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THE HETEROGENEITY OF SOYABEAN LIPOXYGENASE

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SUMMARY

Isolation and purification of soyabean lipoxygenase (linoleate:O_2 oxidoreductase, EC 1.13.1.13), on ion-exchange resins yields two fractions, one showing preferential activity for the free acid, the other for the methyl ester. Each fraction consists of two different components with different isoelectric points, which exhibit, however, the same substrate and product specificity. Isoelectric focusing on polyacrylamide gels and on sucrose density gradients show that all isolated components are still heterogeneous. Each subcomponent is shown to be enzymically active by a specific staining technique.

INTRODUCTION

Lipoxygenase (linoleate:O_2 oxidoreductase, EC 1.13.1.13), a plant enzyme, catalyzes the oxidation of various unsaturated fatty acids and derivatives containing the *cis,cis*-1,4-pentadiene system into hydroperoxides by means of molecular oxygen[1]. Evidence has been mounting to show that, in soyabeans, lipoxygenase is not a single entity but a complex system. Two papers have described evidence that soyabeans contain a triglyceride or ester lipoxygenase as well as a fatty acid lipoxygenase[2,3]. Other authors have found lipoxygenase to be heterogeneous on the basis of disc electrophoresis[4] or Ca^{2+} stimulation[5,6]. Finally Kies[7,8] showed that lipoxygenase is not identical with the enzyme that destroys carotene in the coupled reaction with methyl linoleate. We have further investigated the heterogeneity of the soyabean lipoxygenase system.

MATERIALS

CM- and DEAE-Sephadex were supplied by Pharmacia Fine Chemicals, Uppsala, Sweden, while acrylamide (purum) and riboflavin were obtained from Fluka A.G., Switzerland. The acrylamide was recrystallized from chloroform before use. *N,N'*-Methylene-bis(acrylamide) (98%) and *N,N,N',N'*-tetramethylethylenediamine (98%) were products from EGA-chemie KG, Steinheim-Albuch, Western Germany.

Tween 20 was obtained from Atlas Chemie GmbH, Essen, Western Germany.

Linoleic acid and methyl linoleate were of 98% purity. The other chemicals were of analytical grade, unless stated otherwise.

METHODS

Enzyme activity determinations

The term "acid" enzyme (A) is used for the lipoxygenase which, at pH 9.0, is more active towards linoleic acid than towards the ester. This enzyme is the one described by Theorell *et al.*[9]. By "ester" enzyme (E) we mean the lipoxygenase which, at pH 9.0, shows higher activity towards methyl linoleate.

Assay of the "acid" enzyme

The activity of the "acid" enzyme is routinely determined as follows: an aliquot is taken from a stock solution of 0.35 M linoleic acid in benzene. The solvent is removed *in vacuo* and the acid suspended in 0.1 M sodium borate buffer (pH 9) to a final concentration of $3.5 \cdot 10^{-3}$ M. The emulsion is clarified by adding a trace amount of sodium deoxycholate; 0.5 ml of this solution is filled into two 2-mm quartz cuvettes, which are placed in a double-beam spectrophotometer (Model 124, Hitachi Perkin Elmer). An appropriate amount of enzyme solution, from 2 to 20 μl, is added to the sample cuvette with a micropipette and mixed by inversion. The increase in absorbance at 234 nm is recorded as a function of time, an increase of 0.001 per min being defined as one unit of activity.

Assay of the "ester" enzyme

Method 1. The activity of the ester enzyme towards linoleic acid at pH 7 is used for a series of determinations, following the procedure outlined by Graveland[10].

Method 2. A different assay is used for substrate specificity studies. The substrate solution, either linoleic acid or methyl linoleate, is prepared as described for the assay of the "acid" enzyme, but contains an additional 0.001% of Tween 20. The mixture is emulsified in a Bühler homogenizer (E. Bühler, Tübingen, Germany) for 2 min at maximum speed, with tap water cooling. An appropriate amount of enzyme solution is added to the emulsion and, at regular periods of time, 0.5-ml aliquots are taken out and added to 2.5 ml methanol to stop the oxygenation. The oxygenation reaction is carried out at 23 °C under a steady flow of oxygen. A blank is prepared by adding a corresponding amount of enzyme solution to 2.5 ml of methanol, followed by the corresponding amount of substrate solution. The absorbance at 234 nm, is measured against water–methanol (1:5, by vol.).

