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Isolation and Characterization of Subcellular Organelles from Young and Mature Leaves of Olive Tree *

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Subcellular organelles from young and mature leaves of olive tree have been isolated by differential centrifugation of leaf homogenates, and further purified by sucrose gradient centrifugation. Chlorophyll content was used for detecting chloroplasts after both the differential and density gradient centrifugation; aricase and catalase activities for peroxisomal identification, and the antimycin A sensitive and the antimycin-insensitive NADH-cytochrome C reductase as enzyme markers for mitochondria and microsomes, respectively. Chloroplast and mitochondrial respiration were tested polarographically by measuring the oxygen evolved or consumed, respectively, in the noncyclic electron transport chain of photosynthesis with an electron acceptor, and in the respiratory chain when succinate was used as substrate. No remarkable differences were observed between organelles from young and mature leaves, suggesting that the former can be used as a suitable material to study the lipidic biosynthetic pathways of this plant.

Young leaves from olive tree sprouts are suitable material to study lipidic biosynthesis in this plant (5, 6). They show a high metabolic rate, as well as a comparatively lower lipidic content than the fruit and mature leaves. In this paper subcellular organelles involved in lipidic biosynthesis from both types of leaves were compared. Lipid synthesis take place mainly in the chloroplast, the *in vitro* experiments with isolated chloroplasts showing a biosynthetic rate proportional to the intact ones (18, 20). On the other hand, the desaturating activities concerned with the biosynthesis of mono and polyunsatured fatty acids are mainly located in the mitochrondial and microsomal fractions (8, 17). Because chloroplast and mitochondrial preparations often appear contaminated with peroxisomes, these organelles must be also considered (7, 12).

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Materials and Methods

Plant Material. Sprouts from olive trees *(Olea europaea* cv. Marteño) in vegetative phase were grown in the greenhouse with weekly irrigation, supplemented every two months with the HEWITT's nutritive solution (11). The temperature range was 15-22°, with a photoperiod of 16 hours under a light intensity of 3,000 lux. For the experiments, young (2-3 months) and mature (1 year) leaves were collected.

Isolation of subcellular organelles. 25 g of young and mature leaves were thoroughly washed with distilled water, and then cut into short segments. All operations were then performed between 0° and 4°. The excised leaves were blended for 10 seconds at top speed (Sorvall Omnimixer) with 100 ml of 0.1 M Tris-HCl buffer (pH 7.5), 0.4 M sucrose, 1 mM $MgCl_2$, 10 mM EDTA, 10 mM KCl, 4 mM DTT[2], 3 mM cysteine, 1.5 % polyvinylpyrolidone and 0.1 % bovine serum albumin. Homogenates were filtered through eight layers of nylon cloth, resuspended in a Potter-Elvehjem homogenized and centrifuged at 3,000 × *g* for 1 min (Sorvall RC2-B); the supernatant was decanted and the pellet suspended in 3 ml of 50 mM HEPES * buffer (pH 8.3), 0.4 M sucrose, 5 mM $MgCl_2$, 1 mM $MnCl_2$, 1mM EDTA, and 1 mM DTT (suspension A). The turbid supernatant was again centrifuged at the same speed for 5 min, and the residue collected in 2 ml of above solution (suspension B). The 3,000 × *g* supernatant was then centrifuged at 25,000 × *g* for 25 min, the tube decanted and the residue washed with 15 ml of 0.1 M Tris-HCl buffer (pH 7.5), 0.4 M sucrose, 1 mM $MgCl_2$, 10 mM EDTA, 10 mM KH_2PO_4, 2.5 mM DTT, and 0.1 % bovine serum albumin, and again centrifuged at the same speed for 10 min; the pellet was resuspended in 1.5 ml of the washing solution (suspension C). The 25,000 × *g* supernatant was finally centrifuged at 105,000 × *g* for 60 min (Beckman L-5-65 ultracentrifuge), the supernatant discarded and the pellet collected in 1.5 ml of the preceding solution (suspension D).

