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LIPOXYGENASE FROM PEAS, PURIFICATION AND PROPERTIES OF THE ENZYME

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SUMMARY

1. Lipoxygenase (linoleate:oxygen oxidoreductase, EC 1.13.1.13) from peas was extensively purified by precipitation with $(NH_4)_2SO_4$, gel filtration with Sephadex G-150, and ion-exchange chromatography on DEAE-cellulose.

2. The final enzyme preparation proved homogeneous by ultracentrifugation of a 0.6% protein solution (pH 7.0) but separated into two main and narrow fractions on isoelectric focusing, p*I* values 5.80–5.82.

3. The molecular weight, as calculated on the basis of amino acid analysis and ultracentrifugation, was found to be 72 000 and 67 000, respectively.

4. Pea lipoxygenase contains 7 half-cystine residues, different from the soybean enzyme which has been reported to contain none.

INTRODUCTION

The enzyme lipoxygenase (linoleate:oxygen oxidoreductase, EC 1.13.1.13) catalyzes the oxidation, by molecular oxygen, of *cis,cis*-1,4-pentadiene systems to 5-hydroperoxy compounds. In vegetables, the system resides mainly in linoleic and linolenic acids. Each of these acids is oxidized *in vitro* by catalysis of lipoxygenase to known ratios of isomeric hydroperoxides[1], which in turn give rise to scission products, mainly aldehydes, which are strongly odorous. Comprehensive reviews on the properties of lipoxygenase and the reactions of hydroperoxides are available[2,3].

The accumulation of carbonyl compounds in frozen peas[4] and of alcohols in fresh peas[5] can thus be ascribed to the action of lipoxygenase[6] solely in the former case and to the combined action of lipoxygenase and alcohol dehydrogenase in the latter[7].

To minimize the activity of enzymes in stored vegetables, the latter are blanched before being frozen or dried. The inactivation of peroxidase is commonly used as a criterion of sufficient heat treatment due to its heat stability. However, individual enzymes, differing in their heat response, are not evenly distributed in plant material. In peas, the activity of lipoxygenase was found to be highest in the centre and lowest in the skin[8], whereas for peroxidase the ratio is the reverse (own unpublished results).

Lipoxygenase is not even the only catalyst capable of forming carbonyl compounds from unsaturated fatty acids. Hemoproteins of higher plants, such as peroxidase, catalase and cytochrome *c*, are powerful lipid peroxidizers thanks to their ability to accelerate autoxidation, which is followed by the production of the same main scission products as in the lipoxygenase reaction. Both types of reactions have low activation energies, about 3 and 4 kcal/mole for the hemoprotein- and the lipoxygenase-catalyzed reaction, respectively[9]. The hemoproteins, but not lipoxygenase, are inhibited by CN^-, N_3^- and F^-, a difference used to distinguish between hemoprotein and lipoxygenase catalysis[10].

In our studies of the biosynthesis of flavor compounds in vegetables and heat inactivation of vegetable enzymes, there was a common need for a purified lipoxygenase from peas that was essentially free from hemoproteins. In the biosynthesis work a pure enzyme was needed for studying the development of flavor compounds from the purest possible model systems, free from interference by hemoproteins and their inhibitors. In the heat inactivation work we wanted to study the inactivation of the pure enzyme and the effect of various additives on the inactivation process.

Lipoxygenase has been purified earlier. This was performed on a large scale by extraction from soybean flour, by precipitation of impurities with barium acetate, basic lead acetate and acetone, by fractionated precipitation of the enzyme using ammonium sulfate and alcohol, and by final purification by preparative electrophoresis. The enzyme was crystallized by dialyzing the solution against $(NH_4)_2SO_4$ in increasing concentration[11,12]. This procedure, slightly modified, is widely used for preparation of soybean lipoxygenase for further purification by ion exchange[13], large scale electrophoresis and ion-exchange chromatography[14]. These procedures yielded lipoxygenase presumably free from other enzyme activities, but no assays were made except for lipoxygenase. Commercial crystalline soybean lipoxygenase preparations contain considerable peroxidase activity.

This work was thus started in order to prepare hemoprotein-free lipoxygenase from peas by a procedure simpler than those described earlier for the soybean enzyme.

