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New method of plant mitochondria isolation and sub-fractionation for proteomic analyses

Tomáš Hájek*, David Honys, Věra Čapková

Institute of Experimental Botany, AS CR, Rozvojová 135, CZ-165 02 Praha 6, Czech Republic

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Abstract

A new method for the isolation and sub-fractionation of mitochondria from plant tissues is described. This method was used for the analysis of proteins isolated from a pure total mitochondrial fraction and of protein complexes obtained from two mitochondrial sub-fractions, membrane-bound and matrix, from in vitro cultivated tobacco pollen tubes. The method comprised: a new plant tissue homogenization procedure; differential centrifugation of the homogenates in a Percoll gradient; mitochondria immobilization together with Percoll removal by filtration through an uncharged nylon membrane. Mitochondria trapped on the nylon membrane were used for (1) rapid protein extraction; (2) quantitative phenol–chloroform protein extraction; (3) mitochondria sub-fractionation into membrane-bound and matrix fractions. The new method was found to be more efficient than previously published protocols, requiring the use of as little as 180 mg of fresh plant material. It is very fast, reliable, and suitable for proteomic analyses of plant organs and tissue samples with low biomass.

Keywords: Plant mitochondria isolation; Sub-fractionation; Protein analysis; SDS-PAGE

1. Introduction

In a cell integral system, mitochondria play a pivotal role in energy metabolism and in the maintenance of cellular homeostasis [1,2]. Recent advances in proteomic approaches leading to the identification of large sets of mitochondrial proteins [3–7], as well as proteomic analyses of particular mitochondrial protein complexes [8–10] provide important information about mitochondrial function as well as about the species and tissue specificity of mitochondrial protein composition.

Currently, development of techniques for the isolation of plant mitochondria lag far behind the progress made in protein detection methods. The high resolving power of 2-D electrophoresis allows easy separation of complex mixtures of very many different proteins in tissue extracts, provided that high purity protein samples are available [11]. In most cases, protocols for the purification of mitochondrial fractions are based on the blender tissue homogenization and differential centrifugation of a cell homogenate in a Percoll gradient [4,12]. The starting amount of plant material required is ordinarily of the order of tens of grams or kilograms of fresh tissue [4,12]. The purity of such mitochondrial fractions is usually demonstrated by enzymatic marker detection assay [13]. Although Percoll gradient centrifugation is the crucial step in the purification of mitochondria, removal of Percoll, necessary before further analyses, represents the limiting factor in use of such protocols. Some workers have circumvented difficulties in Percoll removal from the isolated mitochondrial band by replacing Percoll in the gradient buffer with sucrose [14,15]. However, this itself may cause problems with the physiological interpretation of the results obtained because disaccharides are able to enter the mitochondrial matrix through membrane porin channels [16,17].

Here we report a new, improved protocol for the isolation and further sub-fractionation of plant mitochondria. We have developed an original method for cell homogenization, sub-fractionation of mitochondrial compartments and removal of Percoll residues followed by the extraction of mitochondrial proteins. Our method has been successfully used to identify mitochondrial protein spectra in to-

* Corresponding author. Tel.: +420-2-20390451; fax: +420-2-20390461.
E-mail addresses: hajtom@ueb.cas.cz, hajtom@mbox.cesnet.cz (T. Hájek).

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bacco pollen tubes and we have been able to obtain sufficient amounts of mitochondrial proteins to do this from as little as 180 mg of fresh starting material. In vitro cultivated pollen tubes were selected as an example of a plant tissue containing a high portion of mitochondria as well as a rigid cell wall often resistant to standard homogenization techniques.

2. Materials and methods

2.1. Plant material and tissue homogenization

Pollen was collected from *Nicotiana tabacum* L. cv. Samsun plants grown in a greenhouse under natural light condition. Buds were harvested one day before anthesis. Isolated anthers were surface sterilized and allowed to dehisce in a laminar flow box [18]. Pollen grains were stored at −20 °C.

