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Co-oxidation of Fat-Soluble Vitamins by Soybean Lipoxygenase

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ABSTRACT

Lipoxygenase-catalysed oxidation of linoleic acid can be accompanied by considerable losses of fat-soluble vitamins. Vitamins A, D_2, D_3 and E are all susceptible to rapid co-oxidation at neutral pH in the presence of soybean lipoxygenase. Retinal appears to be more stable towards co-oxidation than retinol, which is similar in stability to β-carotene. It is concluded that extensive conjugation is not required for co-oxidation of lipids, but the nature of polar substituents may have a significant effect on the rate of co-oxidation.

INTRODUCTION

The enzyme lipoxygenase (linoleate: oxygen oxidoreductase EC 1.13.11.12) occurs in a wide variety of plant tissues where it catalyses the oxygenation of polyunsaturated fatty acids containing a *cis*, *cis*-1, 4-pentadiene unit to a conjugated dienoic hydroperoxide (Eskin *et al.*, 1977). Other products including ketodienes and volatile degradation products may also be formed, and pigments such as carotenoids and chlorophyll can suffer co-oxidation. Type-2 lipoxygenase isoenzymes, which have optimum activity at pH 6–7, are more effective at catalysing co-oxidation than type-1 isoenzymes which

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have optimum activity at pH9. Very little is known about the active site of lipoxygenase involved in co-oxidation reactions, but considerable progress has been made in understanding the mechanisms of the primary oxidation reaction under aerobic and anaerobic conditions (Klein *et al.*, 1985). It is known that lipoxygenase-catalysed oxidation is sensitive to inhibition by antioxidants including tocopherol, hydroquinone, propyl gallate, nordihydroguaiaretic acid, butylated hydroxytoluene and butylated hydroxyanisole (Eskin *et al.*, 1977). It has been shown that α-tocopherol is destroyed during the lipoxygenase-catalysed oxidation of linoleic acid (Tappel *et al.*, 1953). Although vitamins A and D do not occur naturally in plant tissues, they may be present in food products containing animal components and therefore this study was concerned with a detailed investigation of the co-oxidation of vitamins A, E and D by soybean lipoxygenase. It was also considered that this investigation would provide further information about selectivity in the co-oxidation reaction.

MATERIALS AND METHODS

Soybean lipoxygenase (Sigma Type I, lyophilised, 125 000–175 000 units mg^{-1} protein), α-tocopherol (synthetic, 95%), vitamins A (synthetic 85%), D_2 (synthetic), D_3 (synthetic), β-carotene (Type 1) and linoleic acid (99%) were purchased from Sigma Chemical Company Limited. *N,O*-bis (trimethylsilyl) acetamide was purchased from the Pierce Chemical Co., and Tween 80 was purchased from BDH Chemicals Ltd.

Aqueous linoleic acid solution was prepared by mixing linoleic acid in ethanol (1 ml, 2·25% m/v), Tween 80 (0·3 ml, 10% in ethanol) and EDTA (5 ml, 0·25% m/v in water). Sodium hydroxide (1M) was added until the pH reached 9·0, and the volume was increased to 10 ml with water. Co-oxidation of α-tocopherol was studied by mixing aqueous linoleic acid solution (10 ml), α-tocopherol solution (220 μg in ethanol, 1 ml) with citrate-phosphate buffer (0·2M, 27 ml, pH 7·4) at 30°C. Lipoxygenase solution (2 ml, 50 μg ml^{-1}) was added and air was bubbled through the mixture for 30 s. Analysis of unreacted tocopherol was performed by removing samples (2 ml) and extraction with chloroform–ethanol mixture (10 ml, 10:3). The chloroform layer was separated, washed with water and dried with anhydrous sodium sulphate. An aliquot (1 ml) of the chloroform solution was mixed with a solution of 5 α-cholestane in chloroform (0·05 ml, 0·4 mg/ml) and evaporated to dryness under nitrogen. The residue was silylated in pyridine (0·1 ml) with *N,O*-bis (trimethylsilyl) acetamide (0·05 ml) at 40°C for 15 min. The solution was diluted to 0·2 ml with chloroform and analysed by capillary GLC, using a BP-1 W.C.O.T. column (25 m × 0·25 mm i.d.) in a Perkin-Elmer Sigma 3B

chromatograph. On-column injection was used, with a temperature programme of 50°C (1 min) rising at 25°C min^{-1} to a final temperature of 270°C. The flame ionisation detector temperature was 300°C, and helium was the carrier gas. Linearity of the detector was demonstrated up to concentrations of 0·2 $\mu g\, \mu l^{-1}$ by analysing silylated mixtures of α-tocopherol and 5α-cholestane and plotting the ratio of peak areas against tocopherol concentration. This standard curve was used to calculate unreacted α-tocopherol in the lipoxygenase solutions. Efficiency of extraction of α-tocopherol from the lipoxygenase solution was confirmed as 100% by analysing a sample of tocopherol extracted from a solution which was identical to the lipoxygenase solution except for the absence of the enzyme.