This paper reports the purification procedure of lipoxygenase from peas by gel chromatography and ion-exchange chromatography. The purity of the final preparation was checked by spectral analysis, ultracentrifugation and isoelectric fractionation. As the procedures led to a homogeneous enzyme preparation, purified to a degree above the main intention, an attempt was made to determine the amino acid composition of the enzyme.

MATERIAL AND METHODS

The enzyme was obtained from ripe dry seeds of peas (*Pisum sativum* L. "Whitham wonder", a wrinkled type).

Lipoxygenase activity was determined by a polarographic method[8]. From a diluted enzyme preparation, 0.500 ml was added to 6.35 ml of 7.5 mM linoleic acid emulsion in 0.14 M potassium phosphate buffer (pH 6.5) containing 2.5 ml Tween-20 per l, reaction temperature 14°. The reactions took place in a closed liquid system in a thermostatically controlled vessel. The oxygen analyzer used was a pO_2 electrode Type E5046 placed in the reaction vessel, and Gas monitor Type PHA 927 b connected

to a pH-meter PHM 25 (Radiometer, Denmark) and a 20-mV compensation recorder. One unit of enzyme corresponds to the initial reaction rate of 1 μmole O_2 per min at 14°. Before measurement of its activity, the enzyme was always diluted until it contained about 0.70 unit/ml.

Peroxidase activity was determined spectrophotometrically at 460 nm, using *o*-dianisidine as hydrogen donor.

Catalase activity was measured by a polarographic method, similar to that used for measuring lipoxygenase activity[15].

Protein determinations were made according to the method of LOWRY *et al.*[16] using bovine albumin for calibration.

Spectral analysis was carried out using a Beckman DB-G spectrophotometer.

Dextran gel for chromatography, Sephadex G-150, water-regain 15 g/g gel and the glass column Sephadex K-50 were supplied by Pharmacia Fine Chemicals, Sweden.

DEAE-cellulose DE 52 weak ion exchanger, microgranular and preswollen, was obtained from Whatman, England.

EXPERIMENTAL

Extraction of enzyme

The purification procedure was performed according to the flow scheme shown in Fig. 1. The pea seeds, 3000 g, were ground in batches of 300 g in a Waring blender together with solid CO_2. The cold powder obtained was defatted and decolorized by washing it with cold acetone and diethyl ether (−20°) on a glass filter funnel. After being dried in a desiccator under vacuum for 6 h, the enzyme was extracted from the dry powder by stirring it for 16 h in 10 l of 0.1 M Tris–HCl buffer (pH 7.2) at 4°.

The slurry obtained was forced through cheesecloth and centrifuged at 1000 × *g* for 30 min. The supernatant solution was further fractionated by $(NH_4)_2SO_4$ precipitation at 0°. Inactive material was precipitated at 25% satn., the enzyme by increasing the salt concentration to 50% satn. The precipitate was centrifuged down at 13 000 × *g* for 20 min at 0° and then dissolved in 900 ml of 0.05 M Tris–HCl buffer (pH 7.2).

Gel chromatography

Part of the crude enzyme preparation, 30 ml, was dialyzed against 0.05 M Tris–HCl buffer (pH 7.2) and then passed through the dextran gel column (5 cm × 90 cm) equilibrated with the same buffer. The elution rate was kept constant with a peristaltic pump, and fractions of 10 ml were collected every 20 min by means of a time-operated fraction collector. The content of ultraviolet-absorbing (280 nm) material in the fractions was determined, after which all fractions containing such material were assayed for lipoxygenase, peroxidase and catalase activity.

The fractions containing lipoxygenase were pooled, and the enzyme was precipitated by addition of solid $(NH_4)_2SO_4$ to 50% satn. at 0° and centrifuged down at 15 000 × *g* for 20 min at 0°. The precipitate obtained was dissolved in about 5 ml of 0.01 M Tris–HCl buffer (pH 6.5) containing 2 mM $CaCl_2$ and 1 mM reduced glutathione, (Tris–$CaCl_2$–GSH buffer). This solution was dialyzed against the same buffer 3 times, each time for 5 h at 4°.

