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ENZYMIC OXIDATION OF CAROTENE AND LINOLEATE BY ALFALFA: EXTRACTION AND SEPARATION OF ACTIVE FRACTIONS

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Abstract—The activity of aqueous extracts from alfalfa leaves toward the oxidation of linoleate and the coupled oxidation of carotene was measured by polarographic and colorimetric methods, respectively. Highest activities were achieved by using as extractant a solution of 1 per cent Triton X-100, in phosphate buffer pH 6·8. Chromatography on CM-cellulose resulted in the separation of four distinct protein peaks (A–D), which coincided with peaks of lipid oxidizing activity. The bulk of the activity of the extract was recovered in peaks B and C. All fractions exhibited activity maxima at pH 6·5–6·8. A second activity peak at pH 4·0–4·5 was also observed in fractions A and B in the carotene oxidation test.

INTRODUCTION

CAROTENOIDS present in the green part of most plants are highly susceptible to rapid oxidative degradation.[1] This is the case particularly for alfalfa, which is an economically important fodder plant and constitutes a rich source of carotenoids. In the production of dehydrated alfalfa meal it is important that carotenoid losses be kept low, following harvesting, before the material reaches the drier. Scalding or autoclaving of the fresh alfalfa stabilizes the carotenoids to a marked extent. This was established first by Hauge and Aitkenhead[2] who used the biological vitamin A assay as a criterion, and it has since been demonstrated by numerous workers by direct measurement of carotenoid concentration.

Concurrently with the early observations on the beneficial effect of heat on the stability of carotenoids in fresh alfalfa, the idea developed that the destructive processes were of an enzymic nature. Sumner[3] reported that alfalfa had slight lipoxygenase activity (about 400 times less than soybeans), and Mitchell and Hauge[4] showed that addition of cottonseed oil, which is rich in linoleic acid, accelerated the destruction of carotenoids in alfalfa suspensions, an observation which was interpreted as indicating the presence of lipoxygenase. This enzyme specifically catalyzes the peroxidation of the *cis-cis* 1,4-pentadiene system, and the process causes the co-oxidation of various accompanying compounds, such as carotene. Siddiqi and Tappel[5] investigated the effect of alfalfa press juice on the oxygen absorption by

[1] H. BOOTH, *J. Sci. Food Agric.* **11**, 8 (1960).
[2] S. M. HAUGE and W. AITKENHEAD, *J. Biol. Chem.* **93**, 657 (1931).
[3] R. J. SUMNER, *Ind. Eng. Chem. Anal. Ed.* **15**, 14 (1943).
[4] H. L. MITCHELL and S. M. HAUGE, *J. Biol. Chem.* **163**, 7 (1946).
[5] A. M. SIDDIQI and A. L. TAPPEL, *Plant Physiol.* **31**, 320 (1956).

linoleate. On the basis of substrate specificity, and inhibition by antioxidants but not by cyanide, they reached the conclusion that alfalfa contains a true lipoxygenase. This enzyme, being apparently devoid of a prosthetic group, is insensitive to cyanide, as pointed out by Holman and Bergström.[6] However, Mitchell and Hauge[4] found that cyanide inhibited the carotene-destroying system in alfalfa, and this has been also reported for sugar beet leaves by Dicks and Friend.[7]

The question of the nature of the lipid-oxidizing factors in alfalfa is further complicated by recent reports concerning the carotenoid-destroying role of heme proteins present in chloroplasts and mitochondria.[7,8] The question of whether or not the rapid destruction of carotenoids in alfalfa homogenates is due to lipoxygenase cannot be considered as settled. In the work described here, an attempt was made to extract and fractionate the carotene-destroying and linoleate-oxidizing factors present in homogenates from fresh alfalfa leaves. The two criteria used for this purpose were (*a*) uptake of oxygen by linoleate, measured by a polarographic method similar to that described by Mitsuda *et al.*[9] and (*b*) the coupled oxidation of carotene in the presence of linoleate, measured by a spectrophotometric method.

