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ENZYMIC OXIDATION OF CAROTENE AND LINOLEATE BY ALFALFA: PROPERTIES OF ACTIVE FRACTIONS

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Abstract—An aqueous Triton X-100 extract, prepared from alfalfa leaves, was fractionated on carboxymethylcellulose into four fractions designated *A–D*. Fractions *B* and *C*, which contained the bulk of the carotene-bleaching and linoleate-oxidizing activity, resembled soybean lipoxygenase in several respects. The most striking among these were the high specific activities with regard to the formation of conjugated dienes and oxygen absorption by linoleate; the inability to oxidize oleate; and the insensitivity to cyanide. Fraction *C*, like lipoxygenase, exhibited the absorption spectrum of a pure protein devoid of a prosthetic chromophore, whereas the spectrum of fraction *B* hinted at some contamination with a heme protein. Fraction *A* resembled peroxidase in its high specific peroxidase and indoleacetic acid oxidase activities, coupled with weak linoleate oxidizing and carotene-bleaching activities. Like peroxidase, fraction *A* was able to effect some bleaching of carotene in the presence of oleate and also in the absence of polyunsaturated fatty acids, with a characteristic rapid decline in activity. Additional points of resemblance of fraction *A* with peroxidase were its sensitivity to cyanide and ascorbic acid and its pH optimum in the carotene-bleaching test. Finally, fraction *A* could be separated on carboxymethylcellulose into two subfractions, which exhibited heme protein absorption spectra with typical redox shifts. Fraction *D* was only weakly active. It exhibited some of the properties of heme proteins, but no clear picture as to its nature could be obtained by the tests used.

INTRODUCTION

WE HAVE previously reported that aqueous extracts of alfalfa leaves exhibit pronounced carotene and linoleate oxidizing activity.[1] Furthermore, two of the protein fractions obtained by chromatography on carboxymethylcellulose (CMC) had high specific activities, and on the basis of their chromatographic behaviour, resembled soybean lipoxygenase (E.C. 1.13.1.13). The purpose of the present study was to characterize further these fractions. The assay procedures used in this work permit the measurement of initial oxidation rates and are applicable to the study of the catalytic effects of both lipoxygenase and heme proteins. They include the measurement of oxygen absorption by linoleate by a polarographic procedure,[1] the determination of the increase in conjugated dienes in linoleate by a spectrophotometric technique,[2] and the spectrophotometric recording of the rate of carotene bleaching.[1,3]

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RESULTS

Substrate Specificity

An aqueous Triton X-100 extract was prepared from alfalfa leaves and fractionated by chromatography on CMC. The activity of the four fractions, designated *A–D*,[1] was determined with oleate and linoleate as substrates, using as criteria of activity oxygen absorption and carotene bleaching. Commercial soybean lipoxygenase, peroxidase and cytochrome c were assayed under the same conditions. The results of these tests are presented in Table 1.

TABLE 1. OXIDATION OF OLEATE AND LINOLEATE AND DESTRUCTION OF CAROTENE BY ALFALFA FRACTIONS *A–D* AND SOME OTHER ENZYMES

	O_2 Absorption, μl/min/mg protein		Carotene destruction, μg/min/mg protein	
Enzyme fraction	+Oleate	+Linoleate	+Oleate	+Linoleate
A	0	3	45	70
B	0	153	88	705
C	0	577	91	1150
D	1	8	12	80
Lipoxygenase	3	288	24	566
Peroxidase	0	0	40	56
Cytochrome c	3	102	300	600

Carotene destruction: citrate–phosphate buffer 0·2 M, pH 6·5; 25°; fatty acid concentration 2×10^{-3} M.
Oxygen absorption: citrate–phosphate buffer 0·2 M pH 6·5; 30°; fatty acid concentration $6{\cdot}25 \times 10^{-3}$ M.

