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Cooxidation of β-Carotene by Soybean Lipoxygenase

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The cooxidation behavior of a natural mixture of soybean lipoxygenase isoenzymes has been studied at pH 7.4. The ratio of oxidation rates of linoleic acid and β-carotene was found to be 23.3:1. However, BHT and α-tocopherol reduce the rate of oxidation of β-carotene to a greater extent than that of linoleic acid. BHT is a more effective antioxidant than α-tocopherol, while ascorbic acid has no significant antioxidant properties. α-Tocopherol (10^{-6} M) retards the initial cooxidation of β-carotene and retinyl acetate, but cooxidation proceeds rapidly after this period. The rate of carotene bleaching increases with carotene concentration, but the ratio of the rate of linoleic acid oxidation to β-carotene oxidation is independent of temperature since the two reactions have an identical activation energy within experimental error. These observations are consistent with the accepted mechanism for lipoxygenase-catalyzed cooxidation.

Lipoxygenase, which catalyzes the oxidation of polyunsaturated fatty acids, occurs in a wide variety of plants (Eskin et al., 1977). There are two distinct groups of enzymes described as types 1 and 2. Type 1 lipoxygenase has been reported in relatively few plants and has optimum activity at pH 9 with little tendency to cause cooxidation of other lipids during the reaction. Type 2 lipoxygenase occurs widely with optimum activity at pH 6.5–7.0 and a strong tendency to catalyze the cooxidation of other compounds. Chlorophyll, carotenoids, cholesterol, cytochrome *c*, and thiols in dough are among the substances reported to suffer cooxidation (Eskin et al., 1977). The cooxidation of molecules during the lipoxygenase-catalyzed oxidation of linoleic acid has been ascribed to the fact that a large proportion of the peroxyl radicals is not directly converted to hydroperoxides by the enzyme (Weber and Grosch, 1976). The cooxidation of lipids during lipoxygenase-catalyzed oxidation can lead to the formation of off-flavors (Rackis et al., 1972) and a loss of nutrients (e.g., β-carotene) in foodstuffs, and therefore this study was concerned with increasing the understanding of the factors affecting the rate of the cooxidation reaction catalyzed by a natural mixture of soybean lipoxygenase isoenzymes at neutral pH. Neutral pH was selected, since this is close to the pH of soybean flour dispersed in water.

MATERIALS AND METHODS

Soybean lipoxygenase (Sigma Type 1, lyophilized, a natural mixture of isoenzymes, 125 000–175 000 units/mg protein), linoleic acid (99%), β-carotene (synthetic), retinyl acetate, L-α-tocopherol, and butylated hydroxytoluene (BHT) were purchased from Sigma Chemical Co.

Lipoxygenase-catalyzed 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 (LOOH) concentration was calculated with the molar absorptivity of 26 000 L mol^{-1} cm^{-1} (Matthew et al., 1977).

β-Carotene and retinyl acetate were determined spectrophotometrically from the absorbances at 460 and 325 nm, respectively. The molar absorptivities in the assay medium were found to be 88 000 and 37 313 L mol^{-1} cm^{-1}, respectively.

A typical cooxidation reaction required the preparation of aqueous linoleate and aqueous β-carotene solutions. The linoleate 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 ethylenediaminetetraacetic acid (EDTA) (5 mL, 0.5%) was added before being adjusted to pH 9 with sodium hydroxide solution (1 M). The β-carotene solution was prepared by dissolving β-carotene (25 mg) and Tween 80 (0.9 mL) in chloroform (25 mL). A sample of the chloroform solution was evaporated to dryness, and EDTA solution (10 mL, 0.25%) was added. The linoleate solution (0.5 mL) was mixed with the β-carotene solution (0.5 mL), and citric acid (0.1 M)–disodium hydrogen phosphate (0.2 M) buffer (9 mL, pH 7.4) was added. A sample of the buffered linoleate/β-carotene 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 of enzyme) were added.

