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How to Document the Purity of Mitochondria Prepared from Green Tissue of Pea, Tobacco and *Arabidopsis thaliana*

By

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S u m m a r y

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It is a difficult task to isolate mitochondria from green tissue, especially from the widely used model plant *Arabidopsis thaliana*. However, for several physiological queries very pure and physiologically intact mitochondria are needed. From three plant species, *Pisum sativum* L., *Nicotiana tabacum* L. and *Arabidopsis thaliana* HEYNH., mitochondria were isolated according to different purification protocols described before or by an own optimised protocol. The methods involved differential centrifugation and density gradient centrifugation on self-generating gradients or step-gradients of Percoll. The quality of the purified mitochondria were tested by different methods to prove intactness of the membrane system, the physiological activity and the

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contamination by other organelles. In summary, it was possible to isolate mitochondria from the three different plant species according to all criteria investigated. However, the reliability of the organelle preparations and the purity and intactness varied between the methods. To demonstrate the quality of the purified mitochondria with respect to different traits a combination of several tests is recommendable. Determination of chlorophyll contents and enzyme activities, functional analysis of the respiratory chain, and the use of the powerful technique of Blue-native/Tricine SDS polyacrylamide gel electrophoresis are necessary to fully characterize the mitochondria. Especially combining a visual result by comparing the separation of protein complexes in gels with physiological measurements seems to be very useful for a comprehensive characterization of the organelle preparation.

Zusammenfassung

HAUSMANN N., WERHAHN W., HUCHZERMAYER B., BRAUN H.P. & PAPENBROCK J. 2003. Methoden zur Isolierung und Prüfung der Reinheit von Mitochondrien aus Erbsen-, Tabak- und *Arabidopsis*-Blättern. – *Phyton* (Horn, Austria) 43 (2): 215 – 229, 3 Abbildungen. – Englisch mit deutscher Zusammenfassung.

Es ist eine schwierige Aufgabe, Mitochondrien aus grünem Gewebe zu isolieren, vor allem aus der Modellpflanze *Arabidopsis thaliana*. Jedoch ist für zahlreiche physiologische Untersuchungen der Einsatz von sehr reinen und physiologisch intakten Mitochondrien notwendig. Hier wurden Mitochondrien aus drei Pflanzenarten, *Pisum sativum* L., *Nicotiana tabacum* L. und *Arabidopsis thaliana* L. nach bereits veröffentlichten Protokollen oder nach einer modifizierten Arbeitsmethode isoliert. Die Methoden beinhalten eine Fraktionierung über differentielle Zentrifugation sowie Zentrifugation in kontinuierlichen oder diskontinuierlichen Percoll-Dichtegradienten. Die Qualität der gereinigten Mitochondrienfraktionen wurde mit verschiedenen Methoden getestet, um die Intaktheit des Membransystems, die physiologische Aktivität sowie die Kontamination mit anderen Organellen zu prüfen. Nach den verwendeten Kriterien ist es möglich, Mitochondrien aus allen drei Pflanzenarten zu isolieren; jedoch unterschieden sich die Methoden hinsichtlich ihrer Reproduzierbarkeit sowie der Reinheit und Intaktheit der Organellenpräparationen. Für eine umfassende Charakterisierung der gereinigten Organellen ist eine Kombination verschiedener Methoden sinnvoll, nämlich die Messung von Enzymaktivitäten, eine Funktionsanalyse der Atmungskette, Chlorophyllbestimmungen und der Einsatz der sensitiven Methode der Blau-nativen/Tricin-SDS-Gelelektrophorese. Vor allem der Vergleich eines visuellen Ergebnisses durch die Auftrennung von Proteinkomplexen über zweidimensionale Gele und der physiologischen Messungen ist sehr hilfreich und empfehlenswert.

Introduction

In contrast to cells of heterotrophic eukaryotes, plant cells contain besides mitochondria one additional type of semi-autonomous organelles enveloped by two membranes, the plastids. Both organelle types are believed to be derived from prokaryotic cells and have a similar shape, size and density. Consequently the separation of plastids and mitochondria during subcellular fractionations, which in general are based on differential centrifugations and/or density gradient centrifugations, is a difficult task. Furthermore plastids occur in various forms in plant cells (KIRK & TILNEY-BASSETT 1978, LAMPPA & al. 1980, ELLIS & LEECH 1985). Proplastids are small and represent plastids in a rather undifferentiated state; amyloplasts, chromoplasts, leucoplasts, etioplasts and chloroplasts form a continuous spectrum of larger plastid forms with varying density. The density of

chloroplasts depends on the state of photosynthesis: it usually increases at daytime due to light driven carbon fixation, activity of the Calvin cycle and subsequent starch biosynthesis, and it decreases at night because of the lower abundance of the Calvin cycle enzymes. Last not least the size, shape and density of plastids not only depend on the type of plastid, but also on the species investigated (KIRK & TILNEY-BASSETT 1978, PYKE 1999). As a consequence the separation of plastids and mitochondria is not only difficult, it is almost impossible to be achieved by procedures of general validity and reproducibility. Purification procedures for plant mitochondria or plastids have to be adapted depending on the selected plant species, the tissue type and the physiological state of the plants.

