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Research Article

A simple, economical and reproducible protein extraction protocol for proteomics studies of soybean roots

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Abstract

Sample preparation is a critical step in two-dimensional gel electrophoresis (2-DE) of plant tissues. Here we describe a phenol/SDS procedure that, although greatly simplified, produced well-resolved and reproducible 2-DE profiles of protein extracts from soybean [*Glycine max* (L.) Merril] roots. Extractions were made in three replicates using both the original and simplified procedure. To evaluate the quality of the extracted proteins, ten spots were randomly selected and identified by mass spectrometry (MS). The 2-DE gels were equally well resolved, with no streaks or smears, and no significant differences were observed in protein yield, reproducibility, resolution or number of spots. Mass spectra of the ten selected spots were compared with database entries and allowed high-quality identification of proteins. The simplified protocol described here presents considerable savings of time and reagents without compromising the quality of 2-DE protein profiles and compatibility with MS analysis, and may facilitate the progress of proteomics studies of legume-rhizobia interactions.

Key words: 2-DE, protein extraction, proteomics, soybean roots, symbiosis.

Introduction

Sample preparation, a critical step in proteomics research using two-dimensional gel electrophoresis (2-DE) (Saravanan and Rose, 2004) is particularly troublesome in studies with plants, the tissues of which are often rich in proteases and interfering compounds such as polysaccharides, lipids and phenols (Carpentier *et al.*, 2005). Such contaminants interfere with protein separation and analysis, resulting in horizontal and vertical streaking, smearing, and in decreased number of distinctly resolved spots. In addition, plant tissues have low protein content in comparison to animal and bacterial sources, therefore effective extraction of proteins is essential to obtain successful results (Saravanan and Rose, 2004; Carpentier *et al.*, 2005).

Proteomics studies allow quantitative and qualitative comparisons of proteins, therefore any loss of protein at any step in the analysis process is critical. Furthermore, it is essential to obtain high-quality gels with well-resolved spots showing reproducible protein patterns (Hurkman and Tanaka, 1986). The extraction method must preserve the quality and quantity of the proteins extracted and for that sample treatment must be kept to a minimum number of steps, to minimize protein loss (Hurkman and Tanaka, 1986; Westermeier and Naven, 2002).

Root-nodule symbioses of rhizobia with legumes, resulting in biological nitrogen fixation, represent a major subject of study, due to their contribution to the global nitrogen cycle and to the economic importance of legume crops such as soybean [*Glycine max* (L.) Merr.]. Research in our laboratory is focused mainly on soybean and common bean (*Phaseolus vulgaris* L.) symbioses, generally involving nodulated roots. Although a one-step procedure for protein extraction would be highly desirable, no single method of sample preparation can be universally applied to 2-DE analysis (Dunn, 1999). Due to the need for a simple method that could be applied routinely to proteomics studies of symbiotic interactions, we optimized a phenol/SDS based method described previously (Wang *et al.*, 2006). The simplified method presented here was able to produce well-resolved and reproducible 2-DE protein profiles of soybean roots and it was also compatible with matrix-assisted laser desorption ionization time-of-flight/mass spectrometry (MALDI-TOF/MS) analysis, enabling the successful identification of the proteins. This study offers a

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useful workflow for proteomics of legume-rhizobia symbioses using 2-DE and MS analysis.

Materials and Methods

Plant material

Surface-sterilized soybean seeds were inoculated with *Bradyrhizobium japonicum* strain CPAC 15 (= SEMIA 5079) and grown aseptically receiving sterile N-free nutrient solution. Roots of 13-day-old soybean seedlings were harvested, immediately frozen in liquid nitrogen and stored at -80 °C until extraction. At this stage only nodule primordia were present, with no fully developed nodules. Roots were finely powdered by grinding in a mortar and pestle with liquid nitrogen, which, as emphasized earlier (Giavalisco *et al.*, 2003), is a critical step in obtaining high resolution of proteins in 2-DE gels.

