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PURIFICATION AND PROPERTIES OF A CAROTENE CO-OXIDIZING LIPOXYGENASE FROM PEAS

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SUMMARY

1. An isoenzyme of pea lipoxygenase (linoleate:oxygen oxidoreductase, EC 1.13.1.13) has been purified to essential homogeneity by precipitation with $(NH_4)_2SO_4$, gel filtration, and ion-exchange chromatography on CM-Sephadex and DEAE-cellulose. The isoenzyme (optimum pH 6.3) bleaches carotene and chlorophyll *a* in the presence of linoleic acid and O_2.

2. The molecular weight is 78 000 as determined by gel filtration. The amino acid composition was analyzed, and it was shown that the enzyme contains four residues of free sulfhydryl groups and no cystine per molecule.

3. Isoelectric fractionation of the enzyme resulted in two proteins with lipoxygenase and carotene bleaching activity, pI values 6.00–6.15. The proteins do not differ in the amino acid composition and in the positional specificity of linoleic acid hydroperoxidation. The ratio of the 9- to the 13-hydroperoxy acid was 58:42.

4. The catalytic constants for 1 mole of this pea lipoxygenase (temp. 23 °C; pH 6.3): 4600 moles·min^{-1} linoleic acid and about 1200 moles·min^{-1} carotene. The rate of chlorophyll degradation is very much lower than the carotene bleaching velocity.

INTRODUCTION

Lipoxygenase (EC 1.13.1.13) catalyzes the oxidation of fatty acids such as linoleic acid, containing the *cis,cis*-methylene interrupted diene system, forming the conjugated *cis,trans*-diene hydroperoxide. The enzyme is known to occur in legume seeds, in some cereal grains and oil seeds[1]. Its presence has been reported also in leaves and other plant tissues[2]. Isoenzymes were isolated and extensively purified from soybeans[3–7] and peas[8]. The isoenzymes from soybeans and peas with a pH optimum of 6.5 oxidize β-carotene with linoleic acid as co-substrate[9] very quickly. In contrast, the isoenzyme from soybeans with a pH optimum of 9.0 (Theorell enzyme) is a poor carotene oxidizing enzyme[9,10].

We undertook the present study to show the identity of a lipoxygenase iso-

enzyme from peas with a potent carotene bleaching enzyme. The work described below deals with the purification and some properties of the enzyme.

MATERIALS AND METHODS

Peas (*Pisum sativum* L. "Mignon"); linoleic acid was obtained from Nu Chek Prep. CM-Sephadex C-50, Sephadex G-200 and G-150 were purchased from Pharmacia. DEAE-cellulose was obtained from Serva. β-Carotene was a gift of Hoffmann-La Roche, Basel. Tween 20 and Tween 80 were purchased from Schuchardt. Calibration proteins to determine the molecular weights were obtained from Boehringer Mannheim. The other chemicals were of analytical grade.

Enzyme assays

Lipoxygenase

During purification, fractions were assayed at 23 °C at pH 6.5 using a modification of the Surrey[11] substrate. The assay mixture contained 1.75 mM linoleic acid, 50 μl/l Tween 20, 80 mM sodium phosphate. One unit caused a $\Delta A^{1\text{ cm}}_{234\text{ nm}}$ of 1.0 per min in a 3-ml reaction mixture.

Carotene bleaching activity

The coupled oxidation of β-carotene was determined according Ben Aziz *et al.*[12]. One unit caused a $\Delta A^{1\text{ cm}}_{460\text{ nm}}$ of 1.0/min in a 2-ml reaction mixture.

In the legends of the figures and tables further assay conditions are described.

Chlorophyll bleaching activity

Isolation of chlorophyll a: The pigments from a crude chlorophyll preparation (Chlorophyll, native, Roth) were purified on thin layers of cellulose using a mixture of light petroleum (b.p. 60–80 °C) and acetone (80 : 20, v/v). Chlorophyll *a* was eluted with acetone from the cellulose. The concentration of this pigment was determined spectrophotometrically, by using the correlation *viz.* 1 μg chlorophyll *a*, dissolved in 1 ml acetone causes an absorbance, $A^{1\text{ cm}}_{430\text{ nm}} = 0.106$ (ref. 14).

