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ORIGINAL PAPER

Efficient protocol for hydrophobic and hydrophilic protein solubilization of free cells from plants for two-dimensional electrophoresis analysis

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Abstract Two dimensional electrophoresis (2-DE) remains as the principal technology for resolving complex protein mixtures, prior to mass spectrometry characterization. Protein extraction and solubilization are the most important steps for revealing a proteome by 2-DE. For any kind of tissue, urea/thiourea-based protein solubilization buffers in combination with detergents and reducing agents are usually employed for maximizing solubilization and resolution of different types of proteins. Because protein composition in plants is species-specific and dependent on growing conditions, development of efficient protocols for improving the proteomic analysis in studies focusing to study the response of plants (whole plants and free cells) to environmental factors is always required. Our study model, chlorophyllic free cells of blue grama grass *Bouteloua gracilis* can be used as a unique model for studying the response of grasses to water and osmotic stress at cellular level, due to they are the only one chlorophyllic free cells in Gramineae family. Using these blue grama grass cells, we tested 16 different solubilizations buffers containing different chaotropes, zwitterionic and nonionic detergents and, reducing agents for maximizing both the number of spots and spot resolution in 2-D gels. The most efficient solubilization buffer developed in this work functioned well in a wide pH range utilized in proteomic studies (3–10). This buffer contained two chaotropic agents (7 M urea and 2 M thiourea), two reducing agents [100 mM DTT (dithiothreitol) and 2 mM TBP (tributyl phosphate)], two zwitterionic detergents [2.65% CHAPS (3-3[cholamidolpropyl]-dimethylammonio-1-propane sulfonate), 2% ASB 14 (amidesulfobetaine-14)] and a nonionic detergent (2% Triton X-100).

Keywords proteomics, 2-D gel electrophoresis, proteome, free cells, proteins, solubilization buffer, reducing agents, zwitterionic detergent.

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Introduction

Two-dimensional gel electrophoresis (2-DE) technique, in combination with Mass Spectrometry (MS), still remains as one of the most important methods for revealing complex protein profiles (Rabilloud 2002), despite all the described and known limitations pertaining reproducibility and difficulty in efficient separation of low abundance proteins, low and high molecular weight proteins, highly acidic, basic and hydrophobic proteins (Gygi et al 2000; Wall et al 2000).

Protein extraction has become the most important step in proteome studies. However, to reach a satisfactory 2-DE resolution and a good representation of the proteome, a high-quality protein solubilization is required (Görg et al 2004). Because each sample has unique features related to plant protein composition and growing conditions, an appropriate protocol is always required (Shaw and Riederer 2003). Currently, the most popular sample solubilization buffer is based on O'Farrell's lysis buffer and modifications thereof (9 M urea, 2–4% CHAPS

[3-3(cholamidolpropyl)-dimethylammonio-1-propane sulfo-nate], 1% DTT (dithiothreitol), and 2% (v/v) carrier ampholytes; O'Farrell 1975). Unfortunately, this urea-based lysis buffer is not ideal for the solubilization of all proteins, particularly for membrane or other highly hydrophobic proteins. Improvement in the solubilization of hydrophobic proteins has been achieved using thiourea (Rabilloud 1998) and new zwitterionic detergents such as sulfobetaines (Chevallet et al 1998).