To minimize preparation problems, plant mitochondria are usually purified using non-green tissues like tubers, roots or etiolated seedlings because these tissues lack chloroplasts, and amyloplasts and etioplasts can be separated by centrifugation (MILLAR & al. 2001). Standard purification procedures for plant mitochondria of non-green tissue are based on the combination of differential centrifugations and Percoll density centrifugations and lead to reliable results (DOUCE & al. 1987, reviewed in MILLAR & al. 2001). However, the isolation of plant mitochondria from green plant tissue is important in order to understand mitochondrial functions during photosynthesis. Some procedures for the purification of mitochondria from green tissue were published, but the methods are usually restricted to single plant species or plant tissues. DAY & al. 1985 employed self-generating Percoll gradients which contained a linear gradient of polyvinyl pyrrolidone-25 (PVP-25) to purify pea leaf mitochondria. Protocols were published for spinach leaf mitochondria also using a self-generating Percoll gradient (HAMASUR & al. 1990). CHAUMONT & al. 1994 successfully isolated tobacco mitochondria by the use of two successive Percoll step-gradients and BERKEMEYER & al. 1998 reported the isolation of *Arabidopsis* leaf mitochondria by a continuous Percoll density gradient (0-33%).

Publications reporting the isolation of mitochondria from green tissue often lack a comprehensive documentation of their purity and intactness. To demonstrate the quality of isolated mitochondria usually selected marker enzymes for different cellular compartments were assayed, e.g. fumarase, succinate dehydrogenase, NAD-dependant malate dehydrogenase or cytochrome c oxidase for mitochondria, 2-hydroxypyruvate reductase or glycolate oxidase for peroxisomes, UDP-glucose pyrophosphorylase for the cytoplasm and NADP-dependant glyceraldehyde-3-phosphate dehydrogenase for plastids (reviewed in MILLAR & al. 2001). However, plastid contaminations of mitochondrial fractions might not become obvious on the basis of selected enzyme assays depending on the physiological state and intactness of plastids. For example NADP-dependent glyceraldehyde-3-phosphate dehydrogenase activity does not allow to monitor contaminating thylakoid vesicles in mitochondrial fractions. Furthermore many enzymes of the Calvin cycle are not present in proplastids, etioplasts and amyloplasts. An alternative possibility to monitor plastid contaminations in mitochondrial fractions are the measurements of the chlorophyll contents in the case of green tissue or the carotenoid contents in etiolated tissue (DOUCE & al. 1987).

During the isolation procedure the membranes of the organelles become often leaky and the organelles loose soluble proteins and metabolites, and are filled at the same time by components from the surrounding isolation medium. Therefore both the activities of soluble and membrane-dependent enzymatic reactions need to be measured. For the successful determination of state-3 respiration rates using an O_2 electrode the inner membrane needs to be intact and soluble components have to be available. For the characterization of chloroplasts the light-dependent CO_2 fixation could be used to control membrane intactness as well as availability of the respective solutes. Unfortunately, there is no method reported to judge the ratio of mitochondrial and plastid proteins after subcellular fractionation by one single analytical experiment. Recently, Blue-native/Tricine SDS polyacrylamide gel electrophoresis (BN/Tricine SDS PAGE) was introduced to document the purity of mitochondrial fractions on the basis of detectable protein complexes (KRUFT & al. 2001).

Here we report a strategy for a comprehensive documentation of the purity of mitochondrial fractions prepared from green plant tissue, which is based on marker enzyme assays, chlorophyll determinations, O_2 electrode measurements and BN/Tricine SDS PAGE. The capacity of the strategy is shown for mitochondrial fractions prepared from green tissues of pea, tobacco and *Arabidopsis*. Only the combination of different methods characterizes the quality of the purified mitochondria comprehensively.

Material and Methods

Seeds of *Arabidopsis thaliana*, ecotype Columbia, were originally obtained from the *Arabidopsis* stock centre at the Ohio State University. Seeds were germinated on substrate TKS1 (Florigard, Germany) and grown for 18 days. *Pisum sativum* var. Schöne Rheinländerin plants were cultivated for 18 days. *Nicotiana tabacum* var. Samsun was sown and grown on substrate TKS1 for 3 weeks. All plants were cultivated in the greenhouse in a 16 h light/8 h dark rhythm at a temperature of 23°C/21°C. When necessary, additional light was switched on for 16 h per day to obtain a constant quantum fluence rate of $300 \mu\text{mol m}^{-2} \text{s}^{-1}$ (sodium vapor lamps, SON-T Agro 400, Philips).

