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CAROTENE-BLEACHING ACTIVITIES OF LIPOXYGENASE AND HEME PROTEINS AS STUDIED BY A DIRECT SPECTROPHOTOMETRIC METHOD

A. BEN AZIZ, S. GROSSMAN,* I. ASCARELLI and P. BUDOWSKI

Faculty of Agriculture, Hebrew University, Rehovot, Israel

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Abstract—A procedure is described for the direct spectrophotometric study of carotene-bleaching in aqueous solution. Beta-carotene, with or without added linoleate, is dissolved in suitable buffers with Tween 80, the decrease in absorbance at 460 nm is recorded, and the initial rate of bleaching is computed from the bleaching curves. The technique was applied to a comparative study of the catalytic effects of lipoxygenase, cytochrome *c* and peroxidase. The latter enzyme, which displayed much less activity in the presence of linoleate, compared to the other two catalysts, was the only one to cause significant carotene bleaching in the absence of added fatty acids. Activity was linearly related to the concentrations of lipoxygenase and cytochrome *c*, but not to the amount of peroxidase. The inhibitory effects of some antioxidants on carotene bleaching in the presence of the three catalysts were studied.

INTRODUCTION

THE COUPLED oxidation of β-carotene has frequently served as a criterion for determining the activity of enzymes causing the oxidation of polyunsaturated fatty acids and their esters[1–3] and for the comparative evaluation of antioxidants in both enzymic[4,5] and non-enzymic oxidations.[6,7] The insolubility of carotene in the aqueous medium and the pH-dependent solubility of the fatty acid substrate constitute obvious difficulties which have been overcome to some extent by the use of fine dispersions of lipid substrates, periodic removal of aliquots from the reaction mixture, extraction of carotene and spectrophotometric determination of the yellow pigment in the extract.

We have recently modified the conditions for the spectrophotometric assay of linoleate oxidation by solubilizing the linoleic acid substrate with Tween 20.[8] This allowed the direct recording of initial reaction rates of linoleate oxidation within a broad range of pH values. The present report describes a similar procedure for bringing linoleate and carotene into aqueous solution, so that the disappearance of carotene can be followed continuously. The method was successfully applied to a study of linoleate and carotene-oxidizing fractions of alfalfa.[9] In the present investigation the procedure has been more fully evaluated, and a comparison has been made between the catalytic effects of lipoxygenase, cytochrome *c* and peroxidase on the bleaching of carotene in the presence of unsaturated fatty acids.

* Present address: Department of Life Sciences, Bar Ilan University, Ramat Gan, Israel.

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RESULTS

Absorption Spectrum of the aq. Carotene–Linoleate Solution

The absorption spectrum of the aq. carotene–linoleate solution before and after partial bleaching is presented in Fig. 1. It is seen that the spectrum of β-carotene in aqueous solution (λ_{max} 460 nm) resembles that of β-carotene in chloroform (λ_{max} 462 nm). The maximum after partial bleaching in the presence of peroxidase is also at 460 nm. Thus the decrease in absorbance at this wavelength is a suitable quantitative criterion for carotene bleaching.

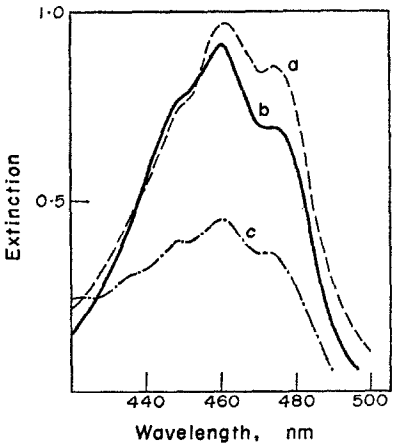

FIG. 1. ABSORPTION SPECTRA OF β-CAROTENE AT A CONCENTRATION OF 5 μg/ml; (a) IN CHLOROFORM; (b) IN THE TWEEN–LINOLEATE REACTION MIXTURE, pH 6·5; (c) AS IN (b), AFTER 1-min REACTION IN THE PRESENCE OF 50 μg PEROXIDASE/ml REACTION MIXTURE.

