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Natural Senescence of Pea Leaves[1]

An Activated Oxygen-Mediated Function for Peroxisomes

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We studied the activated oxygen metabolism of peroxisomes in naturally and dark-induced senescent leaves of pea (*Pisum sativum* L.). Peroxisomes were purified from three different types of senescent leaves and the activities of different peroxisomal and glyoxysomal enzymes were measured. The activities of the $O_2^{\cdot -}$- and H_2O_2-producing enzymes were enhanced by natural senescence. Senescence also produced an increase in the generation of active oxygen species ($O_2^{\cdot -}$ and H_2O_2) in leaf peroxisomes and in the activities of two glyoxylate-cycle marker enzymes. A new fraction of peroxisomes was detected at an advanced stage of dark-induced senescence. Electron microscopy revealed that this new peroxisomal fraction varied in size and electron density. During senescence, the constitutive Mn-superoxide dismutase (SOD) activity of peroxisomes increased and two new CuZn-SODs were induced, one of which cross-reacted with an antibody against glyoxysomal CuZn-SOD. This fact and the presence of glyoxylate-cycle enzymes support the idea that foliar senescence is associated with the transition of peroxisomes into glyoxysomes. Our results indicate that natural senescence causes the same changes in peroxisome-activated oxygen metabolism as dark-induced senescence, and reinforce the hypothesis of an effective role of peroxisomes and their activated oxygen metabolism in this stage of the life cycle.

Senescence is an oxidative process that involves a general deterioration of cellular metabolism. In plants, chlorophyll and protein loss and increases in lipid peroxidation and membrane permeability, along with other changes, are common symptoms of this irreversible process in which the enhanced metabolism of activated oxygen produces severe cellular damage (Kar and Feierabend, 1984; Trippi and De Luca d'Oro, 1985; Thompson et al., 1987; Halliwell and Gutteridge, 1989).

In a previous study, we suggested an activated oxygen-mediated role for peroxisomes in the mechanism of dark-induced senescence of pea (*Pisum sativum* L.) leaves (Pastori and del Río, 1994a). Increased activities of the $O_2^{\cdot -}$-producing XOD and the H_2O_2-generating Mn-SOD and UO were determined in peroxisomes, but CAT, which is the characteristic H_2O_2-scavenging enzyme of peroxisomes, was strongly depressed by senescence (Pastori and del Río, 1994a). An overproduction of $O_2^{\cdot -}$ radicals and H_2O_2 took place in leaf peroxisomes during senescence, as did the induction of two new SOD isozymes identified as CuZn-SODs (Pastori and del Río, 1994a, 1994b). Ultrastructural studies of intact pea leaves showed that the cellular populations of peroxisomes and mitochondria increased with senescence.

The question of whether natural and dark-induced senescence are the same or different phenomena is still under discussion. At the physiological level, natural and dark-induced senescence have many things in common, since both imply Suc starvation and damage to chlorophylls, proteins, lipids, and nucleic acids, as well as other changes. However, considering senescence as a programmed process, the mechanisms of action of both types of senescence could be different at the molecular level. Becker and Apel (1993) found differences in gene expression between natural and dark-induced senescence, concluding that in the latter type these changes could be attributed to a stress situation rather than to a senescence process. In contrast, King et al. (1995) reported the accumulation of transcripts coding for enzymes involved in specific carbon and nitrogen remobilization in both natural and dark-induced senescence, suggesting that the underlying regulatory mechanisms might be similar in both conditions.

The induction of senescence in detached leaves by incubation in complete darkness is a good system for the rapid development of characteristic symptoms of senescence. However, the artificially induced senescence of excised leaves has the disadvantage of producing simultaneously other types of leaf stress such as wound damage and nutrient, hydric, and dark stress. These added stresses overlap with senescence symptoms, making it difficult to know precisely the physiological process ultimately responsible for the metabolic changes observed in the plant tissue.

In this study we analyzed the metabolism of activated oxygen during natural senescence using peroxisomes pu-

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Abbreviations: CAT, catalase; CuZn-SOD, copper,zinc-containing superoxide dismutase; ICL, isocitrate lyase; MDA, malondialdehyde; Mn-SOD, manganese-containing superoxide dismutase; MS, malate synthase; SOD, superoxide dismutase; UO, urate oxidase; XOD, xanthine oxidase.

rified from naturally senescent pea leaves. A comparative study with peroxisomes from dark-induced senescent pea leaves was also carried out. We also studied the transition of leaf peroxisomes into glyoxysomes from the activated oxygen viewpoint and the role of SOD in this process.

