicated that isopropanol extraction resulted in the depletion of the abundant seed storage proteins, we hypothesized that this solvent could be used to enrich seed proteins that are different from the main storage proteins. To separate the maximum number of low abundance proteins that are different from the main storage proteins in soybean seed, we have evaluated the effectiveness of different concentrations of isopropanol as an extraction solvent. The development of this extraction protocol is important for identification of new low abundance proteins that may occur in genetically modified crops, and is also potentially useful for biotech industries interested in modification of soybean protein quality.

Materials and methods

Chemicals

Acrylamide, bis-acrylamide, SDS, TEMED, ammonium persulfate, thiourea, dithiothreitol, IPG buffer (pH 4–7), CHAPS for electrophoresis were purchased from GE Healthcare (Piscataway, NJ). Urea, ammonium sulfate, and glycine were purchased from USB Corporation (Cleveland, OH). Tris–HCl (pH 8.8), 2-mercaptoethanol, and glycerol were purchased from Sigma (St. Louis, MO). α-Canohydroxycinnamic acid (CHCA) matrix was purchased from Bruker Daltonics (Billerica, MA). All other chemicals were standard reagent grade laboratory chemicals. Water from a Millipore Milli-RO4 reverse osmosis system was used for making all solution.

Plant materials

Soybean seeds [*Glycine max* (L.) Merr.] of cultivar Williams 82 were obtained from the USDA soybean germplasm collection, Urbana, Illinois. The soybean seeds used in the current study were planted in the Beltsville greenhouse. The environmental/growth conditions were controlled using a computer program for photoperiod and temperature similar to field conditions to minimize protein variations due to environmental conditions. Seeds were stored at −80 °C until used.

Protein extraction using isopropanol

Total protein extracts were prepared from dry mature soybean seeds. Seeds were powdered in liquid nitrogen using a chilled mortar and pestle and 500 mg of the soybean seed powder was weighed and placed in a tube with 5 ml of a solution containing known concentrations (30%, 40%, 50%, 60%, and 70%) of isopropanol. Protein extraction was carried out on an orbital shaker (Sorvell Instrument, Dupont) for 1 h at 18,500 rpm. The protein extract was centrifuged at 13,000 rpm for 10 min at 4 °C. The supernatant was removed and placed in a clean 15-ml glass tube. To this supernatant, 10 ml of cold acetone was added and vortexed thoroughly. The extract was further incubated at −20 °C overnight. Next day, the samples were centrifuged for 20 min at 8000 rpm at 4 °C and the pellet was dried at room temperature for 30 min. The pellet was resuspended in 0.5 ml of lysis buffer [9 Murea, 1% CHAPS, 0.5% IPG buffer (pH 4–7) and 1% DTT] followed by vortexing till the pellet completely dissolved. Then the protein quantity was determined for 2D-PAGE analysis, according to Natarajan and coworkers [9] using a commercial dye reagent (Bio-Rad).

1D-electrophoresis

The protocol for 1D-electrophoresis was performed according to Krishnan [18]. Proteins extracted with different concentrations of isopropanol were resolved by SDS–PAGE on a 15% resolving gel at 20 mA using a Hoefer SE 260 minigel apparatus (Amersham Biosciences, Piscataway, NJ). Proteins were visualized by staining overnight with Coomassie blue G-250.

2D-electrophoresis

The protocols and conditions were similar to those described by Natarajan and co-workers [9]. The first-dimension IEF was performed using 13 cm pH 4–7 linear IPG strips in the IPGphor system (GE Healthcare). All IPG strips were rehydrated with 250 μl rehydration buffer (8 M urea, 2% CHAPS, 0.5% IPG buffer, 0.002% bromophenol blue), containing 100 μg of protein and DTT. The voltage settings for isoelectric focusing were 500 V for 1 h, 1000 V for 1 h, and 8000 V to a total 14.5 kV h. The focused strips were either immediately run on a second-dimension gel electrophoresis or stored at −80 °C. For the second-dimension gel electrophoresis, the gel strips were incubated with equilibration buffer 1 (50 mM Tris–HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 0.002% bromophenol blue, 1% DTT) and equilibration buffer 2 (50 mM Tris–HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 0.002% bromophenol blue, 2.5% iodoacetamide) for 15 min each on a shaker. After decanting the equilibration buffer, the strips were washed with water and placed on to 12% polyacrylamide gel (18 × 16 cm) with Tris–glycine buffer system. Strips were overlayed with agarose sealing solution. The Hoefer SE 600 Ruby electrophoresis unit (GE Healthcare) was used according to the manufacturer's recommendations. The 2D-PAGE gels were visualized by staining with colloidal Coomassie blue G-250. The gels were fixed overnight in 50% ethanol and 10% acetic acid followed by 3× 30 min washes with deionized water. Then the gels were prestained for 1 h in 34% methanol, 17% ammonium sulfate, and 3% phosphoric acid, and then stained in the same solution containing Coomassie blue G-250 (0.066%) for 2 days. After that the gels were washed with distilled water and stored in 20% ammonium sulfate solution. All gels were scanned using laser densitometry (PDSI, GE Healthcare) and the images were analyzed with Image Master 2D-Elite (version 4.01) software. Triplicate samples were used for soybean seed protein extraction and 2D-PAGE analysis.

