Co-oxidation of β-Carotene Catalyzed by Soybean and Recombinant Pea Lipoxygenases

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A number of products including apocarotenal, epoxycarotenal, apocarotenone, and epoxycarotenone generated by lipoxygenase (LOX) catalyzed co-oxidation of β-carotene have been tentatively identified through the use of GC/MS and HPLC combined with photodiode array detection. Because of the large number of high molecular weight products detected and their probable chemical structures, a co-oxidation mechanism is proposed that involves random attack along the alkene chain of the carotenoid by a LOX-generated linoleoylperoxyl radical. It is suggested that a direct release from the enzyme of the radical, which initiates the co-oxidation of β-carotene, is greater for pea LOX-3 than for pea LOX-2 or soybean LOX-1. It is proposed that further products may be formed by free radical propagated reactions and that the formation of 1,10- and 1,14-dicarbonyl compounds may arise by secondary oxidation of the primary products.

Keywords: *β-Carotene; lipoxygenase (LOX); antioxidant; co-oxidation; HPLC/MS; GC/MS*

INTRODUCTION

Carotenoids are attracting considerable interest in very diverse fields of research. These include not only food science and nutrition but also immunology, epidemiology, public health, radiobiology, and biochemistry (Bendich, 1989) because of their antioxidant activity and possible roles in the treatment of human diseases. For instance, some carotenoids have been found to be effective against skin and lung cancers, possibly because of their antioxidant and free radical trapping ability (Chen and Chen, 1994; Ziegler, 1989). Although recently it has been claimed that β-carotene could have exacerbation effects on lung cancers of heavy smokers (ATBC, 1994; Omenn et al., 1996), it is still one of the most common antioxidants and β-carotene supplementation is entirely safe for normal subjects who do not smoke (Diplock et al., 1998). Both in vivo and in vitro experiments have shown that β-carotene can protect phagocytic cells against damage from autoxidation (Bendich, 1989). β-Carotene, cryptoxanthin, and α-carotene are the major dietary precursors of vitamin A in mammals, whereas fish can convert a number of other carotenoids to vitamin A (Olson, 1989). β-Carotene is unique among the provitamin A carotenoids as it has the theoretical potential to form two molecules of vitamin A, but in reality the oxidative and reductive reactions are equated to one-sixth of the theoretical yield (Sivell et al., 1984).

It is well-known that lipid oxidation has one of the most detrimental effects on food quality. To reduce autoxidation of fats, carotenoids, especially β-carotene, vitamin E, and other chemical additives, are used as antioxidants due to their free radical scavenging ability. However, the extensive system of conjugated double bonds present in β-carotene also imparts a pro-oxidant character and may also make the molecule particularly susceptible to attack by the addition of peroxyl radicals. It has been known since 1940 (Whitaker, 1991) that co-oxidation of carotenoids takes place when lipoxygenase (LOX) oxidizes polyunsaturated fatty acids. This is generally believed to be due to an attack either by an activated form of LOX (Robinson et al., 1995) or by a product of LOX and fatty acids (Yeum et al., 1995). Sanz et al. (1994) have reported a high co-oxidation activity for the type-2 chick pea isoenzymes, and potato LOX has been shown to be a good co-oxidizer for β-carotene (Aziz et al., 1999). Also, it has been reported that soybean LOX-1 is a poor catalyst for co-oxidation of β-carotene and linoleic acid under aerobic conditions (Arens et al., 1973). Yoon and Klein (1979) have shown that one of two pea seed LOXs is more effective for co-oxidation. LOXs have been shown to contribute several beneficial effects to bread-making (Casey, 1997). A soybean extract containing LOX isoforms is frequently used commercially as a "flour improver" and an understanding of the co-oxidation reaction of recombinant pea LOXs, which are becoming available, will allow the properties of individual LOX isoforms and their suitability for use in bread-making to be further assessed.

One potential mechanism for co-oxidation of carotenoids may involve the leakage from an enzyme–substrate complex of a linoleylperoxyl radical (Robinson et al., 1995), which can then either dismutate to form keto and hydroxy acids (Hughes et al., 1998) or might, in the presence of carotenoid, initiate its co-oxidation.

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Table 1. Hydroperoxidation and Co-oxidation Activities of Soybean LOX-1 and *r*-LOX-2 and -3

	soybean LOX-1	*r*-LOX-2	*r*-LOX-3
hydroperoxidation activity	380	64	16.4
co-oxidation activity	61.2	10.5	7.2
ratio, A_{HP}/A_{CO}	6.2	6.1	2.3

However, enzymically derived products formed by co-oxidation of carotenoids (Cabibel and Nicolas, 1991) have not been fully identified and attributed to LOX-catalyzed co-oxidation. Therefore, it has not been possible to confidently propose mechanisms for enzymic oxidation of carotenoids. On the other hand, a number of products, including short-chain volatile compounds (Enzell, 1981; Drawert et al., 1981) and longer chain carotenals (Marty and Berset, 1990; Yeum et al., 1995), have been identified from the thermal degradation and/or oxidation of β-carotene in the presence and absence of chemical free radical initiators. Here we report the identification by HPLC combined with photodiode array (PDA) detection, GC/MS, and HPLC/MS of oxidized products formed during the co-oxidation of β-carotene catalyzed by soybean LOX-1 and recombinant pea seed LOX-2 and LOX-3 (pea *r*-LOX-2 and pea *r*-LOX-3, respectively). A mechanism for LOX-initiated co-oxidation of β-carotene from a generated linoleoylperoxyl radical followed by further chemical oxidation and rearrangements is proposed.