Proteomics techniques have been employed in different plant samples such as whole plants, plant parts, tissues, somatic embryos and calluses. For example, leaves were used in sugarcane and maize (Zhou et al 2012; Wu et al 2012), somatic embryos in coffee (*Coffea Arabica*) and cyclamen (*Cyclamen persicum*) (Tonietto et al 2012; Bian et al 2009), and calluses in vanilla (*Vanilla planifolia*) (Chin Tan et al 2013) for protein analysis. In this study, chlorophyllous cell cultures of blue grama grass, established by Aguado-Santacruz et al in 2001, were used. This cell cultures contain high chlorophyll levels and well-developed chloroplasts, representing an unique model for studying photosynthesis and analyzing the effects of different stresses such as osmotic, cold or heat on chloroplast development and photosynthesis (García-Valenzuela et al 2005; Aguado-Santacruz et al 2011). In the work presented here, this cell model was utilized for optimizing samples for 2-D gel electrophoresis analysis by testing 16 solubilization buffers, containing two chaotropes (7 M urea and 2 M thiourea), and different concentrations of zwitterionic and nonionic detergents and, reducing agents. Our results showed that the most efficient buffer for solubilizing the total protein of the chlorophyllic cells contained, besides the two chaotropic agents, two reducing agents (100 mM DTT [dithiothreitol] and 2 mM TBP [tributyl phosphate]), two zwitterionic detergents (2.65% CHAPS [3-3(cholamidolpropyl)-dimethylammonio-1-propane sulfonate], 2% ASB 14 [amidesulfobetaine-14]) and a nonionic detergent (2% Triton X-100). This optimized buffer worked well in the wide pH range utilized in proteomic studies (3–10).

Materials and Methods

Sample preparation

A chlorophyllic suspension cell (`TADH-XO´) was obtained by culturing shoot tips of blue grama as described before by Aguado-Santacruz et al 2001 and García-Valenzuela et al 2005. This chlorophyllic cell line was routinely grown in liquid propagation medium (PM), which consisted of basal salts and vitamins of MS medium (Murashige and Skoog 1962), 3% sucrose, 1 mg l^{-1} 2,4-D (2,4 Dichlorophenoxyacetic acid), 2 mg l^{-1} BAP (Benzylaminopurine) and 40 mg l^{-1} Adenine (Aguado-Santacruz et al 2001). An initial inoculum of chlorophyllic cells was prepared by culturing 1 g fresh weight cells into 50 ml of PM. After growing for eight days, 125 ml-Erlenmeyer flasks containing 25 ml of PM were inoculated with 500 mg fresh weight of green cells. These flasks were agitated at 95 r.p.m. on a gyratory shaker in a growth chamber (33±1ºC and 33.8% relative humidity) under continuous light (photon flux density = 77 μmol s^{-1} m^{-2}) provided by 30 watts fluorescent daylight lamps (General Electric, model F30T8-D). After six days, chlorophyllic cells were harvested by vacuum filtration system and then frozen in liquid nitrogen for further protein extraction.

Protein extraction

Three hundred and fifty mg fresh weight frozen cells were suspended in 800 μl of the extraction buffer cited in Issacson et al (2006). This extraction buffer was composed of 0.7 M sucrose, 0.1 M KCl, 0.5 M Tris-HCl, pH 7.5, and 50 mM EDTA. Immediately before using, a reducing agent (β-mercaptoethanol) and a cocktail protease inhibitor (Complete mini, EDTA-free, 11836170001, Roche Diagnostic, Germany) were added to the extraction buffer to final concentrations of 2% (v/v) and 1 mM, respectively. After the cells were mixed with the extraction buffer, 800 μl cold phenol saturated with Tris-HCl, pH 7.5, were added. The mixture was stirred on a vortex mixer for 30 min at 4ºC and later centrifuged at 5,000 x g for 30 min at 4ºC. The upper phenolic phase was collected in a new tube, and then 800 μl of the extraction buffer were newly added to the remnant pellet in order to repeat the extraction process two more times. To precipitate the protein, 5 volumes of cold 0.1 M ammonium acetate in methanol were added to the collected phenolic phase which was then stored at -20ºC overnight. Washing steps were carried out two times with methanol and once with acetone. Finally, the samples were air dried for five min. Subsequently, 150 μl of each solubilization buffers tested were added to the samples. Total protein was quantified by 2-D Quant kit (80-6483-56, Amersham Biosciences, Piscataway, NJ). A similar protocol for protein extraction including phenol was used by Tonietto et al (2012) in coffee (*Coffea arabica*) and Zhou et al (2012) in sugarcane.