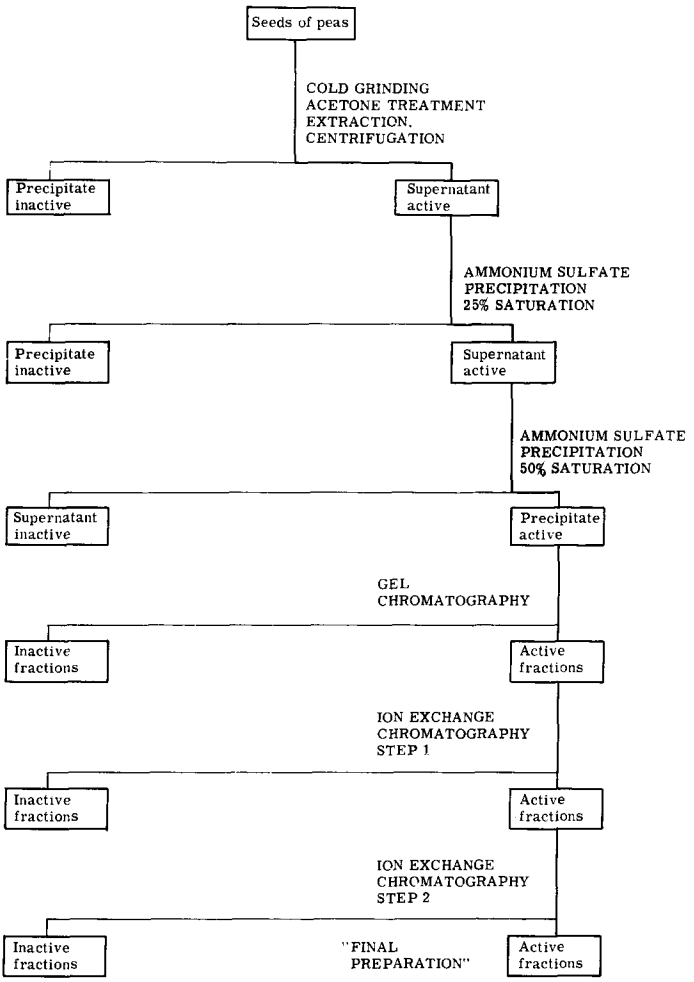

Fig. 1. Scheme of the purification procedure of pea lipoxygenase.

Ion-exchange chromatography

Step 1

The DEAE-cellulose column (2.5 cm × 45 cm) was pretreated with 1% (w/v) NaOH followed by a strong chloride solution[17]. In this case 0.1 M pyridinium chloride solution (pH 4.4) containing 20% (w/v) NaCl was used. After this treatment the ion exchanger was equilibrated with Tris–$CaCl_2$–GSH buffer.

The dialyzed lipoxygenase solution obtained from the gel chromatography step—about 6 ml—was placed on top of the ion-exchange column. Elution was performed by applying a linear salt gradient increasing from 0.01 to 0.61 M Cl^- concentration in a total volume of 700 ml Tris–$CaCl_2$–GSH buffer.

Fractions of 6 ml were collected every 20 min and assayed for ultraviolet-absorbing material, lipoxygenase, peroxidase and catalase activity. Lipoxygenase-active fractions were again pooled, concentrated and dialyzed as before.

Ion-exchange chromatography

Step 2

The DEAE-cellulose column (1.2 cm × 20 cm) was pretreated and equilibrated in the manner described above. The lipoxygenase-active material from *Step 1* was fractionated by linear gradient elution, this time by increasing the Cl^- concentration from 0.01 to 0.41 M in a total volume of 250 ml Tris–$CaCl_2$–GSH buffer. Fractions of 4.5 ml were taken every 25 min and assayed for ultraviolet-absorbing material and lipoxygenase activity. The pooled fractions from this step, which shall be referred to as the final preparation, was then checked to prove that the combination contained no peroxidase and catalase activity.

Properties

The purity of the final preparation was checked, and the molecular weight, isoelectric properties and amino acid composition were determined, by spectral, ultracentrifugation, isoelectric focusing, and amino acid analysis.

The ultraviolet spectrum was run on a solution of 0.63 mg protein per ml of 0.05 M Tris–HCl buffer (pH 7.2) against the same buffer as reference. The visible spectrum, run to check whether any hemoproteins were present, was obtained from 3.0 mg of protein treated with pyridine and sodium dithionite to convert any protein-bound hematin present into pyridine ferrohemochrome[18].

A homogeneity test was made in an analytical ultracentrifuge on a 0.6% protein solution in 0.1 M potassium phosphate buffer (pH 7.0) at 59 780 rev./min. Exposures were taken at 58, 72, 86, 102, 126 and 134 min. Sedimentation equilibrium ultracentrifugation was carried out at a speed of 12 590 rev./min for 21 h at 20° (refs. 19, 20). The protein concentration was 0.1%.