Ten milligram of pollen was dispersed in SMM-MES cultivation media (175 mM sucrose (Sigma); 3 mM $Ca(NO_3)_2{\cdot}4H_2O$; 0.8 mM $MgSO_4{\cdot}7H_2O$; 1.6 mM H_3BO_3; 1 mM KNO_3; 23 mM MES, pH 5.9 adjusted with KOH) [19] at a final concentration of 1 mg/ml. Pollen tubes were cultivated in the dark at 26 °C as a submersed culture and shaken at 190 rpm for first 3 h and then at 90 rpm for a further 1 h. After 4 h, pollen tube cultures were separated from the media by filtration on 45 μm nylon mesh, weighed and immediately homogenized. A total of 180 mg fresh mass of mesh-filtered pollen tubes represented the standard sample routinely used for mitochondria isolation.

The rotary 1 mm nylon mesh was fixed on the blender piston and the stationary 45 μm nylon mesh covered with pollen tubes was placed on Saran foil (Fig. 1). Pollen tubes were overlaid with mitochondria isolation buffer (0.3 M mannitol; 4 mM cystein; 1 mM EDTA; 20 mM cocarboxylase; 0.4 mM PMSF; 1 tablet per 50 ml of proteinase inhibitor mixture (Roche), pH 7.5 adjusted with KOH) [12] and homogenized for 30 s. Cell homogenates containing particles less than

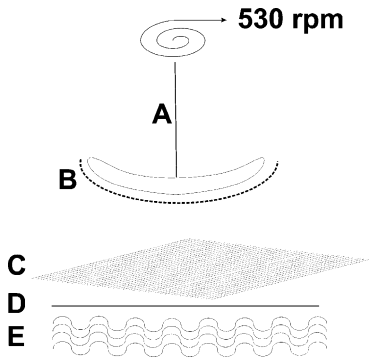

Fig. 1. Schematic representation of the blender used for plant tissue homogenization. The rotary part consisted of a piston (A) with attached 1 mm nylon mesh (B). The stationary part consisted of a 45 μm nylon mesh (C) and Saran foil (D) attached to the soft support (E).

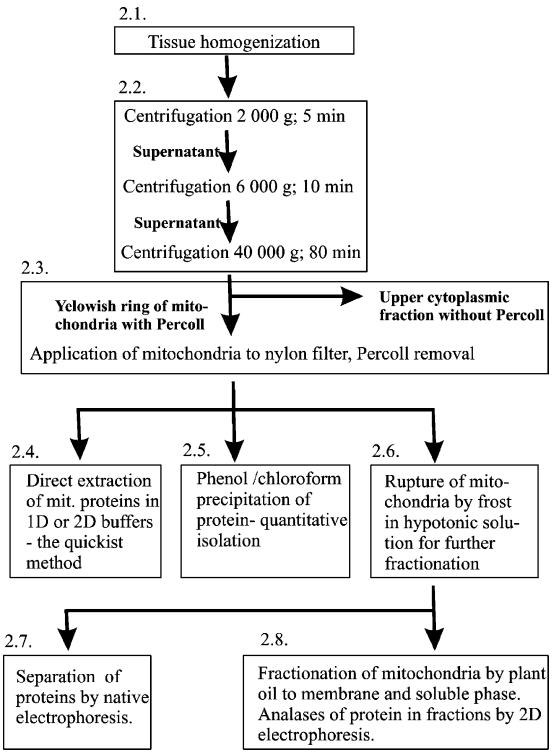

Fig. 2. Scheme for the isolation and sub-fractionation of mitochondria. Boxed labels correspond to sections as described in Section 2.

45 μm in diameter were collected under the stationary mesh on Saran foil.

