BBA 66015

# ISOLATION OF AN ISOZYME OF SOYBEAN LIPOXYGENASE*

JOHN CHRISTOPHER, ELFRIEDE PISTORIUS AND BERNARD AXELROD
*Department of Biochemistry, Purdue University, Lafayette, Ind. (U.S.A.)*

(Received August 4th, 1969)

SUMMARY

1 An isozyme, distinct from the well-known crystalline lipoxygenase (linoleate oxygen oxidoreductase, EC 1.13.1.13, formerly known as lipoxidase) of Theorell, has been isolated from soybeans, as an electrophoretically homogeneous protein.

2 Its substrate specificity over a range of pH values indicates that the newly purified enzyme may be responsible for the "triglyceride" lipoxidase activity reported by Koch.

3 The new enzyme is distinct from the originally described lipoxidase of Theorell by the following criteria: elution profile from DEAE-Sephadex, disc gel electrophoresis, pH activity profile, specificity and heat stability.

## INTRODUCTION

The existence of an enzyme "carotene oxidase" in soybeans, which catalyzes the oxidative destruction of carotene was reported by BOHN AND HAAS[1] in 1928. Four years later, ANDRE AND HOU[2] found that soybeans contained an enzyme, lipoxygenase (linoleate: oxygen oxidoreductase, EC 1.13.1.13), which they termed "lipoxidase", which catalyzed the peroxidation of certain unsaturated fatty acids. SUMNER AND SUMNER[3] subsequently published evidence showing that lipoxygenase and "carotene oxidase" were identical. Finally, THEORELL *et al.*[4] succeeded in crystallizing and characterizing lipoxygenase from soybeans.

Nevertheless, the possibility of the existence of multiple lipoxygenases in soybeans was intimated by several independent observations. Thus, SMITH[5] and SUMNER AND DOUNCE[6], employing methyl linoleate and cotton seed oil as substrates, found a pH optimum of 6.5 for the lipoxygenase activity of crude soybean extracts. HOLMAN[7] reported a pH optimum of 9.0 for crystalline lipoxygenase, using sodium linoleate as substrate.

SMITH[5] suggested that the difference in pH optimum could be attributed to the substrate system employed, and that it reflected the influence of pH on the solubility of the substrate. The identity of lipoxygenase and "carotene oxidase" was questioned.

* This is Journal Paper No. 3773 of the Purdue University Agricultural Experiment Station.

by KIES[8] who discovered different susceptibilities to heat for the two activities, when she employed a partially purified soybean lipoxidase It was later established, using the Theorell lipoxidase, that the "carotene oxidase" activity as observed by KIES *et al* [9] could not be assigned to this enzyme

KOCH *et al* [10] working with partially purified extracts of soybeans, obtained data which indicated the differences in pH optima were not a reflection of substrate solubility but were actually due to two types of lipoxidase activity He suggested that the results could be explained by the existence of two enzymes, one of which utilized linoleic acid and for which he proposed the name "fatty acid lipoxidase" For the other which appeared to be effective on trilinolein he offered the designation "triglyceride lipoxidase "

More recently, GUSS *et al* [11] suggested that four lipoxygenases existed in aqueous extracts of soybeans, on the basis of disc electrophoresis followed by specific enzyme staining

The present study reports the purification and identification of an isozyme of lipoxygenase which is distinct from the classical crystalline lipoxygenase originally characterized and crystallized by THEORELL *et al* [4] Some of the properties of the pure Theorell enzyme and the newly isolated isozyme are reported and compared

## MATERIALS AND METHODS

### *Materials*

Defatted soybean flakes were provided through the kindness of Dr E W Meyer of the Chemurgy Division of Central Soya (Chicago) Linoleic acid, methyl linoleate, and trilinolein were purchased from the Hormel Institute (University of Minnesota) DEAE-Sephadex was obtained from Pharmacia and hydroxylapatite (Biogel HT) from Calbiochem The Theorell enzyme was prepared according to the procedure of SCHROEDER[12] All other materials used in this study were commercial products of reagent grade

### *Enzyme 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 $O_2$ (ref 13) concentrations in solution were not corrected for the effect of ionic solutes 1 unit of enzyme activity corresponded to the consumption of 1 $\mu$mole $O_2$ per min Assays were carried out under one of the following three conditions