RESULTS

Optimal Conditions for Extraction

A number of extraction media were tried for the homogenization of the alfalfa leaves, and the activities of the resulting extracts were determined with regard to linoleate and carotene oxidation. The results (Table 1) show that the extraction medium yielding the highest

TABLE 1. COMPARISON OF LIPID-OXIDIZING ACTIVITIES OF THE SUPERNATANT (20,000 × *g*) OBTAINED FROM ALFALFA LEAVES HOMOGENIZED WITH VARIOUS EXTRACTANTS. THE ACTIVITIES, DETERMINED AT pH 6·8, ARE GIVEN AS PERCENTAGES OF THE ACTIVITY OF THE TRITON X-100 EXTRACT

Extractant	Relative oxidative activity	
	Linoleate oxidation	Carotene destruction
Triton X-100, 1 % in phosphate buffer, pH 6·8	100	100
Tween 80, 1 % in phosphate buffer, pH 6·8	32	85
NaCl, 1 % in phosphate buffer, pH 6·8	12	26
Phosphate buffer, pH 7·0	3	65
Distilled water, followed by adjustment to pH 4·5	3	83

The extracts were prepared as described in the Experimental section, except for the use of different extractants and the omission of the acidification and dialysis steps.

activities was a solution of the detergent Triton X-100 at pH 6·8. Linoleate oxidation was highly dependent on the type of extracting medium used, little activity being recovered in the supernatant in the absence of Triton or Tween. On the other hand, with carotene disappearance as the criterion, considerable activity was obtained in the supernatant even in the absence

[6] R. T. HOLMAN and S. BERGSTRÖM, in *The Enzymes* (edited by J. B. SUMNER and K. MYRBÄCK), Vol. II, Part 1, p. 559, Academic Press, New York (1951).
[7] J. W. DICKS and J. FRIEND, *Phytochem.* **6**, 1193 (1967).
[8] J. FRIEND and A. M. MAYER, *Biochim. Biophys. Acta* **41**, 422 (1960).
[9] H. MITSUDA, K. YASUMOTO and A. YAMAMOTO, *Agr. Biol. Chem.* **31**, 115 (1967).

of detergents. Table 2 shows that relatively inactive insoluble material which interfered with the subsequent chromatography could be removed by acidifying the crude homogenate to pH 4·5 before centrifugation; furthermore, the supernatant thus obtained was more active than the original homogenate.

TABLE 2. EFFECT OF ACIDIFICATION ON THE LIPID-OXIDIZING ACTIVITY OF ALFALFA LEAF HOMOGENATE

	Relative oxidative activity*	
	Linoleate oxidation, pH 6·2	Carotene destruction, pH 4·8
Triton alfalfa homogenate	100	100
After acidification to pH 4·5 and centrifugation:		
Supernatant	116	225
Precipitate†	10	37

* Extracts were diluted 5-fold and 10-fold for assay of linoleate oxidation and carotene destruction, respectively.

† Resuspended in amount of phosphate buffer 0·005 M, pH 6·8, equal to initial volume of homogenate.

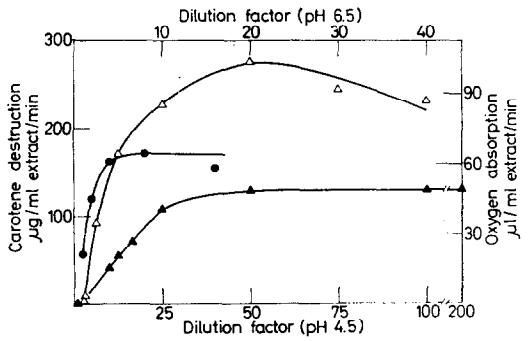

FIG. 1. EFFECT OF DILUTION ON INITIAL RATE OF CAROTENE DESTRUCTION AND LINOLEATE OXIDATION.

▲ Carotene destruction, pH 4·5;
△ Carotene destruction, pH 6·5;
● Oxygen absorption by linoleate, pH 6·5.

The Triton extract became considerably more active upon dilution, as shown in Fig. 1. In the case of carotene destruction the undiluted extract exhibited a very low activity. With increasing dilution, the rate of destruction increased to such an extent that by the use of a smaller amount of extract a greater absolute destruction was obtained. The rate of carotene destruction, expressed per ml original extract, reached a constant value, at pH 4·5, at a 40-fold dilution. At pH 6·5, the rate of carotene destruction and oxygen absorption also increased with dilution, but reached a constant value at lower dilutions.

