weight \(^1\); means \(\pm\) S.D.), demonstrating a role for this enzyme in the maintenance of cellular LHPO levels.

**Conclusion**

The FOX2 method readily detected the LHPO content of plant tissues. The levels of LHPO in plant tissues are maintained at a basal level of between 0.6 and 1.7 \(\%\), of total fatty acids in a wide range of tissues examined. The method of LHPO determination reported here is a rapid and simple technique and may be useful for the determination of LHPO in tissues where the levels of these compounds are expected to vary, such as in plants grown under conditions of abiotic and biotic stress or during the course of senescence in plant organs.

We thank the BBSRC, the MAFF, the British Council and the Spanish Ministryo de Educacion y Ciencia Acciones Integradas Programme.

---

**References**


Received 23 June 2000

---

**Lipoxygenase-catalysed degradation of carotenoids from tomato in the presence of antioxidant vitamins**

P. A. Biacs and H. G. Daoood

Central Food Research Institute, Herman Ottó u. 15, 1022 Budapest, Hungary

**Abstract**

Carotenoid extract from ripe tomato fruit was subjected to a lipoxygenase-catalysed co-oxidation in the presence of vitamin C and vitamin E at different concentrations. Relative retention (\(\%\)) of major carotenoids by the experimental mixture was used as an index of their degradation and interaction with the antioxidants. Oxidation-prevention activity of each antioxidant against pigment co-oxidation as impacted by their molar concentration was studied. \(\beta\)-Carotene was found to be the most sensitive pigment, followed by lycopene and lycoblitin. Ascorbic acid when added in the range of 0–1.8 mM interacted with the different carotenoids by different modes. Evidence was given on regeneration, by ascorbic acid, of lycopene during the course of co-oxidation. The concentration required for \(\alpha\)-tocopherol acetate to exhibit antioxidative effect was 10 times higher than that of ascorbic acid. \(\beta\)-Carotene was prevented, by \(\alpha\)-tocopherol acetate, faster than lycoblitin and lycopene. The latter carotenoids differed substantially in their interaction with the lipophilic antioxidant at only the lowest concentration (0.3 mM). It was established that under the given conditions there is no synergism between vitamin C and vitamin E that improves their oxidation prevention against co-oxidation of carotenoids. Moreover, the combined use of antioxidants caused more oxidative degradation of \(\beta\)-carotene.

**Introduction**

The colour of ripe tomato fruit is attributed to carotenoid-type pigments that have technological, nutritional and biological importance. Most of the carotenoids from tomatoes have been found in different tissues of human and animal bodies \([1–3]\). Owing to their potential vitamin A activity some carotenoids are of nutritional importance. It is essential to be able to distinguish among different carotenoids because, due to structural differences, the biological activity of all carotenoids is not the same \([3,4]\). This becomes particularly important in products such as tomatoes where the major carotenoid, lycopene, has no vitamin A activity.
Biochemical Society Transactions (2000), Volume 28, part 6

Also, interest in carotenoids has increased recently because of their possible association with the process and progress of carcinogenesis [6,7].

In addition to carotenoids, tomato fruit distributes considerable quantities of other bioactive compounds such as α-tocopherol and ascorbic acid, the most important antioxidant vitamins [8]. These compounds together with carotenoids determine antioxidation potency in tomato products.

Oxidizing enzymes are of increasing interest because of their effect on both the colour and flavour of plant foods. Of these enzymes, lipoxygenase (EC 1.13.11.12; LOX, linoleate:oxygen oxidoreductase) is of special interest. The enzyme catalyses the oxidation of polyunsaturated fatty acids containing a 1,4-cis-cis-pentadiene system. The enzyme may also catalyse the co-oxidation of carotenoids, resulting in the loss of natural colourants and essential nutrients [9]. Two LOXs have been found in tomato fruits, soluble and membrane-associated [10]. Both isozymes have carotenoid co-oxidation activity [11–13]. As stated by Klein et al. [14,15] and Ludwig et al. [16] co-oxidation of carotenoids by LOX may be catalysed by a free-radical mechanism, and it is possible that the enzyme is an integral part of the system for co-oxidation.

The objective of this work was to assay LOX-catalysed co-oxidation of carotenoids from tomato extract using a HPLC procedure and to study in depth how α-tocopherol and ascorbic acid interact with the different carotenoids in the course of linoleic acid oxidation. The effect of antioxidant concentration on the prevention efficiency against co-oxidative damage of carotenoids was also studied.