Aqueous chlorophyll solution. 3.6 μl Tween 80 was added to a solution of 100 μg chlorophyll *a* in acetone. This mixture was evaporated to dryness under vacuum and the residue dissolved in 10 ml 0.1 M phosphate buffer (pH 6.3) which contained 5.2 mg EDTA.

Assay procedure. The reaction was carried out at 23 °C in a spectrophotometer. In a 1 cm cuvette 1 ml aqueous chlorophyll solution was mixed with 1 ml 0.1 M sodium phosphate buffer (pH 6.3) which contained 564 μg linoleic acid and 0.62 μl Tween 80. After adding 1 ml of the enzyme solution the decrease of the absorbance at 430 nm was measured.

Purification of lipoxygenase-2

All steps were performed at 4 °C.

Acetone-defatted ground pea seeds (100 g) were extracted with 1 l of (0.1 M) sodium acetate buffer (pH 4.5). The extract was filtered through cheesecloth, and the resulting filtrate centrifuged (30 min, 13 000 $\times$ *g*). The supernatant was treated with $(NH_4)_2SO_4$ and the fraction precipitating between 30 and 60% satn was dissolved in 0.05 M Tris–HCl buffer pH 7.5. After concentration by ultrafiltration the crude enzyme preparation (3 ml) was chromatographed on a Sephadex G-200 column

(2.6 cm × 85 cm) which was equilibrated with the same buffer. The fractions containing lipoxygenase were pooled, concentrated by ultrafiltration (Amicon; membrane PM-10) and washed with 0.05 M sodium acetate buffer (pH 4.8). The resulting solution (5 ml) was applied to a CM-Sephadex column and chromatographed as described in the legend of Fig. 2. The combined fractions were concentrated and washed with 0.01 M sodium phosphate buffer (pH 7.0) by ultrafiltration. The enzyme solution (5 ml) was applied to a DEAE-cellulose column and chromatographed as described in the legend of Fig. 3. For isoelectric focusing the fractions with the lipoxygenase-2 were pooled. The solution of the enzyme was concentrated and washed with water by ultrafiltration.

Protein determination

Protein was determined routinely by measuring the absorbance at 280 nm or 230 nm. For more accurate measurements especially when dealing with crude and semipurified preparations, the biuret method as described by Beisenherz[13] was used.

Isoelectric focusing on sucrose density gradient

The experiments were carried out with an LKB 8101 electrofocusing column of 110-ml capacity (LKB Producter AB, Bromma, Sweden). Electrofocusing was performed as described in the LKB instruction manual (pH 5–7; 4 °C; 60 h; final potential 500 V).

Disc gel electrophoresis

Polyacrylamide gel electrophoresis was performed (using 5% gels with a triethanolamine–*N*-tris(hydroxylmethyl)methyl-2-aminoethane sulfonic acid buffer) as described by Orr *et al.*[15], samples were applied to the top of the gel in the cathode buffer containing 34% sucrose and 0.2% bromophenol blue. The electrophoresis was performed at 4 °C for 3 h, at 3 mM per tube in 5.5 cm × 0.5 cm tubes. The proteins were stained with Amino Black.

The disc electrophoresis in the presence of sodium dodecylsulfate was performed according to Weber and Osborne[16].

Cellulose acetate gel electrophoresis

The electrophoresis was performed as described by Grossman *et al.*[17]. To estimate the position of the lipoxygenase the strips were cut into pieces (2 mm). Each piece was put into a cuvette with 2 ml 0.1 M sodium phosphate buffer (pH 6.5). After 30 min 0.5 ml of the linoleic acid–Tween 20 substrate of Surrey[11] was added and the increase in absorption at 234 nm was followed.

Amino acid analyses

Samples of the purified lipoxygenases were thoroughly dialyzed against deionized water. After concentration by ultrafiltration, aliquots were hydrolyzed for 24 h under a vacuum at 110 °C: (a) in 6 M HCl; (b) in 3 M *p*-toluenesulfonic acid containing 0.2% 3-(2-aminoethyl)indole according to Liu and Chang[18].

The amino acids corresponding to about 1 mg protein were fractionated according to the procedure of Spackman *et al.*[19] with the amino acid analyzer Unichrom (Beckman).

The half-cystine content was determined after performic acid oxidation by the method of Hirs[20]. Free sulfhydryl groups were determined with 4,4'-dithiopyridine as reagent as described by Grasetti and Murray[21]. Before the reaction the proteins were denaturated by incubation with 5 M urea.

