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A rapid method for depletion of Rubisco from soybean (*Glycine max*) leaf for proteomic analysis of lower abundance proteins

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ABSTRACT

2-DE analysis of complex plant proteomes has limited dynamic resolution because only abundant proteins can be detected. Proteomic assessment of the low abundance proteins within leaf tissue is difficult when it is comprised of 30–50% of the CO_2 fixation enzyme Rubisco. Resolution can be improved through depletion of Rubisco using fractionation techniques based upon different physiological or biochemical principles. We have developed a fast and simple fractionation technique using 10 mM Ca^{2+} and 10 mM phytate to precipitate Rubisco from soybean leaf soluble protein extract. This method is not only rapid, but also inexpensive, and capable of removing 85% of the extremely abundant Rubisco enzyme from soybean leaf soluble protein extract. This method allowed for roughly 230 previously inconspicuous protein spots in soybean leaf to be more easily detectable (3-fold increase in vol%) using fluorescent detection and allowed 28 phosphorylated proteins previously undetected, to be isolated and identified by MALDI-TOF-MS.

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1. Introduction

The majority of protein species within most tissue detected by two-dimensional gel electrophoretic analysis are those "housekeeping" proteins present in very high copy number per cell (Görg et al., 2004). This fact alone can make those low abundance protein species, such as regulatory and signaling factors, hard or even impossible to detect using 2D gel electrophoresis. In addition, electrophoretic separation and assessment of some tissues is confounded further due to one or several extremely abundant proteins. These sometimes "overabundant" protein species can not only limit the dynamic resolution and yield of those inconspicuous low abundance proteins of interest (Herman et al., 2003), but also due to their massive quantity, can mask other proteins or affect the electrophoretic migration of neighboring protein species as well (Cho et al., 2008; Shaw and Riederer, 2003).

For example, most plant leaf tissues are heavily comprised of the photosynthetic CO_2 fixation enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) (Ellis, 1979). In the leaf, Rubisco dominates the C3 and C4 protein species profile, exhibiting roughly 50% and 30% of the total proteins respectively (Feller et al., 2008). 2-DE of leaf tissue for proteomic assessment of low abundance proteins is highly hindered by Rubisco in nearly all species, not only through its inherent limitation it places on capacity protein load (Xi et al., 2006), but by its co-migratory masking ability of neighboring species (Corthalis et al., 2000). However, 2-DE resolution can be improved using fractionation techniques of the protein isolate based upon varying physiological and biochemical principles (van Wijk, 2001). Many techniques have been developed to remove or reduce a substantial portion of Rubisco from leaf protein isolate of several species of plant (Cellar et al., 2008; Cho et al., 2008; Espagne et al., 2007; Kim et al., 2001; Kwanyuen et al., 2002; Lee et al., 2007; Widjaja et al., 2009; Xi et al., 2006). However, these methods can entail complex steps, take significant amounts of time or be costly.

When preparing proteins for 2-DE, the amount of time between tissue disruption and protein denaturation is of critical importance. Development of a simpler, less expensive and most importantly,

Abbreviations: Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; 1-DE, one-dimensional SDS–PAGE; 2-DE, two-dimensional SDS–PAGE; SYPRO Ruby, SYPRO® Ruby fluorescent protein dye (registered trademark of Molecular Probes); Pro-Q Diamond, ProQ® Diamond fluorescent phosphoprotein gel stain (registered trademark of Molecular Probes).

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faster method to remove Rubisco from plant leaf protein extract would significantly enhance the study of those low abundance proteins. Such a methodology would allow for those low abundance proteins to be assessed for post-translational modifications, relative protein quantity, mass or charge properties, complex formation, immunoreactivity, and protein–protein interactions. Even more importantly, it would aid in the isolation of higher amounts of those low abundance proteins, making peptide mass analysis more justifiable and allowing for the discovery of novel proteins within the leaf proteome. Development of such fractionation techniques for agronomically important and commercially valuable crops such as soybean would be highly useful, allowing more information about their nutritive value, yield potential and responses to environmental stresses to be ascertained.

Our approach was to simplify the removal of the vast majority of Rubisco from the soluble protein fraction of soybean leaf, in an effort to have a fast and effective way of reducing or possibly excluding it from our protein preparations for 2-DE separation and analysis. Earlier work had shown that phytate has the ability to complex with proteins, resulting in their reduced solubility (Cheryan, 1980; Prattly et al., 1982). Phytate is a highly charged electronegative molecule, capable of binding minerals and proteins very strongly (Lah and Cheryan, 1980). Protein binding by phytate however, is very dependent on the pH of the medium. Direct protein binding only occurs at pH values below the isoelectric point of a protein. At pH values above the isoelectric point of a protein, phytate–protein binding occurs through an alkaline-earth metal, such as calcium (Cheryan, 1980). Phytate alone has limited solubility above pH 5; hence, phytate–protein complexes in the presence of a multivalent cation, in a more neutral pH solution, can result in protein insolubility (Cheryan, 1980; Nolan et al., 1987).

Keeping in mind that the isoelectric point of soybean's large subunit of Rubisco is 6, our intent was to use a near neutral buffer, pH 6.8, in an effort to precipitate Rubisco using Ca^{2+} and phytate. The results of this simple Ca^{2+}-phytate fractionation step and electrophoretic analysis of soybean leaf proteins carried out with 1-D and the more complex 2-D separation are reported in detail here. In addition, our success in utilizing this approach with cultivated soybean brought us to hypothesize that the same or a similar technique might be universally applicable to other economically important seed crops such as corn, wheat and rice, and a model plant like *Arabidopsis*. Our studies suggest successful depletion of Rubisco can be achieved using a similar fractionation protocol.