There was a striking lack of oxygen absorption by oleate in the presence of the alfalfa fractions. Lipoxygenase and the two heme proteins also failed to display any significant activity under these conditions. Oxygen absorption by linoleate, on the other hand, was most pronounced in the presence of fraction *C* and lipoxygenase. Fraction *B* and cytochrome c were also active in this respect, but fractions *D* and *A*, and peroxidase, induced little or no oxygen absorption by linoleate.

Carotene-bleaching activity, on the other hand, was observed in all cases also in the presence of oleate, though this effect was always weaker than that observed in the presence of linoleate. Table 1 shows that, on the basis of the relative bleaching efficiencies in the presence of these two fatty acids, fractions *B* and *C* resembled lipoxygenase. Fraction *A* was similar to the heme proteins, in that its carotene-bleaching activity with oleate was more than half the activity induced by linoleate. Cytochrome c was remarkably effective in bleaching carotene, even when linoleate was replaced by oleate.

Effect of Enzyme Concentration

The relations between the amounts of the different catalysts and the rates of linoleate oxidation and carotene bleaching are shown graphically in Fig. 1.

The rate of linoleate oxidation appears to be a linear function of the amount of fractions *A*, *B* and *C*, but fraction *D* does not conform to this pattern (Fig. 1a). With carotene bleaching as the criterion (Fig. 1b), fractions *A* and *D* exhibit linear relations, but deviations from linearity are observed for fraction *C* and, particularly, fraction *B*. Commercial lipoxygenase and cytochrome c were tested under the same conditions. The results (Fig. 1c) indicate a linear relation between the concentration of either catalyst and oxygen absorption,

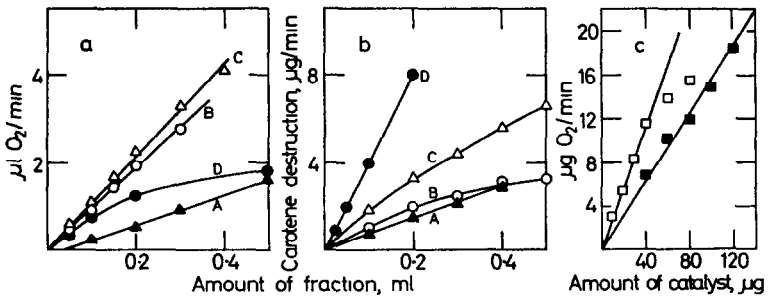

FIG. 1. RELATIONS BETWEEN ENZYME CONCENTRATION AND INITIAL VELOCITY OF REACTION.
(a) Linoleate oxidation by fractions A–D; (b) carotene destruction by fractions A–D; (c) linoleate oxidation by lipoxygenase (□) and cytochrome c (■). All determinations were carried out at pH 6·5, with the exception of cytochrome c which was tested at pH 7·5.

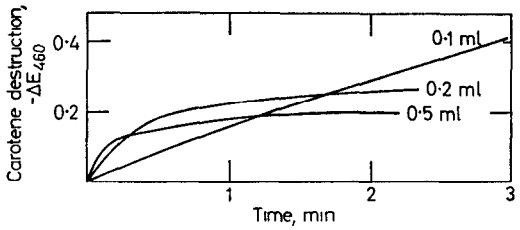

FIG. 2. TIME COURSE OF CAROTENE DESTRUCTION IN THE PRESENCE OF DIFFERENT AMOUNTS OF FRACTION A.
Citrate–phosphate buffer 0·2 M, pH 4·0; 25°.

and similar linear relations were found with carotene bleaching as a criterion, as reported by us elsewhere.[2]

The time course of carotene bleaching by fraction *A* is characterized by the rapid decline in activity, as illustrated in Fig. 2. As the amount of fraction *A* in the reaction mixture is raised, carotene bleaching declines after increasingly shorter time intervals. Thus, after 2 min, more carotene is bleached by 0·1 ml than by 0·5 ml of fraction *A*, although the *initial* rate of carotene bleaching is proportional to the amount of fraction *A*. We have found that peroxidase, among several catalysts tested, exhibits the same characteristic behavior toward carotene bleaching.[3]