In-gel digestion of protein spots

Protein spots were excised from the stained gel and washed first with distilled water to remove ammonium sulfate and then with 50% acetonitrile containing 25 mM ammonium bicarbonate to destain the gel plug. The gel plug was dehydrated with 100% acetonitrile, dried under vacuum, and then reswollen with 20 μl of 10 μg/ml trypsin (modified porcine trypsin, sequencing grade, Promega, Madison, WI) in 25 mM ammonium bicarbonate. Digestion was performed overnight at 37 °C. The resulting tryptic fragments were extracted with 50% acetonitrile and 5% trifluoroacetic acid with sonication. The extract was dried to completeness and then dissolved in 50% acetonitrile and 0.1% trifluoroacetic acid.

Protein identification

MALDI-TOF-MS analysis of tryptic peptides

Peptide mass fingerprints of typtic digests were acquired with a Voyager DE-STR MALDI-TOF mass spectrometer (Applied Biosystems, Framingham, MA) operated in positive-ion reflector mode using CHCA as the matrix. Spectra were calibrated using the trypsin autolysis peaks at *m/z* 842.51 and 2211.10.

MS/MS analysis of tryptic peptides

A Thermo Finnigan LCQ Deca XP plus Ion Trap mass spectrometer was used to analyze proteins that were not positively identi-

fied by MALDI-TOF mass spectrometry. Peptides were separated by reverse-phase chromatography on a 100 × 0.18 mm BioBasic-18 column using a 30 min linear gradient from 5% to 40% ACN in 0.1% formic acid at a flow rate of 3 μl/min. The instrument was operated in data-dependent mode with a duty cycle that acquired and recorded MS/MS spectra of the three most abundant ions determined by a survey scan over the range of 400–1600 *m/z*. Dynamic exclusion was employed to prevent the continuous analysis of the same ions. Once two MS/MS spectra had been acquired from any given ion, the parent mass was placed on an exclusion list for the duration of 3 min.

Protein identification was performed by searching the NCBI nonredundant database using the Mascot search engine (http://www.matrixscience.com), which uses a probability-based scoring system. The following parameters were used for database searches with MALDI-TOF peptide mass fingerprinting data: monoisotopic mass, 25 ppm mass accuracy, trypsin as digesting enzyme with 1 missed cleavage allowed, carbamidomethylation of cysteine as a fixed modification, oxidation of methionine, N-terminal pyroglutamic acid from glutamic acid or glutamine as allowable variable modifications. For database searches with MS/MS spectra the following parameters were used: average mass, 1 Da peptide and MS/MS mass tolerance, peptide charge of +1, +2, or +3, trypsin as digesting enzyme with 1 missed cleavage allowed, and the same fixed and variable modifications as were used for searching MALDI-TOF-MS data. Taxonomy was limited to green plants for both MALDI-TOF-MS and MS/MS ion searches. For MALDI-TOF-MS data to qualify as a positive identification, a protein's score had to equal or exceed the minimum significant score. Positive identification of proteins by MS/MS analysis required a minimum of two unique peptides, with at least one peptide having a significant ion score.

Results and discussion

Various methods involving solvent extraction have been used to analyze and enrich low abundant proteins in various crops [17–19]. Ferro and co-workers [19] used chloroform/methanol extraction to enrich low abundant proteins and chloroplast membrane proteins in *Arabidopsis thaliana*. Likewise, Marmagne and co-workers [17] were able to identify about 100 low abundant plasma membrane integral proteins from *Arabidopsis* plasma membrane-enriched fractions following treatment with chloroform/methanol. Cole and co-workers [20] used size-exclusion chromatography to separate proteins. Hurkman and Tanaka [15] used phenol to solubilize soybean proteins and reported separation of more abundant proteins and some lower abundance proteins. We compared eight different concentrations (10%, 20%, 30%, 40%, 50%, 60%, 70%, and 80%) of isopropanol in water to determine their ability to preferentially solubilize lower abundance soybean seed proteins for analysis by 1D-PAGE. In 1D-PAGE, 10% and 20% isopropanol extracts contained abundant proteins including the storage proteins, β-conglycinin and glycinin (Fig. 1. lanes 1 and 2). Above 20% isopropanol concentrations, extraction of abundant storage proteins decreased, improving the observance of lower abundance protein bands. In our 1D-PAGE analysis, we observed that 30%, 40%, and 50% isopropanol extracts exhibited the lower abundance proteins. At higher concentrations of isopropanol there was a decrease in the number of protein bands, and almost no protein was found in the 80% isopropanol extract. Therefore, we have chosen concentrations between 30% and 60% isopropanol as optimal for further characterization of the low abundance proteins using 2D-PAGE.

Several different solubilization protocols have been used in attempts to analyze soybean seed and other crop proteins. Hu and Esen [21] used exhaustive sequential extraction to extract proteins from defatted soybean meal and subjected to 2D-PAGE. The authors reported that three solubility fractions such as water-soluble, NaCl-soluble, and acetic acid-soluble fractions yielded 647, 543, and 346 protein spots, respectively. The water-, salt-, and acetic acid-soluble fractions cumulatively represented 91% of the total seed proteins with poor content of sulfur amino acids. In contrast, the isopropanol-soluble fraction, which represented less than 1% of the total seed protein, had a substantial fraction of the overall sulfur amino acid containing seed proteins. Flengsrud and Kobro [22] used three-step extraction procedures to extract barley and potato leaves and spruce needles proteins and obtained good and reproducible separation using 2D gel electrophoresis. The three steps included grinding the tissues in pH 5.0 buffer with thiourea and precipitation with acetone followed by dialysis. Thiering and co-workers [23] manipulated solvent properties to remove some of the abundant soybean globulins. Rao and co-workers [24] used calcium ions to bind soybean proteins and Deak and co-workers [25] further fractionated soybean storage proteins using calcium and $NaHSO_3$.