The chloroplast preparation (suspension A) was further purified by sucrose gradient centrifugation, for 10 min at 1,000 × *g*, in a discontinuous gradient made up by layering 10 ml each of 1.5 M, 1 M, 0.75 M and 0.4 M sucrose in the buffer of the sample (23). Suspensions C and D enriched in mitochondrial organelles and microsomal membranes, respectively, were also purified by sucrose gradient centrifugation at 27,000 × *g* for 60 min, in a discontinuous gradient prepared by layering 4 ml each of 68, 65, 58, 55, 48, 41 and 34 % sucrose in 0.1 M Tris-CHl buffer (pH 7.0), 10 mM KH_2PO_4, 10 mM KCl, and 0.075 % bovine serum albumin. In all cases the tubes were drained off, and the separately collected bands tested for chlorophyll content and enzymatic activities. After dilution to 0.4 M sucrose concentration with the buffer solution, the active bands were centrifuged at 27,000 × *g* for 30 min, and the pellets collected in this buffer as purified organelles.

Assay of activity and estimation of organelle purity. Catalase and uricase activities linked to peroxisomes have been determined according to LUCK (16) and LEONE (14), respectively. The NADH-cytochrome C reductase activities, both the mitochondrial-sensitive and the microsomal-insensitive to the antimycin A, were tested by the methods descrived by HACKETT *et al.* (10). Chlorophyll was determined by the BRUINSMA's methods (3), and proteins according to LOWRY *et al.* (15) on samples smaller than 40 µl, in

* Abbreviations used: HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; DTT, dithiothreitol.

order to avoid the interference by high sucrose concentrations (9).

The activity of isolated chloroplasts was checked by measuring the oxygen release by the Clark electrode method (Gilson KM oxygraph), in the presence of potassium ferricyanide or p-benzoquinone as electron acceptors (19). The organelles were incubated in 50 mM HEPES buffer (pH 8.3), 0.4 M sucrose, 2 mM EDTA, 10 mM KCl, 10 mM $MgCl_2$, 1 mM $MnCl_2$, 20 mM $NaHCO_3$, 1 mM DTT, 10 mM KH_2PO_4, 1.5 mM $K_3[Fe(CN)_6]$ (or 0.25 mM p-benzoquinone), and chloroplast preparation equivalent to 25 μg/ml of chlorophyll. The electron flow inhibition by cyanide was tested in presence of 60 mM KCN.

The activity of mitochondrial preparations were checked by measuring polarographycally the oxygen intake with succinate as substrate, according to CHANCE and WILLIAMS (4). The incubation mixture was as follows: 10 mM Tris-HCl buffer (pH 7.0), 0.25 M sucrose, 5 mM $MgCl_2$, 0.5 mM EDTA, 10 mM KH_2PO_4, 3.33 mM ADP, 0.75 % bovine serum albumin, 6 mM sodium succinate, and mitochondrial preparation equivalent to 1 mg protein. ADP noteworthy enhances the respiratory rate, and because of its phosphorylation to ATP, the above concentration was often restored.

Results and Discussion

Table I shows the chlorophyll content and enzymatic activities of the organelle suspensions prepared by differential centrifugation of young and mature leaf homogenates. The pellet obtained after centrifugation for 1 min fat 3,000 × *g* (suspension A) accounts for the 60-70 % of the whole chlorophyll content.

The 3,000 and 25,000 × *g* pellets show high levels of catalase and uricate oxidase activities, probably due to contamination by the peroxisomal enzymes released during the preparation of the homogenates and later centrifugations (2, 22). Suspensions B, C and D showed NADH-cytochrome C reductase activity; this is a mitochondrial and microsomal enzyme, even though it ras been also described in peroxisomal organelles from some plant tissues (7).

In accordance with these results, suspensions A and D were used for further chloroplast and microsome purification. Likewise, suspension C was employed for further purification of mitochondria and peroxisomes. Broken chloroplasts — The

Table I. *Isolation of subcellular organelles from young and mature leaves of olive tree by differential centrifugation.*
Chlorophyll content and catalase, uricase and NADH-cytochrome C reductase activities were used as organelle markers.

