

SUB-CELLULAR FRACTIONATION AND GEL-BASED PROTEOMICS OF *HABERLEA RHODOPENSIS*: A PROMISING APPROACH TO OPEN THE BLACK BOX OF RESURRECTION PLANTS

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Abstract

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Haberlea rhodopensis (Gesneriaceae) belongs to the group of the resurrection plants. Their ability to survive extreme desiccation renders them excellent models to study plant response to dehydration. Plant metabolic networks are very complex and sub-cellular proteomics has proven to be a powerful approach to gain functional knowledge about the sub-cellular compartments. Here we present a protocol for the simultaneous isolation of pure and intact chloroplasts and mitochondria from *Haberlea rhodopensis* with subsequent gel-based proteomics including BN PAGE and 2-D gel electrophoresis. Differential detergent – based solubilization of chloroplast membranes allows separation of distinct activities in sub-organellar compartments and contributes for drastic reduction in overall sample complexity improving resolution and sensitivity of further proteomic analyses.

Key words: BN-PAGE, detergent-based subcellular fractionation, gel-based proteomics, *Haberlea rhodopensis*, resurrection plants

Abbreviations: 2-D gel electrophoresis – two-dimensional gel electrophoresis; SDS-PAGE – sodium dodecylsulphate polyacrylamide gel electrophoresis, BN-PAGE – Blue Native Gel Electrophoresis; DMM – dodecyl maltoside

Introduction

Haberlea rhodopensis is a Balkanic endemite and glacial relict present in several populations in Rhodopi Mountains and Balkan Mountains in Bulgaria and Bozdag Mountain in Greece. The species belongs to the *Gesneriaceae* family of resurrection plants whose unique ability to withstand the loss of almost all tissue water and to recover very fast upon re-watering renders them a suitable model to study drought stress response in plants.

Despite many studies on desiccation tolerance of *H. rhodopensis*, the regulatory mechanisms for its extreme toler-

ance remain largely unknown. It has been shown that during moderate-severe drought the integrity of the photosynthetic apparatus is being maintained in parallel with observed disconnection between the photosystems (Georgieva et al., 2007; Strasser et al., 2010). A pronounced accumulation of sucrose in fully dry plants has been observed, independent from the rate of starch hydrolysis and decrease of CO₂ fixation rates during the early stages of desiccation (Muller et al., 1997; Djilianov et al., 2011). On the other hand, the reported overexpression of succinate dehydrogenase as a part of mitochondrial photorespiratory chain and Krebs cycle, suggests functionality of mitochondria during drought stress (Geor-

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gieva et al., 2012). It is well known that metabolic processes in plant cells are highly compartmentalized, in this respect cellular fractionation techniques combined with proteomic analysis and metabolic profiling may provide additional knowledge about the specific functions and regulatory roles of subcellular compartments during the different stages of desiccation of *Haberlea rhodopensis*. Moreover, fractionation of total cellular proteins into subproteomes, allows subsequent detection and identification of proteins from samples with manageable complexity and thus considerably improves resolution and sensitivity of analyses. In the present study, we present a procedure for the simultaneous isolation of intact chloroplasts and mitochondria from *Haberlea rhodopensis* and gel-based proteomic analyses.

Materials and Methods

Simultaneous preparation of chloroplasts and mitochondria

75 g leaves were harvested and homogenized three times for two seconds each in Warring blender with 50 ml ice cold grinding buffer (0.33 M sucrose, 10 mM Tricine, 30 mM MOPS-KOH pH 7.9, 5 mM EDTA, 10 mM NaHCO₃, 2 mM MgCl₂, 0.1% BSA, 2 mM DTT, 20 mM ascorbate, 1% PVP 40). The homogenate was filtered through nylon mesh (20- μ m pore size) and three layers of Miracloth and then centrifuged at 1500xg for 6 min at 4°C. The pellet containing crude chloroplasts was resuspended gently in chloroplast wash buffer (Seigneurin-Berny et al., 2008) and loaded onto three-step Percoll gradient consisting of 90%, 60%, 40% Percoll in wash buffer and then centrifuged at 4000xg for 10 min at 4°C. The green bands from the 40%–60% and 60%–90% layers were collected and washed twice in wash buffer with centrifugation 1500xg for 6 min at 4°C. The pellet was resuspended in hypotonic buffer (Seigneurin-Berny et al., 2008) for disruption of intact chloroplasts and then stored in liquid nitrogen. The subsequent chloroplast fractionation into stroma and thylakoids was performed according to Salvi et al. (2008). For mitochondria isolation, the supernatant from the first centrifugation was centrifuged again at 6000xg for 5 min at 4°C in order to pellet the residual part of the chloroplasts. Then the supernatant was centrifuged at 20000xg for 10 min at 4°C. The mitochondria from the pellet were isolated essentially according to Millar et al. (2001).