Vitamins D_2 and D_3 were co-oxidised and analysed in an identical procedure to that of α-tocopherol, except that silylation was only performed for 5 min. Reaction media were prepared using Vitamin D_2 (202 μg) and Vitamin D_3 (196 μg) instead of α-tocopherol (220 μg). Thermal decomposition in the GLC column gave two peaks for each vitamin corresponding to pyro and isopyro products (*c.* 2:1) as reported by Ziffer *et al.* (1960). The sum of the two peaks was used in the quantification of each vitamin. The detector response was shown to be linear up to $50 \times 10^{-3}\, \mu g\, \mu l^{-1}$.

The co-oxidation of retinol, retinyl acetate, retinal and β-carotene was studied on a small scale in a spectrophotometer cuvette. Lipid (e.g. 25 mg for β-carotene) mixed with Tween 80 (0·9 ml) was dissolved in chloroform (25 ml). A sample of the chloroform solution (2 ml) was evaporated to dryness and EDTA solution (10 ml, 0·25%) was added. Aqueous linoleic acid solution was prepared from a solution of linoleic acid in ethanol (1 ml, 7·5%) mixed with Tween 80 in ethanol (0·3 ml, 10%) to which aqueous EDTA (5 ml, 0·5%) was added before the pH was adjusted to 9 with sodium hydroxide solution (1 M). The volume was increased to 10 ml with water.

Aqueous linoleic acid solution (0·5 ml) was mixed with solutions of β-carotene or the vitamin A derivatives (0·5 ml) and citric acid–disodium hydrogen phosphate buffer (0·3M, 9 ml, pH 7·4) was added. A sample of the buffered solution (1·5 ml) was transferred to a spectrophotometer cuvette and distilled water (0·4 ml) and lipoxygenase solution (0·1 ml containing 12 μg enzyme) were added. β-carotene, retinyl acetate, retinol and retinal were determined spectrophotometrically with a Perkin-Elmer 552 spectrophotometer from the absorbance at 460 nm, 325 nm, 325 nm and 382 nm, respectively, using values of 88 000, 37 313, 20 000 and 32 394 litres mol^{-1} cm^{-1} for the molar absorptivity. Molar absorptivity values were determined for solutions of the nutrients in the assay medium.

Lipoxygenase-catalysed oxidation of linoleic acid was monitored by spectrophotometric determination of the increase in absorbance at 234 nm, according to the method of Ben Aziz *et al.* (1970). The hydroperoxide

concentration was calculated using the molar absorptivity 28 000 litres mol^{-1} cm^{-1} (Privett *et al.*, 1955).

RESULTS AND DISCUSSION

Vitamins D_2 and D_3 did not appear to suffer any co-oxidation at 20°C (possibly due to solubility problems) and therefore the co-oxidation of the vitamins was compared with that of α-tocopherol at 30°C (see Fig. 1). It was evident that most of the oxidation occurred within the first 10 mins of the reaction. The importance of bubbling air into the sample was checked and it

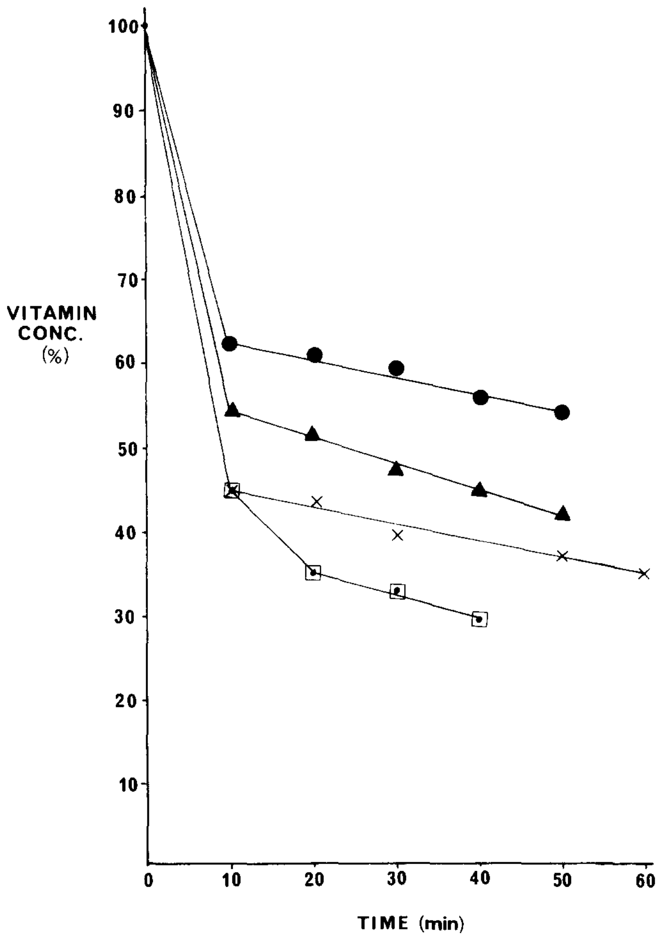

Fig. 1. Co-oxidation of fat-soluble vitamins in the presence of soybean lipoxygenase and linoleic acid (2×10^{-3}M) at 30°C. ●: Vitamin D_2 ($1{\cdot}28 \times 10^{-4}$M); ▲: Vitamin D_3 ($1{\cdot}28 \times 10^{-4}$M); ×: Vitamin E ($2{\cdot}56 \times 10^{-4}$M); ⊡: Vitamin E ($1{\cdot}28 \times 10^{-4}$M).