MATERIALS AND METHODS

Pea (*Pisum sativum* L. cv Lincoln) plants were grown in vermiculite in a growth chamber under optimal conditions for 15 and 50 d, as described by del Río et al. (1985).

Induction of Senescence

Leaves of 50-d-old plants were used for natural senescence. Dark-induced senescence was carried out by placing excised leaves (about 50 g fresh weight) from 15-d-old pea plants (control) in trays floating in air-saturated, distilled water and incubating them in permanent darkness at 26°C for up to 11 d (Pastori and del Río, 1994a).

Purification of Peroxisomes

Peroxisomes were isolated from naturally and dark-induced senescent leaves by differential centrifugation, and the washed, 12,000*g* particulate pellet, enriched in peroxisomes and mitochondria, was centrifuged in density gradients of Percoll (15–53%, v/v), as previously described (Sandalio et al., 1987). All operations were performed at 0 to 4°C. After centrifugation, the gradients were fractionated by upward displacement with 45% (w/w) Suc using a fractionator (model 185, Isco, Lincoln, NE) equipped with an optical unit and an absorbance detector. The purified peroxisomes had intactness percentages between 70 and 90% and were free of chloroplasts and mitochondria, as verified by marker enzyme evaluation (Sandalio et al., 1987). For the preparation of membranes, peroxisomes were broken by hypotonic shock in 50 mM potassium-phosphate buffer, pH 7.8, containing 0.02 mM FAD and 0.1 mM diethylenetriaminepentacetic acid, and were centrifuged at 237,000*g*, as described by del Río et al. (1989). Peroxisomal membranes were then washed with 0.1 M sodium carbonate, as described by López-Huertas et al. (1995).

Enzyme Assays

CAT (EC 1.11.1.6) was assayed according to the method of Aebi (1984). Hydroxypyruvate reductase (EC 1.1.1.29) was assayed according to the method of Schwitz-guébel and Siegenthaler (1984). MS (EC 4.1.3.2) and ICL (EC 4.1.3.1) were assayed according to the methods of Dixon and Kornberg (1962) and Kornberg and Collins (1958), respectively. Fumarase was assayed by the method of Walk and Hock (1977).

For the separation of SOD (EC 1.15.1.1) isozymes, nondenaturing PAGE was performed on 10% acrylamide disc gels. SOD isozymes were detected in gels by the photochemical nitroblue tetrazolium staining method (Beauchamp and Fridovich, 1971). The different types of SOD were differentiated by performing the activity stain in gels previously incubated for 20 min at 25°C in 50 mM potassium-phosphate buffer, pH 7.8, containing either 2 mM KCN or 5 mM H_2O_2. CuZn-SODs are inhibited by KCN and H_2O_2, Fe-SODs are resistant to CN^- but inactivated by H_2O_2, and Mn-SODs are resistant to both inhibitors (Halliwell and Gutteridge, 1989). The isozyme activity in the gels and the cross-reactivity bands in the nitrocellulose sheets were recorded by measuring at 560 nm the relative transmission and absorbance of samples, respectively, in a densitometer (model CS9000, Shimadzu, Columbia, MD).

Western Blotting

Peroxisomes from control, naturally induced, and dark-induced senescent leaves were subjected to a nondenaturing PAGE using a slab cell (Mini-Protean II, Bio-Rad). Protein transferring onto nitrocellulose sheets and cross-reactivity assays with an antibody against glyoxysomal CuZn-SOD were carried out according to the method of Bueno et al. (1995).

Electron Microscopy and CAT Cytochemistry

Fractions corresponding to mitochondria and peroxisomes were taken from the Percoll density gradients of control and dark-induced senescent leaves. After removing Percoll by centrifugation, samples were included in 2% agar and fixed according to the method of Sautter et al. (1981). Peroxisomes were identified by CAT staining using an electron-cytochemical method involving the peroxidatic action of CAT on 3,3′-diaminobenzidine-HCl (Müller and Beckman, 1978). Samples of mitochondria and peroxisomes were incubated with 3,3′-diaminobenzidine-HCl, processed as described by Palma et al. (1991), and examined with an electron microscope (EM 10C, Zeiss) at 60 kV.

Other Analytical Methods

H_2O_2 concentration was determined in peroxisomes purified from naturally senescent leaves by a peroxidase-coupled assay using 4-amino antipyrine and phenol as donor substrates (Frew et al., 1983). Intact peroxisomes (50–200 μL) were added to a reaction mixture containing 25 mM phenol, 5 mM 4-aminoantipyrine, 0.1 M potassium-phosphate buffer (pH 6.9), 0.02 μM peroxidase, and 2.5 μM H_2O_2. Quinone-imine formation was measured at 505 nm.

