Lipid-Phenolic Radical Adducts as a Plausible Mechanism of „Plant Ageing" Pigment Formation

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Abstract. Co-oxidation of chlorogenic acid, caffeic acid, aesculetin and lucigenin with linoleic acid and egg phosphatidyl choline leads to the formation of fluorescent polymer materials. The fluorescent products are more lipophylic, they have lower elution volumes on Sephadex LH-20 column than related phenols and they differ by their fluorescence and chromatographic properties considerably from polymer lipid peroxidation products. From the presence in the excitation fluorescence spectra of a band corresponding to the phenols it was concluded that the fluorophoric groups were similar in both cases. The data are discussed in terms of liquid phase peroxidation and the appearance of the fluorescent species are attributed to the production of molecular adducts as a result of lipid and phenoxyl radical recombination. The characteristics of products obtained are compared with properties of fluorescent "plant ageing" pigments accumulated in aged and damaged plant cells.

Key words: Phenols — Lipids — Peroxidation — Ageing — Fluorescence

Introduction

Recent works from several laboratories have demonstrated the accumulation of fluorescent compounds in the lipid extracts from ageing, diseased and herbicide-treated plants (Maquire and Haard 1975; Merzlyak et al. 1982a, b; 1983; Shevchenko et al. 1980; Wilhelm and Wilhelmova 1981). These compounds with excitation and emission fluorescent maxima et 350—370 nm and 420—440 nm, respectively, are eluted in small volumes with Sephadex LH-20 column chromatography and show thin layer mobility similar to those of polar plant lipids (Merzlyak et al. 1982a, b; 1983). In herbicide-treated plants, these substances are sedimented in particulate (e.g. plastids and microsomes) fractions as well as polar lipid peroxides (Shevchenko et al. 1981). The formation of similar fluorescent substances has been observed after induction of NAD(P)H-dependent lipid peroxidation in pea microsomes in the dark (Pogosyan et al. 1981) and after illumination of chloroplast suspension (Rubin et al. 1976). Based on the above data and on resemblance with liposoluble fractions of animal lipofuscine pigments, the role of

lipid peroxidation in the fluorescent compound formation has been proposed (Maquire and Haard 1975; Merzlyak et al. 1982a, b).

Several reactions have been suggested as the mechanism of fluorescent lipid peroxidation production in biological membranes (Tappel 1975; Vertushkov 1977; Tabata et al. 1979). According to the generally accepted hypothesis of Tappel (1975), the principal reaction in the lipofuscine-like material formation is the condensation of malon dialdehyde with amino-containing phospholipids. However, interaction of the lipid peroxidation product with dipalmitoyl- or lysophosphatidyl ethanolamine produced fluorescent species with considerably more long-wave excitation maxima (Merzlyak and Rumyantseva 1983) as compared with those in senescing plants. Thus, the mechanism does not seem quite convincing to explain liposoluble fluorescent product formation in this case.

Plant tissues contain considerable amounts of phenolic compounds, many of which are fluorescent, and Knee (1982) has suggested similarity of fluorescent products accumulated in ripening fruits with cinnamic acid derivatives. However, we have not been able to detect any phenolic compounds in lipid preparations under our extraction procedure (Merzlyak et al. 1982a). Indeed, the identification with simple phenolics seems inconsistent with their relatively high polarity and relatively low molecular weight.

Many of plant phenols possess pronounced antioxidative action and some of them, e.g. α-tocopherol (Merzlyak and Yuferova 1975) or kaempferol (Takahama 1982) suppress lipid peroxidation in illuminated chloroplasts. Free radical phenol oxidation produces a number of products as a result of reactions following hydroxyl hydrogen abstraction (arylation, alkylation, dimerization, etc.). Some of these oxidation products can pricipally be fluorescent. Hence, to ascertain the nature of the liposoluble products in ageing tissues, we have studied fluorescent species arising from peroxidizing linoleic acid and lecithin interactions with some widespread plant phenols and strongly fluorescent lucigenin.

