Evidence for the Accumulation of Peroxidized Lipids in Membranes of Senescing Cotyledons

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ABSTRACT

Fluorescent products of lipid peroxidation accumulate with age in microsomal membranes from senescing cotyledons of Phaseolus vulgaris. The temporal pattern of accumulation is closely correlated with a rise in the lipid phase transition temperature reflecting the formation of gel phase lipid. Increased levels of fluorescent peroxidation products are also detectable in total lipid extracts of senescent cotyledons. Lipoxigenase activity increases with advancing age by about 3-fold on a fresh weight basis and 4-fold on a dry weight basis indicating that the tissue acquires elevated levels of lipid hydroperoxides. As well, levels of glutathione and superoxide dismutase activity decline on a dry weight basis as the cotyledons age, rendering the tissue more susceptible to oxidative damage. Catalase activity rises initially and then declines during senescence, but peroxidase activity rises steeply. Thus, apart from this increase in peroxidase, which would scavenge H₂O₂ only if appropriate cosubstrates were available, the defense mechanisms for coping with activated oxygen species (O₂·⁻, H₂O₂, OH⁻) are less effective in the older tissue. The observations support the contention that formation of gel phase lipid in senescing membranes is attributable to lipid peroxidation and suggest that the reactions of lipid peroxidation are utilized by the cotyledons to mediate deteriorative changes accompanying the mobilization and transport of metabolites from the storage tissue to the developing embryo.

Disruption of cell membrane integrity is an inherent feature of senescence in plants. This is evident from ultrastructural studies showing progressive deteriorative changes in the organelles and membranes of plant tissues and also from permeability studies indicating increased leakage of solutes (25). Large changes in the physical properties of membrane lipids accompanying senescence appear to contribute to this loss of selective permeability. In particular, increasing proportions of the membrane lipid assume the gel phase, resulting in a mixture of discrete liquid-crystalline gel phase domains in the membrane matrix (18–20). For microsomal membranes from senescing bean cotyledons, the lipid phase transition temperature increases by more than 50°C between days 2 and 9 after germination under etiolating conditions (18). By day 4 after germination, these membranes contain a mixture of gel and liquid crystalline lipid phases at the growth temperature; and this coexistence of lipid phases gives rise to discontinuities in the bilayer that result in greatly increased permeability (1).

Recent in vitro studies indicate that gel phase lipid forms when lipid peroxidation is induced in isolated membranes (22, 24). In fact, several lines of evidence indicate that free radical-induced lipid peroxidation plays a role in bringing about many of the deteriorative changes associated with plant senescence. For example, increased levels of H₂O₂ and lipid peroxides have been measured in ripening fruits, and inhibition of H₂O₂ formation has been shown to delay the onset of ripening (8). The axes of aged soybean seeds have been found to contain high levels of malondialdehyde (27), and increased levels of oxygenated fatty acids have been observed in aged seeds of Cichorium and Crepis (26). Fluorescent products of lipid peroxidation have been reported in pear and banana pulp as well as in a mitochondrial fraction isolated from banana, and the levels of these pigments appear to increase with natural or ethylene-induced ripening (17). Senescing leaves also accumulate fluorescent pigments (29), and Dhindsa et al. (5) have reported a correlation between lipid peroxidation and increased membrane permeability in senescing leaf tissue.

In the present study, we have examined the extent to which lipid peroxidation reactions can be correlated with membrane deterioration in senescing cotyledons of Phaseolus vulgaris. In particular, the time course of fluorescent pigment accumulation in lipid extracts of microsomal membranes has been compared with the temporal pattern of gel phase lipid formation in the membranes. The fluorescence intensity of lipid extracts can be used to quantitatively assess accumulated peroxidative damage (28). The effects of senescence on the activities of enzymes that regulate levels of reactive oxygen species able to initiate lipid peroxidation and on the levels of reduced and oxidized glutathione, a cysteine-containing tripeptide thought to afford protection against oxidative stress (7), have also been examined.

