Suppression of Radical-Induced Lipid Peroxidation in a Model System by Alkyl Esters of Cinnamate Quaternary Ammonium Salts

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Three new groups of phenolic antioxidants, quaternary ammonium salts with a phenol ring and alkyl chains of different length (pyrrolidine ethyl esters of 3,5-di-*t*-butyl-4-hydroxy-dihydrocinnamic acid n-alkoxymethylchlorides (PYE-n) or n-alkylbromides (PYA-n) and 2-dimethylaminoethyl ester n-alkylbromides (PPA-n), were synthesized. Some of them were previously found to efficiently protect yeast cells against oxidants and to inhibit the production of thiobarbituric acid-reactive substances in whole yeast cells and in isolated membrane lipids. The new antioxidants (at 1–100 μM) abolished or diminished peroxidation of oliwe oil emulsions caused by the OH$^\bullet$-producing Fe^{2+} and RO$^\bullet$ and ROO$^\bullet$-producing *tert*-butylhydroperoxide (TBHP) and the azo compounds 2,2′-azobis-(amidinopropane)dihydronitrile (AAPH) and 1,1′-azobis-(1-cyclohexanecarbonitrile) (ACHN): all present at 10 mM. The efficiency of individual both antioxidants was examined in relation to the type of lipid peroxidation inducer, the site of antioxidant incorporation into the emulsion lipid phase, the length of the alkyl chain, and the maximum concentration of effective antioxidant monomers given by its critical micelle concentration. PYA-n class compounds were highly efficient against all peroxidation inducers and their efficiency did not depend on the position of their molecules in the lipid phase and/or on the aliphatic chain length. In contrast, the efficiency of PYE-n and PPA-n class compounds depended both on the type of oxidant and on the length of their aliphatic chain. Their potency against Fe^{2+} and ACHN increased with increasing alkyl chain length whereas with AAPH it dropped with increasing alkyl chain length. A similar pattern was found with the action of PYE-n against TBHP whereas in the PPA-n group an extending alkyl chain reduced the anti-TBHP efficiency. These relationships may not be entirely straightforward and other factors (chemical nature of each compound, its possible interaction with fluorescent probes used for diagnostics, etc.) may play a considerable and not yet quite clear role. PPA-n class antioxidants have the lowest critical micelle concentration, which may limit their efficiency. Nevertheless, these phenolic antioxidants can be conveniently employed as highly efficient inhibitors of lipid peroxidation.

Introduction

Lipid peroxidation occurs in cells under normal conditions on exposure of cell lipids to oxidants. It is initiated by reactive oxygen species (ROS) such as superoxide anion, hydrogen peroxide, singlet oxygen or hydroxyl radical abstracting a hydrogen atom from a methylene group in a lipid chain (Halliwell and Gutteridge, 1999). The chain reaction that follows produces large amounts of secondary radicals (Alscher *et al.*, 1997) and leads to lipid degradation. In phospholipids, the process brings about a drastic alteration of the properties of lipid bilayers and consequently impaired function of biological membranes. Polyunsaturated fatty acids (PUFA) are the most susceptible to peroxidation but the process can be initiated with any unsaturated fatty acid, including monounsaturated ones, e.g., oleic and palmitoleic acids. Its products are similar in both cases (Evans *et al.*, 1998). The end products of the peroxidation process, dialdehydes such as malonedialdehyde (MDA), can attack amino groups on protein molecules and cause cell death (Halliwell and Gut-

teridge, 1999). Lipid peroxidation can be terminated by antioxidants that act as hydrogen donors and protect cell membranes and other cellular lipids against the reactive oxygen species (ROS). Highly efficient natural antioxidants are phenolic compounds such as tocopherols that protect cell lipids *in vivo* by reacting with $OH^{\bullet}$, lipid peroxyl and alkoxyl radicals, donating labile hydrogen to them and terminating the chain reaction of peroxidation by scavenging chain-propagating radicals (Halliwell and Gutteridge, 1999). Artificial antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) or propylgallate are widely used as foodstuff additives. Their mechanism of action is similar to that of tocopherol. In many cases (especially at a high level of consumption) their use is limited by serious adverse effects on the organism and new antioxidants are being sought that are more effective at lower concentrations so that their level as, e.g., additives in food products can be reduced.