Materials and Methods

Linoleic and stearic acids (80—100 μmoles), egg phosphatidyl choline (20 μmoles) separately or in a mixture with chlorogenic acid, caffeic acid, aesculetin and lucigenin, were incubated in 8 mm (internal diameter) glass tubes at 40 ± 1 °C over 15—20 days in the dark. The substances were dissolved in methanol, mixed in required proportions, and the solvent was removed under vacuum. The phenol and lucigenin concentrations in the samples were 2—5 mol%.

After the incubation, the samples were dissolved in methanol or in a mixture of chloroform-methanol (1:1) and aliquots were partitioned on a Sephadex LH-20 (1.0×100 cm) column using chloroform-methanol (1:1) as the mobile phase. Continuous registration of fluorescence excited at 365 nm was conducted during the separation (Merzlyak et al. 1982a; 1983). Consecutive fractions corresponding to the selected curve regions were collected for further analysis. Thin layer chromatography was performed on Silufol (Kavalier) plates with chloroform-methanol-water (65:25:4) as a solvent.

Fluorescence spectra were determined with a Hitachi MPF-4 spectrophotofluorimeter under

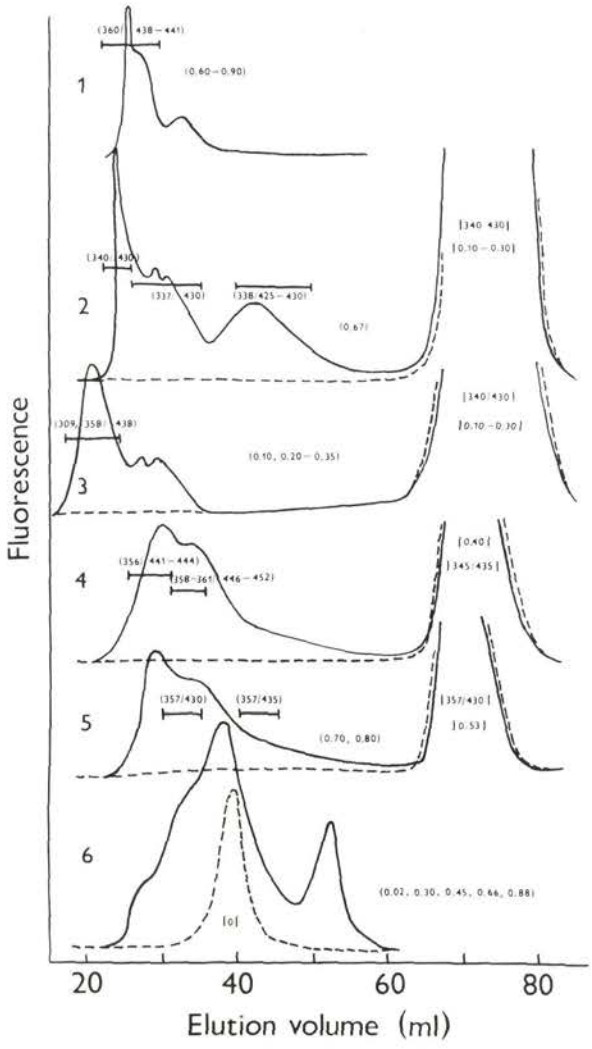

Fig. 1. Chromatographic and spectral properties of polymerized lipids, phenols and fluorescent products formed after their co-incubation. Chromatography on Sephadex LH-20. 1 — linoleic acid, 2 — linoleic acid + chlorogenic acid, 3 — lecithin + chlorogenic acid, 4 — linoleic acid + caffeic acid, 5 — linoleic acid + aesculetin, 6 — linoleic acid + lucigenin. The elution curves of original phenols are represented by broken lines and those in peroxidized samples by solid lines. Excitation/emission maxima (nm) in uncorrected fluorescence spectra and thin layer R_f's values are given in square brackets for original phenols and in round brackets for those in peroxidized samples. ⟷ — volume intervals of collected fractions for which fluorescence data are presented.

conditions described previously (Merzlyak et al. 1982a, b; 1983), and absorption data were obtained with a Specord UV-Vis and a Beckman-26 spectrophotometers. Both the absorption and fluorescence spectra were measured in chloroform-methanol (1:1). Mass-spectra were recorded with a MS-1302 (SKB AP AN SSSR) device. The samples were evaporated in the ionization region at 120—150 °C and at a ionization electron energy of about 70 eV.

