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Antioxidant synergy between phosphatidyl ethanolamine and α-tocopherylquinone

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Neither phosphatidyl ethanolamine (PE) nor α-tocopherylquinone shows any significant antioxidant activity when added individually to lard. However, a mixture of these compounds shows very strong antioxidant activity at 100°C. The antioxidant activity of the mixture was increased greatly when it had been refluxed in toluene before addition to the lard. One of the major products isolated from the refluxed mixture was identified as α-tocopherol. This finding indicates that PE acts as a secondary antioxidant by reducing quinones formed by the autoxidation of phenolic antioxidants.

INTRODUCTION

Phospholipids are minor components in edible oils and fats but are reduced to low levels during the degumming stage of a refining process. This is necessary in order to reduce refining losses by emulsification, to avoid foaming in the deodoriser, to avoid charring at high temperatures due to the gums associated with the phospholipids, and to avoid carry-through of metal ions as phospholipid complexes. However, phospholipids are added back to oils as emulsifiers in some food products.

The antioxidant activity of phospholipids has been studied extensively (Olcott & Mattill, 1936; Bunyatyn & Kamalyan, 1948; King, Boyd & Sheldon, 1992). Some reports claimed that phosphatidyl ethanolamine (PE) and other phospholipids have primary antioxidant activity (Chipault, 1965). Most reports have shown that phospholipids are not primary antioxidants for fats. However, PE does have a strong synergistic effect with phenolic antioxidants, such as tocopherols, butylated hydroxyanisole, butylated hydroxytoluene, *t*-butylhydroquinone, and flavonoids (Weng, 1991; Hudson & Lewis, 1983; Hudson & Ghavami, 1984; Dziedzic & Hudson, 1984, 1986; Kashima *et al.*, 1991).

The mechanism of the synergistic effect of PE remains unclear. Some reports claim that PE can regenerate a phenolic antioxidant (Olcott & Mattill, 1936, Hudson & Lewis, 1983) just as ascorbic acid regenerates tocopherols (Packer, Slater & Wilson, 1979). Linow and Mieth (1976) suggested that PE acts as a synergist by chelating metals. Other reports suggested that PE has a strong synergistic effect because it can regenerate lipid radicals owing to the presence of an acidic proton (Olcott & Mattill, 1936; Issidorides, 1951; Hudson & Lewis, 1983).

This study was concerned with clarifying the mechanism by which PE acts as a synergist with primary antioxidants in retarding lipid oxidation.

MATERIALS AND METHODS

Materials

α-Tocopherol (95%) and L-α-dilauryl phosphatidyl ethanolamine (DLPE) (98%) were purchased from Sigma Chemical Co.

Pig fat was purchased at Reading Slaughter House, UK, and rendered in the laboratory.

Methods

Induction periods were measured with a Metrohm Rancimat model 617 in lard at 100°C.

Preparation of tocopherylquinone

α-Tocopherylquinone (2·5 g crude) was prepared by oxidation of α-tocopherol (2·616 g) with lead tetraacetate, based on the method of Issidorides (1951). The products was analysed by two-dimensional thin-layer chromatography (TLC) with a prepared-foil silica-gel plate (20 × 20 cm) with fluorescence at 254 nm. In the first direction, the plate was developed with hexane–ethyl acetate (8 : 1) and in the second with toluene–acetone (6 : 1).

Preparative TLC plates coated with silica gel (0·75 mm thick) were used to separate and purify

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α-tocopherylquinone. The separation was performed with hexane–ethyl acetate (4 : 1), *Rf* 0·38, and a second separation was performed with toluene–acetone (10 : 1), *Rf* 0·49. The pure α-tocopherylquinone was a lemon-coloured viscous liquid. The yield of α-tocopherylquinone from oxidised α-tocopherol was 49·4%, after two TLC separations.

Isolation of product from the reaction of α-tocopherylquinone and DLPE

α-Tocopherylquinone (72 mg) and DLPE (92·7 mg) (equimolar) were refluxed in toluene for 42·5 h. After removing the solvent under vacuum, the product was separated by preparative TLC. The plates were developed for 7·0 cm with hexane–chloroform–acetic acid (20 : 20 : 5·2), dried for 5 min in a fume cupboard, and then developed with hexane–ethyl acetate–acetone (30 : 5 : 3). Seven bands appeared after spraying with dichlorofluorescein under UV radiation. The *Rf* values were 0, 0·08, 0·4 (broad black band), 0·53, 0·69, 0·78, 0·89, and these bands are coded 1·7 with increasing *Rf* value.