The lipid peroxidation-suppressing activity of different phenolic antioxidants depends on a number of factors. These include the number and position of hindered hydroxyl groups in the molecule (Chen *et al.*, 1996), localisation of the molecules in the lipid phase (Kleszczynska *et al.*, 1998) and factors governing the effective concentration of the antioxidant in the lipid. The last factor corresponds to the concentration of antioxidant monomers capable of incorporating into the lipid, i.e. to the critical micelle concentration (CMC) (Lichtenberg *et al.*, 1983). We have synthesised new phenolic antioxidants with various lengths of the alkyl side chain, which have been found to be highly efficient in protecting yeast cells of different species from oxidants (Krasowska *et al.*, 1999) and inhibiting oxidant-generated lipid peroxidation in whole yeast cells and in isolated membrane lipids (Krasowska *et al.*, 2000). In this study, we used a model lipophilicity to obtain information on the relationship between the structure of the PYE-n, PYA-n and PPA-n antioxidants and their inhibitory action on lipid peroxidation. $OH^{\bullet}$ is a powerful oxidant that reacts with most biomolecules including cell lipids (triacyglycerols, phospholipids) (Storz and Imlay, 1999).

Materials and Methods

Materials

The quaternary ammonium salts with antioxidant function (Fig. 1) were synthesised in our laboratory by quaternization of pyrrolidine ethyl esters of 3,5-di-*t*-butyl-4-hydroxy-dihydrocinnamic acid by n-alkoxymethylchlorides (PYE-n) or n-alkylbromides (PYA-n). Quaternization of 2-dimethylaminoethyl ester by n-alkylbromides produced a third group of antioxidants (PPA-n). These compounds have a low toxicity since the LD50 for the representative compound PPA16 administered orally to rats was 1722 mg/kg body weight; when injected hypodermally, it was over 2000 mg/kg. PPA16 was found to moderately irritate the rabbit skin (Toxicological report, 2000).

All compounds studied were of analytical grade purity checked by ^1H NMR spectra (Bruker Avance DRX_{300} instrument in deutero-chloroform, TMS as internal standard). Based on our previous studies (Krasowska *et al.*, 1999, 2000), the antioxidants were used at concentrations up to 100 μM.

Olive oil (Aceites Ybarra S. A., Spain) containing 2 g saturated lipids, 1.5 g polyunsaturated lipids and 10 g monounsaturated lipids in 15 ml was purchased in a retail outlet. The oxidation inducers included AAPH (2,2′-azobis-(amidinopropane)dihydronitrile) (Polyscience, USA), ACHN (1,1′-azobis-(1-cyclohexanecarbonitrile) (Fluka, Poland), TBHP (*tert*-butylhydroperoxide) and $FeSO_4.7H_2O$ (Sigma-Aldrich, Poland). They were used at 10 mM concentration, which was previously found to be the highest concentration at which lipid peroxidation in yeast cells and membranes still depends on oxidant level; at higher concentrations a "saturation" of TBRS production was observed (Krasowska *et al.*, 2000). Thiobarbituric acid (TBA) and trichloroacetic acid (TCA) were obtained from Fluka, Poland.

The fluorescent dyes 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH) and N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadekanoyl-*sn*-glycero-3-phosphoethanolamine (NBD-PE) were from Molecular Probes, Eugene, Ore. USA, 8-anilino-1-naphthalenesulfonic acid (ANS) was from Sigma, St. Louis, USA and dipalmitoylphosphatidylcholine (DPPC) was obtained from Lipid Products, UK.