Protein extraction

The simplified protocol is based on the phenol/SDS method described previously (Wang *et al.*, 2006), which comprises three successive washes with trichloroacetic acid (TCA) in acetone, methanol and acetone, followed by a vacuum-drying step before the phenol/SDS extraction. The modifications in our method are the elimination of three preliminary wash steps and of the vacuum-drying step, representing considerable economy of reagents and time (Figure 1).

For comparison, proteins were extracted from soybean root samples using the simplified method described here and the original phenol/SDS method (Wang *et al.*, 2006). For both methods, protein extraction and 2-DE were performed with three replicates and comparisons were made for protein yields and 2-DE gel resolution and reproducibility.

Pulverized samples (250 mg) were resuspended in 0.8 mL of Tris-buffered phenol, pH 8.0, and 0.8 mL of SDS buffer [0.1 M Tris-HCl pH 8.0, 2% SDS, 5% β-mercaptoethanol, 30% sucrose, 1 mM phenylmethylsulfonyl fluoride (PMSF)]. The samples were vigorously vortexed for 10 min and centrifuged at 16,000 *g* for 5 min at 4 °C, and the top phenol layer (0.5 mL) was transferred to a new tube. Proteins were precipitated for 2 h at -20 °C with three volumes of pre-cooled 0.1 M ammonium acetate in absolute methanol and then pelleted by centrifugation (16,000 *g* for 5 min at 4 °C). The pellet was washed once with pre-cooled 0.1 M ammonium acetate in methanol and once with pre-cooled 80% v/v acetone, followed by vacuum drying.

The protein pellets were solubilized in isoelectric focusing (IEF) buffer (7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 20 mM *dithio*threitol (DTT), 0.5% immobilized pH gradient (IPG) buffer, pH 3-10 or 4-7), by pipetting followed by sonication for 30 s (2x) in a water bath sonicator at 15-20 °C. The solubilized proteins were quantified using the Bradford protein assay (Bradford, 1976) with a bovine serum albumin (BSA) standard curve.

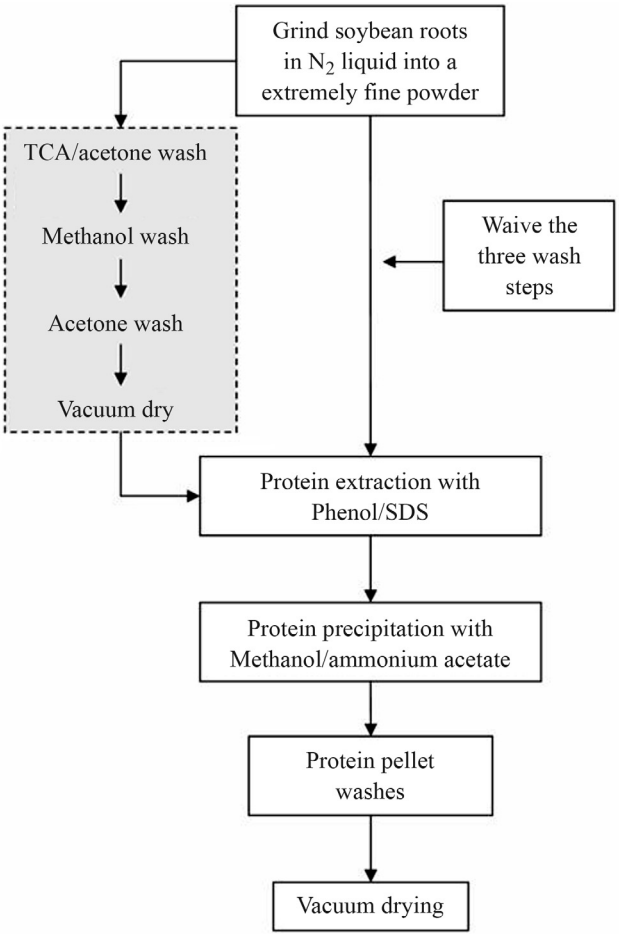

Figure 1 - Schematic outline of the protocol presented here. The modifications performed on the original phenol/SDS method (Wang *et al.*, 2006) are shown in the dashed frames with a gray background.