2. Results and discussion

2.1. Fractionation and 1-DE analysis

Empirical work on fractionation of the soluble leaf proteins from soybean was performed using freshly prepared calcium and phytate stock solutions. Soluble proteins were isolated from soybean leaf and fractionated as outlined in Section 4. Those proteins remaining in solution were combined 1:1 with SDS–PAGE sample buffer, quantified and adjusted volumetrically to achieve an equal protein load for all 1-DE samples. Early fractionation trials using 1-DE analysis demonstrated a significant reduction in the amount of large and small subunit of Rubisco in the soluble protein fraction using increasing amounts of Ca^{2+} and phytate, combined in an equimolar fashion (Fig. 1A). These initial experiments revealed that neither Ca^{2+} nor phytate separately gave the desired reduction in the amount of Rubisco, and that the minimum amount of both that were needed is 10 mM (Fig. 1B). 1-DE results also showed that a considerable reduction in Rubisco is achieved with a minimum incubation time of 6 min (Fig. 2A). Further fractionation trials

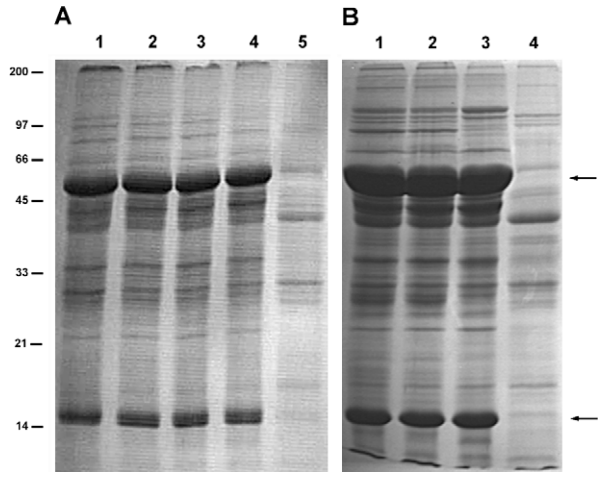

Fig. 1. 1-DE analysis of soybean leaf proteins fractionated with varying concentrations of Ca^{2+} and phytate. Soluble proteins were extracted from soybean leaf using a low ionic strength buffer and the effects of increasing equimolar concentrations of Ca^{2+} and phytate on protein fractionation were examined (**Panel A**: lane 1, control; lane 2, 1 mM Ca^{2+} and 1 mM phytate; lane 3, 2 mM Ca^{2+} and 2 mM phytate; lane 4, 5 mM Ca^{2+} and 5 mM phytate; lane 5, 10 mM Ca^{2+} and 10 mM phytate). Additional testing demonstrated that neither Ca^{2+} nor phytate alone at the highest concentration needed could result in depletion of Rubisco (**Panel B**: lane 1, control; lane 2, 10 mM Ca^{2+}; lane 3, 10 mM phytate; lane 4, 10 mM Ca^{2+} and 10 mM phytate). Fractionation and electrophoresis were performed as outlined in Section 4. Gels were 13.5% and protein molecular weight markers are in kDa. Arrows indicate both the large and small subunit of Rubisco.

(not shown) demonstrated that maximal depletion of Rubisco was achieved with a 10 min incubation of the leaf extract with 10 mM Ca^{2+} and 10 mM phytate. After determination of the optimum quantity of fractionation agents and incubation time that was required for the desired results, a significant amount of effort went into testing for optimum fractionation conditions. It was empirically determined that to achieve a significant reduction in Rubisco an incubation temperature of 42 °C is required (Fig. 2B). A cooler temperature would have been ideal; however this fractionation temperature was achieved in the presence of protease inhibitors to eliminate unwanted proteolysis.

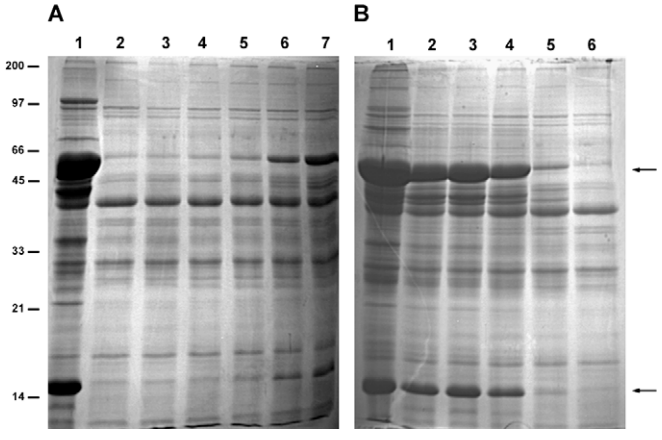

Fig. 2. 1-DE analysis of soybean leaf proteins fractionated with 10 mM Ca^{2+} and 10 mM phytate using varying conditions. Soluble proteins were extracted from soybean leaf using a low ionic strength buffer and the effects of incubation time (**Panel A**) and incubation temperature (**Panel B**) on depletion of Rubisco examined. **Panel A**: lane 1, control; lane 2, 10 min; lane 3, 8 min; lane 4, 6 min; lane 5, 4 min; lane 6, 2 min; lane 7, 1 min. **Panel B**: lane 1, control; lane 2, 4 °C; lane 3, 10 °C; lane 4, 20 °C; lane 5, 30 °C; lane 6, 42 °C. Fractionation and electrophoresis were performed as outlined in Section 4. Gels were 13.5% and protein molecular weight markers are in kDa. Arrows indicate both the large and small subunit of Rubisco.

