Available online at www.sciencedirect.com
ScienceDirect
Phytochemistry 67 (2006) 2341–2348
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Polyethylene glycol fractionation improved detection of low-abundant proteins by two-dimensional electrophoresis analysis of plant proteome

Jinghui Xi [a,b], Xu Wang [a], Shanyu Li [a,c], Xin Zhou [a], Lin Yue [a], Jia Fan [a], Dongyun Hao [a,*]

[a] *Key Laboratory for Molecular Enzymology and Engineering of the Ministry of Education (Jilin University), Changchun 130021, PR China*
[b] *Plant Science College, Jilin University, Changchun 130062, PR China*
[c] *Department of Pediatrics, The First Hospital of Jilin University, Changchun 130021, PR China*

Received 20 December 2005; received in revised form 2 June 2006
Available online 14 September 2006

Abstract

Poor detection of low-abundant proteins is a common problem in two-dimensional electrophoresis (2-DE) for separation of proteins in a proteome analysis. This is attributed partially, at least, to the existence of high-abundant proteins, e.g. ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) in plants. They engage a large proportion of the whole-cell proteins and thus prevent low-abundant proteins from being up-taken by immobilized pH gradient (IPG) strip, consequently making the latter poorly detectable by 2-DE. In this work, we report a straightforward protocol for preparation of whole-cell proteins through differential polyethylene glycol (PEG) precipitation aiming at elimination of Rubisco from plant protein samples. In comparison with 2-DE analysis of protein samples prepared using a conventional TCA/acetone method, a relatively high reproducibility of proteins was achieved using a PEG fractionation protocol in terms of protein yield and protein species. As expected, the large subunit of Rubisco was precipitated predominantly in the 16% PEG fraction. This allowed proteins of the Rubisco-containing fraction to be analyzed separately from those of other PEG fractions. After taking into account the overlapping protein spots among 2-DE gels of all fractions through image and statistical analyses, we detected with this protocol a total 5077 protein spots, among which ca. 80% are proteins undetectable with the TCA/acetone method, while the rest of proteins exhibited a significant increase in their abundance. This protocol was developed using Arabidopsis as a source of protein and thus may also be applicable to protein preparations of other plants.

Keywords: *Arabidopsis thaliana*; Cruciferae; Polyethylene glycol; Protein preparation; Two-dimensional electrophoresis; Low-abundant proteins; Rubisco

1. Introduction

Proteomics is developing rapidly in the post-genomic era and has become increasingly desirable for exploration of gene function (Phizicky et al., 2003). Preparation of proteins at the proteome scale is however essential for proteomic analysis. Although many techniques have been used successfully for proteomic analysis, such as chromatography, isotope coded affinity tag (ICAT) and protein micro-arrays (Adam et al., 2002; Templin et al., 2003), two-dimensional electrophoresis (2-DE) is currently the only technique that can be routinely applied for parallel quantitative profiling of large sets of complex protein mixtures. To perform proteome analysis using 2-DE, sample preparation is crucial to achieve efficient protein resolution, which in turn affects 2-DE results in terms of image quality and protein species distribution (Lilley et al., 2002). It is noteworthy that the majority of proteins revealed by 2-DE are abundant "housekeeping" proteins that are present in copy number per cell from 10^5 to 10^6 (Patlerson and Aebersold, 2003; Görg et al., 2004). However, many low-abundant proteins, in particular those that are regulatory

* Corresponding author. Tel.: +86 431 8498038x601; fax: +86 431 8498440.
E-mail address: hao@jlu.edu.cn (D. Hao).

0031-9422/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2006.08.005

factors and receptor molecules are present in cells at relatively low concentrations (typically, 100 molecules per cell), and are thus usually not detectable (Gygi et al., 2000; Tirumalai et al., 2003). Development of efficient methods or strategies for protein sample preparation has therefore become an important goal in applications of 2-DE for proteome analysis.

Gene expression of a large copy number of high-abundant proteins is essential for living organisms to maintain their fundamental physiological processes. In plants, for example, ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) is the most abundant protein and is involved in CO_2 fixation of photosynthesis (Gutteridge and Gatenby, 1995); indeed, it makes up more than half of the total leaf proteins in some species (Ellis, 1979). Since these high-abundant proteins usually do not participate in gene regulatory events, they become interfering factors in 2-DE proteomic analysis of regulatory proteins in two ways: (1) Rubisco co-migrates with the low-abundant proteins during 2-DE causing the latter to be undetectable (Corthals et al., 2000); (2) since Rubisco accounts for a large proportion of total protein sample and protein loading capacity for a given immobilized pH gradient (IPG) strip is limited, low-abundant proteins may only be up-taken by the IPG strip in a small quantity and hence hardly be visualized by 2-DE analysis. It is apparent that one of the considerations to improve the detection of low-abundant proteins by 2-DE is to minimize the high-abundant proteins.