Protein determination

Protein is determined routinely by measuring the absorbance at 280 nm. For more accurate measurement, especially when dealing with crude and semipurified preparations, the method of Kalckar[11] is used.

Isoelectric focusing on sucrose density gradient

The experiments are carried out with an LKB 8101 electrofocusing column of 110 ml capacity (LKB-Produkter AB, Bromma, Sweden). A carrier ampholyte with a pH span of 5.5–6.5 is prepared from the commercially available product having a pH

range of 5–7, according to Method B of the LKB Ampholine Instruction Manual. The sucrose density gradient is prepared manually as described in the same Manual.

The enzyme sample is dialysed against 10^{-4} M $CaCl_2$ and centrifuged. 2 ml of the enzyme solution (27 mg protein) are mixed with the appropriate amounts of Ampholine solution and water to a volume of 10 ml, which constitutes the less dense solution in the middle of the pH range in preparing the sucrose density gradient.

Electrofocusing is performed at 5 °C for 40 h, with a final potential of 800 V. The column is drained through the bottom tubing by slowly pumping water on top of the density gradient. Fractions of 2 ml are collected, which are analyzed manually for absorbance at 280 nm, pH and enzyme activity of the "acid" and the "ester" enzyme (Method 1).

Isoelectric focusing on polyacrylamide gels

A required number of Pyrex glass tubes (80 mm × 6 mm) are supported on a layer of Plasticine and filled by means of a Pasteur pipette, up to about 15 mm from the rim, with a solution containing 7% (w/v) acrylamide, 0.2% (w/v) *N,N'*-methylenebis(acrylamide), 0.05% (v/v) tetramethylethylenediamine, 1% Ampholine of the required pH range and 0.005% (w/v) riboflavin. A few drops of water are layered on top and the gels are photopolymerized by daylight illumination. The liquid remaining on top of the gel is removed, the glass walls are rinsed with 1% Ampholine solution and the tubes are filled completely with fresh 1% Ampholine solution. They are inserted into an Acrylophor apparatus (Pleuger N.V., Antwerp, Belgium). The enzyme solution, not more than 0.2 ml, containing 10–20% sucrose, is slowly layered on the gel surface, beneath the Ampholine layer. The anode (lower) and cathode (upper) compartments are filled with 300 ml of 0.2% (v/v) H_2SO_4 and 150 ml of 0.4% (v/v) ethylenediamine respectively. The apparatus is connected to an LKB Power Supply, Type 3371 C. The starting voltage is usually about 80 V with a current of no more than 1 mA per tube. As the current drops, voltage is gradually increased to 200 V. This final voltage is maintained for 1 h. After the power has been switched off, the gels are removed from the tubes by inserting water between glass wall and gel with a blunt hypodermic needle. The gels are rinsed with distilled water and stained.

Staining of the gels

The gels are stained for protein in 0.2% bromphenol blue, dissolved in ethanol–water–acetic acid (50:45:5, by vol.), for 90 min. The excess dye is removed by repeated washing in ethanol–water–acetic acid (30:65:5, by vol.). The gels can be stored indefinitely in this liquid.

Staining for enzyme activity is done in test tubes by soaking the gels, at room temperature, in a medium containing $3.5 \cdot 10^{-3}$ M linoleic acid, 15% (v/v) ethanol, 0.1 M sodium borate buffer, pH 8.5, 10^{-3} M KCN and 0.02% (w/v) *o*-dianisidine (from a 1% stock solution in ethanol). Incubation time may vary from several minutes to several hours, depending on the enzyme activity present. During the incubation, oxygen supply is ascertained by frequent inversion of the tubes. When the orange band pattern is sufficiently visible, the gels are washed repeatedly with distilled water and stored in a solution containing 15% (v/v) ethanol and 0.1 M borate buffer, pH 8.5. A photograph is taken as soon as possible, because the intensity and resolution of the

bands diminish slowly. It is possible to stain the gels first for enzyme activity, take a photograph, and then stain the same gels for protein.