Materials and methods

Materials

Tomato fruits were obtained from the University of Horticulture and Food Industry, Budapest, Hungary. The samples were stored at -20 °C in vacuumed nylon sacs when not analysed immediately.

Analytical-grade methanol and carbon tetrachloride were from Reanal (Budapest, Hungary). The HPLC-grade acetonitrile, propan-2-ol and methanol were purchased from Merck. Purified soya LOX, linoleic acid, Tween-20 and ascorbyl palmitate (96 %) were from Sigma. α-Tocopherol (97 %), α-tocopherol acetate (98 %) and L-ascorbic acid (99 %) were from Aldrich.

Pigment extraction

Lipid fraction including fat-soluble pigments was extracted by a previously described procedure [16a]. After disintegration of 20 g of ripe tomato fruit in a crucible mortar in the presence of 1 g of quartz sand, the water was cached by adding 25 ml of methanol. The mixture was transferred to a 100 ml conical flask and 60 ml of 5:1 CCl<sub>4</sub>/methanol was added. The mixture was separated into two phases by adding a few drops of distilled water. The complete transfer of fat-soluble pigment to the CCl<sub>4</sub> layer was achieved by shaking for 15 min. The mixture was then transferred to a separatory funnel and the CCl<sub>4</sub> layer was dried on anhydrous Na<sub>2</sub>SO<sub>4</sub>. The solvent was then removed under vacuum using a rotary evaporator at 40 °C.

Preparation of LOX solution

Purified soya LOX (1 mg) containing 123000 units was dissolved in 100 ml of phosphate buffer, pH 7. One unit is the amount that will cause an increase in absorbance at 234 nm of 0.001/min at pH 9.0 and 25 °C when linoleic acid is used as substrate.

Co-oxidation protocol

To the extracted carotenoids 50 μl of Tween-20, 50 μl of linoleic acid and 10 ml of chloroform were added and mixed. The solvent was evaporated under vacuum at 40 °C. The residues were suspended in 18 ml of 0.01 M phosphate buffer, pH 9, with the help of ultrasonication. The suspension was divided into five portions; each was transferred to a small flask and agitated with an electric vibrator for 1 min to insert a sufficient amount of air into the mixture. The co-oxidation reaction was induced by adding 100 μl of freshly prepared solution of soya LOX (123 units). Adding 20 ml of methanol to the mixture at 0, 15, 30, 45 and 60 min of incubation stopped the reaction. The pigments were extracted by the same procedure as described above. A blank experiment was done in the same way except that no enzyme preparation was added.

To study the effect of antioxidant vitamins on the oxidative degradation of pigments, ascorbic acid and α-tocopherol acetate were added to the suspension before the enzyme's addition at concentrations of 0–1.8 and 0–10 mM respectively.

HPLC determination

A Beckman-series liquid chromatograph consisting of a model 114 solvent-delivery pump, a
model 421 controller and a model 165 variable-wavelength UV/visible detector was used. A model C-R2A recorded the detector signals. A C-R2A Shimadzu integrator was used. To study the spectral characteristics of the individual carotenoids a Waters model 990 photodiode-array detector was used. The data were stored and processed by means of an NEC.APCIV power Meta 2 IBM computing system. The absorption spectra of carotenoids were recorded between 190 and 700 nm at the ratio of 2 spectra/s.

Separation of carotenoids was performed on columns (25 cm length × 4.6 mm inner diameter) packed with lichrosorb C₁₈ of 6 μm particles. The mobile phase was acetonitrile/isopropanol/methanol/water (39:52:6:4) and the flow rate was 0.9 ml/min [16b].

**Peak identification**
Retention times and spectral characteristics of the carotenoids were compared with those of the standard (β-carotene) and authentic materials (lycopene and lycxanthin) prepared according to the method of Daoed et al. [16a].

**Calculation of formulae**
Determination of the oxidative degradation of each pigment was based on measurement of its percentage retention by the system, taking retention at time 0 to be 100%.

The retention was calculated from the change in the peak area of the component on the HPLC chromatogram at 0, 15, 30, 45 and 60 min of the oxidation reaction:

\[
\% \text{ Retention} = \frac{\text{Peak area at time 0} \times 100}{\text{Peak area at time intervals}}
\]

Antioxidant performance of antioxidant vitamins was expressed as the increase in % retention of the pigment caused by addition of the antioxidant after 30 min of incubation with the enzyme.