Lipid peroxidation assay

Lipid peroxidation was measured in olive oil emulsion as the oxidation of triacylglycerols present in the oil. The oxidants (AAPH, ACHN, TBHP and Fe^{2+}), all at a final concentration of 10 mM, were allowed to induce the oxidation of lipid emulsions containing the antioxidants under study. The end products of lipid peroxidation, i.e. malonedialdehyde (MDA) and other TBA-reactive substances, were quantified by determining the level of thiobarbituric acid-reactive substances (TBRS) by a method adapted from Aust (1994).

In all experiments, 1 ml samples containing 37.5 mg olive oil and 1–100 μM antioxidants in 10 mM Tris-HCl (tris[hydroxymethyl]aminomethane), pH 7.4, were incubated for 15 min at 37 °C. The oxidation inducers were added to a concentration of 10 mM and the samples were incubated for another 15 min at the same temperature. Two ml of reagent A (15% TCA and 0.37% TBA in 0.25 M HCl) was added and the mixture was thoroughly blended. Test tubes containing the samples were stoppered with glass marbles, heated at 100 °C for 15 min, cooled under running tap water and centrifuged for 10 min at 2000 × g. Sample absorbance was measured at 535 nm on a Cecil CE-2020 spectrophotometer against a reference blank containing the TBA reagent and distilled water.

Estimation of antioxidant localisation in the lipid phase

This was done by determining the effect of the antioxidants on the fluorescence of two probes, TMA-DPH and NBD-PE, incorporated in small unilamellar vesicles (SUV) produced from DPPC. The SUV were prepared according to (Lentz, 1988). DPPC was dissolved and mixed in chloroform together with TMA-DPH or NBD-PE; the solvent was removed by a stream of nitrogen and then under vacuum at room temperature. An appropriate aqueous buffer solution was added and the sample was vortexed to obtain a milky suspension of multilamellar vesicles. This suspension was sonicated for 30 min at 20 kHz to obtain a suspension of transparent SUV. Each sample of vesicle suspension was prepared shortly before measurements and was kept on ice during experiments.

The effects of the antioxidants on fluorescent probes variously immersed in the SUV were as-

sayed by measuring the fluorescence quenching of the probes on a Kontron SFM 25 spectrofluorimeter at the following excitation and emission wavelengths: TMA-DPH – Ex = 355 nm, Em = 450 nm, for NBD-PE – Ex = 460 nm, Em = 534 nm. The fluorescence intensity measurements yielded polarisation coefficients C_p which were calculated according to the formula (Lentz, 1988):

$$Cp = \frac{I_{II} - GI_\perp}{I_{II} + GI_\perp}$$

where I_{II} - intensity of fluorescence emitted in the direction parallel to the polarisation plane of excitation light, $I_\perp$ – intensity of fluorescence emitted in the direction perpendicular to the polarisation plane of the excitation light, G – wavelength-dependent diffraction constant. Interaction of an antioxidant with the lipid phase in the vicinity of the probe resulting in lipid fluidisation or rigidisation should bring about a change in the C_p value. Changes in NBD-PE fluorescence reflect changes occurring in the hydrophilic part of the lipid bilayer, i.e. at the level of the phosphate residues of phospholipids whereas TMA-DPH fluorescence reports on interactions inside the bilayer – below the C4 atom in the phospholipid alkyl chains (Jemiola-Rzeminska *et al.*, 1996). In all experiments, 0.5 mg of liposomes was added to 2.7 ml 140 mM NaCl in 10 mM Tris-HCl (pH 7.4). The aqueous solutions of antioxidants were added at 5 min. intervals to a final concentration of 4.3–43 μM.

Determination of antioxidant critical micelle concentration (CMC) from surface tension

The measurements were performed according to Devinsky *et al.* (1985) at room temperature in a Nima Technology ST 9000 tensiometer by using a 24 cm² teflon vessel and Wilhelm's plates. Solutions of the PYA, PYE and PPA antioxidants in 2% aqueous ethanol were added to the water at 5 min intervals to a final concentration of 0.1 μM – 1 mM.

