Screening of extraction buffers for 2-D DIGE analysis of plant proteins

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Sample preparation is a crucial part of the 2-D electrophoresis workflow to achieve a large number of well-resolved protein spots. Plant tissue is a challenging material to work with due to the high content of interfering components and low protein content. A novel workflow for common bean leaves was developed using 2-D Protein Extraction Buffer Trial Kit in combination with 2-D Clean-Up Kit. The purified protein fractions were analyzed by 2-D DIGE and DeCyder™ image analysis software. All tested buffers resulted in overall well-resolved gel images having the majority of relative spot intensities at similar levels. However, some spot intensities depended on the extraction buffer used.

Introduction

Plant tissue often contains high levels of polysaccharides, lipids, and phenolic compounds, which can interfere with protein extraction. A preparation workflow for 2-D DIGE analysis needs a fast and effective cell lysis and protein extraction protocol that ensures the highest solubility, efficient removal of interfering substances, and a minimum of proteolytic activity.

The buffers in the 2-D Protein Extraction Buffer Trial Kit are based on urea or urea/thiourea with different detergents including CHAPS, SB 3-10, and ASB-16 all of which are known to give high solubilization power. The buffers are easily prepared by mixing a dry powder with the supplied diluent solution. Table 1 shows the composition of the six buffers included in the kit.

Table 1. Composition of extraction buffers included in 2-D Protein Extraction Buffer Trial Kit. Buffers highlighted in blue were included in the study together with standard 2-D lysis buffer (7 M urea, 2 M thiourea, and 4% CHAPS)

2-D Protein Extraction Buffer-I	Urea (< 10 M) and NP-40* (< 10%)
2-D Protein Extraction Buffer-II	Urea (< 10 M) and CHAPS† (< 10%)
2-D Protein Extraction Buffer-III	Urea (< 8 M), thiourea (< 5 M), CHAPS (< 5%), and ASB-16‡ (< 5%)
2-D Protein Extraction Buffer-IV	Urea (< 8 M), thiourea (< 5 M), CHAPS (< 5%), and SB 3-10§ (< 5%)
2-D Protein Extraction Buffer-V	Urea (< 8 M), thiourea (< 5 M), and CHAPS (< 10%)
2-D Protein Extraction Buffer-VI	Urea (< 8 M), thiourea (< 5 M), CHAPS (< 5%), and NDSB-201¶ (< 4%)

* *Nonylphenyl polyethylene glycol*
† *3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate*
‡ *Amidosulfobetaine-16*
§ *n-Decyl-N,N-dimethyl-3-ammonio-1-propanesulfonate*
¶ *3-(1-Pyridino)-1-propane sulfonate*

In this study, leaves from *Phaseolus vulgaris* (common bean) at seedling stage were used. Buffers III, IV, V, and VI from the 2-D Protein Extraction Buffer Trial Kit and a standard 2-D lysis reference buffer (7 M urea, 2 M thiourea, and 4% CHAPS) were tested. The 2-D Clean-Up Kit was subsequently used for removal of the contaminants, followed by 2-D-DIGE analysis using precast low fluorescent DIGE gels and DIGE Buffer Kit. Figure 1 shows the overall workflow of the study.

Fig 1. Workflow for plant protein extraction.

Protein extraction

Extraction buffers were supplemented with 1% DTT, 2% IPG buffer pH 3 to 10 and 10 µl/ml Protease Inhibitor Cocktail VI (P-1545, A. G. Scientific) prior to use.

Two leaf sample replicates of approx. 1.1 g each were ground to powder in liquid nitrogen for 3 min. Each sample was divided into five parts, rapidly mixed with 1 ml of the buffers included in the study and kept on ice until all samples were ground. Following that, all samples were tempered to 30°C to completely dissolve all buffer components. The samples were incubated for 30 min with occasional mixing before centrifugation at 16 100 × g for 30 min at 20°C. The supernatant was collected and stored at -20°C.

Protein clean-up

2-D Clean-Up Kit was used according to the kit instructions for samples of >100 µg protein (1). The procedure was optimized as follows: 150 µl of extract was mixed with 450 µl precipitant followed by 450 µl of co-precipitant in 2 ml microtubes with round bottoms to spread out the precipitate. The precipitate was washed with 100 µl of of co-precipitant in step 7 and 100 µl of ultrapure water in step 9. In the latter step, a pipette tip was initially used to disperse the pellet prior to vortex mixing in order to wash the protein precipitate.

The pellet was then washed in 1.5 ml of wash buffer and centrifuged at 16 100 × g at 20°C to collect the precipitated protein. The precipitate was solubilized in 50 µl of extraction buffer supplemented with 30 mM Tris, pH 8.5. The solubilization was aided by heating to 30°C for 10 min and mixed by pipetting the sample up and down several times. Finally, the solubilized samples were centrifuged for 5 min and transferred to new tubes to remove any insoluble material. The protein concentration was 2 ± 0.5 mg/ml before and 3.9 ± 1.1 mg/ml after the clean-up step (2-D Quant Kit).

The samples were also analyzed by SDS-PAGE to confirm integrity prior to 2-D DIGE labeling (Fig 2).