To further analyze and characterize the lower abundance soybean seed proteins extracted with isopropanol, we utilized 2D-PAGE. Extraction with four different concentrations of isopropanol (30%, 40%, 50%, and 60%) solubilized different proportions of the major seed proteins and the lesser abundant proteins (Fig. 2, I, II,

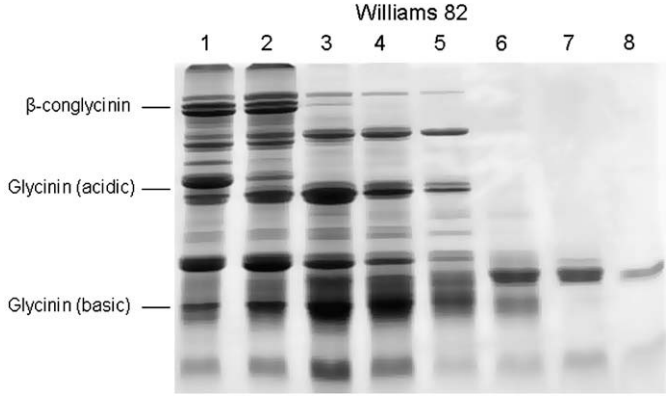

Fig. 1. One-dimensional electrophoretogram of soybean proteins extracted using various concentrations of isopropanol. Equal volumes of protein samples extracted from equivalent amounts of seed powder were loaded onto lanes of a 12% SDS–PAGE. Gels were stained with Coomassie blue stain G-250. Lane 1, 10%; lane 2, 20%; lane 3, 30%; lane 4, 40%; lane 5, 50%; lane 6, 60%; lane 7, 70%; and lane 8, 80% isopropanol extracted proteins.

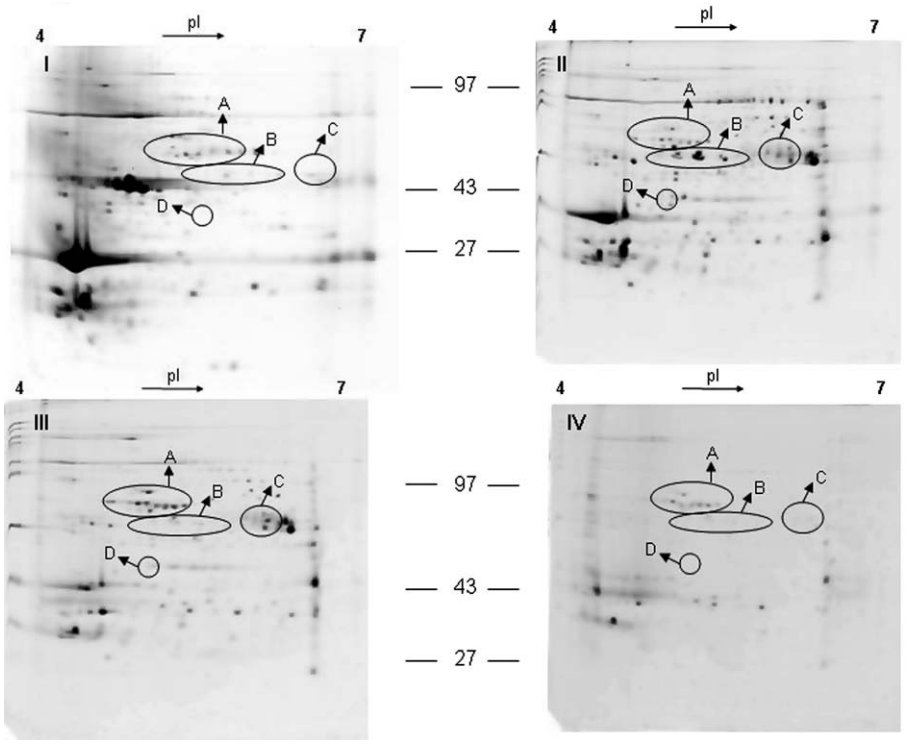

Fig. 2. 2D-PAGE comparison of *G. max* soybean seeds extracted with different concentrations of isopropanol. I, 30% isopropanol; II, 40% isopropanol; III, 50% isopropanol; IV, 60% isopropanol. The first dimension was run using a pH gradient from 4.0 to 7.0. The second dimension was a 12% SDS–PAGE. Gels were stained with colloidal Coomassie blue stain G-250. Circles indicate different groups of protein spots that were compared and analyzed.

III, and IV). Comparison of the protein profiles on gels indicated that 40% isopropanol extracts consistently resolved a larger number of lower abundance protein spots (562) compared to the other concentrations 30% (440), 50% (338), and 60% (230) tested (Fig. 2). At 30%, 50%, and 60% isopropanol, protein resolution was poor in several areas. Some spots were diffuse in the high and low molecular weight regions (Fig. 2, I, III, and IV).