2.2. Centrifugation steps

Cell homogenates (Section 2.1; Fig. 2) were pre-cleaned by two-step centrifugation (2000 × *g*, 5 min, 4 °C and 6000 × *g*, 10 min, 4 °C). The supernatant was layered above a Percoll cushion (28% Percoll; 0.3 M sucrose; 1 mM EDTA; 10 mM phosphate buffer, pH 7.2) and centrifuged (40,000 × *g*, 80 min, 4 °C, Beckman SW60 rotor). The centrifugation resulted in the formation of a yellowish mitochondrial ring in the middle of the Percoll cushion. 4′,6-Diamino-phenylindole (DAPI) [20] staining of the ring revealed contamination of its lower part by nuclei; only the upper part formed by a pure mitochondrial fraction was further analyzed. The upper aqueous phase was collected as the “cytoplasmic fraction”.

2.3. Percoll removal

The Percoll medium containing the mitochondria was filtered under pressure through uncharged nylon membrane (diameter 2.5 cm, pore size 0.22 μm, Whatman). Mitochondria were trapped on the filter whilst smaller particles together with Percoll remnants were rinsed with washing media (0.3 M mannitol, 1 mM EDTA, 10 mM phosphate buffer, pH 7.2).

2.4. Rapid protein extraction with electrophoretic sample buffers

The filter containing trapped mitochondria (Section 2.3) was cut into small pieces and shaken in Eppendorf tube with 1-D or 2-D sample buffer for 30 min, sedimented by centrifugation (2000 × *g*, 5 min, 4 °C, Sigma) and mitochondrial proteins released into the sample buffer were prepared for electrophoretic separation (Section 2.9).

2.5. Quantitative protein extraction with phenol

Mitochondrial proteins (Section 2.3) were quantitatively extracted by dissolving the whole nylon filters covered with mitochondria in phenol. Filters placed in Eppendorf tubes and overlaid with 0.5 ml phenol were completely dissolved and proteins were extracted after 10 min shaking. Proteins were precipitated by another 10 min shaking with 0.5 ml chloroform and were separated by centrifugation (20,000 × *g*, 1 h, 4 °C) in the interphase between aqueous and phenol/chloroform phases. The protein fraction was washed once with phenol/chloroform (1:1) and twice with pure chloroform to remove remaining phenol. After each washing step, proteins were precipitated by centrifugation (20,000 × *g*, 20 min, 4 °C). Finally, the sedimented protein precipitate was lyophilized.

2.6. Deep freeze-rupture of mitochondria

Nylon filters covered with mitochondria were cut into small pieces and shaken for 2 min in Eppendorf tubes with 0.7 ml of hypotonic buffer (50 mM Bis–Tris/HCl, pH 7.0; 0.5 M aminohexanoic acid; 0.4 mM PMFS; 1 tablet per 50 ml proteinase inhibitor mixture (ROCHE)) prepared with modifications according to Schagger et al. [21]. After deep freezing at −70 °C and successive thawing, mitochondria were disrupted and preserved protein complexes were released.

2.7. Native electrophoresis of mitochondrial protein complexes

Native protein complexes released from deep freeze-ruptured mitochondria (Section 2.6) were separated by native protein gel electrophoresis as described previously [21].

2.8. Mitochondria sub-fractionation

Samples prepared according to Section 2.6 were supplemented with 0.5 ml refined sunflower oil and shaken vigorously at room temperature for 5 min. Mitochondria samples were separated by centrifugation (20,000 × *g*, 10 min, 4 °C) into three phases according to their polarity: (1) protein-free oil phase; (2) interphase containing mitochondrial membrane structures with bound proteins; (3) aqueous phase enriched with mitochondrial matrix proteins. Two mitochondrial sub-fractions were re-centrifuged (20,000 × *g*, 10 min, 4 °C) and proteins of both fractions were isolated by phenol/chloroform extractions as described in Section 2.5.