For the preparation of mitochondria from *Pisum sativum* the method described by DAY & al. 1985 was followed in principal. Modifications given in brackets were done to improve the quality of the purified mitochondria. Approximately 130 g (original protocol 300 g) of pea leaves were disrupted with a tissue grinder (Waring blender) in 300 ml of ice cold grinding buffer containing 0.3 M mannitol, 25 mM pyrophosphate, 10 mM KH_2PO_4 , pH 7.6, 1 mM EDTA, 0.2% (w/v) bovine serum albumine (BSA), 0.5% (w/v) PVP-40 and 4 mM cysteine. The brei was filtered through 4 layers (instead of 8 layers in the original method) of muslin and a 50 μm nylon net and then centrifuged for 10 min at 2,000g in a Sorvall RC-5B centrifuge (Sorvall GSA rotor, radius 14.61 cm, tube size: 250ml, fixed angle). The supernatant was centrifuged for 20 min at 12,000g and the pellet was resuspended in 100 ml of washing buffer containing 0.3 M mannitol, 10 mM KH_2PO_4 , pH 7.2, 2 mM glycine and 0.1% BSA. The resuspended pellet was centrifuged at 1,500g for 5 min and the supernatant was centrifuged again at 12,000g for 20 min to yield crude mitochondria. This pellet was resuspended in 10 ml of washing buffer, aliquots of 1.5 ml (instead of 2 ml as given by DAY & al. 1985) were layered over a 32 ml self-generating Percoll gradient containing 0.3 M sucrose, 10 mM KH_2PO_4 , pH 7.2, 0.1% (w/v) BSA, 28% (v/v) Percoll and a linear gradient of 0-10% (w/v) PVP-25 (top to bottom) in a Beckman SW28 rotor (tube size 34 ml, swing

out rotor) instead of a fixed angle rotor and centrifuged for 45 min at 40,000g. The mitochondria were localized in a yellow-white band near the bottom of the tube and were carefully removed. After diluting at least five-fold with suspension buffer (washing buffer without BSA), the mitochondria were washed by centrifuging twice at 15,000g for 20 min in a Sorvall RC-5B centrifuge (Sorvall SS34 rotor, radius 10.8 cm, tube size 34 ml, fixed angle). The same method was also used for the preparation of mitochondria from *Arabidopsis*.

To purify mitochondria from *Nicotiana tabacum* the method described by CHAUMONT & al. 1994 was modified. 8 g leaves were disrupted using a tissue grinder (Ultra-Turrax T25, Janke & Kunkel, Staufen, Germany) with 35 ml of cold grinding buffer 0.33 M sucrose, 50 mM Tris-HCl, pH 8.0, 0.2% (w/v) BSA and 0.04% (v/v) 2-mercaptoethanol. After filtration through two layers of Miracloth and a 50- μ m nylon net, 1 ml of the homogenate was centrifuged for 5 min at 16,000g (Eppendorf-centrifuge) to get a crude cytosolic supernatant and a crude organellar pellet. The remaining homogenate was centrifuged again for 30 s at 4,500g (Sorvall SS34 rotor). The pellet was resuspended in 2 ml of washing buffer (0.4 M mannitol, 10 mM KH_2PO_4 , pH 7.2, 0.2% (w/v) BSA). Aliquots of 1 ml were then layered onto a two-step Percoll gradient (4 ml 80% Percoll, 0.25 M sucrose, 0.2% (w/v) BSA and 5 ml 40% Percoll, 0.25 M sucrose, 0.2% (w/v) BSA) and centrifuged for 10 min at 14,400g (Beckman SW40 rotor, swing out rotor, tube size: 10ml). The interface enriched in chloroplasts was removed. The supernatant of the first differential centrifugation was again centrifuged for 12 min at 27,000g (Sorvall SS34 rotor). The pellet was resuspended in 2 ml of the washing buffer, layered onto a two-step Percoll gradient (3 ml 45% Percoll, 0.25 M sucrose, 0.2% (w/v) BSA and 4.5 ml 21% Percoll, 2.5 M sucrose, 0.2% (w/v) BSA) and centrifuged for 30 min at 78,500g (Beckman SW40 rotor). The interface enriched in mitochondria was carefully recovered. The mitochondrial fractions were washed in suspension buffer (washing buffer without BSA).