Analyses of the positional specificity by hydroperoxidation

5 mg linoleic acid and 20 μg Tween 20 were dissolved in 20 ml 0.1 M sodium phosphate buffer (pH 6.3), as described by Surrey[11]. 1 mg lipoxygenase-2,1 (and in a second experiment lipoxygenase-2,2) was added. The mixture was incubated 30 min at 0 °C under O_2 degassing. The extent of the reaction was followed by measuring the increase in absorption of the aliquots at 234 nm. After the incubation the reaction mixture was acidified (pH 3.0) with diluted HCl and extracted 3 times with 50 ml diethyl ether. The diethyl ether solution was washed twice with 50 ml water and dried over Na_2SO_4. Ethanol, 20 ml, was added and the diethyl ether removed under vacuum. 50 mg $NaBH_4$ was added and the solution stirred for 1 h under N_2. 20 ml water was added to the ethanolic solution acidified to pH 3.0 with diluted HCl and extracted 3 times with 30 ml diethyl ether. The diethyl ether solution was washed and dried as above described.

The hydroxy fatty acids were converted into their methyl esters by the method of Schlenk and Gellerman[22].

The 9-hydroxyoctadienoic methyl ester was separated from the 13-isomer by thin-layer chromatography on silica gel HF_{254} (Merck) twice developed with the solvent system *n*-hexane–diethyl ether (7:3, v/v).

The 9- and 13-isomer were located on the plates by ultraviolet absorption, scraped off, eluted with diethyl ether and were dissolved in a definite volume. The concentration of each isomer was estimated by measuring the absorbance at 234 nm.

RESULTS

Crude extracts of peas were chromatographed on DEAE-cellulose to obtain information about the occurrence of lipoxygenase isoenzymes. The elution diagram (Fig. 1) shows three peaks with lipoxygenase and carotene bleaching activity. The peaks were designated as lipoxygenase-1 (L-1), lipoxygenase-2 (L-2) and lipoxygenase-3 (L-3).

Purification of lipoxygenase-2

Separation of a $(NH_4)_2SO_4$ precipitate by gel chromatography resulted in one peak with lipoxygenase activity. This peak was subjected to chromatography on CM-Sephadex. Lipoxygenase was eluted with 0.2–0.3 M NaCl in the sodium acetate buffer (Fig. 2). The combined fractions were chromatographed on the above described DEAE-cellulose-column and thus purified further. The diagram (Fig. 3) demonstrates that only one lipoxygenase was eluted, which was identical with the isoenzyme-2. Lipoxygenase-1 is not so stable as the lipoxygenase-2, it denatures during the acid extraction and chromatography. As seen in the summary of the purification steps (Table I) the ratio of lipoxygenase activity to carotene bleaching activity was not changed by the separation procedures.

The isolated lipoxygenase-2 revealed one protein band on cellulose acetate gel

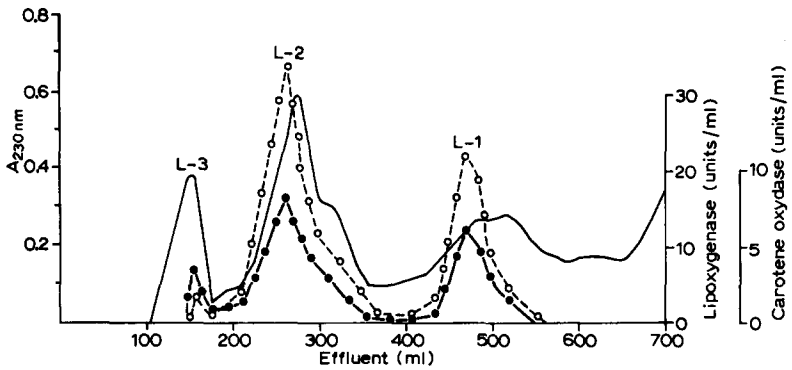

Fig. 1. DEAE-cellulose chromatography of an extract from peas. 5 g of ground peas were extracted with 50 ml 0.05 M sodium phosphate buffer (pH 7.0). After centrifugation the supernatant was applied to a Sephadex G-50 column which was equilibrated with the same buffer. The eluate containing the proteins (80 mg) and other high molecular substances was applied to a DEAE-cellulose column (32 cm × 2.5 cm) which was equilibrated against the 0.01 M sodium phosphate buffer (pH 7.0). Elution (32 ml/h) was performed with a linear NaCl gradient (0–0.18 M) in the buffer. ———, protein; ○– – ○, lipoxygenase activity at pH 6.5, ●—●, carotene oxidation at pH 6.5.

