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ISOLATION OF A THIRD ISOENZYME OF SOYBEAN LIPOXYGENASE*

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SUMMARY

1. A third isoenzyme of soybean lipoxygenase (linoleate: oxygen oxidoreductase, EC 1.13.1.13) has been purified to essential homogeneity by Sephadex DEAE-A50 column chromatography and isoelectric focusing.

2. The enzyme differs from the two previously described pure isoenzymes (lipoxygenases-1 and -2)[1] by the following criteria: elution profile from DEAE-Sephadex, isoelectric point, pH activity profile, and the effect of Ca^{2+} on activity.

3. The new isoenzyme also shows an anomalous, inverse dependence of activity on enzyme concentration.

INTRODUCTION

The possible occurrence of multiple forms of soybean lipoxygenase (linoleate: oxygen oxidoreductase, EC 1.13.1.13) has been intimated by several independent observations. Smith[2] and Sumner and Dounce[3], employing methyl linoleate and cottonseed oil as substrates, respectively, reported a pH optimum of 6.5 for the lipoxygenase activity of crude soybean extracts. Holman[4] showed that crystalline lipoxygenase had a pH optimum of 9.0 with sodium linoleate as substrate.

In 1947, Kies[5], utilizing the coupled destruction of carotene as an assay, encountered anomalies which suggested the complex nature of soybean lipoxygenase. Employing a partially purified extract and trilinolein and linoleic acid as substrates, Koch *et al.*[6] obtained data which could best be explained by the existence of two enzymes. In 1967, Guss *et al.*[7] suggested that four lipoxygenases existed in aqueous extracts of soybeans on the basis of disc gel electrophoresis followed by a specific stain for enzyme activity.

More recently, three groups have reported on the isolation and partial characterization of an isoenzyme of soybean lipoxygenase. Investigating a guaiacol–linoleic acid hydroperoxide oxidoreductase activity in soybeans, Schormüller *et al.*[8] isolated two such activities. Both were homogeneous on cellulose acetate electrophoresis and also possessed lipoxygenase activity. The latter was not further charac-

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terized. Yamamoto *et al.*[9] have reported the isolation of an isoenzyme of lipoxygenase which was distinct from the classical enzyme[10] in electrophoretic mobility, pH profile, and response to calcium ions. Christopher *et al.*[1] have also reported the isolation of an ioenzyme (lipoxygenase-2) and established its identity as different from the Theorell enzyme (hereafter referred to as lipoxygenase-1) on the basis of pH profile, substrate specificity, electrophoretic mobility, and heat stability.

We now report on the isolation and partial characterization of a third isoenzyme of lipoxygenase which has been shown to be different from lipoxygenases-1 and -2.

MATERIALS AND METHODS

Materials

Soybeans of Hawkeye variety (1970 crop) were used to prepare the enzymes. Linoleic acid was obtained from Hormel Inst. (U. of Minn.), Sephadex DEAE-A50 and G-150 from Pharmacia, and isoelectric focusing equipment from LKB. Commercial lipoxygenase was purchased from Seravac.

Assays

Lipoxygenase activities were determined using a Clark oxygen electrode (Yellow Springs) in a Gilson Medical Electronics Oxygraph, Model KM. The reaction vessel had a volume of 1.7 ml. Values for oxygen concentrations[11] in solution were not corrected for the effect of ionic solutes. One unit of enzyme activity corresponded to the consumption of 1 μmole O_2 per min. Assays were carried out under one of the following conditions.

Method I. Samples were assayed at 15 °C and at two pH values (6.8 and 9.0) using linoleic acid dispersed in Tween 20 (modification of Surrey's substrate)[12]. At pH 9.0, mixtures contained 165 mM sodium borate and 1.23 mM linoleic acid. At pH 6.8, assays were performed with 165 mM sodium phosphate and 1.23 mM linoleic acid, in the absence or presence of Ca^{2+} (0.59 mM).