The NADH-dependent production of superoxide radicals by peroxisomal membranes was determined by the method of the SOD-inhibitable reduction of ferricytochrome *c* (Fridovich, 1985). The assay was carried out at 25°C in a spectrophotometer (DU-7, Beckman) under conditions previously described (del Río et al., 1989). Proteins were assayed by the method of Bradford (1976) using crystalline BSA to standardize the assay procedure.

RESULTS

The specific activities of enzymes involved in the metabolism of activated oxygen were analyzed in peroxisomes isolated from naturally senescent pea leaves, and results obtained were consistent with those previously found in

Table I. *Specific activities of activated oxygen-related enzymes in peroxisomes of naturally senescent pea leaves*

Peroxisomes were purified from leaves of 15- and 50-d-old plants. Each value is the mean of six different experiments ± SE.

Age	XOD	XDH	UO	SOD	CAT
d	*nmol uric acid min^{-1} mg^{-1} protein*			*units mg^{-1} protein*	*μmol H_2O_2 min^{-1} mg^{-1} protein*
15	3.4 ± 0.9	1.2 ± 0.5	2.8 ± 1.0	7.8 ± 1.3	870 ± 90
50	27.4 ± 6.7	8.7 ± 2.9	6.7 ± 1.9	28.1 ± 3.1	170 ± 20

dark-induced senescent pea leaves (Pastori and del Río, 1994a). The $O_2^{\cdot -}$-producing XOD activity increased significantly with senescence, as did xanthine dehydrogenase and UO activity (Table I). Natural senescence produced an increase in SOD activity, which was accompanied by a strong decrease in CAT activity (Table I). The glyoxylate-cycle enzymes MS and ICL, which are characteristic of glyoxysomes and are absent in peroxisomes of control leaves, were clearly detected in peroxisomes of naturally senescent leaves (Table II).

The NADH-dependent production of $O_2^{\cdot -}$ radicals by peroxisomal membranes and the H_2O_2 concentration in peroxisomal matrices were increased as a consequence of natural senescence (Table III), a situation similar to that found in dark-induced senescent pea leaves (Pastori and del Río, 1994a, 1994b). The rate of lipid peroxidation, an indicator of oxidative damage, was also increased significantly in peroxisomes of naturally senescent leaves (Table III).

A comparison of Percoll density gradients of control, dark-induced, and naturally senescent leaves was conducted using marker enzymes of peroxisomes, glyoxysomes, and mitochondria. In the Percoll density gradients of control leaves (Fig. 1), the characteristic peak of peroxisomes was found in fractions 17 to 19 and had an equilibrium density of 1.091 g cm^{-3}, a value similar to that previously determined for pea leaf peroxisomes (Sandalio et al., 1987). The activity of fumarase, a mitochondrial marker enzyme, was undetectable in the peroxisomal peak, indicating the absence of contamination by mitochondria. MS and ICL, two marker enzymes of glyoxysomes, were not detected in the peroxisomal fractions.

In dark-induced senescent leaves, the pattern of the Percoll density gradients showed important changes (Fig. 2). When leaves from 30-d-old plants were incubated in the dark, two peroxisomal peaks were found in fractions 17 and 19 to 22, with equilibrium densities of 1.089 and 1.098 g cm^{-3}, respectively. The equilibrium density of the first peroxisomal fraction was similar to that of peroxisomes from control leaves, whereas the second peroxisomal peak (fractions 19–22) showed an equilibrium density higher than that of control leaves. CAT and hydroxypyruvate reductase activities of both peroxisomal peaks were smaller than those of peroxisomes from control leaves. The glyoxysomal enzymes MS and ICL were detected in both peroxisomal fractions, whereas fumarase was practically absent in those fractions.

In naturally senescent leaves, analysis of Percoll density gradients only showed the presence of a broad peak of peroxisomes in fractions 18 to 22, with an equilibrium density of 1.093 g cm^{-3} (Fig. 3). In naturally senescent pea leaves, the presence of the glyoxylate cycle enzymes MS and ICL was also clearly detected (Fig. 3).

To analyze the purity and morphology of the peroxisomal and mitochondrial fractions obtained from control and dark-induced senescent leaves, an electron microscopy study was carried out (Fig. 4). Senescence brought about a dramatic deterioration of mitochondria, affecting both their membranes and matrices (Fig. 4B). In peroxisomes, clear morphological differences between the two peroxisomal fractions of senescent leaves were observed. Peroxisomes from fraction 17 of the first peak (Fig. 2) showed a typical size but a lower matrix electron density (Fig. 4D) than peroxisomes from control leaves (Fig. 4C). In contrast, peroxisomes from fractions of the second peak (Fig. 2) had a smaller size but a higher matrix electron density (Fig. 4E).