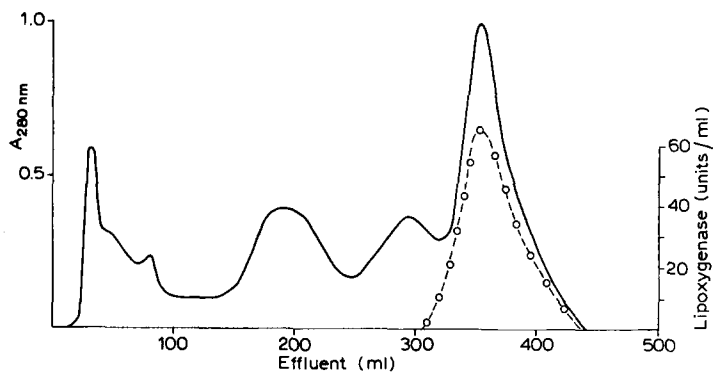

Fig. 2. Chromatography on CM-Sephadex C-50 of the gel-chromatographed lipoxygenase (150 mg, dissolved in 0.05 M sodium acetate buffer (pH 4.8). The column (1.6 cm × 35 cm) with the ion exchanger was equilibrated against the same buffer. Elution (20 ml/h) was performed with a linear NaCl gradient (0.06–0.4 M) in the buffer. ———, protein; ○— – ○, lipoxygenase activity at pH 6.5.

electrophoresis and on disc gel electrophoresis (with and without sodium dodecyl sulfate). After cellulose acetate gel electrophoresis the lipoxygenase activity of this protein could be detected. In contrast to the crude pea extract the purified lipoxygenase-2 lost the activity by the applied disc gel electrophoresis procedures.

Isoelectric fractionation of the lipoxygenase-2 on the pH gradient 5–7 resulted on two peaks with lipoxygenase and carotene oxidation activity (Fig. 4). The two species of lipoxygenase molecules were designated as lipoxygenase-2,1 (pI = 6.00) and lipoxygenase-2,2 (pI = 6.15).

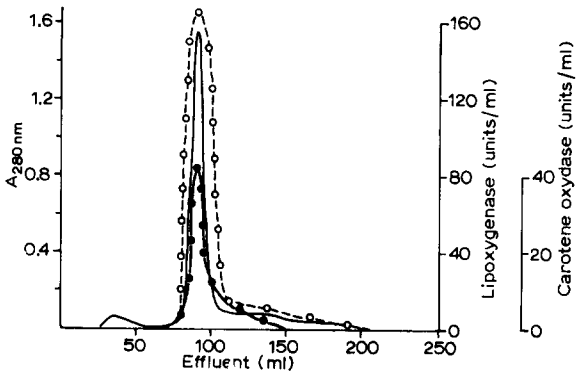

Fig. 3. Chromatography on DEAE-cellulose. The lipoxygenase active proteins from the CM-Sephadex effluent were dissolved in 5 ml 0.01 M sodium phosphate buffer (pH 7.0). The column (1.5 cm × 30 cm) with the ion exchanger was equilibrated against the same buffer. Elution (15 ml/h) was performed with a linear NaCl gradient (0.0–0.07 M) in the buffer. ———, protein; ○— —○, lipoxygenase activity at pH 6.5; ●—●, carotene oxidation at pH 6.5.

TABLE I

CONCENTRATION OF LIPOXYGENASE FROM PEAS

Assay conditions: Lipoxygenase: The enzyme dissolved in 0.1 ml elution buffer was added to 2 ml 0.05 M sodium phosphate buffer (pH 6.3) containing 2.01 μmoles linoleic acid, 0.25 mg EDTA and 0.98 μl Tween 80. 1 μmole diene causes an increase in $A^{1\,cm}_{234\,nm}$ of 11.9. Carotene bleaching: The enzyme dissolved in 0.1 ml elution buffer was added to 2 ml of a substrate containing $1.9 \cdot 10^{-2}$ μmoles β-carotene and additional the ingredients of the lipoxygenase activity test. Bleaching of 1 μmole β-carotene causes a decrease of $A^{1\,cm}_{460\,nm}$ of 28.6.