Method II. Assays for pH optimum study were performed at 15 °C using linoleic acid dispersed in Tween 20. Reaction mixtures contained 1.23 mM linoleic acid and 50 mM buffer of constant ionic strength (0.2).

Disc gel electrophoresis

Electrophoresis of protein was done on 7% acrylamide gels at pH 4.3, using the method of Reisfeld *et al.*[13]. A 25% glycerol solution was substituted for the stacking gel. Runs were performed at 5 °C for 2 h at a constant current of 3 mA per tube. Protein was stained with Amido Schwarz and gels were scanned at 600 nm.

Protein determination

Protein concentrations were calculated from absorbance values obtained at 280 nm. Measurement of the dry weight of purified lipoxygenase gave a value of 0.7 mg protein per ml per absorbance unit.

Elution profile on Sephadex G-150

An indication of molecular weight was obtained by gel filtration at room temperature. A column (2.5 cm × 48 cm) of Sephadex G-150 was poured and equili-

brated with 0.05 M sodium phosphate (pH 6.8) containing 0.15 M NaCl. Void volume was determined with 2 ml of 0.2% Dextran Blue. Protein samples (3–4 mg) were applied and eluted with the above buffer. The flow rate was adjusted to 30 ml/h and fractions of 2.5 ml were collected. Fractions were assayed for activity at pH values 6.8 and 9.0.

EXPERIMENTAL

Purification

Hexane-defatted soybean meal (75 g) was extracted with 750 ml of water at room temperature for 1 h with stirring. The suspension was passed through one layer of cheesecloth and the resulting filtrate centrifuged at 16 000 × *g* for 10 min. The precipitate was discarded and the supernatant treated as described below. All subsequent steps were performed at 0–5 °C and centrifugations at 16 000 × *g* for 10 min. Sodium phosphate buffers were employed unless otherwise specified. To the supernatant (600 ml) from above, 50 ml of a 0.58 M $CaCl_2$ solution were added. The resulting precipitate was centrifuged and discarded. The supernatant (600 ml), which