Mitochondria from *Arabidopsis* were isolated by differential centrifugation and Percoll density gradient centrifugation as described in WERHAHN & al. 2001 and KRUFFT & al. 2001. About 75 g *Arabidopsis* leaves and stems were disrupted in 450 ml ice-cold grinding buffer (450 mM sucrose, 1.5 mM EDTA, 0.2 % (w/v) BSA, 0.6 % (w/v) PVP-40, 10 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 15 mM MOPS-KOH pH 7.4) with an Ultra-Turrax. The brei was filtered through 4 layers of muslin and a 50- μ m nylon net. To remove cell debris and some chloroplasts the filtered brei was centrifuged in a Sorvall SLA3000 rotor at 3,500g for 10 min, the supernatant was first centrifuged at 3,500g for 5 min and then at 6,000g for 5 min. The mitochondria were pelleted at 17,000g for 10 min, resuspended in washing buffer (300 mM sucrose, 1 mM EDTA, 0.2 mM PMSF, 10 mM MOPS-KOH, pH 7.2) and carefully homogenized by two strokes in a Dounce homogenisator. The solution containing mitochondria was layered on top of three-step Percoll gradients (6 gradients 30 ml each containing 10 ml 18%, 10 ml 29% and 10 ml 40% Percoll in 0.3 M sucrose, 10 mM MOPS-KOH, pH 7.2). After centrifugation for 45 min at 70,000g (Beckman SW28 rotor) the mitochondria can be isolated from the 29%/ 40% interphase. To remove the Percoll the purified mitochondria were centrifuged twice in suspension buffer (0.4 M mannitol, 1 mM EDTA, 0.2 mM PMSF, 10 mM Tricine-KOH, pH 7.2) for 10 min at 14,500g using a Sorvall SS34 rotor and once in a Sorvall F-20/Micro rotor (tubes size 1.5 ml, fixed angle). The pellet was weighed and resuspended in 1.5 ml suspension buffer.

To check the purity of isolated organelles the activities of specific marker enzymes were measured. Mitochondrial fumarase (EC 4.2.1.2) was measured photometrically in a double beam spectrophotometer (Uvicon, Bio-Tek Instruments, Neufahrn, Germany) as described by BERKEMEYER & al. 1998. The standard reaction medium contained 100 mM KH_2PO_4 and 50 mM L-malate in a total volume of 3 ml; for the determination of activities in lysed mitochondria 5% Triton X-100 (w/v) was added when appropriate. The pH was adjusted to pH 7.4 and the temperature was kept constant at 25°C. The reaction was started by addition of 20 μ l isolated mitochondria suspension (average protein concentrations: pea 1 μ g/ μ l, tobacco 0.2 μ g/ μ l, *Arabidopsis* 0.25 μ g/ μ l). Production of fumarate was monitored by reading the absorbance at 240 nm. For calculations we used the millimolar absorbance coefficient $E_{240} = 2.44 \text{ cm}^2 \mu\text{mol}^{-1}$ (BERGMEYER 1974).

Respiratory O_2 consumption was measured in a Clark-type electrode at 25°C. The O_2 concentration in air-saturated water was assumed to be 240 μM (DAY & al. 1985). The standard assay medium contained 0.3 M mannitol, 5 mM $MgCl_2$, 10 mM KCl, 10 mM phosphate buffer (KH_2PO_4 , adjusted to pH 7.2), 0.1% (w/v) BSA; for the determination of activities in lysed mitochondria 5% Triton X-100 (w/v) was added when appropriate. Unless indicated otherwise, in all experiments the electrode was filled with 2 ml of this medium and 50 μl of mitochondria (average protein concentration see above) suspended in isolation buffer minus BSA. In a first test mitochondria were incubated in this mixture in the presence of 1 mM of added ADP. Then the stimulation of respiration by addition of succinate (at a final concentration of 10 mM) was measured. According to NEUBURGER & al. 1982 the integrity of isolated mitochondria was determined by the addition of pre-reduced cytochrome c and measurement of its stimulatory effect on respiration. The reaction mixture was the same as described above. As a marker for contaminating peroxisomes in the mitochondria preparation by we finally measured glycolate oxidase (EC 1.1.3.15) dependent O_2 consumption. In this test isolated mitochondria were incubated in the above mixture in the Clark-type electrode in the presence of 0.3 mM added KCN prior to the addition of glycolate at a final concentration of 10 mM according to DAY & al. 1985.

Two-dimensional BN/Tricine SDS PAGE (SCHÄGGER & al. 1994) was used to check the purity and intactness of the mitochondria prepared from different plants. About 100 μg protein were resuspended in 75 μl buffer containing 0.5 mM EDTA, 750 mM aminocaproic acid, and 50 mM Bis-Tris, pH 7.0, and solubilized with 15 μl of 10% n-dodecylmaltoside. After centrifugation for 30 min at 20,000g in a SIGMA 2K15 centrifuge (Rotor 12145, tube size: 1.5 ml) the supernatant was supplemented with 20 μl of a Coomassie-blue solution (5% Serva blue G, 750 mM aminocaproic acid) and loaded onto a 4.5-16% acrylamide gradient gel. Electrophoresis was carried out as described in JÄNSCH & al. 1996. The gels were silver-stained according to HEUKESHOVEN & DERNICK 1988.