Effect of the Concentration of Tween 80

In order to obtain a substrate solution which would remain clear at different pH values, it was found necessary to prepare separately aqueous solutions of carotene and linoleate. Such solutions were obtained with the help of Tween 80 (see Experimental). Preliminary tests showed that no turbidity developed, provided the final concentration of this detergent in the substrate mixture was at least 0·05%. Since Tween might be an inhibitor of the reaction its effect was tested at various concentrations. Only a negligible inhibition is caused by raising the Tween concentration in the reaction mixture from 0·05 to 0·1%, when either lipoxygenase or cytochrome *c* are used as catalyst. However, at a Tween concentration of 0·2%, there was a strong inhibition of carotene destruction, activity being reduced to 36% for lipoxygenase and 5% for cytochrome *c*.

Effect of the Concentrations of Carotene and Linoleate

The effect of different carotene concentrations on the initial bleaching velocity, in the presence of constant linoleate and lipoxygenase concentrations, was investigated. In order to obtain clear solutions at the higher carotene concentrations, the amount of Tween had also to be raised, however, this was done within the upper limit of 0·1% that had been shown to have no appreciable inhibitory effect. There was no change in the initial bleaching rate when the amount of carotene was raised from 15 to 30 μg/2 ml reaction mixture, the velocity being 10 μg/min over this range (reaction conditions as in Table 1).

The effect of linoleate concentration on the initial rate of carotene destruction was studied on lipoxygenase at pH 6·5 at concentrations up to 3×10^{-3} M linoleate. Maximum activity was found at 2×10^{-3} M linoleate. The same optimal linoleate concentration was found for cytochrome *c* under the same conditions whereas peroxidase was most active at 5×10^{-4} M linoleate.

The pH profile of Lipoxygenase, Cytochrome c *and Peroxidase*

The pH-activity curves for the coupled oxidation of carotene by these three catalysts are presented in Fig. 2. Optimal pH values were at 8·5, 7·0 and 4·0 for cytochrome *c*, lipoxygenase and peroxidase, respectively. Peroxidase, in addition, showed a secondary peak at pH 8·0.

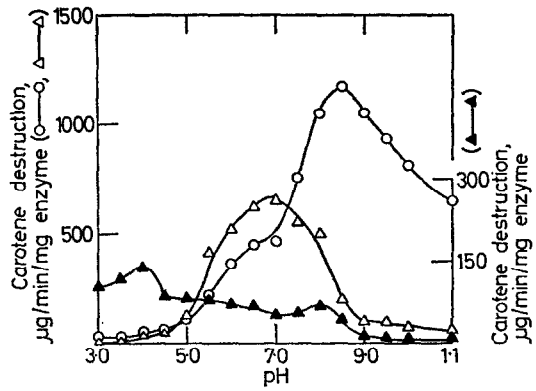

FIG. 2. THE pH PROFILES OF LIPOXYGENASE, CYTOCHROME *c* AND PEROXIDASE. CONCENTRATIONS IN REACTION MIXTURE: CATALYSTS 12·5 μg/ml; LINOLEATE 2×10^{-3} M. △ LIPOXYGENASE; ○ CYTOCHROME *c*; ▲ PEROXIDASE.

Time Course of Carotene Bleaching

Carotene bleaching as a function of time, obtained with lipoxygenase, cytochrome *c* and peroxidase, in the presence and absence of linoleate, is presented in Fig. 3. The rapid initial bleaching velocity by cytochrome *c* in the carotene–linoleate system was more than twice that found for lipoxygenase. However, the activity of cytochrome *c* declined more rapidly, compared to lipoxygenase, so that after a time interval of 40 sec, more carotene appeared to have been destroyed by lipoxygenase than by cytochrome *c*. Peroxidase was relatively less active in this system; however, unlike the two other catalysts studied, it bleached also in the absence of linoleate, especially during the first minute of the reaction.

An additional difference between peroxidase and the other two catalysts is shown by the pronounced effect of peroxidase concentration on the time course of carotene bleaching in the presence of linoleate. The initial reaction rate was correlated with the catalyst concentration but a more rapid decline in activity was found at higher catalyst concentrations. As a result of this effect, an enzyme concentration of 10 μg/ml, after a reaction period of 3 min, caused nearly twice as much carotene destruction as a concentration of 50 μg/ml. This effect was not observed with the other catalysts examined.