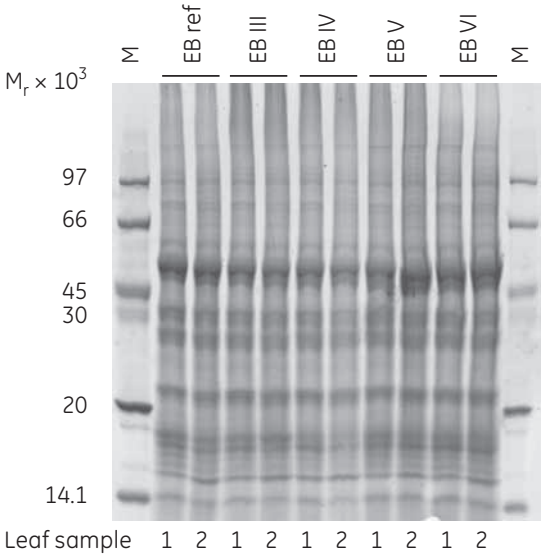

Fig 2. 1-D SDS-PAGE (12% Tris-glycine) of samples (10 µg/lane) extracted by the buffers annotated on the top (EB = 2-D Protein Extraction Buffer) followed by 2-D Clean-Up Kit procedure described in the text. The gel was stained with Deep Purple™ Total Protein Stain.

2-D DIGE analysis

Labeling was performed with CyDye DIGE Fluor minimal dye labeling kit according to the DIGE manual (2); 35 µg protein of each sample was labeled with Cy™5 and Cy3, respectively, and a pooled standard was labeled with Cy2. A total of 500 pmol dye was used per sample in a final volume of 8 to 14 µl.

Immobiline™ DryStrip gels, pH 3–11 NL (24 cm) were reswollen in 450 µl DeStreak Rehydration Solution. Sample mixes were adjusted to 100 µl final volume with 2-D Protein Extraction buffer VI supplemented with DTT (10 mM final concentration), and applied by cup-loading at the anodic end. The strips were run in a manifold on an Ettan IPGphor 3 instrument. Precast DIGE gels and DIGE Buffer Kit were used for the second-dimension run in Ettan DALT*twelve* electrophoresis unit according to the DIGE gel instruction.

The gels were stored overnight at 4°C before scanning in Typhoon™ FLA 9000 Biomolecular Imager. Gel images were evaluated with DeCyder 2-D Differential Analysis Software, v7.0 by the protein statistics function based on Student's t-test.

All five buffers resulted in well-resolved gel images with the majority of protein spot intensities at similar levels. However, some protein spot intensities depended on the extraction buffer used. Figure 3 shows the gel pattern obtained by Extraction Buffer VI with examples of differentially extracted proteins marked A to E and the corresponding graph view showing the relative abundance of protein in a certain gel spot. The largest difference was found between 2-D Extraction Buffer III and the other buffers. This buffer was favorable to some spots (D, E) and less effective on the rest of the marked spots while the other buffers were more comparable to each other. The reference buffer and 2-D Protein Extraction Buffer V behaved in a very similar way, which is expected since both buffers are based on thiourea, urea, and CHAPS.

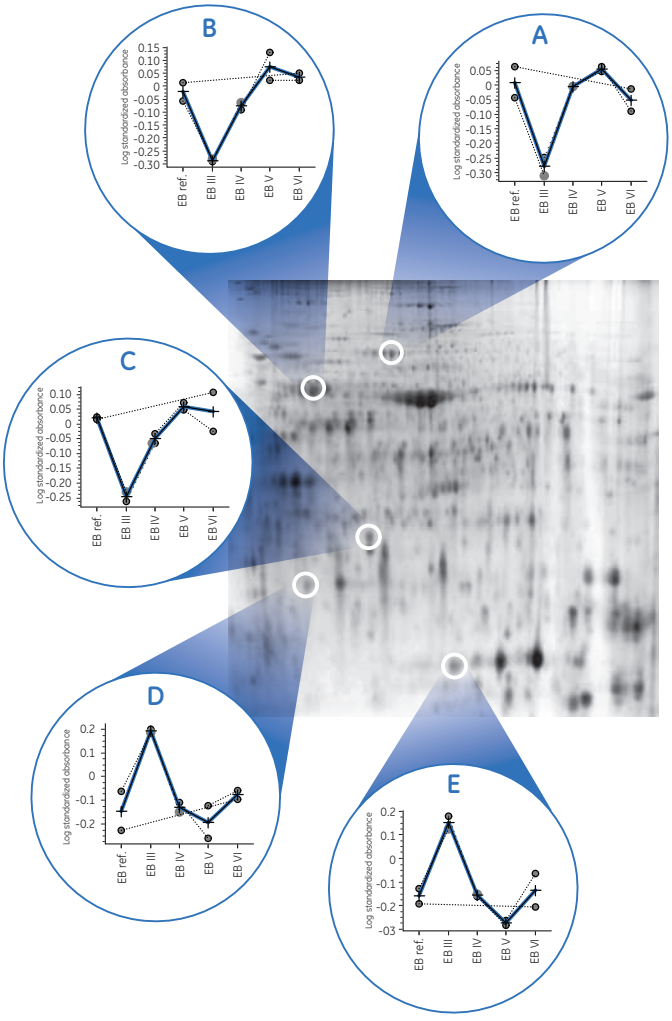

Fig 3. The spots marked A to E in the gel image are examples of proteins extracted to different levels depending on the buffer used during extraction. The graph view shows the relative abundance of a protein obtained by use of the buffers, respectively.

Summary

2-D Protein Extraction Buffer and 2-D Clean-Up Kit give a suitable preparation workflow for 2-D DIGE analysis of plant proteins from *Phaseolus vulgaris*, and will most likely be useful for protein extraction from other types of leaves. The screening study described compared five different extraction buffers, and some proteins spots showed different relative abundance depending on the buffer used during extraction, which may be beneficial for preparative work of certain proteins of interest.

References

1. User Manual: Ettan DIGE System, 18-1173-17, Edition AB (2006).
2. Instructions: 2-D Clean-Up Kit, 80-6486-60, Edition AE (2009).

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