The protein concentration was measured according to STOSCHECK 1990 using BSA as a standard. Chlorophyll was determined as described by ARNON 1949.

Results and Discussion

The purity of mitochondrial and chloroplast preparations is usually monitored by activity measurements of specific marker enzymes in addition to chlorophyll determinations (DAY & al. 1985, DOUCE & al. 1987, BERKEMEYER & al. 1998). However, these results reflect only a small part of the whole organellar protein set. It was shown before that Blue-native PAGE is a well-suited tool for the separation of membrane-bound and soluble protein complexes (reviewed in SCHÄGGER 2001). If combined with a second gel dimension in the presence of SDS, the subunits of protein complexes can be resolved forming vertical rows. To make the interpretation of the following gels easier, the results of typical two-dimensional separations of total chloroplast and mitochondrial protein contents are given in figure 1A (taken from KRUFT & al. 2001) and figure 1B (taken from KÜGLER & al. 1997), respectively. Protein complexes were localized on the basis of subunit compositions or the identification of single subunits by Edman degradation or mass spectrometry (JÄNSCH & al. 1996, KÜGLER & al. 1997).

In an attempt to document the purity of mitochondria prepared from pea and tobacco leaves established procedures were employed for preparations of organelles as outlined in DAY & al. 1985 and CHAUMONT & al. 1994. Analysis of isolated pea mitochondria by BN/Tricine SDS PAGE revealed the presence of respiratory chain complexes and the ATP synthase complex (Fig. 2A). The

presence of the matrix localized soluble HSP60 complex indicates that the isolated organelles were closely sealed during the preparation. The most dominant chloroplast protein complexes, the ribulosebiphosphate carboxylase/oxygenase complex (RubisCO) and the light harvesting complex IIb (LHCIIb) trimer are visible on the gel, but none of the protein complexes of the photosynthetic electron transport chain (the photosystems I and II and the b_6f complex) can be seen (also in comparison with Fig. 1B). The b_6f complex is a good indicator for the occurrence of plastids in general because this protein complex is also present in etioplasts (HERRMANN & al. 1992). Similar results were obtained for mitochondria isolated from tobacco leaves (Fig. 2B): besides the protein complexes of the respiratory chain, the ATP synthase complex and the HSP60 complex, and a slight contamination of the LHCIIb complex are visible to different extents. Since the detected mitochondrial and plastid protein complexes are of high stability, the dilution of the total protein concentration during sample preparation should not lead to dissociation of protein complexes and therefore not effect the estimation of contaminations.

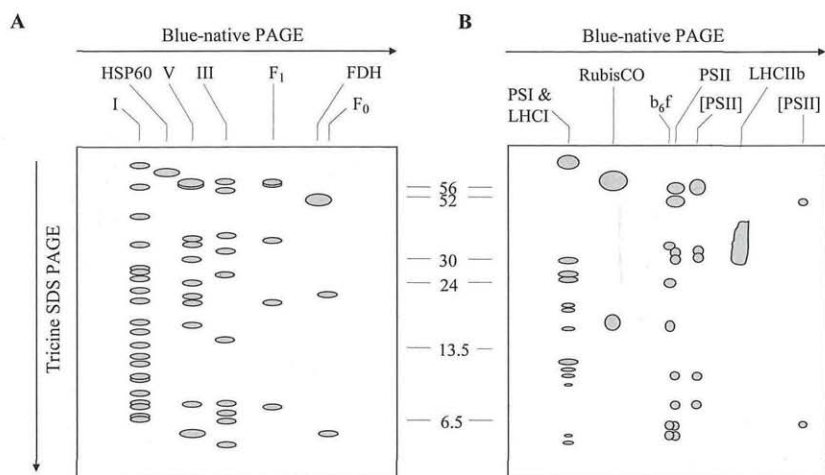


Fig. 1. Scheme of organellar protein complexes as resolved by two-dimensional Blue-native/Tricine SDS polyacrylamide gel electrophoresis. (A) Mitochondrial protein complexes, (B) chloroplast protein complexes. The designations on the top indicate the identity of the separated protein complexes: I: NADH-dehydrogenase; HSP60: heat stress protein 60; V: F_0F_1 ATP synthase complex; III: cytochrome c reductase; F_1 , F_0 : F_1 and F_0 parts of the ATP synthase complex; FDH: formate dehydrogenase; PSI: photosystem I; LHCI: light harvesting complex I; RubisCO: ribulosebiphosphate carboxylase/oxygenase; b_6f : cytochrome b_6f complex; PSII: photosystem II; [PSII]: subcomplex of photosystem II; LHCIIb: light harvesting complex IIb. The numbers in the middle indicate the molecular mass of standard proteins (in kDa). The schemes are adapted from two-dimensional gels as given in JÄNSCH & al. 1996 and KÜGLER & al. 1997.