Isoelectric focusing

For the first dimension, 350 µg of solubilized protein was transferred to a fresh tube and the volume was brought up to 0.25 mL with De Streak buffer (GE Biosciences). The protein suspensions were rehydrated overnight into IPG strips (linear pH 3-10 or 4-7, 13 cm, GE Biosciences). Next, IEF was carried out on the IPGphor II (Amersham Biosciences) with a current limit of 50 µA/strip at 20 °C using a focusing protocol: 1 h at 200 V, 1 h at 500 V, 1 h at 1,000 V (gradient), 2.5 h at 8,000 V (gradient) and then 1.5 h at 8,000 V, reaching a total of 24.8 kVh.

SDS-PAGE

Prior to second dimension analysis, the IPG strips were gently agitated for 30 min in equilibration buffer emended with 1% (w/v) of DTT followed by agitation for 30 min in buffer emended with 3.5% (w/v) iodoacetamide. Then, the IPG strips were loaded onto acrylamide gels

(11.6%) and overlaid with agarose solution. Second dimension electrophoresis was carried out using an SE 600 Ruby (GE Biosciences) with the first current cycle at 15 mA per gel (30 min) and a second cycle at 30 mA per gel (4 h). Next, gels were stained overnight with Comassie Brilliant Blue (CBB) R-350 (GE Biosciences), destained in a solution of 40% ethanol and 10% acetic acid and scanned (ImageScanner LabScan v. 5.0). The 2-DE gel images were analyzed using the Image Master 2-D Platinum v.5.0 software (GE Biosciences).

MALDI-TOF/MS analysis and protein identification

Randomly selected spots were excised and processed as described before (Chaves *et al.*, 2009), with slight modifications on the digestion procedure, done with trypsin (Gold, mass spectrometry grade, Promega, Madison, WI) at 37 °C overnight. For MALDI-TOF analysis, 0.5 μL of the tryptic peptide was mixed with a matrix solution of saturated α-cyano-4-hydroxycinnamic acid (HCCA) in acetonitrile 50%/trifluoroacetic acid (TFA) 0.1%. The mixture was spotted onto a MALDI sample plate and allowed to crystallize at room temperature. The same procedure was used for the standard peptide calibration mix (Bruker Daltonics).

Mass spectra were acquired using a MALDI-TOF-MS Autoflex spectrometer (Bruker Daltonics) operated in the reflector for MALDI-TOF peptide mass fingerprint (PMF) with a fully manual mode, using FlexControl software. For protein identification, PMFs generated were searched against the NCBInr database using the MASCOT software (Matrix Science). For the protein search, monoisotopic masses were used and a peptide tolerance of 100 ppm and one missed cleavage was allowed. Carbamidomethylation of cysteine and oxidation of methionine were taken into consideration as fixed and variable modifications, respectively.

Results and Discussion

Quantitative comparison of protein samples obtained with the two extraction protocols indicated that protein yields were statistically equal (ANOVA, $p < 0.05$) (Table 1). The protein values recovered by both methods were within the range of those reported in the literature for plant roots (Westermeier and Naven, 2002; Saravanan and Rose, 2004); however, the slightly higher protein yield obtained in our modified method might be attributed to the removal of the preliminary washes, which were part of the original method, and potentially preventing protein loss. Any increase in protein yield is important when the material under analysis is limited or when the protein content is low, as is the case with soybean roots.

A prerequisite, but also a major challenge in proteomic analysis, is the separation of proteins from complex biological samples with high resolution and reproducibly (Görg *et al.*, 2004). Both the original and the modified methods tested in this study have proven to be suitable to obtain proteins from soybean roots, resulting in high-quality 2-DE gels (Figure 2). The proteins were well resolved on gels, with no streaks or smears, indicating insignificant degradation of proteins and efficient removal of contaminant compounds.

