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Partial Purification of a Lipoxygenase from Apples

In-Sook Kim and Werner Grosch*

A membrane-bound lipoxygenase (EC 1.13.11.12) was partially purified from apples by differential centrifugation and gel chromatography. The enzyme had a pH optimum at 6.0 and converted linoleic acid predominantly into the 13-hydroperoxyoctadeca-9,11-dienoic acid. Reversible inhibition was obtained with ethylenediaminetetraacetic acid disodium salt and cyanide. Hemoproteins were not involved in the lipid peroxidation activity.

According to a proposal of Tressel et al. (1970) a lipoxygenase-catalyzed formation of hydroperoxides and a subsequent cleavage by an "aldehyde lyase" in apples is the pathway involved in the breakdown of unsaturated fatty acids to volatile carbonyl compounds like hexanal and 2-*trans*-hexenal. In agreement with the first postulated reaction, we recently found (Grosch et al., 1977) that a homogenate of apples is highly specific in peroxidizing linoleic acid to the 13-hydroperoxyoctadeca-9,11-dienoic acid (13-LOOH). In this paper we present further evidence for the occurrence of a lipoxygenase (linoleate:oxygen oxidoreductase; EC 1.13.11.12) in apples.

EXPERIMENTAL SECTION

Materials and Reagents. The following materials were used: apples ("Golden Delicious" from a local market), linoleic acid, and linolenic acid (99%, Nu Chek Prep), dithioerythritol (Sigma), Tween 20 (Schuchardt), ethylenediaminetetraacetic acid disodium salt (EDTA; Merck), Sepharose CL 2 B (Pharmacia), and Triton X-100 (Serva). The other chemicals were of analytical grade.

Methods. *Determination of Lipoxygenase Activity.* The linoleate peroxidation was assayed at 25 °C by two different techniques. Method I: The oxygen consumption was determined polarographically with a Clark oxygen electrode according to Delieu and Walker (1972). The apparatus was purchased from Bachofer, Reutlingen (Germany). Unless otherwise stated incubation mixtures contained 0.64 mM linoleic acid, 0.2 μL/mL of Tween 20 and 2 mL of 0.1 M sodium acetate buffer (pH 6.0). The mixture was stirred continuously, and after attaining equilibrium at 25 °C, enzyme solution was injected. Enzyme activities were calculated from initial rates of O_2 uptake, assuming an initial dissolved oxygen concentration of 0.25 μmol/mL. One unit of lipoxygenase catalyzed the uptake of 1 μmol of O_2/min. Method II: From the substrate described unter method I, 3 mL was pipetted into a 1-cm silica glass cuvette. After addition of the enzyme solution, the change in absorbance at 234 nm was measured.

Protein Determination. During chromatography protein was measured by the absorbance at 280 nm. For more accurate measurements the method of Lowry et al. (1951) with bovine serum albumin as standard was used.

Detection of Hematin. Using the method of Hartree (1955), the Sepharose fraction (1.2 mg of protein) was treated with 3 mL of a reagent containing 0.1 N NaOH, pyridine, and sodium dithionite. The visible spectrum was recorded between 400 and 650 nm.

Determination of the Hydroperoxide Isomers. *Fatty Acid Emulsion.* Ten milligrams of linoleic acid was (with the addition of 8 μL of Tween 20 and some drops of 1 N NaOH) dissolved in 10 mL of H_2O. The solution was diluted to 50 mL with 0.1 N sodium acetate buffer (pH 6.0). The pH of the emulsion was corrected to 6.0 with dilute HCl.

