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INTRACELLULAR DISTRIBUTION OF LIPOXYGENASE-LIKE ACTIVITY OF ALFALFA LEAVES

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Abstract—Particulate fractions were prepared from alfalfa leaf homogenates and tested for carotene and linoleate oxidizing activity. Mitochondria were the most active of the fractions tested, followed by chloroplasts. Microsomes contributed but little activity. Extracts obtained from particulate fractions with 1% aqueous Triton X-100 were submitted to chromatography on carboxymethylcellulose. Four active fractions were eluted in all cases. Their relative activities and other properties were similar to those observed for the corresponding fractions previously obtained from whole homogenates, i.e. the two fractions found to be most active exhibited the properties of lipoxygenase. None of the chromatographic fractions appeared to be specifically associated with any one subcellular particle.

INTRODUCTION

ALFALFA leaves contain a lipoxygenase-like enzyme system able to oxidise linoleate and carotene. We have previously reported details on the extraction of the active principles into aqueous solution and the preparation of active fractions by chromatography on carboxymethylcellulose,[1] and we have also described the properties of the fractions thus obtained.[2] The objectives of the present report are to provide information on the distribution of the activity among the cell components and the properties of the extracts obtained from them.

RESULTS

Distribution of Linoleate-oxidizing Activity among Subcellular Fractions

Chloroplasts, mitochondria, microsomes and the cytosol were prepared as described in Experimental. All fractions exhibited activity with regard to linoleate oxidation and boiling destroyed the activity. Data on the specific activities of the subcellular fractions and on the percentage distribution of activity between the fractions are presented in Table 1.

Chloroplasts contributed only a small percentage to the total oxidizing activity of the homogenate, whereas mitochondria constituted the main source of activity. An appreciable proportion of the activity was recovered in the cytosol and discarded fractions, mainly mitochondrial debris. Specific activity was found to be highest in the chloroplast fraction, due to the apparently low protein content of the latter. Indeed, the chloroplastic protein may have been underestimated by Lowry's method because of the presence of insoluble material in the reaction mixture, which required centrifugation before readings.

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TABLE 1. LINOLEATE-OXIDIZING ACTIVITY OF THE VARIOUS SUBCELLULAR FRACTIONS FROM ALFALFA LEAVES

Fraction	Volume (ml)	Protein content (mg/ml)	Linoleate oxidation (μl O_2 absorbed/ml/min)	Linoleate oxidation Specific activity (μl O_2 absorbed/mg protein/min)	Linoleate oxidation Total activity per fraction	(%)
Whole homogenate	170		13·2		2244	100
Chloroplasts	20	4·6	6·9	1·5	138	6
Mitochondria	20	17·7	14·4	0·8	288	13
Microsomes	10	2·6	2·0	0·8	20	1
Cytosol	166	7·4	5·1	0·7	850	38
Discarded fractions*					480	22

* Washings and debris from particulate fractions; most of the activity originated from mitochondrial debris obtained at 30,000 *g*.

Extraction of Enzyme Activity from Particles

In order to study the properties of the linoleate-oxidizing enzyme systems present in the subcellular particles, various extraction methods were tried. One per cent Triton X-100 was the most efficient of the solubilizing agents tested and extracted 41 and 80 per cent of the activities present in chloroplasts and mitochondria respectively. Higher concentrations of the detergent, or repeated extractions of the particles did not improve the yield. It was also found that part of the enzymic activity in chloroplasts could be brought into solution by sonication, the efficiency of the extraction depending on the duration of the treatment. However, even under the best conditions (sonication for 20 min) only 28 per cent of the activity in whole chloroplasts was extractable. In view of these results, and of earlier data[1] on the efficiency of Triton in extracting linoleate-oxidizing activity from whole homogenates, Triton X-100 was selected as the extracting agent for further studies.

Properties of the Triton Extracts

The pH curves were determined for Triton extracts of mitochondria, since these were the most active among the particles. The optimal pH for mitochondrial extracts was about 6·7 in both linoleate oxidation and carotene-bleaching tests, with a shoulder at 8·0. Whole mitochondria yielded the same maximum as the extracts. Difficulties were experienced in obtaining consistent pH-activity curves from chloroplast and microsome extracts because of their low overall activity and the low efficiency of extraction of chloroplasts, referred to above.