We further analyzed four gel regions to determine which isopropanol concentration was optimal for mass spectrometry analysis of protein spots. These regions are circled in Fig. 2 and labeled A, B, C, and D. Area A showed distinct separation with good intensity of the protein spots in all isopropanol concentrations (Fig. 2, I, II, III, and IV). However, at 40% isopropanol concentration, all the areas including B, C, and D showed a higher number of visible protein spots than at 30%, 50%, and 60% isopropanol concentrations (Fig. 2, I, II, III, and IV). Therefore, we selected 40% isopropanol extraction for further analytical studies to isolate and identify specific protein spots. Spots were manually picked from Coomassie-stained gels, digested with trypsin, and analyzed by MS. We have used both MALDI-TOF-MS and LC–MS/MS to identify most of the proteins visualized. MALDI-TOF-MS and LC–MS/MS of all the excised spots resulted in good-quality spectra, indicating the compatibility of the isopropanol extraction method for MS analysis. Data listed in Table 1 include the assigned protein spot number from Fig. 3, the calculated isoelectric point and molecular weight, protein identity, the number of peptides matched, percentage sequence coverage, MOWSE score, the expected value, and the NCBInr accession number of the best match using the NCBI-nonredundant, UniprotKB, and MSDB databases.

Soybean seeds contain highly abundant storage proteins, β-conglycinin, and glycinin [26]. Glycinin consists of acidic (A) and basic (B) polypeptides and was originally reported to be made up of five subunits G1, G2, G3, G4, and G5 [27]. However, Beilinson and coworkers [28] identified two additional glycinin subunits in the soybean variety Resnik. Based on physical properties, the original five subunits are classified into two distinct major groups; group I consists of G1 (A1aBx), G2 (A2B1a), and G3 (A1aB1b) proteins and group II contains G4 (A5A4B3) and G5 (A3B4) subunits. Other known seed proteins of lower abundance include β-amylase, cytochrome *c*, lectin, lipoxygenase, urease, and the Bowman–Birk inhibitor (BBI) of chymotrypsin and trypsin [29]. In our studies, we observed some glycinin isomers (Fig. 3, spots 1–14) using our isopropanol extraction procedures; however, there appeared to be complete removal of all β-conglycinin. Isopropanol solubilized a higher proportion of group I glycinin subunits than group II subunits. Group I subunits contain more methionine residues than group II, an important feature for plant breeders desiring to increase the methionine content in soybean seeds to improve their nutritional quality [30]. Earlier, we reported the efficient extraction of group I and II glycinin subunits from 16 soybean genotypes using a modified TCA/acetone method [12].

Soybean lectins (agglutinins), anti-nutritional proteins present in soybeans, account for about 10% of the total protein in some legumes. These carbohydrate-binding proteins are present in moderate levels in soybean, pea, and clover [31]. We observed 10 spots (spots 15–24) of soybean lectins in isopropanol extracts. In our previous study using the modified TCA extraction method, we found only three spots of lectin [11]. Pull and co-workers [32] reported that soybean varieties showed wide variation in the content of lectin, including lines that had no lectin.

Dehydrins are a family of late embryogenesis-abundant proteins (LEA) that commonly accumulate to high levels during the late stages of seed maturation [33]. The major features of LEA proteins are low sequence complexity, occurrence of repeat motifs, high hydrophilicity, heat solubility, and an apparent lack of defined structure [34]. Dehydrins are expressed during seed maturation and drying, are induced by environmental stresses associated with low temperature or dehydration, and are reported to protect other

Table 1
Soybean seed proteins identified by MS method.