These initial trials demonstrated that a very small amount of Rubisco is retained after fractionation, and that other proteins not targeted for removal do precipitate along with Rubisco. However, our goal of a fast and efficient method for Rubisco depletion was achieved with minimal cost and effort by using both 10 mM calcium and 10 mM phytate, with 10 min incubation at 42 °C. It is worth noting that portions of those non-target proteins can be retained if both calcium and phytate concentrations are reduced slightly, and/or either incubation time or temperature is reduced. This as shown in both Figs. 1 and 2, and suggests this methodology can be modified to suit applications that require less depletion of Rubisco and more retention of a specific target protein. Notably, with reference to fractionation temperature, a 44% reduction compared to an 86% reduction in total soluble proteins results from fractionation performed at 4 °C and 42 °C, respectively.

2.2. 2-DE separation and analysis

The goal for using Ca^{2+}-phytate fractionation was to have a sample enriched for the low abundance proteins in soybean leaf, through reduction or complete elimination of the highly abundant Rubisco protein. Ultimately, we wanted to uncover those inconspicuous proteins in leaf using 2-DE separation and have another tool for verifying proteome changes resulting from genetic manipulation. To test if this Ca^{2+}-phytate fractionation methodology was an effective approach for enhancing 2-DE analysis, proteins were resolved from pH 3 to 10 using a mid-size IEF and second dimension format, then visualized with a sensitive fluorescent stain SYPRO Ruby. The resulting images clearly demonstrated the removal of an overwhelming majority of both the large and small subunit of Rubisco from soybean leaf and the enhancement of many protein spots not seen in the non-fractionated sample (Fig. 3). Two additional fractionations and 2-DE separations were performed, again using SYPRO Ruby stain. All images were analyzed with Delta2D image analysis software, capable of generating intensity based spot volumes (normalized) of each individual protein spot. Delta2D revealed an 85% reduction of Rubisco (6.67-fold reduction ± 0.29, $n = 3$) along with an increase of $\geqslant$3-fold in 227 protein spots (±9 spots; range 3–259, mean 11.84, $n = 3$) in the Ca^{2+}-phytate fractionated sample. A total of 187 protein spots were reduced $\geqslant$3-fold (±24 spots; range 3–250, mean 18.28, $n = 3$), but Rubisco comprised more than 26% of the total precipitated protein. In the original soluble leaf protein extract, Rubisco comprised 25% of the total protein separated via 2-DE.

The resulting vol% data generated from those images analyzed using Delta2D software also revealed that of the 1330 total spots recognized on the SYPRO Ruby replicates ($n = 3$), nearly 480 protein spots increased more than 2-fold (50% spot volume increase) in the Ca^{2+}-phytate fractionated sample. Although many non-target proteins were removed with Ca^{2+}-phytate fractionation, this fractionation procedure causes an 85% reduction of Rubisco enabling many of the inconspicuous proteins of interest to be detected. For the enhanced protein spots, with two times the amount of protein, those nearly 480 proteins have a better chance of being isolated and analyzed via MALDI-TOF-MS since greater amounts of protein should yield higher populations of individual peptide fragments. These data demonstrate not only the reproducibility but also the usefulness of this methodology towards our goal of enrichment of low abundance protein spots for future proteome analysis and protein discovery.

In addition, identically prepared and fractionated soybean leaf samples were separated with 2-DE using the same pH range (3–10) and analyzed for the presence of phosphoproteins using Pro-Q Diamond in-gel phosphorylated protein stain. This was an effort to establish if either the appearance or enhancement of signal from those low abundance phosphorylated proteins, possibly masked or limited by Rubisco could be detected. Previously undetected phosphorylated proteins (11 spots) were visible after fractionation, while those difficult to detect phosphorylated proteins (17 spots) had enhanced signal making them easier to discern from background (images not shown). Several phosphorylated protein spots in the non-fractionated sample were depleted along with Rubisco; however, isolation of these protein spots from a control gel would be possible. Hence, while Ca^{2+}-phytate fractionation would interfere with actual global quantization of protein phosphorylation, since depletion of Rubisco is not exclusive, it clearly provides a method for detecting novel phosphorylated protein spots. This result also demonstrates the usefulness of this method-