Effect of Enzyme Concentration on the Initial Velocity of the Reaction

In the case of cytochrome *c* and lipoxygenase a linear relationship was found between enzyme concentration and initial velocity of the coupled oxidation of carotene. Cytochrome

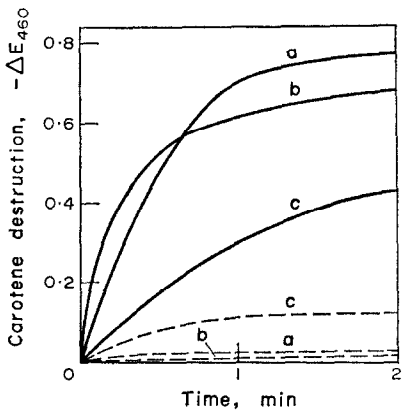

FIG. 3. TIME COURSE OF CAROTENE DESTRUCTION: (a) LIPOXYGENASE, 10 μg/ml, pH 7·0; (b) CYTOCHROME *c*, 10 μg/ml, pH 8·5; (c) PEROXIDASE, 20 μg/ml, pH 4·0; CITRATE–PHOSPHATE BUFFER, 0·2 M. - - - NO LINOLEATE; —— LINOLEATE 2×10^{-3} M.

c was more active than lipoxygenase under these conditions. In the case of the less active peroxidase, the relation between enzyme concentration and activity was not linear. Further tests showed that in this case the initial rate of carotene oxidation was linearly related to the square root of enzyme concentration over the concentration range tested (up to 50 μg/ml).

Effect of Different Unsaturated Fatty Acids on the Coupled Oxidation of Carotene

The initial velocities of carotene destruction for the three catalysts in the presence of oleic, linoleic or linolenic acid are given in Table 1. In the presence of oleate, there was only a negligible destruction of carotene by lipoxygenase, whereas for peroxidase and cytochrome *c* the activities were of the order of 50 and 70%, respectively, of those found in the presence of linoleate. In the presence of linolenate, the activities expressed as percentages of the activities obtained in the presence of linoleate, were 50, 80 and 90% for lipoxygenase, cytochrome *c* and peroxidase, respectively.

TABLE 1. INFLUENCE OF DIFFERENT UNSATURATED FATTY ACIDS ON THE COUPLED OXIDATION OF CAROTENE IN THE PRESENCE OF LIPOXYGENASE, CYTOCHROME *c* AND PEROXIDASE

	Fatty acid			
Enzyme	None	Oleic	Linoleic	Linolenic
	(μg carotene destroyed/min/mg enzyme)			
Lipoxygenase	7	24	576	288
Cytochrome *c*	4	300	600	480
Peroxidase	28	40	56	50

Concentrations in reaction mixture: Lipoxygenase and cytochrome *c* 12·5 μg/ml; peroxidase 25 μg/ml; fatty acids 2×10^{-3} M; citrate–phosphate buffer 0·2 M, pH 6·5.

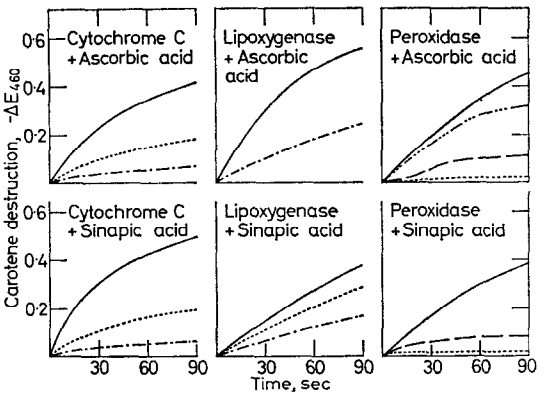

FIG. 4. INFLUENCE OF ASCORBIC AND SINAPIC ACIDS ON THE TIME COURSE OF CAROTENE DESTRUCTION. CONCENTRATIONS IN THE REACTION MIXTURE: LIPOXYGENASE 12·5 μg/ml; CYTOCHROME *c* 12·5 μg/ml; PEROXIDASE 60 μg/ml; LINOLEATE 2 × 10^{-3} M. CITRATE–PHOSPHATE BUFFER 0·2 M, pH 6·5 FOR LIPOXYGENASE, pH 8·5 FOR CYTOCHROME *c* AND pH 5·5 FOR PEROXIDASE. ANTIOXIDANT CONCENTRATION: —— CONTROL, —·—·— 10^{-3} M, - - - 10^{-4} M, — — — 10^{-5} M, ——··—— 10^{-6} M.