Preparation of a partially purified soyabean extract

Soyaflour obtained by milling 1.5 kg of soyabeans in an Overcross–Beat mill, (Peppink, Amsterdam, the Netherlands) is defatted by three extractions with 3, 2 and 1 l of cold pentane (technical grade), respectively. 1 kg dry defatted flour is extracted overnight with 10 l of 0.2 M sodium acetate buffer at pH 4.5. This and all subsequent steps are performed at 4 °C.

The suspension is centrifuged and the supernatant brought to 60% saturation with solid $(NH_4)_2SO_4$ (Merck A.G., Darmstadt, Germany, grade: "for biochemical purposes"). The resulting suspension is centrifuged after standing overnight. The precipitate is taken up in 1 l of tridistilled water and dialysed exhaustively against 0.125 M sodium acetate buffer at pH 5.5.

All proteins having an isoelectric point lower than 5.5 are then eliminated by a batchwise adsorption of the non-diffusables onto 10 g (dry weight) of CM-Sephadex C 50, equilibrated against the same buffer. After the suspension has been transferred to a column, it is eluted frontally by means of the acetate buffer at a concentration of 0.5 M.

RESULTS

The elution diagram of a crude aqueous soyabean extract treated with $CaCl_2 \cdot 2H_2O$ (see Fig. 1) shows a triple protein peak (E) which has higher enzyme activity towards linoleic acid at pH 7 than at pH 9. The reverse is true for the single protein peak (A), which exhibits preferential activity towards linoleic acid at pH 9.

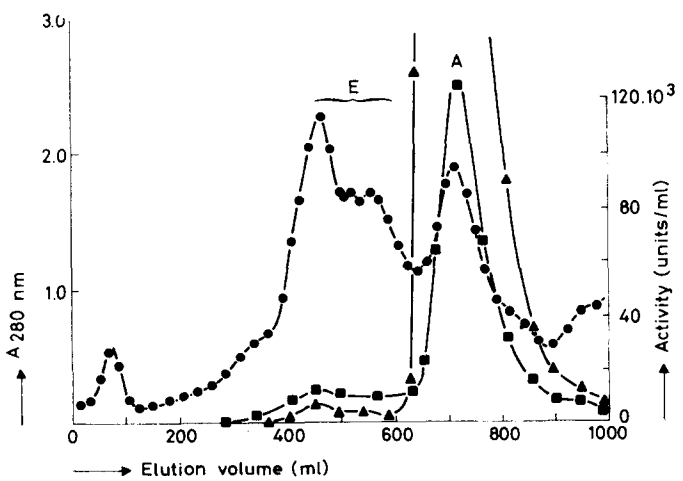


Fig. 1. Chromatography on DEAE-Sephadex A-50 of a crude aqueous soyabean extract (1/10, w/v) treated with 1/16 volume of 8.5% $CaCl_2 \cdot 2\ H_2O$, 265 ml of enzyme solution (1.5 g protein) containing 0.05 M imidazole–HCl buffer (pH 6.8) and $5 \cdot 10^{-4}$ M $CaCl_2$, adsorbed onto a 2.5 cm × 30 cm bed of the ion exchanger, equilibrated against the same buffer. Elution (30 ml/h) was performed wit a linear NaCl gradient (0–0.25 M) in the buffer. ●—●, protein; ▲—▲, activity at pH 9; ■—■, activity at pH 7 (Method 1).

Fractionation of the "ester" enzyme

Further chromatography of the triple peak (E) (Fig. 2) showed that the first peak had no lipoxygenase activity. Linoleic acid was oxidized preferentially at pH 7 by the two other components E_1 and E_2, although the ascending part of E_2 showed some activity at pH 9.