Step	*Specific lipoxygenase activity (μmole diene per min·mg protein)*	*Purification (-fold)*	*Specific carotene bleaching activity (μmole β-carotene per min·mg protein)*	*Ratio: lipoxygenase/carotene bleaching* $\left(\frac{\mu mole\ diene}{\mu mole\ \beta\text{-}carotene}\right)$
Crude extract	0.26	—	0.067	3.9
$(NH_4)_2SO_4$ (30–60%)	0.62	2.4		
Sephadex G-200	1.17	4.5		
CM-Sephadex	5.71	22		
DEAE-cellulose	19	73	4.87	3.8

Molecular weight

The molecular weight of the lipoxygenase-2 was determined by gel chromatography. It was calculated from the calibration curve (Fig. 5) as 78 000.

Amino acid composition

Table II presents the amino acid composition of the lipoxygenase-2 (hydrolysis with *p*-toluenesulfonic acid) and the lipoxygenases-2,1 and -2,2 (hydrolysis with 6 M HCl). There was not significant difference detected in the amino acid composition of the two lipoxygenases. The difference in the pI values of the lipoxygenases results perhaps from a different conformation of the polypeptide chains or from a relatively higher content of glutamine or asparagine in the lipoxygenase-2,2.

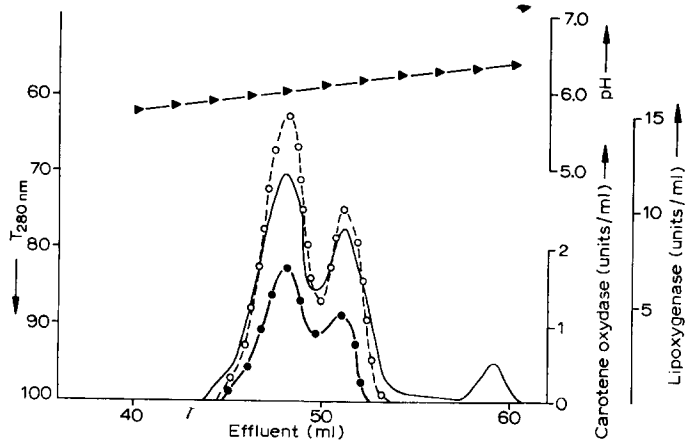

Fig. 4. Isoelectric focusing of lipoxygenase-2 between pH 5 and 7 in a sucrose density gradient. Column loaded with 8 mg protein. ———, protein; ○ – – ○, lipoxygenase activity at pH 6.5; ●—●, carotene oxidation at pH 6.5; ▲—▲, pH.

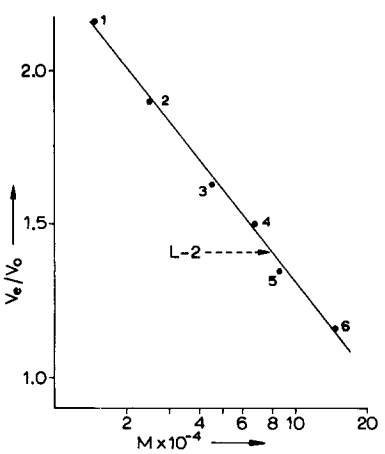

Fig. 5. Calibration curve of molecular weights of proteins. Sephadex G-150 column (98 cm × 1.6 cm); 0.05 M sodium phosphate buffer (pH 6.5) containing 0.01% NaN_3. Calibration proteins: 1, cytochrome *c*; 2, chymotrypsinogen A; 3, albumin from hen egg; 4, albumin from bovine serum; 5, conalbumin; 6, aldolase.

In the lipoxygenases-2,1 and -2,2 the same content of sulfhydryl groups could be detected. After treatment with urea 3.5 sulfhydryl groups per molecule of protein were found with the reagent of Grasetti and Murray[21]. This number of sulfhydryl groups is in agreement with the value of half-cystine, estimated after oxidation to cysteic acid. Thus, there appear to be only four residues of cysteine and no cystine per molecule of lipoxygenase-2. On the basis of a molecular weight of 78 000 the number of amino acid residues was calculated for lipoxygenase-2 (Table III).