Pellet		Chlorophyll	Catalase		Uricase		NADH-cytochrome c	Reductase (pH 7.0)
		mg/100 of leaf	μmoles/ min^{-1}/ mg protein^{-1}	μmoles/ 100 g of leaf	nmoles/ min^{-1}/ mg protein^{-1}	nmoles/ 100 g of leaf	nmoles/ min^{-1}/ mg protein^{-1}	nmoles/ 100 g of leaf
Suspension A	a	12.2	15	3495	3.4	782	—	—
(3.000 × *g*/1 min)	b	20.4	23	7153	4.0	1244	—	—
Suspension B	a	3.5	16	2720	20.5	3485	1.0	170
(3.000 × *g*/5 min)	b	9.3	29	3248	12.0	1344	0.9	100
Suspension C	a	1.3	15	1125	26.0	1950	2.8	210
(25.000 × *g*/25 min)	b	4.9	20	2040	11.0	1122	2.3	235
Suspension D	a	0.8	11	770	1.6	112	0.7	49
(105.000 × *g*/60 min)	b	1.8	—	—	4.6	331	0.9	65

[a] Young leaf; [b] Mature leaf.

Class II chloroplasts of SPENCER and UNT (21) — were located at botton of the 0.75 M sucrose layer, whereas intact chloroplasts — Class I chloroplasts — floated just on the 1.5 M sucrose band. The latter showed their typical morphology when visualized under phase contrast microscopy, and good oxygen evolving capability, as shown in table II. When p-benzoquinone was used as electron acceptor the rate of evolved oxygen is higher than that with ferricyanide, surely due to a higher permeability of the chloroplast envelopes to the former, as it has been described in some Gramineae (19). Although ferricyanide acts at pH 7 as an electron acceptor mainly at the level of photosystem II. BEN HAYYIM *et al.* (1) have showed that it can also be reduced by the photosystem I at the higher pH 8.3 that we have used. Since we have found a 70 % inhibition when cyanide — a specific inhibitor of photosystem I — is added to the incubation mixture, it is clear that the complete electron transport system is in operation in our purified chloroplast preparations. Both young and mature leaf chloroplasts showed similar evolving activity and inhibition susceptibility by cyanide.

The chlorophyll content and enzymatic activities of the organelles purified by density gradient centrifugation are shown in table III. The low chlorophyll level of the nonchloroplastidic organelles indicates a low contamination by photosynthetic particles. Peroxisomas take a position in the 1.6 M sucrose band (1.20 density) (7), and they account for the 70-80 % of total uricase activity, but only around 30 % of the total catalase. The catalase level is indeed lower than that of peroxisomes from other sources (2, 22), which can be

Table II. *Oxygen evolution capability of chloroplasts purified by sucrose gradient centrifugation.*

The incubation mixture was: 50 mM HEPES buffer pH 8.3, 0.4 M sucrose, 2 mM EDTA, 10 mM KCl, 10 mM $MgCl_2$, 1 mM $MnCl_2$, 20 mM $NaHCO_3$, 1 mM DTT, 10 mM KH_2PO_4, 1.5 mM K_3 [Fe (CN_6] (or alternatively 0.25 mM p-benzoquinone), and chloroplast preparation equivalent to 75 μg of chorophyll. The cyanide electron flow inhibition was tested in the presence of 60 mMKCN. Figures in parenthesis show the percentage inhibition by cyanide.

	nmoles of evolved O_2/h/mg of chlorophyll With K_3 [Fe(CN)$_6$]	With p-benzoquinone
Young leaf	5.6 (70)	14.7
Mature leaf	8.9 (73)	18.6

Table III. *Chlorophyll content and catalase, uricase and NADH-cytochrome C reductase activities of organelles from young and mature leaves of olive tree, purified by sucrose gradient centrifugation.*

Figures in parenthesis show the percentage inhibition by antimycin A.