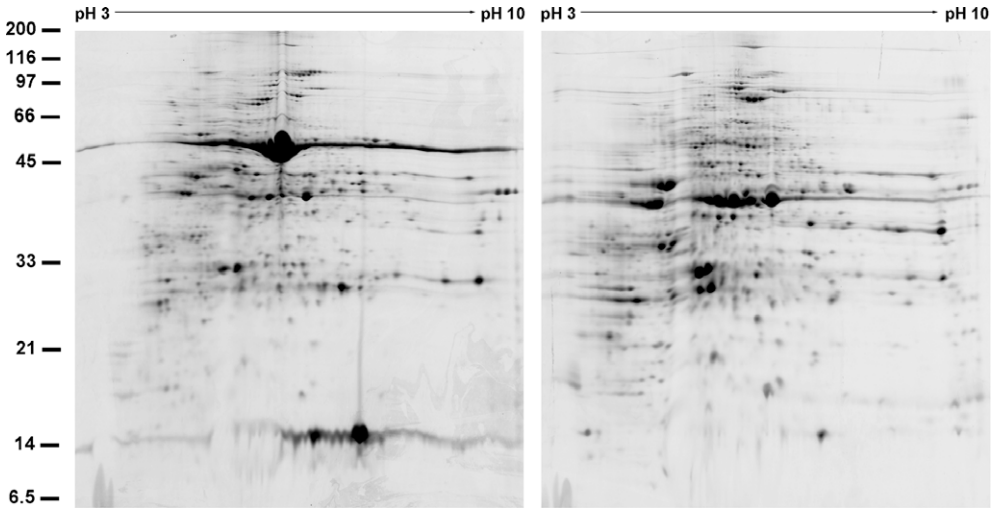

Fig. 3. 2-DE analysis of non-fractionated soybean leaf proteins (**Panel A**) and Ca^{2+}/phytate fractionated soybean leaf proteins (**Panel B**). Protein extract was fractionated and the remaining proteins in solution isolated for 2-DE separation and analysis as outlined in Section 4. Gels were incubated with the sensitive fluorescent protein stain SYPRO Ruby to demonstrate the enhancement of signal from those low abundance proteins usually masked by the massive amount of Rubisco, seen as heavily stained proteins spots in **Panel A** (52 kDa large subunit and 16 kDa small subunit). Clearly, depletion of Rubisco is evident, as is the many previously undetectable proteins spots visible now in **Panel B**. In addition, other protein spots have enhanced signal making them easier to discern from background. Image was analyzed using Delta2D image analysis software. IEF was from pH 3–10 and protein molecular weight markers are in kDa.

ology towards future discovery of specific low abundance leaf proteins that posses other post-translational modifications, that possibly have been previously undetectable.

Several additional fractionations and electrophoretic separations were performed, but gels were stained with Coomassie to compare with previous fluorescent staining results. Our goal was to ascertain how effective this method would be for obtaining gels conducive to visual spot detection and manual spot isolation. The results were very similar to the previous SYPRO Ruby analysis, showing a large reduction in Rubisco and a similar enhancement of those low abundance proteins when Ca^{2+}-phytate fractionation of the extracted leaf proteins was used prior to separation (images not shown). The 28 proteins previously identified as phosphorylated proteins spots, were manually isolated from one of these sample preparations and separations, along with an additional 24 protein spots identified as enhanced, novel proteins. These proteins spots were identified with peptide mass fingerprinting MALDI-TOF-MS (Table 1) and locations mapped within the fractionated leaf proteome (Fig. 4). Many of the phosphorylated protein spots were identified as regulatory factors (kinase, transcription factor, etc.) or are known to be key elements in biochemical pathways. This clearly demonstrates the effectiveness of this fractionation

Table 1
Proteins identified from soybean leaf protein extract after using Ca^{2+}/phytate fractionation method, 2-DE separation and peptide mass fingerprinting (MALD-TOF MS). Spot identification #'s (SID) correspond to those protein spots labeled in Fig. 4. Spots 1–28 were recognized by Pro-Q Diamond in-gel phosphorylated protein stain. Spots 29–52 were recognized in as novel, enhanced. Isoelectric point (pI) and molecular weight (M_r) values are given as theoretical/experimental values. MOWSE scores (MO) in italics represent those searches performed via Protein Prospector using a peptide mass tolerance of 20 ppm. Mascot "probability based MOWSE scores" $\geqslant 57$ represent those above 95% confidence limit ($p \leqslant 0.05$) using a peptide mass tolerance of 20 ppm. Peptides matched (PM), percentage sequence coverage (SC) and accession numbers within each respective database are given. Searches were confined to *Glycine max* databases unless no clear match could be found. Databases are as follows: National Center for Biotechnological Information (NCBI non-redundant); Matrix Sciences Database-Mascot (MSDB); Universal Protein Knowledgebase (UniProtKB).