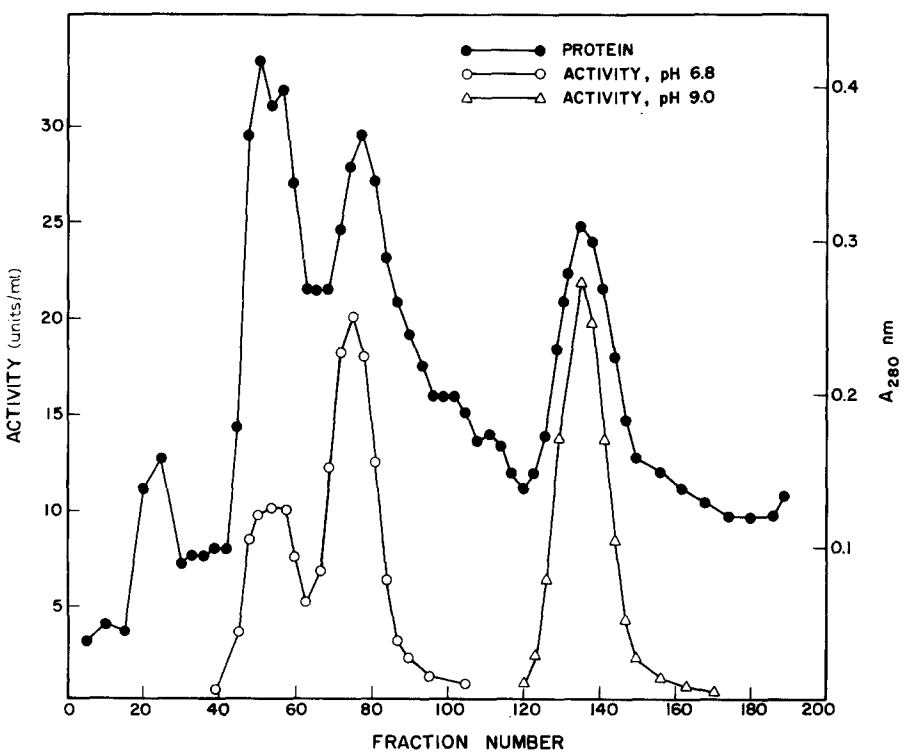

Fig. 1. Sephadex DEAE-A50 column chromatography. A column (4 cm × 38 cm) was packed with DEAE-A50 and equilibrated with 0.02 M sodium phosphate (pH 6.8). The enzyme sample was applied in the same buffer and elution was effected (70 ml/h) with a linear gradient formed from equal volumes of 0.02 M and 0.22 M sodium phosphate (pH 6.8). Fractions of 17 ml were collected and protein content determined by absorbance at 280 nm. Activity was assayed by Method I in the presence of 0.59 mM Ca^{2+}.

constituted the crude extract, was made to 75% saturation with respect to $(NH_4)_2SO_4$ by the addition of 310 g of the solid salt. The pH was adjusted to 6.8 ± 0.2 with 2 M NaOH. The precipitated protein was centrifuged and then dissolved in 0.05 M buffer (pH 6.8) to give a volume of 145 ml. This protein solution was dialyzed overnight against 40 volumes of 0.01 M buffer (pH 6.8) containing 0.5 mM Ca^{2+}. The dialysate from above was collected and centrifuged. The inactive precipitate was discarded, and the supernatant (145 ml) was successively fractionated with solid $(NH_4)_2SO_4$ to fractions corresponding to 0–25%, 25–55%, and 55–75% saturation. The bulk of the desired activity was in the 25–55% fraction which was dissolved in 70 ml 0.05 M buffer (pH 6.8) and dialyzed overnight against 40 volumes of 0.02 M buffer (pH 6.8) containing 0.5 mM Ca^{2+}.

After centrifugation, the 25–55% fraction (73 ml and 730 $A_{280\ nm}$ units) was subjected to chromatography on a column of DEAE-Sephadex. The elution pattern obtained is illustrated in Fig. 1. The first enzyme peak to emerge represents the new isoenzyme (lipoxygenase-3), the second, lipoxygenase-2 and the last, lipoxygenase-1. Fractions 45–60 were pooled.

A portion (50 ml) of these pooled fractions was dialyzed overnight against 20 volumes of deionized water to reduce salt content. This solution was subjected to isoelectric focusing in an LKB 110 ml column at 4 °C, according to the method of Vesterberg and Svensson[14]. The sucrose gradient was prepared with the aid of a gradient mixer. The protein solution (15.2 $A_{280\ nm}$ units) was incorporated into the light solution. LKB ampholytes (40%) of pH range 3–10 were employed at a final column concentration of 1%. The bottom electrode served as the anode. The column