Isoelectric fractionation was carried out on 10 mg of protein on a pH gradient ranging from 3 to 10 and in another experiment on a pH gradient range of 5–7 (refs. 21, 22).

For determination of amino acid composition, dialyzed and lyophilized protein from the final preparation was hydrolyzed in 6 M hydrochloric acid in evacuated glass ampoules at 105° for 24 and 37 h. Hydrolysates from both times were treated with H_2O_2 and formic acid for 20 h. The amino acids, both oxidized and unoxidized, corresponding to about 1 mg of original protein were fractionated according to the procedure of SPACKMAN *et al.*[23]. The analytical data were fed into a computer to calculate

TABLE I

CONCENTRATION OF LIPOXYGENASE FROM PEAS BY $(NH_4)_2SO_4$ PRECIPITATION

	Total activity (units)	*Total protein (mg)*	*Specific activity (units/mg)*	*Purification relative to crude enzyme*	*Recovery (%)*
Crude enzyme	534 000	221 000	2.4	1.0	100
$(NH_4)_2SO_4$ fractionation 25% sat. supernatant	473 000	185 000	2.6	1.1	89
50% sat. precipitate	499 000	48 400	10	4.2	93

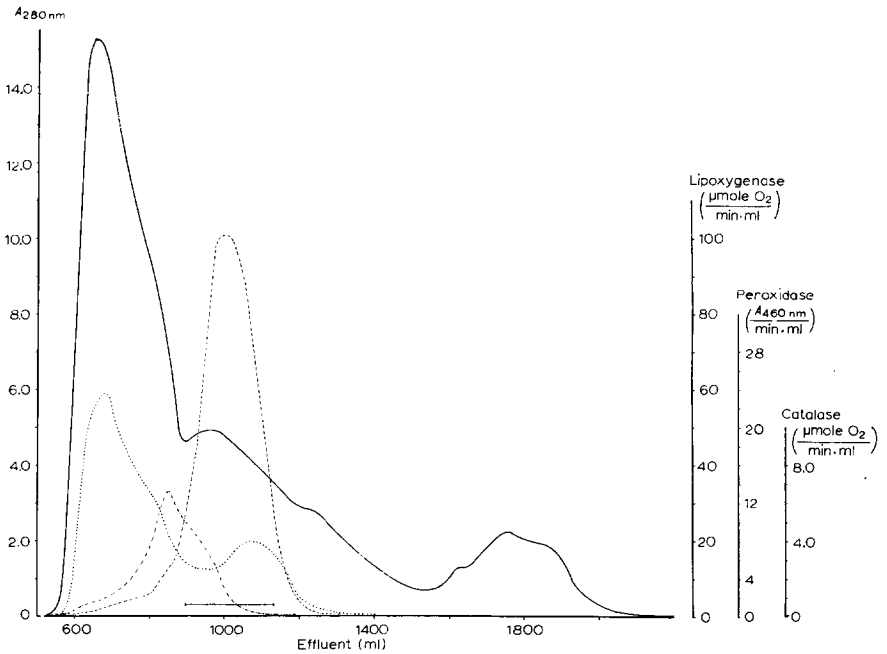

Fig. 2. Gel chromatography of pea lipoxygenase. Column: Sephadex G-150 (5 cm × 90 cm) in 0.05 M Tris–HCl (pH 7.2). Sample: $(NH_4)_2SO_4$ precipitated enzyme. ———, $A_{280\,nm}$; — — —, lipoxygenase activity; . . . . . , peroxidase activity; —·—·—, catalase activity.