Solubilization buffers

In this analysis, 16 different solubilization buffers methods were tested for protein solubilization of chlorophyllic cells of blue grama (Table 1) by one dimension SDS-PAGE

(sodium dodecyl sulfate-polyacrylamide gel electrophoresis). Formulation of these new buffers were based on the published protocols by Di-Ciero et al (2004), Gu-Kang et al (2004), Hye-Kyoung et al (2005), Natarajan et al (2005), Parker (2006), Rabilloud et al (2007), Martins et al (2007) and Rabilloud (2009).

One dimension SDS-PAGE analysis was carried out with Laemmli electrophoresis running buffer (Laemmli 1970), loading 100 µg protein per lane and using 5 µl protein ladder 220 kDa (Benchmarck^{TM}10747-012 Life Technologies, Gaithers-burg, Md). Gels were elaborated using 12% acrylamide and the samples were run at 100 V for approximately 3 h and then stained with PhastGel Blue Coomassie R-350 (PhastGel Blue^{TM} 17051-801 Amersham Bioscience, Piscataway, NJ). All one dimension SDS-PAGE gel separations were repeated at least five times for each solubilization buffer. The most efficient buffers were selected in terms of intensity and number of bands by GelQuant Software (Biosistematica, Tavistock, Devon, UK) and then the samples prepared with these buffers were utilized for running two-dimensional gel electrophoresis.

Two-dimensional gel electrophoresis (2-DE)

Total protein was quantified by 2-D Quant kit (80-6483-56, Amersham Biosciences, Piscataway, NJ). Solubilized samples containing 1500 µg of protein were applied to 18 cm IPG gel strips with a pH separation range of 3–10 (GE Life Sciences, Piscataway, NJ). Due to the concentration of sample per µl, were used 18 cm IPG gel strips. After 14 h of rehydration, isoelectric focusing (IEF) was carried out at 22ºC, first applying 100 V for 2 h, later 250 V for one hour, subsequently 0.5 h at 500 V, 0.5 h at 1000 V, and finally 8000 V for 13 h in an IPGphor apparatus (GE Life Sciences, Piscataway, NJ), maintaining a limiting current of 50 mA per strip. First dimension strips were subjected to the standard reduction step with 0.05% DTT, (15 min), and the alkylation step with 1.25% 2-iodoacetamide (IAA, 15 min) prior to running the second dimension electrophoresis. The second dimension (SDS-PAGE) was performed on 12% polyacrylamide gels using an Ettan DALTsix electrophoresis unit connected to a refrigerating system (GE Life Sciences, Piscataway, NJ). The electrophoresis was carried out for 1 h at 100 V, and later on at a constant current of 30 mA until the dye front reached the bottom of the gel and the proteins were detected by a colloidal Coomassie blue staining (PhastGel Blue Coomassie R-350). Five independent samples prepared with each solubilization buffer were run and then the resulting spots were analyzed (Table 2).

2-DE image analysis

The stained gels were scanned by transmittance at 300 dpi of resolution using Lab Scan software (GE Life Sciences, Piscataway, NJ). Images were saved as *.mel files. The software ImageMaster 2D platinum version 6.0 (GE Life Sciences, Piscataway, NJ) was used for spot detection, counting and quantification.

Statistical analysis

For high reliability, the same parameters, based on the default settings, were applied to each set of gels stained simultaneously in the same tray. After calculating the standard deviation (SD) and coefficient of variation (CV), the statistical significance of the variation in PhastGel Blue Coomassie R-350-stained spot intensity across three replicates was calculated (t-test, $p < 0.05$).

Table 1. Efficiency of different buffers for solubilizing the total protein of chlorophyllous free cells of blue grama as measured by the number and intensity of bands revealed by one dimensional electrophoresis SDS-PAGE. All one dimension SDS-PAGE gel separations were repeated at least five times for each solubilization buffer.