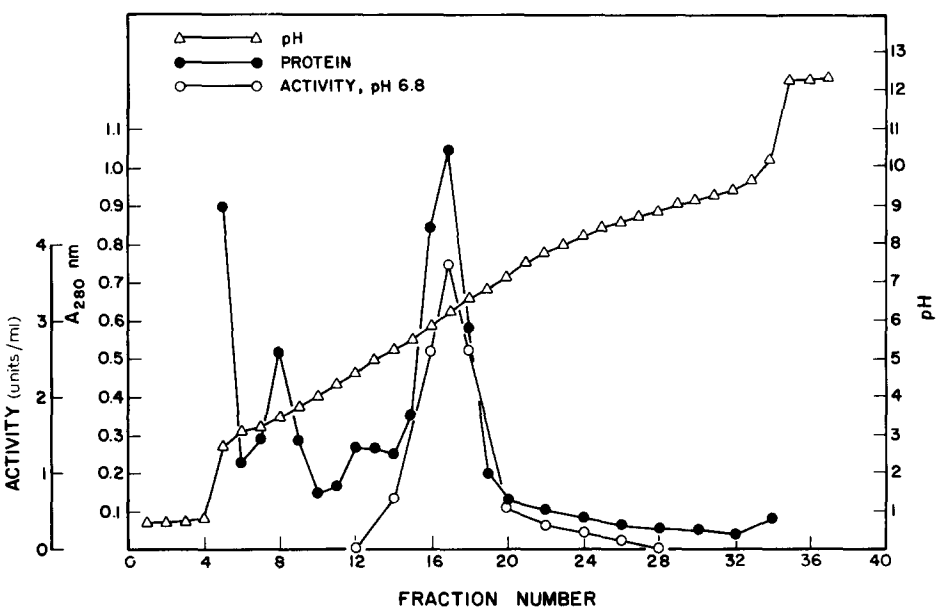

Fig. 2. Isoelectric focusing. Isoelectric focusing was performed on an LKB 8201 apparatus at 4 °C for 41 h. The protein sample was focused in a pH gradient of 3–10 at a final ampholyte concentration of 1%. Fractions of 3 ml were collected. Protein was determined by absorbance at 280 nm and activity by Method I in the presence of 0.59 mM Ca^{2+}. The pH of each fraction was measured at 23 °C.

was charged at a flow rate of 3 ml/min and emptied at 1.5 ml/min. Voltage was maintained at 200 V for 17 h and then increased to 300 V for 24 h; initial current was 6.8 mA. Fractions of 3 ml were collected. The elution profile obtained on isoelectric focusing of the material represented by the first peak of the DEAE column exhibited one peak of lipoxygenase activity (see Fig. 2), with an isoelectric point of 6.15. Fractions 16–18 were combined. Ampholytes were removed by adding 0.3 g NaCl to the sample (9 ml) and dialyzing this solution overnight against 75 volumes 0.05 M buffer, pH 6.8, containing 0.15 M NaCl. The dialysate was collected, 0.3 g NaCl was added, and the solution dialyzed overnight against the above buffer. The dialysate was removed and dialyzed against 75 volumes of fresh 0.05 M buffer (pH 6.8) for 48 h. Protein was concentrated by ultrafiltration employing a 10-ml capacity Amicon cell equipped with a PM-10 filter. Ultrafiltration was performed at room temperature under 40 lb/inch^2 nitrogen. This concentrated protein solution was employed for subsequent studies. The purification of lipoxygenase-3 is summarized in Table I. An explanation for the discrepancy in the values for total activity at pH 6.8 is presented below (see activity as a function of enzyme concentration).

TABLE I

SUMMARY OF PURIFICATION

Step	*Vol. (ml)*	*Total protein ($A_{280\ nm}$ units)*	*Total activity (units)*	
			*pH 6.8**	*pH 9.0*
Crude extract	600	11 400	12 200	13 000
$(NH_4)_2SO_4$ concentrate	145	1 600	18 700	8 000
$(NH_4)_2SO_4$ fractionation	73	730	14 100	6 500
Sephadex DEAE-A50	255	76.5	841 (663)**	—
Isoelectric focusing	9	4.4	51 (180)**	—

* Values refer to lipoxygenase-3 units, assuming linearity between enzyme content and O_2 consumption.

** Values in parentheses refer to lipoxygenase-3 units as defined in the text.

RESULTS

Disc gel electrophoresis

Electrophoresis at pH 4.3 revealed the presence of one major band (R_F 0.307) and two trace bands (R_F values 0.240 and 0.173). Comparison of appropriate peak areas (Fig. 3) indicated that the two trace bands together comprised less than 5% of the total protein.

Additional experiments, subsequent to the completion of this work have shown that chromatography on a column of Sephadex CM separates the material corresponding to the band of R_F 0.307 from the trace impurities. Furthermore, the former was shown to possess lipoxygenase activity whereas the latter are inactive.

pH profile

The enzyme activity as a function of pH, using linoleic acid as substrate (see *Assay*: *Method II*) is shown in Fig. 4. Activity was seen at all pH values tested except pH 9.0. The pH optimum is very broad under these conditions.