Incubation. The combined fractions from the Sepharose column with lipoxygenase activity (7 units) were added to the fatty acid emulsion after degassing with O_2 (5 min). The reaction mixture was stirred for 20 min at 25 °C. Immediately after incubation the reaction mixture was acidified (pH 4.0) with diluted HCl and extracted twice with 50 mL of diethyl ether. The diethyl ether solution was washed twice with 30 mL of water, dried over Na_2SO_4, and concentrated to 27 mL. After addition of 3 mL of methanol, the fatty acids were methylated with diazomethane as described by Schlenk and Gellerman (1960). For purification the methyl esters were chromatographed on silica gel HF_{254} by developing with isooctane/diethyl

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Table I. Partial Purification[a]

enzyme preparation	vol, mL	total protein, mg	total activity, unit	sp act., units/mg of protein
1. supernatant of the centrifugation at 10^4g	20	70	3.7 (3.8)[b]	0.053
2. residue of the centrifugation at $(1.35 \times 10^5)g$	2	20	8.2	0.410
3. Sepharose fraction	18	5.2	9.0	1.730

[a] The values quoted were obtained during purification of 17 g of apples. [b] The value in brackets was measured with an extract which contained no EDTA.

ether (7:3, v/v). The monohydroperoxides were located on the plates by ultraviolet absorption, scraped off, and eluted with diethyl ether. After removing the solvent in a stream of nitrogen, the residue was dissolved in some hexane containing 0.7 vol % ethanol. The ratio of the 9- and 13-hydroperoxides was estimated by high-performance liquid chromatography according to Chan and Prescott (1975). The system was calibrated with a mixture of 9- and 13-hydroperoxides produced with soybean lipoxygenase as described earlier (Grosch, 1977).

Partial Purification of the Lipoxygenase. All steps were performed at 0–2 °C. After washing the apples, they were cut into small pieces. The pieces (50 g) were soaked in 30 mL of 0.1 M Tris-HCl buffer (pH 7.0) containing 2 mM EDTA, 4 mM dithioerythritol, and 0.2% Triton X-100 and then homogenized with an Ultra-Turrax three times for 10 s. The slurry obtained was filtered through two layers of cheesecloth and centrifuged at 10000g for 30 min. The pellet was discarded and the supernatant centrifuged at 135000g for 1 h. The pellet, suspended in 7 mL of Tris-HCl buffer (pH 7.0), containing 4 mM dithioerythritol and 0.2% Triton X-100, was homogenized with a Potter-Elvehjem. After centrifuging at 5000g for 20 min, 2.0 mL of the supernatant was chromatographed on Sepharose CL 2B column (1.5 × 45 cm), which was equilibrated and eluated with a 0.1 M Tris-HCl buffer (pH 7.0). Fractions of 3 mL were collected and assayed for protein by absorption at 280 nm and for lipoxygenase activity. For convenience the combined eluate containing the partially purified lipoxygenase activity was designated "Sepharose fraction".

RESULTS AND DISCUSSION

Paritial Purification. As shown in Table I the enzyme was isolated by two centrifugation steps which are generally carried out for the separation of the microsomal fraction from other components of plant cells. After homogenization of the microsomal fraction further purification of the lipoxygenase is possible by chromatography on a Sepharose CL 2B column. Figure 1 indicates that a fraction with lipoxygenase activity is eluted with the void volume in the range of 40 × 10^6 daltons. From this result it may be concluded that the enzyme is associated with membrane fragments of high particle weights.

During the first two stages of purification the lipoxygenase activity is stabilized by EDTA and dithioerythritol. In the absence of these substances the total activity of the 10^4g supernatant decreases by 10%, that of the residue of the $(1.35 \times 10^5)g$ centrifugation by 30–50%. The stabilizing effect of EDTA is remarkable since experiments described below show that the lipoxygenase activity of the Sepharose fraction is reversibly inhibited by EDTA. EDTA, Triton X-100, and dithioerythritol are separated from lipoxygenase by chromatography on Sepharose.

Hematin was not detected in the partially purified lipoxygenase preparation in the range of ca. 0.1 μg of hematin in 1 mg of protein (Hartree, 1955). Thus the possibility that hemoproteins are involved in the lipoxygenase reaction catalyzed by the apple enzyme can be discounted.