Effect of Substrate Concentration

The effect of linoleate concentration on its initial rate of oxidation was tested in the presence of fraction *C*, the most active of the four fractions obtained by CMC chromatography. In these tests, the concentration of Tween 20 was adjusted, so that the weight ratio of Tween to linoleate remained equal to 1·0, a value found to be optimal for linoleate oxidation. Two criteria were used in these experiments: oxygen absorption by the polarographic method, and formation of conjugated dienes, by the spectrophotometric procedure.[2] With both techniques, the kinetics followed the Michaelis–Menten equation, as shown by the straight lines obtained in the Lineweaver–Burk plot. The following K_m values were calculated from these plots: $5{\cdot}0.10^{-3}$ M with the polarographic method; and $5{\cdot}3.10^{-4}$ M with the spectrophotometric diene procedure.

Inhibition by Cyanide

The effect of the four alfalfa fractions and three commercial catalysts on linoleate oxidation and carotene bleaching was studied, following preincubation of the catalysts with 10^{-2} M sodium cyanide during 10 min. The preincubation was carried out in citrate–phosphate buffer, pH 7·5, since in the absence of buffer, addition of sodium cyanide to the catalyst solutions raised the pH to about 10·5, which resulted in a pronounced inactivation of lipoxygenase.

Table 2 shows the percentage inhibition caused by cyanide in the case of the various fractions and catalysts. Assays were carried out at pH 6·7, where alfalfa fractions exhibit activity peaks, and also at slightly alkaline pH values. The data presented in Table 2 indicate that, with linoleate oxidation as a criterion, cyanide appreciably inhibited fractions *A* and *D*, as well as cytochrome c. Neither lipoxygenase, nor fractions *B* and *C*, were inhibited by

TABLE 2. EFFECT OF CYANIDE ON THE OXIDATION OF LINOLEATE AND ON CAROTENE DESTRUCTION IN THE PRESENCE OF ALFALFA FRACTIONS *A–D*, SOYA BEAN LIPOXYGENASE, PEROXIDASE AND CYTOCHROME C

		% Inhibition by cyanide			
		Oxygen absorption		Carotene destruction	
Enzyme	pH	6·7	7·2	6·7	7·7
A		36	—	26	14
B		3	−2	8	0
C		−10	5	12	4
D		17	33	15	0
Lipoxygenase		0	0	0	0
Peroxidase		—	—	46	41
Cytochrome c		50	50	0	1

Preincubation for 10 min with 10^{-2} M NaCN. Controls without CN^- were similarly incubated with 10^{-2} M NaOH. Citrate–phosphate buffer 0·2M, pH 7·5.

cyanide. When carotene bleaching served as the criterion, the alfalfa fractions were all slightly inhibited at pH 6·7, but there was no inhibition at pH 7·7, except for fraction *A*, which exhibited a 14% decrease in activity. Carotene bleaching by peroxidase was markedly inhibited by cyanide, at both pH 6·7 and 7·7, but neither lipoxygenase nor cytochrome c displayed any sensitivity to cyanide at these pH values. Thus, when cytochrome c is the catalyst, cyanide inhibits oxygen absorption by linoleate, but not the coupled oxidation of carotene.

Absorption Spectra of Alfalfa Fractions

Fraction *C* exhibited only a protein peak at 275 nm. Fraction *B* exhibited the same protein peak and also a shoulder at 410 nm. Fraction *D* yielded, in addition to the protein peak, a very small peak at 405 nm. Fraction *A* was rechromatographed on CMC (see Experimental), and the absorption spectra of the subfractions A_1 and A_2 are presented in Fig. 3.

Fraction A_1 in its oxidized form is seen to exhibit a clear peak at 417 nm, whereas in its reduced form, it shows three peaks at 427, 530 and 562 nm. Fraction A_2 has a peak at 405 nm in its oxidized state; upon reduction, this peak is shifted to 425 nm, while a small peak also appears at 560 nm.