*Method I* During purification, fractions were assayed at 15° and at two pH's (6 8 and 9 0) using a modification of SURREY's[14] substrate in linoleic acid dispersed with Tween 20 The assay mixture used at pH 9 0 contained 165 mM sodium borate and 1 23 mM linoleic acid That employed at pH 6 8, included 165 mM sodium phosphate, 1 23 mM linoleic acid, and 0 59 mM $Ca^{2+}$

*Method II* Assay mixtures employed in studies of substrate specificity were identical to those of KOCH *et al* [10] except that all the buffers were 0 05 M as opposed to 0 01 M since it was found that the lower concentration lacked sufficient buffering capacity in this system

*Method III* Assays for pH optimum and heat stability studies were performed

at 15° using linoleic acid dispersed with Tween 20 Reaction mixtures used in the studies of pH contained 50 mM buffer Constant ionic strength (0 2) was maintained Linoleic acid was present at a concentration of 1 23 mM Assays made in connection with the studies of heat stability were performed under optimal pH conditions for each isozyme

*Disc gel electrophoresis*

Electrophoresis of protein was done on 7% polyacrylamide gels at pH 9 5, using the methods of DAVIS[15] Instead of a stacking gel, 40% sucrose or 25% glycerol was used Runs were carried out at 6° for 2 h at a constant current of 3 mA per tube Gels were stained with Amido Schwarz

*Protein determination*

Protein concentrations were determined from absorbance values obtained at 280 nm Independent measurement of the dry weights of purified lipoxidase gives a value of 0 70 mg protein per ml per absorbance unit

## RESULTS

*Isolation of isozyme of lipoxidase*

Defatted soybean flakes (1500 g) were extracted for 1–1 5 h at room temperature with 10 vol of sodium phosphate buffer (0 05 M, pH 6 8) containing 0 1 mM $Ca^{2+}$ After the initial extraction, all steps were performed at 4° The extract was filtered through cheesecloth, and the resulting filtrate centrifuged The supernatant solution was brought to 70% saturation with solid $(NH_4)_2SO_4$ The resulting precipitate was dissolved in 0 01 M sodium phosphate buffer (pH 6 8) containing 0 5 mM $Ca^{2+}$ and dialyzed against 0 01 M solution of the same buffer An inactive precipitate formed, which was removed by centrifugation The supernatant was treated with $(NH_4)_2SO_4$ and the fraction precipitating between 30 and 50% saturation was collected and dialyzed as before The sediment which formed on dialysis was discarded

The supernatant was next fractionated with DEAE-Sephadex A50 in a batchwise manner The solution (1080 ml) containing 4 5 $10^4$ $A_{280\ nm}$ units, was mixed with 4500 ml of a thick slurry of Sephadex equilibrated with 0 01 M phosphate buffer (pH 6 8) and stirred intermittently for 30 min The material was filtered on a Buchner funnel under gentle suction to yield a filtrate of 3300 ml Elution was repeated successively with 3 l each of 0 05, 0 10, 0 20, and 0 50 M sodium phosphate buffers, pH 6 8 The first three filtrates (0 01, 0 05, and 0 10 M) having the best ratios of pH 6 8–9 0 activities were combined and brought to 65% saturation with $(NH_4)_2SO_4$ The batchwise procedure was employed to minimize the time consuming fractionation In subsequent practice we omit this step and go directly to large diameter columns The resulting precipitate, after being dissolved and dialyzed as described above, was applied to a DEAE-Sephadex A50 column (4 25 cm × 80 cm) which had been previously equilibrated with sodium phosphate buffer (0 01 M, pH 6 8) The column was flushed with 1 l of the same buffer Elution was then continued with an increasing linear gradient consisting of 1 5 l of 0 01 M, and 1 5 l of 0 2 M, sodium phosphate buffers (pH 6 8) Fractions of 18 ml were collected and assayed for protein by absorption at 280 nm and for lipoxygenase activities at pH 6 8 and 9 0 *(Method I)* The