The removal of interfering compounds prior to protein extraction may also be critical (Saravanan and Rose, 2004; Carpentier *et al.*, 2005), and washes with TCA and acetone are usually performed to eliminate salts, polyphenols, and also to minimize protein degradation (Görg *et al.*, 2004). However, no evident improvements were observed by preliminary TCA/acetone, methanol and acetone washes (Figure 2), proving that our modified protocol is suitable both for removing interfering compounds and minimizing protein degradation in extracts from soybean root tissues.

Another limitation may result from changes in factors such as pH, ionic strength, and temperature that may lead to selective losses of proteins; the more steps in the extraction process, the greater are the chances of losing or erroneously detecting differentially expressed protein subsets (Rose *et al.*, 2004). Decreasing the number of treatment steps in our method may help to avoid protein losses and, at the same time, enable high-quality images, with no need of cleanup steps to reduce background smear or to remove horizontal and/or vertical streaks.

Both protocols gave well-resolved and reproducible spot patterns (Figure 2). Fully automatic spot detection and matching were performed, and the data indicating the reproducibility of each protocol are shown in Table 1. Statistically similar numbers (ANOVA, $p < 0.05$) of detected spots were observed when both methods were compared. Spots matched in all three replicate gels ranged from 84% in the original phenol/SDS (Wang *et al.*, 2006) to 88% in our modified method (Table 1).

Table 1 - Recovered protein yield and reproducibility from soybean root proteins using two protein extraction methods and IPG strips (13 cm) with a linear pH range 3-10. a: Values represent the means of three independent biological replicates with standard deviation in parentheses; NS: Values show no statistically significant difference (ANOVA test, $p < 0.05$).

Extraction method	Protein yield (mg/g roots)[a]	Number of detected spots[a]	Matched spots (%)[a]	Spots with CV value < 30% (%)	Correlation value (R^2) of matched spots
Original phenol/SDS	1.61 (±0.11)[NS]	646 (±33)[NS]	84	91	0.963
Modified phenol/SDS	1.82 (±0.09)[NS]	715 (±40)[NS]	88	88	0.924

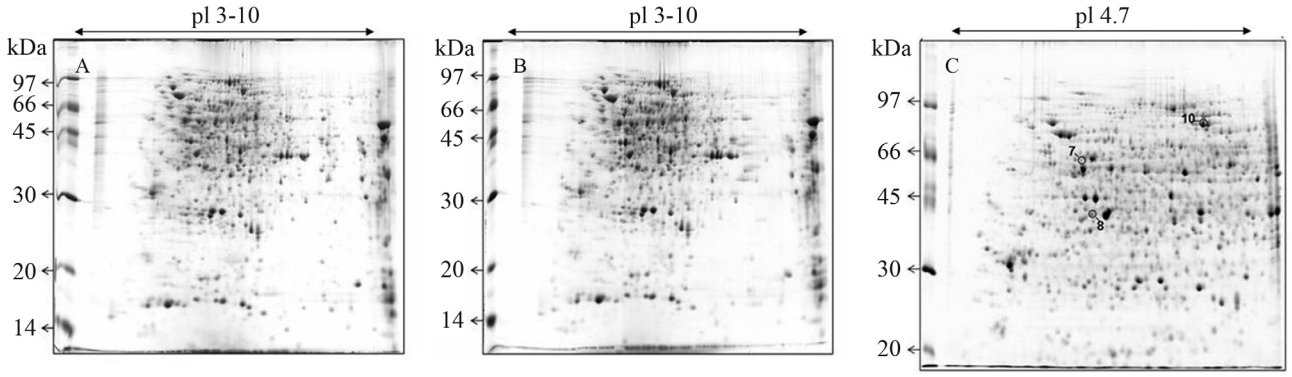

Figure 2 - 2-DE analysis from soybean root proteins extracted using the modified phenol/SDS extraction method (A, C) and original phenol/SDS method (B) (Wang *et al.*, 2006). Solubilized proteins (350 µg) were focalized using IPG strips (13 cm) with a linear pH range 3-10 (A, B) or a linear pH range 4-7 (C). SDS-PAGE was performed in a vertical 11.6% acrylamide gel and stained with CBB R 350. The molecular weight of protein standards is indicated on the left.