Determination of antioxidant CMC by ANS fluorescence (Kozubek, 1996)

The PYA, PYE and PPA antioxidants were added to 2 ml of aqueous 10 μM ANS solution of (Ex = 370 nm, Em = 480 nm) to a final concentration of 0.1 μM – 1 mM. PYA and PYE were dissolved in 10 mM Tris-HCl (pH = 7.4) and PPA in a

Fig. 1. The chemical structure of PYA-n, PYE-n and PPA-n anti-oxidants.
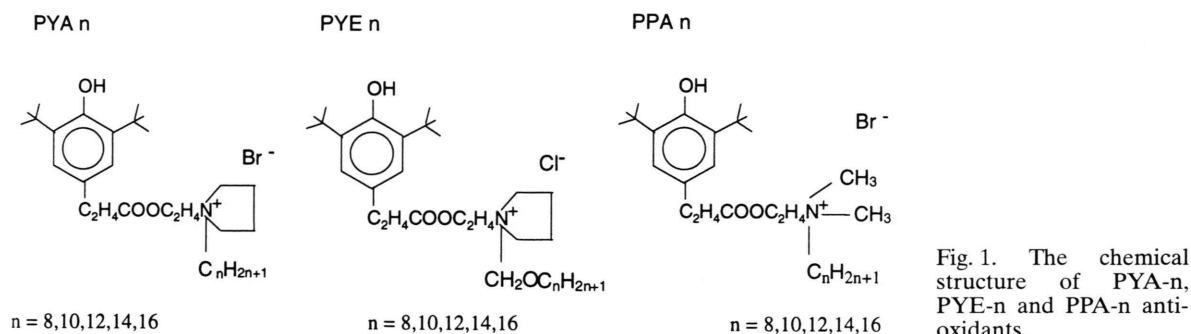
water/ethanol mixture (1:1). The mixture itself did not influence the ANS fluorescence (data not shown). The measurements on a Kontron SFM 25 spectrofluorimeter were done at room temperature.

Results

Lipid peroxidation was induced by four compounds with different solubility in lipids. AAPH (2,2′-azobis-(amidinopropane) dihydronitrile) and Fe^{2+} are hydrophilic and water-soluble whereas TBHP is amphiphilic and thus both partially water- and lipid-soluble (Evans *et al.*, 1998) and ACHN (1,1′-azobis-(cyclohexacarbonitrile)) is soluble in lipids. At equal concentrations of all oxidants, the intensity of lipid peroxidation, as reflected in TBRS production, decreased in the sequence Fe(II) > ACHN ~ TBHP > AAPH (Fig. 2). Thus in this system the $OH^{\bullet}$-producing
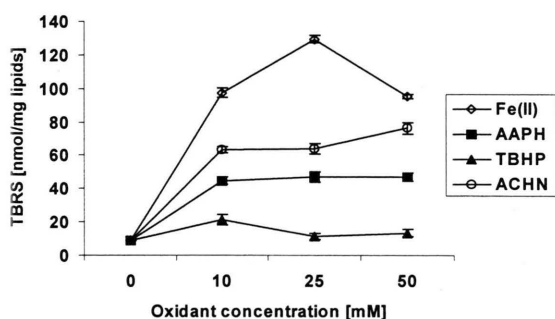

Fig. 2. Spontaneous and oxidant-induced TBRS production in olive oil.
37,5 mg of olive oil emulsion in Tris-HCl buffer (pH 7.4) was exposed to increasing concentrations of Fe(II) (◊), AAPH (■), TBHP(▲) and ACHN (○). The data are means ± SD (n = 3). Value at zero oxidant concentration stands for spontaneous autooxidation.