Purification and Fractionation on Carboxymethylcellulose (CM-cellulose)

Attempts to isolate the active principles by ammonium sulfate precipitation or adsorption on DEAE-cellulose were unsuccessful because of considerable losses of activity. Chromatography on CM-cellulose was satisfactory, but required removal of salts from the extract

prior to application to the column. When the salts were removed by dialysis against cold distilled water, there were considerable losses of activity, but this difficulty was overcome by dialyzing against cold dilute sodium acetate buffer at pH 5·5. Under these conditions, only 5–10 per cent of the activity was lost. The latter pH was also optimal for subsequent CM-cellulose chromatography. At lower pHs, adsorption was too strong and relatively high buffer concentrations were required for elution of the active fractions, resulting in a low recovery of total activity. At pH values above 6·0, the adsorptive capacity of the column was low and all enzymic activity emerged with the starting buffer. The detergent Triton X-100, which contributes extinction at 280 nm, thus interfering with the determination of the protein elution pattern, could be washed out of the CM-cellulose column with approximately 2 l. of starting buffer at pH 5·5.

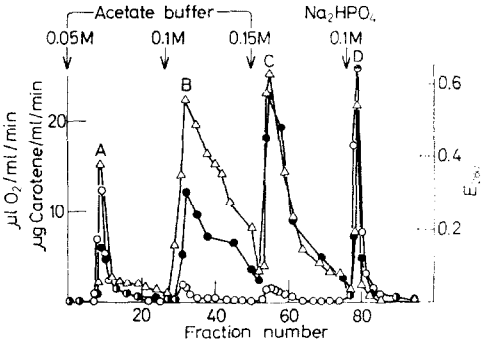

FIG. 2. CHROMATOGRAPHY OF ALFALFA EXTRACT ON CM-CELLULOSE.

○ Protein, extinction at 280 nm;
△ Carotene destruction, μg/ml/min;
● Linoleate oxidation, μl O_2/ml/min.

Figure 2 shows that three distinct protein peaks (A, B and C) were obtained by using sodium acetate buffer, pH 5·5, of increasing molarity. Concentrations above 0·15 M did not elute any additional activity from the column. A fourth peak (D) was obtained by elution with 0·1 M disodium phosphate. Further washing of the column with Tris buffer 0·1 M, pH 10, did not elute any further activity. Figure 2 also shows that each of the protein peaks corresponds to a peak of carotene and linoleate-oxidizing activity. All fractions were stable and could be stored for several months at $-20°$ as CM-cellulose eluates. Freeze-drying destroyed most of the activity of the fractions.

Activities of the Fractions toward Linoleate and Carotene Oxidation

The recovery and distribution of carotene and linoleate-oxidizing activity among the four fractions obtained by chromatography on CM-cellulose are presented in Table 3, which also includes the specific activities for those fractions collected near the elution peaks. The bulk of the activities was found in fractions B and C, with fraction A and D contributing only little to the total activity. There were also great differences in the specific activities: fraction C exhibited the highest specific activity toward both linoleate and carotene oxidation, followed by fraction B. Fractions A and D had much lower specific activities under the conditions of the test.

TABLE 3. ACTIVITIES TOWARD OXIDATION OF LINOLEATE AND CAROTENE OF DIFFERENT FRACTIONS OBTAINED BY CHROMATOGRAPHY ON CM-CELLULOSE. ASSAYS CARRIED OUT AT pH 6·7

	Linoleate oxidation		Carotene destruction	
Fraction*	μl O_2 absorbed/min/mg protein†	Total activity recovered (%)	μg/min/mg protein†	Total activity recovered (%)
Unchromatographed extract	—	100	—	100
A	19	14	48	5
B	300	29	607	43
C	651	42	771	29
D	41	9	37	8
All fractions	—	84	—	85

* Fractions A–D are designated as in Fig. 2.
† For the peak fractions. Protein calculated from absorbance readings at 280 nm.

Effect of pH

The pH activity curves were determined for each of the four fractions, and also for the unchromatographed extract, using both linoleate oxidation and carotene destruction as criteria (Fig. 3). Linoleate oxidation exhibits a maximum at pH 6·5–6·8 for all fractions and for the unchromatographed extract. Except for fraction A, activity declines sharply toward both acid and alkaline pH values. In the coupled oxidation of carotene, there is a single sharp activity peak around pH 6·5 for fractions C and D. The unchromatographed extract, and fractions A and B also exhibit a maximum at pH 6·5–6·8. However, fraction A shows an even more pronounced peak at pH 4·5, whereas fraction B and the unchromatographed extract exhibit smaller peaks at pH 4·0.