SID	Protein identification [species]	pI (Thr./Exp.)	M_r (Thr./Exp.)	MO	PM	SC	Accession	Database
1	Methionine synthase [*Glycine max*]	5.93/6.02	84,401/82,254	176	22	35	gi|33325957	NCBInr
2	Enolase [*Glycine max*]	5.30/5.40	47,720/47,310	*7.6e⁴*	13	48	gi|42521309	NCBInr
3	NFR1b receptor-like kinase [*Glycine max*]	5.20/4.66	66,424/42,707	*9709*	9	23	gi|148362058	NCBInr
4	Phosphatidylinositol-3-kinase (isoform) [*Glycine max*]	6.50/6.36	93,003/93,931	79	4	20	gi|1171966	NCBInr
5	Chalcone synthase 2 [*Glycine max*]	6.40/4.17	42,505/41,390	*570*	6	23	P17957	UniProtKB
6	Fructose-1,6-bisphosphatase [*Glycine max*]	5.86/4.50	42,149/39,927	80	23	46	Glyma11g34900.1	MSDB
7	Fructose-1,6-bisphosphatase [*Glycine max*]	5.86/4.72	42,149/39,830	89	23	44	Glyma11g34900.1	MSDB
8	NAD(P)H-Flavin oxidoreductase [*Glycine max*]	8.04/5.52	40,887/38,122	63	15	40	Glyma16g23710.1	MSDB
9	Gamma-glutamyl hydrolase [*Glycine max*]	6.08/7.65	37,824/37,049	61	8	25	gi|6016129	NCBInr
10	S-locus receptor kinase [*Raphanus sativus*]	5.30/3.73	33,026/36,610	*199*	4	14	gi|46410677	UniProtKB
11	Ca^{2+}-Dependent lipid binding protein [*Glycine max*]	8.24/4.06	49,013/34,707	67	9	24	Glyma12g03620.1	MSDB
12	Phytochrome B-2 (ATPase fragment) [*Glycine max*]	5.70/3.91	115,419/33,098	68	4	5	gi|190586165	NCBInr
13	Mitogen activated protein kinase 2 [*Glycine max*]	5.50/5.61	44,745/34,024	*493*	7	17	gi|33340593	NCBInr
14	4-coumarate-CoA ligase 1 [*Glycine max*]	7.20/6.60	32,028/33,000	*76*	6	19	P31686	UniProtKB
15	Transcription factor MYB173 [*Glycine max*]	7.10/7.15	31,821/31,926	*217*	4	25	Q0PJI0	UniProtKB
16	Acid phosphatase [*Glycine max*]	6.90/7.06	30,105/30,787	*1808*	8	36	gi|3341443	NCBInr
17	Transcription factor (bZIP homology) [*Glycine max*]	9.40/8.55	26,923/37,249	*678*	9	28	gi|113367178	NCBInr
18	Fe-Superoxide dismutase, chloroplast [*Glycine max*]	5.60/5.51	27,881/30,539	74	8	33	gi|134646	NCBInr
19	Fe-Superoxide dismutase [*Glycine max*]	5.45/5.50	27,506/28,518	68	6	29	Q71UA1_SOYBN	MSDB
20	Transcription factor bZIP116 [*Glycine max*]	5.50/5.49	25,665/26,781	*2100*	6	24	A4ZGT2	UniProtKB
21	Transcription factor MYB139 [*Glycine max*]	5.60/5.93	29,971/27,384	*237*	6	19	Q0PJI3	UniProtKB
22	Cysteine synthase [*Glycine max*]	6.60/6.87	40,125/27,243	*471*	7	24	gi|148562451	NCBInr
23	Proteosome subunit α type-5 [*Glycine max*]	4.70/4.06	25,980/28,554	*837*	5	24	Q9M4T8_SOYBN	SwissProt
24	Proteasome subunit α type-5 (20S) [*Glycine max*]	4.70/4.18	26,135/28,448	79	8	35	gi|12229923	NCBInr
25	Ribose-5-phosphate isomerase [*Glycine max*]	5.50/4.33	29,821/28,483	84	12	42	Glyma03g40640.1	MSDB
26	NADP-Sorbitol-6-PO_4 dehydrogenase [*Prunus* sp.]	9.16/3.87	28,526/27,420	67	8	47	gi|21842196	NCBInr
27	Chalcone isomerase (putative) [*Glycine max*]	4.90/4.32	23,496/25,577	*207*	6	29	gi|51039630	NCBInr
28	Uncharacterized protein [*Picea sitchensis*]	4.18/3.50	17,520/18,696	81	6	49	gi|116782900	NCBInr
29	Lipoxygenase L-5 [*Glycine max*]	6.08/5.84	91,337/92,553	193	21	30	gi|161318161	NCBInr
30	Methionine synthase [*Glycine max*]	5.93/6.12	84,401/82,254	173	24	38	gi|33325957	NCBInr
31	Malic enzyme (homolog) [*Glycine max*]	5.83/6.09	65,328/63,754	100	28	43	Glyma01g01180.1	Gly.-max
32	Enolase [*Glycine max*]	5.30/5.66	47,720/47,446	147	15	44	gi|42521309	NCBInr
33	NADP-Isocitrate dehydrogenase [*Glycine max*]	5.87/5.83	46,363/47,582	63	8	23	IDHC_SOYBN	SwissProt
34	PEP carboxylase (subunit) [*Glycine max*]	5.70/5.52	110,687/40,317	*2.6e⁴*	15	17	Q02909	UniProtKB
35	PEP carboxylase (subunit) [*Glycine max*]	5.89/5.68	110,687/40,024	*1.5e⁶*	22	20	gi|399182	NCBInr
36	PEP carboxylase (subunit) [*Glycine max*]	5.89/5.91	110,761/39,927	*3.5e⁴*	18	15	gi|1705587	NCBInr
37	Actin-1 [*Glycine max*]	5.40/5.29	41,363/38,317	*165*	4	16	P02582_SOYBN	SwissProt
38	Gamma-glutamyl hydrolase precursor [*Glycine max*]	6.08/7.06	37,824/37,000	74	9	28	gi|6016129	NCBInr
39	Gamma-glutamyl hydrolase precursor [*Glycine max*]	6.08/8.22	37,824/36,220	*1445*	7	26	gi|6016129	NCBInr
40	Gamma-glutamyl hydrolase precursor [*Glycine max*]	6.08/8.41	37,824/36,024	82	11	33	gi|6016129	NCBInr
41	PHD4 (metal binding, zinc finger) [*Glycine max*]	5.00/4.80	28,648/33,634	*597*	5	21	Q06A76	UniProtKB
42	Inorganic pyrophosphatase [*Glycine max*]	6.04/4.91	32,506/33,195	89	15	46	Glyma15g06670.1	MSDB
43	Predicted sugar phosphatase (HAD family) [*Oryza sativa*]	6.70/4.97	39,494/34,073	*2927*	6	18	gi|115459134	NCBInr
44	Elongation factor 1alpha [*Pleodorina* sp.]	7.75/3.82	36,143/30,043	98	15	40	gi|29539324	NCBInr
45	Fe-Superoxide dismutase [*Glycine max*]	5.45/5.37	27,506/29,972	74	9	36	gi|37654895	NCBInr
46	No match	3.67	14,519					
47	Kunitz trypsin inhibitor (putative) [*Glycine max*]	5.20/4.00	23,655/23,414	*477*	6	22	P27991	UniProtKB
48	Predicted N-acetylglucosylaminyl phosphotidylinositol de-acetylase protein [*Oryza sativa*]	7.80/4.71	29,314/22,351	*8413*	4	24	Q7XKF2	UniProtKB
49	NAD(P)H-Quinone oxidoreductase [*Piper cenocladum*]	5.94/4.66	21,554/19,501	71	7	35	gi|115605072	NCBInr
50	Trypsin inhibitor [*Glycine max*]	6.10/4.79	18,000/19,173	*3663*	4	39	gi|9858468	NCBInr
51	Glutathione S-transferase GST8 [*Glycine max*]	5.70/6.60	25,852/19,113	64	4	27	Q9FQF0	UniProtKB
52	AP-2 DRE binding factor DBF1 [*Oryza sativa*]	7.07/7.13	29,483/21,535	87	6	22	gi|31745669	NCBInr