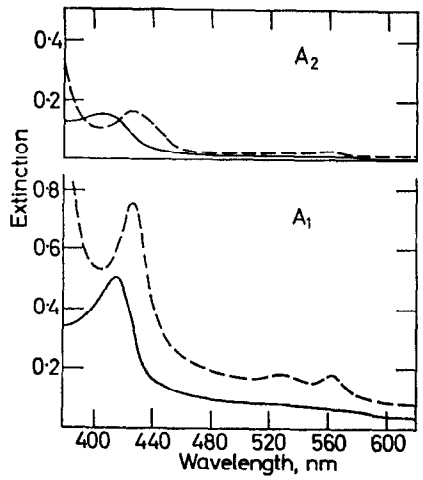

FIG. 3. ABSORPTION SPECTRA OF FRACTIONS A_1 AND A_2 BEFORE AND AFTER REDUCTION WITH $Na_2S_2O_4$. The reduction was carried out by addition of a few crystals of the reducing agent. The spectra were measured in acetate buffer 0·4 M, pH 5·5, against the pure buffer. ——— Oxidized; - - - - - - reduced.

Enzymic Activities of Fractions A–D

Fractions *A–D* were assayed for linoleate oxidation, carotene bleaching, and peroxidase and indoleacetic acid (IAA) oxidase activity. The results are presented in Table 3, together with those obtained with commercial soybean lipoxygenase, peroxidase and cytochrome c. Catalase, ascorbic acid oxidase and polyphenol oxidase activities were also assayed, but results were negative in all cases.

It is seen from Table 3 (see also Table 1) that fractions *B* and *C* and lipoxygenase had high specific activities toward linoleate oxidation and carotene bleaching. On the other hand, the specific activities of fraction *A* and peroxidase toward linoleate oxidation and

TABLE 3. RELATIVE SPECIFIC ACTIVITIES OF ALFALFA FRACTIONS *A–D* AND LIPOXYGENASE, PEROXIDASE AND CYTOCHROME C*

Enzyme	Linoleate oxidation		Carotene destruction	Peroxidase activity	IAA oxidase activity
	Oxygen absorption	Conjugated diene formation			
A	7	3	8	29	11
B	104	32	107	42	25
C	226	88	136	2	21
D	14	3	6	1	0
Lipoxygenase	100	100	100	0	0
Peroxidase	—	1	10	100	100
Cytochrome c	35	3	106	—	—

* Linoleate and carotene oxidizing activity = 100 for commercial lipoxygenase; peroxidase and IAA oxidase activity = 100 for commercial peroxidase.

carotene bleaching were very low. Both fractions *A* and *B* exhibited marked peroxidase activity, in contrast to the previously mentioned difference in activity with respect to carotene and linoleate oxidation. Comparison of total (rather than specific) activites shows that peroxidase activity in fraction *A* is 3·5 times greater than in fraction *B*, whereas total carotene bleaching activity by fraction *B* is 8 times greater as compared to fraction *A*.

Indoleacetic acid oxidase roughly paralleled peroxidase activity, except that fraction *C* displayed marked IAA oxidase activity, but no peroxidase activity. Fraction *D* yielded very weak activities in all tests and did not resemble any of the commercial enzymes tested.

Influence of Antioxidants

The following antioxidants markedly inhibited all alfalfa fractions with regard to carotene and linoleate oxidation: α-tocopherol, 2,6-di-tert.-butyl-4-methylphenol ('butylated hydroxytoluene', BHT), quercetin, ascorbic acid, and sinapic acid. The results obtained with fraction *C* in the presence of different levels of BHT are typical and are presented in Fig. 4.

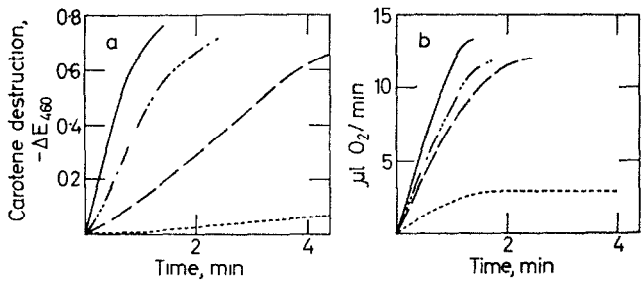

FIG. 4. EFFECT OF BHT ON ACTIVITY OF FRACTION C.