The SOD activity of peroxisomes was analyzed by native PAGE and showed significant changes with natural leaf senescence very similar to those observed in dark-induced senescent leaves (Pastori and del Río, 1994b). The constitutive Mn-SOD activity of leaf peroxisomes increased significantly in naturally senescent leaves, and two new SOD isozymes were detected (Fig. 5). These SODs were identified as CuZn-SODs on the basis of their sensitivity to KCN and H_2O_2. The SOD activity of the two peroxisomal peaks from dark-induced senescent leaves (Fig. 2) was analyzed. Constitutive Mn-SOD was mainly present in the lower-density peroxisomal peak (Fig. 2, fraction 17), whereas the new CuZn-SODs occurred predominantly in the higher-density peroxisomal peak (Fig. 2, fractions 19–22).

Table II. *Specific activities of glyoxylate-cycle enzymes in peroxisomes of naturally senescent pea leaves*

Peroxisomes were purified from leaves of 15- and 50-d-old plants. Each value is the mean of six different experiments ± SE.

Age	MS	ICL
d	*nmol malate min^{-1} mg^{-1} protein*	*nmol glyoxylate min^{-1} mg^{-1} protein*
15	–[a]	–
50	33.5 ± 2.3	68.3 ± 6.1

[a] –, No activity was detected.

Table III. *Lipid peroxidation, superoxide production, and H_2O_2 concentration in peroxisomes of naturally senescent pea leaves*

MDA and H_2O_2 concentrations were determined in intact leaf peroxisomes purified from 15- and 50-d-old plants. The NADH-dependent generation of $O_2^{\cdot-}$ radicals was estimated in peroxisomal membranes. Each value is the mean of six (MDA and H_2O_2) and three ($O_2^{\cdot-}$) different experiments ± SE.

Age	Lipid Peroxidation	Superoxide Production	H_2O_2 Content
d	*nmol MDA mg^{-1} protein*	*nmol $O_2^{\cdot-}$ min^{-1} mg^{-1} protein*	*nmol H_2O_2 mg^{-1} protein*
15	0.78 ± 0.1	4.6 ± 1.1	1.9 ± 0.2
50	2.53 ± 0.3	9.8 ± 3.5	3.2 ± 0.4

Peroxisomes from naturally induced and dark-induced senescent leaves were examined by western blotting for reactivity with an antibody against glyoxysomal CuZn-SOD from watermelon cotyledons. One of the two CuZn-SODs induced by senescence cross-reacted with the antibody; the reaction was stronger in samples of the higher-density peroxisomal fraction from dark-induced senescent leaves and almost undetectable in peroxisomes from naturally senescent leaves (Fig. 5, F and G).

DISCUSSION

In a previous study of dark-induced senescence of pea leaves, it was proposed that peroxisomes could play an activated oxygen-mediated role in the oxidative mechanism of this type of senescence (Pastori and del Río, 1994a).

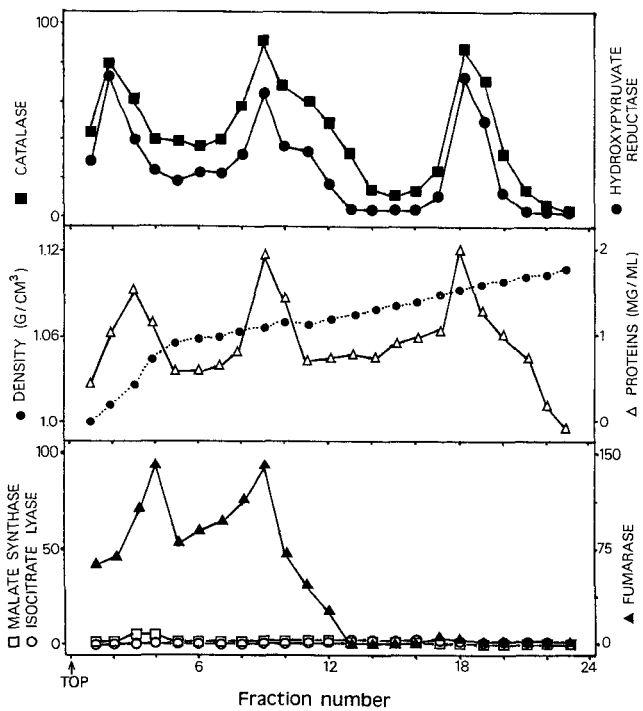

Figure 1. Purification of peroxisomes and mitochondria from control leaves. Cell organelles were purified from 15-d-old pea leaves by Percoll density gradient centrifugation. Fractions of 1.5 mL were eluted with a gradient fractionator and the activity of different marker enzymes was assayed. Enzyme activities are expressed in nmol min^{-1} mg^{-1} protein for MS and ICL, and in μmol min^{-1} mg^{-1} protein for hydroxypyruvate reductase, CAT, and fumarase.