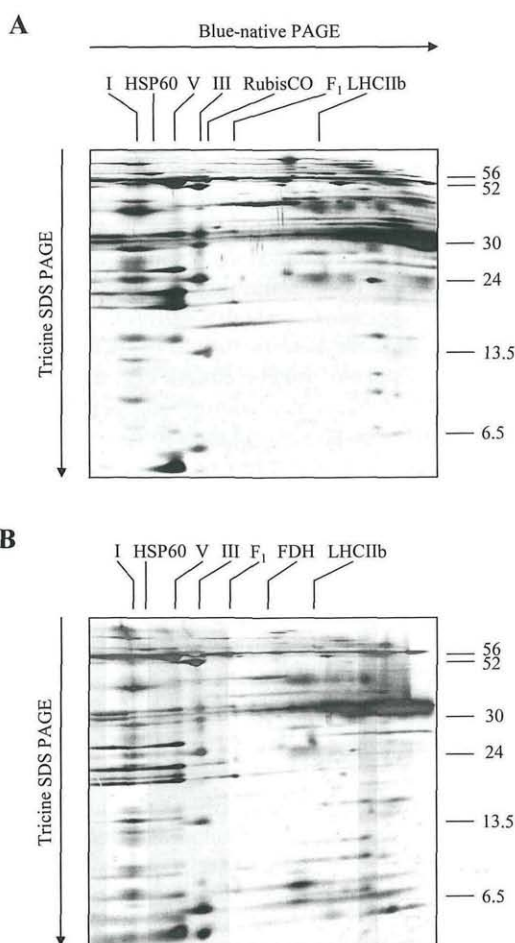


Fig. 2. Documentation of the purity of mitochondria prepared from green tissue of pea (A) and tobacco (B) The designations on the top indicate the identity of the separated protein complexes: I: NADH-dehydrogenase; HSP60: heat stress protein 60; V: F₀F₁ ATP synthase complex; III: cytochrome c reductase; RubisCO: ribulosebispophosphate carboxylase/oxygenase; F₁, F₀: F₁ and F₀ parts of the ATP synthase complex; FDH: formate dehydrogenase; LHCIIb: light harvesting complex IIb. The numbers on the right indicate the molecular mass of standard proteins (in kDa). Note: the identification of the separated protein complexes is based on the molecular masses on the first gel dimension (cytochrome c reductase, 480 kDa; ATP synthase, 580 kDa; HSP60 complex, 750 kDa; complex I, ca. 1,000 kDa; RubisCO, 450 kDa) and the subunit compositions on the second gel dimensions as reported previously (JÄNSCH & al. 1996, KÜGLER & al. 1997). The following subunits are useful markers for the identification of protein complexes: the 2 "core" subunits (ca. 51–56 kDa) for cytochrome c reductase, the α - and β -subunit (ca. 55 kDa) and the subunit c (ca. 7 kDa) for the ATP synthase complex, the 75 kDa FeS subunit for complex I, the 60 kDa subunit for the HSP60 complex, the "large" and "small" subunit (ca. 53 and 14 kDa) for RubisCO.