(a) Carotene destruction; (b) oxygen absorption by linoleate. Concentrations of BHT: ——— control; —·—·— 10^{-6} M; — — — 10^{-5} M; - - - - - 10^{-4} M.

It is seen that the rate of carotene bleaching decreased progressively, as the concentration of BHT is increased from 0 to 10^{-4} M. Higher levels of BHT completely inhibit carotene bleaching during the time interval tested. In the case of linoleate oxidation, the antioxidant causes, in addition to decreased initial rates of oxidation, a marked decline in oxygen uptake after short time intervals: thus, at a BHT concentration of 10^{-4} M, there is some slight initial oxygen uptake which stops completely after about 1 min. The picture is essentially the same with the other antixoxidants and alfalfa fractions. However, some quantitative differences were observed. Among the antioxidants tested, α-tocopherol was the most effective in inhibiting carotene bleaching, but its antioxidant activity in linoleate oxidation was relatively weak. It was also found that ascorbic and sinapic acids were very effective inhibitors of fraction *A*, but they were relatively weakly effective with regard to the other alfalfa fractions.

DISCUSSION AND CONCLUSIONS

Previous work[1] has established that the bulk of the carotene and linoleate-oxidizing activity of alfalfa leaves resides in fractions *B* and *C* eluted from CMC with 0·1 and 0·15 M sodium acetate buffer, pH 5·5. The results of the tests carried out in the present study indicate that these two fractions resemble soybean lipoxygenase.

The high specific activities of fractions *B* and *C* with regard to the formation of conjugated dienes and oxygen absorption by linoleate places them in the same category with soybean lipoxygenase (Tables 1 and 3). The latter enzyme, used for comparative purposes, contained a large proportion of inactive protein and had, in fact, a lower specific activity than the above two fractions. Peroxidase and cytochrome c were only weakly active in the diene test, an observation which we have reported and discussed earlier.[2]

The insensitivity of fractions *B* and *C* toward the inhibitory action of cyanide (Table 2) is typical of soybean lipoxygenase. Under the same conditions, heme proteins are markedly inhibited. It is not known why carotene bleaching in the presence of cytochrome c is not affected by cyanide (Table 2).

Substrate specificity is a further indication of the nature of fractions *B* and *C*. Like lipoxygenase, these fractions are unable to oxidize oleate. Their carotene-bleaching activity in the presence of oleate is only about one-tenth that found in the presence of linoleate (Table 1), whereas the corresponding ratio is much higher for the heme proteins tested.

The absorption spectrum of fraction *C* is that of a pure protein, devoid of a prosthetic group, which again, is typical of soybean lipoxygenase. A similar spectrum is observed for fraction *B*, except for a shoulder at 410 nm, possibly due to some contamination with a heme protein.

Fractions *B* and *C*, like lipoxygenase, are only weakly inhibited by ascorbic acid, in the carotene-bleaching test, in contrast to the strong inhibition found in the case of peroxidase and also cytochrome c.[3] Blain *et al.*[4] have used the inhibition by ascorbic acid as a criterion for differentiating between lipoxygenase and heme proteins.

The pH optima for fractions *B* and *C* in carotene bleaching was previously[1] reported to be around 6·5–7, and a value of 7 was found for soybean lipoxygenase,[3] whereas heme proteins had quite different pH optima under these conditions. Again, pH optima of 6·7–7 are observed for fractions *B* and *C* and for lipoxygenase in the polarographic measurement of oxygen uptake by linoleate (Ref. 1 and unpublished observations).

