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Comparison of two methods for preparing mitochondria from tomato leaves to study the ascorbate-glutathione cycle activity

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Abstract

Mitochondria were purified from tomato leaves by a differential centrifugation method and a Percoll density-gradient method. A comparative study on the ascorbate-glutathione cycle activity revealed that there was no difference in the specific activity of the antioxidative enzymes and ascorbate and glutathione concentrations between mitochondria prepared by these two methods. However, the contents of dehydroascorbic acid and oxidised glutathione in Percoll-separated organelles were 260 and 210 %, respectively, of those found in mitochondria purified by the differential centrifugation method.

Additional key words: antioxidants, *Lycopersicon esculentum*, mitochondria isolation.

Introduction

In plant cells a relevant role of an antioxidant defence system has been attributed to the ascorbate-glutathione cycle (AA-GSH cycle). Recent studies have focus on the changes in activity of the AA-GSH cycle under oxidative stress imposed by environmental constraints as a good indicator of the degree of stress experienced by plants (Hernández *et al*. 1993, Kuźniak and Skłodowska 1999, Blokhina *et al.* 2003, Drążkiewicz *et al.* 2003). All components of the AA-GSH cycle have been detected in cytosol, chloroplasts, mitochondria and peroxisomes (Jiménez *et al.* 1997, Mittova *et al.* 2000, Kuźniak and Skłodowska 2001). Hence, measurement of the activity/ concentration of the cycle components at the crude homogenate level may not adequately reflect their role in the protection mechanism as well as the interplay between the antioxidant systems that are distributed in different cell organelles. A prerequisite to study the compartment-specific role of the AA-GSH cycle in plant response to changing environmental conditions is to develop a highly reproducible method for isolating pure, stable, intact and metabolically competent individual organelles.

Mitochondria have been suggested to play a unique role in the mechanism by which plant cells receive and respond to the oxidative stress (Jones 2000, Møller 2001, Tiwari *et al*. 2002, Virolainen *et al.* 2002). Many different methods that have been described for isolation of plant mitochondria were originally developed for etiolated seedlings or storage tissues (Douce *et al.* 1972, Petit *et al.* 1987, Rasmusson and Møller 1990). Separation of mitochondria from green tissues is a much more difficult task because of the necessity to overcome the problem of separation of mitochondria from chloroplasts. Procedures developed for isolation of leaf mitochondria are based on fractionation by differential centrifugation and/or sucrose or Percoll density gradient centrifugation. As to the differential centrifugation methods they have often been considered to be of limited suitability due to an unsatisfactory removing of contaminating chloroplasts (Nishimura *et al.* 1982). However, the density gradient methods are time consuming and can produce mitochondria that are less stable (Liang *et al.* 1982) hence a loss in enzyme activity can occur during purification. Moreover, the yield is

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Abbreviations: AA - reduced ascorbate; APX - ascorbate peroxidase; CAT - catalase; DHA - dehydroascorbate; DHAR - dehydroascorbate reductase; GR - glutathione reductase; GSH - reduced glutathione; GSSG - oxidized glutathione; MDHAR - monodehydroascorbate reductase.

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markedly reduced as compared to the differential centrifugation method (Peckmann and Herpich 1998). In this work we compare the applicability of a differential centrifugation method and a Percoll density gradient method to obtain mitochondria from tomato leaves for biochemical studies. A comparison of the antioxidant profiles, *i.e.*, the activities of AA-GSH cycle enzymes and the concentrations of ascorbate and glutathione in mitochondria obtained by these two methods are presented.

Materials and methods

Plants: Tomato plants (*Lycopersicon esculentum* Mill.) cv. Perkoz were grown in a growth chamber for six weeks as described earlier (Kuźniak and Skłodowska 2001).