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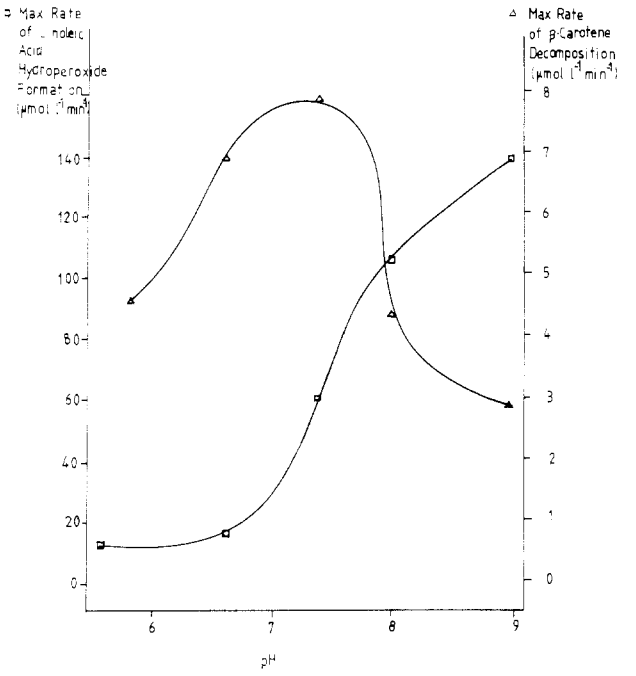

Figure 1. Effect of pH on the lipoxygenase-catalyzed oxidation of linoleic acid (1×10^{-3} M) and cooxidation of β-carotene (7×10^{-6} M). Maximum rates are quoted instead of initial rates since in some cases a lag time was observed before the maximum rate was reached.

The antioxidant studies involved addition of an ethanolic solution of BHT or α-tocopherol (0.1 mL). The ascorbic acid was added as an aqueous solution. All solutions used were water-clear.

All studies were performed in duplicate with freshly prepared solutions at 21 ± 1 °C.

RESULTS AND DISCUSSION

A commercial sample of soya lipoxygenase comprising a mixture of isoenzymes was used for this investigation, since this is relevant to the effects of soya flour in food products. Gel electrophoresis indicated four major protein bands consistent with the presence of four major isoenzymes observed by earlier workers (Grosch et al., 1977). The sample was characterized by its activity over a range of pH values (Figure 1). The rate of oxidation of linoleic acid was greatest at pH 9, indicating that the sample showed strong type 1 activity. However, the rate of cooxidation of β-carotene was maximum at pH 7.4. This is consistent with the presence of a type 2 isoenzyme, which is most active at neutral pH values and is the main isoenzyme catalyzing the cooxidation reaction (Galliard and Chan, 1980). Therefore, it is clear that the lipoxygenase used contained both types 1 and 2 isoenzymes. The cooxidation reaction was studied at pH 7.4. The increase of absorbance at 234 nm was linear for at least 0.6 min for all experiments, indicating that the reaction was aerobic during the initial period. Absorbance at this wavelength remains constant in the absence of pigment or falls in the presence of carotene if the reaction is anaerobic (Klein et al., 1984). β-Carotene was oxidized rapidly in the presence of linoleic acid and lipoxygenase. The ratio of the rate of linoleic acid oxidation to β-carotene oxidation was found to be 23.3:1 on a molar basis at pH 7.4. This value can be compared with 4.0 for purified soybean lipoxygenase type 2 at pH 6.5 [calculated from Grosch et al. (1977)]. The difference in experimental conditions, especially in pH, may contribute to the difference between the two values, but the major factor is likely to be the presence of lipoxygenase type 1 which catalyzes the oxidation of linoleic acid to some extent even though it is well away from its optimum pH.