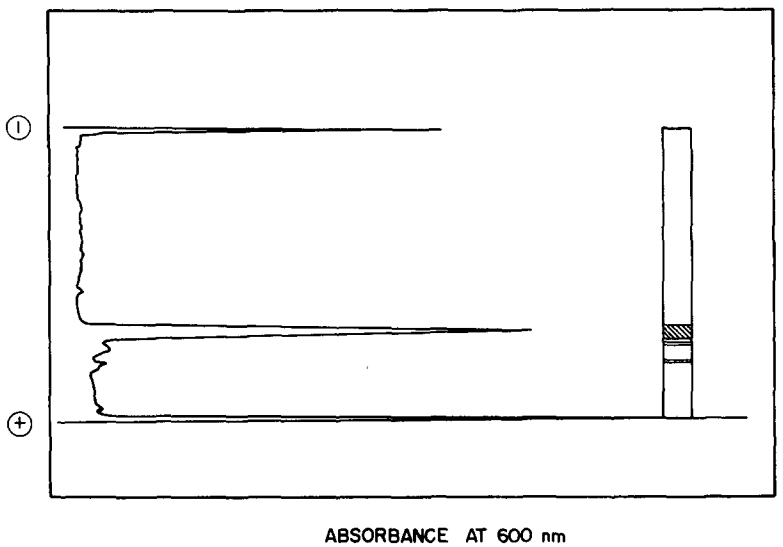

Fig. 3. Polyacrylamide gel electrophoresis. Lipoxygenase-3 (22 μg) was layered on the gel surface in a 25% glycerol solution. Electrophoresis (pH 4.3) was performed in 7% gel for 2 h at 5 °C and at a constant current of 3 mA per tube. Protein was stained with a solution of Amido-Schwarz, and gel was scanned at 600 nm after destaining electrophoretically.

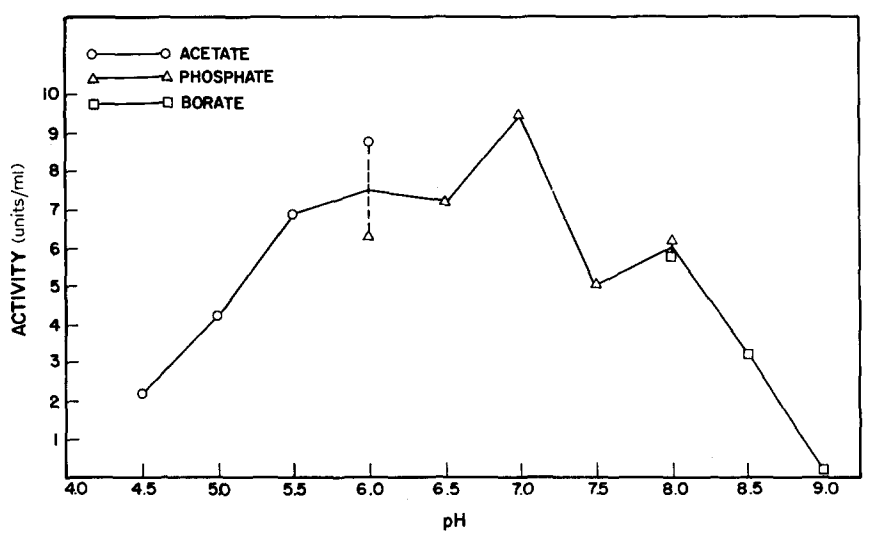

Fig. 4. Activity of lipoxygenase-3 toward linoleic acid as a function of pH at constant ionic strength Assays were performed by Method II and 18 μg of enzyme were employed per test. Average values of activity were plotted in regions of overlapping buffers.