The substrate specificities of the pooled peaks E_1 and E_2 of Fig. 2 were investigated by comparing their activities towards linoleic acid and methyl linoleate. Both enzyme fractions have an activity several times higher towards the ester than the free

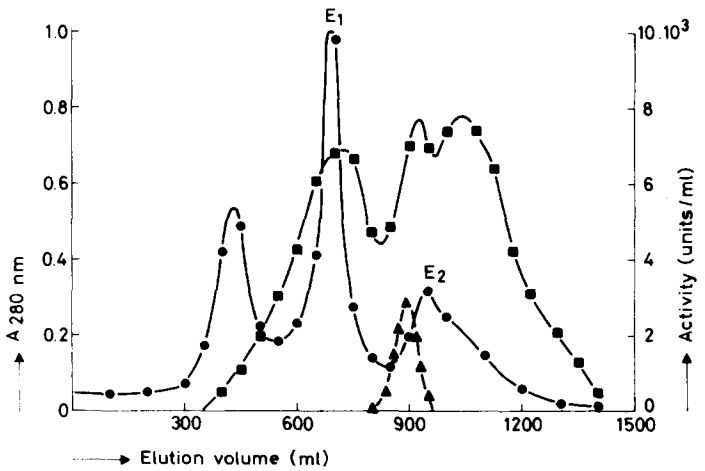


Fig. 2. Chromatography on CM–Sephadex C-50 of the combined peaks E from Fig. 1. 185 ml of enzyme solution (325 mg protein) in 0.1 M sodium acetate buffer (pH 5.5) containing $5 \cdot 10^{-4}$ M $CaCl_2$, adsorbed onto a 2.5 cm × 30 cm bed of the ion exchanger, equilibrated against the same buffer. Elution (30 ml/h) was performed with a linear NaCl gradient (0–0.4 M) in the buffer. ●—●, protein; ▲—▲, activity at pH 9; ■—■, activity at pH 7 (Method 1).

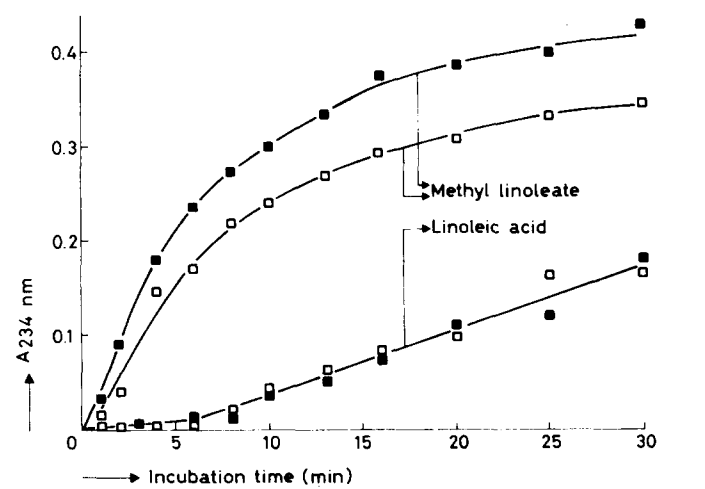


Fig. 3. Activity of the "ester" enzyme fractions E_1 (■—■) and E_2 (□—□) from Fig. 2 towards methyl linoleate and linoleic acid, at pH 8.5 and 23 °C (Method 2). Enzyme concentration, 8 μg/ml.

acid (Fig. 3). Therefore, they are probably identical with the lipoxygenase-2 described by Christopher *et al.*[3].

Fractionation of the "acid" enzyme

Fractionation of a partially purified soyabean extract (see Methods) on CM-Sephadex C-50 (Fig. 4) gave three enzyme peaks: A_1, E and A_2. A_1 with high specific activity, was further purified by two consecutive chromatographic runs on DEAE- and CM-Sephadex respectively until free from "ester" enzyme.