Preparation of mitochondria: Mitochondria were isolated from the first, second and third true leaves. Leaves without the main midribs were cut into pieces, chilled in an ice bath for 20 min, homogenised in a blender in an ice-cold isolation solution (1:5 m/v) and filtered through 4 layers of Miracloth (crude extract). Two different isolation methods were used. According to the differential centrifugation method of Warrilow and Hawkesford (1998) leaves (10 g) were homogenised for 30 s in a homogenisation medium containing 50 mM Tris-HCl, 0.33 M D-mannitol, 2.5 mM EDTA, 2.5 mM sodium ascorbate and 2 mM $MgCl_2$ (pH 8.1). The chloroplasts were sedimented by centrifugation at 1 500 *g* for 10 min. The supernatant was further centrifuged at 8 200 *g* for 10 min and the obtained supernatant was considered the cytosolic fraction. The pellet (mitochondria and peroxisomes) was resuspended twice in a washing medium containing 50 mM Tris-HCl, 2 mM $MgCl_2$ and 2 mM sodium ascorbate (pH 8.1) to give the final mitochondrial fraction.

The second isolation method was essentially the Percoll density gradient method described by Struglics *et al*. (1993) except the following modifications. Leaves (50 g) were homogenised twice for 15 s in a medium (1:5 m/v) containing 0.3 M mannitol, 0.1 % bovine serum albumin, 1 mM EDTA, 20 mM Tris-HCl, 10 mM sodium ascorbate and 2 mM $MgCl_2$ (pH 7.4) and centrifuged at 200 g for 20 min to remove chloroplasts. The supernatant was centrifuged at 10 000 *g* for 10 min (cytosol). The 3 cm^3 of pellet (mitochondria and peroxisomes) was gently resuspended in a solution containing 0.3 M mannitol, 0.05 % bovine serum albumin, 1 mM EDTA, 10 mM Tris-HCl, 1 mM sodium ascorbate, 0.15 M sucrose (pH 7.2), layered on a 35-cm^3 Percoll gradient composed of 0.3 M sucrose, 1 mM EDTA, 10 mM Tris-HCl, 0.05 % bovine serum albumin, 1 mM sodium ascorbate, 28 % Percoll (pH 7.2) and centrifuged at 30 000 *g* for 50 min. The band of intact mitochondria, as verified by marker enzymes activities, was collected with a Pasteur pipette from the band near the top of the gradient (25 - 31 cm^3), diluted 10 times with a washing medium containing 0.3 M mannitol, 10 mM Tris-HCl, 1 mM sodium ascorbate, 1 mM EDTA (pH 7.2) and centrifuged at 12 000 *g* for 10 min. In order to remove Percoll this washing procedure was repeated three times.

Enzyme assays, determination of antioxidants and chlorophyll: Each step of the isolation procedures as well as the quality of the mitochondrial fraction were monitored by measurements of marker enzyme activities and chlorophyll content. To assess contamination by other organelles fumarase (EC 4.2.1.2) and NADH:cytochrome *c* oxidoreductase (EC 1.6.99.3) were used as markers of mitochondria while catalase (EC 1.11.1.6), acid phosphatase (EC 3.1.3.2) and chlorophyll were used as markers of peroxisomes, vacuoles and chloroplasts, respectively. For determining enzyme activities aliquots of the fractions designated as crude extract, chloroplasts, cytosol, mitochondria and peroxisomes and the final mitochondrial fraction were homogenised in a mortar in a medium containing 1 M NaCl, 1 % polyvinylpyrrolidone, 1 mM EDTA, 1 mM sodium ascorbate in 0.05 M phosphate buffer, pH 7.0 and centrifuged at 20 000 *g* for 15 min. The activities of marker enzymes were measured in the obtained supernatants following the earlier published protocol (Kuźniak and Skłodowska 2001) except for NADH:cytochrome *c* oxidoreductase determined as described by Douce *et al*. (1972) and acid phosphatase assayed using *Sigma-Aldrich* (Steinheim, Germany) diagnostic kit. Chlorophyll was extracted with 80 % (v/v) acetone and measured according to Porra *et al*. (1989) using *UNICAM UV300* UV-visible spectrophotometer. The activities of marker enzymes were given as follows: fumarase – mmol(fumarate formed) g^{-1}(protein) min^{-1} (coefficient of absorbance ε = 2.25 mM^{-1} cm^{-1}); NADH:cytochrome *c* oxidoreductase – mmol(cytochrome *c* reduced) g^{-1}(protein) min^{-1} (ε = 21 mM^{-1} cm^{-1}); catalase – mmol(H_2O_2 decomposed) g^{-1}(protein) min^{-1} (ε = 39 M^{-1} cm^{-1}); acid phosphatase – mmol(nitrophenol) g^{-1}(protein) min^{-1}.