MATERIALS AND METHODS

Materials. Soybean LOX-1 (type IB), *all-trans*-β-carotene, linoleic acid, and Tween 80 were from Sigma. Reacti-Gel 6X (6% cross-linked beaded agarose) was from Pierce. Recombinant pea *r*-LOX-2 and -3 were produced in *Escherichia coli* and purified as described by Hughes et al. (1998). Tenax A (60−80 mesh) was from Phase Separations. The specific activities of enzymes used as measured by hydroperoxidation at 234 nm were 5×10^4 units/mg (pea *r*-LOX-2), 1×10^6 units/mg (pea *r*-LOX-3), and 1×10^5 units/mg (soybean LOX-1).

Co-oxidation Rate Measurements. β-Carotene (1 mg) was dissolved in 1 mL of chloroform in a 25 mL filtration flask, and 40 μL of Tween 80 was added. After mixing, the chloroform was evaporated under a water-jet vacuum. The carotene residue in Tween was redissolved in 10 mL of 0.25% (w/v) EDTA solution. All of the solutions were prepared daily before use.

For the co-oxidation rate measurements, 0.9 mL of linoleic acid (3 mM) and 100 μL of the β-carotene solution were added to a 3-mL cuvette containing 1.95 mL of 0.2 M buffer at optimum pH values (borate buffer at pH 9.0 for soybean LOX-1 or phosphate buffer at pH 6.5 for pea *r*-LOX-2 and pea *r*-LOX-3) saturated with oxygen. Finally, 50 μL of enzyme solution, which was equivalent to a hydroperoxidation activity of 380 units (soybean LOX-1), 64 units (pea *r*-LOX-2), or 16.4 units (pea *r*-LOX-3) (Table 1), was added to initiate the reaction, which was measured by reduction in absorbance at 456 nm. One unit of co-oxidation activity is defined as that which will cause a decrease in absorbance at 456 nm of 0.001 per min at pH 9.0 or 6.5 and 25 °C, for a reaction volume of 3.0 mL with a light path length of 1.0 cm, when β-carotene and linoleic acid are used as substrates. The hydroperoxidation activity of LOX was measured in a similar way except that only linoleic acid was used and the absorbance was monitored at 234 nm. The term "hydroperoxidation activity" is used for the normal LOX activity to distinguish it from co-oxidation activity. One unit is defined as that which will cause an increase in absorbance at 234 nm of 0.001 per min at pH 9.0 (or pH 6.5) and room temperature, for a reaction volume of 3.0 mL with a light path length of 1.0 cm (Wu et al., 1995). To determine the effect of substrate and enzyme concentrations on the rate of co-oxidation, assays with differing amounts of linoleic acid, β-carotene, and/or enzymes were carried out in triplicate at room temperature.

Sample Preparation for HPLC and GC/MS Analysis. To slow the overall rate of co-oxidation and to prevent the organic solvent from evaporating (see later section where octane was used), the reaction was carried out at 0 °C with oxygen bubbling. The concentration of linoleic acid was 1.0 mM and that of β-carotene, 3.3 μg/mL (6.2 μM) (the molar ratio of linoleic acid to β-carotene was 160), in the mixture. The reaction was stopped by acidification with 5 M HCl to pH 2. No bleaching of the carotenoid color occurred in the absence of enzyme. For headspace analysis, volatile products were trapped with Tenax columns cooled with ice and eluted with diethyl ether. The eluant was evaporated to ~200 μL before GC/MS. For HPLC analysis, the reaction mixture was extracted twice with an equal volume of diethyl ether and then dried with anhydrous sodium sulfate and evaporated. The residue was dissolved in acetonitrile/chloroform (3:2, v/v) for HPLC analysis.

Sample Preparation for HPLC/MS Analysis. To increase (~10-fold) the yield of products for this coupled analysis, the oxidation was carried out in a single organic phase using immobilized enzyme. Immobilization of LOX has been used for the preparation of hydroperoxides from polyunsaturated fatty acids (Parra-Diaz et al., 1993; Piazza et al., 1994; Drouet et al., 1994; Hsu et al., 1997), but we are not aware of any such application for the co-oxidation reaction. 1,1′-Carbonyldiimidazole-activated matrix Reacti-Gel 6X (6% cross-linked beaded agarose) was used for the immobilization of LOX. Reacti-Gel (6 mL) was placed in a 12 mL disposable polypropylene column fitted with a porous polyethylene disk. The acetone in which the Reacti-Gel was suspended was removed from the gel. The gel was not allowed to dry completely and was washed with water and mixed with 15 mL of a 3 mg/mL solution of soybean LOX-1 (1×10^5 units/mg of solid) in 0.2 M borate buffer, pH 9.0. The mixture was incubated with gentle agitation (~240 rpm) at 4 °C for ~42 h. During the immobilization, the pH of the gel−enzyme mixture was maintained at pH 9 (for soybean LOX-1) or 6.5 (for pea *r*-LOX-2, 2.7×10^4 units/mL, and pea *r*-LOX-3, 1.2×10^5 units/mL) with 20% (w/v) sodium hydroxide solution.

For the co-oxidation of β-carotene with the immobilized LOX, 150 mg of β-carotene was dissolved in 60 mL of octane saturated with water and oxygen in a 250 mL flask. Three milliliters of the appropriate buffer (i.e., borate buffer, pH 9, for soybean LOX-1 and phosphate buffer, pH 6.5, for pea *r*-LOX-2 and pea *r*-LOX-3) was added in the same flask. The immobilized LOX (IMM-LOX), as the gel cake, was added to the solution after removal of the buffer by filtration with the same porous polyethylene disk as used previously. One milliliter of linoleic acid (3.6 mg/mL in octane) was added to initiate the oxidation at 0 °C, and further additions were made at 5 min intervals up to 2 h. The organic phase was separated from the gel and the buffer and evaporated under vacuum. The residue was dissolved in 0.6 mL of acetonitrile/chloroform (3:2 v/v). This sample was used for both HPLC/PDA and HPLC/MS analysis.