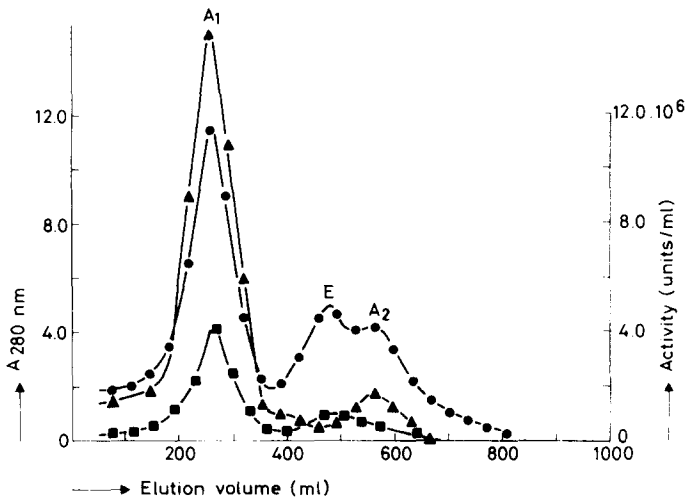


Fig. 4. Chromatography on CM-Sephadex C-50 of a partially purified soyabean extract (see Methods). 220 ml of enzyme solution (8 g protein) in 0.1 M sodium cacodylate buffer, pH 5.8, containing 10^{-4} M $CaCl_2$, adsorbed onto a 2.5 cm × 35 cm bed of the ion exchanger, equilibrated against the same buffer. Elution (30 ml/h) was performed with a linear NaCl gradient (0–0.2 M) in the buffer. ●—●, protein; ▲—▲, activity at pH 9; ■—■, activity at pH 7 (Method 1).

From the partially resolved second and third peaks E and A_2 of Fig. 4, E showed preferential activity towards linoleic acid at pH 7, while A_2 again showed much higher activity at pH 9. The pooled peaks E *plus* A_2 were then further resolved by chromatography at pH 6.8 on DEAE-Sephadex A-50 (Fig. 5). A third chromatography of A_2 on CM-Sephadex C-50 resulted in the elution of one single peak with high specific activity.

Incubation of the extensively purified enzyme fractions A_1 and A_2 with linoleic acid and methyl linoleate (Fig. 6) showed that both fractions have negligible activity towards methyl linoleate and are probably identical with lipoxygenase-1 as described by Christopher *et al.*[3].

Isoelectric fractionation on sucrose density gradient

Isoelectric fractionation of a partially purified soyabean extract (see Methods) on a sucrose density gradient gave the pattern shown in Fig. 7. Peak A, focused around pH 5.6, has by far the highest enzymic activity and represents the "acid" enzymes. This protein peak has a shoulder on its right side which probably represents

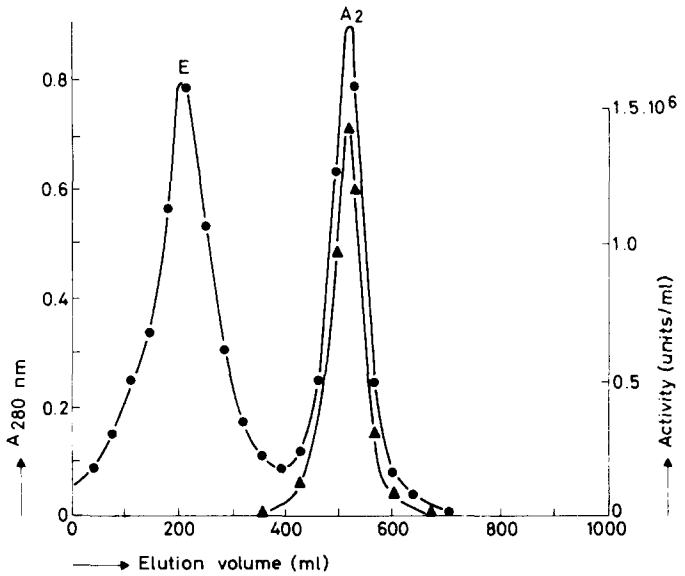


Fig. 5. Rechromatography on DEAE-Sephadex A-50 of the combined peaks E *plus* A_2 from Fig. 4. 116 ml of enzyme solution (250 mg protein) in 0.1 M imidazole–HCl buffer, pH 6.8, containing 10^{-4} M $CaCl_2$, adsorbed onto a 2.5 cm × 35 cm bed of the ion exchanger, equilibrated against the same buffer. Elution (30 ml/h) was performed using a linear NaCl gradient (0–0.2 M) in the buffer. ●—●, protein; ▲—▲, activity at pH 9.