The activities of AA-GSH cycle antioxidant enzymes as well as ascorbate and glutathione contents were determined as described earlier (Kuźniak and Skłodowska 2001). The activities of AA-GSH cycle enzymes were expressed as follows: APX - mmol(ascorbate oxidised) g^{-1}(protein) min^{-1} (ε = 13.7 mM^{-1} cm^{-1}); DHAR - mmol (ascorbate formed) g^{-1}(protein) min^{-1} (ε = 14.6 mM^{-1} cm^{-1}); MDHAR - mmol(NADH oxidised) g^{-1}(protein) min^{-1} (ε = 6.22 mM^{-1} cm^{-1}); GR - mmol (NADPH oxidised) g^{-1}(protein) min^{-1}

(ε = 6.22 mM^{-1} cm^{-1}). Ascorbate content was measured in preparations obtained with buffers lacking sodium ascorbate. The protein content was determined according to Bradford (1976) using serum albumin as a standard.

The latency of enzyme activity was determined by carrying out the assays for mitochondria in an isotonic medium and in the mitochondrial extract. The latency of enzyme activity [%] was calculated using the formula of Burgess *et al.* (1985):

latency = (activity intact/activity burst) × 100.

Results and discussion

The purification profiles obtained by both methods were consistent, except for the distribution of fumarase and catalase in chloroplasts and NADH:cytochrome *c* oxidoreductase in cytosol (Table 1). Thus, the activities of marker enzymes indicated similar organelle separation during the differential centrifugation step shared by the two methods. Moreover, as evaluated by chlorophyll content, the major part of chloroplasts was removed in the first centrifugation step, *i.e*. 1 500 *g*, 10 min for the differential centrifugation method and 200 *g*, 20 min for Percoll density-gradient procedure (data not shown). Similarly, the results obtained for the AA-GSH cycle enzymes were irrespective of the method used, except for GR activity in chloroplasts (Table 2). In general, when correction for different activity units was made, the activities of the ascorbate-glutathione cycle enzymes in crude extracts were similar to those obtained earlier for tomato leaves (Kuźniak and Skłodowska 1999). This indicated that no preferential loss of any AA-GSH cycle enzymes took place in the first homogenisation step of both isolation procedures. For both methods, the activity of the antioxidant enzymes recovered in the mitochondrial and peroxisomal fraction was of the same order of 10 - 15 % of the total activity detected in crude extracts. The relative intraorganellar distribution of the activities of these enzymes, when expressed in μmol g^{-1}(protein) min^{-1}, matched that reported in different plant species (Jiménez *et al.* 1997). There was no difference between the isolation procedures regarding the antioxidant enzymes with respect to both the pattern of intraorganellar distribution and activity level, except for the activity of Percoll-purified chloroplastic DHAR being 39 % lower than that obtained by differential centrifugation method (Table 2). Taking into account both distribution of marker enzymes (Table 1) and AA-GSH cycle enzyme activities (Table 2) it seems that the only difference between these methods concerned the chloroplastic fraction quality, which is of minor importance with regard to the aim of this work.

The disadvantage of the Percoll density-gradient method found in our study was that the maximum yield of only 0.88 mg of mitochondrial protein could be obtained from 50 g of leaves. The yield of tomato leaf mitochondria, calculated on a protein basis, was 3.8 fold

Table 1. Distribution of marker enzyme activities [mmol g^{-1}(protein) min^{-1}]. Means from four independent experiments ± SD. ND - not detected.