Ultrastructural studies of dark-induced senescent pea leaves showed that although other cellular compartments were gradually altered and degraded, peroxisomes conserved their structure and their population in leaf cells was increased about four times compared with control leaves.

Since there is still controversy concerning possible differences between dark-induced senescence and natural senescence, we decided to use naturally senescent leaves to study the activated oxygen-related function of peroxisomes in leaf senescence. The dark treatment previously used (Pastori and del Río, 1994a) could have been too severe and the plant material might have been subjected to different types of stresses unrelated to senescence.

Results obtained in this study show that during natural senescence of pea leaves, very important changes in the metabolism of peroxisomes take place. These changes are similar to those previously observed in peroxisomes of dark-induced senescent leaves (Pastori and del Río, 1994a), and therefore are characteristic of the physiological process of senescence. During natural senescence, there is an enhancement in the activity of activated oxygen-producing enzymes in leaf peroxisomes. XOD, UO, and SOD activities increased significantly, whereas CAT activity was strongly decreased, implying that an accumulation of toxic oxygen species could take place in peroxisomes. Certainly, enhancement of $O_2^{\cdot-}$ production by peroxisomal membranes and of the H_2O_2 concentration in peroxisomes was detected during natural senescence. The increased activity of Mn-SOD and the appearance of two new CuZn-SODs in peroxisomes strongly suggest that SOD has a relevant role in the natural senescence process of pea leaves. Peroxisomal SOD could be one of the last protective actions against the increased production of $O_2^{\cdot-}$ radicals that takes place during senescence. These peroxisomal SODs could also have a role in the generation of H_2O_2 for the senescence process, which requires strong oxidizing agents (del Río et al., 1992; Pastori and del Río, 1994a). Peroxisomal SODs could be induced as a result of the increased $O_2^{\cdot-}$-derived H_2O_2 concentration in the cytosol during senescence, or the observed decrease in CAT activity could be due to proteolysis by peroxisomal endopeptidases, which are significantly enhanced by leaf senescence (Distefano et al., 1996).

Regarding the cell signals that trigger the metabolic changes found in leaf peroxisomes during senescence, Suc starvation is known to be an important factor in the senescence process (Graham et al., 1992), but activated oxygen species could also be involved. Superoxide radical and H_2O_2 may act as specific chemical messengers in cellular

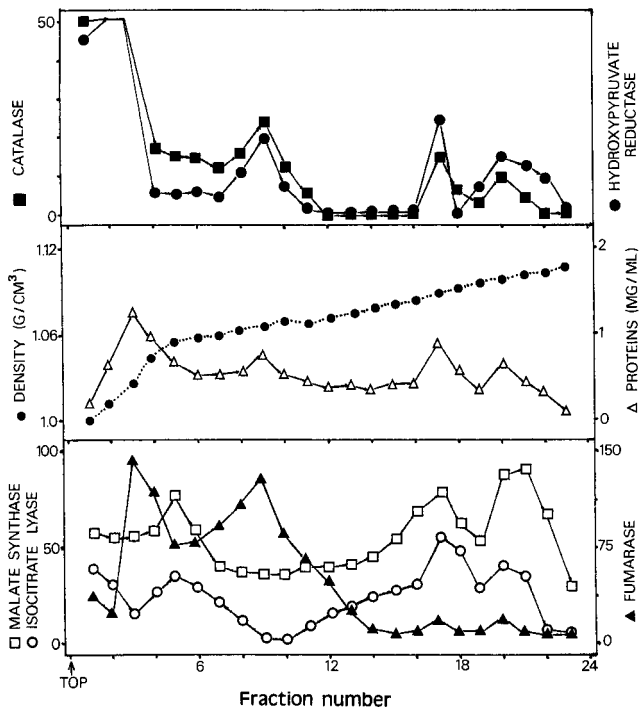

Figure 2. Purification of peroxisomes and mitochondria from 30-d-old leaves induced to senesce by dark incubation. Cell organelles were purified by Percoll density gradient centrifugation. Fractions of 1.5 mL were eluted with a gradient fractionator and the activity of different marker enzymes was assayed. Enzyme activities are expressed in nmol min^{-1} mg^{-1} protein for MS and ICL, and in μmol min^{-1} mg^{-1} protein for hydroxypyruvate reductase, CAT, and fumarase.