Fe(II) is a more efficient inducer of lipid peroxidation than the $RO^{\bullet}$- and $ROO^{\bullet}$-producing compounds irrespective of the degree of their lipophilicity. It is not clear whether this is due to the type of the radical produced or to the quantity of radicals generated during the exposure time. The antioxidant properties of the phenolic PYA-n, PYE-n and PPA-n compounds differing in the substituents at the quaternary nitrogen atom and in the length of the n-alkyl substituent (Fig. 1) were assessed by determining the concentration of the antioxidant at which lipid peroxidation was inhibited by 50% (IC_{50}). The IC_{50} values were used for estimating the relationship between the structure of the phenolic compounds and their antioxidant activity. The CMC values should reflect the highest attainable concentration of the active monomers of antioxidant molecules. They were found to decrease in the sequence PYA > PYE > PPA and, within these groups of antioxidants, with the decreasing alkyl chain length (Table I). The change in the C_p parameters of the two fluidimetric fluorescent probes, NBD-PE and TMA-DPH caused by the oxidants (Fig. 4A,B) should hint at the position in which the antioxidant molecules are incorporated in the lipid phase.

PYA-n antioxidants were found to be fairly effective blockers of lipid peroxidation with all pro-oxidants under study (Fig. 3A–D). Their IC_{50} systematically decreased with increasing aliphatic chain length from 30–70 µM for PYA-8 to 5–30 µM for PYA-12 and PYA-16. In view of the large scatter of C_p values in the absence of PYA antioxidants, these compounds (with the possible exception of PYA 8 in low concentrations) did not seem to have any perceptible systematic effect on the fluorescence polarisation of the two probes in

Table I. The critical micelle concentration (CMC) values for PYA, PYE and PPA antioxidants, ± SD (n = 3)

Compound	CMC [x10^{-3}M] determined by:	
	ANS fluorescence	Surface-tension measurements
PYA 8	6.11 ± 0.3	5.3 ± 0.1
PYA 12	2.82 ± 0.9	3.7 ± 0.3
PYA 16	1.58 ± 0.1	0.93 ± 0.4
PYE 8	0.67 ± 0.1	0.97 ± 0.2
PYE 12	2.19 ± 0.8[a]	0.41 ± 0.1
PYE 16	0.32 ± 0.005	0.19 ± 0.06
PPA 8	0.047 ± 0.03	0.085 ± 0.02
PPA 12	0.00269 ± 0.0008	0.003 ± 0.001
PPA 16	0.00122 ± 0.0003	0.00089 ± 0.0002

[a] The exceptionally high critical micelle concentration (CMC) value for PYE-12 determined by ANS fluorescence may be due to PYE-12/ANS interaction.

SUV (Fig. 4A). Hence the PYA compounds do not appear to have any fixed position in the lipid phase and, in accordance with this wide distribu-tion within the lipids and at its surface, are nearly equally effective against hydrophobic, amphiphilic and hydrophilic oxidants. The efficiency increases if their molecules contain longer aliphatic chains. Their easy incorporation into the lipid phase can be aided by the high CMC values.