	Reducing agent (mM)		Detergent (%)			pH stabilizer (mM)		
Buffer*	DTT	TBP	CHAPS	Triton X-100	ASB 14	K_2CO_3	Average No. of bands	Average band intensity
B1	20		2.65				32	530259
B2	50		2.65				33	612440
B3	100		2.65				26	243278
B4		2	2.65				24	286875
B5		4	2.65				33	471767
B6		5	2.65				34	463486
B7		6	2.65				36	664791
B8		4	2.65			5	36	447155
B9		6	2.65			5	54	790717
B10	100	2	2.65				57	671710
B11		4	2.65	1.35		5	40	713803
B12		4	4.00			5	49	292469
B13		6	2.65		2.00	5	26	486422
B14	100	2	2.65		2.00		24	251293
B15		6	2.00		2.00	5	39	635765
B16	100	2	2.65	2.00	2.00		41	728598

*All buffers contained 7 M urea and 2 M thiourea as chaotropic agents.

Table 2. Number of protein spots revealed in 2-DE profiles of free cells of blue grama using six solubilization buffers for sample preparation. Five independent samples prepared with each solubilization buffer were run.

Buffer	Composition	No. of acid protein spots	No. of basic protein spots	Standard deviation (SD)	Coefficient of variation (CV)
B2	7 M urea, 2 M thiourea, 2.65% CHAPS, 50 mM DTT	228	29	3.51	0.013
B7	7 M urea, 2 M thiourea, 2.65% CHAPS, 6 mM TBP	289	29	4.09	0.012
B9	7 M urea, 2 M thiourea, 2.65% CHAPS, 6 mM TBP, 5 mM K_2CO_3	180	38	3.40	0.015
B10	7 M urea, 2 M thiourea, 2.65% CHAPS, 100 mM DTT, 2 mM TBP	368	119	6.76	0.013
B11	7 M urea, 2 M thiourea, 2.65% CHAPS, 4 mM TBP, 5 mM K_2CO_3, 1.35% Triton X-100	444	99	5.50	0.010
B16	7 M urea, 2 M thiourea, 2.65% CHAPS, 100 mM DTT, 2 mM TBP, 2% Triton X-100, 2% ASB 14	517	251	8.50	0.011

Results and Discussion

In this research, we formulated and compared 16 different buffers for protein solubilization of chlorophyllous free cells. Efficiency of protein solubilization was measured in terms of number of spots, spot resolution and intensity in a 2D–PAGE analysis. The protein extraction process utilized in this work was based on the phenol protocol described by Isaacson et al (2006). This extraction process has been reported as efficient in proteomic research (*e.g.* Grosse et al 2008; Cilia et al 2009; Kelley et al 2010) and in previous work with our chlorophyllous cells, in which comparatively greater protein yields were obtained (up to 2.78 μg mg^{-1} fresh weight) using this phenol-based protocol.

All of the 16 solubilization buffers contained 7 M urea and 2 M thiourea as chaotropic agents, while the differences among them were related to the type (s) and concentration (s) of the reducing agents and detergents incorporated (Table 1). One dimension SDS-PAGE analysis revealed differences in the intensity and number of bands as a function of the buffer employed. Buffers B9, B10 and B11 revealed higher number of bands (Table 1), but the bands obtained with B2, B7 and B16 showed higher intensity. Consequently, we only choose the plant samples processed with six buffers (B2, B7, B9, B10, B11 and B16) for further bidimensional electrophoresis analysis. Before 2-DE analysis, the total protein content quantified in samples processed with buffers B2, B7, B9, B10, B11 and B16 was 8.1, 12.3, 17.9, 13.5, 15.9 and 39.6 μg $μl^{-1}$, respectively, showing that B16 buffer yielded the highest protein concentration. Di-Ciero et al (2004) mentioned that one of the best detergents to solubilize hydrophobic proteins is ASB 14 (amidesulfobetaine-14) followed by CHAPS and finally Triton X-100. Because hydrophobic proteins are grouped into basic pH (Marmagne et al 2004), we decided to analyze two-dimensional electrophoresis in a pH separation range of 3–10.