MATERIALS AND METHODS

Growth Conditions and Membrane Isolation. Seeds of Phaseolus vulgaris (Kinghorn) were germinated under etiolating conditions at 20°C in moist vermiculite, and the cotyledons were harvested from 2-, 4-, 7-, and 9-d-old seedlings. Smooth microsomal membranes were isolated from the cotyledons as described by McKersie and Thompson (18).

Chemical and Enzymic Assays. Cell-free systems for enzyme assays were prepared by homogenizing the cotyledons in 0.3 M sucrose-0.05 M NaHCO₃, pH 7.0, (25% w/v) with a Sorvall Omnimixer and filtering through four layers of cheesecloth. Appropriate dilutions of the filtrate were assayed for catalase, peroxidase, and lipoxigenase as soon as possible after preparation of the homogenates. Peroxidase dismutase activity was assayed in samples that had been frozen overnight at −20°C. Cytosol fractions for isoenzyme determinations were obtained by centrifuging homogenates at 165,000g for 1 h followed by a second centrifugation of the resulting supernatant again at 165,000g for 1 h.

Protein levels were determined as described by Lowry et al.

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(16) using BSA as a standard. Catalase was assayed as described in the Worthington Enzyme Manual (30), superoxide dismutase as described by Beauchamp and Fridovich (2), peroxidase as described by Hart et al. (12), and lipoxigenase as described by Kim and Grosch (14).

Polycrylamide disc gel electrophoresis of cytosol fractions for determinations of peroxidase and superoxide dismutase isoenzymes was carried out as described by Davis (4) except that the stacking and concentration portions of the gels were not included. Superoxide dismutase activity in the gels was localized according to the procedure of Beauchamp and Fridovich (2) and peroxidase activity as described by Hart et al. (12).

Five g of cotyledon tissue were used to prepare homogenates for glutathione determinations. The cotyledons were added to 20 ml of 25% H3PO4 (v/v) and 75 ml of 0.1 m phosphate-5 mm EDTA buffer (pH 8.0) in a Sorvall Omnimixer. Air was flushed from the homogenizer cup with N2, and the tissue was homogenized on ice by running the Omnimixer at maximum speed for five periods of 30 s interspersed with 15-s periods for cooling. The homogenate was centrifuged at 165,000g for 30 min and the supernatant was assayed for reduced and oxidized glutathione by the fluorometric method of Hisasin and Hil (13).

Fluorescence Measurements of Lipid Extracts. Extraction of total lipids from whole cotyledons and smooth microsomal membranes and determinations of lipid phosphate levels were carried out as described previously (20, 24). Chloroform-solvent neutral lipid extracts were isolated from smooth microsomal membranes of 9-d-old cotyledons as previously reported (20). Smooth microsomal membranes from 2-d-old cotyledons were treated with ozone to induce the formation of chloroform-soluble neutral lipids able to convert liquid-crystalline phospholipid into the gel phase as described by Pauls and Thompson (24). The fluorescence of these extracts was measured after treatment with UV light to minimize interference by pigments (6). Interference by pigments was also minimized by using etiolated tissue throughout the study. The spectrofluorometer was standardized by using 0.1 mg ml-1 quinine sulfate (Sigma) in 0.1 m H2SO4.

RESULTS

Fluorescence spectra of lipid extracts from smooth microsomal membranes of cotyledons at various stages of senescence are illustrated in Figure 1. These spectra all show excitation maxima at 370 nm and emission maxima at 430 nm. The intensity of fluorescence in the membrane lipid extracts expressed on a per µmol phospholipid basis increased approximately 10-fold between day 2 and day 9 (Fig. 2). There was also an increase in the fluorescence intensity of total lipid extracts from whole cotyledons over the same period. However, the increment was only 2- to 3-fold depending on whether fluorescence intensity was expressed on a per µmol phospholipid, per mg lipid, or per g cotyledon basis (Table 1), and the intensity at day 9 was only about 50% of the value observed for lipid extracts of smooth microsomal membranes (Table 1; Fig. 2).