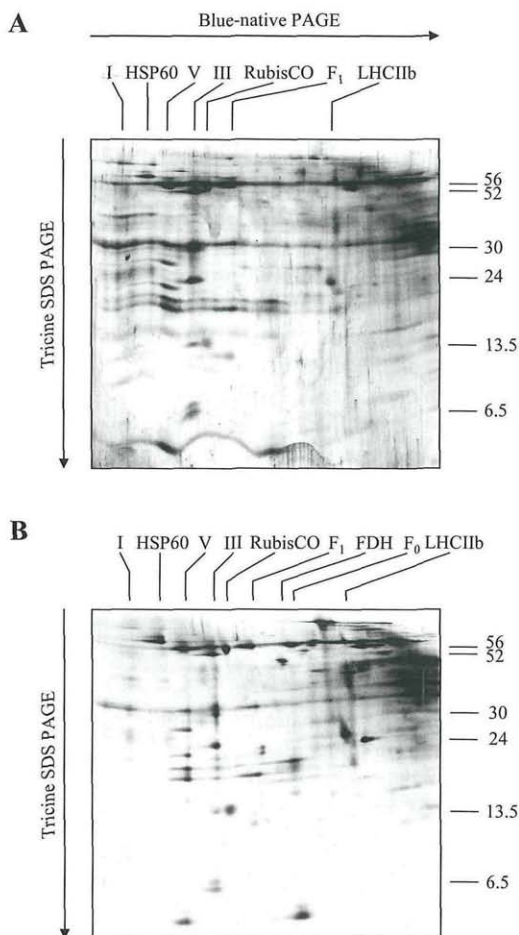


Fig. 3. Documentation of the purity of mitochondria prepared from green tissue of *Arabidopsis thaliana*. Mitochondria were prepared according to DAY & al. 1985 (A) and KRUFT & al. 2001 (B). Designations on top refer to the identity of the resolved protein complexes (see Fig. 2) and numbers on the right to the molecular masses of standard proteins (in kDa).

Mitochondria were also isolated from leaves of the model plant *Arabidopsis* as outlined in KRUFT & al. 2001. Alternatively the procedure for preparing pea leaf mitochondria as given in DAY & al. 1985 was adopted to isolate *Arabidopsis* mitochondria. The purity of the mitochondria prepared according to

DAY & al. 1985 is documented by BN/Tricine SDS PAGE (Fig. 3A). The BN/Tricine SDS PAGE from purified *Arabidopsis mitochondria* as described by KRUFT & al. 2001 is shown in figure 3B. Both preparations have the potential to gain almost pure mitochondria according to the gel analysis: The respiratory chain complexes and the ATP synthase complex can be identified. The presence of the matrix localized soluble HSP60 complex indicates that the isolated organelles were intact, however its abundance is higher in the mitochondria prepared after KRUFT & al. 2001. The most dominant chloroplast protein complexes, the RubisCO complex and the light LHClIb trimer are visible on both gels to different extents, but none of the protein complexes of the photosynthetic electron transport chain can be seen when compared to figure 1B. The contamination of the both mitochondrial fractions with soluble plastid proteins, demonstrated by the relative absence of RubisCO subunits, seems to be low. However, a contamination with higher amounts of thylakoid proteins like LHClIb can not be excluded as indicated by the large spot at 32 kDa on the right side of both gels.

In general the physiological characterization of the isolated organelles from different species confirmed the results obtained by BN/Tricine SDS PAGE with some deviations. All organelle isolation methods were repeated at least 6 times, however with different success. For the physiological results the measurements of two comparable preparations were combined (Table 1). Except for pea, the physiological data were collected from the same samples which were loaded on the respective gels. Because it is very important to have a good reference for all calculations the most reliable protein determination method for samples rich in membrane fragments was tested. The method using bicinchoninic acid according to STOSCHECK 1990 was the most convincing in comparison to methods described by LOWRY & al. 1951 and BRADFORD 1976 because of its linearity. Both other methods are well suited for the estimation of soluble proteins but revealed non-linear results for samples rich in lipids (data not shown).

The physiological data determined for the preparations of mitochondria from pea (DAY & al. 1985) are the most convincing (Table 1): The electron and proton transport along the respiratory chain was functioning according to high O₂ consumption in the presence of succinate. By adding ascorbate-reduced cytochrome c, O₂ consumption was low indicating an intact membrane system. According to NEUBURGER & al. 1982 cytochrome c induced stimulation of respiration can occur only if the mitochondrial membranes were leaky. In this case a loss of mitochondrial matrix proteins during organelle preparation can be assumed. This result is in agreement with the high fumarase activity, a soluble mitochondrial matrix protein, underlining the membrane continuity during our preparation. Determinations of fumarase activity with and without Triton X-100 revealed a percentage of intact mitochondria of about 50%. The preparation was free of peroxisomes because in the presence of glycolate and cyanide O₂ consumption was almost undetectable. The chlorophyll content in the mitochondria preparation was low. A calculation of the thylakoid protein contents shows, assuming a protein to chlorophyll ratio of 10 (ANDERSSON & ANDERSON 1985),

Table 1. Biochemical analysis of the purity of mitochondria from green plant tissue.

Species	Method	Fresh weight	Protein ^a yield	Fumarase	Chlorophyll to protein ratio	O ₂ consumption		
						succinate	reduced cytochrome c	glycolate
		[g]	[mg]	malate formed [nmol (min mg protein) ⁻¹]	[μg mg ⁻¹]	[nmol O ₂ (min mg protein) ⁻¹]		
Pea	DAY & al. 1985	130	0.975	83.5	1.0	804.5	144.5	0.016
Tobacco	CHAUMONT & al. 1994	8	0.170	<0.5 ^b	10.2	33.5	1453.0	0.767
<i>Arabidopsis</i>	DAY & al. 1985	92	0.375	11.7	8.7	1039.0	1352.0	422.6
<i>Arabidopsis</i>	KRUFT & al. 2001	75	0.142	2.9	0.0	685.0	388.0	208.5