Instrumentation. Waters's 600E HPLC and 996 PDA detector were used for HPLC separation and identification of the co-oxidation products. A Vydac reversed-phase C18 column (250 × 4.6 mm) with an LC-C18 guard column (20 × 4.6 mm) was used. A linear gradient was used from methanol/water (9:1, v/v) for the first 5 min to acetonitrile (100%) in 15 min and then to acetonitrile/ethyl acetate (85:15, v/v) for a further 15 min, after which time the mobile phase remained isocratic. The flow rate was 1 mL/min. For the PDA detection, spectra were scanned from 200 to 500 nm. All data were collected and analyzed by Waters Millennium software (version 2.10).

GC/MS was carried out on a Kratos MS80RF mass spectrometer directly coupled to a Carlo Erba GC. Electron impact (EI) mass spectra were recorded using a standard EI ionization box at 150 °C and an electron energy of 70 eV. GC separation was carried out on a BPX5 fused silica (film thickness = 0.25 μm) capillary column (50 m × 0.33 mm i.d.) with helium as carrier gas. The GC was maintained at 50 °C for 5 min and

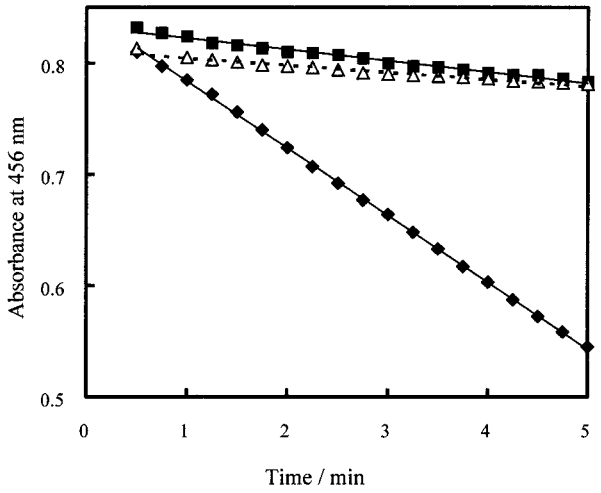

Figure 1. Co-oxidation activity measurements for soybean LOX-1 (380 units, ◆) and pea *r*-LOX-2 (64 units, ■) and -3 (16.4 units, △). Incubation conditions are given under Materials and Methods.

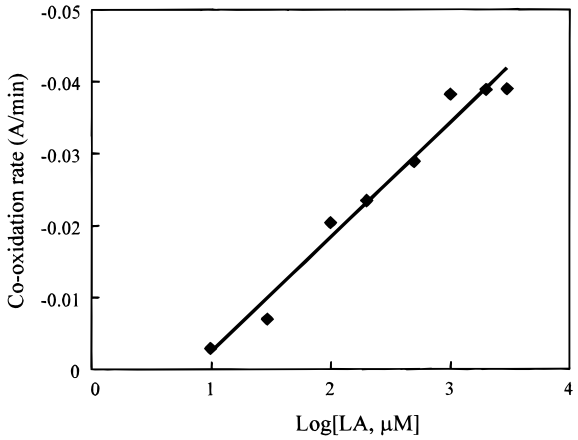

Figure 2. Effect of linoleic acid on rate of co-oxidation of β-carotene. Incubation conditions are given under Materials and Methods.

then programmed from 50 to 280 °C at 5 °C/min. Data were analyzed on a Data General D'Top DG10 computer system.

All LC/MS identifications were performed using a VG Platform single linear quadrupole mass spectrometer (Fisons Instruments, Manchester, U.K.) equipped with atmosphere pressure chemical ionization (APcI). The settings of the main tuning parameters for positive-ion APcI were as follows (Clarke et al., 1996): corona discharge, 3.0 kV; high-voltage lens, 0.0 V; cone, 15 V; offset, 5 V; source temperature, 120 °C; and APcI probe temperature, 500 °C. Mass spectra were acquired by scanning from *m/z* 50 to 650 with a 2 s scan time and a 0.2 s interscan delay. The same HPLC conditions were used as described above.

RESULTS AND DISCUSSION

Co-oxidation of β-Carotene. As shown in Figure 1, soybean LOX-1 catalyzed the bleaching of β-carotene in the presence of linoleic acid under aerobic conditions. Previously it has been reported that the soybean enzyme possessed a poor bleaching activity under aerobic conditions but a higher co-oxidation activity under anaerobic (argon) conditions (Klein et al., 1984). A comparison of the relative hydroperoxidation activity (A_{HP}) and co-oxidation activity (A_{CO}) of soybean LOX-1 with that of pea *r*-LOX-2 and pea *r*-LOX-3 showed that both soybean LOX-1 and pea *r*-LOX-2 gave approximately the same activity ratio, A_{HP}/A_{CO} (6.1−6.2). For pea *r*-LOX-3 this activity ratio (2.3) was smaller, indicating that the enzyme had a relatively higher co-oxidation activity.