Unlike nucleic acids that can be amplified by the polymerase chain reaction (PCR), low-abundant proteins from living organisms are hardly amplified due to the high dynamic range and diversity of their gene expression. Many strategies have been developed to promote detection of low-abundant proteins by 2-DE. For example, enrichment of proteins can be achieved by SDS–PAGE-based size fractionation (Sun et al., 2003) and a combination of complementary multidimensional technologies (Issaq et al., 2002) for proteome analysis. In addition, a three-phase partitioning (TPP) fractionation method was used on a new group of low abundant proteins in the proteome of the stripped thylakoid membrane (Peltier et al., 2004a). Another way to visualize as many proteins as possible in 2-DE is to perform subcellular fractionation for sub-proteome studies (Millar et al., 2001; Ferro et al., 2003; Pendle et al., 2005). As for diminishing the interference of Rubisco, the high-abundant proteins in plants and other organisms, selective precipitation (Sun et al., 2001), sucrose density gradient centrifugation (Peltier et al., 2004b) and FPLC anion-exchange chromatography (Wienkoop et al., 2004), etc. were also reported to deplete Rubisco successfully from different plant materials. However, many of these protocols are either laborious and time-consuming or require expensive equipments (Giavalisco et al., 2003).

In this work, we report a differential precipitation protocol using polyethylene glycol (PEG) to facilitate proteomic analysis by 2-DE. Using this protocol, Rubisco could be enriched into a specific PEG concentration fraction and thus detection of low-abundant proteins was improved significantly. PEG is a nontoxic water soluble synthetic polymer. It is understood that protein solubility (S) is depended on PEG concentration (C) following equation: $\log S = \beta C + \text{constant}$ (Atha and Ingham, 1981 and references therein). It can be seen from the equation that the linearity of C extends over a wide range of S. This advantage has made PEG an excellent differential precipitation reagent for purification of proteins from a variety of sources (Juckes, 1971). This protocol was characterized through detailed studies on protein precipitation efficiency using 2-DE image comparison and computational statistic analysis. The results showed that differential PEG fractionation is a straightforward method that can be used to visualize more proteins in 2-DE analysis of plant proteome.

2. Results and discussion

2.1. Distribution of plant whole-cell proteins upon PEG fractionation

Since protein loading capacity of the IPG strip is limited, one of the considerations to improve the visualization of low-abundant proteins for analysis is to deplete or even eliminate the high-abundant proteins from the whole-cell proteins, enabling more low-abundant proteins to be up-taken by the IPG strip and thus become detectable by 2-DE. To this end, upon the PEG fractionation, we investigated the electrophoresis distribution of plant proteins, in particular the high-abundant proteins such as Rubisco. Arabidopsis was used as a source of proteins and the whole-cell proteins were precipitated with the PEG of various concentrations as described in Section 4 and Fig. 1. Each of the resulting protein precipitants (F1–F5), together with the whole-cell proteins prepared without fractionation (NF), were suspended into a resolubilization/rehydration buffer and analyzed using SDS–PAGE. It can be seen from the gel profile shown in Fig. 2 that protein bands in each fraction distribute differentially throughout the full range of molecular weight, indicating that the whole-cell proteins were effectively fractionated by the PEG. As expected, the large and small subunits of Rubisco (LSU and SSU) in Arabidopsis are present predominantly in the 16% PEG fraction (F3). This provided possibility to eliminate Rubisco from Arabidopsis whole-cell proteins.

To further visualize the detailed protein separation profile upon the PEG fractionation, each PEG fraction, together with the non-fractionated sample (NF), was subjected to 2-DE analysis. The selected 2-DE images presented in Fig. 3 show that, similar to the observations on the SDS–PAGE analysis, the LSU and SSU exist predominantly in F3 (the 16% PEG fraction). The LSU spreads into a horizontal smear over a broad range from ca. pH 5–7 (Fig. 3a) and takes up a big proportion of the total proteins, apparently overlaying many protein spots along its

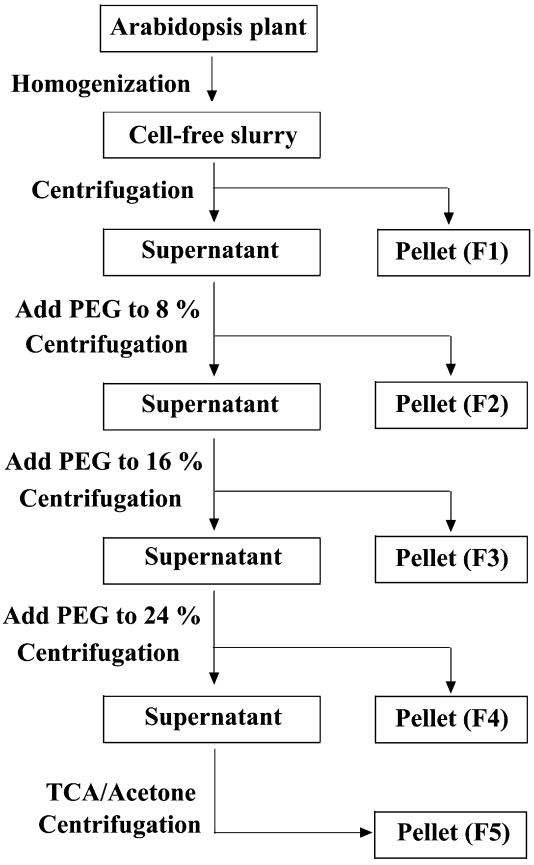

Fig. 1. Schematic work flow of a differential PEG precipitation for the fractionation of plant proteome proteins (see Section 4.2.2 for detailed description).