Overall, the effectiveness of each solubilization buffer can be easily observed by the number and abundance of spots revealed between the isoelectric points 3 to 10 and the molecular weight values within 10 and 220 kDa (Figure 1, Table 2). 768 protein spots were revealed in the gel loaded with the sample treated with B16 buffer consisting of a 7 M urea/2 M thiourea-based buffer combined with the reducing agents, DTT (dithiothreitol) and TBP (tributyl phosphate), at concentrations of 100 mM and 2 mM, respectively, zwitterionic detergents, CHAPS (3-3[cholamidolpropyl]-dimethylammonio-1-propane sulfonate) and ASB 14 (amidesulfobetaine-14), at a concentrations of 2.65% and 2.00%, respectively, and the nonionic detergent Triton X-100 at concentration of 2.00%. Other studies (Chevallet et al 1998; Cordwell et al 2000; Leimgruber et al 2002; Molloy et al 2001; Di-Ciero et al 2004) have also shown the effectiveness of these sulfobetaines detergents for 2-DE analysis. Our results indicate that the method based on B16 buffer enriched the samples for membrane proteins, which represented 32.6% of the total protein (Figure 1). This greater efficiency is the result of the combined action of the different components integrating the buffer. Urea is a chaotropic agent that is quite efficient in disrupting hydrogen bonds, leading to protein unfolding and denaturation. Likewise, thiourea is an organosulfur compound first utilized in proteomic studies by Rabilloud in 1998, which is better suited for breaking hydrophobic interactions; inconveniently, its usefulness is somewhat limited due to its poor solubility in water.

On the other hand, detergents, such as SDS, are utilized to prevent hydrophobic interactions between the hydrophobic protein domains, which result in protein loss due to aggregation and precipitation. Because the anionic detergent SDS is one of the most efficient surfactants, solubilization of proteins in SDS solutions has been recommended for protein solubilization (Boucherie et al 1995; Harder et al 1999). Additionally, zwitterionic detergents, such as CHAPS, and sulfobetaines (SB 3–10 or ASB 14) perform better and have been shown to solubilize -in combination with urea and thiourea chaotropes- several integral membrane proteins (Santoni et al 2000; Molloy 2000).

Since reduction and prevention of re-oxidation of disulfide bonds is also a critical step of the sample preparation procedure, reducing agents are necessary for cleaving intermolecular disulfide bonds to achieve complete protein unfolding. The most commonly used reductants are dithiothreitol (DTT) and dithioerythritol (DTE), which are applied in excess (100 mM). However, DTT and DTE are not well suited for the reduction and solubilization of proteins containing high cysteine content. Herbert et al (1998) have proposed tributylphosphine (TBP) in lower concentrations (2 mM) as an alternative to DTT. Our data show that the solubilization of acid and basic proteins is better achieved when the combination of TBP, ASB 14, CHAPS, and Triton X-100 is considered, making the protein solubilization particularly efficient in free cells of the grass blue grama.