Spot No.	Theoretical PI/Mr	Protein identity	Peptides matched	Sequence coverage (%)	MOWSE score	Expected value	NCBI accession number	MS method
1	5.52/58152	gy5 [*Glycine soja*]	4	9	162		gi|736002	LC–MS/MS
2	5.46/55850	Glycinin A3b4 Subunit	11	22	83	2.20E−03	gi|33357661	MALDI-TOF
3	5.21/63837	Glycinin [*G. max*]	2	5	151		gi|18641	LC–MS/MS
4	5.21/63837	Glycinin [*G. max*]	3	9	156		gi|18641	LC–MS/MS
5	5.89/56299	Glycinin G1 precursor [A1a and Bx subunit]	3	6	148		gi|121276	LC–MS/MS
6	5.89/56299	Glycinin G1 precursor [A1a and Bx subunit]	2	5	79		gi|121276	LC–MS/MS
7	5.46/54357	Glycinin G2 (A2 and B1a subunit)	2	6	146		gi|121277	LC–MS/MS
8	5.46/54927	Glycinin G2 (A2 and B1a subunit)	10	19	73	0.02	gi|121277	MALDI-TOF
9	5.46/54927	Glycinin G2 (A2 and B1a subunit)	8	19	75	0.015	gi|121277	MALDI-TOF
10	5.46/54357	Glycinin G2 (A2 and B1a subunit)	3	22	135		gi|121277	LC–MS/MS
11	5.46/54357	Glycinin G2 (A2 and B1a subunit)	2	6	162		gi|121277	LC–MS/MS
12	5.46/54927	Glycinin G2 (A2 and B1a subunit)	5	10	200		gi|121277	LC–MS/MS
13	5.78/54047	Proglycinin A1ab1b	8	19	93	2.00E−04	gi|15988117	MALDI-TOF
14	5.78/54047	Proglycinin A1ab1b	8	19	82	2.40E−03	gi|15988117	MALDI-TOF
15	5.65/30909	Lectin precursor (agglutinin)	2	10	109		gi|126151	LC–MS/MS
16	5.65/30909	Lectin precursor (agglutinin)	5	22	162		gi|126151	LC–MS/MS
17	5.15/27555	Soybean agglutinin	5	19	237		gi|6729836	LC–MS/MS
18	5.15/27555	Soybean agglutinin	6	22	272		gi|6729836	LC–MS/MS
19	5.15/27555	Soybean agglutinin	6	32	85	0.0013	gi|6729836	MALDI-TOF
20	5.15/27555	Soybean agglutinin	6	30	69	0.054	gi|6729836	MALDI-TOF
21	5.15/27555	Soybean agglutinin	9	44	90	0.00044	gi|6729836	MALDI-TOF
22	5.15/27555	Soybean agglutinin	6	35	85	0.0014	gi|6729836	MALDI-TOF
23	5.15/27555	Soybean agglutinin	6	34	89	0.00057	gi|6729836	MALDI-TOF
24*	5.15/27555	Soybean agglutinin	1	6	63		gi|6729836	LC–MS/MS
25	6.07/23704	Dehydrin-like protein	15	61	136	1.10E−08	gi|497417	MALDI-TOF
26	5.87/23720	Dehydrin[*G. max*]	10	46	88	0.00073	gi|37495455	MALDI-TOF
27	5.87/23720	Dehydrin[*G. max*]	12	51	127	8.40E−08	gi|37495455	MALDI-TOF
28	9.22/17310	Dehydrin [*G. max*]	2	13	50		gi|2270990	LC–MS/MS
29	9.22/17310	Dehydrin [*G. max*]	2	21	110		gi|2270990	LC–MS/MS
30	5.87/23720	Dehydrin [*G. max*]	2	15	128		gi|37495455	LC–MS/MS
31	5.87/23720	Dehydrin [*G. max*]	9	40	73	0.022	gi|37495455	MALDI-TOF
32*	5.87/23720	Dehydrin [*G. max*]	1	6	58		gi|37495455	LC–MS/MS
33*	5.87/23720	Dehydrin [*G. max*]	1	6	44		Q7OEL8_SOYBN	LC–MS/MS
34	5.52/11485	Late embryogenesis-abundant protein [*G. max*]	2	22	165		gi|1762955	LC–MS/MS
35	5.14/26938	Seed maturation protein [*G. max*]	5	25	71	3.20E−02	gi|6648964	MALDI-TOF
36	4.99/25827	Seed maturation protein [*G. max*]	7	40	92	2.80E−04	gi|6648966	MALDI-TOF

(*continued on next page*)

Table 1 (*continued*)

Spot No.	Theoretical PI/Mr	Protein identity	Peptides matched	Sequence coverage (%)	MOWSE score	Expected value	NCBI accession number	MS method
37	5.49/18871	Maturation protein [*G. max*]	4	21	291		gi|5733686	LC–MS/MS
38	5.49/18871	Maturation protein [*G. max*]	4	21	288		gi|5733686	LC–MS/MS
39	6.02/25644	Maturation protein [*G. max*]	10	36	87	0.00092	gi|170020	MALDI-TOF
40	5.49/18871	Maturation protein pPM32 [*G. max*]	2	12	98		Q9SPJ6_SOYBN	LC–MS/MS
41	5.49/18871	Maturation protein pPM32 [*G. max*]	10	48	68	0.043	Q9SPJ6_SOYBN	MALDI-TOF
42	6.07/23700	Maturation-associated protein	3	24	125		gi|170024	LC–MS/MS
43	4.99/25827	Seed maturation protein PM25 [*G. max*]	7	40	74	0.019	gi|6648966	MALDI-TOF
44	5.14/26824	Seed maturation protein PM24 [*G. max*]	7	26	390		gi|6648964	LC–MS/MS
45	4.99/25713	Seed maturation protein PM25 [*G. max*]	2	14	100		gi|6648966	LC–MS/MS
46	6.60/31747	Seed maturation protein PM34 [*G. max*]	3	12	117		gi|9622153	LC–MS/MS
47	8.98/61146	Maturase-like protein	2	3	57		gi|5817720	LC–MS/MS
48	4.69/16762	Stress-induced protein SAM22 (Allergen Gly m 4)	4	14	140		gi|134194	LC–MS/MS
49	4.69/16762	Stress-induced protein SAM22 (Allergen Gly m 4)	8	39	272		gi|134194	LC–MS/MS
50	4.69/16762	Stress-induced protein SAM22 (Allergen Gly m 4)	3	23	153		gi|134194	LC–MS/MS
51	4.69/16762	Stress-induced protein SAM22 (Allergen Gly m 4)	2	13	99		gi|134194	LC–MS/MS
52*	4.69/16762	Stress-induced protein SAM22 (Allergen Gly m 4)	1	7	58		gi|134194	LC–MS/MS
53	4.49/10846	Stress-induced protein SAM22-like (*G. max*)	2	21	61		gi|1863553	LC–MS/MS
54	4.99/24346	Trypsin inhibitor subtype A [G. max]	3	14	145		gi|18770	LC–MS/MS
55	4.99/24346	Trypsin inhibitor subtype A [*G. max*]	2	8	91		gi|18770	LC–MS/MS
56	4.99/24346	Trypsin inhibitor subtype A [*G. max*]	6	29	279		gi|18770	LC–MS/MS
57	4.99/24346	Trypsin inhibitor subtype A [*G. max*]	4	18	197		gi|18770	LC–MS/MS
58	4.99/24346	Trypsin inhibitor subtype A [*G. max*]	5	23	221		gi|18770	LC–MS/MS
59	4.99/24346	Trypsin inhibitor subtype A [*G. max*]	4	18	180		gi|18770	LC–MS/MS
60	4.99/24346	Trypsin inhibitor subtype A [*G. max*]	2	10	96		gi|18770	LC–MS/MS
61	4.99/24346	Trypsin inhibitor subtype A [*G. max*]	3	17	96		gi|18770	LC–MS/MS
62	5.0/24419	Kunitz trypsin inhibitor [*G. max*]	10	44	155	1.7E−10	gi|13375349	MALDI-TOF
63	5.0/24419	Kunitz trypsin inhibitor [*G. max*]	8	31	92	0.00033	gi|13375349	MALDI-TOF
64	5.0/24419	Kunitz trypsin inhibitor [*G. max*]	6	25	76	0.015	gi|13375349	MALDI-TOF
65	4.97/22817	Kunitz-type trypsin inhibitor KTI1 precursor	6	24	249		gi|125722	LC–MS/MS
66	4.97/22817	Kunitz-type trypsin inhibitor KTI1 precursor	2	12	84		gi|125722	LC–MS/MS
67	4.97/22817	Kunitz-type trypsin inhibitor KTI1 precursor	2	12	80		gi|125722	LC–MS/MS
68	5.0/24419	Kunitz trypsin inhibitor [G. max]	6	30	315		gi|13375349	LC–MS/MS
69	4.99/24318	Kunitz trypsin inhibitor [G. max]	5	26	298		gi|15216344	LC–MS/MS
70	4.97/22817	Kunitz-type trypsin inhibitor KTI1 precursor	2	12	85		gi|125722	LC–MS/MS
71	6.70/4503	Bowman–Birk proteinase inhibitor	3	33	240		Q9SBA9	LC–MS/MS
72	6.09/41870	Alcohol dehydrogenase 1	2	3	56		gi|113361	LC–MS/MS
73	6.13/36357	Alcohol-dehydrogenase [*G. max*]	6	16	303		gi|4039115	LC–MS/MS