Elution profile on Sephadex G-150

Simultaneous gel filtration of lipoxygenase-3 and a commercial lipoxygenase, as a source of lipoxygenase-1, resulted in the elution curve shown in Fig. 5. The activities at pH 9.0, characteristic of lipoxygenase-1, and pH 6.8, characteristic of lipoxygenase-3, were both eluted at the same V_e/V_0 ratio. The same result was also

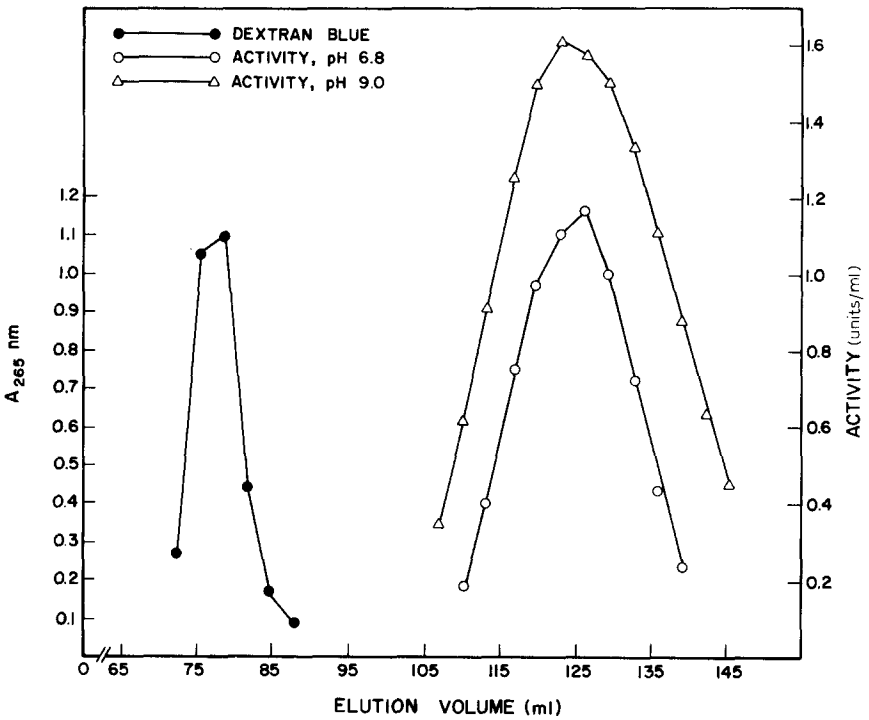

Fig. 5. Elution profile on Sephadex G-150 of lipoxygenase-3. A Sephadex G-150 column (2.5 cm × 48 cm) was equilibrated with 0.05 M sodium phosphate (pH 6.8) containing 0.15 M NaCl. Lipoxygenases-1 and -3 (3–4 mg each) were applied either in admixture or separately and eluted with the above buffer. Fractions (3 ml) were assayed for activity by Method I in the absence of Ca^{2+}.

obtained when the enzymes were chromatographed separately. Thus, it was concluded that lipoxygenase-1 and lipoxygenase-3 had similar Stokes radii. Lipoxygenase-1 has been reported to have a molecular weight of about 100 000 based on sedimentation velocity[10], equilibrium sedimentation[15], and gel filtration[16].

Effect of Ca^{2+} on activity

The response of activity to increasing concentrations of Ca^{2+} is illustrated in Fig. 6. Ca^{2+} did not stimulate activity. Indeed, Ca^{2+} was inhibitory at all concentrations tested.

Activity as a function of enzyme concentration

The striking deviation from a linear relationship between the quantity of lipoxygenase-3 taken for assay and the rate of O_2 consumption is illustrated in Fig. 7. Specific activity varied more than 3.5-fold over a 20-fold range in enzyme concentration. The experimental points were obtained using a series of enzyme concentrations under standard assay conditions during which the rate of O_2 utilization was proportional to reaction time. A unit of lipoxygenase-3 was defined arbitrarily as ten times the quantity of enzyme catalyzing the consumption of precisely 0.10 μmole of O_2 per min under the standard assay conditions.