Neither the extracts from whole homogenates, nor those from chloroplasts and mitochondria, were inhibited by 0·01 M NaCN. Microsomal extracts, in which the activity was low, showed 21 per cent inhibition under these conditions. Triton extracts of particulate fractions and of whole homogenate could be stored at $-10°$ for several weeks, with only 10–20 per cent losses of activity. Dialysis against cold 0·005 M sodium acetate buffer, pH 5·5, did not appreciably decrease the linoleate oxidizing activity. However, Triton could not be removed under these conditions.

Attempts at concentrating or fractionating the activity of Triton extracts from particles generally met with the same difficulties previously encountered in the case of extracts from

whole homogenate.[1] Thus, ammonium sulfate precipitation resulted in partial destruction of linoleate-oxidizing activity and the spreading of the remaining activity over a number of protein fractions. Moreover, some Triton was adsorbed on the protein. Difficulties were also experienced in attempts at purifying Triton extracts by chromatography on diethylaminoethylcellulose columns: active protein was strongly adsorbed, and only a small percentage of linoleate-oxidizing activity could be eluted. On the other hand active fractions could be prepared in high yield by carboxymethylcellulose (CMC) chromatography, following removal of colloidal particles by acidulation and centrifugation, and dialysis of the supernatant (see Experimental). This procedure was identical to the one used for Triton extracts from whole homogenate.[1]

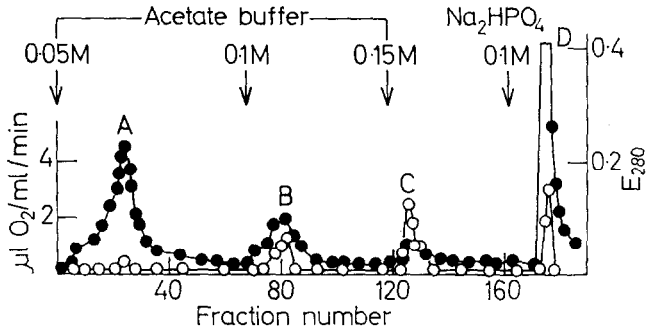

FIG. 1. CHROMATOGRAPHY OF TRITON EXTRACT FROM ALFALFA LEAF CHLOROPLASTS
●—Protein, extinction at 280 nm; ○—linoleate oxidation, μl O_2/ml/min.

Chromatography on Carboxymethylcellulose (CMC)

Chromatograms obtained with Triton extracts from chloroplasts and mitochondria on CMC columns are presented in Figs. 1 and 2. In both cases, four protein fractions were obtained which showed linoleate-oxidizing activity. Carotene-bleaching activity of the eluate

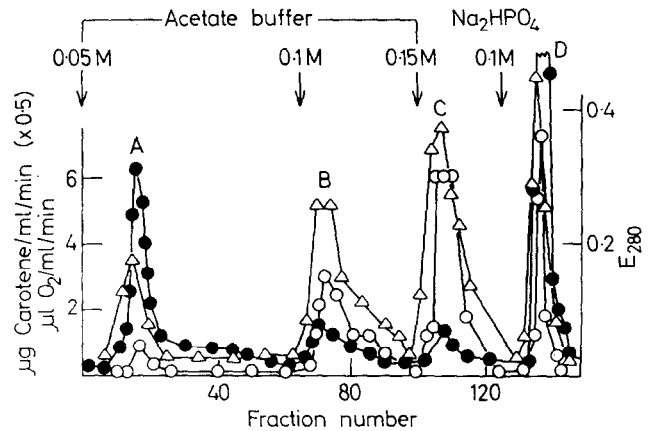

FIG. 2. CHROMATOGRAPHY OF TRITON EXTRACT FROM ALFALFA LEAF MITOCHONDRIA
●—Protein, extinction at 280 nm; ○—linoleate oxidation, μl/ml/min; △—carotene destruction, μg/ml/min.