signal transduction pathways, and different functions have been reported for H_2O_2 as a diffusible signaling molecule (Saran and Bors, 1989; Schreck et al., 1991; Levine et al., 1994; Prasad et al., 1994; del Río et al., 1996). In the light of the results reported in the current study of leaf peroxisomes, and considering senescence as an oxidative process, $O_2^{\cdot -}$ radicals and H_2O_2 generated in peroxisomes during leaf senescence could also have a role in those signal transduction processes that lead to specific gene expression. In peroxisomes the NADH-dependent generation of $O_2^{\cdot -}$ radicals seems to take place at the cytosolic side of the peroxisomal membrane (del Río et al., 1992, 1996; López-Huertas et al., 1996). Senescence could stimulate the extrusion of membrane-generated $O_2^{\cdot -}$ radicals into the cytosol, which could then join the overproduced H_2O_2 that can easily leak out of peroxisomes (Boveris et al., 1972).

Analysis of the peroxisomal fractions from naturally senescent leaves showed that peroxisomes had equilibrium densities that were very similar to the organelles from young control leaves; the same applied to the equilibrium density of mitochondria during senescence.

In the dark-induced senescent leaves, different results were obtained depending on the age of the leaves subjected to the dark treatment. When leaves from 15-d-old plants were used, the gradient profiles were similar to those of naturally senescent leaves, with a unique and broad area of peroxisomes having an equilibrium density very similar to that of control leaves (data not shown). However, when leaves from 30-d-old plants were used for dark incubation, two well-defined areas of peroxisomes were observed. One of them had the same equilibrium density of control leaf peroxisomes, and the other was the most abundant and had a higher equilibrium density. Electron microscopy revealed that neither population of peroxisomes was contaminated with other organelles, and that they were clearly different in size and electron density.

The appearance of a new peroxisomal fraction at advanced stages of senescence suggests that a new population of peroxisomes could originate as a result of generalized degradative processes occurring at the final stages of leaf senescence. The severe senescence conditions prevailing might induce an increase in the density of leaf peroxisomes, probably as a result of the lipid peroxidation of peroxisomal membranes and other drastic changes produced in the metabolism of these oxidative organelles. In plants the proliferation of peroxisomes has been reported in tissues treated with an herbicide (de Felipe et al., 1985), ozone (Morré et al., 1990), and the hypolipidemic drug clofibrate (Palma et al., 1991), and in senescent petals (Droillard and Paulin, 1990) and leaves (Pastori and del Río, 1994a), although there are no reports concerning the appearance of qualitatively different peroxisomal populations.

In animals peroxisomal heterogeneity has been reported as a result of treatment with clofibrate (Flatmark et al.,

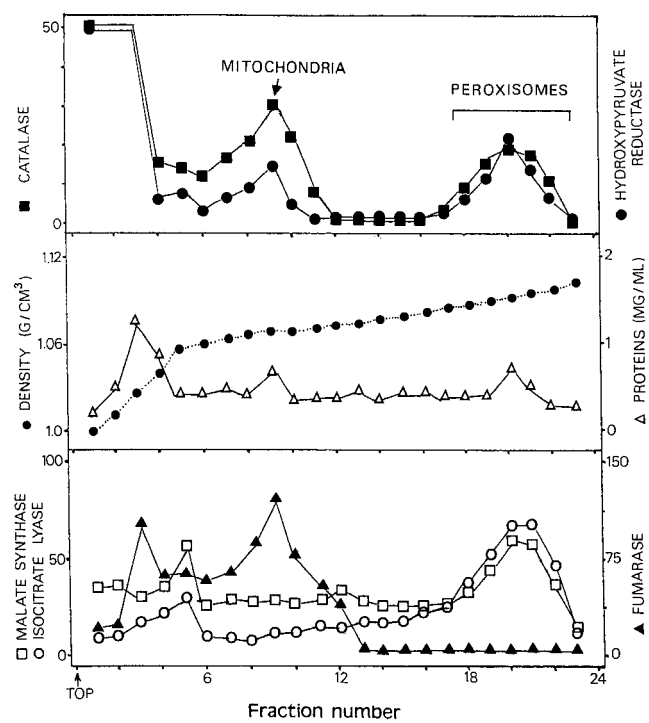

Figure 3. Purification of peroxisomes and mitochondria from naturally senescent leaves. Cell organelles were purified from 50-d-old pea leaves by Percoll density gradient centrifugation. Fractions of 1.5 mL were eluted with a gradient fractionator and the activity of different marker enzymes was assayed. Enzyme activities are expressed in nmol min^{-1} mg^{-1} protein for MS and ICL, and in μmol min^{-1} mg^{-1} protein for hydroxypyruvate reductase, CAT, and fumarase.