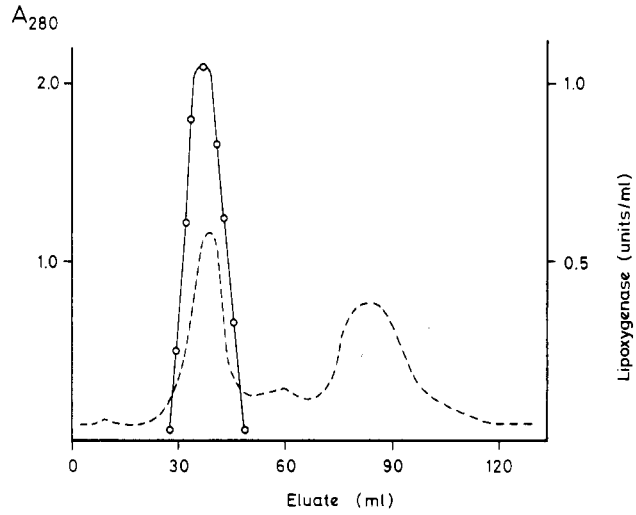

Figure 1. Gel filtration on Sepharose CL 2B. Sepharose column (1.5 × 45 cm) equilibrated with 0.1 M Tris-HCl buffer (pH 7.0); 20 mg of protein dissolved in the same buffer was applied. Elution (15 mL/h) was performed with 0.1 M Tris-HCl buffer (pH 7.0): (---) protein; (O–O) lipoxygenase activity at pH 6.0. Blue Dextran ($2 \cdot 10^6$ daltons) was eluted with V = 69 mL.

Table II. Influence of Apple Components on the Lipoxygenase Activity of the Sepharose Fraction

reaction system		
exp. substrate	Sepharose fraction (protein), μg	activ., μmol of O_2/min
1. linoleic acid (1.28 μmol) and Tween 20 (0.4 μL) dissolved in 2 mL of 0.1 M acetate buffer (pH 6.0)	50	0.084
2. linoleic acid (1.28 μmol) and Tween 20 (0.4 μL) dissolved in 1 mL of 0.1 M acetate buffer (pH 6.0) and 1 mL of 10^4g supernatant[a] of the apple homogenate	50	0.064

[a] The 10^4g supernatant was heated at 50 °C for 10 min and after cooling the pH was adjusted to 6.0. A control experiment indicated that the buffer of the supernatant does not influence the lipoxygenase activity of the Sepharose fraction.

During the purification the specific lipoxygenase activity increases 33-fold (Table I). It is surprising that the total lipoxygenase activity of the Sepharose fraction is higher than that of the 10^4g supernatant of step 1. In order to

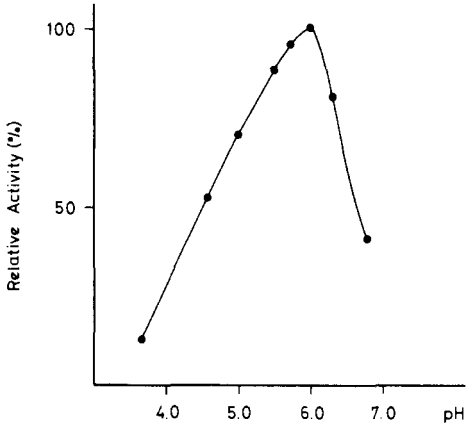

Figure 2. pH response curve for lipoxygenase activity. The lipoxygenase assay was carried out by method I. The following buffer solutions were used: sodium acetate (pH 3.5–6.5), triethanolamine·HCl/NaOH (pH 6.5–8.0).

Table III. Stability of the Lipoxygenase

storage at −20 °C	activity[a] related to the fresh preparation, %
24 h	70
48 h	70
5 days	70

[a] After thawing at 2–4 °C the activity was measured with method I.

study the reason for this difference the activity of the Sepharose fraction was measured in the presence of the heated 10^4g supernatant of an apple homogenate (without EDTA). The results listed in Table II indicate that about 25% of the partial purified lipoxygenase is inhibited by components of the apple. From these results we have to assume that at least a part of the rise in total enzyme activity is due to the separation of inhibitors during purification of the lipoxygenase.