Linoleic and stearic acids and lucigenin were from Reachim, egg lecithin was kind gift by Dr. Yu. N. Kaurov. Chlorogenic and caffeic acids were obtained from Serva, and aesculetin from Chemapol.

Results

Originally, neither linoleic acid nor phosphatidyl choline were fluorescent, and phenols were eluted as single symmetrical peaks at high elution range (Fig. 1) Incubation of the lipids separately or in a mixture with phenols resulted in the appearance of fluorescent compounds eluted between 15 and 50 ml. Simultaneously, a yellowish tinge developed in the lipid mixture. Peroxidized products eluted in the 20—45 ml range had a higher fluorescence intensity when phenols were present in the samples. The fluorescence intensity in the phenol elution volume decreased during their co-incubation with linoleic acid or lecithin, but it did not vary after the same incubation period (up to 20 days) if phenols were incubated in the absence of lipids. Fluorescent products were not formed during incubation of saturated (stearic) fatty acid separately or with chlorogenic acid (up to 20 days) and the fluorescence did not decrease.

The elution patterns of the fluorescent products formed in the systems containing linoleic acid or phosphatidyl choline and chlorogenic acid were similar but differed in their elution ranges. Fluorescent substances produced after co-incubation of chlorogenic acid and lecithin were eluted in lower ranges than those arising from interaction of phenol with linoleic acid. This points to a higher molecular weight of phosphatidyl choline oxidation products. Fluorescent substances formed in the presence of chlorogenic acid had lower elution volumes and different elution patterns than those with caffeic acid (co-oxidation with linoleic acid).

Lucigenin degradation coupled with peroxidation of linoleic acid gave several products with yellow fluorescence. Products eluted in elution volume greater than that of lucigenin were observed in this case.

The chromatographic mobility and fluorescence spectral data are also shown in Fig. 1. High molecular weight fluorescent products from autooxidation of linoleic acid had higher thin layer mobility and those arising from lecithin peroxidation had lower R_f values in the solvent system used. Formation of additional fluorescent zones was found after the incubation of linoleic acid with chlorogenic acid, aesculetin and lucigenin. Phenols and fluorescent products formed in their presence had similar ultra-violet absorption and fluorescent maxima positions. It should be noted that spectral and chromatographic characteris-