Effects of Substrate and Enzyme Concentrations on Co-oxidation of β-Carotene. The rates of oxidation of linoleic acid and co-oxidation of β-carotene and linoleic acid increased gradually with increasing enzyme concentration with a linear range of ~3 orders (correlation coefficient = 0.999). For increasing concentrations of linoleic acid, the rate of co-oxidation increased linearly to the logarithm of linoleic acid concentration (from 1 μM to 1 mM) (Figure 2). Afterward, the co-oxidation rate changed little with increasing amounts of linoleic acid. In contrast, the effect of β-carotene concentration on the rate of co-oxidation at four different concentrations (6.2, 12.4, 18.6, and 24.8 μM) was constant (−0.0118, −0.0110, −0.0118, and −0.0116 min^{-1}, respectively).

Identification of Co-oxidation Products by GC/MS, HPLC/PDA, and LC/MS. From the volatile products trapped with Tenax columns β-cyclocitral and β-ionone were identified by GC/MS (retention times of 17.0 and 25.5 min, respectively). The mass spectrum of β-cyclocitral shows peaks, besides the molecular ion peak at 152, at *m/z* 137 (M − CH_3), 123 (M − CO + H), and 109 [M − (CH_3 + CO)]. The mass spectrum of β-ionone shows the molecular ion at *m/z* 192 and other ions at 177, the base peak (M − CH_3), 149 (M − CH_3-CO), 135 [M − (CH_3CO + CH_3) + H], and 43 (CH_3CO). These identifications are confirmed by both the MS library and previous publications (McLafferty and Turecek, 1993).

Separation by HPLC combined with PDA detection of the nonvolatile co-oxidation products has enabled us to detect >50 peaks absorbing at a wavelength >350 nm (Figure 3). As neither the components of linoleic acid, enzyme, and detergent nor their possible oxidation products absorb at these wavelengths, it is assumed that products absorbing at wavelengths >350 nm were from the oxidation of β-carotene. Although the lack of authentic standards can hinder final confirmation of the identity of co-oxidation products, characteristic differences in the UV spectra of co-oxidation products when compared to β-carotene are helpful. The creation of carbonyl chromophores gives a strong single band. This is shown in Figures 4 and 5. Figure 4 gives the spectra of six apo products, and Figure 5 shows the spectra of seven epoxy products. On the other hand, the loss of the double bond in the terminal ring of β-carotene gives the spectrum a fingerprint fine structure due to greater flexibility as shown in Figure 5 (the last spectrum). Furthermore, the wavelength for maximum absorption is shifted upward with an increase in the carbon chain length, and other useful information can be deduced from the retention time of the products. As shown in Figures 4 and 5, both the retention time and the maximum absorbance wavelength increase with the increase in the chain length of the products when they are in the same group. Assignments for the components detected by HPLC and PDA are summarized in Table 2.

Many protonated molecular ions were obtained from APcI of LC/MS. Some of the *m/z* values (Table 3) correspond to the protonated molecules (molecular weight + 1) of the possible products listed in Figure 6. For the tentatively identified products, it can be seen that the retention time is proportional to the carbon chain length for a series of compounds of increasing size. However, the proposed more polar products containing

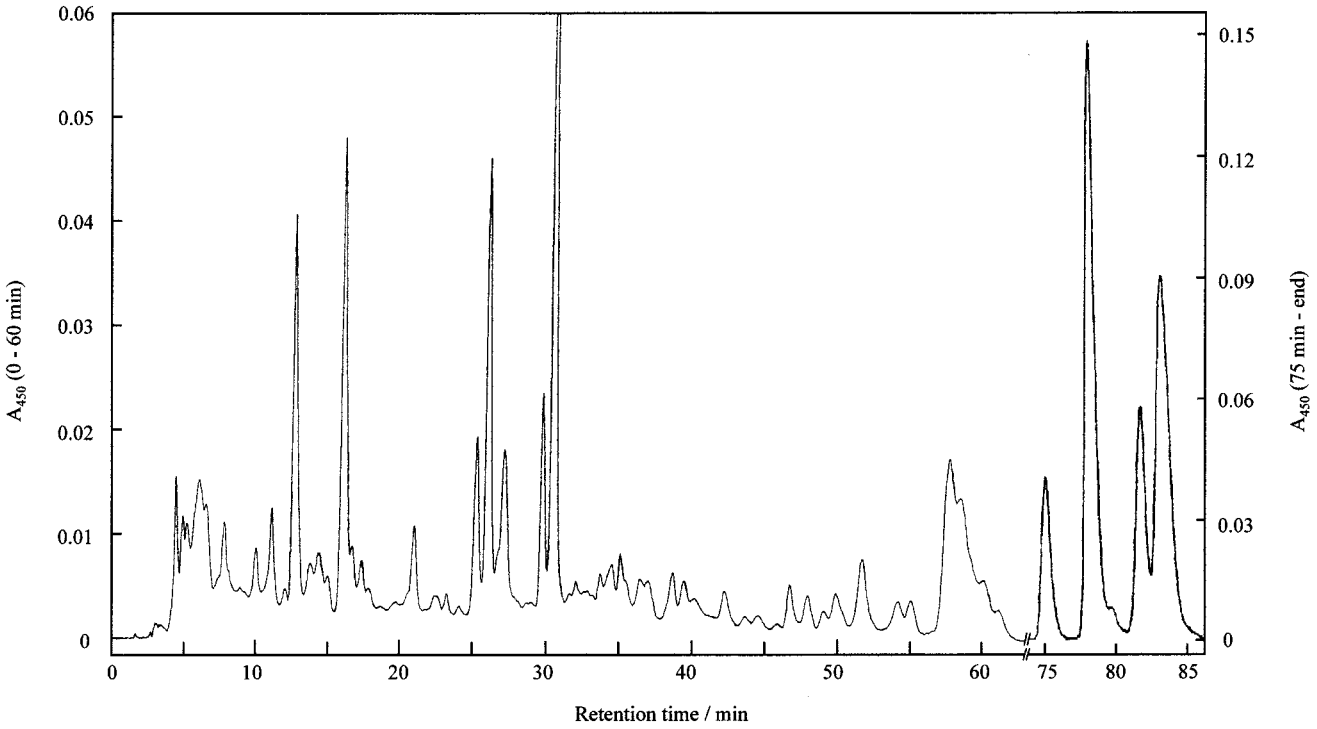

Figure 3. HPLC chromatogram of the co-oxidation products separated on a Vydac reversed-phase C18 column (250 × 4.6 mm) and eluted with methanol/water, acetonitrile, and acetonitrile/ethyl acetate mixture (see Materials and Methods).