TABLE I

SUMMARY OF PURIFICATION

| Step | Total protein ($A_{280\ nm}$) | Total activity (units) | | Ratio of activity at pH 6 8 to activity at pH 9 0 | Specific activity* (units/ $A_{280\ nm}$) | Purification* (-fold) | Yield* (%) |
|---|---|---|---|---|---|---|---|
| | | pH 6 8 | pH 9 0 | | | | |
| Crude extract | 7 17 $10^5$ | 9 21 $10^5$ | 3 06 $10^5$ | 3 0 | 1 3 | — | — |
| $(NH_4)_2SO_4$ (0–70%) | 2 12 $10^5$ | 6 48 $10^5$ | 2 57 $10^5$ | 2 5 | 3 0 | 2 4 | 70 4 |
| $(NH_4)_2SO_4$ (30–50%) | 4 46 $10^4$ | 3 19 $10^5$ | 1 48 $10^5$ | 2 2 | 7 2 | 5 6 | 34 6 |
| DEAE-Sephadex batch eluate | 5 58 $10^3$ | 5 08 $10^4$ | 1 06 $10^4$ | 4 8 | 9 1 | 7 1 | 5 5 |
| DEAE-Sephadex column eluate | 7 95 $10^2$ | 9 0 $10^3$ | 1 16 $10^2$ | 80 | 17 0 | 13 0 | 0 9 |
| Hydroxylapatite eluate | 28 5 | 1 86 $10^3$ | 0 0 | | 65 3 | 50 | 0 2 |

* Refers to lipoxygenase activity at pH 6 8

bulk of the pH 6 8 activity was found in Tubes 106–130, with the peak at Tube 114 Good separation from the pH 9 0 enzyme (Theorell enzyme) was achieved since the latter enzyme requires a high buffer concentration for elution, as shown below

To further purify the pH 6 8 enzyme the combined fractions (Tubes 106–130) were placed on a hydroxylapatite column (5 5 cm × 5 5 cm) which had previously been washed with 0 01 M phosphate, pH 6 8 Elution was performed with 500 ml of 0 1 M phosphate buffer followed by an increasing linear gradient established between 1 l of 0 1 M phosphate buffer (pH 6 8) and 1 l of the same buffer at 0 3 M Fractions of 18 ml were collected Tubes 65–71 which contained material of high specific activity were retained for the comparative studies with the pH 9 0 enzyme (Theorell enzyme) The purification is summarized in Table I

*Chromatographic separation of the lipoxygenase-1 and lipoxygenase-2*

For convenience in discussion the purified enzymewas designated lipoxygenase-2 while the term lipoxygenase-1 was retained for the lipoxidase originally described by THEORELL *et al.* [4]

The procedure described above depends on the difference in behavior between lipoxygenase-1 and lipoxygenase-2 on DEAE-Sephadex chromatography Since no effort was made to separate and identify lipoxygenase-1 in the preparative procedure, additional results are shown which illustrate the sharp separation of lipoxygenase-1 and lipoxygenase-2 and hence their non-identities (Fig 1) The first peak corresponds to the lipoxygenase-2, the second to lipoxygenase-1 The identity of the second peak with lipoxygenase-2 was established by comparison with the behavior of the pure enzyme on the bases of DEAE-Sephadex chromatography, disc gel electrophoresis and pH activity profile In the studies reported below pure lipoxygenase-1 (see *Materials*) was used

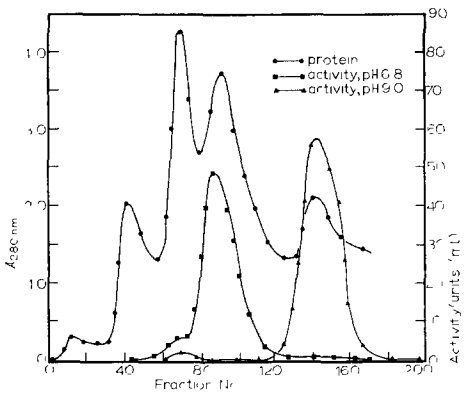


Fig 1 DEAE-Sephadex chromatography of crude soybean lipoxidase A column (4 25 cm 80 cm) was packed with DEAE-Sephadex A50 and equilibrated with 0 01 M sodium phosphate buffer, pH 6 8 Enzyme solution was applied in the same buffer and elution performed (40 ml/h) with a linear gradient of sodium phosphate buffer, pH 6 8 (0 01–0 30 M) at 4° Fractions of 20 ml were collected and protein content was measured by absorbance at 280 nm Enzyme activity was determined as described, by *Method I*

*Disc gel electrophoresis*

The purified lipoxygenase-2 revealed on disc gel electrophoresis only one protein band, $R_F$ 0 25 As seen in Fig 2, this protein is electrophoretically distinct from the Theorell enzyme (lipoxygenase-1) which has an $R_F$ of 0 34