2.9. Electrophoretic separation

Proteins obtained as described in Sections 2.4–2.6 and 2.8 were separated by 1-D and 2-D SDS-PAGE. 1-D SDS-PAGE was performed as described previously [22]. The first run of 2-D SDS-PAGE was performed on Bio-Rad (Protean IEF Cell), strip size 11 cm with ampholyte range 3–10 pH (Bio-Rad) and the second run (15% SDS-PAGE) was done on Biometra apparatus according to Laemmli [22]. Separated proteins were visualized by silver staining of polyacrylamide gels according to Blum et al. [23].

2.10. Detection of cytochrome-c-oxidase as a mitochondrial marker

The intactness of isolated mitochondrial fractions were tested using the modified method described by Douce et al. [13]. The detection solution (30 mM Tris–Mes pH 7.5; 2 mM Digitonin; 54 μM cytochrome-c) was supplemented with $Na_2S_2O_4$ until complete reduction of cytochrome-c. Tested mitochondria fractions were incubated with reduced solution for 10 min and the ratio of oxidized and reduced forms of cytochrome-c was measured spectrophotometrically and expressed as ΔA_{550}. The mitochondrial fraction boiled for 5 min was used as a negative control.

3. Results and discussion

The method of homogenization method usually represents the critical step affecting the yield of mitochondria isolated from plant cells. Whereas, in animal cells the hypotonic extraction solution releases intact mitochondria easily, the wall of plant cells dictates their mechanical disruption. The problem of balancing total disruption of cell wall structures with the need to maximize preservation of intact mitochondria places crucial emphasis on the optimization of both the intensity and the duration of homogenization. All formerly published procedures have started with several kilograms of fresh tissue in order to obtain several milligram of mitochondrial proteins. A published protocol resulting in an output of 150 mg of mitochondrial protein from 3 kg of spinach leaves [24] was considered to be very efficient, representing a 30–70 fold increase in yield compared to previously published protocols [25–27].

The use of plant mitochondria for proteomic studies presents a new challenge as some plant organs or tissues do not produce a high biomass and only microgram quantities of mitochondrial proteins are available. In order to meet the strict requirement for highly efficient extraction techniques, we have developed a new method of homogenization (Fig. 1), in which only 180 mg fresh mass of in vitro cultivated tobacco pollen tubes was used to isolate

mitochondria proteins in sufficient quantity for proteomic studies. The use of low quantities of pollen tube material is possible because a greatly increased density of mitochondria per cell compared to somatic cells [28,29]. The homogenization step occurred between two pieces of nylon mesh of different pore size. A yield of mitochondrial proteins as high as 600 μg per 180 mg pollen tubes was repeatedly achieved with high reproducibility. In the case of somatic tissues the starting fresh mass needed to be raised to 1 g of cell suspension culture BY-2 in order to harvest the same yield of mitochondrial proteins. Homogenization between two pieces of nylon mesh, using a rotating blender piston covered with 1 mm pore size mesh to grind pollen tubes layered on a stationary 45 μm pore size mesh provided three advantages. First, homogenization was very fast, 30 s sufficing for complete cell disruption (determined microscopically). Second, pollen tubes were spread in a very thin layer on the stationary net, substantially increasing the efficiency of their disruption. Third, stationary mesh also functioned as a filter, releasing to the subtending Saran foil only homogenate particles less than 45 μm in diameter. All heavy cell debris, a major source of contamination with high adhesivity to mitochondria, was removed during the first step of isolation procedure.

The homogenate was subsequently centrifuged at 2000 × *g* and 6000 × *g*. Determination of cytochrome-c-oxidase activity, expressed as ΔA_{550}, indicated the presence of mitochondria in the 6000 × *g* sediment (Table 1). Because the homogenate was cleared of heavy structures (see above), presence of mitochondria in 6000 × *g* sediment was unlikely to have been caused by contamination. When no significant and reproducible quantity of mitochondria was released under conditions of different centrifugation speed, the existence of two independent mitochondria compartments suggested by Dai et al. [30] cannot be excluded. Mitochondria present in the 6000 × *g* supernatant were isolated by Percoll gradient centrifugation with a reproducible

Table 1
Levels of cytochrome-c-oxidase activity in subcellular fractions of tobacco pollen tubes

Fraction	ΔA_{550}
Boiled cell homogenate (control)	0.040
6000 × *g* sediment	0.843
Cytoplasmic fraction	0.215
MT separated on filter after Percoll	0.992

The cytochrome-cactivity, expressed as ΔA_{550}, was measured after 10 min of reaction. Subcellular fractions are labeled as described in Fig. 2 (MT: mitochondria).