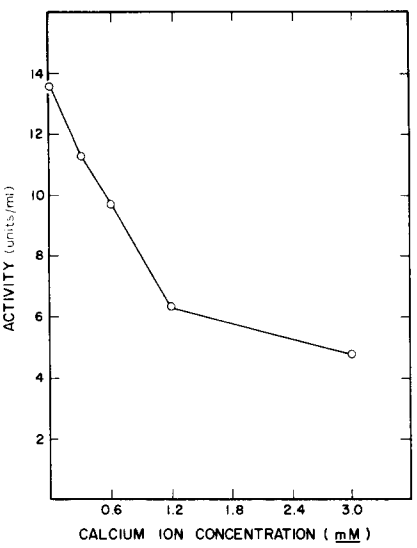

Fig. 6. Effect of calcium ion on activity. Assays were performed by Method I at pH 6.8 under varying concentrations of Ca^{2+} and 18 μg of lipoxygenase-3 per assay.

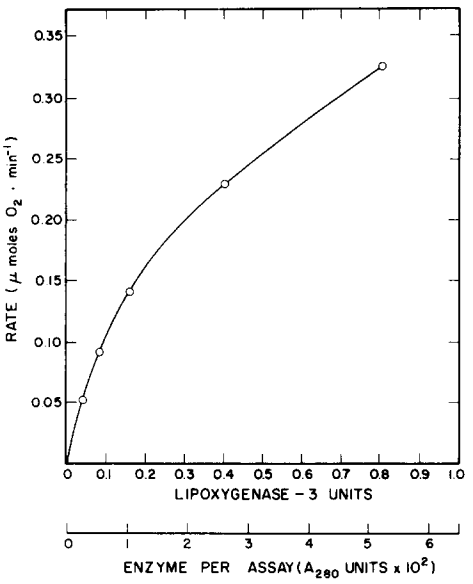

Fig. 7. Activity as a function of enzyme concentration. Varying concentrations of lipoxygenase-3 were employed. Assays were performed according to Method I at pH 6.8 in the absence of Ca^{2+}.

DISCUSSION

The newly-isolated isoenzyme, lipoxygenase-3, appeared to have a molecular weight of 100 000 as judged by gel filtration, the same value as found for the other two isoenzymes. However, lipoxygenase-3 exhibited a number of differences in other respects.

(1) Chromatographic behavior on DEAE–Sephadex clearly differentiated between all three isoenzymes.

(2) Different isoelectric points were found for each isoenzyme: 5.68 for lipoxygenase-1, 6.25 for lipoxygenase-2 (unpublished results) and 6.15 for lipoxygenase-3. The value for lipoxygenase-1 agreed well with the pI of 5.65 obtained by Catsimpoolas[17].

(3) The pH–activity profiles of each were significantly different under similar assay conditions. Lipoxygeanses-1 and -2 exhibited pH optima of 9.5 and 6.5, respectively[1]. Lipoxygenase-3, however, had a broad pH–activity profile throughout the pH range 4.5–9.0 and showed no activity at pH 9.

(4) The specific activities of lipoxygenases-1 and -2 were not dependent on enzyme concentration in contrast to lipoxygenase-3.

(5) Lipoxygenase-3 could be distinguished from lipoyxgenase-2 with respect to the effect of Ca^{2+} on activity. Ca^{2+} stimulated the activity of lipoxygenase-2[9] but inhibited the activity of lipoxygenase-3 under similar conditions.

It is clear that at least three isoenzymes, and possibly four[7], of lipoxygenase occur in soybean extracts. The molecular basis for these differences is unknown. The

possibility that these multiple forms might be hybridized dimers of monomeric species may deserve consideration since Stevens *et al.*[15] have indicated that lipoxygenase-1 consists of two subunits of equal molecular weight. However, we (unpublished results) have observed no dissociation of lipoxygenase-1 on electrophoresis in sodium dodecylsulfate gels after treatment of protein with mercaptoethanol.

The reason for the striking non-linearity of specific activity as a function of enzyme concentration is not known. It cannot be the result of enzyme inactivation nor depletion of substrate because the rates of oxygen consumption are linear throughout the reaction period on which the assay is based. These aspects of the problem as well as other differences between the isoenzymes are currently under investigation.

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