Fraction	Differential centrifugation method				Percoll density gradient method			
	fumarase	NADH:cyt *c* oxidoreductase	CAT	acid phosphatace	fumarase	NADH:cyt *c* oxidoreductase	CAT	acid phosphatace
Crude extract	0.147±0.038	0.069±0.026	71.70±10.74	12.32±2.71	0.097±0.026	0.049±0.012	100.55±24.12	10.29±1.95
Chloroplasts	0.082±0.025	0.017±0.005	13.53± 5.02	4.19±1.13	0.011±004	0.012±0.004	30.11± 7.97	3.94±1.38
Cytosol	0.051±0.010	0.036±0.010	30.03± 7.80	10.55±2.32	0.044±0.007	0.008±0.003	19.77± 2.96	5.72±2.00
Mit. + perox.	0.089±0.035	0.084±0.027	108.89±41.37	ND	0.083±0.028	0.077±0.018	151.26±57.44	ND

Table 2. Distribution of AA-GSH cycle enzyme activities [mmol g^{-1}(protein) min^{-1}]. Means from four independent experiments ± SD.

Fraction	Differential centrifugation method				Percoll density gradient method			
	APX	DHAR	MDHAR	GR	APX	DHAR	MDHAR	GR
Crude extract	0.122±0.025	0.093±0.027	0.028±0.009	0.152±0.047	0.127±0.041	0.058±0.015	0.026±0.008	0.169±0.048
Chloroplasts	0.029±0.060	0.018±0.005	0.014±0.003	0.091±0.030	0.033±0.010	0.011±0.003	0.012±0.002	0.072±0.031
Cytosol	0.053±0.017	0.043±0.011	0.007±0.002	0.113±0.007	0.038±0.008	0.033±0.005	0.009±0.003	0.126±0.040
Mit. + perox.	0.049±0.017	0.024±0.004	0.011±0.004	0.051±0.009	0.044±0.008	0.025±0.007	0.013±0.002	0.055±0.009

higher when the differential centrifugation method was used. Mitochondria were identified by the activity of specific marker enzymes: fumarase and NADH: cytochrome *c* oxidoreductase (Table 3). The specific activities of fumarase calculated for mitochondria obtained by differential centrifugation method and Percoll density-gradient method were 0.16 and 0.19 mmol g^{-1}(protein) min^{-1}, respectively. These data coincide with those reported by Sandalio *et al*. (1987) and Corpas *et al.* (1998) for pea mitochondria. Likewise, the activity of NADH:cytochrome *c* oxidoreductase, was similar to that found by other authors (Schwitzguebel and Siegenthaler 1984). To assess the membrane integrity of the purified mitochondria the latency of fumarase and NADH:cytochrome *c* oxidoreductase was determined (Table 3). In both mitochondrial preparations the latency of fumarase was approximately the same, and was found to be between 94 and 98 %. Based upon the measurement of NADH: cytochrome *c* oxidoreductase activity mitochondria with 80 and 71 % integrity were obtained by the differential centrifugation method and Percoll density-gradient method, respectively. The latency data reflect the high yield of intact mitochondria isolated by both methods. Hence, our results did not confirm those of Schwitzguebel and Siegenthaler (1984) and Bergman *et al*. (1980) who reported that the organelle intactness was improved by Percoll gradient centrifugation. Our results indicated that the pure mitochondrial preparations were free from vacuoles, negligibly contaminated by broken chloroplasts and peroxisomes, as judged from acid phosphatase activity, chlorophyll content and catalase activity, respectively (Table 3). These results coincide with other reports showing that further purification on sucrose/Percoll density gradient did not prevent the mitochondrial fraction from contamination by other organelles (Sandalio *et al.* 1987, Corpas *et al.* 1998). According to Bartoli *et al.* (2000) the mitochondrial- and peroxisomal-enriched fraction obtained from potato leaves and purified using Percoll density-gradient contained 17 % of chlorophyll. Similarly, in the purified mitochondrial fraction from pea leaves an analysis of the distribution profile of the mitochondrial marker – cytochrome *c* oxidase and chlorophyll revealed that the highest activity and the chlorophyll maximum content partly overlapped. Moreover, on the basis of hydroxypyruvate reductase activity pea mitochondria were contaminated with peroxisomes by about 13 % (Jiménez *et al.* 1997).

Irrespective of the isolation procedure used in our study, the specific activities of the AA-GSH cycle enzymes were almost identical, except for a 30 % lower activity of DHAR in mitochondria prepared by differential centrifugation method in comparison with the Percoll-gradient method (Table 4). Thus it seems that differences in the activity levels of antioxidant enzymes between our study and those of other authors were not

Table 3. Marker enzyme activities [mmol g^{-1}(protein) min^{-1}] and chlorophyll content [μg cm^{-3}] in the mitochondrial fraction. ND - not detected. The percentage of latency calculated for mitochondrial marker enzymes as described in Materials and methods is given in parenthesis. Means from four independent experiments ± SD.