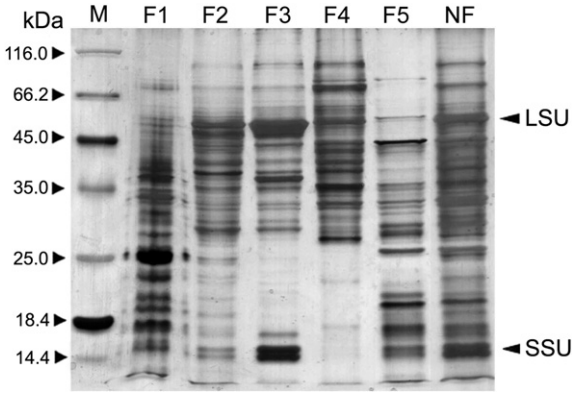

Fig. 2. SDS–PAGE analysis of proteins from the PEG fractionation and the non-fractionation. Lanes markings are made corresponding to those shown in Fig. 1, i.e. F1, F2, F3, F4 and F5 for the PEG fractionations and, the lane NF represents protein sample without fractionation serving as a control. Molecular weight markers (M) are shown on the left. Arrows on the right indicate the large subunit (LSU) and small subunit (SSU) of Rubisco, respectively. SDS–PAGE was performed on a 12.5% gel which was silver-stained.

smearing region. The 2-DE image of F4 (Fig. 3b) exhibits more protein spots than that of the NF (Fig. 3c). Statistical analysis indicated that overlapping protein spots are

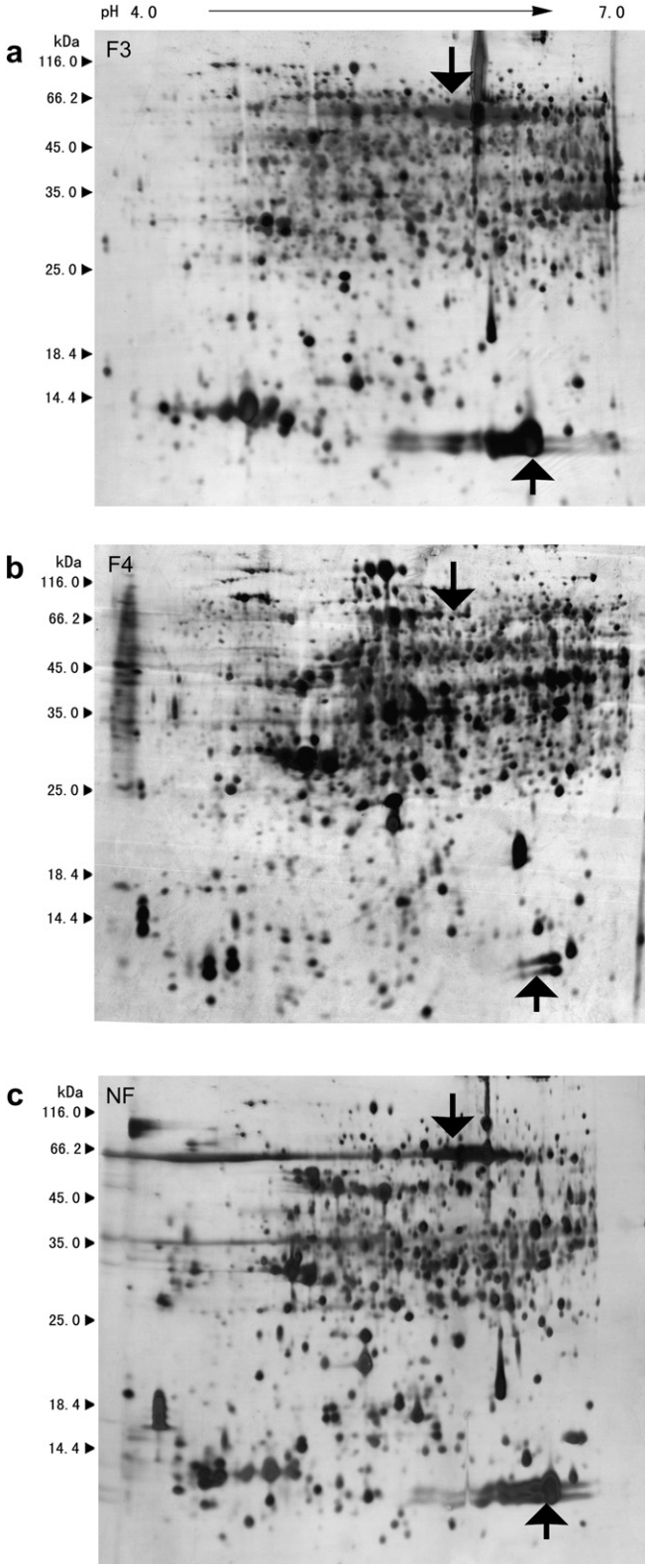

Fig. 3. 2-DE analyses of proteins from the PEG fractionation and the non-fractionation. Three representative protein profiles are illustrated, i.e. F3 (a), F4 (b) and NF (c). 200 μg of protein samples was loaded on an 18 cm IPG strip with a linear gradient of pH 4–7 for IEF, following electrophoresis of 12.5% SDS–PAGE and silver staining. The large subunit (LSU) and the small subunit (SSU) of Rubisco are pointed with downward and upward arrows, respectively, inside the images.