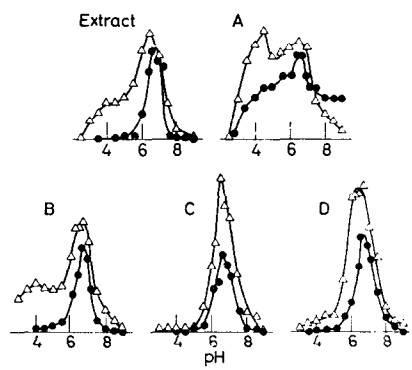

FIG. 3. EFFECT OF pH ON LIPID-OXIDIZING ACTIVITIES OF ALFALFA EXTRACT AND OF FRACTIONS A–D OBTAINED BY CHROMATOGRAPHY ON CM-CELLULOSE.

△ Carotene destruction;
● Linoleate oxidation.
In the case of alfalfa extract the amounts used were 0·2 and 0·5 ml of a 10-fold dilution for carotene destruction and linoleate oxidation, respectively.

DISCUSSION

The present work represents an attempt to extract and fractionate the linoleate and carotene-oxidizing factors present in alfalfa leaves. Oxygen absorption by linoleate was measured by a polarographic technique similar to that described by Mitsuda *et al.*,[9] whereas carotene oxidation in the presence of linoleate was followed spectrophotometrically. Both methods were adapted for direct recording of initial reaction rates. Full evaluation of these techniques will be reported elsewhere.

Using these two criteria for assaying the activity of extracts and fractions, it was found that efficient extraction of carotene and linoleate-oxidizing factors from alfalfa leaves requires the use of Triton solutions. This points to the probable involvement of subcellular particles as sources of activity. Solubilization of active particulate material from leaves by Triton has been achieved by Dicks and Friend.[7] Dilution of the Triton extract caused an increase in activity, as was also reported recently by Blain *et al.*[10] for the carotene-bleaching activity of tomato extracts. This dilution effect is possibly related to the presence of Triton and also of a water-soluble antioxidant,[11]

Chromatography on CM-cellulose yielded four distinct fractions active toward the oxidation of linoleate and carotene. Two of these fractions, designated B and C, amounted to 71–72 per cent of the total activity of the leaves and also exhibited the highest specific activities toward linoleate oxidation and carotene destruction. All four fractions had pH optima around 6·5–6·8, with negligible activity above pH 8·0. Under similar conditions of chromatography, an aqueous soybean extract yielded four protein peaks in positions corresponding to the four alfalfa fractions A–D, but only the two peaks corresponding to B and C displayed linoleate oxidizing activity. Thus, on the basis of chromatographic behaviour on CM-cellulose, alfalfa fractions B and C resemble soybean lipoxygenase.

Additional purification and characterization attempts are being made, in order to define the nature, properties and intracellular localization of the four fractions obtained from alfalfa leaves.

EXPERIMENTAL

Materials

Leaves were taken from "Hairy Peruvian" alfalfa, *Medicago sativa* (Leguminosae), grown under irrigation and cut at the first appearance of bloom.

Preparation of Extracts

The preparation of extracts was carried out at 4°. 100 g alfalfa leaves were placed in the cup of a Waring Blendor, together with 200 ml 0·005 M phosphate buffer, pH 6·8, containing 1% Triton X-100. The Blendor was operated at top speed, three times for 2 min, with 10-min intervals between the blendings. The homogenate was passed through two layers of cheese-cloth and acidified with HCl to pH 4·5. The precipitate was removed by centrifugation at 20,000 *g* during 10 min. The pH of the supernatant was immediately raised to 5·5 by addition of NaOH, and the solution was dialyzed against 0·005 M NaOAc buffer, pH 5·5, during 6 hr with three changes of buffer. This dialyzed solution, which still contained Triton X-100, is designated "alfalfa extract".