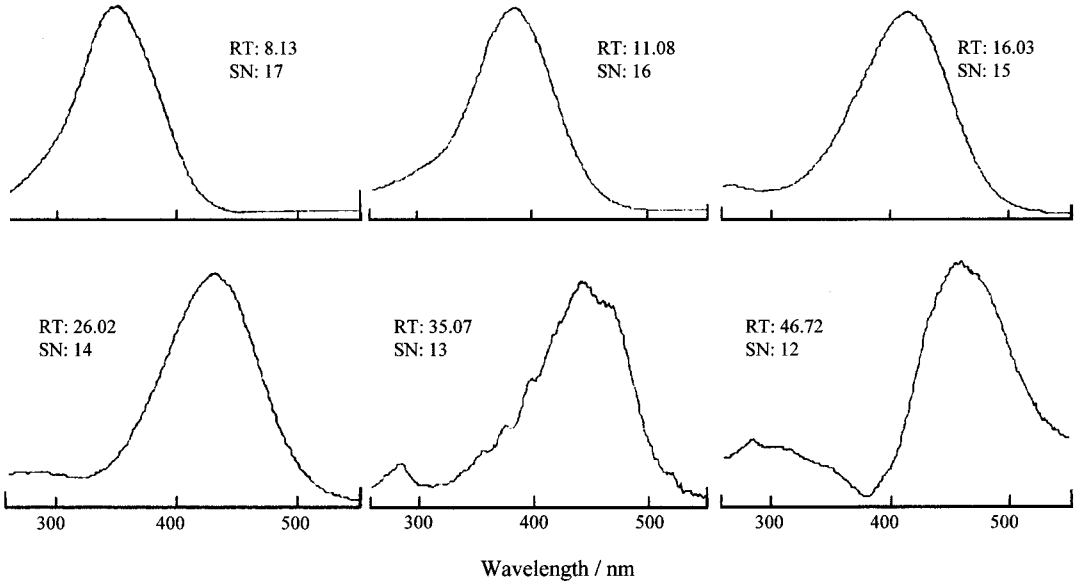

Figure 4. UV spectra of the apo products from the co-oxidation of β-carotene. Both the retention time (t_R, in minutes) of the corresponding peak in the HPLC chromatogram (Figure 3) and the structure number (SN) of the assigned molecule in Figure 6 are given.

either two carbonyl or epoxy groups were eluted earlier. Moreover, the formation of two dicarbonyl compounds (Figure 7) was indicated by HPLC/MS, and these may have arisen by secondary oxidation of the cyclohexene ring.

Co-oxidation Mechanism. The most likely explanation for the large number of products separated by HPLC (Figure 3) and tentatively identified is that LOX-initiated oxidation of β-carotene may occur randomly at different positions along the conjugated unsaturated hydrocarbon chain. The extensive system of conjugated double bonds in the molecule is likely to increase the susceptibility of the carotenoid to attack by linoleyl-peroxyl radicals, generated by enzymic oxidation of the polyunsaturated fatty acid. It has been generally accepted that the most likely LOX-generated oxidizing agent is the peroxyl radical from which a carotenoid and peroxycarotenoid radicals are produced (Burton, 1989):

$$\beta\text{-carotene} + \mathrm{ROO}^{\bullet} \rightarrow \beta\text{-carotene}^{\bullet} + \mathrm{ROOH} \quad (1)$$

$$\beta\text{-carotene}^{\bullet} + \mathrm{O_2} \rightarrow \beta\text{-carotene}{-}\mathrm{OO}^{\bullet} \quad (2)$$

It is possible that these primary products may also serve as the substrates for both further LOX-catalyzed co-oxidation and further random chemical oxidation by enzyme-released linoleylperoxyl radicals and result in oxidation at various positions along the chain and the