The increase in pigment content (%) as a function of antioxidant addition was expressed as Δ pigment retention, which was calculated by the following expression:

\[
\Delta \text{Retention} = \% \text{ retention without antioxidant} - \% \text{ retention with antioxidant}
\]

**Results and discussion**
The use of LOX in the accelerated oxidation system described in this work was based on the fact that the enzyme is a free-radical-generating biocatalyst bleaching fat-soluble pigments through its aerobic or anaerobic pathway [17]. Furthermore, availability of its substrate (unsaturated lipids) and occurrence of different iso-enzymes in tomatoes make it possible that the loss of carotenoids as a whole at post-harvest and during processing is related to LOX activity.

To avoid interference of non-enzymatic proteins in the course of pigment co-oxidation, highly purified soya LOX-1 was used. LOX-1 from soya has been reported to have similar co-oxidative properties to both the soluble and membrane-associated tomato LOXs [11,12], which are not available commercially.

To check the accuracy of the procedure applied to determine retention of pigment in the reaction mixture at time 0 and after a period, four parallel measurements were performed. The coefficient of variation ranged between 4.2 and 7.6 depending on the type of the pigment examined. The experimental error within this range makes the level of confidence and accuracy of the assay method acceptable from the analytical point of view.

Figure 1 shows the susceptibility of the major carotenoids of tomato extract to LOX-catalysed co-oxidation. β-Carotene was found to be the most sensitive component, followed by lycxanthin and lycopene. This agrees with the findings of Cabibel...
and Nicolas [13] that β-carotene is oxidized faster than other carotenoids by purified tomato LOX. These results also implied that β-carotene can actively perform its antioxidant function during the course of lipid oxidation. In a previous work (results not shown) the rate of degradation of β-carotene has been found to be lower than that of chlorophyll when an extract of parsley leaves has been subjected to LOX-catalysed co-oxidation. It therefore seems that oxidative degradation and, accordingly, antioxidant activity of each carotenoid depends on the rate of its interaction with the peroxyl radical produced through the LOX pathway.

Ascorbic acid, when added at different concentrations to the mixture prior to the addition of LOX, inhibited co-oxidative damage of lycopene (Figure 2) and in a similar way lycopethin (results not shown). Increasing the concentration of ascorbic acid up to 1.8 mM prevented most 93% and 79% of the original lycopene and lycopethin, respectively, against co-oxidative degradation. It was remarkable that at low doses of ascorbic acid there was slight antioxidant effect in the first 30 min of the reaction time followed by a marked increase in the content of lycopene, and to some extent lycopethin, in the experimental system. Convincing evidence is given here that ascorbic acid interacts weakly with the free radicals of lipids as compared with carotenoids, but in a time-dependent reaction it interacts with the oxidized forms of lycopene and lycopethin to generate their original structures. The rate of such an interaction was increased by increasing the ascorbic acid concentration so that at 1.8 mM the regeneration process seems to be taking place immediately after the initiation of the co-oxidation reaction.

Shown in Figure 3 is the change in the retention of β-carotene by the reaction mixture as a function of time and concentration of added ascorbic acid. The degradation rate of β-carotene could be decreased slightly by addition of ascorbic acid even at the highest dose (1.8 mM). This reveals on the one hand the high antioxidant activity of β-carotene, and on the other hand the low ability of ascorbic acid to compete with carotenoids as an antioxidant under the conditions used. From these results it was also evident that there is a weak radical exchange between oxidized forms of β-carotene and ascorbic acid, as occurred with lycopene and lycopethin. This supported the suggestion of Regdel et al. [18] that different fat-soluble pigments are co-oxidized by LOX with different mechanisms. The results led to the hypothesis that β-carotene in a fully oxygenated system would react with dioxygen to form peroxyl radicals that exchange very slowly with the bulk.
aqueous solvent. It is also possible that under the conditions used in this study β-carotene reacts with carbon-centred radicals to produce stable carbon-centred radicals that might not abstract a hydrogen atom from the surrounding H-donors such as ascorbic acid and fatty acids.

From the practical and economical points of view it is important to estimate the concentration of any antioxidant at which the maximum preventive effect towards carotenoid oxidation can be approached. As shown in Figure 4, increasing the concentration of ascorbic acid from 0 to 1.8 mM resulted in an increase in the retention of carotenoids by the mixture. However, at the lowest dose, ascorbic acid interacted with the different carotenoids by different modes. It was remarkable and surprising that addition of ascorbic acid at 0.3 mM caused more oxidative degradation of β-carotene. At higher doses ascorbic acid tended to protect β-carotene against oxidative degradation. Also, with lycoxanthin there was no protective effect of ascorbic acid up to 0.3 mM. The best protective effect was on lycopene, which exhibited quick interaction with the added ascorbic acid. Over 0.3 mM, the rates of ascorbic-lycopene and ascorbic-lycoxanthin interactions were almost similar and, in each case, higher than that of the ascorbic-β-carotene interaction. These results implied that much more ascorbic acid should be added in order to prevent the entire β-carotene content oxidizing.