was also studied in the case of mitochondrial extract (Fig. 2) and found to parallel linoleate-oxidizing activity. Similar results had been obtained earlier with Triton extracts from whole homogenate.[1] Table 2 illustrates the yield of linoleate-oxidizing and carotene-bleaching activities following CMC chromatography of the Triton extracts from mitochondria. From this table it can be seen that fractions B and C were the major oxidizing fractions of mitochondria, containing about 80 per cent of the total enzymic activity of the Triton extract. Fractions A and D, especially the former, contained only a small percentage of the total oxidizing activity of mitochondria. Also shown are the specific activities of the peak fractions obtained by CMC chromatography of the Triton extract of mitochondria. It is clear that

TABLE 2. LINOLEATE AND CAROTENE OXIDIZING ACTIVITIES OF DIFFERENT FRACTIONS OBTAINED BY CMC CHROMATOGRAPHY OF TRITON EXTRACT OF ALFALFA LEAF MITOCHONDRIA

	O_2 absorption		Carotene destruction	
Fraction*	Total activity recovered (%)	Specific activity† (μg/min/mg protein)	Total activity recovered (%)	Specific activity (μg/min/mg protein)
Unchromatographed extract	100	1·6	100	7·6
A	4	4·5	10	22·1
B	30	42·9	24	140·5
C	48	103·5	26	223·2
D	18	6·6	9	16·3

* Fractions A–D are designated as in Fig. 1.
† For the peak fractions. Protein calculated from absorbance readings at 280 nm.

fraction C had the strongest specific activity for both linoleate oxidation and carotene destruction. Fraction B also was very active, but fractions A and D had weak oxidizing activity. Similar pictures regarding the distribution of activity among fractions A–D and their specific activities were presented by extracts obtained from chloroplasts (Fig. 1) and also from whole homogenates, as shown previously.[1]

The cytosol lost 70 per cent of its activity upon dialysis against 0·005 M acetate buffer, pH 5·5, but CMC chromatography of the remaining activity again indicated the presence of four fractions similar to those obtained from particles and whole homogenate.

Additional tests were carried out with fractions A–D obtained from mitochondrial extracts, since the latter provided higher activities than chloroplasts and microsomes. Fractions B–D exhibited the same substrate specificity toward linoleate as previously reported for the analogous fractions from whole homogenate.[1,2] Oleate was not oxidized and did not induce carotene bleaching. Linoleate caused equal rates of oxygen absorption as linolenate.

Further points of resemblance between the chromatographic fractions prepared from mitochondria and whole homogenate were obtained from the pH optima at around 6·5 and from inhibition studies. The latter revealed that fractions B and C obtained from mitochondria were not inhibited by 0·01 M sodium cyanide in the linoleate oxidation and carotene bleaching tests. Other inhibitors (sodium azide 0·01 M, sodium fluoride 0·001 M and *p*-chloromer curibenzoic acid 0·001 M) were similarly without effect.

DISCUSSION

Previous work[1] on the extraction of oxidizing activity from alfalfa leaf homogenates had shown that only little activity remained in the supernatant fraction after centrifugation; thus, 0·2 M phosphate buffer, pH 7·0, extracted 3 per cent of the total linoleate-oxidizing activity of the homogenate, whereas various salt solutions yielded about 10 per cent soluble activity. In the present work, a much higher percentage of activity appeared in the cytosol. This was presumably due to the lengthy differential centrifugation procedure in 0·4 M sucrose solution resulting in the leakage of part of the activity in the solution. Nevertheless, it is clear from the data presented in Table 2 that among the subcellular particle fractions, the mitochondria constituted the main source of activity, particularly if the 'discarded' fractions, which were mainly mitochondrial debris, are taken into consideration. It is interesting that none of the 4 chromatographic fractions A–D was associated with a specific subcellular particle. Friend *et al.* have shown that the crocin-bleaching activity of sugar-beet leaves is distributed among mitochondria, chloroplasts and supernatant fraction.[3,4] According to Dicks and Friend,[5,6] Triton X-100 extracts of sugar-beet mitochondria oxidized crocin according to kinetics and inhibition characteristic of haem proteins. We have shown elsewhere[7,8] that the kinetic criterion does not necessarily permit an unequivocal distinction between catalysis caused by haem proteins or lipoxygenase. Our results obtained by CMC chromatography indicate that at least one of the fractions (fraction A) obtained from mitochondria, chloroplasts, cytosol, and also from whole homogenates[2] of alfalfa leaves, contains a peroxidase-like haem protein, whereas most of the activity (fractions B and C) is provided by enzymes behaving like soybean lipoxygenase. It is possible that the ratio of haem protein to lipoxygenase is different in leaves from different plant species. Recent work by Holden[9] has shown the presence of lipoxygenase in the leaves of a number of species.