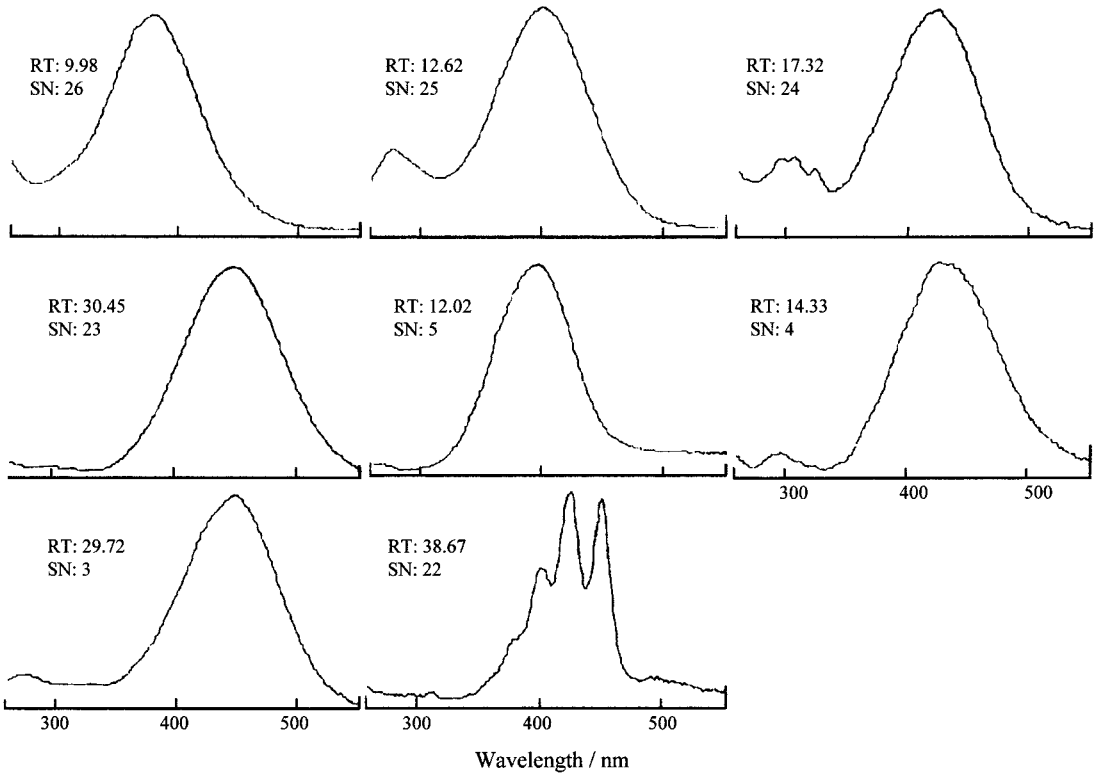

Figure 5. UV spectra of the epoxy products and diepoxy product from the co-oxidation of β-carotene. Both the retention time (t_R, in minutes) of the corresponding peak in the HPLC chromatogram (Figure 3) and the structure number (SN) of the assigned molecule in Figure 6 are given.

Table 2. Tentative Assignment of HPLC Peaks from PDA Detection

t_R (min)	λ_{max} (nm)	reported λ_{max} (nm)	assigned to (tentatively)	no. of CDB[a]	identified by other methods	structure no. in Figure 6
			Apo Products			
8.13	349.5	352	β-apo-13-carotenone	5	HPLC/MS	17
11.08	386.2	384	β-apo-15-carotenal (retinal, or vitamin A aldehyde)	6	HPLC/MS	16
16.03	412.6	409	β-apo-14′-carotenal	7	HPLC/MS	15
26.02	429.4	429	β-apo-12′-carotenal	8	HPLC/MS	14
35.07	445.1	451	β-apo-10′-carotenal	9	HPLC/MS	13
46.72	459.5	462	β-apo-8′-carotenal	10		12
			5,6-Epoxy Products			
9.98	381.4		5,6-epoxy-β-apo-14′-carotenal	6	HPLC/MS	26
12.62	404.2		5,6-epoxy-β-apo-12′-carotenal	7	HPLC/MS	25
17.32	425.8		5,6-epoxy-β-apo-10′-carotenal	8	HPLC/MS	24
30.45	451.1		5,6-epoxy-β-apo-8′-carotenal	9	HPLC/MS	23
			5,8-Epoxy Products			
12.02	397.0		5,8-epoxy-β-apo-12′-carotenal	6	HPLC/MS	5
14.33	425.8		5,8-epoxy-β-apo-10′-carotenal	7	HPLC/MS	4
29.72	446.3		5,8-epoxy-β-apo-8′-carotenal	8	HPLC/MS	3
			Unoxidized Substrates			
78.28	454.7	455	*all-trans*-β-carotene	11		
81.83	448.7	450	9-*cis*-β-carotene[b]	11		
83.30	447.5	445	15-*cis*-β-carotene[b]	11		

[a] CDB, conjugated double bonds. [b] The UV spectrum of a *cis*-isomer of the parent *all-trans*-β-carotene is characterized by a "*cis*-peak", which is located (142 ± 2) nm (in hexane) lower than the peak of the longest waveband of the *trans*-compound. The *cis*-peak of 15-*cis*-β-carotene is much stronger than that of the 9-*cis*-β-carotene, but both of them are located at ~340 nm.

β-ionone rings to form secondary oxidation products. The detection of a number of 5,6- and 5,8-epoxy products as well as apocarotenals (Tables 2 and 3) supports the proposal of random attack on the β-carotene molecule by the linoleylperoxyl radical and other free radicals, possibly including the products of reactions 1 and 2 as opposed to a more controlled sequential enzymatic degradation of the carotenoid.

Alternatively, it has been suggested that during chemical oxidation of β-carotene the addition of an ROO• radical to the conjugated system of β-carotene (reaction 3) occurs, rather than an H-atom abstraction (reaction 1) (Burton and Ingold, 1984; Samokyszyn and Marnett, 1987; Mordi et al., 1993; Yamauchi et al., 1993; Yeum et al., 1995). This addition reaction might be more energetically favorable at the C-4 position in the ring of β-carotene (Woodall et al., 1997).

$$\mathrm{ROO^{\bullet}} + \beta\text{-carotene} \rightarrow \mathrm{ROO}-\beta\text{-carotene}^{\bullet} \quad (3)$$

From this addition product further products including epoxides may be formed by chemical rearrangement