Effect of Antioxidants on Carotene Destruction

In order to compare the inhibitory effects of ascorbic and sinapic acids on carotene destruction, enzyme concentrations were chosen to give similar initial velocities. Ascorbic and sinapic acids, at 10^{-3} M concentration, caused about 50% inhibition of carotene destruction by lipoxygenase (Fig. 4). Their inhibitory effect on the cytochrome *c*-induced reaction was 10 times stronger, since a similar inhibition was obtained at 10^{-4} M concentration. In the case of peroxidase, the inhibition was even stronger, since both acids caused an almost complete inhibition at a concentration of 10^{-4} M and the inhibition was very strong even at 10^{-5} M. Ascorbic acid, at this latter concentration, produced a definite induction period in the peroxidase-catalysed carotene oxidation, followed by a short period of relatively rapid bleaching, which quickly ended.

The antioxidant effects of α-tocopherol, 2,6-di-*tert*-butyl-4-methylphenol ('Butylated Hydroxytoluene', BHT) and 1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline (ethoxyquin)

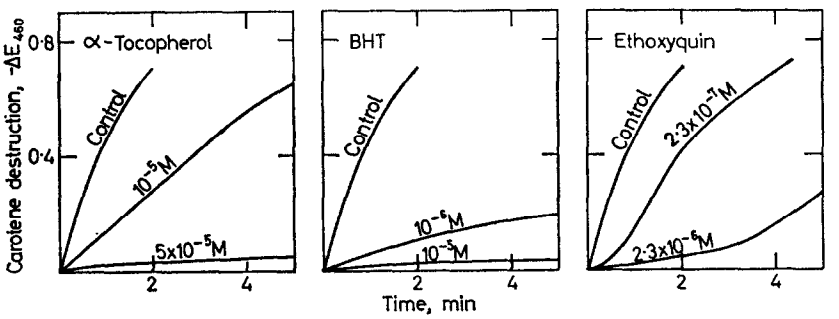

FIG. 5. INFLUENCE OF α-TOCOPHEROL, BHT AND ETHOXYQUIN ON THE TIME COURSE OF CAROTENE DESTRUCTION IN THE PRESENCE OF LIPOXYGENASE. CONCENTRATIONS IN THE REACTION MIXTURE: LIPOXYGENASE 12·5 μg/ml; LINOLEATE 2 × 10^{-3} M. CITRATE–PHOSPHATE BUFFER 0·2 M, pH 6·5. ANTIOXIDANTS CONCENTRATIONS HIGHER THAN THOSE SHOWN CAUSED COMPLETE INHIBITION OVER THE TIME INTERVAL TESTED.

in the coupled oxidation induced by lipoxygenase were evaluated at different inhibitor concentrations (Fig. 5). There is a clear induction period in the presence of ethoxyquin but no such effect was found for the other two antioxidants. The antioxidant activities of ethoxyquin and BHT were about equal, while that of α-tocopherol was lower by an order of 10.

DISCUSSION

The technique used in the present investigation for measuring the disappearance of carotene in the coupled oxidation of linoleate and carotene has several advantages. Inclusion of Tween 80 produces a water-soluble linoleate–carotene substrate the absorption spectrum of which is very similar to that of a carotene solution in chloroform, even after partial bleaching of the pigment, and by studying the bleaching reaction in a spectrophotometer, initial reaction rates can be determined accurately. Furthermore, the influence of pH on the reaction can be studied, since the substrate remains soluble within a wide range of pH values. Possible inhibitory effects caused by Tween 80 are prevented when the concentration is below 0·1 %.

Application of this technique to a comparative study of the catalytic effect of lipoxygenase, cytochrome *c* and peroxidase revealed significant differences in the way these biocatalysts of lipid oxidation operate.