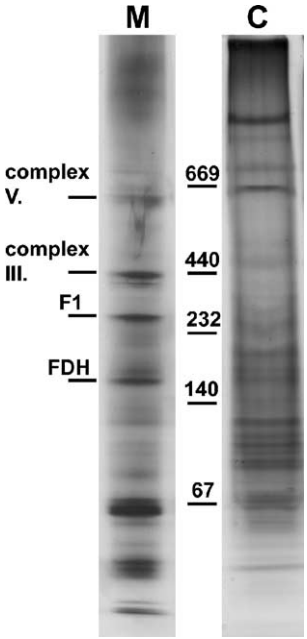

Fig. 3. Native polyacrylamide gel electrophoresis of mitochondrial and cytoplasmic protein complexes (see Section 2.7). M: mitochondrial fraction; C: cytoplasmic fraction; F1–F1-ATPase; FDH: formate dehydrogenase; the numbers refer to the size of standard protein complexes (669 kDa: thyroglobulin; 440 kDa: ferritin; 232 kDa: catalase; 140 kDa: lactate dehydrogenase; 67 kDa: bovine serum albumin).

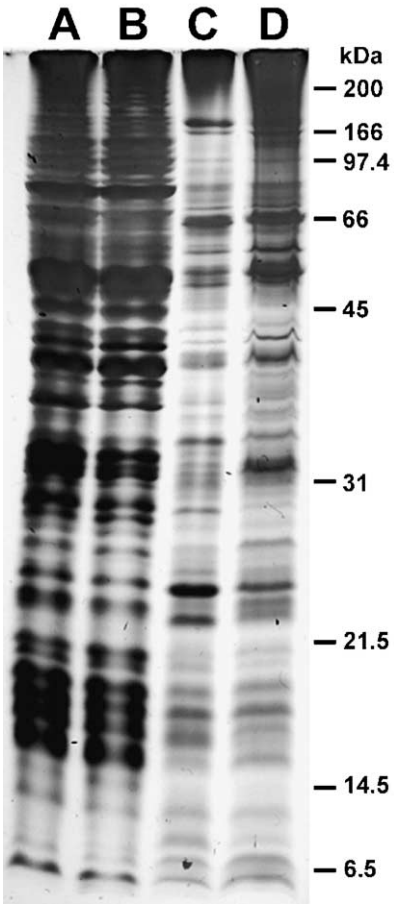

Fig. 4. 1-D SDS-PAGE of protein fractions obtained during isolation of mitochondria from in vitro cultivated pollen. A: crude homogenate; B: cytoplasmic fraction (supernatant; see Sections 2.2 and 2.3); C: mitochondrial fraction separated by Percoll and trapped on nylon filter (see Sections 2.2 and 2.3); D: mitochondrial matrix fraction after sunflower oil sub-fractionation (see Section 2.8).