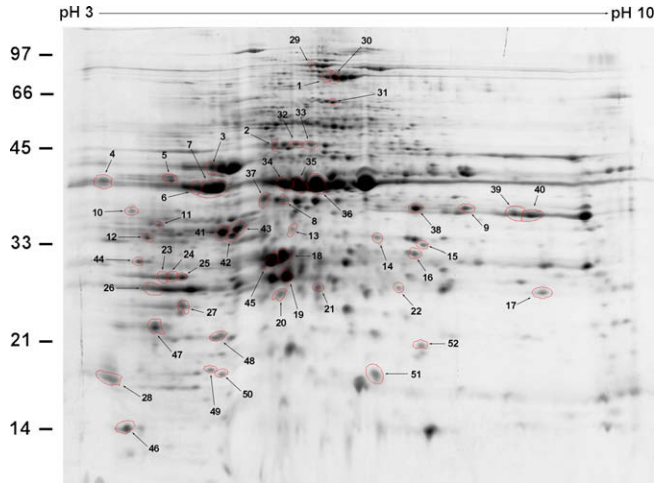

Fig. 4. 2-DE image of fractionated soluble soybean leaf proteins stained with SYPRO Ruby. Those low abundance proteins, now more evident after using calcium fractionation, can be seen here in more detail. Those outlined spots were previously identified as phosphorylated (1–28) or novel (29–52) and were isolated from a replicate gel stained with Coomassie. Protein spot numbers correspond to those listed in Table 1. IEF separation was from pH 3–10 and protein molecular weight markers are in kDa.

methodology towards acquisition of key elements within the leaf proteome.

2.3. Application towards other plants

The successful reduction of Rubisco in leaf protein extracts from cultivated soybean brought us to hypothesize this technique might be universally applicable to other important crop plants as well. Mature leaves from corn, wheat, rice and the model plant *Arabidopsis* were collected, ground into a fine powder and extracted using the same low ionic strength buffer that had been used with soybean. Samples were fractionated using 10 mM Ca^{2+}/10 mM phytate, for 10 min at 42 °C, exactly as with soybean and after clarification of the solution, samples of the supernatant were analyzed using 1-DE. The results demonstrate the removal of several highly abundant proteins, along with substantial amounts of Rubisco, within all leaf samples used (data not shown). This suggests that this methodology is applicable to more than just soybean, and if used in conjunction with 2-DE protein separation, could aid in the analysis and discovery of those less abundant seed proteins within each species.

3. Concluding remarks

From a proteome standpoint, soybean leaf consists of thousands of proteins. With a good isolation technique, preparation and separation, at times roughly 3000 spots can be detected on our soybean leaf 2-DE gels using a sensitive fluorescent stain. In our typical 2-DE separation of soybean soluble leaf proteins, roughly 1500–2000 proteins can be detected on a gel image using our standard protocols and visual stains. However, of those proteins only the most abundant proteins can be readily detected (<500), since the vast majority of the total loaded and separated protein is made up of Rubisco. Obviously, not only does Rubisco limit the amount of protein that one can load and separate using 2-DE, thereby limiting the accessibility of the less abundant proteins, but from our vantage point it greatly interferes with the overall electrophoretic separation of the other proteins in both dimensions as well. In addition, as can be observed in comparing images from our non-fractionated and fractionated samples, it severely masks proteins in the same pI and size range. All these facts are clearly evident upon examination of the 2-DE image from the non-fractionated soybean leaf (Fig. 3A) or to anyone who has done 2-DE on proteins isolated from leaf.