Table 3. Tentative Assignment of HPLC Peaks from LC/MS Detection

m/z (M + 1)	tentative assignment	identified by other methods	structure no. in Figure 6
139	2,6,6-trimethyl-2-cyclohexene-1-one		
157	2-hydroxy-2,6,6-trimethylcyclohexanone		
191	$C_{12}H_{14}O_2$[a] (see Figure 7)		
181	dihydroactinidiolide		
169	5,6-epoxy-β-cyclocitral		31
257	$C_{17}H_{20}O_2$[a] (see Figure 7)		
209	5,6(or 5,8)-epoxy-β-ionone		30 or 10
235	5,6(or 5,8)-epoxy-β-apo-11-carotenal		29 or 9
193	β-ionone	GC/MS	30
153	β-cyclocitral	GC/MS	20
275	5,6(or 5,8)-epoxy-β-apo-13-carotenone		28 or 8
301	5,6(or 5,8-)-epoxy-β-apo-15-carotenal		27 or 7
219	β-apo-11-carotenal		18
327	5,6(or 5,8)-epoxy-β-apo-14′-carotenal		26 or 6
259	β-apo-13-carotenone	HPLC/PDA	17
367	5,6(or 5,8)-epoxy-β-apo-12′-carotenal	HPLC/PDA	25 or 5
285	β-apo-15-carotenal (retinal, vitamin A aldehyde)	HPLC/PDA	16
393	5,6(or 5,8)-epoxy-β-apo-10′-carotenal	HPLC/PDA	24 or 4
311	β-apo-14′-carotenal	HPLC/PDA	15
433	5,6(or 5,8)-epoxy-β-apo-8′-carotenal	HPLC/PDA	23 or 3
351	β-apo-12′-carotenal	HPLC/PDA	14
377	β-apo-10′-carotenal	HPLC/PDA	13
553	5,6(or 5,8)-epoxy-β-carotene		21 or 1
537	*all-trans*-β-carotene		
537	15-*cis*-β-carotene		

[a] The secondary oxidation products.

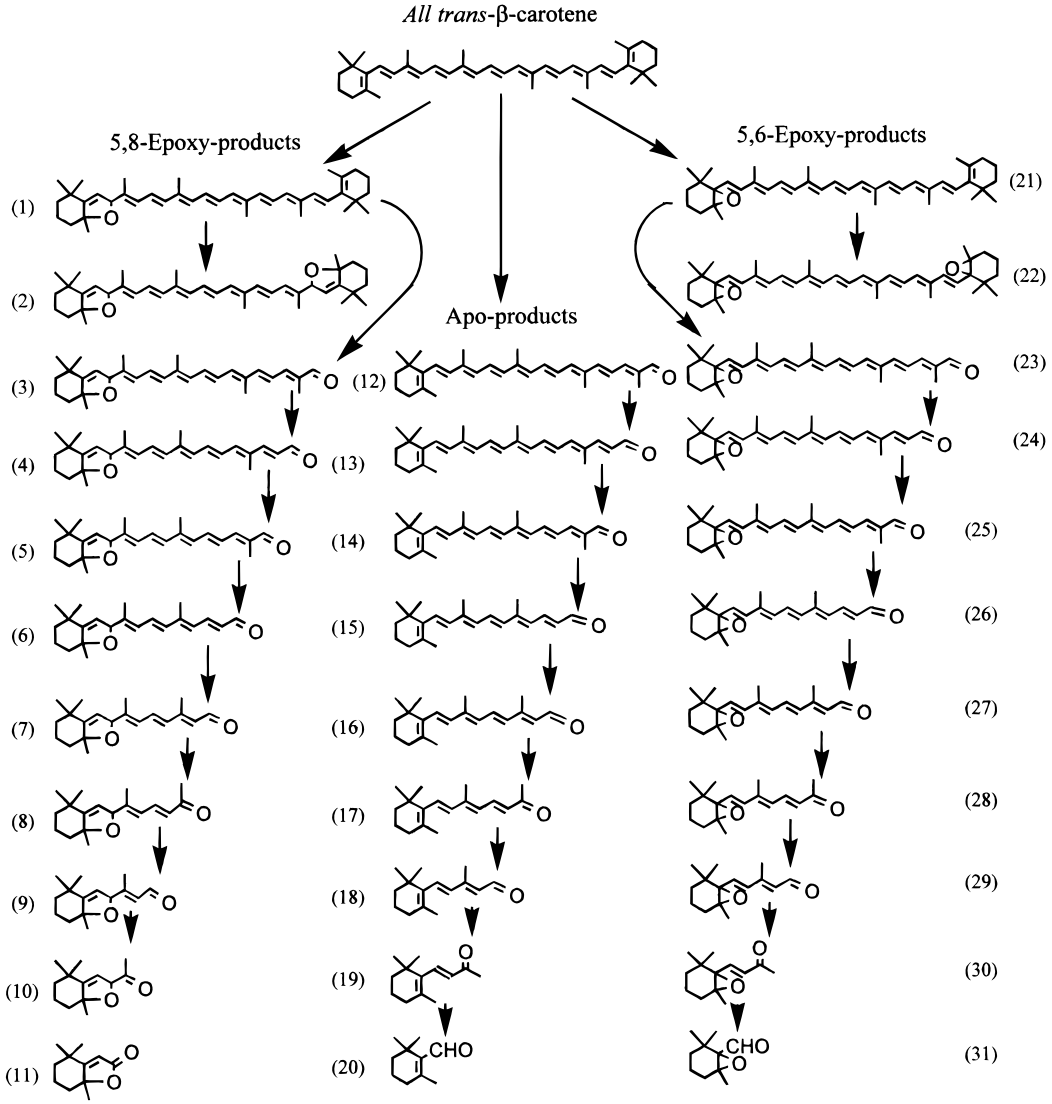

Figure 6. Possible products formed during the co-oxidation of β-carotene.