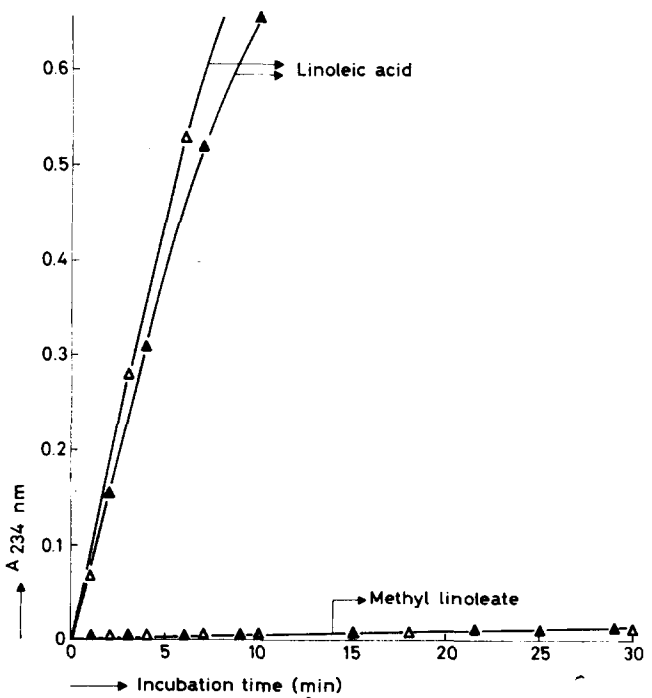


Fig. 6. Activity of the purified "acid" enzyme fractions A_1 (▲—▲) and A_2 (△—△) towards methyl linoleate and linoleic acid, at pH 8.5 and 23 °C (Method 2). Enzyme concentration, 0.2 μg/ml.

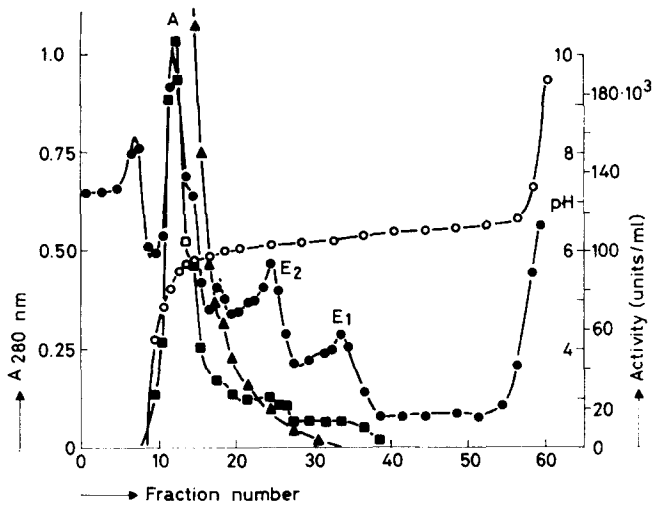


Fig. 7. Isoelectric focusing of a partially purified soyabean extract (see Methods) between pH 5.5 and 6.5 in a sucrose density gradient. Column loaded with 2 ml of enzyme solution (27 mg protein). ●—●, protein; ▲—▲, activity at pH 9; ■—■, activity at pH 7 (Method 1); ○—○, pH.

fraction A_2 in Figs 4 and 5. The activity of the "ester" enzymes towards linoleic acid at pH 7 is rather low, but higher than at pH 9. Since gel-electrofocusing pictures (see below) show that the "ester" enzyme E_1 contains a component with a higher isoelectric point than E_2, we can assume that the protein peak with an isoelectric point at 6.28 represents E_1. The isoelectric point of E_2 then is 6.1.