The PYE compounds were most effective against the hydrophobic ACHN (IC$_{50}$ < 10 μM) ir-respective of the aliphatic chain length. Com-pounds with the shortest and the longest alkyl chain were also active against lipid peroxidation induced by the amphiphilic TBHP. Lipid peroxida-tion caused by the hydrophilic AAPH was inhib-ited by all PYE-n except PYE-14 (Fig. 3D) and the antioxidant efficiency against Fe^{2+} increased with increasing aliphatic chain length (Fig. 3A). PYE compounds had a more conspicuous effect on the C_p values of the two fluorescent probes than PYA-n ones. The C_p for TMA-DPH decreased with increasing concentration of PYE 12 and PYE 16, PYE 8 having no effect (Fig. 4B). Thus the former
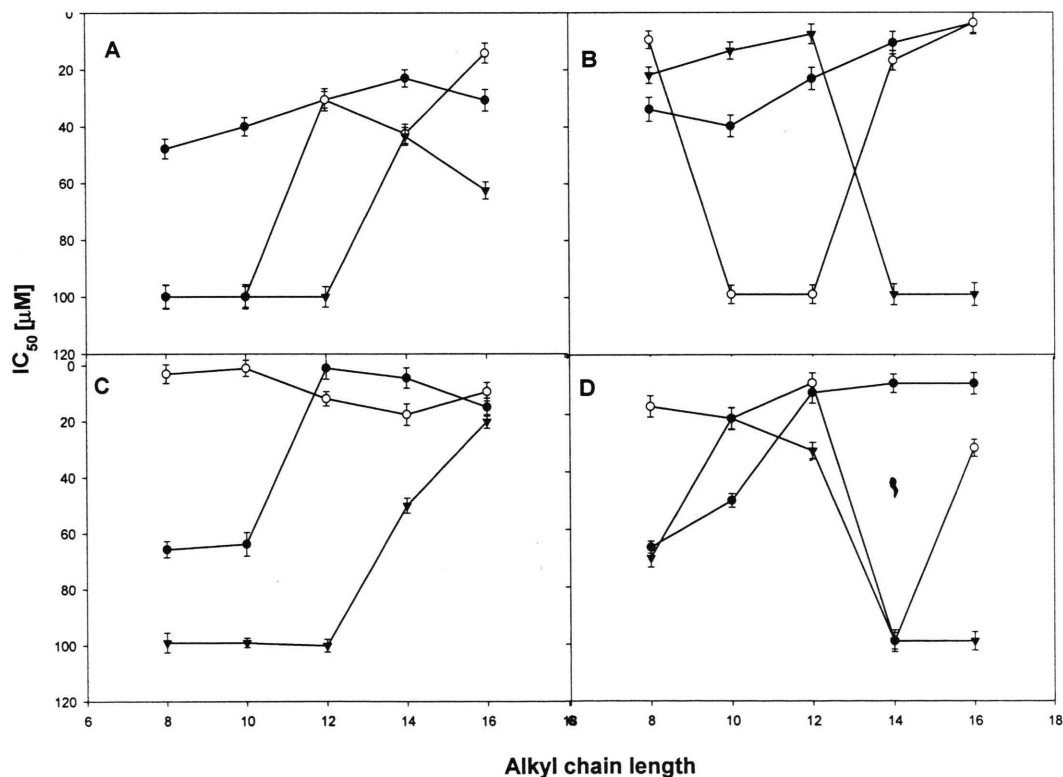