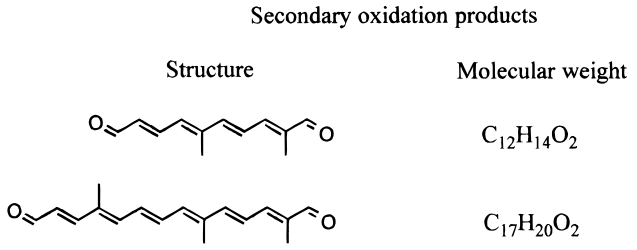

Figure 7. Dicarbonyl compounds from the secondary oxidation of the primary co-oxidation products.

requiring first homolytic cleavage of the O−O bond to release a lipoxy radical (Mordi et al., 1993; Yamauchi et al., 1993). From the epoxides carbonyl compounds may be formed (Yamauchi et al., 1993). Accordingly, the rate of co-oxidation would be mainly dependent on the rate of linoleylperoxy radical formation and the release of the lipoxy radical (LO•) from the addition product. For pea *r*-LOX-3, carbonyl compounds, the maximum absorption wavelength of which is 280 nm under our assay conditions, were dominant products of linoleic acid oxidation (Hughes et al., 1998). It has been suggested that these arise by a dismutation type reaction of released peroxyl radicals, and this distinguishes pea *r*-LOX-3 from pea *r*-LOX-2. The activity ratios, A_{HP}/A_{CO}, for pea *r*-LOX-3 are smaller than those of soybean LOX-1 and pea *r*-LOX-2. Retention of the peroxyl radical by pea *r*-LOX-2 and soybean LOX-1 to subsequently form the more stable hydroperoxide may explain the similar activity ratios, A_{HP}/A_{CO}, for soybean LOX-1 and pea *r*-LOX-2.

In the presence of β-carotene, linoleylperoxyl radicals are expected to be consumed in the co-oxidation reaction, and thus the propensity for hydroperoxide formation would be less. The ability of β-carotene to inhibit lipid oxidation in this way could be due to its lower activation energy: 5 kcal lower than that of linoleic acid (El-Tinay and Chichester, 1970). A proposed mechanism of LOX-catalyzed co-oxidation is illustrated in Figure 8, which shows a combined scheme for both hydroperoxidation of linoleic acid (Hughes et al., 1998) and co-oxidation of β-carotene. The removal of LOO• through addition to β-carotene offers an explanation as to how β-carotene may reduce the pea *r*-LOX-3-catalyzed formation of the hydroperoxide (LOOH). The primary oxidized products (possibly β-carotene epoxides) could undergo further oxidation by either pea *r*-LOX-3-generated linoleylperoxyl radicals or more slowly by autoxidation to form the secondary oxidized products (Figure 7) or other isomerized products. Thus, the overall effect would seem to be diversion of a potentially damaging lipid chain reaction by pea *r*-LOX-3 into a much less deleterious side reaction involving the expendable β-carotene (Burton and Ingold, 1984; Burton, 1989).

The above findings tempt us to suggest that β-carotene is an effective antioxidant not only for the peroxy radicals released directly in our work by the action of pea *r*-LOX-3 on linoleic acid but possibly for other plant LOXs and similar mammalian enzymes. The studies suggest that pea *r*-LOX may act as an improved biocatalyst in the bread-making process, and this is currently being investigated. Pea *r*-LOX-2 and pea *r*-LOX-3 are also highly suitable for probing the primary determinants of co-oxidation behavior in LOX catalysis, because their primary structures are 86% similar but have distinct co-oxidation mechanisms. Although we have not studied the co-oxidation behavior of the major

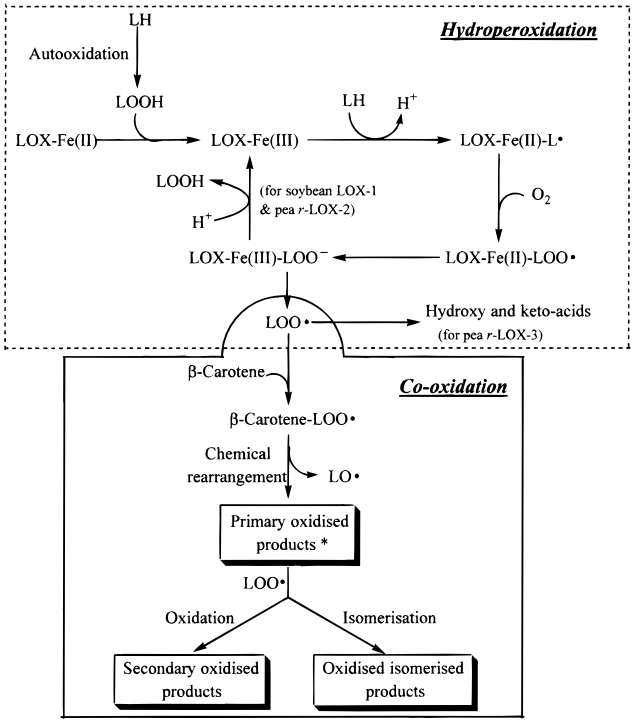

Figure 8. Proposed schemes for the LOX-catalyzed oxidation of linoleic acid and the co-oxidation of β-carotene by soybean LOX-1 and pea LOX-2 and -3 enzymes.

LOX purified from pea seeds, in every respect the properties of the recombinant LOX were indistinguishable from their major counterparts in pea seeds (Hughes et al., 1998). Thus, an increased understanding of the co-oxidation behavior of the recombinant pea LOX will permit the development of rational programs for pea improvement by convential breeding or genetic engineering (Casey, 1999). Both the identification of the oxidation products of β-carotene and the mechanism proposed for the co-oxidation of β-carotene will be helpful in future studies of β-carotene, either as an antioxidant or as a pro-oxidant.

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