*Substrate specificity*

The two enzymes were compared on an equal protein basis for their relative ability to utilize linoleic acid, methyl linoleate, and trilinolein as substrates using the assay conditions *(Method II)* of KOCH *et al* [10] in order to permit comparison with the results they obtained with partially purified preparations The results are summarized

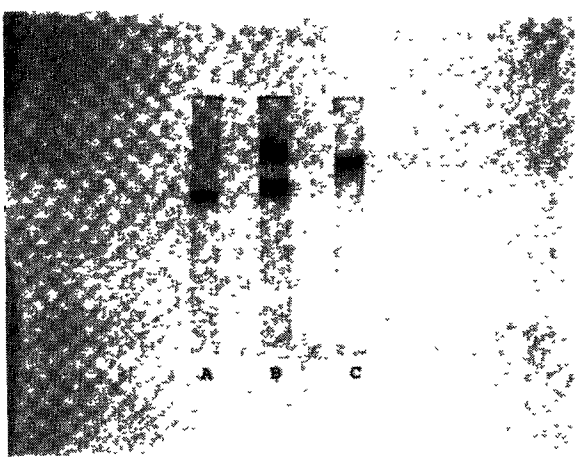


Fig 2 Polyacrylamide gel electrophoresis of the two isozymes Electrophoresis was carried out in 7% polyacrylamide gel for 2 h at 6° and 2–3 mA per tube A 56 μg of lipoxygenase-1 B 56 μg of lipogenase-1 and 48 μg of lipoxygenase-2 C 64 μg of lipoxygenase-2

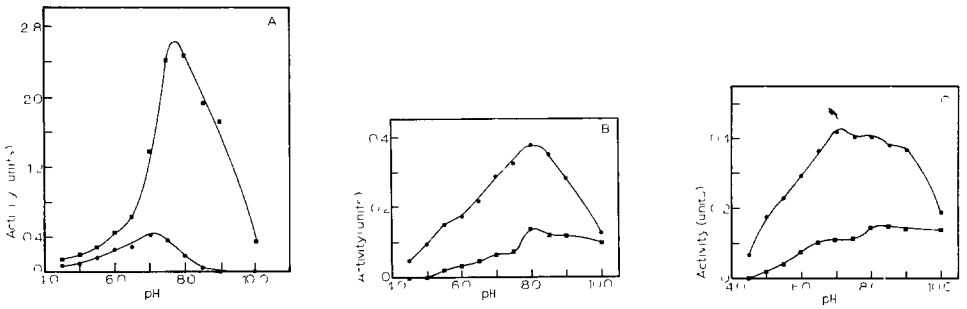


Fig 3 Lipoxygenase-1 (■—■) and lipoxygenase-2 (●—●) activities as function of pH using three different substrates under conditions of KOCH *et al* [10] Equal amounts (16 4 μg) of each enzyme were used as in *Method II* A Linoleic acid B Methyl linoleate C Trilinolein

in Fig 3 Throughout the pH range 4 5–10 0, lipoxygenase-1 is more active towards linoleic acid than is lipoxygenase-2 However, when either methyl linoleate or trilinolein is used, the new enzyme proved to be more active than the Theorell lipoxygenase throughout the same range

The buffer systems employed above resulted in varying ionic strength throughout the pH range studied Since it was known that the lipoxygenase activities were sensitive to ionic strength, pH-activity profiles for lipoxygenase-1 and lipoxygenase-2 were obtained at constant ionic strength Linoleate was employed as substrate, using Tween 20 as a dispersing agent *(Method III)* Lipoxygenase-1 exhibited an optimum at pH 9 5 while lipoxygenase-2 showed an optimum at pH 6 6 but had no detectable activity at pH 9 5 (Fig 4)

*Heat stability*

Difference in heat stabilities further differentiated the two enzymes Activities were measured at the pH optimum of each enzyme Lipoxygenase-1 had a half-time of survival of 25 min at 69°, while lipoxygenase-2 was at least 36 times less stable,