Table I. Effect of Antioxidants on the Cooxidation of β-Carotene (7×10^{-6} M) in the Presence of Linoleic Acid (1.0×10^{-3} M) at pH 7.4

	carotene oxidn rate (μmol L^{-1} min^{-1}) at antioxidant concn			% inhibition	
antioxidant	control	10^{-6} M	10^{-5} M	10^{-6} M	10^{-5} M
BHT	2.5	1.7	0.3	31.6	86.4
α-tocopherol	2.5	3.0[a]	1.1		54.4
ascorbic acid	5.1	[b]	4.8	[b]	5.9

[a] α-Tocopherol (10^{-6} M) introduced a slow initial oxidation period (ca. 1 min) during which the oxidation rate was 2.0 μmol L^{-1} min^{-1}. [b] Not measured.

Table II. Effect of Antioxidants on the Rate of Oxidation of Linoleic Acid (1.0×10^{-3} M) at pH 7.4 in the Absence of β-Carotene

	linoleic acid oxidn rate (μmol L^{-1} min^{-1}) at antioxidant concn			% inhibition	
antioxidant	control	10^{-6} M	10^{-5} M	10^{-6} M	10^{-5} M
BHT	58.2	54.9	33.4	5.6	42.6
α-tocopherol	58.2	53.8	40.9	7.4	29.6
ascorbic acid	92.6	93.7	[a]	0	[a]

[a] Not measured.

When BHT was added as an antioxidant, the rate of cooxidation of β-carotene was reduced, with a reduction of 86% in the presence of 10^{-5} M BHT (Table I). α-Tocopherol also reduced the rate of cooxidation, but the effect was less with a reduction of 54% at 10^{-5} M α-tocopherol. Since BHT is a more effective antioxidant than α-tocopherol despite being considerably more hindered in the region of the active phenol group, it can be deduced that β-carotene is oxidized by reaction with relatively unhindered radicals. Ascorbic acid, which is water soluble, did not produce a significant reduction in β-carotene oxidation rate. BHT and α-tocopherol are less effective at reducing the rate of linoleic acid oxidation (Table II). Hence, it appears that linoleic acid is oxidized via two pathways, one of which is less sensitive to antioxidants than that leading to β-carotene oxidation. These observations are consistent with the mechanism for cooxidation proposed by Weber and Grosch (1976). It was noted that when α-tocopherol was present at 10^{-6} M, it reduced the rate of loss of β-carotene during the first minute but was unable to prevent the cooxidation of β-carotene proceeding within a few minutes at a rate similar to the maximum rate in the absence of antioxidant. The introduction of an induction period in lipoxygenase-catalyzed oxidation in the presence of α-tocopherol was observed by Reinton and Rogstad (1981) but not by Ben Aziz et al. (1971). In this study an induction period was observed with α-tocopherol at 10^{-6} M, but not at 10^{-5} M. It appears that the relative concentrations of the components in the reaction medium affect the induction period. The reactivity of the cooxidant also has an effect since retinyl acetate, which is oxidized more slowly than β-carotene, shows a more pronounced induction period with 10^{-6} M α-tocopherol (Figure 2).

The rate of carotene oxidation increased with pigment concentration (Figure 3). Within experimental error, this plot appears to be linear as expected for a reaction in which two components (β-carotene, linoleic acid) are competing for slowly formed peroxy radicals. The reaction of β-carotene with peroxy radicals is fast and single order in β-carotene at fixed linoleic acid concentrations. A higher