Fig. 3. Dependence of the efficiency of inhibition of prooxidant-induced (**A.** Fe^{2+}, **B.** TBHP, **C.** ACHN, **D.** AAPH) olive oil peroxidation by PYA-a (●), PYE-n (○) and PPA-n (▼) antioxidants (IC$_{50}$ values, μM) on the aliphatic side chain length.
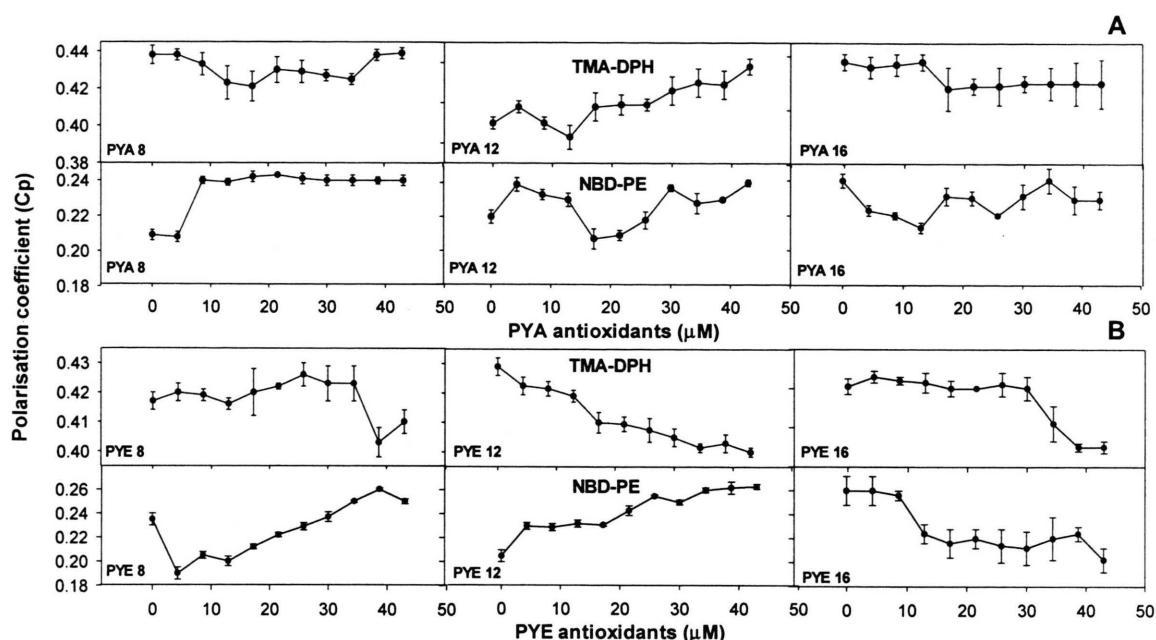
Fig. 4 A, B. Polarisation coefficient C_p of NBD-PE and TMA-DPH versus concentrations of PYA antioxidants. **A.** PYA-n compounds, **B.** PYE-n compounds. The data are given as mean ± SD (n = 3).

two compounds seem to interact with the deeper-immersed TMA-DPH whereas PYE 8 appears to be surface-localised, as is also seen from the effect of its low concentrations on the C_p value of the lipid surface-located NBD-PE. Increasing concentrations of PYE 12 substantially enhanced the C_p of this probe while PYE 16 strongly decreased it. PYE-n antioxidants are strongly amphiphilic. As seen in Table I, the CMC values for PYE are lower than for PYA and this may affect the concentration of the PYA monomers that can incorporate into the lipid phase and exert an antioxidant action there.

PPA-n compounds with 8- to 12-membered alkyl chains were very active against TBHP- and AAPH-induced oxidation of triacylglycerols whereas PPA-14 and PPA-16 blocked effectively ACHN- and Fe^{2+}-induced peroxidation (Figs. 3A-D). We could not determine the interaction of the PPA-n compounds with fluorescent probes in the lipid phase owing to a low solubility of these compounds in the Tris-HCl buffer and large fluctuations in fluorescence characteristics reading observed in ethanol solutions of these compounds.

The CMC values for the PPA-n series were the lowest among all antioxidants (Table I) which may

negatively affect the PPA activity against the oxidants.

Discussion

In our previous studies, PYA-n and PYE-n were found to strongly increase the survival of oxidant-exposed cells of yeast cells lacking different antioxidant enzymes (Krasowska *et al.,* 1999) and to inhibit the TBRS production in whole cells and in membrane lipids isolated from three yeast species differing in oxidant sensitivity (Krasowska *et al.,* 2000).

The present study, performed in a model system, should yield some data on the mode and site of action of these antioxidants, and also of another class of compounds, PPA-n, against different radicals.

In our search for new phenolic antioxidants that could incorporate into, and act on, lipid aggregates (emulsions, micelles, bilayers) at a required depth we have synthesised three groups of compounds with a hindered phenol substituent. Their graded incorporation into the lipid phase was made possible by introducing in their molecules alkyl chains with different number of carbon atoms (Kleszc-

zynska *et al.*, 1998). Using four kinds of oxidation inducers with different hydrophobicity, we assessed the inhibition of lipid peroxidation by these antioxidants.