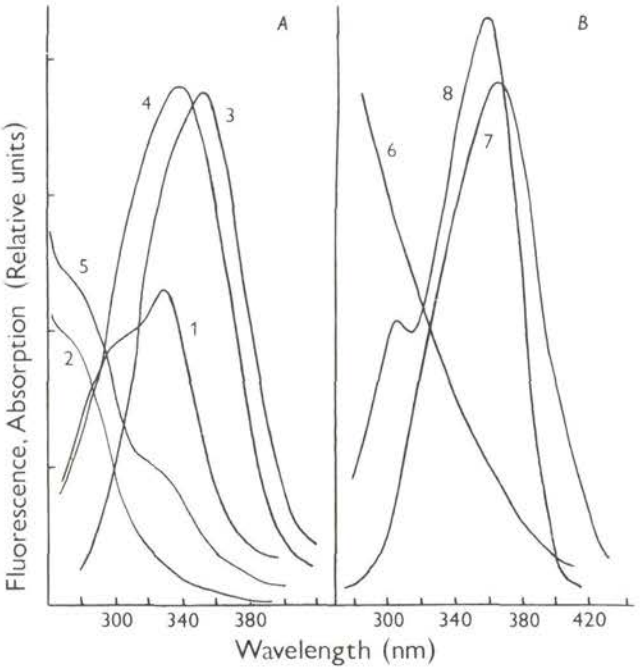

Fig. 2. Absorption and fluorescence spectra of fluorescent products derived from co-incubation of chlorogenic acid with linoleic acid (*A*) or phosphatidyl choline (*B*). 1 — absorption spectra of chlorogenic acid, 2,6 — absorption spectra of peroxidized linoleic acid and lecithin, 3,7 — corrected excitation fluorescence spectra of peroxidized linoleic acid and lecithin, 4,8 — corrected excitation fluorescence spectra of linoleic acid (fraction 24—30 ml) and lecithin co-incubated with chlorogenic acid, 5 — absorption spectra of the 24—30 ml fraction after co-incubation of linoleic acid with chlorogenic acid.

tics of lecithin and linoleic acid oxidation products were different from those of lipid-phenol co-oxidation. Several fluorescent compounds with different thin layer mobility but retaining lucigenin resolved electronic spectra (not shown) were found after incubation of the fluorescent compounds with linoleic acid. At the same time, some differences in the maxima positions in individual fractions obtained with Sephadex LH-20 chromatography were observed.

Electronic absorption spectra of peroxidized linoleic acid heavy fractions and lecithin (Fig. 2) were characterized by a smooth decrease in the absorption between 280 and 400 nm. Though there was no marked absorption in this band the peroxidized lipids showed intense fluorescence with excitation maxima at 360—367 nm (Fig. 1, 2). "Heavy" fractions obtained after separation of fatty substances co-incubated with chlorogenic acid had, in addition to the absorption of peroxidized lipids, a distinct shoulder near 300—310 nm. The corrected fluores-

cence excitation spectra of the fractions resembled in this case absorption spectra of chlorogenic acid. Thus, it may be suggested that some of the fluorophore groups of the products formed were close enough to the parent phenols.

The mass-spectra of the products in the fluorescent (24—30 ml) fractions of peroxidized linoleic acid were compared with that of linoleic acid-caffeic acid co-incubation products. As compared with the autooxidized fatty acid, additional peaks in the ranges 300—500 m/e were characteristic of lipid phenol oxidation products. Also, it is noteworthy that free caffeic acid ion was not recorded in linoleic acid-caffeic acid mass-spectrum. Free chlorogenic acid was not found in the eluate from Sephadex LH-20 column between 26 and 32 ml (absence of the corresponding fluorescent zone on chromatoplates under long-wave ultra-violet). Thus, although the fluorescent properties of the oxidized products resembled those of the native phenols, our data have provided evidence for absence of free phenols in high-molecular fractions of the peroxidized samples.

As noted above, the fluorescence intensity increased during linoleic acid incubation. This fluorescence was very likely caused by the formation of polymerized lipid oxidation products (Orlov et al. 1975). Even a short-time (10—20 minutes) interaction of peroxidized (autooxidation for 14 days) linoleic acid with chlorogenic acid and with lucigenin led to the formation of fluorescent products as it was the case during prolonged co-incubation. These products had spectral and chromatographic properties similar to the compounds formed during long time interaction in mixture.

Thus the absence of free phenol in the fluorescent heavy fractions, data on the interaction of peroxidized lipids with phenols, and also the appearance of additional chromatographically different compounds, suggest covalent binding of the fluorescent phenols with lipid peroxidation products.

Discussion

Autoxidation of unsaturated fatty acid or egg phosphatidyl choline with widespread plant phenols results in formation of fluorescent oxidation products. By their behaviour on Sephadex LH-20 column, thin layer mobility and excitation and emission spectra, these compounds are principally similar to fluorescent species found previously in ageing plant tissues (cf. Merzlyak et al. 1982a,b; 1983). Although the presence of polymerized lipids is necessary for the product formation, their chromatographic and spectral data considerably differ from those of lipid polymer products.

Liposoluble fractions of animal lipofuscine pigments are able to form micelles under conditions of gel-filtration (Purdy and Tappel 1979) and, probably, to solubilize phenols. However, the absence of free phenols in the "heavy" fractions

and the appearance of additional fluorescent sports on thin layer plates indicates covalent binding between phenols and lipid polymers.