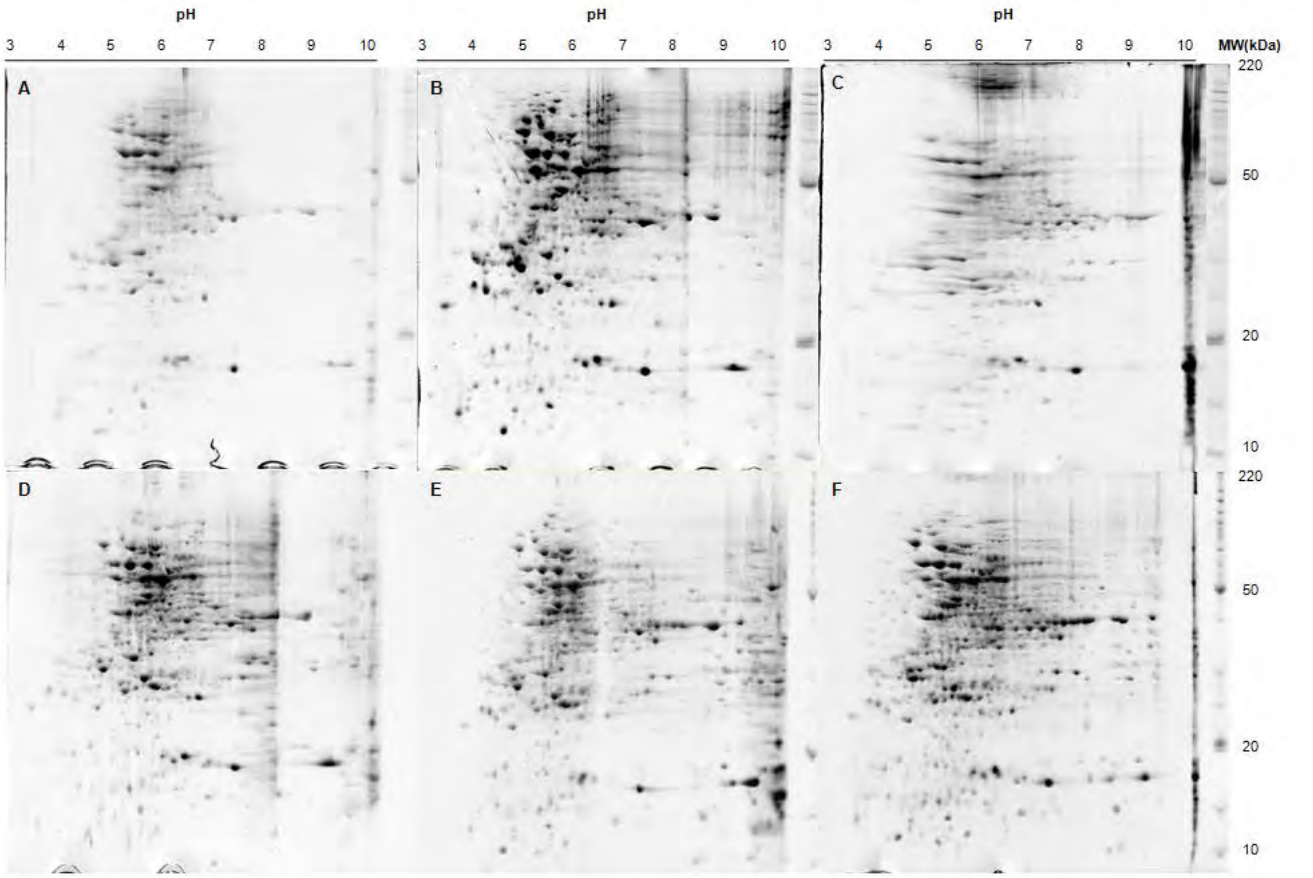

Fig. 1 Two-dimensional electrophoresis of chlorophyllic cells proteins from blue grama extracted, using various methods: (A) B2, (B) B7, (C) B9, (D) B10, (E) B11, and (F) B16 buffers. Protein (1500 μg) was run used 18 cm IPG strips (pH 3–10) in first dimension, and the second-dimension run used 12% SDS–PAGE. Gels were stained with PhastGel Blue Coomassie R-350. Five independent samples prepared with each solubilization buffer were run.

Finally, we expect this efficient and improved protocol for obtaining protein samples with satisfactory quality for 2-D analysis be instrumental in analyzing the cellular response to biotic and abiotic stresses in the chlorophyllous free cells as in similar plant models. Our research group has used PEG 8000 to study the effect of osmotic stress on these free cells. PEG has been demonstrated in the past to be imperfect in respect of permeability (Lawlor 1970; Yaniv and Werker 1983; Newton et al 1990) and several reports suggest that it may be taken up by plants (Mexal et al 1975; Woolley 1963) and that it causes detrimental effects other than osmotic ones (Lagerwerff et al 1961; Lawlor 1970; Leshem 1966). Although other reports suggest that PEG has been used as a nonpenetrating osmoticum (Appelgate 1960; Mexal et al 1975). For this reason and for future research, we tested full optimized protocol in free cells treated with 14% PEG 8000 and there was a good resolution of proteins (data not show).

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