Isoelectric focusing on polyacrylamide gels

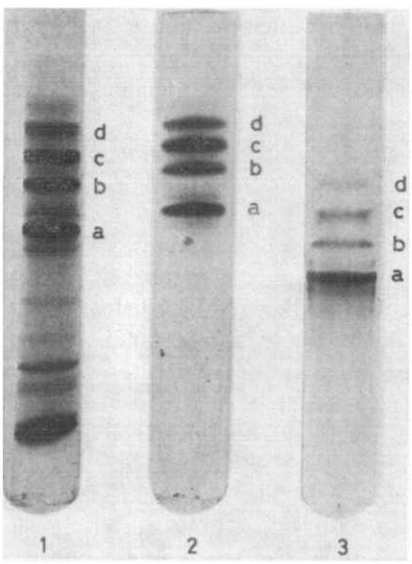
In order to obtain a more detailed picture of the heterogeneity of the soyabean lipoxygenase system, the soyabean extracts were also focused isoelectrically on polyacrylamide gels. The protein components showing lipoxygenase activity were localized by a specific staining technique (see Methods). Nonspecific oxidation by heme proteins was completely inhibited by the presence of KCN in the incubation medium.

Fig. 8 shows three polyacrylamide gels on which crude and partially purified soyabean extracts have been focused. A complex of mainly three bands, designated a for all the gels, represents the "acid" lipoxygenases A_1 and A_2, since the band pattern coincided when purified "acid" lipoxygenase was superimposed on the crude extract. Bands b, c and d also showed enzymic activity; as will be seen from Fig. 10, they represent the "ester" enzymes.

Focusing of the "acid" lipoxygenase A_1 in Fig. 5 and of the extensively purified lipoxygenase A_2 (Fig. 5) shows that three protein components per enzyme fraction are present and that the fractions are slightly contaminated by each other, since a mixture of both (gel No. 3) only shows 4 components (Fig. 9). All components exhibited enzymic activity.

The "ester" enzymes were localized by focusing the protein peaks E and A of Fig. 1. As a mixture of both peaks again constitutes the original partially purified soyabean extract, its protein pattern is identical to that shown on gel 2 in Fig. 8.

Finally, peaks E_1 and E_2, shown in Fig. 2, were electrofocused in the same way



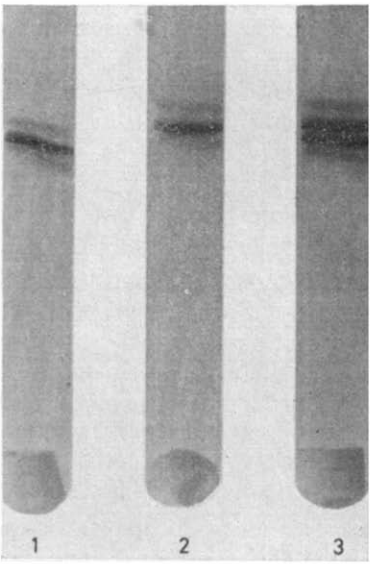


Fig. 8. Isoelectric focusing on polyacrylamide gel between pH 5 and 7 of: (1) crude soyabean extract, stained for protein; (2) partially purified soyabean extract, stained for protein; (3) crude soyabean extract, stained for enzyme activity.

Fig. 9. Isoelectric focusing on polyacrylamide gel between pH 5 and 7 of the purified "acid" lopoxygenase fractions, stained for protein: (1) lipoxygenase fraction A_1 from Fig. 4 further purified; (2) extensively purified A_2; (3) A_1 and A_2.