The possible mechanism of the fluorescent product formation may be discussed in terms of liquid phase peroxidation of unsaturated compounds (Nonhebel et al. 1980) and it is probably related to the production of molecular adducts as a result of lipid and phenoxyl free radical recombination;

$$\begin{aligned} R^{\cdot} + H\text{—}Ph &\rightarrow RH + Ph^{\cdot}, \\ R^{\cdot} + Ph^{\cdot} &\rightarrow R\text{—}Ph, \\ R^{\cdot} + RH &\rightarrow R^{\cdot}\text{—}RH, \\ R^{\cdot}\text{—}RH + Ph^{\cdot} &\rightarrow HR\text{—}R\text{—}Ph, \\ Ph^{\cdot} + Ph^{\cdot} &\rightarrow Ph\text{—}Ph, \end{aligned}$$

where RH is the fatty acid substrate molecule; H-Ph is phenol molecule; R˙ and Ph˙ are lipid and phenoxyl radicals, respectively.

The formation of phynoxyl (semiquinone) radicals under one-electron enzymatic phenol oxidation (catalyzed by polyphenol oxidase or by peroxidase) as well as quinone reduction may be suggested to occur in plant tissues. In this case, following reaction is also of interest:

$$Ph^{\cdot} + RH \rightarrow Ph^{\cdot}\text{—}RH$$

The most thorough study of formation of molecular adducts (R-Ph) has been conducted with α-tocopherol during lipid autooxidation. Co-oxidation of phenol with linoleic acid applied as monolayer on silica gel resulted in as much as 40% of initial α-tocopherol quantity recovered as 1:1 oxidation adduct compound for which the formation of a second chroman ring has been assumed (Porter et al. 1971). A similar tocopherol oxidation product could be separated and identified after incubation of α-tocopherol with soybean lecithin under the same conditions. The possibility that α-tocopherol interacts with phosphatidyl choline dimers and trimers was proposed (Porter et al. 1973). Moreover, up to 50—60% of α-tocopherol were included in isomeric tocopheryl-octadecanoate ethers formed as a result of primary alkyl fatty acid radicals interception by the antioxidant molecule (Koch et al. 1976).

Such oxidation products (e. g. Ph-R, Ph-R-R) retain the π-electron structure; this can be confirmed by the similarity of ultra-violet absorption spectra of tocopherol and the adducts (Porter et al. 1971, 1973; Koch et al. 1976). They also may be fluorescent as both hydroxy- and methoxy-monosubstituted benzene derivatives fluoresce (Udenfriend 1965). According to this mechanism, phenol hydroxyl group become lost. Thus, such oxidation products could probably not be revealed on chromatograms by usual "phenol" reagents. Phenolics cross-linked to

lipid polymers become more lipophilic and have therefore higher thin layer mobility in a polar solvent system. It may also be suggested that similar products can be extracted from tissues with relatively nonpolar (("lipid") solvents. At the same time, rapid binding of phenols and peroxidized lipid do not rule out other covalent binding mechanism, since steady-state free-radical concentrations should not be very high.

Cross-linking and covalent binding between biologically important molecules seems to be an important sequence of lipid peroxidation in the cell (Tappel 1975; Jain and Hochstein 1980; Nielsen 1981). Phenol participation in lipid peroxidation can result in the formation of unusual for normal tissues "lipophenols". Plant cell phenolic compounds are localized predominantly in vacuoli and data on their presence in other cell organelles are available (Swain 1965; Zaprometov 1974; Charriere-Ladreix and Tissut 1971). From this point of view, the *in vivo* formation of liposoluble fluorescent products may be interpreted as a result of lipid peroxidation directly in membranes, or as a consequence of unspecific binding of phenols released from the vacuoles with preformed lipid peroxidation polymers. The above data principally suggest that products derived from fluorescent phenols may be present in the polymer materials in ageing and damaged plant cells. Experiments with lucigenin indicate that other non-phenolic fluorescent compounds may interact with lipid peroxidation products and form new lipophilic fluorescent species.

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