Cytochrome *c* was found to be more effective than lipoxygenase with regard to the initial velocity of carotene bleaching. In previous work[8] we found cytochrome *c* and other heme proteins to be much less active than lipoxygenase, on measuring the conjugated dienes formed by oxidation of linoleate. This apparent contradiction is readily resolved by considering the primary reaction catalysed by heme proteins, namely, the homolytic scission of hydroperoxides,[10] resulting in the formation of free radicals. Free radicals, by attacking the highly susceptible conjugated polyene systems, would then produce a low yield of dienes in the heme protein-catalysed oxidation of linoleate,[8] or increased bleaching of carotene, as found in the present investigation.

With both lipoxygenase and cytochrome *c*, Tween 80 concentrations in excess of 0·1 % were found to inhibit carotene bleaching. Previously, using the formation of conjugated dienes as a criterion, we reported that Tween 20 stimulated the oxidation of linoleate induced by heme proteins.[8] The same argument used in the preceding paragraph leads us to the conclusion that the detergent exerts some kind of protective action on polyene systems, causing increased yields of dienes, or decreased bleaching of carotene, in the heme protein-catalysed reactions. This effect is different from the competitive inhibition of lipoxygenase by Tween 20 reported by Ben Aziz *et al.*[8]

The carotene-bleaching action of lipoxygenase is dependent on the presence of linoleic acid or a similar compound having the *cis,cis*-1,4-pentadiene structure.[11] In agreement with this fact, substitution of linoleate by oleate caused almost complete loss of activity. On the other hand, cytochrome *c* was able to bleach carotene in the presence of oleate, although the speed of the reaction was reduced to half its value. As a result, cytochrome *c* was *ca.* 10 times more active than lipoxygenase, when carotene was bleached in the presence of oleate. The bleaching activity of peroxidase was only slightly affected by substituting oleate for linoleate.

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The relatively weak effect of linolenate on carotene bleaching (Table 1) is noteworthy. Tappel[10] reported that the rate of oxidation of linolenate is greater than for linoleate, in the presence of hemoglobin, and that with lipoxygenase as a catalyst, both substrates oxidize at an equal rate.[12] Kendrick and Watts,[13] on the other hand, found that heme compounds caused less rapid oxidation of linolenate as compared to linoleate. It is clear that different techniques and different criteria of 'oxidation' do not yield comparable results.

Among the three enzymes studied, only peroxidase showed significant bleaching activity in the absence of added lipids. Dicks and Friend,[14] in a study of crocin oxidation by sugar beet leaves, tested commercial peroxidase and found that this enzyme alone did not bleach crocin in the presence of oxygen. These authors determined the amount of carotenoid remaining after an incubation period of 20 min, whereas the present study involved the measurement of initial reaction rates. In view of our observation that the bleaching effect, observed initially, practically ceased after about 1 min, it is quite likely that the percentage of carotenoid actually destroyed under the conditions used by Dicks and Friend would be very small.

The pH profiles (Fig. 2) reveal further differences between the three catalysts. Peroxidase exhibited maximal activity at pH 4·0, i.e. at the same pH found when diene formation was used as a criterion for linoleate oxidation.[8] The pH optimum for lipoxygenase was 7·0, as compared to 9·0 observed in the diene assay, but, as pointed out earlier,[9] the pH optimum of lipoxygenase tends to shift toward 7 with increasing concentrations of linoleate. Blain *et al.*[2] found three activity peaks for soybean extracts at pH 5·0, 6·5 and 7·7. On the other hand, Dicks and Friend,[15] in a study of crocin oxidation, found a pH optimum near 8·5 for lipoxygenase. The latter authors also reported that cytochrome *c* was most active at pH 4·0, in contrast to the optimum of pH 8·5 found in the present work. It is likely that the state of dispersion of the lipid substrate influences the position of the pH optimum. In the present experiments Tween 80 was used to dissolve the substrate; Blain *et al.*[2] did not use a detergent but an acetone emulsion; and Dicks and Friend[15] included Triton X-100 in their system. In addition, the absence of a chelating agent in many of the assay systems described in the literature may also account for some of the discrepancies. We have previously emphasized the importance of traces of metal ions and of EDTA in the oxidation of linoleate and carotene.[5]