Table 1 (*continued*)

Spot No.	Theoretical PI/Mr	Protein identity	Peptides matched	Sequence coverage (%)	MOWSE score	Expected value	NCBI accession number	MS method
74	8.23/36119	Malate dehydrogenase [*G. max*]	3	9	159		gi|5929964	LC–MS/MS
75	8.23/36119	Malate dehydrogenase [*G. max*]	7	13	226		gi|5929964	LC–MS/MS
76	8.23/36119	Malate dehydrogenase [*G. max*]	5	17	346		gi|5929964	LC–MS/MS
77	8.23/36119	Malate dehydrogenase [*G. max*]	5	17	287		gi|5929964	LC–MS/MS
78	8.11/43156	Malate dehydrogenase precursor	4	10	227		gi|2827084	LC–MS/MS
79	5.27/15298	Superoxide dismutase [Cu–Zn]	3	15	132		gi|47117142	LC–MS/MS
80	5.05/15029	Copper–zinc superoxide dismutase [*Citrullus lanatus*]	3	26	172		gi|45643751	LC–MS/MS
81	5.64/15175	Superoxide dismutase [Cu–Zn] 4AP	3	15	121		gi|134598	LC–MS/MS
82	5.77/22214	Superoxide dismutase [Cu–Zn], chloroplast precursor	3	12	117		gi|134682	LC–MS/MS
83	5.60/27881	Superoxide dismutase [Fe], chloroplast precursor	4	16	154		gi|134646	LC–MS/MS
84	6.0/18393	Napin-type 2S albumin 1 precursor	2	12	85		gi|4097894	LC–MS/MS
85	6.0/18393	Napin-type 2S albumin 1 precursor	2	13	119		gi|4097894	LC–MS/MS
86*	6.0/18393	Napin-type 2S albumin 1 precursor	1	5	42		Q9ZNZ4_SOYBN	LC–MS/MS
87	5.20/19018	2S albumin	4	22	171		gi|5902685	LC–MS/MS
88	5.20/19018	2S albumin	4	22	171		gi|5902685	LC–MS/MS
89	4.70/25964	Proteasome subunit alpha type 5	4	21	204		gi|12229923	LC–MS/MS
90	5.83/27489	Proteasome subunit alpha type-6	10	43	111	3.30E−06	gi|12229897	MALDI-TOF
91	6.24/40081	Type IIIa membrane protein cp-wap13	7	21	302		gi|2218152	LC–MS/MS
92	6.24/39396	Type IIIa membrane protein cp-wap13	3	7	144		gi|2218152	LC–MS/MS
93	8.76/36030	Type IIIa membrane protein cp-wap11	3	8	91		gi|2218150	LC–MS/MS
94	4.96/37087	Class III acidic endochitinase [*G. max*]	2	6	99		gi|2934696	LC–MS/MS
95*	4.96/37543	Class III acidic endochitinase [*G. max*]	1	3	46		gi|2934696	LC–MS/MS
96	4.96/37543	Class III acidic endochitinase [*G. max*]	2	5	77		gi|2934696	LC–MS/MS
97*	4.96/37543	Class III acidic endochitinase [*G. max*]	1	3	59		gi|2934696	LC–MS/MS
98	5.93/16489	Nucleoside diphosphate kinase 1	5	30	80	0.0037	gi|2498078	MALDI-TOF
99	7.67/33325	Triose-phosphate isomerase	3	14	184		gi|15226479	LC–MS/MS
100	7.05/46806	Dihydroorotate dehydrogenase 1	3	7	95		Q1SIZ4_MEDTR	LC–MS/MS
101	4.61/33553	Chain A, the structure of soybean peroxidase	4	10	140		gi|13399943	LC–MS/MS
102	5.61/20946	Glyoxalase I [*G. max*]	5	32	198		gi|4127862	LC–MS/MS
103	5.26/25905	Dienelactone hydrolase [*Arabidopsis thaliana*]	2	8	104		gi|15225693	LC–MS/MS
104	5.10/139721	Disease resistance gene [*Lycopersicon esculentum*]	2	2	55		gi|3426260	LC–MS/MS
105*	5.36/113347	Unnamed protein product [Vitis vinifera]	1	1	48		gi|157342139	LC–MS/MS
106	7.95/66664	Hypothetical protein [Vitis vinifera]	3	6	71		gi|147841400	LC–MS/MS
107	9.63/27496	Hypothetical protein [Medicago truncatula]	2	10	43		Q1SP99_MEDTR	LC–MS/MS