Purified Organelles		Chlorophyll	Catalase		Uricase		NADH-cytochrome c	Reductase (pH 7.0)
		mg/100 g of leaf	μmoles/ min^{-1}/ mg protein^{-1}	μmoles/ 100 g of leaf	nmoles/ min^{-1}/ mg protein^{-1}	nmoles/ 100 g of leaf	nmoles/ min^{-1}/ mg protein^{-1}	nmoles/ 100 g of leaf
Chloroplasts	a	2.0	23	418	4.0	73	3.2	58.2
	b	2.4	33	663	6.8	137	1.5	30.1
Peroxisomes	a	0.4	73	233	103.0	330	5.4	17.4 (15)
	b	0.5	88	387	98.0	431	8.8	38.7 (15)
Mitochondria	a	0.1	16	53	2.3	8	22.0	72.6 (70)
	b	0.2	33	158	3.2	15	27.0	129.6 (70)
Microsomes	a	trace	6	10	1.6	3	6.0	9.6 (5)
	b	trace	8	18	4.6	10	7.5	16.5 (5)

[a] Young leaf; [b] Mature leaf.

explained, as stressed above, because of peroxisomal disintegration during purification.

Mitochondria were located in the 1.4 M sucrose layer (1.18 density), with a NADH-cytochrome C reductase activity similar to that found by DONALDSON *et al.* (7) in the organelles from spinach leaves. The 70 % inactivation by antimycin A shows the mitochondrial origin of this enzymatic activity. Table IV shows that the specific respiratory rate of mitochondrial increases 2-3 times after purification by sucrose gradient centrifugation. The «respiratory control ratio» of both young and mature leaf mitochondria — defined according to CHANCE and WILLIAMS (4) as the quotient between the respiratory rate in the presence of added ADP and that obtained when ADP is exhausted — is in the range 2.0-2.4, similar values to those found with mitochondria from other plants (13). According with the results found by DONALSON in spinach leaves, microsomes settle on the 1.2 M sucrose layer (1.15 density), as shown by the low inhibition of the NADH-cytochrome C reductase by antimycin A.

Table IV. *Respiratory activity of mitochondria isolated by differential centrifugation (25.000 g pellet), and after further purification by gradient centrifugation.*
The incubation mixture was: 10 mM Tris-HCl buffer pH 7.0, 0.25 M sucrose, 5 mM $MgCl_2$, 0.5 mM EDTA, 10 mM KH_2PO_4, 3.33 mM ADP, 0.075 % bovine serum albumin, 6 mM sodium succinate, and mitochondrial preparation equivalent to 1 mg of protein. The ADP concentration was repeatedly restored.

	nmoles of O_2 intake/h/mg of protein	Respiratory control ratio
Young leaf		
25.000 × *g* pellet	90	2.0
purified mitochondria	167	2.3
Mature leaf		
25.000 × *g* pellet	51	2.2
purified mitochondria	154	2.4

In conclussion there are no remarkable differences in the behaviour of the subcellular organelles from young and mature leaves of the olive tree. Accordingly, young leaves are a suitable material to study the lipidic biosynthetic mechanism in this plant.

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Resumen

Se han aislado gránulos subcelulares de hojas jóvenes y maduras de plántula de olivo, por centrifugación diferencial de homogenados de hoja y posterior purificación por centrifugación en gradiente de sacarosa. El contenido en clorofila se utilizó para determinar la conducta de los cloroplastos en la centrifugación diferencial y en gradiente de densidad; las actividades uricasa y catalasa para identificar los peroxisomas, y la NADH-citocromo C-reductasa, sensible e insensible a la antimicina A, como enzimas marcadores de mitocondrias y microsomas, respectivamente. La operatividad de cloroplastos y mitocondrias se determinó polarográficamente, según el oxígeno liberado o consumido, respectivamente, en la cadena de transporte electrónico no cíclica de la fotosíntesis, utilizando un aceptor de electrones y en la cadena respiratoria partiendo de succinato como substrato.

No se han encontrado diferencias importantes en la funcionalidad de los orgánulos celulares aislados de ambos tipos de hojas, sugiriendo que la hoja joven puede ser utilizada como material básico para un estudio del metabolismo lipídico en esta planta.

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