^aBicinchoninic acid method, ^bless than 2times above background

that the contamination with thylakoid protein is about 1% on a protein basis. This value is in the range of the estimated percentage of the chloroplast proteins separated in the BN-gels when the soluble protein contaminations like RubisCO are taken into account (about 2-5 %). The BN/Tricine SDS PAGE stresses the physiological results shown and in addition documents the completeness of several soluble and membrane mitochondrial complexes and the low abundance of plastid proteins. In addition the protein yield was relatively high. Many scientists have chosen pea plants for their mitochondria preparations working on several aspects of metabolism (NASH & WISKICH 1983); obviously the separation of chloroplasts, mitochondria and peroxisomes is easier from pea tissue than from green tissue of the two other species used in this study, a fact confirmed by our analysis.

Nicotiana tabacum can be easily genetically engineered; the *Arabidopsis* genome is sequenced and there are many interesting *Arabidopsis* mutants existing. Therefore the demand to isolate mitochondria from green non-pea tissue has to be considered. As described above the BN/Tricine SDS PAGE from isolated tobacco mitochondria revealed a high purity, only the HSP60 complex is low abundant (Fig. 2B). However, the mitochondria did not consume any O₂ indicating a missing membrane integrity. The result was confirmed by the high values for O₂ consumption in the presence of reduced cytochrome c. The combination of all methods revealed a low quality of the tobacco preparations.

Both methods, the adopted method by DAY & al. 1985 using a linear gradient and the modified method for the isolation of *Arabidopsis* mitochondria from cell cultures using a step-gradient (KRUFT & al. 2001) were applicable for the preparation of *Arabidopsis* mitochondria. During the optimization of the purification protocols for the successful isolation of mitochondria from green tissue we can point a number of crucial steps: The younger the plants the better the yield of intact mitochondria, the homogenization procedure should be as fast and as effective as possible, and the lower the volume loaded on the gradients the better the quality of the mitochondria. In general, the isolated *Arabidopsis* organelles respired with relatively high rates in comparison to data from the literature (e.g. DAY & al. 1985). The mitochondrial membranes prepared according to KRUFT & al. 2001 were fully intact as shown by low O₂ consumption after the addition of reduced cytochrome c whereas the overall intactness of the organelles had suffered during the preparation after DAY & al. 1985. The soluble matrix protein fumarase could be determined indicating the preparation of sealed organelles in agreement with the presence of the HSP60 complex visible on the gel. In both mitochondria preparations some active peroxisomes were detected demonstrated by the determination of glycolate oxidase in Triton-lysed organelle preparations. The optimized isolation procedure for mitochondria from green *Arabidopsis* tissue (KRUFT & al. 2001) seems to be the best method to date. It probably needs some optimization to eliminate the peroxisomal contaminations.

To estimate the amount of material needed for the quality check of purified mitochondria following numbers are given: Assuming an average yield of 500 µg mitochondrial protein from 100 g fresh weight of plant material, about 150 µg protein was used for the O₂ consumption measurements, 50 µg for the fumarase

assay, 10 µg each for protein and chlorophyll determinations and 250 µg for the BN/Tricine SDS PAGE. Therefore one should start with at least 200 g fresh weight to be able to keep half of the preparation after the quality check for the actual analysis. One also has to keep in mind that the analysis of the samples by BN/Tricine SDS PAGE lasts about three days to obtain the final results. In general, conclusions drawn from results obtained with well-characterized mitochondria preparations are more reliable and satisfying.

Conclusions

In the mid-age of proteomics reproducible fractionation of cell compartments to investigate subproteomes has gained even more importance and interest. Also for a complete characterization of the many available *Arabidopsis* mutants the prerequisite is the reliable isolation of organelles from green tissue. The isolation and investigation of mitochondria from cell cultures is only a compromise. With respect to the quality control all methods applied here have advantages and disadvantages, e.g. BN-PAGE is a very suitable procedure for the detection of mitochondrial and plastid cross-contaminations, but of limited value for the determination of contaminations by peroxisomes, because the latter organelles lack stable protein complexes which can be monitored on the native gels. Therefore only a combination of physiological methods, in which membrane integrity and soluble components are required, and visualization of protein complexes separated by gel electrophoresis unequivocally demonstrates the intactness and purity of the purified mitochondria. For general physiological investigations plant mitochondria should be isolated from *Pisum sativum* instead of *Arabidopsis thaliana*. However, absolute purity of mitochondria from green tissue may never be reached due to the principal that in the continuous spectrum of plastids in a cell a number have very similar size, density and shape corresponding exactly to the properties of typical mitochondria (KIRK & TILNEY-BASSETT 1978, LAMPPA & al. 1980, ELLIS & LEECH 1985).

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