Depending on leaf location, growth stage and overall plant health, cultivated soybean leaf protein extract can consist of nearly 50% of the enzyme Rubisco. It has been shown here with this methodology, and by others, that electrophoretic resolution can be improved through fractionation of the leaf proteome using customized separation techniques. Here, we have demonstrated a simple, fast and inexpensive method to remove this overabundant protein from soybean leaf soluble protein extract. With the simple addition of 10 mM Ca^{2+} and 10 mM phytate to the leaf protein extract in low ionic strength buffer, 85% of Rubisco can be depleted from the total leaf protein extract. As demonstrated here, this methodology allows for those low abundance proteins in soybean leaf to be more accessible for assessment of post-translational modifications and ultimately spot isolation and protein identification. Even though the procedure described is a simple and an efficient way to deplete Rubisco, it does remove a significant amount of non-target proteins. Further refining of our fractionation technique may be necessary to minimize the loss of non-target proteins. Subsequently, other methods such as immunoaffinity depletions may be still necessary to specifically remove only the target proteins.

Along with our results with other plant leaf tissue, we are additionally suggesting that our methodology will work with other agronomically important plants. Such a fractionation technique has the potential to allow more clues about protein expression, modification and properties for far more proteins than ever before. This will ultimately help many of those who struggle with analysis of plant nutritive potential, yield potential or environmental stress response on a protein expression level.

4. Experimental

4.1. Reagents

Sodium phytate, calcium chloride dihydrate, 3-(4-heptyl)phenyl-3-hydroxypropyl)-dimethylammoniopropanesulfonate (C7BzO), 2-hydroxyethyl disulfide (2-HED), and protease inhibitor cocktail (P-9599) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Pro-Q Diamond in-gel phosphorylated protein stain, SYPRO Ruby fluorescent protein stain, and EZQ protein quantification kit were obtained from Molecular Probes (Invitrogen, Carlsbad, CA, USA). Immobilized pH gradient (IPG) strips, IPG Buffer 3–10, were obtained from GE Healthcare (Piscataway, NJ, USA). Modified porcine trypsin was acquired from Promega (Madison, WI, USA). R-cyano-4-hydroxycinnamic was acquired from Bruker Daltonics (Billerica, MA, USA).

4.2. Plant material

Fractionation experiments included soluble proteins extracted from the leaves of the following plants: soybean [*Glycine max* (L.) Merr. cv Williams 82], corn [*Zea mays* (L.). ssp. *mays*], rice [*Oryza sativa* (L.)], wheat [*Triticum aestivum* (L.)], and thale cress [*Arabidopsis thaliana* (L.) Heynh.].

4.3. Protein isolation and fractionation

Mature, freshly harvested leaves were frozen in liquid N_2 and ground into a fine powder using a mortar and pestle. Leaf tissue powder (500 mg) was placed into a clean mortar and pestle and ground further using 1 mL of 50 mM Tris–Cl, pH 6.8 containing

plant protease inhibitors. Liquefied tissue was clarified with centrifugation at 16,100×*g* for 10 min. Supernatant was removed and placed into a clean tube for fractionation. For fractionation, a 100 mM $CaCl_2{\cdot}2H_2O$ stock solution and 100 mM sodium phytate stock solution were prepared and used to bring each leaf extract to the final concentration. After addition of the fractionation agents, gentle mixing and incubation, each solution was clarified with centrifugation at 16,100×*g* for 10 min. Supernatant was removed, placed into a clean tube, and placed immediately on ice.

4.4. 1-D electrophoresis

For 1-DE analysis the clarified supernatant was combined 1:1 with 2× SDS–PAGE sample buffer. A typical gel contained about 40 μg of protein for each sample. One-dimensional separation followed the method of Laemmli (Laemmli, 1970) using 13.5% T gels run using a Mini250 apparatus (GE Healthcare). Electrophoretic separation was achieved with 20 mA per gel (constant current) and a 1.5 h run time. For visualization of proteins, gels were removed from the cassette and placed immediately in Coomassie staining solution (40% v/v MeOH, 7% v/v AcOH, 10% w/v Coomassie Blue R-250).

4.5. 2-D electrophoresis

After initial leaf protein isolation and calcium/phytate fractionation, clarified supernatant was placed on ice and brought to 0.9 M sucrose and 0.4% β-mercaptoethanol, and adjusted to 0.1 M Tris–Cl, pH 8.8. An equal volume of Tris-equilibrated phenol was added followed by vortexing. Solution was then mixed vigorously for 30 min at 22 °C followed immediately by centrifugation at 4000×*g* for 20 min at 15 °C in a swing-bucket rotor. The upper phase (phenolic) was removed and added to five volumes of freshly prepared MeOH with 0.1 M ammonium acetate (pre-chilled to −80 °C). Protein precipitation progressed for 2 h at −80 °C. Solution was centrifuged at 6000×*g* for 10 min at 4 °C, supernatant discarded and protein pellet resuspended vigorously in a freshly prepared solution of 100% methanol with 0.1 M ammonium acetate and 0.01 M DTT (pre-chilled to −20 °C). Insoluble proteins were repeatedly washed (3×) with the MeOH/ammonium acetate/DTT solution, then washed (2×) with a freshly prepared solution of 80% acetone–H_2O containing 0.01 M DTT. In between each vigorous wash step, the solution of insoluble proteins was incubated at −20 °C for 20 min prior to centrifugation at 12,000×*g* for 10 min at 4 °C. After the final centrifugation, the protein pellet was allowed to dry (near dryness), then solubilized in a small volume of 7 M urea, 2 M thiourea, 1% CHAPS and 2% C7BzO with vortexing. Samples were never frozen, only stored on ice, until protein concentration obtained following the method of Bradford (Bradford, 1976). Each solution was then brought to 0.1 M DTT.