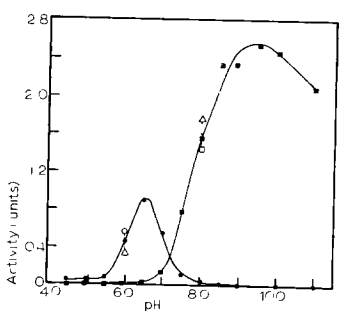


Fig 4 Action of Lipoxygenase-1 (■—■) and lipoxygenase-2 (●—●) on linoleic acid as function of pH, at constant ionic strength *Method III*, with 16 4 μg of pure enzyme per test, was employed Acetate buffer employed at pH 6 5 and below, phosphate, pH 6 5–8 0, borate, pH 8 0 and above Average values for activity were plotted in regions of overlapping buffers At pH 6 5 ○, acetate, ◿, phosphate, at pH 8 0 △, phosphate, □, borate

having a half time of 0 7 min or less under the same conditions The enzymes were present at a concentration of approx 0 3 mg/ml in 0 1 M phosphate buffer, pH 6 8

## DISCUSSION

A fairly simple procedure has been described for the isolation of a second isozyme of soybean lipoxidase The enzyme (lipoxygenase-2) which appears to be homogeneous by disc gel electrophoreses, has been shown to be distinct from the species of lipoxygenase, which was first isolated and crystallized by THEORELL *et al* [4] Lipoxygenase-2 shows a more acidic pH optimum in the oxidation of linoleic acid, is far less heat stable, and differs in its mobility in disc gel electrophoresis and its elution from DEAE-Sephadex

KOCH *et al* [10] obtained partially purified fractions from soybean extracts, which they tested against linoleic acid and trilinolein at various pH values They interpreted their results as indicating the existence of a "fatty acid (or linoleic acid) lipoxidase" and a "triglyceride lipoxidase", despite the evidence of some overlap While our results with the Theorell and the purified lipoxygenase may appear to correspond roughly to the classification of "linoleic acid" lipoxidase and "trilinolein" lipoxidase, respectively, it is clear that each of the pure enzymes are active on both substrates The relative activities of the enzymes on the two substrates are a function of pH

There seems no reason to assume the existence of a specific "linoleic acid" lipoxygenase and a "trilinolein" lipoxygenase, although we would not wish to exclude the possibility of additional isozymes, especially in view of the electrophoretic results of GUSS *et al* [11]

The molecular weight of lipoxygenase-1 was shown by THEORELL *et al* [4] to be 102 000 SCHROEDER[12] confirmed this value by means of exclusion chromatography on Sephadex G-150 We found that when a mixture of lipoxygenase-1 and lipoxygenase-2 was subjected to exclusion chromatography the elution peaks for the two enzymes coincided within experimental error Therefore assuming approximately the same molecular weight for both enzymes and making comparisons at the most favorable pH for each enzyme (Fig 4), lipoxygenase-1 is about 2 5 times as effective as lipoxygenase-2 in peroxidizing linoleic acid The respective turnover numbers are 15 800 and 6500 moles of substrate $min^{-1}$ $mole^{-1}$

## REFERENCES

1 R M BOHN AND L W HAAS, cited in J B SUMNER AND R J SUMNER, *J Biol Chem*, 134 (1940) 531
2 E ANDRE AND K HOU, *Compt Rend*, 194 (1932) 645
3 J B SUMNER AND R J SUMNER, *J Biol Chem*, 134 (1940) 531
4 H THEORELL, R T HOLMAN AND Å ÅKESON, *Acta Chem Scand*, 1 (1947) 571
5 G N SMITH, *Arch Biochem Biophys*, 19 (1948) 133
6 J B SUMNER AND A L DOUNCE, *Enzymologia*, 7 (1939) 130
7 R T HOLMAN, *Arch Biochem Biophys*, 15 (1947) 403
8 M W KIES, *Federation Proc*, 6 (1947) 267
9 M W KIES, J L HAINING, E PISTORIUS, D H SCHROEDER, AND B AXELROD, *Biochem Biophys Res Commun*, 36 (1969) 312
10 R B KOCH, B STERN AND C G FERRARI, *Arch Biochem Biophys*, 78 (1958) 165
11 P L GUSS, T RICHARDSON AND M A STAHMANN, *Cereal Chem*, 44 (1967) 607

12 D H SCHROEDER, *Some Properties of Soybean Lipoxidase*, Ph D Thesis, Purdue University, West Lafayette, Ind , 1968
13 C D HODGMAN, *Handbook of Chemistry and Physics*, Chemical Rubber, Cleveland, Ohio, 36th ed , 1954–1955, p 1610
14 K SURREY, *Plant Physiol* , 39 (1964) 65
15 B J DAVIS, *Ann N Y Acad Sci* , 121 (1964) 404