Of particular significance is the finding that the accumulation of fluorescent products of lipid peroxidation in the microsomal membranes during senescence shows a close temporal correlation with the rise in lipid-phase transition temperature reflecting formation of gel phase lipids in the membranes (Fig. 2). This rise in transition temperature has been shown previously to be attributable to chloroform-soluble neutral lipids that are formed during free radical-mediated lipid peroxidation and are able to order membrane phospholipid into the gel phase (3, 20, 23, 24). The transition temperature data shown in Figure 2 are from McKersie and Thompson (18), but several checks of the membrane preparations used in the present study showed that their transition temperatures increased with age in the same way. The transition temperature is defined as the highest temperature at which gel phase lipid can be detected, and the rise in transition temperature with advancing senescence reflects the progressive addition to the gel phase domains of lipids with inherently higher melting points (18). Moreover, the source of fluorescence in the total lipid extracts of senescent membranes proved to be the chloroform-soluble neutral lipids that accumulate with advancing senescence, are responsible for ordering membrane phospholipid into the gel phase and are products of lipid peroxidation (3, 20, 23, 24). The excitation and emission spectra of these rigidifying neutral lipids, which were isolated from the total lipid extracts of senescent 9-d-old membranes by silica gel chromatography as

![Graph](https://via.placeholder.com/150)
Table 1. Changes during Senescence in the Fluorescence Intensity of Lipid Extracts from Whole Cotyledons of \textit{P. vulgaris}

Differences between 2-d-old and 9-d-old cotyledons were found to be significant at the 5% level when analyzed with a \textit{t} test. Excitation, 370 nm; emission, 465 nm.

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\textbf{Time} & \textbf{Fluorescence Intensity} & \textbf{Age (days)} & \hline
\textbf{after Germination} & \textbf{\(\mu\text{mol phospholipid} / \text{mg lipid} / \text{g cotyledon}\)} & \hline
2 & 0.61 \pm 0.09\text{*} & 0.37 \pm 0.11 & 3.32 \pm 0.32 & \\
4 & 0.72 \pm 0.15 & 0.41 \pm 0.14 & 3.37 \pm 0.90 & \\
7 & 1.80 \pm 1.16 & 0.70 \pm 0.45 & 6.47 \pm 4.05 & \\
9 & 1.93 \pm 0.45 & 0.57 \pm 0.09 & 4.57 \pm 0.99 & \\
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\text{* Mean \(\pm\) se; \(n = 3\).

Fig. 3. Levels of reduced (\(\bigcirc\)) and oxidized (\(\square\)) glutathione in senescing cotyledons of \textit{P. vulgaris}. Se are shown by bars; \(n = 5-7\).

Levels of glutathione declined dramatically during senescence on both a cotyledon basis (Fig. 3) and per unit fresh or dry weight (Fig. 10). In young 2-d-old cotyledons, the level of reduced glutathione exceeded oxidized glutathione by about 25\%, but by day 4, levels of reduced glutathione were lower than levels of oxidized glutathione (Fig. 3). In 9-d-old cotyledons, both reduced and oxidized glutathione had decreased to less than 5\% of their levels in young 2-d-old tissue (Fig. 3). Lipoxynagenase activity rose by 50\% between days 2 and 4 when expressed on a per cotyledon basis, but then declined between days 4 and 7 to levels just slightly lower than those obtained for 2-d-old cotyledons (Fig. 4). However, the activity per unit fresh weight and per unit dry weight increased between days 2 and 9 by 3-fold and 4-fold, respectively (Fig. 10). By contrast, superoxide dismutase, an enzyme that catalyzes the dismutation of \(\text{O}_2^-\) to \(\text{H}_2\text{O}_2\), declined during senescence such that the activity per cotyledon at day 9 was only 10\% of that found in 2-d-old tissue (Fig. 5). This was also reflected in decreased activities per g fresh weight and per g dry weight (Fig. 10). Cytosol fractions contain three isoenzymes of superoxide dismutase (Fig. 6). The isoenzymes appeared as achromatic bands on a blue stained background and were manifest as negative peaks in densitometer scans of the gels. Identical patterns were obtained for cytosol fractions from all ages of cotyledon. Band II, which comprised the largest proportion of the total activity (Fig. 6), proved to be asymmetrical and may in fact be two or more unresolved isoenzymes. The two fastest moving isoenzymes (bands I and II) were sensitive to KCN, but band III was not (Fig. 6).