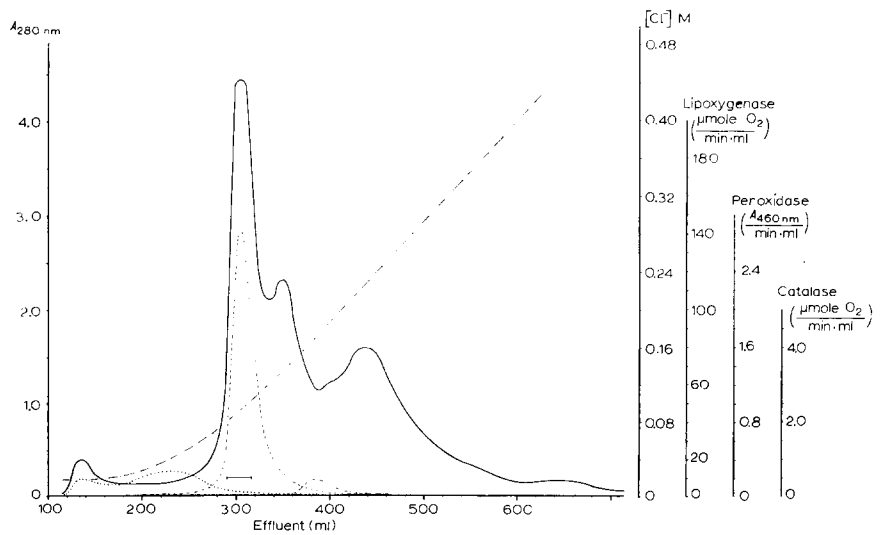

Fig. 3. Ion-exchange chromatography. *Step 1.* Column: DEAE-cellulose (2.5 cm × 45 cm) in Tris–$CaCl_2$–GSH buffer (pH 6.5). Sample: Gel-chromatographed lipoxygenase; eluated by a NaCl gradient. ———, $A_{280\,nm}$; — — —, lipoxygenase activity; . . . . . , peroxidase activity; –·–·–·–·–·–, catalase activity; —·—·—, [Cl^-].

the amino acid composition and the molecular weight of lipoxygenase. The computer program was worked out by Assist. Prof. B. Lindqvist, Mjölkcentralen, Stockholm, Sweden.

RESULTS AND DISCUSSION

The extraction and precipitation before further fractionation by chromatography gave a good yield of a crude stable enzyme preparation, concentrated about 4 times (Table I). The preparation maintained its original activity for several months when stored as a precipitate in 25% $(NH_4)_2SO_4$ solution at −20°. It contained considerable peroxidase and catalase activity.

Separation by gel chromatography resulted in one major fast-running fraction followed by several less well-separated ones (Fig. 2). Lipoxygenase activity was found in the first part of the fraction following the major one, indicating a medium molecular weight of the enzyme. Already in this step, most of the lipoxygenase was separated from the peroxidase and catalase originally present.

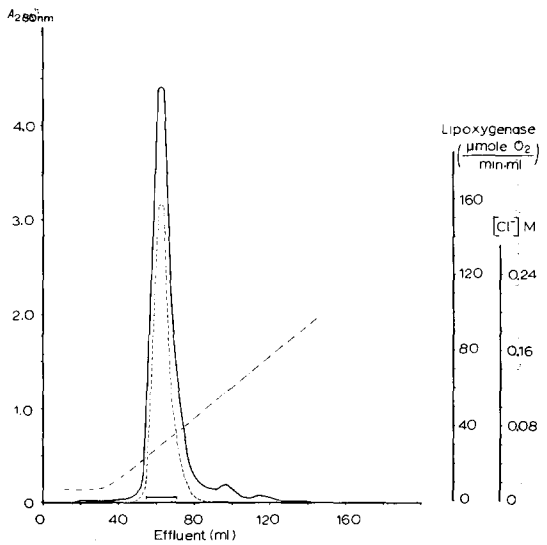

Fig. 4. Ion exchange chromatography. *Step 2*. Column: DEAE-cellulose (1.2 cm × 20 cm) in Tris–$CaCl_2$–GSH buffer (pH 6.5). Sample: concentrated lipoxygenase from ion exchange chromatography, *Step 1*; eluted by a NaCl gradient. ———, $A_{280\ nm}$; – – – – –, lipoxygenase activity; —·—·—, $[Cl^-]$.

When subjected to *Step 1* of ion-exchange chromatography the fractions containing concentrated lipoxygenase were separated further into a small fast-running fraction containing peroxidase followed by three major fractions. Lipoxygenase was eluted in the first well-defined major peak at *I* 0.07–0.11 (Fig. 3). The decrease in activity at this step depends on a partial coprecipitation of active material thanks to the drop in ionic strength during the dialysis of the material before putting it on the column. Only minor peroxidase and catalase activity was left in the concentrated lipoxygenase fraction, which, when rechromatographed on a less steep salt gradient