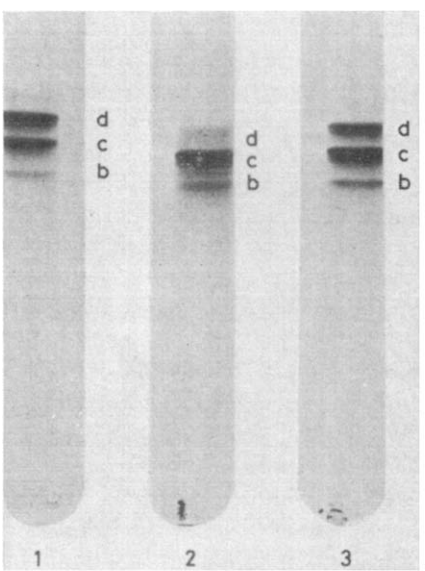


Fig. 10. Isoelectric focusing on polyacrylamide gel, between pH 5 and 7, of the lipoxygenase fractions obtained as shown in Fig. 2. (1) "ester" enzyme peak E_1; (2) "ester" enzyme peak E_2; (3) E_1 and E_2.

(Fig. 9). The same three main protein bands, albeit in different proportions, were present in both peaks E_1 and E_2. This means that the ionexchange chromatography, illustrated in Fig. 2, gave no complete resolution into different components. Fig. 10 shows additional splitting of band d on gel 1 and band c on gel 2.

DISCUSSION

Our results confirm the observation by Christopher *et al.*[3] that soyabeans, contain a lipoxygenase with a different substrate specificity in addition to the lipoxygenase described by Theorell *et al.*[9]. This difference between the "acid" and the "ester" lipoxygenase is due to a different way of binding the substrate, since the "ester" enzyme is capable of catalyzing the oxygenation of linoleic acid at neutral pH (non-ionized) but is practically inactive at pH 9 when the acid is completely dissociated. We can only speculate whether this heterogeneity of both the "acid" and the "ester" lipoxygenase is at all functional in the soyabean. The oxidation of linoleic acid by the "acid" lipoxygenase usually gives rise to 10–30% 9-hydroperoxylinoleic acid[12]. This is not an autoxidation product, as Veldink *et al.*[13] have shown, since it is optically active, which confirms its enzymic origin.

Dolev *et al.*[14] described the conversion of linoleic acid into 100% 13-hydroperoxylinoleic acid and Christopher and Axelrod[15] obtained conversion into the 13-isomer almost exclusively with a purified "acid" lipoxygenase from soyabeans. Since the second fraction (A_2) of the "acid" lipoxygenase constitutes about 25% of the total "acid" lipoxygenase in the soyabean, we checked whether this fraction might be responsible for the formation of the 9-hydroperoxylinoleic acid.

Although both fractions A_1 and A_2 were extensively purified, their incubation with linoleic acid at pH 9 gave the 13- and 9-hydroperoxy isomers in the ratio of approximately 70:30. This was shown by thin-layer chromatography following the reduction with $NaBH_4$ of the peroxy acids and methylation with diazomethane of the unsaturated hydroxy acids (unpublished results).

Christopher and Axelrod[15] found that the "ester" enzyme catalyzes the formation of the 9- and 13-hydroperoxy isomers in a 50:50 ratio. It is, however, unlikely that the presence of some "ester" enzyme as an impurity in preparations of the "acid" enzyme is responsible for the formation of the 9-hydroperoxylinoleic acid, since the ester enzyme is practically inactive at pH 9[3].

Again, no fundamental differences in substrate specificity were noticed between the "ester" enzymes. Although Peak E_1 (Fig. 2) is slightly less active towards methyl linoleate (Fig. 3) than Peak E_2, the difference is too small to be significant. This is probably due to considerable cross contamination, as demonstrated by the gel-electrofocusing patterns (Figs 8–10).

It is of course possible to envisage that small variations on the surface of the protein molecule, causing a small change in iso-electric point, are allowable, without affecting the structure and conformation of the active site of the enzyme. In this way the multiplicity of components would not necessarily point to a multifunctional behaviour. It is also possible that some of the enzyme components catalyze the formation of compounds which hitherto have not been recognized as products of the lipoxygenase reaction, since they are present in minor amounts and may have looked upon

as autoxidation products[16]. That the course of the lipoxygenase reaction may be more complex than generally accepted, has been suggested before[17].

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