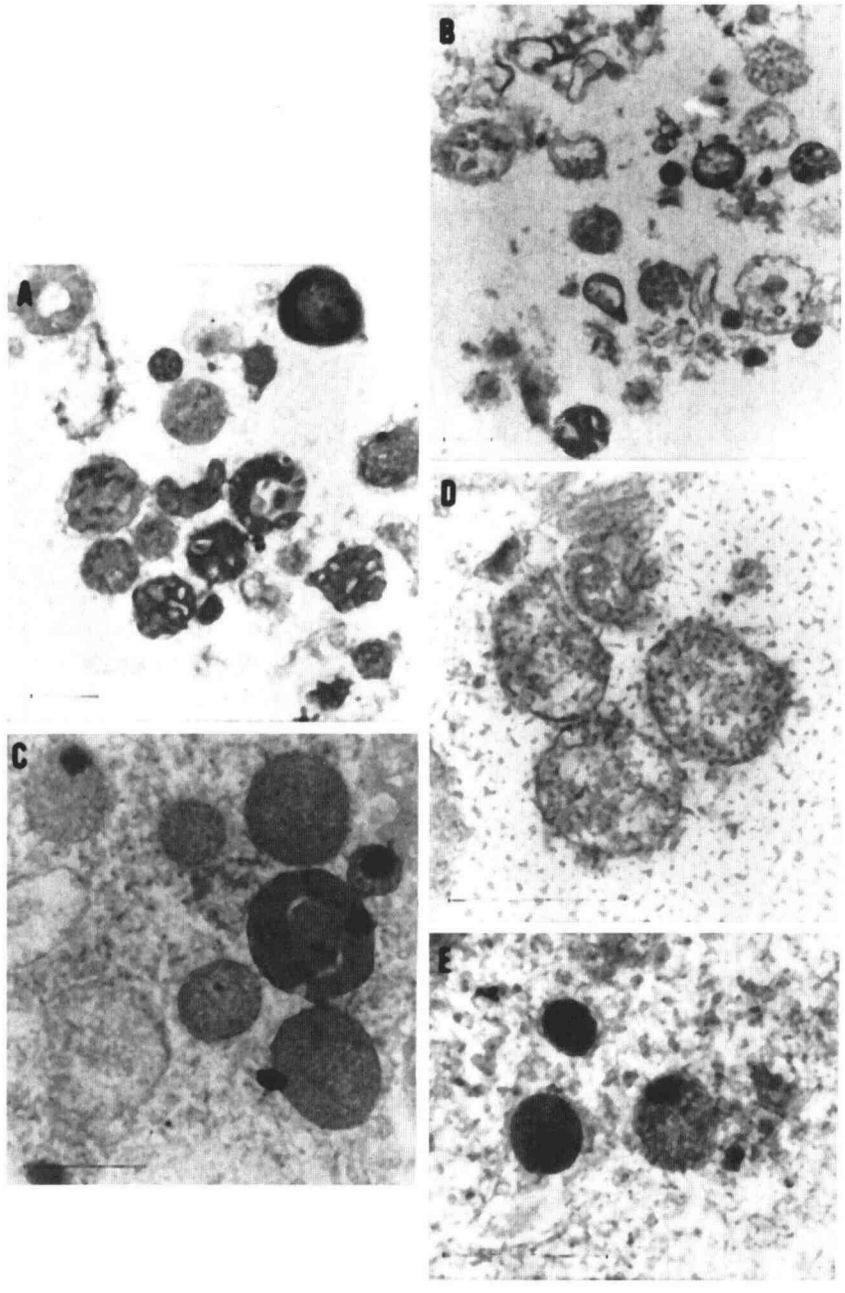

Figure 4. Electron micrographs of purified mitochondria and peroxisomes from 30-d-old leaves induced to senesce by dark incubation. A, Mitochondria from control leaves (×25,000); B, mitochondria from dark-induced senescent leaves (×20,000); C, peroxisomes from control leaves (×47,500); D, peroxisomes from dark-induced senescent leaves (fraction 17 in Fig. 2) (×50,000); and E, peroxisomes from dark-induced senescent leaves (fractions 19–22 in Fig. 2) (×50,000). Bars = 1 μm.