TABLE II

PURIFICATION OF LIPOXYGENASE BY CHROMATOGRAPHY

	Total activity (units)	*Total protein (mg)*	*Specific activity (units/mg)*	*Purification relative to crude enzyme*	*Recovery (%)*
Sample	16 300	1580	10	4.2	93
Gel chromatography	15 200	360	42	18	86
Ion-exchange chromatography					
Step 1	3800	49	78	33	22
Step 2	1770	21	84	35	10

(Fig. 4), was completely freed from these enzymes. Only these two hemoproteins were determined during the purification procedure because they are easy to follow by their normal action on H_2O_2. It was, however, checked by spectral analysis that the final preparation contained no hemoproteins at all. No pyridine ferrohemochrome absorption bands thus occurred in the visible part of the spectrum. From the ultraviolet part, $E^{1\%}_{1cm}$ at 278 nm was calculated as 13.2, the figure being based on the dry weight of lyophilized protein.

The purification of the lipoxygenase by the chromatographic procedure, which showed to be highly reproducible, thus yielded a hemoprotein-free lipoxygenase preparation with a specific activity of 84 enzyme units per mg protein, purified about 35 times (Table II).

The result of the last purification step indicated that a rather pure enzyme had been obtained, for the shape of the activity curve corresponded closely to that of the protein absorption curve. This was confirmed by the ultracentrifugation analysis of the final fraction, which showed only one component (Fig. 5). A preliminary molecular weight of this component was calculated as 75 000 assuming a partial specific volume of 0.75 for the protein. The sedimentation constant was found to be 5.2 S.

Isoelectric fractionation of the purified lipoxygenase from ion-exchange *Step 2* on the steep pH gradient (3–10) gave a single peak at an isoelectric pH of 5.8. The lipoxygenase activity curve corresponded closely to the protein absorption curve.

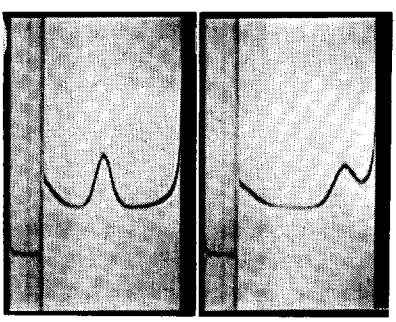

Fig. 5. Ultracentrifugational pattern of purified pea lipoxygenase. 0.6% protein solution in 0.1 M potassium phosphate buffer (pH 7.0) was run at 59 780 rev./min. Exposures shown were taken 58 and 102 min after reaching full speed. The sedimentation coefficient obtained, $s_{20,w} = 5.2$ S.

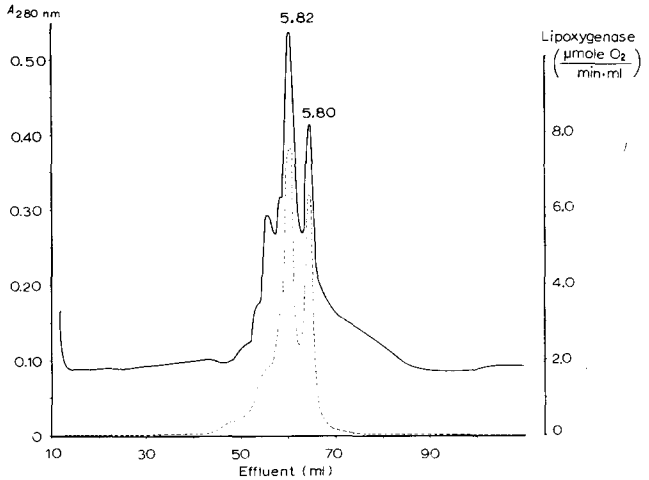

Fig. 6. Isoelectric fractionation of purified pea lipoxygenase on a gradient range of pH 5–7. p*I* values are given for the two main components. ———, $A_{280\ nm}$; – – – –, lipoxygenase activity.

However, on the pH gradient 5–7, the same material separated into two major protein bands and a minor one, which differed very little in their p*I* values (Fig. 6). The two major peaks occurred at pH's 5.80 and 5.82. The lipoxygenase activity curve matched all parts of the protein absorption curve, and reflects some minor differences between various species of lipoxygenase molecules. This is in agreement with recent results from disc electrophoresis of crude pea extracts, where 2–3 lipoxygenase isoenzymes appeared[24].