The protein content of a lipoxygenase-2 solution with known absorption at 280 nm was determined by amino acid analyses. From these data $A^{1\%}_{1\,cm}$ at 280 nm was calculated as 14.2.

TABLE II

RESULTS OF THE AMINO ACID ANALYSES (MOLE %)

Amino acid	*Lipoxygenase-2** *(mole %)*	*Lipoxygenase-2,1*** *(mole %)*	*Lipoxygenase-2,2*** *(mole %)*
Lys	6.9	7.3	7.0
His	3.1	3.4	3.3
Arg	4.7	5.0	4.9
Trp	1.6		
Asp	11.3	11.5	11.4
Thr	5.5	5.4	5.4
Ser	7.3	7.0	7.2
Glu	10.0	10.5	10.3
Pro	7.0	6.9	7.1
Gly	7.4	7.4	7.1
Ala	5.7	5.5	5.4
Cys	Trace	Trace	Trace
Val	4.5	4.9	5.0
Met	1.8	1.1	1.0
Ile	4.5	4.9	5.1
Leu	10.3	10.3	10.9
Tyr	4.2	4.2	4.2
Phe	4.1	4.3	4.2

* Values determined by hydrolysis with *p*-toluene sulfonic acid according to Liu and Chang[18].

** Values determined by hydrolysis with 6 M HCl.

TABLE III

AMINO ACID COMPOSITION OF PEA LIPOXYGENASE-2

Amino acid	*Residues in moles per 78 000 g of protein**
Lys	49
His	23
Arg	34
Trp	11
Asp	78
Thr	37
Ser	48
Glu	71
Pro	48
Gly	49
Ala	37
Cys	4
Val	34
Met	7
Ile	34
Leu	72
Tyr	29
Phe	29
Total	694

* Calculated to nearest integer.

Properties of the lipoxygenase-2

When linoleic acid-Tween 20 is used as substrate both lipoxygenases-2,1 and -2,2 exhibited an optimum at pH 6.3. In the presence of $4 \cdot 10^{-4}$ M Ca^{2+} the enzyme showed an optimum at pH 7.8 but its activity was about 10-fold lower than in the presence of Tween 20. The lipoxygenases-2,1 and -2,2 oxidize linoleic acid (substrate in O_2-gassed solution pH 6.3 at 0 °C) to hydroperoxy acids with the same positional specificity. The ratio of the 9- to the 13-hydroperoxy acid was 58:42.

The substrate specificity of lipoxygenase-2 was investigated by comparing the activity towards linoleic acid and methyl linoleate. In this experiment the substrates were prepared in the same way. Fig. 6 shows that both substrates were oxidized by

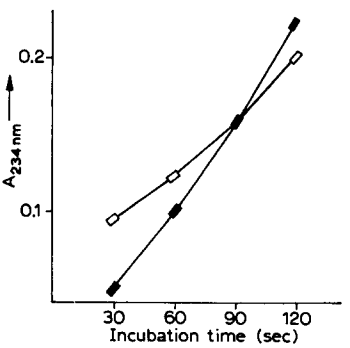

Fig. 6. Activity of the lipoxygenase-2 towards linoleic acid and methyl linoleate at pH 6.3 and 23 °C. Substrates: 125 mg linoleic acid or methyl linoleate and 125 µl Tween 80 were dissolved in 2.5 ml acetone. This solution was evaporated in a N_2 stream to dryness and the residue dissolved immediately in 100 ml 0.1 M sodium phosphate buffer (pH 6.3). Test: 4 ml of substrate were mixed with a solution of 0.43 mg lipoxygenase-2 in 0.5 ml 0.1 M sodium phosphate buffer (pH 6.3). During incubation the emulsion was flushed with O_2 and stirred. At regular periods of time 0.5-ml aliquots were taken out and added to 2.5 ml methanol. The absorbance at 234 nm was measured against methanol. ■—■, linoleic acid; □—□, methyllinoleate.