The initiators AAPH or ACHN produce radicals at a constant rate (Niki *et al.*, 1991). In systems such as emulsions of triacylglycerols (Parthasarathy *et al.*, 1990), these radicals (via secondary peroxyl radicals) induce the formation of lipid hydroperoxides and consumption of antioxidants. Ferrous ions (Fe^{+2}) in the presence of oxygen produce $OH^\bullet$ radicals and can also react with lipid peroxides to form an alkoxyl radical: $L\text{-}OOH + Fe^{2+}\text{-complex} \rightarrow Fe^{3+}\text{-complex} + OH^- + L\text{-}O^\bullet$. This reaction of Fe^{2+} ions with lipid hydroperoxides is very fast – k_2 for $L\text{-}OOH + Fe^{2+}$ is about 1.5×10^3 M^{-1} s^{-1} (Halliwell and Gutteridge 1999). The amphiphilic *tert*-butylhydroperoxide (TBHP) (Evans *et al.*, 1998) produces $t\text{-}BuO^\bullet$ or $t\text{-}BuOO^\bullet$ radicals (Kennedy *et al.*, 1992).

Inhibition of lipid peroxidation by phenolic antioxidants involves scavenging or interception of peroxyl or alkoxyl radicals. In addition, phenolic antioxidants can react very fast with $OH^\bullet$ (Halliwell and Gutteridge, 1999) and also with $O_2^{\bullet-}$ radicals. The reaction of the phenolic group with peroxyl or alkoxyl radicals (Migliavacca *et al.*, 1997) can yield non-radical products: $L\text{-}OO^\bullet + ph\text{-}O^\bullet \Leftrightarrow$ non-radical products. According to Migliavacca *et al.* (1997) one molecule of the phenolic antioxidant can scavenge two free radicals in this way.

When incorporated in the lipid phase, the centre of antioxidant activity, viz. the phenolic ring, should be near the prooxidant molecules to ensure maximum antioxidant activity. As a rule, the shortest-chain compounds should be most active on or near the lipid surface (against both hydrophilic AAPH and amphiphilic TBHP) and the longest-chain molecules of antioxidants, mostly incorporated inside the lipid phase, should have the strongest effect on both hydrophobic ACHN and Fe^{2+}-induced hydroxyl radicals. This has indeed been observed; in particular, lipid peroxidation caused by Fe^{2+} was perceptibly suppressed by long-chain compounds of all three classes

(Fig. 3A). This indicates that, despite its ionic character and hydrophilicity that would imply an especially strong impact of Fe^{2+}-generated perferryl and $OH^\bullet$ radicals at the surface of the lipid phase, Fe^{2+} induces lipid peroxidation that is in fact most intensive within this lipid phase.

Possible mechanisms participating in the very high antioxidant efficiency of PYA, PYE and PPA class compounds (up to 100% suppression of lipid peroxidation caused by a 100-fold excess of the oxidants) may include a fast repair of oxidized lipids and/or regeneration of the antioxidant molecules by reduction of the $ph\text{-}O^\bullet$ radicals.

The differences observed between and inside individual groups of antioxidants indicate that their physical properties, such as critical micellar concentration and/or the partition between the aqueous and the lipid phase, can play an important role in their antioxidant properties. However, these properties do not seem to fully account for the actual antioxidant potency displayed by a particular antioxidant against a given oxidant. Thus the antioxidant action of PYA-n compounds seems to be the same against all oxidants used, and to be independent of the position of the antioxidant molecules within the lipid matrix (as illustrated by the lack of significant effects on the lipid-incorporated fluorescent probes and by the relative independence of action on the aliphatic chain length). On the other hand, the action of PYE-n and PPA-n depends both on the type of oxidant and on the length of their aliphatic chain. However, this relationship may not be simple, and the nature of each of the compounds seems to play a considerable (and not quite clear) role. For instance, the exceedingly large CMC value for PYE-12 obtained by ANS fluorimetry may stem from an interaction of the antioxidant with the fluorescent probe.

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