Catalase activity per cotyledon rose to day 4, then declined by day 9 to about 25\% of that observed in young 2-d-old tissue (Fig. 7). On a fresh weight and dry weight basis, there was little change in catalase activity between days 2 and 9 (Fig. 10), but between day 4 (when the activity per cotyledon had peaked; see Fig. 7) and day 9, the activity declined by 2.2-fold on a fresh weight basis and by 2.4-fold on a dry weight basis (data not shown). Peroxidase activity per cotyledon increased about 7-fold between days 2 and 7 before declining to a level by day 9 that was about 4-fold greater than the activity for day 2 (Fig. 8). On a fresh...
weight and dry weight basis, peroxidase activity increased by 12-fold and 17-fold, respectively, between days 2 and 9 (Fig. 10). Seven isoenzymes of peroxidase were detectable in cytosol fractions from the cotyledons, and one of those (labeled B in Fig. 9) increased dramatically with advancing senescence (Fig. 9). In fact, based on the relative areas of absorbance peaks in the gel scans, this particular isoenzyme increased from 25% of the total peroxidase activity in 2-d-old tissue to 74% by day 9 (Table II).

DISCUSSION

The fluorescence spectra obtained from the lipid extracts of senescing membranes and corresponding intact tissue have emission and excitation maxima that are very similar to those measured previously for compounds that accumulate with age in certain animal tissues and in vitro after induction of lipid peroxidation reactions (28). There is good evidence that these fluorescent products are a family of compounds with a characteristic Schiff base structure (—N=C—C=N) that is formed by the reaction of peroxidized lipids with compounds containing free amino groups (28). Fluorescent compounds of this type have been shown to increase in ripening fruit (17) and senescing leaves (29), and from the present study, it is clear that they also accumulate in the membranes of senescing bean cotyledon tissue. Moreover, the increased fluorescence of lipid extracts from senescing membranes can be interpreted as reflecting peroxidative damage since fluorescent compounds, which resemble those found in senescent membranes to the extent that they can order phospholipid into the gel phase and have identical fluorescence excitation and emission maxima, are formed during ozone-induced lipid peroxidation of isolated membranes.

Of particular significance is the finding that the accumulation of peroxidized lipids in senescing membranes of the cotyledon correlates with a rise in the lipid phase transition temperature reflecting the formation of gel phase lipid. The presence of gel phase lipid has been documented for membranes from a number of senescing tissues (18, 19) and has been attributed to free radical-mediated lipid peroxidation. For example, treatment of isolated membranes with ozone under conditions in which free radicals are produced induces lipid peroxidation and formation...
of gel phase (23, 24). Similarly, treatment of photosynthetic tissue with the herbicide paraquat, which short-circuits photosynthetic electron transport and facilitates the catalytic production of \( \text{O}_2^\cdot \), induces lipid peroxidation and the parallel appearance of gel phase lipid in chloroplast and microsomal membranes (3). The close parallel between the accumulation of peroxidized lipids and the rise in lipid phase transition temperature supports the contention that the formation of gel phase lipid during senescence is attributable to lipid peroxidation.

Reduced glutathione functions as an antioxidant by scavenging oxidizing substances and is thought to play a role in protecting cells against oxidative damage. In addition, reduced glutathione repairs sustained oxidative damage by regenerating sulphydryl groups from disulfide bonds through a glutathione-disulfide exchange mechanism and by participating in reactions mediated by glutathione peroxidase that reduce hydroperoxides, including lipid hydroperoxides, to alcohols (7). The decline in the endogenous titer of reduced glutathione to low levels in aged cotyledons presumably signifies a progressively diminishing capacity of the tissue to resist and repair oxidative damage. Measurements of the relative levels of reduced and oxidized glutathione in the bean cotyledon tissue showed a shift to the oxidized form, particularly during the early stages of senescence, which can be interpreted as reflecting an accentuated oxidative challenge to the tissue as senescence intensifies.