Among the other fractions obtained from alfalfa leaf extract by CMC chromatography, fractions A_1 and A_2 can be definitely identified as heme proteins, with a strong resemblance to peroxidase. This is seen first of all by the relatively high specific peroxidase and IAA oxidase activities of fraction *A* and the low specific activity of this fraction in linoleate oxidation and carotene bleaching, and also by the absorption spectra of subfractions A_1 and A_2, which are typical of heme proteins (Fig. 3). Additional purification experiments (to be published) have further shown that fraction *A* contains inactive protein, while fraction *B* is contaminated with a peroxidase-like protein.

Fraction *A*, like peroxidase, is able to stimulate carotene bleaching in the presence of oleate, albeit to a lesser extent than in the presence of linoleate. Fraction *A* is markedly inhibited by cyanide[5] and by low concentrations of ascorbic acid, as is the case also for peroxidase. The optimal pH for carotene bleaching is around 4 for fraction A_1 as well as for peroxidase.[3] The rapid decline in the rate of carotene oxidation, especially at higher concentrations of fraction *A* (Fig. 2), is also typical of peroxidase.[3] Fraction *A*, alone among the alfalfa fractions, is able to induce carotene bleaching also in the absence of added fatty acids (unpublished), a property also found for peroxidase.[3]

The fourth main fraction of alfalfa, designated *D*, cannot be readily identified on the

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basis of the tests conducted. Partial inhibition by cyanide indicates the presence of a heme protein, but other properties failed to yield a clear picture of the nature of this fraction. Total and specific activities toward linoleate oxidation and carotene bleaching were relatively low.

A kinetic criterion has been used frequently for distinguishing between lipoxygenase and heme proteins. Thus Tappel[5] has reported that, while linoleate oxidation varies in direct proportion to the concentration of lipoxygenase, in the case of catalysis by heme proteins, the rate of the reaction is proportional to the square root of the catalyst concentration. This kinetic criterion was of no help in the present study of alfalfa fractions, since both lipoxygenase and cytochrome c were found to yield straight-line relationships between rate of linoleate oxidation and catalyst concentration. A similar result was obtained when the oxidizing activity was measured by the formation of conjugated dienes[2] and by carotene bleaching.[3] It is possible that measurement of initial reaction rates yields the simpler kinetics observed in the case of heme protein catalysis.

Our observations concerning the presence of a powerful lipoxygenase-like enzyme system in alfalfa leaves are in agreement with the recent findings by Holden[6] who detected lipoxygenase in the leaves from many different plants. Siddiqi and Tappel[7] had earlier reported the presence of lipoxygenase in the press juice of fresh alfalfa. It appears therefore that the presence of lipoxygenase is not restricted to seeds and that the enzyme is also found in leaves.

EXPERIMENTAL

Enzymes

Enzymes were obtained from the same commercial sources and were prepared as described in our previous work.[2]

Chromatography on Carboxymethylcellulose (CM-cellulose)

This chromatography was carried out as described by Grossman *et al.*[1] For further purification of fraction *A*, 1 l. of alfalfa extract was chromatographed on a 4 × 50 cm CM-cellulose column. The eluate obtained with 0·05 M sodium acetate buffer, pH 5·5 was diluted 1:4 and this solution was again applied to a 3 × 20 cm column of the same adsorbent. No enzymic activity was detected in the eluate obtained with the 0·01 M sodium acetate buffer; the fractions eluted with the same buffer at 0·02 M and 0·05 M concentration were enzymically active and were designated A_1 and A_2, respectively.

Enzyme Assays

Carotene destruction was determined by the spectrophotometric measurement of the decrease in absorbance of an aqueous carotene solution, according to Ben-Aziz *et al.*[3]

Linoleate oxidation was assayed by two different techniques: (a) oxygen absorption, measured polarographically,[1] and (b) measurement of conjugated diene formation.[2]

Peroxidase activity was assayed according to Chance and Maehly,[8] catalase according to Beers and Sizer,[9] IAA oxidase according to Meudt,[10] ascorbic acid oxidase according to Dawson and Magee[11] and polyphenol oxidase according to Alberghina [12]

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