The manner of α-tocopherol acetate in prevention of oxidation of tomato carotenoids is different from that of ascorbic acid. Figure 5 shows the change in retention of β-carotene as a function of time of LOX-catalysed co-oxidation and concentration of α-tocopherol acetate. The degradation rate of β-carotene was lowered markedly by addition of α-tocopherol acetate. Retention of β-carotene by the mixture after 60 min of co-oxidation reaction could be increased from 18% to 72% by adding the antioxidant at a concentration of 7.5 mM. This concentration is 5.6 times higher than the concentration required for ascorbic acid to show maximum prevention of β-carotene against co-oxidative damage. As stated by Pryor et al. [19] α-tocopherol shows a different interaction with the oxidized lipids from that of other antioxidants. It should be realized that the mobility of α-tocopherol acetate in a non-homogenous aqueous system can make its prevention efficiency much less than that of ascorbic acid.
As compared with β-carotene, lycopene and lycoxanthin were protected to a lower extent by α-tocopherol acetate when added to the reaction mixture, particularly at 5 mM (Figure 6). This may reflect the fact that interaction of α-tocopherol acetate is easier with β-carotene than with other pigments. At concentrations higher than 5 mM, α-tocopherol acetate was scarcely effective in prevention of β-carotene oxidation and caused a gradual increase in the stability of the other pigment towards co-oxidative damage. Under the given conditions and applied concentrations no pro-oxidant effect of α-tocopherol was observed. As stated by Cillard and Cillard [20,21], the pro-oxidant effect of α-tocopherol in linoleic acid dispersion can be influenced by several factors that affect the difference between the resonance energies of the tocopheroxyl radical and tocopherol.

It seems that α-tocopherol interacted with the different pigments by the same mode most probably because the functional parts of the antioxidant and the pigments have similar lipophilic properties in the aqueous dispersion. Despite the high effectiveness of α-tocopherol at the given concentrations towards pigment oxidation, the maximum

**Figure 6**
Prevention of lycopene from LOX-catalysed degradation by addition of α-tocopherol acetate at different concentrations

Prevention from LOX-catalysed degradation is expressed as the increase in % retention for each pigment in the experimental system.

**Figure 7**
Stability of lycopene (upper panel) and β-carotene (lower panel) in the experimental system towards LOX-catalysed co-oxidative degradation in the presence of 0.6 mM ascorbic acid, 5 mM α-tocopherol acetate, or a mixture of them both

Abbreviations: No ant., no antioxidant added; ASC, ascorbic acid; Toc., α-tocopherol acetate; mixt., mixture.
Combined use of antioxidants

Interactions between vitamin C and vitamin E have aroused great interest lately. According to speculations and experiments of many authors, these interactions result in an improved antioxidant effect during lipid oxidation [22,23]. It has been confirmed repeatedly that ascorbic acid regenerates α-tocopherol by intercepting the primary radical formed in the initial step of lipid oxidation [24]. Using chemically induced radical-generating systems in an oil-in-water emulsion Niki et al. [25] and Barclay et al. [26] showed the synergism between α-tocopherol and ascorbic acid. In the present study we aimed at investigating whether the combined use of α-tocopherol and ascorbic acid results in a synergism between them in a system of LOX-catalysed oxidation of unsaturated fatty acid.

With regard to prevention of co-oxidative damage of lycopene, adding 0.6 mM of ascorbic acid together with 2.5 mM α-tocopherol acetate slightly lowered the rate of pigment degradation as compared with that obtained when only α-tocopherol acetate was added (Figure 7, upper panel). Surprisingly, the combined use of antioxidants worsened the stability of β-carotene in the system. From these results it is evident that synergism between α-tocopherol and ascorbic acid scarcely takes place in a system containing carotenoids, as the interaction happens actively between the added antioxidants and radical species of fat-soluble pigments (Figure 7, lower panel). This supported what Bruun-Jensen et al. [27] found in their work on antioxidant effects of tocopherol and ascorbic acid in stored turkey meatballs. The authors stated that separate addition of these antioxidants has a better antioxidant effect than their combined use.

References


Received 7 August 2000