To assess reproducibility of the protein patterns, we performed a correlation analysis and estimated the coefficient of variation (CV) of the calculated normalized spot volumes (% vol) from pairwise matched spots obtained in each extraction protocol (Table 1). Considering the similarities between gels, the correlation (R^2) of the %vol of spots was higher than 0.92 for both protocols, indicating high reproducibility. In agreement with previously reported data (Molloy *et al.*, 2003), CV values lower than 30% were considered as indicative of the quality of the experiment, and for our modified method and the original phenol/SDS method (Wang *et al.*, 2006), the CV% for the %vol was lower than 30% for 88% and 91% of matched spots, respectively. CV values higher than 30% were obtained only for spots prone to mismatches due to local pattern distortions.

In 2-DE gels using 3-10 pH IPG strips, about 65% of total spots were concentrated in the 4-7 pH range, prompting us to choose this narrower range to increase the resolution of the proteins concentrated in this pH region and to facilitate comparative studies of soybean root proteins. A total of 512 ± 42 spots were detected on the gel by using a pH 4-7 IPG strip (Figure 2). Three of these spots were randomly selected (Figure 2) and subjected to MALDI-TOF mass spectrometry analysis. The searching of MS data against the NCBInr protein database using Mascot allowed the protein identification of all selected spots, as listed (Table 2). In all cases, both significant protein score values in Mascot identifications and a high percentage of sequence coverage were obtained. The high quality of MS data indicated the compatibility of the optimized protein extraction method with MALDI-TOF/MS.

Among the proteins identified, one is related to the peroxisomal betaine-aldehyde dehydrogenase that catalyzes the conversion of betaine aldehyde to betaine, a carbon or nitrogen source and an important osmoprotectant agent (Pocard *et al.*, 1997). Betaine was also known to be one of the non-flavonoid compounds that is able to induce *nod* genes in rhizobia (Subramanian *et al.*, 2007).

We have also identified a cytosolic glutamine synthetase β1 (GS- β1), a key enzyme responsible for the primary assimilation of ammonia in root nodules of legumes. The β1-form is considered the constitutive isoform, but it is expressed in relatively high levels in nodules. It has been demonstrated that, in soybean, the GS- β1 gene (*gln*β1) is induced in nodules by reduced N or its assimilation products, showing that its expression is regulated by physiological changes (Morey *et al.*, 2002). Methionine synthase was the third identified protein.

In conclusion, the protocol presented here, although greatly simplified, allowed well-resolved and reproducible spot patterns on 2-DE gels of soybean root proteins. Our sample preparation helped avoid protein modifications or losses and facilitated reproducibility. The modified method presented in this study proved to be efficient, simple, economical and appropriate for proteomics studies with

Table 2 - Selected protein spots identified by MALDI-TOF.

Spot no.	Protein name and organism	GenBank accession number	Mascot score	N[1]	Cov[2]	pI[3]		MW[4] (kDa)	
						Exp.[5]	The.[6]	Exp.[5]	The.[6]
7	Peroxisomal betaine-aldehyde dehydrogenase (*Glycine max*)	gi|167962545	79	10	22	5.03	5.23	65	55.4
8	Cytosolic glutamine synthetase (*Glycine max*)	gi|10946357	78	9	29	5.15	5.48	45	39.1
10	Methionine synthase (*Glycine max*)	gi|33325957	107	13	18	6.26	5.93	85	84.4

[1]Number of peptides identified; [2]Sequence coverage (%); [3]Isoelectric point; [4]Molecular weight (kDa); [5]Experimental value; [6]Theoretical value.

soybean roots, and will likely be applicable to the root tissues of other plants.

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