present differently between the neighboring fractions in a range from ca. 5% to 10% (see Table 1). After taking into account those overlapping protein spots among all fractions (i.e. F1–F5), more than 5000 protein spots were detected upon the PEG fractionation in comparison with ca. 1000 protein spots for NF (Table 2). This is ca. twice the number of protein spots reported previously in rice leaf by 2-DE gel analysis (Sun et al., 2001). These results suggested that separation of the F3 from the other PEG fractions would improve visualization of low-abundant proteins.

There exist a few studies dealing with proteome analysis of diverse plant samples (Giavalisco et al., 2003; Saravanan and Rose, 2004) and depletion of high-abundant proteins using affinity chromatography (Fountoulakis et al., 2004). Marchand et al. (2004) reported a partial removal of Rubisco from a crude protein preparation by size exclusion chromatography. In addition, sucrose density gradient centrifugation was used successfully to remove a majority of Rubisco from stromal complexes (Peltier et al., 2004b). Using our protocol, more than 90% of Rubisco can be selectively precipitated by the PEG into a specific fraction (i.e. F3) and thus analyzed by 2-DE separately from other cellular proteins in Arabidopsis.

Table 1
Percentage overlap of protein spots between the neighboring fractions

Neighboring fractions	Mean of protein spots[a]	NbOverlap[b]	% Overlap[c]
F1/F2	1122 ± 22	64 ± 4	5.7
F2/F3	1192 ± 7	121 ± 9	10.2
F3/F4	1149 ± 18	103 ± 6	9.0
F4/F5	1236 ± 3	130 ± 7	10.5

[a] The mean of protein spots detected from the neighboring fractions.
[b] The number of overlapping protein spots between the neighboring fractions (Nb) calculated using the ImageMaster™ 2D Platinum.
[c] % Overlap was calculated as $(b/a) \times 100\%$.

Table 2
Correspondence of proteins identified between the PEG fractionations and the non-fractionation

Samples	F spots number[a]	NF spots number[b]	Matched spots number[c]	% Match[d]
1	5048	1008	926	91.9
2	5108	973	902	92.7
3	5076	1022	937	91.7
Mean	5077 ± 30	1001 ± 25	922 ± 18	92.1

[a] The sum of all protein spots from the five PEG fractions (F) after taking into account the overlapping spots on 2-DE according to the inclusion–exclusion principle (Ian, 1989).
[b] The number of protein spots detected from the non-fractionated samples (NF) by 2-DE.
[c] The number of protein spots in the fractionated samples (F) that match those in the non-fractionated samples (NF).
[d] % Match is the percentage of the Matched spots number[c] over the NF spots number[b].

2.2. Reproducibility of whole-cell proteins upon the PEG fractionation

To examine if the whole-cell proteins may be reproduced upon the PEG fractionation, two parameters were tested, i.e. yield of proteins and homogeneity of protein species between fractionated and non-fractionated samples. Table 3 summarizes the yield of proteins upon the PEG fractionations (F1–F5) and the non-fractionation (NF) from three independent experiments. Among all fractions, the highest protein yield (ca. 2.0 mg protein/g fr.wt) was achieved at the 16% PEG fraction (F3), while the 40% PEG fraction (F5) exhibits the least yield of ca. 0.38 mg protein/g fr.wt. The sum of protein yields from all fractions is ca. 4.44 mg protein/g fr.wt. This is similar to that of the non-fractionation (NF) (ca. 4.59 mg protein/g fr.wt) which was directly precipitated using the TCA/acetone method. These results indicate that the PEG fractionation may recover the whole-cell proteins with a relatively high reproducibility. The marginal loss of proteins may likely be due to the increased complication of fractionation steps.