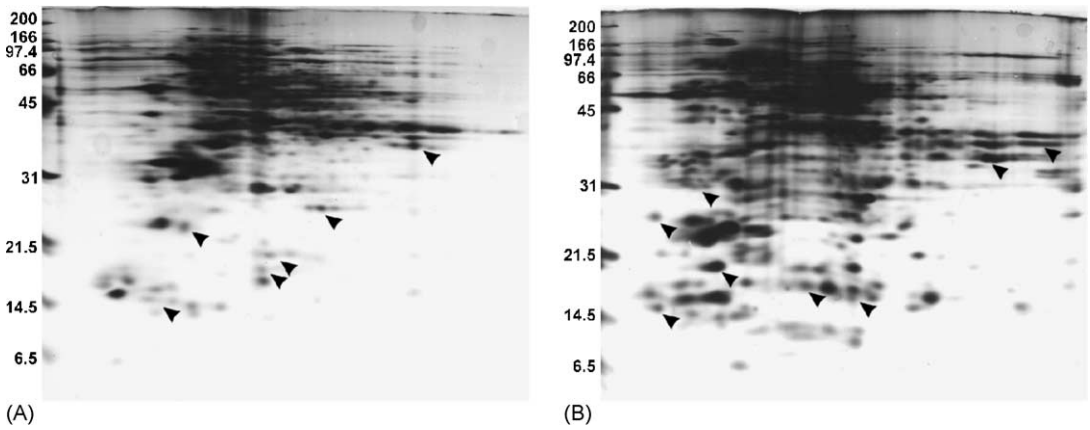

Fig. 5. 2-D SDS-PAGE spectra of two protein fractions obtained from in vitro cultivated pollen tubes. (A) Cytoplasmic and (B) mitochondrial fractions are compared. Arrowheads indicate the most abundant differentially localized proteins.

high efficiency. The major contribution to the efficiency of mitochondria isolation resulting from removal of Percoll from the mitochondrial fraction by filtration and the collection of pure mitochondria on nylon filters (third step in the extraction procedure) is clearly illustrated by the results shown in Table 1.

Following the isolation of intact mitochondria on nylon filters, three subsequent approaches to mitochondrial protein analysis become possible: (1) rapid protein extraction in buffer; (2) quantitative phenol extraction of mitochondrial proteins; (3) mitochondrial protein sub-fractionation into membrane and matrix fractions followed by phenol extraction.

Rapid (30 min) extraction (Section 2.4; Fig. 2) of mitochondria-bearing filters in 1-D or 2-D SDS-PAGE sample buffer, is simple and especially suitable for initial checking of mitochondrial protein patterns by electrophoresis. Although it is not quantitative, there were no qualitative differences observed between spectra of proteins released by rapid (Section 2.4) and phenol/chloroform (Section 2.5) protocols.

The phenol/chloroform extraction (Section 2.5) is quantitative and is the most appropriate method of sample preparation for mitochondria proteomic studies. Ten minutes treatment of the nylon filters with phenol led to the complete dissolution of the nylon filters. It guaranteed the quantitative extraction of total mitochondrial proteins and qualitative and quantitative completeness of 2-D SDS-PAGE spectra with the high reproducibility. Moreover, phenol extraction even increased the quality of 2-D protein separation by removal of nucleic acids, lipids and most of salts.

The quantity of mitochondria accumulated on the nylon filter enabled further analysis of mitochondria sub-fractions (Section 2.6). Two minutes shaking of the filters and their trapped mitochondria in the modified sample buffer for native electrophoresis, followed by freezing (−70 °C) and thawing, released intact complexes of mitochondrial proteins. Isolated complexes were analyzed by two alternative methods. First, the mitochondrial complexes were immediately separated by native electrophoresis (Section 2.7) according to Schagger et al. [21] (Fig. 3). The native