The 13 cm IPG strips (linear) used throughout this study were loaded maximally with 250 μg of protein using in-gel passive rehydration. Rehydration solutions were brought to volume with 7 M urea, 2 M thiourea, 1% CHAPS and 2% C7BzO with a final concentration of 5% glycerol, 2.2% 2-HED and 0.5% 3–10 IPG buffer. The final concentration of DTT (0.06 M) to be used in conjunction with 2-HED in each strip was optimized previously (Sarma et al., 2008). Strips were rehydrated passively, overnight at 22 °C. Isoelectric focusing was performed with a Protean II IEF (BioRad) and method was as follows: 50 V, 1 h, fast ramp; 250 V, 250 Vhr, fast ramp; 1000 V, 500 Vhr, fast ramp; 8000 V, 2 h, linear ramp; 8000 V, 65,000 Vhr, fast ramp. IPG strips were equilibrated with 5% SDS in a urea based solution (0.05 M Tris–Cl pH 8.8, 6 M urea, 30% glycerol and 0.1% bromophenol blue) containing 2% DTT for 20 min, followed with 2.5% iodoacetamide for 20 min prior to the second dimension. Strips were carefully placed onto a Hoeffer SE600 (GE Healthcare) 16% T vertical second dimension and secured into place with warm SDS–PAGE running buffer (0.2% SDS) containing 1% agarose. Gels were run at an initial 10 mA/gel followed by 25 mA/gel for the remainder of the run. 2-DE gels for Coomassie staining were immediately removed and fixed in 5:4:1 (MeOH:-H_2O:AcOH) for 30 min, followed by staining in Coomassie G-250 for overnight. 2-DE gels for Pro-Q Diamond or SYPRO Ruby staining were immediately removed and fixed in 2:7:1 (MeOH:H_2O:AcOH) for overnight (with two changes of fixative) prior to staining.

4.6. Pro-Q Diamond and SYPRO Ruby analysis

For fluorescent staining, a more accurate sample protein concentration was determined using the EZQ system. Sample preparation and electrophoresis for Pro-Q Diamond in-gel phosphorylated protein analysis and SYPRO Ruby fluorescent stain was same as described above. However, for in-gel phosphorylated protein spot analysis, 200 μg of each protein sample was run per gel. Pro-Q Diamond staining and processing followed a modified methodology (Agrawal and Thelen, 2005). After scanning, gels were rinsed with ultrapure H_2O, and stained for total protein using SYPRO Ruby according to the manufacturer's protocol.

4.7. Image acquisition and analysis

Coomassie gels were destained with multiple changes of ultrapure H_2O and scanned using a HP Scanjet 5470c controlled with Adobe Photoshop. Gels stained with Pro-Q Diamond and SYPRO Ruby were scanned using a Fuji FLA5000 v3.0, at 532 nm excitation with a 575 long pass green filter and at 473 nm excitation with a 510 nm long pass blue filter respectively. Image quality for both was optimized using Fuji Multi Gauge v2.3. Images from Coomassie, Pro-Q Diamond and SYPRO Ruby stained gels were analyzed for proteome differences using Delta2D v3.6 image analysis software (Decodon, Greifswald, Germany). Delta2D provided vol% data used throughout this report. Parameters were set to maximize detection of every spot and images were globally warped to a master, non-fractionated image, using exact spot matching and reference proteins which were present in all gels. No background subtraction was used. Percent spot volume ratio [non-fractionated]/[Ca^{2+}-phytate fractionated] differences were taken from the spot quantitation table and cutoffs were determined according to fold changes. Since nearly all protein spots were kept for calculation purposes, and the total quantity of all spots on the gel is 100%, the relative quantity of the spot gave an accurate determination of the percentage change between non-fractionated and fractionated samples.

4.8. Protein identification

Protein gel spots for identification were excised with a 1.5 mm Spot Picker (The Gel Company, San Francisco, CA, USA) from a Coomassie G-250 stained gel. Gel pieces were washed briefly in distilled H_2O and destained in CH_3CN–H_2O (1:1, containing 25 mM ammonium bicarbonate. A final CH_3CN acetonitrile wash was performed) and the protein contained in the acrylamide gel was digested using 20 μL (10 μg/mL) of modified porcine trypsin in 25 mM ammonium bicarbonate. Peptides resulting from the tryptic digest were analyzed using a Voyager DE-STR (Applied Biosystems, Framingham, MA, USA) matrix-assisted-laser-desorption-time-of-flight mass spectrometer. The peptides were co-crystallized with R-cyano-4-hydroxycinnamic acid matrix on a MALDI-TOF-MS plate, briefly dried, and ionized using a 337 nm nitrogen laser operating at 20 Hz. Trypsin autolysis peaks of charge/mass ratios 842.51, 1045.56 and 2211.10 served as internal calibrants. Peptide mass searches were performed via Mascot (Matrix Sci-

ences, http://www.matrixscience.com) and/or Protein Prospector (University of California – San Francisco, http://prospector.ucsf.edu) using NCBI non-redundant *Glycine max* database and UniProtKB *Glycine max* database. All searches were performed with a fragment mass tolerance of 20 ppm, allowance of only one missed cleavage, and carbamidomethyl fixed modification. Peptides from unmatched proteins were searched using identical search criteria, but within "all-plant" databases.

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