Spectroscopic methods

IR spectra of KBr discs were recorded with a Perkin-Elmer 577 spectrophotometer; UV spectra were determined in ethanol with a Perkin-Elmer Lamda 5 UV/Vis Spectrophotometer; 400-MHz ^{1}H NMR and 100-MHZ ^{13}C NMR were recorded in deuteriochloroform by the SERC NMR service at the University of Warwick; MS were recorded with a Kratos MS 80 RFA spectrometer with electron–impact ionization, electron energy 70 eV.

RESULTS AND DISCUSSION

The oxidised α-tocopherol was a very complex mixture. There were twelve components appearing when the mixture was analysed by two-dimensional TLC. The major compound was α-tocopherylquinone.

The structure of α-tocopherylquinone was confirmed by the UV, IR, MS, ^{1}H NMR and ^{13}C NMR spectra. Its IR spectrum agreed well with that reported by Issidorides (1951). The *m/z* value of the molecular ion was 446 (Table 1), which agrees with the calculated value.

Both α-tocopherylquinone (0·1%) and DLPE (0·26%) had little primary antioxidant effect in lard (5·0- and 4·2-h induction periods) compared with the control (3·2 h), whereas lard containing the primary antioxidant α-tocopherol (0·1%) had an induction period of 21·8 h (Table 2).

However, when 0·1% α-tocopherylquinone and 0·26% DLPE (molar ratio 1:2) were added to lard as a mixture, the induction period was extended to 22·6 h, and, when mixtures of tocopherylquinone and DLPE were refluxed in toluene for 12 h before addition to lard, the induction periods were even longer. Lard containing the products from α-tocopherylquinone and DLPE, in the ratio 1 : 2·6, had induction periods of 54·3 h and 65·7 h for product added at 0·27% and 0·36%, respectively. When the product from a mixture of 0·019% α-tocopherylquinone and 0·025% DLPE (equimolar) was refluxed in toluene for 37·5 h before addition to lard, the induction period was extended to 24·6 h, and this was more effective than 0·1% α-tocopherol added to lard (21·8 h).

It was remarkable that the mixture of α-tocopherylquinone and DLPE had such a strong antioxidant effect, since neither had a significant antioxidant effect when added to lard alone. The antioxidant activity of the mixture was dramatically increased when it was refluxed in toluene before addition to the lard.

The product from α-tocopherylquinone and DLPE refluxed in toluene was separated as described in the Materials and Methods section.

The TLC bands from the separation were extracted with ethyl acetate. After storage overnight in a refrigerator, one-third of the solutions from bands 2, 3, 4 and 5 was added to the lard to test their antioxidant activity. The induction periods at 100°C were 3·5 h, 8·5 h, 22·5 h, and 24·4 h, respectively. The induction periods of dichlorofluorescein solution (0·5 ml), silica gel (0·1%) added to lard, and a lard control were also determined in order to eliminate the possibility that the antioxidant activity came from contamination with silica gel or dichlorofluorescein. The induction periods were only 2·5 h, 7·4 h, and 3·0 h, respectively, demonstrating that the antioxidant effects of bands 4 and 5 were not caused by contaminants but were due to compounds isolated from the refluxed mixture. However, it is possible that a small part of the antioxidant effect was due to contamination with silica gel.

Band 4 was purified by TLC, eluting with hexane–ethyl–acetate–acetone (8 : 1 : 1). One of the main bands

Table 1. Spectroscopic data for α-tocopherylquinone

Mass spectrum (*m/z*): 447(M+1, 2·0), 446(M, 6·4), 431(5·2), 430(11), 428(11·7), 221(99·8), 203(18·1), 180(22), 179(19·7), 178(76·6), 165(36) 150(23·4) 135(9·2).
m = mass; *z* = charge; M = molecular ion.
^{1}H NMR (δ ppm): 2·51(m, 2H), 2·00(s, 3H), 1·97(s, 6H), 1·47(m, 3H), 1·33(m, 3H), 1·22(t, 2H), 1·20(s, 3H), 1·09(m, 10H), 1·04(m, 6H), 0·80–0·84(12H).
m = multiplet, *s* = singlet, *t* = triplet.
^{13}C NMR (δ ppm): 187·40, 187·07, 144·31, 140·37, 140·26, 140·2, 72·47, 42·3, 40·08, 40·05, 39·21, 37·56, 37·28, 32·63, 27·81, 26·44, 26·41, 24·65, 24·36, 22·58, 22·48, 21·27, 21·17, 19·60, 19·53, 12·22, 12·14, 11·81.
Ultra-violet (nm, absorbance): 367·3(1·985), 259·2(1·995), 199·1(0·558)
Infra-red (cm^{-1}): 3520, 2960, 2936, 2873, 1645, 1463, 1376.