was found that vitamin D_2 was oxidised at an initial rate of 4·60 μmol litre^{-1} min^{-1} when air was bubbled into the sample for 30 s and 4·37 μmol litre^{-1} min^{-1} when no air was bubbled into the sample. Hence it appears that the bubbling of air into the sample does not have a significant effect on the rate of co-oxidation, and it can be concluded that the reaction is occurring under aerobic conditions in both cases.

The three vitamins D_2, D_3 and E were all strongly co-oxidised with 38%, 46·5%, and 55% of the vitamins being lost within the first 10 mins. Increasing the concentration of vitamin E from $1{\cdot}28 \times 10^{-4}$M to $2{\cdot}56 \times 10^{-4}$M increased the rate of co-oxidation of the vitamin from 69·8 μmol litre^{-1} min^{-1} to 139·6 μmol litre^{-1} min^{-1} but the percentage of the vitamin lost within the first 10 mins remained the same. The three vitamins all inhibited the oxidation of linoleic acid by lipoxygenase (Table 1), but vitamin E was most effective at a concentration of 10^{-5}M. Klein *et al.* (1985) suggest that co-oxidation may proceed by interaction of co-oxidant with an enzyme–peroxyl radical, or an alkoxyl radical or even an enzyme-oxyl radical formed by cleavage of the enzyme–peroxyl radical complex. If a free alkoxyl radical was involved it might be expected that vitamin E would be much more effective at inhibiting the reaction than vitamin D, since vitamin E is an effective antioxidant for autoxidation. Since the difference in effectiveness of the vitamins is small, it appears more likely that they compete with linoleic acid for an enzyme–radical complex. It is notable that vitamins D_2 and D_3 inhibit the oxidation of linoleic acid (Table 1) at 10^{-6} or 10^{-5}M, while no co-oxidation of these vitamins was detected at 20°C. This reflects the relative insensitivity of the methods used for monitoring loss of

TABLE 1
Effect of Additives on the Initial Rate of Oxidation of Linoleic Acid at 21°C.

Additive	*Rate in absence of additive*[a] (*μmol litre^{-1} min^{-1}*)	*% Inhibition*	
		10^{-6}M	*10^{-5}M*
Vitamin D_2	45·4	11·9	13·6
Vitamin D_3	51·9	5·2	11·8
Vitamin E	58·2	7·4	29·6

[a] Differences in maximum rate in the absence of additives were observed but the reaction was reproducible when the same aqueous linoleic acid solution was used, and therefore the same aqueous linoleic acid solution was used for the control and the solutions containing a particular added vitamin. The aqueous linoleic acid solution was prepared freshly each day and it appears that differences in droplet size in the emulsion have an effect on the oxidation rate.

TABLE 2
Rate of Co-oxidation of Additives ($1\cdot4 \times 10^{-5}$M) in the Presence of Linoleic Acid (10^{-3}M) at 21°C

Additive	*Initial Co-oxidation rate* ($\mu mol\ litre^{-1}\ min^{-1}$)
β-carotene	14·4
Retinol	12·7
Retinyl acetate	8·3
Retinal	1·9

vitamins D_2 and D_3. All studies of co-oxidation of vitamins D_2 and D_3 involved the use of vitamin concentrations in excess of 10^{-4}M and solubility problems appeared to occur with these vitamins at 20°C at 10^{-4}M.

In order to gain more information about the nature of co-oxidation, the rates of co-oxidation of β-carotene and various vitamin A derivatives were compared. It appeared that β-carotene and retinol were similar in co-oxidation rate, while retinyl acetate was oxidised more slowly and retinal was the most stable towards co-oxidation (Table 2). These results suggest that a wide range of polyenes may be susceptible to co-oxidation. The precise rate of co-oxidation is likely to depend on the stability of the polyene–enzyme–radical complex. It has been shown that β-carotene is oxidised about three times as fast as the polyene glycoside crocin by the isoenzymes with a pH optimum of 6·5 (Weber *et al.*, 1974) while canthaxanthin is oxidised at the same speed as β-carotene (Grosch *et al.*, 1977). The products from the co-oxidation of β-carotene indicate that oxidation proceeds at several positions along the polyene chain (Grosch *et al.*, 1977). Since vitamin D is oxidised at quite a fast rate with only three conjugated double bonds, it is clear that extensive conjugation is not required for co-oxidation. The slow rate of co-oxidation of retinal compared with retinol suggests that the nature of polar substituents can have a significant effect on the rate of co-oxidation.

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