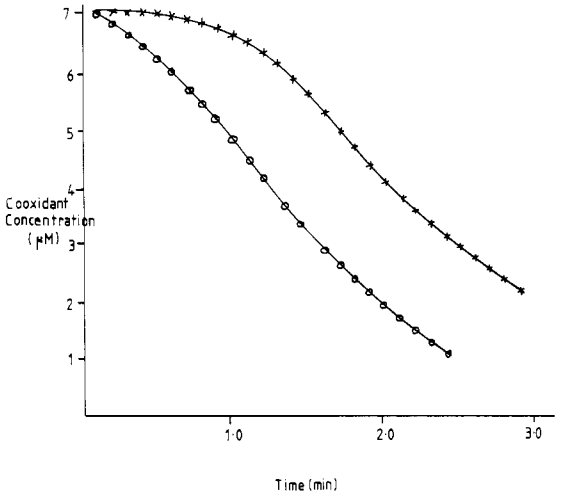

Figure 2. Effect of α-tocopherol (10^{-6} M) on the cooxidation of β-carotene (O) and retinyl acetate (×) by soybean lipoxygenase in the presence of linoleic acid (1×10^{-3} M).

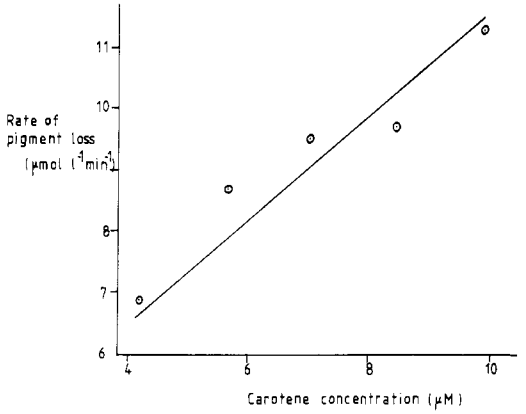

Figure 3. Effect of β-carotene concentration on the maximum rate of loss of β-carotene in the presence of linoleic acid (1×10^{-3} M) and lipoxygenase.

concentration of β-carotene caused more rapid loss of β-carotene by reaction with free radicals, but the rate of linoleic acid oxidation was reduced. It was observed that the rate of conjugation of linoleic acid fell in the ratio of 183.1:100.1:99.1 for solutions containing 0, 1.4×10^{-5}, and 1.68×10^{-5} M β-carotene, respectively.

The effect of temperature on the relative rates of linoleic acid oxidation and β-carotene cooxidation was also investigated. The Arrhenius plots of logarithm of the reaction rate against reciprocal of the absolute temperature were straight lines (Figure 4), and the activation energies were identical, allowing for experimental error. Values of 20.8 and 20.0 kJ mol^{-1} were determined for the activation energies of linoleic acid oxidation and β-carotene cooxidation, respectively. The activation energy for the lipoxygenase-catalyzed oxidation of linoleic acid is similar to the value of 18.0 kJ mol^{-1} reported by Tappel et al. (1953) from studies at pH 9.0. The coincidence of the

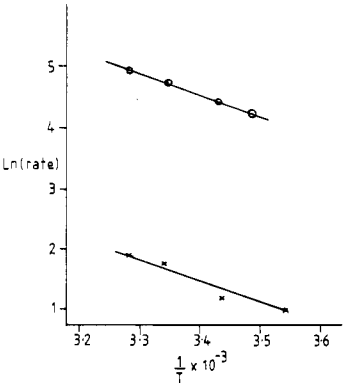

Figure 4. Effect of temperature on the rate of oxidation of linoleic acid (O) and β-carotene (×) in the presence of soybean lipoxygenase.

activation energies for linoleic acid and β-carotene oxidation is consistent with the accepted mechanism for lipoxygenase-catalyzed oxidation in which the rate-limiting step is believed to be abstraction of a hydrogen atom from the fatty acid (Egmond et al., 1973). This step precedes the reaction of peroxy radicals with lipids, leading to cooxidation, and hence both oxidation of linoleic acid and cooxidation of β-carotene share the same rate-limiting step. Consequently the activation energies are identical, and the ratio of the rate of linoleic acid oxidation to that of pigment bleaching is independent of temperature.

Registry No. BHT, 128-37-0; lipoxygenase, 9029-60-1; β-carotene, 7235-40-7; linoleic acid, 60-33-3; retinyl acetate, 127-47-9; L-α-tocopherol, 59-02-9; ascorbic acid, 50-81-7.

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