Method	Chlorophyll content	Fumarase	NADH:cyt *c* oxidoreductase	Catalase	Acid phosphatase
Differential centrifugation	13.25±3.57	0.16±0.04 (94%)	0.07±0.03 (80%)	67.14±16.63	ND
Percoll density gradient	29.79±8.64	0.19±0.05 (98%)	0.08±0.03 (71%)	29.51±6.41	ND

Table 4. Ascorbate-glutathione cycle enzyme activities [mmol g^{-1}(protein) min^{-1}] in mitochondria from tomato leaves. Means from at least four independent experiments ± SD.

Method	APX	DHAR	MDHAR	GR
Differential centrifugation	0.129±0.030	0.039±0.010	0.023±0.003	0.044±0.005
Percoll density gradient	0.118±0.026	0.050±0.008	0.027±0.003	0.044±0.007

Table 5. Ascorbate and glutathione contents [μmol g^{-1}(protein)] in mitochondria from tomato leaves. Means from at least four independent experiments ± SD.

Method	AA	DHA	AA total	AA/total	GSH	GSSG	GSH total	GSH/total
Differential centrifugation	35.80±6.72	1.32±0.23	37.12±6.58	0.96	5.59±1.32	0.31±0.087	5.90±1.14	0.95
Percoll density gradient	32.65±5.55	3.43±0.37	36.08±5.05	0.90	5.90±1.58	0.65±0.11	6.55±1.54	0.90

attributable to the isolation procedure but to the differences in plant material, *e.g.*, age of the parent tissue, seasonal variations, growth conditions, especially with regard to the inconsistency in literature data on the activity of the AA-GSH cycle in plant mitochondria (Jiménez *et al.* 1997, Mittova *et al.* 2000). The reduced ascorbate and glutathione contents determined in the mitochondrial fraction prepared by the differential centrifugation method perfectly matched those in organelles separated by the Percoll method. Therefore it seems reasonable that the mitochondrial non-enzymatic antioxidant concentrations estimated in our study were independent of the isolation procedure used (Table 5). However, the levels of DHA and GSSG in Percoll-purified mitochondria were about twice the contents determined in preparations obtained by the differential centrifugation method. We suggest that the Percoll procedure, which requires more handling, is not short enough to preserve ascorbate and glutathione oxidation. Along with the increase in DHA and GSSG concentrations the decrease in redox state of the mitochondrial non-enzymatic antioxidant pools was observed down to 0.90 as compared with 0.95 - 0.96 found in mitochondria purified by the differential centrifugation method (Table 5). The latter seem to coincide better with the literature data on the high ascorbate and glutathione redox status in plants (Karpinski *et al.* 1997, Veljovic-Jovanovic *et al.* 2001).

Taken together, our results indicated that further purification on a Percoll gradient did not improve the yield, intactness and the antioxidant enzyme activities as well as antioxidant contents in the mitochondrial fraction. Similar results were presented by Peckmann and Herpich (1998). Although mitochondria obtained by the simple and quick differential centrifugation method were not perfectly satisfactory with respect to the relatively high chlorophyll content and catalase activity, they were sufficiently pure, intact and metabolically competent for biochemical and functional studies. In the case of the Percoll-gradient procedure better separation of mitochondria from contaminating peroxisomes was observed. However, this method was found to be time consuming and ensured lower final recovery of the mitochondrial protein and lower redox status of the non-enzymatic antioxidants as compared with the differential centrifugation one. The latter disadvantage seems to be of particular importance for studies aimed to dissect the role of mitochondrial redox system in plant response to stress. Taking into account the advantages and disadvantages of each method, on the basis of *1*) the intactness of the isolated mitochondria, *2*) the activities of marker enzymes, as well as *3*) the activities of antioxidant enzymes and ascorbate and glutathione concentrations we found that both methods could be recommended for biochemical studies on the AA-GSH cycle activity in this organelle fraction.

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