A straight-line relation was found between the initial rate of carotene bleaching and the concentration of either lipoxygenase or cytochrome *c*, similar to that reported between catalyst concentrations and linoleate oxidation as measured by the formation of conjugated dienes,[8] and by the decrease in pO_2 by polarography.[16] On the other hand, the kinetics of peroxidase-catalysed carotene bleaching are different: activity appears to be proportional to the square root of catalyst concentration. It is possible that the early inactivation of the catalyst is responsible for the observed kinetics of peroxidase-catalysed carotene bleaching since, as the concentration of the catalyst is increased, its activity falls off after increasingly shorter time intervals. In the case of cytochrome *c* and other heme proteins, the relation between activity and catalyst concentration appears to depend on the technique employed in the measurement of the rate of reaction. While there is general agreement that lipoxy-

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genase exhibits a direct proportionality between activity and enzyme concentration, it has been reported that the catalytic activity of heme proteins is proportional to the square root of their concentration. Dicks and Friend[15] used this difference in an activity–concentration relationship to define the type of catalyst present in Triton extracts of sugarbeet mitochondria. The present results obtained by measuring the initial reaction rates indicate that the kinetics of carotene bleaching do not permit differentiation between cytochrome *c* and lipoxygenase.

Inhibition of carotene bleaching by antioxidants reveals additional differences between lipoxygenase, cytochrome *c* and peroxidase: peroxidase-catalysed carotene bleaching is much more sensitive to ascorbic and sinapic acids, compared to the lipoxygenase-catalysed reaction, while the effect of the antioxidants on cytochrome *c* is intermediate. Furthermore, peroxidase exhibits a distinct induction period in the presence of ascorbic acid.

EXPERIMENTAL

Enzyme solutions. Lipoxygenase (Fluka AG), horse radish peroxidase (California Corporation for Biochemical Research) and cytochrome *c* (Sigma Chemical Co.) were dissolved in freshly distilled water at 1 mg/ml and further dilutions were made in distilled water.

Assay of Carotene-oxidizing Activity

Aqueous linoleate solution. Linoleic acid (Fluka AG) in ethanol (7·5%, w/v; 1 ml) was mixed with Tween 80 in ethanol (10%, v/v; 0·3 ml). Aqueous EDTA (0·5%; 5 ml) was added, the pH adjusted to 9·0 by dropwise addition of 0·1 N NaOH, and the volume adjusted to 10 ml with distilled water. The solution was refrigerated under N_2 for up to 1 week.

Aqueous carotene solution. β-Carotene (25 mg) (Eastman Organic Chemicals) and Tween 80 (0·9 ml) were dissolved in chloroform (25 ml). When not used immediately, the solution was refrigerated under N_2, protected from light, up to 48 hr. This solution (1 ml) was evaporated to dryness under vacuum and the residue dissolved immediately in EDTA solution (0·25%; 10 ml). This solution was prepared daily.

Aqueous buffered carotene–linoleate solution. Aqueous linoleate (1 ml) was mixed with aqueous carotene (1 ml), and the volume adjusted to 10 ml with buffer. This solution, prepared immediately before use, contained 750 μg linoleic acid, 10 μg β-carotene, 0·66 μl Tween 80 and 0·5 mg EDTA per ml.

Assay procedure. The reaction was carried out at room temp. (25 ± 2°) in the cuvette of a double-beam recording spectrophotometer set at 460 nm. The test sample contained 1·5 ml buffered carotene–linoleate, 0·1–0·4 ml enzyme solution, and distilled water to a final volume of 2·0 ml. The enzyme solution was added at zero time, after balancing the instrument with water in place of the enzyme solution. The initial rate of decrease in absorbance was computed from the recorded graph and converted into μg carotene/min. The concentrations in the initial reaction mixture were as follows: β-carotene $1{\cdot}4 \times 10^{-5}$ M; linoleate 2×10^{-3} M; EDTA 10^{-3} M; and Tween 80 0·05%. Antioxidants were added for evaluation in 0·1 ml ethanol, except for ascorbic acid, which was dissolved in 0·1 ml water. Corresponding amounts of ethanol were added to the controls not containing antioxidants.