To assess the homogeneity of protein species between samples of PEG fractionation and that of non-fractionation, we performed pair-wise comparison of all 2-DE images with the ImageMaster™ 2D Platinum software together with statistical analysis to singularize the overlapping protein spots from gels of neighboring fractions. The results in Table 2 show that an average of 922 protein spots from each PEG fraction may be matched up with the corresponding spots in NF which exhibited 1001 spots in total. This gave rise to over 92% homogeneity of proteins made by the PEG fractionation over those without fractionation,

Table 3
Protein yields upon the PEG fractionations

Sample[a]	Protein yields (mg/g fr. wt.)						
	F1	F2	F3	F4	F5	Sum[b]	NF
1	0.58 ± 0.01	0.92 ± 0.01	2.04 ± 0.05	0.52 ± 0.02	0.38 ± 0.02	4.45 ± 0.05	4.58 ± 0.04
2	0.58 ± 0.01	0.95 ± 0.05	2.05 ± 0.05	0.50 ± 0.03	0.36 ± 0.03	4.44 ± 0.08	4.59 ± 0.03
3	0.60 ± 0.01	0.98 ± 0.05	1.99 ± 0.06	0.49 ± 0.02	0.41 ± 0.02	4.48 ± 0.03	4.61 ± 0.04

Data are represented as the mean values ± SD of three independent experiments, and the statistic analyses show that the results are the least significantly different among independent experiments ($p < 0.05$).
[a] Samples F1–F5 indicate the fractions produced from the PEG fractionation; the NF is the protein sample without fractionation.
[b] The sum of protein yields from the F1–F5.

suggesting that the majority of proteins produced using the conventional TCA/acetone method may also be precipitated by the PEG fractionation protocol. The small disparity of protein species may be due to three reasons: (1) Proteins may be lost during the multi-step PEG fractionation, as similar observation was reported previously (Shefcheck et al., 2003); (2) proteins precipitated by PEG may slightly be different from those precipitated by the TCA/acetone; (3) the PEG fractionation may likely cause irreversible precipitation of proteins (Rabilloud, 1996) which were not up-taken onto IPG strip of 2-DE. In this PEG fractionation protocol, nevertheless, a relatively high reproducibility of protein was achieved, and the gel profiles of the SDS–PAGE and 2-DE coincided with each other.

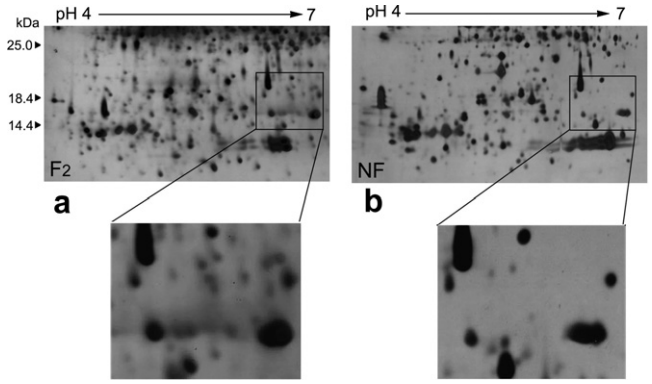

Fig. 4. PEG fractionation increased detectable proteins in 2-DE analysis. PEG fractionation and 2-DE analyses were performed the same as that described in Section 4 and elsewhere in the text. 2-DE protein profile detected upon 8% PEG fraction (F2, a) is selected as an example to compare with those non-fractionated protein samples (NF, b).

2.3. PEG fractionation improves detection of low-abundant proteins

The ImageMaster™ 2-DE Platinum software was used to analyze the 2-DE images so as to evaluate the PEG fractionation protocol in detection of low-abundant proteins in the Arabidopsis proteome. The results in Fig. 3 show that the number of visualized protein spots by 2-DE varies among different PEG precipitation samples, with the LSU predominantly in the 16% PEG fraction (F3). This allowed 2-DE analysis of the F3 to be performed separately from the other PEG fractions. This result is similar to the observation in fractionation of rice leaf proteins (Sun et al., 2001), in which the LSU was found to be exclusively in the 20% PEG fraction. In other PEG fractions (Fig. 3b), interestedly, there were many protein spots appeared in the area corresponding to the lengthy LSU smearing of F3 (Fig. 3a) and, apparently, those proteins contribute to the increased number of total detectable proteins by the PEG fractionation. In fact, a total of 5681 proteins were detected by 2-DE from the sum of proteins in all the five PEG fractions. After singularizing the overlapping protein spots from 2-DE images using the inclusion–exclusion principle (Ian, 1989), 5077 proteins were found to be unique and thus the PEG fractionation has accounted for fivefold more proteins than those without fractionation (i.e. NF).

We further investigated the changes in abundance of protein spots upon the PEG fractionation by means of relative spots volume measurements using the ImageMaster™ 2D Platinum software. Fig. 5 exhibits a pair-wise comparison of representative protein spots between each PEG fraction