Proteins were analyzed using the NCBI-nonredundant, UniprotKB, and MSDB databases.
* Spots of low molecular weight search (MOWSE) scores/low peptide matches.

proteins under abiotic stress [35,36]. They have been proposed to play an important role in membrane and protein stability and osmotic adjustment [37]. It has been hypothesized that dehydrins function as surfactant molecules, acting synergistically with com-

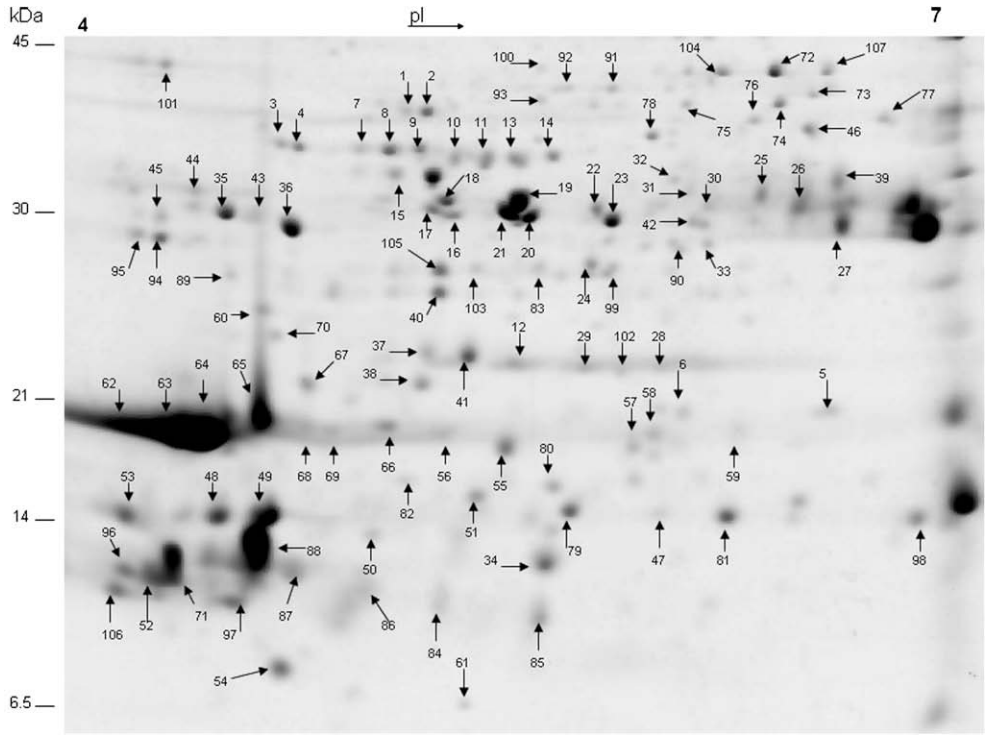

Fig. 3. Two-dimensional electrophoresis of soybean proteins extracted in 40% isopropanol. The first dimension was run using a pH gradient from 4.0 to 7.0. The second dimension was a 12% SDS–PAGE. Gels were stained with colloidal Coomassie blue stain G-250. Arrows indicate the protein spots that were analyzed by MS (Table 1).

patible solutes to prevent coagulation of colloids and a range of macromolecules [36]. We observed 9 protein spots of various sizes and isoelectric points identified as dehydrin (spots 25–33) in the 40% isopropanol extracts.

Seed maturation proteins (SMPs) are also synthesized during the later stages of seed development. Maturation proteins are different from LEA proteins in that the messages for maturation proteins are not necessarily present at high levels during late embryogenesis [38]. A total of 14 spots (spots 34–47) identified as maturation-associated proteins were found in our isopropanol extract, whereas 7 spots of seed maturation proteins were extracted using urea/thiourea [39].