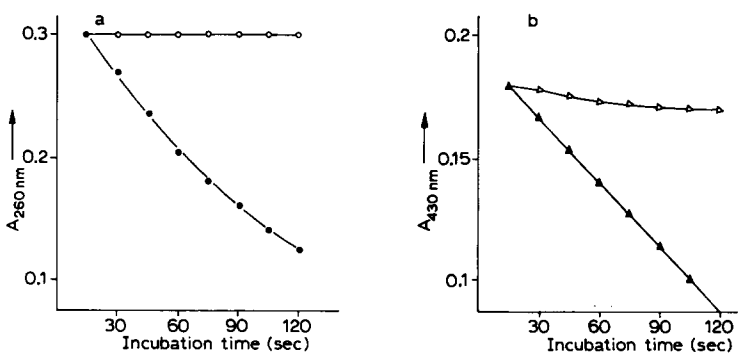

Fig. 7. (a). Carotene oxidation by the lipoxygenase-2. Assay conditions: 2.1 ml 0.1 M sodium phosphate buffer (pH 6.3) contains 564 µg linoleic acid, 250 µg EDTA, 10 µg β-carotene, 0.98 µl Tween 80 and 0.71 µg lipoxygenase-2. ○—○, without enzyme; ●—●, with enzyme. (b). Chlorophyll bleaching by the lipoxygenase-2. Assay conditions: 2.1 ml 0.1 M sodium phosphate buffer (pH 6.3) contains 564 µg linoleic acid, 250 µg EDTA, 10 µg chlorophyll *a*, 0.98 µl Tween 80 and 71 µg lipoxygenase-2 (assay procedure see Materials and Methods). △—△, without enzyme; ▲—▲, with enzyme.

the enzyme, but its activity towards the acid was about 50% higher than towards the ester.

In the presence of O_2 and linoleic acid the lipoxygenase-2 does not destruct only carotene (Fig. 7a), but also chlorophyll (Fig. 7b). In the case of chlorophyll bleaching the enzyme exhibited only a very low activity. Therefore, we must increase the concentrations of the lipoxygenase-2 100-fold with respect to the carotene oxidizing test to obtain the measured chlorophyll disappearance.

DISCUSSION

The possible occurrence of multiple forms of pea lipoxygenase has been shown by Hale *et al.*[23] on the basis of disc electrophoresis. One isoenzyme has been purified by Eriksson and Svensson[8]. We have demonstrated the multiple forms of pea lipoxygenase by the chromatography of a crude extract on DEAE-cellulose. Three protein zones were eluted which exhibit lipoxygenase and carotene bleaching activities. The enzyme which was designated as lipoxygenase-2 was purified to essential homogeneity.

The newly isolated isoenzyme appeared to have a molecular weight of 78 000 as judged by gel filtration, a similar value (72 000) was found by Eriksson and Svensson[8] for their pea lipoxygenase. Differences exist also in the amino acid composition and in the p*I* values. Specially the contents of aspartic acid and half-cystine differ significantly. Lipoxygenase-2 contains less of these amino acids than the previously described isoenzyme. Lipoxygenase-2 gave two peaks by isoelectric fractionation but the separated proteins (designated as lipoxygenase-2,1 and -2,2) exhibit the same amino acid composition. The smaller content of acidic amino acids in the lipoxygenase-2,1 and -2,2 may cause the higher p*I* values (6.00 and 6.15) of these proteins in comparison with the isoenzyme purified by Eriksson and Svensson[8].

The positional specificity of the newly isolated isoenzyme is in agreement with the other enzyme[24]. The lipoxygenases which were separated by isoelectric focusing form the same ratio of 9- to 13-hydroperoxides (58:42) during the oxidation of linoleic acid. Besides hydroperoxides small concentrations of volatile aldehydes are formed. These results were published in a separate paper[25].

The lipoxygenase-2 oxidizes linoleic acid somewhat faster than methyl linoleate. Also it is a very potent carotene oxidizing enzyme in the presence of linoleic acid and O_2. It is most likely that the oxidation of carotene is due to radicals which are produced by the lipoxygenase reaction with linoleic acid, since the pigment was not bleached with linoleic acid hydroperoxides[9]. The activity ratio of carotene bleaching to linoleic acid peroxydation is 1:3.8 at pH 6.3 (optimum pH). The respective turnover numbers are about 1200 moles β-carotene per min/mole and 4600 moles linoleic acid per min/mole. Lipoxygenase-2 destructs also chlorophyll in a co-oxidation reaction with linoleic acid, but this activity is only one hundredth of the carotene bleaching activity.

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