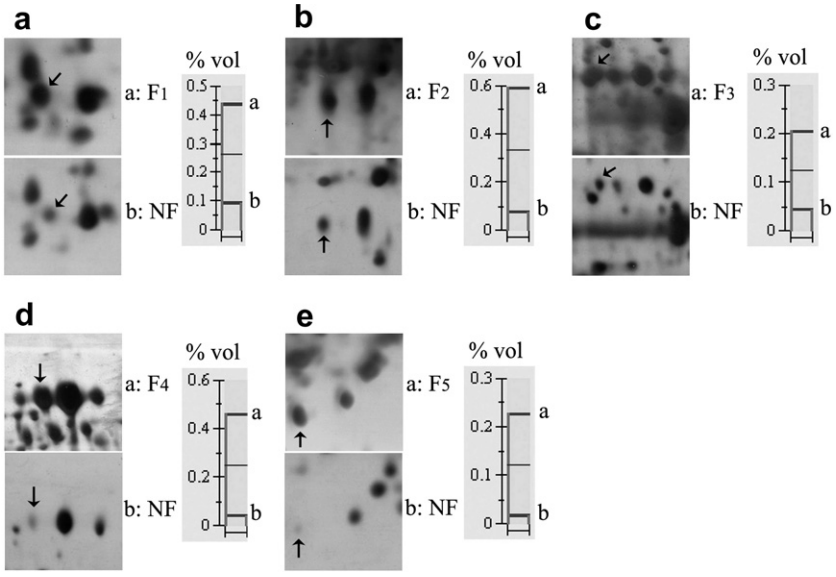

Fig. 5. Change in protein abundance upon the PEG fractionation. The local images (a) from each PEG fractions (F1–F5 which are labeled with A, B, C, D and E, respectively) are lined up pair-wised with their correspondences (b) from non-fractionated protein samples (NF). The change in % volume of each pair-wised spots (arrowed in each pair of images) was used as a measure of changes in proteins abundance upon the PEG fractionation and the spots of fourfold greater % volume difference were taken into account. % volume of spots in gel was calculated using software the ImageMaster™ 2D platinum (the software instruction manual is referred for the definition of % volume).

and the non-fractionation. It is noted that the relative volume of individual protein spots in each PEG fraction increased in magnitude from 4- to 12-folds (Fig. 5) over that of the non-fractionation including the Rubisco enriched fraction (F3). Indeed, the PEG fractionation enabled visualization of many protein spots that were not detectable without fractionation (Fig. 4). These results suggested that an increase in total protein number upon PEG fractionation is the consequence of amplification in protein abundance. The major drawback of plant proteomic analysis is that the copy number of gene product shows a wide distribution of ca. $1–10^6$ orders in magnitude (Görg et al., 2004), while the detectable protein range by the standard 2-DE is less than $1–10^4$ (Rabilloud, 2002). This makes the detection of low-abundant proteins a difficult task. However, the depletion of Rubisco in our protocol resulted in visualization of additional weak spots and ''hidden'' spots, leading to a dramatic increase in total detectable protein number.

3. Conclusions

In this study, we presented a simple protocol through fractionation of the whole-cell proteins into five different fractions upon the differential PEG precipitation. Through depletion of the high-abundant protein Rubisco from protein samples in Arabidopsis, the scheme was chosen for its merit in increasing the amount of individual proteins in each fraction. This allowed an enhanced quantity of proteins to be up-taken by the IPG strip and was also proven to decrease the complexity of proteins in cell-free extracts, leading to a higher protein resolution in 2-DE analysis. Using this protocol, the total number of proteins detected in 2-DE analysis were fivefolds over that of non-fractionated proteins, giving rise to more than 5000 protein spots. Since Rubisco is a common high copy number gene product existing in plants as well as other photosynthetic organisms, this protocol may be extended to a broader applications for proteomic analysis by 2-DE. However, pilot experiments are always recommended due to the complexity of cell-free protein extracts in plants.

4. Experimental

4.1. Plant materials

Seeds of *Arabidopsis thaliana* Columbia ecotype were obtained from the Arabidopsis Biological Resource Center (The Ohio State University, USA). Seeds were geminated in mixture soil and the seedlings routinely grew in a climate-simulated chamber at 75% humidity with 16 h light (80 μE/s m^2) at 22 °C and 8 h dark at 19 °C. The plants were harvested after four weeks of growth and washed with Milli-Q water to remove the soil attached. Immediately, the intact plants were frozen in liquid nitrogen and stored at −80 °C prior to protein preparation.

4.2. Protein preparations

4.2.1. Whole-cell protein extraction

Four grams of the frozen plants were crashed down in a pre-chilled mortar and homogenized in 20 ml of ice-cold protein extraction buffer containing 0.5 M Tris–HCl (pH 7.8), 2% (v/v) NP-40, 20 mM $MgCl_2$, 2% (v/v) β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM EDTA and 1% (w/v) polyvinylpolypyrrolidone (PVPP). The cell-free slurry was made by sonication and a four-volume of cold 10% TCA/acetone containing 0.07% (v/v) β-mercaptoethanol was added to allow proteins precipitated at −20 °C for 1 h. After centrifugation of the sample solution at 15,000*g* at 4 °C for 10 min, the resulting pellet was saved, rinsed twice with ice-cold acetone containing 0.07% (v/v) β-mercaptoethanol and dried up under vacuum. Finally, the dried pellet was resuspended in resolubilization/rehydration buffer (RB) (5 M urea, 2 M thiourea, 4% CHAPS, 20 mM dithiothreitol, 2% IPG-buffer (Amersham Biosciences, Sweden) and 0.002% bromophenol blue), taking as the non-fractionated protein sample which is designated as the NF in this paper.