Lipoxygenase per unit fresh weight and per unit dry weight increased by 3- to 4-fold during senescence. Increased activities of this enzyme have been observed previously during senescence (11) and presumably give rise to elevated levels of lipid hydroperoxides. These compounds are potentially unstable and are susceptible to a variety of isomerization reduction or free radical cleavage and polymerization reactions that can lead to membrane damage (10). In addition, because of declining levels of reduced glutathione, the potential capacity of the tissue to remove lipid peroxides through glutathione reductase would decrease. However, it should be noted that we were unable to detect glutathione reductase activity in cotyledon tissue of any age (data not shown), and this raises the possibility that this line of defense against oxidative damage is not operable in this tissue.

The capacity of the tissue to enzymically scavenge \( \text{O}_2^\cdot \) also declines with age. Total superoxide dismutase activity per cotyledon falls off between days 2 and 9, and this is also reflected in dramatically reduced activities per unit fresh weight and per unit dry weight. The three isoenzymes of superoxide dismutase in this tissue have been previously identified as a Cu,Zn-enzyme (bands I and II) and a Mn-enzyme (band III) (15). The Cu,Zn-enzyme is found in the cytosol of eukaryotic cells whereas the Mn form is localized in the mitochondrial matrix (9). Since there was no change in the isozyme pattern during senescence, it appears that the rate of decline in superoxide dismutase activity in these two cellular compartments is very similar.

The decrease in catalase activity observed between days 4 and 9 suggests that the tissue acquires an increased load of \( \text{H}_2\text{O}_2 \) during the later stages of senescence. Decreased activity of this enzyme has been noted previously and has been correlated with the buildup of \( \text{H}_2\text{O}_2 \) (8). This together with a presumed accumulation of \( \text{O}_2^\cdot \) in the face of reduced superoxide dismutase activity would favor formation of \( \text{OH}^- \), and any accumulation of this extremely potenti oxidant would clearly result in free radical-mediated damage, in particular, the initiation of lipid peroxidation in membranes (21). By contrast, peroxidase activity in the cotyledons increases with advancing senescence, particularly one of the six isoenzymes. Increases in peroxidase activity have been noted previously in senescing plant tissue (22). A role for these elevated levels has not been identified; they may tend to ameliorate \( \text{H}_2\text{O}_2 \) accumulation, but it is conceivable that the effect of gel phase (23, 24). Similarly, treatment of photosynthetic tissue with the herbicide paraquat, which short-circuits photosynthetic electron transport and facilitates the catalytic production of \( \text{O}_2^\cdot \), induces lipid peroxidation and the parallel appearance of gel phase lipid in chloroplast and microsomal membranes (3). The close parallel between the accumulation of peroxidized lipids and the rise in lipid phase transition temperature supports the contention that the formation of gel phase lipid during senescence is attributable to lipid peroxidation.

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* Mean ± SE; \( n = 2 \).
tiveness of peroxidase as a scavenger of H₂O₂ is curtailed with advancing senescence by the unavailability of specific reducing cosubstrates. Thus, apart from the increase in peroxidase activity and its possible role in scavenging H₂O₂, there appears to be a progressive diminution in cellular defences against activated oxygen species and lipid peroxides. This together with an enhanced propensity to produce lipid hydroperoxides through lipoygenase presumably accounts, at least in part, for the accumulation of fluorescent products of lipid peroxidation in membranes.

Theories of aging fall into two broad categories, viz. programmed senescence, or senescence as random deterioration. In plants, senescence is normally a precisely controlled event executed for a specific purpose. Nonetheless, it is conceivable that within this framework the degenerative and essentially random process of free radical-induced lipid peroxidation could be mediating many of the deteriorative changes occurring during senescence. Cells normally possess checks and balances in the form of enzyme scavengers and antioxidants to control the titer of free radicals, but any relaxation of these defence mechanisms could initiate tissue degradation. In bean cotyledons, it would appear that one consequence of this relaxation is the accumulation of peroxidized lipids in membranes and ensuing membrane dysfunction.

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