The amino acid composition and molecular weight of lipoxygenase were calculated from the computer data where the sum of minimum molecular weight times | number of residues – nearest integral number | showed two deep minima at mol. wt. 61 773 and 71 845 (ref. 25). If these two values are used in the Svedberg Eqn. 1,

$$M = \frac{RT\, s_w}{D(1 - \bar{\nu}\varrho)} \qquad (1)$$

$$T = 293.2,\ \varrho = 1.00$$

the partial specific volume ($\bar{\nu}$) becomes 0.70 and 0.74 ml/g, respectively. The former value is less probable and the mol. wt. of 71 845 was chosen for further calculations. These calculations (Table III) give a final mol. wt. of 71 871. The partial specific volume calculated from the amino acid composition data was found to be 0.72 ml/g (ref. 26), a value which when employed in Eqn. 1 gives a mol. wt. of 67 000 from the ultracentrifugation data.

This analysis, which gave 637 amino acid residues per protein molecule, showed that the enzyme is rich in aspartic acid, part of it in the form of asparagine. Tryptophan is lacking because of the acid hydrolysis. The presence of half-cystine residues is of interest for the further studies of the heat inactivation of the enzyme. In the chromatograms, only amino acid peaks appeared. Thus, there were no indications that carbohydrate material was present in the final preparation, a fact that makes the

TABLE III

AMINO ACID COMPOSITION OF PEA LIPOXYGENASE

Amino acid residue	*Residue (g/100 g of protein)*	*Minimal mol. wt.*	*Residues per 100 g of protein* (Mol. wt. = 71 845)*	*Nearest integral number × minimal mol. wt.*	*Residues in moles per 71 871 g of protein*	*Integral numbers of residues per 71 871 g of protein*
Asp	20.997 ± 0.010	548.1	131.08	71 801	131.13	131
Thr	4.947 ± 0.006	2043.7	35.15	71 530	35.16	35
Ser	4.843 ± 0.017	1798.1	39.96	71 924	39.97	40
Glu	10.430 ± 0.017	1237.9	58.04	71 798	58.06	58
Pro	4.561 ± 0.017	2129.1	33.74	72 389	33.75	34
Gly	3.038 ± 0.004	1877.9	38.26	71 360	38.27	38
Ala	2.858 ± 0.005	2487.1	28.89	72 126	28.90	29
Cyś	0.989 ± 0.001	10 326.6	6.96	72 286	6.96	7
Val	3.893 ± 0.019	2546.4	28.21	71 299	28.22	28
Met	1.198 ± 0.004	10 950.8	6.56	(76 656)	6.56	7
Ile	4.919 ± 0.010	2300.3	31.23	71 309	31.24	31
Leu	9.552 ± 0.024	1184.6	60.65	72 261	60.67	61
Tyr	5.805 ± 0.001	2810.9	25.56	73 083	25.57	26
Phe	4.731 ± 0.001	3110.8	23.10	71 548	23.11	23
Lys	6.444 ± 0.020	1989.0	36.12	71 604	36.13	36
His	4.776 ± 0.007	2871.4	25.02	71 785	25.03	25
Ammonia (amide)	−0.0865 ± 0.0005	1144.5	62.77	72 104	62.79	63
Arg	6.108 ± 0.017	2557.1	28.10	71 599	28.11	28
Total				71 871**		637

* From computer data.
** The value of Met omitted from the total.

amino acid analysis more reliable, especially concerning the value of lysine which would be influenced by the presence of such material during hydrolysis.

Some differences exist between the soybean and the pea enzyme, the molecular weight of the latter, 72 000, being lower than that of the former, 102 000, based on ultracentrifugation data[12]. However, our calculations based upon the previously reported amino acid analysis[27] suggest a somewhat lower molecular weight—around 95 000—for the soybean enzyme. The two enzymes also differ in amino acid composition mainly for the acidic and neutral amino acids. The basic amino acids occurred to nearly the same extent in both proteins. The most obvious difference, however, concerns the sulphur amino acids. Cysteine or cystine were not found in soybean lipoxygenase in contrast to the seven residues in pea lipoxygenase, but the former contained more methionine.

The isoelectric pH of soybean lipoxygenase, pI = 5.4, obtained by electrophoretic mobility measurements[12] is more acid than that found for the pea enzyme by isolectric focusing.

The pea lipoxygenase purified by the reported procedure is now routinely used for investigations of the biosynthesis of flavor compounds and the heat inactivation of the enzyme.

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