4.2.2. PEG fractionation of proteins

A schematic work flow for PEG fractionation of the Arabidopsis whole-cell proteins is illustrated in Fig. 1. Proteins in frozen plants were prepared the same way as described in Section 4.2.1, leading to the cell-free slurry. After centrifugation of the slurry at 12,000*g*, 4 °C for 15 min, the pellet was rinsed with ice-cold acetone containing 0.07% (v/v) β-mercaptoethanol and designated as the fraction one (F1). To the supernatant, a 50% (w/v) PEG (PEG 4000) stock solution was added to give a final concentration of 8% PEG. The PEG suspended solution was placed on ice for 30 min to ensure protein precipitation and then centrifuged at 1500*g*, 4 °C for 10 min. The resulting pellet was taken as the fraction two (F2). The supernatant was further treated the same way as for the F2 to produce the 16% and the 24% PEG precipitation fractions, which are designated as the fraction three (F3) and the fraction four (F4) respectively. A difference from the F2 was that the centrifugations for the F3 and the F4 were performed at 12,000*g* for 15 min. The final supernatant from the 24% PEG precipitation was collected and further precipitated with four volumes of the cold TCA/acetone. The resulting pellet collected gave rise to the fraction five (F5). All fractions obtained were resolved into the RB for protein assay and 2-DE analysis. The protein concentration for all samples was determined prior to the electrophoresis analysis using the Bradford method (Bradford, 1976). For all experiments, three replications were performed including the extraction of proteins.

4.3. Two-dimensional gel electrophoresis

The sample was diluted with RB to 0.5 μg/μl in a final volume of 400 μl and an IPG strip of 18 cm (pH 4–7)

(Amersham Biosciences, Sweden) was rehydrated for 16 h to allow proteins to be up-taken. Iso-electric focusing (IEF) was performed using the Investigator™ 5000 (Genomic Solutions, USA) at 20 °C with a current limit of 50 μA per strip. Prior to second dimension analysis, the strips were equilibrated in 10 ml of 1% (w/v) dithiothreitol (DTT)-containing an equilibration buffer (6 M urea, 30% (w/v) glycerol, 2% SDS, 0.002% bromophenol blue, 50 mM Tris, pH 8.8) for 15 min, and subsequently, in 10 ml of 2.5% (w/v) iodoacetamide (IAA)-containing the same equilibration buffer for 15 min. Protein separation of the second dimension was carried out on a 12.5% SDS–PAGE. After 2-DE separation, the proteins on gel were visualized by silver staining as described by Méchin et al. (2003).

4.4. Image analysis

The gel profile of protein spots was scanned using the ImageScanner (Amersham Bioscience, Sweden), following the image analysis in the ImageMaster™ 2D Platinum software (Amersham Bioscience, Sweden). Protein spots were detected and quantified by software into their relative volume. A mean of three replicates was made for each gel to produce a master image. Overlapping and quantitative comparison of protein spots between two master gel images were carried out using the ImageMaster™ 2D Platinum. Normalization of the gels was done using the total spot density of each gel. The abundance of individual protein spots was determined as % volume and calculated using the ImageMaster™ 2D Platinum (for definition of % volume, the software instruction manual is referred).

Acknowledgements

We thank Dr. Kevin Lee of University of California at Los Angeles for his critical proofreading of this manuscript. This work was financially supported with a grant issued by the National Natural Science Foundation of China (Grant No. 30470159/C01020304).

References

Adam, G.C., Sorensen, E.J., Cravatt, B.F., 2002. Chemical strategies for functional proteomics. Mol. Cell Proteomics 1, 781–790.

Atha, D.H., Ingham, K.C., 1981. Mechanism of precipitation of proteins by polyethylene glycols. J. Biol. Chem. 256, 12108–12117.

Bradford, M.M., 1976. A rapid and sensitive for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Corthals, G.L., Wasinger, V.C., Hochstrasser, D.F., Sanchez, J.C., 2000. The dynamic range of protein expression: a challenge for proteomic research. Electrophoresis 21, 1104–1115.

Ellis, R.J., 1979. The most abundant protein in the world. Trends Biochem. Sci. 4, 241–244.

Ferro, M., Salvi, D., Brugiere, S., Miras, S., Kowalski, S., Louwagie, M., Garin, J., Joyard, J., Rolland, N., 2003. Proteomics of the chloroplast envelope membranes from *Arabidopsis thaliana*. Mol. Cell. Proteomics 5, 325–345.

Fountoulakis, M., Juranville, J.F., Jiang, L., Avila, D., Röder, D., Jakob, P., Berndt, P., Evers, S., Langen, H., 2004. Depletion of the high-abundance plasma proteins. Amino Acids 27, 249–259.

Giavalisco, P., Nordhoff, E., Lehrach, H., Gobom, J., Klose, J., 2003. Extraction of proteins from plant tissues for two-dimensional electrophoresis analysis. Electrophoresis 24, 207–216.

Görg, A., Weiss, W., Dunn, M.J., 2004. Current two-dimensional electrophoresis technology for proteomics. Proteomics 4, 3665–3685.

Gutteridge, S., Gatenby, A.A., 1995. Rubisco systhesis, assembly, mechanism, and regulation. Plant Cell 7, 809–819.