Detergent-based solubilization of chloroplast and mitochondrial membranes

For selective detergent-based fractionation of thylakoid proteins into stromal and granal membrane proteins an aliquot from de-enveloped chloroplasts corresponding to 750 μ g protein was centrifuged at 8000xg for 6 min at 4°C. The pellet was

treated successively by 1.25% Digitonin and 1.25% DDM as described (Sirpiö et al., 2011) in BN PAGE solubilization buffer (Wittig et al., 2006). For mitochondrial membranes, 1 mg mitochondrial protein was solubilized with 1.5 mg DDM.

BN PAGE and 2D BN/SDS PAGE

The protein complexes from the differentially detergent-fractionated thylakoid membranes were separated on 1D BN PAGE. Both strips each containing thylakoid proteins from the grana or stromal domains were loaded simultaneously and separated on 2D BN/SDS PAGE according to Braun et al. (2006).

NADH dehydrogenase zymography

After separation of the mitochondrial fraction on 1D BN PAGE, NADH dehydrogenase (EC 1.6.99.3) activity was detected using zymogram analysis according to Chen et al. (2009).

2-D gel electrophoresis

Polyacrylamide gel containing ampholines in the pH range of 3.5 to 9.5 (GE Healthcare) was used for analytical flatbed isoelectric focusing (IEF) of thylakoid proteins and after IEF the excised lanes were separated by SDS-PAGE in the second dimension (Tchorbadjieva et al., 2005). Proteins were stained with silver nitrate according to Blum et al. (1987). Precision Plus Protein™ Dual Color Standards (BioRad) were used as molecular mass standards.

Results and Discussion

Based on previous reports suggesting an increased density of chloroplasts of resurrection plants (Koonjul et al., 2000; Georgieva et al., 2010), we used an additional layer of 60% Percoll for more efficient separation of the intact organelles from the disrupted ones. As a result, two fractions of chloroplasts were observed between the 90%–60% and 40%–60% Percoll layers (Figure 1A). For further analyses, we used the denser band. Using sucrose density centrifugation, the purified chloroplasts were fractionated into stroma and thylakoid fraction and resolved on 1D SDS PAGE (Figure 2A). Further, the thylakoid fraction was differentially solubilized after subsequent treatment with two detergents – digitonin and DDM. Considering the higher detergent resistance of thylakoids from *Haberlea* as compared to spinach (Georgieva et al., 2010) we used slightly higher concentrations of detergents to efficiently solubilize the membrane proteins. The resolved supercomplexes showed different patterns on 1D BN PAGE (Figure 2B). After the first mild solubilization with Digitonin, nine protein complexes were resolved on BN PAGE corresponding to PSI LHC; ATP synthetase; PSII dimers; PSII monomer; cytb6f; LHC as well as three

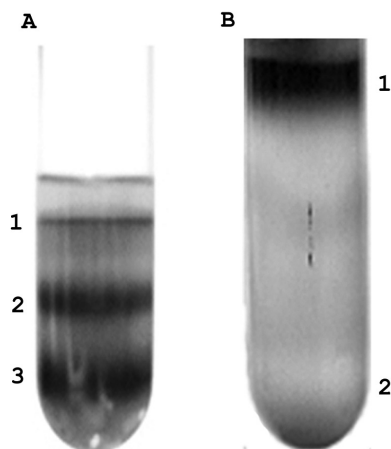


Fig. 1. Percoll density purification of leaf *Haberlea rhodopensis* chloroplasts and mitochondria.

A) Chloroplasts: 1 – broken chloroplasts; 2 – 40%–60% fraction, 3 – 90%–60% fraction.