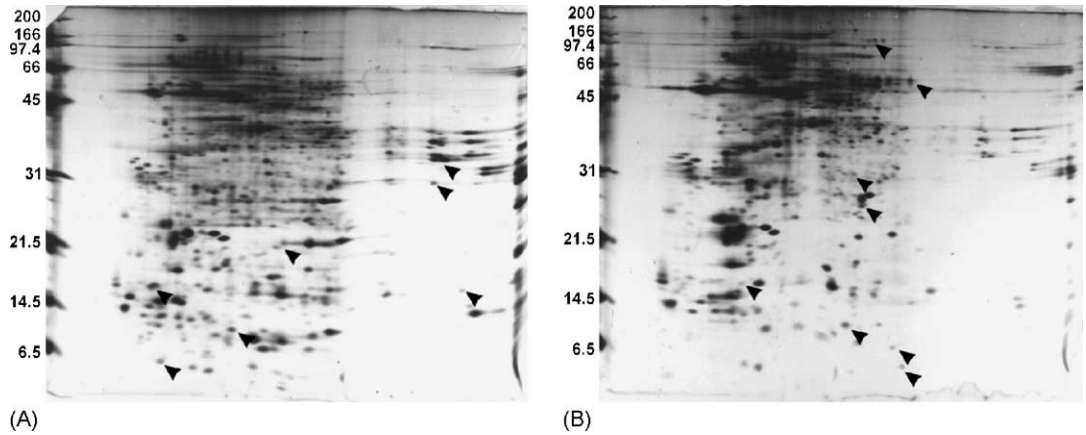

Fig. 6. 2-D SDS-PAGE spectra of proteins comprising two mitochondrial sub-fractions obtained from in vitro cultivated pollen tubes. (A) Membrane and (B) matrix fractions are compared. Arrowheads indicate the most abundant differentially localized proteins.

electrophoresis was performed on 5–16% gradient gel comparing both mitochondrial and cytoplasmic fractions. Both fractions showed clearly different spectra of protein complexes. The number and distribution of bands corresponding to the most abundant known mitochondrial protein complexes (complex III, complex V, F1-ATPase, formate dehydrogenase) was in accordance with previously published data obtained under the same experimental conditions [10]. Second, crushed mitochondria were further fractionated to obtain matrix and membrane-bound fractions. Such sub-fractionation of mitochondria complexes into membrane and matrix fractions was readily achieved by their distribution between polar and non-polar phases after shaking homogenates with oil for 5 min (Section 2.8).

The method of rapid mitochondria protein extraction and 1-D SDS-PAGE was used to verify at the qualitative level the purity of the mitochondria isolated using the procedure described here (Fig. 4). Crude cell homogenate and the cytosolic fraction after Percoll centrifugation were nearly identical. On the contrary, proteins isolated from mitochondria purified by Percoll and collected on nylon filter and proteins separated from the sub-mitochondrial matrix fraction showed significant differences. Quantitative phenol extraction in combination with 2-D SDS-PAGE (Figs. 5 and 6) revealed specific protein patterns in the cytosolic fraction, intact mitochondria and in the mitochondria membrane and matrix sub-fractions. This analysis also confirmed the purity of isolated mitochondria (Fig. 5) and their sub-fractions (Fig. 6), as well as their suitability for subsequent proteomic analyses.

Our results demonstrate that the method presented here allows the isolation and purification of proteins from two mitochondrial sub-fractions, from the mitochondrial matrix and proteins tightly bound to mitochondrial membranes. With the method used it is feasible to separate different cell compartments, that is usually correlated with complicated and time-consuming protocols. Pure mitochondria trapped on nylon filters are accessible to other treatments such as extraction by different buffers and destruction of one organelle structure and solubilization by phenol. Use of phenol, as the last step, is suitable for the successfully clarification of protein spectra by removal of traces of nucleic acids, lipids and salts dissolved in discarding solutions surrounding the protein precipitate.

4. Conclusion

We present here a new protocol for the quick and efficient isolation of mitochondria and their immobilization in native form on nylon membranes. This protocol also allows the purification of mitochondrial proteins by their solubilization in appropriate buffers or by phenol extraction. The mitochondrial proteins can be further sub-fractionated into matrix and membrane-bound fractions by freeze-rupture followed by a sunflower oil-based fractionation step. Membrane structures are not destroyed because of the natural characteristics of sunflower oil. The protocol described increases significantly the efficiency of mitochondrial isolation from very small amounts of plant material. It is especially suitable for proteomic analyses of mitochondria isolated from individual plant organs and tissues.

Acknowledgements

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