Gygi, S.P., Corthals, G.L., Zhang, Y.N., Rochon, Y., Aebersold, R., 2000. Evaluation of two-dimensional gel electrophoresis-based proteome analysis technology. PNAS 97, 9393–9395.

Ian, A., 1989. A First Course in Combinatorial Mathematics, second edn. Oxford University Press, Oxford, pp. 67–71.

Issaq, H.J., Conrads, T.P., Janini, G.M., Veenstra, T.D., 2002. Methods for fractionation, separation and profiling of proteins and peptides. Electrophoresis 23, 3048–3061.

Juckes, I.R., 1971. Fractionation of proteins and viruses with polyethylene glycol. Biochim. Biophys. Acta 229, 535–546.

Lilley, K.S., Razzaq, A., Dupree, P., 2002. Two-dimensional gel electrophoresis: recent advances in sample preparation, detection and quantitation. Curr. Opin. Chem. Biol. 6, 46–50.

Marchand, C., Maréchal, P.L., Meyer, Y., Miginiac-Maslow, M., Issakidis-Bourguet, E., Decottignies, P., 2004. New targets of Arabidopsis thioredoxins revealed by proteomic analysis. Proteomics 4, 2696–2706.

Méchin, V., Consoli, L., Guilloux, M.L., Damerval, C., 2003. An efficient solubilization buffer for plant proteins focused in immobilized pH gradients. Proteomics 3, 1299–1302.

Millar, A.H., Sweetlove, L.J., Giege, P., Leaver, C.J., 2001. Analysis of the Arabidopsis mitochondrial proteome. Plant Physiol. 127, 1711–1727.

Patlerson, S.D., Aebersold, R., 2003. Proteomics: the first decade and beyond. Nat. Genet. 33, 311–323.

Peltier, J-B., Ytterberg, A.J., Sun, Q., van Wijk, K.J., 2004a. New functions of the thylakoid membrane proteome of *Arabidopsis thaliana* revealed by a simple, fast, and versatile fractionation strategy. J. Biol. Chem. 279 (47), 49367–49383.

Peltier, J-B., Ripoll, D.R., Friso, G., Rudella, A., Cai, Y., Ytterberg, J., Giacomelli, L., Pillardy, J., van Wijk, K.J., 2004b. Clp protease complexes from photosynthetic and non-photosynthetic plastids and mitochondria of plants, their predicted three-dimensional structures, and functional implications. J. Biol. Chem. 279 (6), 4768–4781.

Pendle, A.F., Clark, G.P., Boon, R., Lewandowska, D., Lam, Y.W., Andersen, J., Mann, M., Lamond, A.I., Brown, J.W., Shaw, P.J., 2005. Proteomic analysis of the Arabidopsis nucleolus suggests novel nucleolar functions. Mol. Biol. Cell 16, 260–269.

Phizicky, E., Bastiaens, P.I.H., Zhu, H., Snyder, M., Fields, S., 2003. Protein analysis on a proteomic scale. Nature 422, 208–215.

Rabilloud, T., 1996. Solubilization of proteins for electrophoretic analysis. Electrophoresis 17, 813–829.

Rabilloud, T., 2002. Two-dimensional gel electrophoresis in proteomics: old, old fashioned, but it still climbs up the mountains. Proteomics 2, 3–10.

Saravanan, R.S., Rose, J.K.C., 2004. A critical evaluation of sample extraction techniques for enhanced proteomic analysis of recalcitrant plant tissues. Proteomics 4, 2522–2532.

Shefcheck, K., Yao, X.D., Fenselau, C., 2003. Fractionation of cytosolic proteins on an immobilized heparin column. Anal. Chem. 75, 1691–1698.

Sun, T.K., Kyu, S.C., Yu, S.J., Kyu, Y.K., 2001. Two-dimensional electrophoresis analysis of rice proteins by polyethylene glycol fractionation for protein arrays. Electrophoresis 22, 2103–2109.

Sun, T.K., Hyu, S.K., Han, J.K., Sang, G.K., Sun, Y.K., Dong, B.L., Kyu, Y.K., 2003. Prefractionation of protein samples for proteome analysis by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Mol. Cells 16, 316–322.

Templin, M.F., Stoll, D., Schwenk, J.M., Potz, O., Kramer, S., Joos, T.O., 2003. Protein microarrays: promising tools for proteomic research. Proteomics 3, 2155–2166.

Tirumalai, R.S., Chan, K.C., Prieto, D.A., Issaq, H.J., Conrads, T.P., Veenstra, T.D., 2003. Characterization of the low molecular weight human serum proteome. Mol. Cell. Proteomics 2, 1096–1103.

Wienkoop, S., Glinski, M., Tanaka, N., Tolstikov, V., Fiehn, O., Weckwerth, W., 2004. Linking protein fractionation with multidimensional monolithic reversed-phase peptide chromatography/mass spectrometry enhances protein identification from complex mixtures even in the presence of abundant proteins. Rapid Commun. Mass Spectrom. 18, 643–650.