B) Mitochondria: 1 – broken chloroplasts; 2 – mitochondrial fraction

unidentified supercomplexes, all located in the stromal thylakoid domains accessible to low concentration of Digitonin. The electrophoretic mobility patterns of the resolved protein complexes after the second treatment with DDM showed typical photosynthetic components for grana stacks enriched

in: PSII dimers; PSII monomer; cytb6f; LHCII assemblies; LHCII, as well as high molecular weight supercomplexes consisting of PSII LHCII dimers and tetramers. The different mobility of the protein complexes derived from the differential detergent treatments allows simultaneous separation of the protein subunits from the large photosynthetic protein complexes in the second dimension using 2D tandem strip BN/SDS PAGE system proposed in the present study. Thus, it is possible to analyze their phosphorylation and abundance in different structural domains of thylakoid membranes on one gel (Figure 2B). Fractionation of the thylakoid domains from one sample allows for the direct comparison of the photosynthetic machinery structure and dynamic quantification of protein migration between the domains typical for state transitions of thylakoids under stress conditions (Lemeille et al., 2009). We used 2D IEF/SDS PAGE also for resolving the thylakoid proteins (Figure 2C). Approximately 30 protein spots were detected on the gel with molecular masses and pIs in the range between 10–150 kDa and 3–10, respectively. For the mitochondrial fraction (Figure 1B), we used BN PAGE separation with subsequent colorimetric enzymatic staining of gels for detection of NADH dehydrogenase activity as the most abundant enzyme in mitochondria membranes (Figure 3).

Recently, development of accurate gel-based proteomics tools allowed more sophisticated approach to genome-wide protein dynamics and biochemical regulation. At present, profiling distinct cell organelle proteomes is

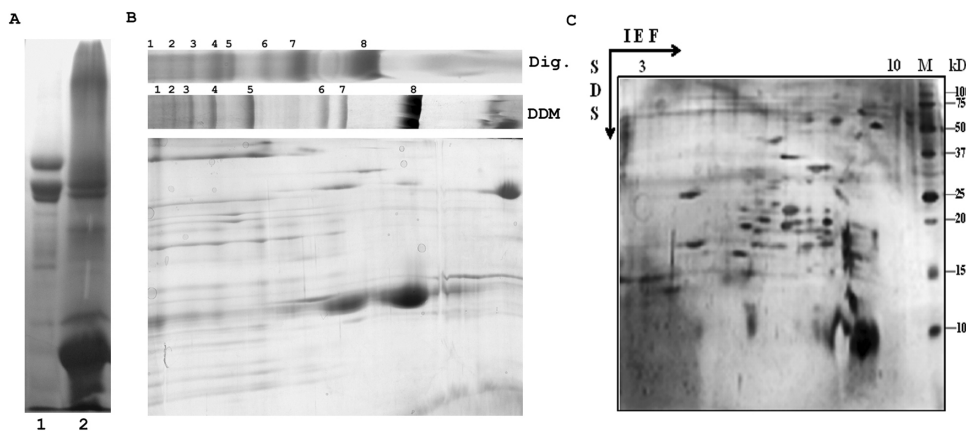


Fig. 2. Electrophoretic analysis of chloroplast proteins. **A)** 1D SDS PAGE after sub-fractionation of chloroplast proteins: lane 1 – stromal proteins; lane 2 – thylakoid proteins.

B) 2D tandem strip BN/SDS PAGE of detergent-based differential fractionation of thylakoid proteins. Dig: 1,2,3 – unidentified supercomplexes, 4 – PSI LHC, 5 – ATP synth, 6 – PSII dimers, 7 – PSII monomer and cytb6f; 8 – LHC. DDM: 1,2,3,4 – high molecular weight supercomplexes consisting of PSII LHCII dimers and tetramers, 5 – PSII dimers, 6 – PSII monomer and cytb6f, 7 – LHCII assemblies, 8 – LHCII. **C)** 2D IEF/SDS PAGE of thylakoid proteins. M, molecular mass markers

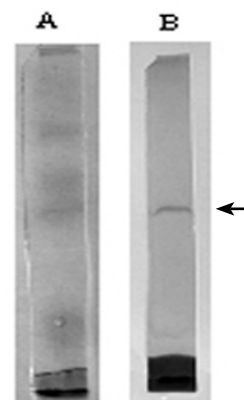


Fig. 3. 1D BN PAGE of mitochondrial proteins.

A) CBB-stained gel. **B)** Zymogram of NADH dehydrogenase activity (arrow)

one of the most active proteomic approaches. It will refine our knowledge of cellular processes by pinpointing certain activities to specific organelles from system biology perspective.

Conclusions

The protocol presented in this study enables the isolation of intact chloroplasts and mitochondria and ensures protein yields that are sufficient for sub-cellular proteomic studies in resurrection plants, including *Haberlea rhodopensis*.

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