1981) and thyroxine (Just et al., 1982), as well as in ischemia-reperfusion injury (Gulati et al., 1992) and cold exposure (Goglia et al., 1989). Wilcke et al. (1995) found novel peroxisomal populations in the livers of rats treated with di(2-ethylhexyl)phthalate, with a very important heterogeneity in protein content and size, varying from normal peroxisomal size to very small vesicles. It was suggested that these novel peroxisomal fractions could be subcompartments of a larger peroxisomal structure involved in protein import and biogenesis (Wilcke et al., 1995).

A transition of leaf peroxisomes into glyoxysomes during leaf senescence has been proposed. The presence of the glyoxysomal enzymes MS and ICL in peroxisomes from senescent leaves has been described in different plant species (De Bellis et al., 1990, 1991; Landolt and Matile, 1990; De Bellis and Nishimura, 1991). Results reported in the present study on the presence of MS and ICL in peroxisomes from naturally senescent pea leaves agree with results previously obtained in dark-induced senescent pea leaves (Pastori and del Río, 1994a) and in naturally senescent rice and wheat leaves (Pistelli et al., 1991) and pumpkin cotyledons (Nishimura et al., 1993).

In the present study, we showed that one of the two CuZn-SODs induced in leaf peroxisomes by senescence cross-reacted with an antibody against glyoxysomal CuZn-SOD. This reaction was clearly observed in peroxisomes from dark-induced senescent leaves, whereas in those from

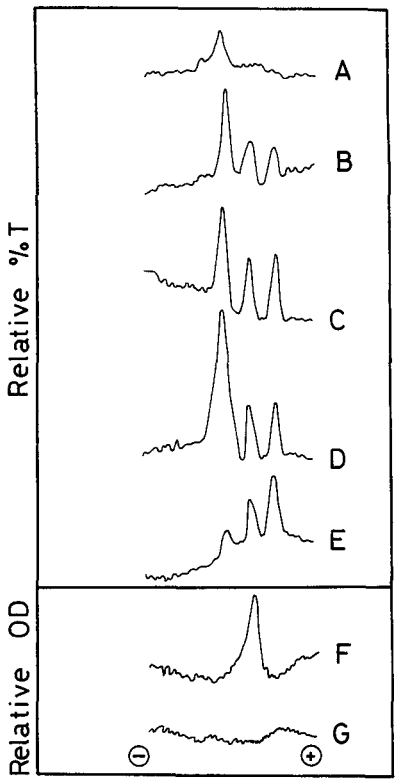

Figure 5. Isozyme pattern of SOD and western blot analysis of peroxisomes from senescent pea leaves. Gels A to E were stained for SOD activity by the photochemical nitroblue tetrazolium method and then scanned with a densitometer. A, Peroxisomes of control leaves (15-d-old plants; 20 μg of protein); B, leaf peroxisomes of naturally senescent leaves (7 μg of protein); C, peroxisomes of dark-induced senescent leaves (15-d-old leaves induced to senesce by dark incubation; 7 μg of protein); D, peroxisomes of dark-induced senescent leaves (30-d-old leaves; fraction 17 of Fig. 2; 7 μg of protein); and E, peroxisomes of dark-induced senescent leaves (30-d-old leaves; fractions 19–22 of Fig. 2; 7 μg of protein). For the western blot assays, peroxisomes of senescent leaves were subjected to nondenaturing-PAGE on 10% gels and then transferred to nitrocellulose sheets incubated with a polyclonal antibody against glyoxysomal CuZn-SOD. The relative absorbance of nitrocellulose membranes was recorded by densitometry. F, Peroxisomes of dark-induced senescent leaves (fractions 19–22 of Fig. 2; 15 μg of protein); G, peroxisomes of naturally senescent leaves (15 μg of protein).

naturally senescent leaves it was extremely weak, probably due to a very low concentration of antigen. These results support the hypothesis of a senescence-driven transition of leaf peroxisomes to glyoxysomes, since in addition to the presence of the glyoxylate-cycle enzymes MS and ICL in senescent peroxisomes, a new CuZn-SOD is induced and is recognized by an antibody against glyoxysomal CuZn-SOD. The absence of the constitutive Mn-SOD in the second population of peroxisomes in dark-induced senescent leaves suggests that this new peroxisomal fraction could correspond to the final form of "leaf glyoxysomes" produced by the senescence-induced transformation of leaf peroxisomes.

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