Chromatography on Carboxymethylcellulose (CM-cellulose)

Alfalfa extract, in amount not exceeding 100 ml, was applied to a CM-cellulose column (2 × 30 cm) previously equilibrated with 0·005 M NaOAc, pH 5·5, starting buffer. Elution was performed by the starting buffer (approximately 2 l.) until the E_{280} value of the eluate began to return to the baseline value. The Triton and inactive proteins were thus eluted. Elution was then continued with successive portions of 0·05 M, 0·1 M and 0·15 M NaOAc buffer, pH 5·5, and finally with 0·1 M Na_2HPO_4. Changes of buffer were carried

[10] J. A. BLAIN, J. D. E. PATTERSON and M. PEARCE, *J. Sci. Food Agric.* **19**, 713 (1968).
[11] A. BEN AZIZ, S. GROSSMAN, P. BUDOWSKI, I. ASCARELLI and A. BONDI, *J. Sci. Food Agric.* **19**, 605 (1968).

out when the E_{280} readings approached the baseline. The flow rate was about 1·5 ml/min for the starting buffer and 2·5–3·0 ml/min for the subsequent eluents, and 8-ml fractions were collected. All operations were carried out at 4°. At least every third tube was assayed for protein content and enzyme activity.

Determination of Protein

Protein was estimated from the E_{280} readings by assuming that a reading of 1·0 corresponds to a protein concentration of 1 mg/ml. The protein values thus obtained were used for the calculation of specific enzyme activities.

Assay of Linoleate Oxidation

Linoleate oxidation was determined at 30° using a YSI Biological Oxygen Monitor (Yellow Springs Instrument Co., Yellow Springs, Ohio). A volume of 2·5 ml buffered $7{\cdot}5 \times 10^{-3}$ M linoleate solution, containing Tween 20 and prepared as described by Ben-Aziz *et al.*,[11] was placed in the reaction cell, 0·5 ml enzyme solution was added, and the oxygen uptake (ratio of oxygen taken up by the sample to total oxygen dissolved in the initial reaction mixture) was recorded. Under these conditions boiled enzyme solutions did not show any activity.

Assay of Carotene-oxidizing Activity

(*a*) *Aqueous linoleate solution.* 1 ml of linoleic acid (N.B. Co., Cleveland, Ohio) in ethanol (75 mg/ml) was mixed with 0·3 ml of a solution of Tween 80 in ethanol (0·1 ml/ml). 5 ml aqueous EDTA (5·0 mg/ml) were added, the pH was adjusted to 9·0 by dropwise addition of 0·1 N NaOH and the volume of the solution was brought to 10 ml with distilled water. This solution may be kept refrigerated under N_2 for 1 week.

(*b*) *Aqueous carotene solution.* 25 mg β-carotene and 0·9 ml Tween 80 were dissolved in 25 ml $CHCl_3$. This solution was stable for 2 days if kept refrigerated in the dark under N_2. 1 ml of this solution was evaporated to dryness under vacuum. The residue was immediately taken up in 10 ml EDTA solution (2·5 mg/ml). This solution had to be prepared daily.

(*c*) *Aqueous buffered carotene-linoleate solution.* 1 ml aqueous linoleate was mixed with 1 ml aqueous carotene and the volume was brought to 10 ml with the desired buffer. This solution contained, per ml, 750 μg linoleic acid, 10 μg β-carotene, 0·66 μl Tween 80 and 0·5 mg EDTA, and its final pH was that of the buffer used in its preparation. This solution was stable for about 0·5 hr.

Assay procedure. The reaction was carried out at room temp (*ca.* 25°) in the cuvette (1 cm light path) of a double-beam recording spectrophotometer, with the wavelength adjusted to 460 nm, where the aqueous carotene exhibits its maximum absorbance. 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 concentrations of the components of the initial reaction mixtures were: beta-carotene, 7·5 μg/ml; linoleate, 2×10^{-3} M; Tween 80, 0·05 per cent (v/v); and EDTA, 10^{-3} M.

Prior to the start of the reaction the instrument was balanced at zero extinction, with both reference and test cuvettes filled with enzyme solution, at the same concentration as in the reaction mixture. The test cuvette was then filled with 1·5 ml carotene-linoleate substrate and the required amount of water, the reaction was started by addition of enzyme, and the initial rate of decrease of absorbance was recorded. Boiled enzyme solutions did not show any activity under the conditions of this test.

In those instances in which the unchromatographed extract was assayed, particularly before dialysis, it was found to be of low activity. This difficulty, due probably to the presence of inhibitors, could be overcome by dilution.

Specific activities. The specific activities for both linoleate oxidation and carotene destruction were expressed per mg protein.