Table 2. Rancimat induction periods of lard containing additives at 100°C

Additive		Induction period (h)
(1)	— (Control 1)	3·2
(2)	α-Tocopherylquinone (0·1%)	5·0
(3)	DLPE (0·26%)	4·2
(4)	α-Tocopherol (0·1%)	21·8
(5)	α-Tocopherylquinone (0·1%) + DLPE (0·26%)	22·6
(6)	Product from α-tocopherylquinone and DLPE (1:2·6) heated in toluene for 12 h (0·27%)	54·3 h
(7)	As (6) at a concentration of 0·36%	65·7 h
(8)	Product from α-tocopherylquinone and DLPE (1:1·3) refluxed in toluene for 37·5 h (0·044%)	24·6 h
(9)	— (Control 2)	3·0 h
(10)	TLC Band 2 from separation of product from α-tocopherylquinone and DLPE	3·5 h
(11)	TLC Band 3	8·5 h
(12)	TLC Band 4	22·5 h
(13)	TLC Band 5	24·4 h
(14)	Dichlorofluorescein (0·5 ml)	2·5 h
(15)	Silica gel (0·1%)	7·4 h

Table 3. Spectroscopic data for α-tocopherol

Mass spectrum (*m/z*): 431(M+1, 32·8), 430(M, 100), 205(9·3), 203(5·3), 166(10·6), 165(83·9), 164(26·9), 121(4·8).
^{1}H NMR (δ ppm): 4·16(s, 2H), 2·59(t, 2H), 2·15(s, 3H), 2·10(s, 6H), 1·77(m, 2H), 1·53(m, 2H), 1·13(m, 10H), 1·22(s, 3H), 1·06(m, 6H), 0·82–0·87(m, 12H).
^{13}C NMR (δ ppm): 145·44, 144·42, 122·52, 120·88, 118·33, 117·26, 74·43, 39·77, 39·70, 39·27, 37·35, 37·28, 37·18, 32·68, 32·59, 27·88, 24·71, 23·61, 22·62, 22·53, 20·95, 20·65, 19·65, 19·58, 12·11, 11·67, 11·17.
Ultra-violet (nm, absorbance): 292·1(0·995), 224·8(2·093)

was blue under UV radiation with *Rf* 0·47 after spraying with dichlorofluorescein; band 5 was purified by TLC elution with the same solvents to give a main band that had a similar *Rf* value to that of band 4. These two were therefore mixed and purified further by TLC eluting with hexane–toluene–chloroform (1 : 1 : 2). One very strong blue band appeared under UV radiation with *Rf* 0·47, which was a similar *Rf* value to that of a tocopherol standard. This band became pink under natural light after spraying with dichlorofluorescein. On the same plate, the *Rf* values of α-tocopherylquinone and DLPE were 0·067 and 0.

The compound isolated from the refluxed mixture had almost the same UV spectrum as that of standard α-tocopherol (Table 3) with maximum absorptions at 292·1 nm (0·995 A) and 224·8 nm (2·093 A). After adding potassium hydroxide, the maximum absorptions were shifted to 311·4 nm. This bathochromic shift of 19·3 nm was similar to that of a sample of α-tocopherol which had a shift of 20·2 nm on adding potassium hydroxide.

The molecular ion of the mass spectrum was at *m/z* 430, as observed for α-tocopherol. ^{1}H NMR and ^{13}C NMR spectral data also confirmed the compound to be α-tocopherol.

It is well known that phenolic antioxidants are oxidised to a mixture of products including quinones during autoxidation in oils. Kikugawa, Kungi, and Kurechi (1990) reviewed this area comprehensively. One of the degraded compounds from α-tocopherol is α-tocopherylquinone. It is clear from our research that PE can reduce α-tocopherylquinone to α-tocopherol, and this must be at least part of the reason for its antioxidant effect at elevated temperatures.

It should be noted that traces of iron or copper eliminated the antioxidant synergistic properties of PE (Rahman, 1990) and this is likely to be the cause of some of the confusion in the literature about whether PE is an antioxidant synergist.

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