We also found 6 spots (spots 48–53) identified as stress-induced protein SAM22 (starvation-associated message 22). SAM22 is currently called allergen Gly m 4 and is a major soybean allergen. It is a homologue of the Birch tree pollen allergen, Bet v 1. and Herian and co-workers [40] demonstrated Bet v 1-homologue cross-reactivity between Gly m 4, Ara h 8 from peanut, and Pru av 1 from cherry. Mittag and co-workers [41] reported that Gly m 4 caused severe symptoms localized in the oral cavity of soybean-sensitive patients. In our laboratory, we could not identify Gly m 4 when we extracted seed proteins using the TCA/acetone solubilization [10].

Other soybean anti-nutritional proteins are protease inhibitors, of which the Kunitz trypsin inhibitor (KTI) and the Bowman–Birk inhibitor (BBI) are well studied [42]. KTI is an abundant protein that can inhibit trypsin, an important animal digestive enzyme. KTIs have been characterized as food allergens in humans and have 32% sequence homology with a rye grass pollen allergen [43]. The 40% isopropanol extraction not only enriched generally for lesser abundant proteins but also resulted in a several fold increase of KTI protein extraction. We found 8 spots of trypsin inhibitor subtype A (spots 54–61) and 9 spots of KTI (spots 62–70). Bowman–Birk proteinase inhibitors are cys-rich protease inhibitors with molecular masses of about 8–16 kDa. These protease inhibitors are double-headed, with two reactive sites in a single inhibitor molecule. We have observed one protein spot of BBI (spot 71). BBIs have been identified in the Fabaceae including soybean (*G. max*) and Lima beans (*Phaseolus lunatus*), and are encoded by a family of related genes [44]. Krishnan [18] reported that isopropanol extraction is efficient in solubilizing low molecular weight proteinase inhibitors, KTI and BBI, in soybean.

Alcohol dehydrogenase (ADHs) enzymes are necessary for successful germination under low oxygen conditions and can accumulate to about 1% of the total protein in soybean seeds [45]. We observed 2 spots (spots 72 and 73) of ADH using the isopropanol solubilization method. Mooney and co-workers [45], in studies on soybean seed maturation, reported 3 spots of ADHs using phenol in their extraction buffer. In addition, the authors reported the presence of 7 spots identified as malate dehydrogenase. We previously reported the presence of two polypeptides of malate dehydrogenase in soybean leaf samples extracted by the TCA/acetone method [2]. Using 40% isopropanol extraction, we observed 5 spots (spots 74–78) of malate dehydrogenase with comparable molecular mass and location in 2D-PAGE. Herman and co-workers [39] reported the absence of ADHs and malate dehydrogenase when they extracted the protein with urea/ thiourea extraction buffer after defatting the soybean meal twice with hexane.

Superoxide dismutases (SODs) are ubiquitous metalloenzymes which catalytically scavenge the superoxide radical to hydrogen peroxide and molecular oxygen and thus prevent oxidative damage in all organisms [46]. Three classes of SODs, such as CuZnSOD, MnSODs, and FeSODs differing in the metals at their catalytic active site, are known in plants [47]. We have observed 3 polypeptides (spots 79–82) of copper–zinc superoxide dismutase (CuZnSOD) and one polypeptide of Fe superoxide dismutase (spot 83) with the isopropanol extraction method. Mooney and co-workers [45] found one polypeptide of Fe superoxide dismutase and one polypeptide of manganese superoxide dismutase using their phenol extraction method.

Additional protein spots identified on gels of proteins extracted with isopropanol included three spots of napin type 2S albumin 1 precursor (spots 84, 85, and 86); two spots of 2S albumin (87 and 88); two spots of proteasome subunit alpha type 5 and 6 (spots 89 and 90); three spots of type IIIa membrane protein (91, 92, and 93); four spots of class III acidic endochitinase (spots 94, 95, 96, and 97), one spot of nucleoside diphosphate kinase 1 (spot 98); one spot of triose phosphate isomerase (spot 99); one spot of dihydroorotate dehydrogenase (100); one spot of chain A soybean peroxidase (101); one spot of glyoxalase (102); one spot of dienelactone hydrolase (spot 103); and one spot of a disease resistance gene like protein (104). Three spots were identified as unnamed/hypothetical proteins (105, 106, and 107). The molecular mass, isoelectric point, and location on a 2D-gel of most of these polypeptides are comparable with earlier published results [45]. Variation from previously published results was primarily in terms of gel location, and this could be due to the method of extraction or to the soybean genotype we selected. Some protein spots (spots 24, 32, 33, 52, 86, 95, 97, and 105) have low MOWSE scores, which could result from the presence of impurities in the sample or the low amount of protein on the 2D-PAGE gel. In the future, the application of this technique will enable identification of less abundant proteins masked by abundant storage proteins in soybean seeds.

Conclusion

Less abundant proteins and low molecular weight proteinase inhibitors are traditionally purified by ammonium sulfate precipitation, gel filtration, column chromatography, or HPLC prior to analysis. These methods are often time consuming and result in limited amounts of purified material. In this study, we compared and evaluated different concentrations of isopropanol to enrich the extracts in seed components that are different from the main storage proteins from soybeans. A 40% isopropanol extraction as a first step prior to precipitation is efficient and suitable for the isolation and separation of low abundant proteins in terms of spot intensity as well as total number of spots. In addition, this method enriches for proteinase inhibitors which are rich in methionine and cysteine and contribute a majority of the sulfur amino acids in soybean. Because sulfur amino acids are limited in soybean, industries currently must add these amino acids to animal feed. Using isopropanol extraction we can easily screen soybean genotypes and select genotypes with more of these sulfur-containing proteins.

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