Siddiqi and Tappel[10] reported the presence of a lipoxygenase in alfalfa press juice, but were unable to concentrate the enzyme. It appears from the present results that the activity is associated with particulate material and that solubilization (e.g. with Triton X-100) is required for purification and characterization of the enzyme.

EXPERIMENTAL

Separation of subcellular fractions. The leaves were homogenized in a 0·4 M sucrose solution containing 0·067 M phosphate buffer, pH 7·2, 0·01 M KCl, 0·01 M ascorbic acid and 0·0006 M EDTA. The ratio of sucrose solution to leaves was 3:1 (v:w). Ascorbic acid and EDTA were added to the medium after preliminary experiments indicated that they reduced loss of enzymic activity in the homogenate. The homogenate was filtered through a double layer of gauze.

Conditions of centrifugation for the separation of subcellular fractions were as follows. Whole and broken cells were removed by a 1-min centrifugation at 200 *g*. A chloroplast fraction was obtained by centrifugation at 1200 *g* for 10 min, and an additional 10 min centrifugation at 4000 *g* removed chloroplastic debris. Mitochondria were obtained after 20 min at 20,000 *g*, followed by mitochondrial debris after 20 min at 30,000 *g*. A final centrifugation at 105,000 *g* for 60 min yielded a microsomal fraction and supernatant cytosol. Chloroplasts, mitochondria and microsomes were washed by resuspension in the sucrose medium and recentrifugation. Determination of enzymic activity of particles was carried out on freshly prepared suspensions, because of rapid loss of activity upon storage.

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Extraction of enzymic activity from subcellular particles. Chloroplasts or mitochondria were suspended in ce-cold phosphate buffer, pH 6·8, containing 1 % Triton X-100, or other detergents as specified, and placed in a precooled blender. The suspension was homogenized three times for 2 min with 10 min intervals between homogenizations. Small amounts of particles were homogenized in a Potter–Elvehjem glass homogenizer. The homogenates were centrifuged at 20,000 *g* for 10 min and the supernatants were tested for enzymic activity. Unlike particle suspensions, Triton extracts retained their activity for several weeks when stored at or near 0°.

Extraction of activity for further purification. Extracts for further purification were prepared in phosphate buffer, pH 6·8, containing 1 % Triton X-100, 0·01 M ascorbic acid and 0·0006 M EDTA. The resultant homogenate was acidified to pH 4·5 with 1 N HCl and centrifuged at 20,000 *g* for 10 min. The clear supernatant, designated 'Triton extract', was brought to pH 5·5 with 1 N NaOH and dialysed at 4° against 0·005 M sodium acetate buffer, pH 5·5, for 6 hr. During this period the buffer was changed three times.

Sonication of chloroplasts. The chloroplasts were suspended in phosphate buffer, pH 6·8, and sonicated for different lengths of time in the cold in an MSE sonicator. The sonicated suspension was centrifuged at 20,000 *g* for 10 min.

Column chromatography. Chromatography of the Triton extracts of the subcellular particles on CMC columns and designation of the protein fractions A–D were as described by Grossman *et al.*[1]

Determination of linoleate oxidizing activity. This was done by measuring the rate of oxygen absorption by linoleate, as described by Grossman *et al.*[1]

Determination of protein and calculation of specific activities were also carried out according to Grossman *et al.*[1]

Determination of carotene-bleaching activity. The direct spectrophotometric method described by Ben-Aziz *et al.*[7] was used.

Key Word Index—Medicago sativa; Leguminosae; alfalfa; lipoxygenase; intracellular distribution.