The Sepharose fraction was incubated with various concentrations of a 9-/13-LOOH mixture (25:75) prepared as described earlier (Grosch, 1977). No breakdown of the hydroperoxides at different pH values was detectable. The results indicate that the enzyme preparation was free of a hydroperoxide cleaving factor.

Properties of the Partially Purified Enzyme. The lipoxygenase activity of the Sepharose fraction is higher in the presence of a detergent. For 0.64 mM linoleic acid a maximum activity is observed in the presence of 0.2 μl/mL of Tween 20.

The pH response curve (Figure 2) shows an optimum at pH 6.0. At the pH of apple pulp (3.6), lipoxygenase activity is only 12% of the maximum activity.

A plot of the protein concentration vs. diene formation was linear up to 200 μg of protein (data not shown). In 0.1 M acetate buffer pH 6.0, the enzyme lost 50% activity in 5 min at 40 °C and was completely inactivated by treatment at 50 °C for 10 min. The frozen enzyme was relatively stable; an ca. 30% loss of activity is observed after thawing, however (Table III).

The substrate specificity of the Sepharose fraction shows a preference for linolenic acid as compared to linoleic acid (Table IV).

The monohydroperoxides resulting from the oxidation of linoleic acid by the partially purified enzyme were isolated and analyzed by high-pressure liquid chromatography. The chromatogram (Figure 3) shows that the apple lipoxygenase predominantly catalyzes the formation

Table IV. Substrate Specificity[a]

substrate	relative lipoxygenase activity, %
linoleic acid	100
linolenic acid	122

[a] The lipoxygenase activity of the Sepharose fraction was measured by method II.

Table V. Inhibition Experiments[a]

inhibitor	relative lipoxygenase activity, % substrate with inhibitor[b]	substrate without inhibitor
without		100
EDTA 10^{-5} M	82	
10^{-4} M	54	90
10^{-3} M	33	
KCN 10^{-5} M	88	
10^{-4} M[c]	70	95
10^{-3} M[c]	58	

[a] Tubes containing the Sepharose fraction and the inhibitor were placed in an ice bath for 10 min before measuring the lipoxygenase activity with method II. [b] The substrate of the lipoxygenase assay contained the same inhibitor concentration as the preincubation system. [c] The pH was adjusted to 6.0 with dilute HCl.

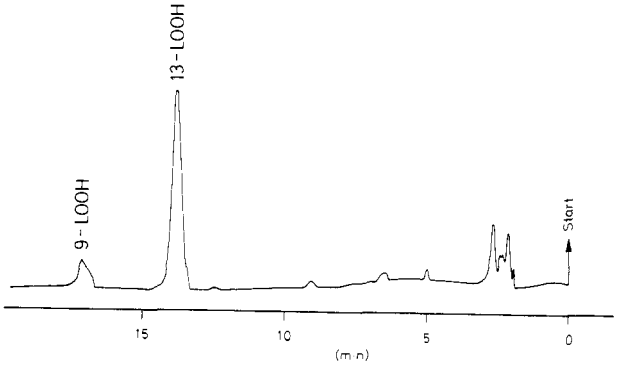

Figure 3. High-performance liquid chromatography of methyl esters of 9- and 13-hydroperoxides obtained from incubating linoleic acid with the Sepharose fraction.

of 13-hydroperoxyoctadeca-9,11-dienoic acid. It is possible that the small amount of the 9-isomer formed is due to some autoxidation during incubation and extraction procedures. The specificity of the partially purified enzyme agrees with an earlier report (Grosch et al., 1977) concerning apple homogenate.

The lipoxygenase activity of the Sepharose fraction is inhibited by EDTA and cyanide, EDTA being more effective (Table V). Preincubation of the Sepharose fraction in the presence of inhibitor but in the absence of the substrate yields considerably less inhibition (Table V). This difference suggests that the inhibition by EDTA and cyanide is reversible. The inhibition experiments furthermore provide evidence that a metal ion is involved in the catalysis of lipid peroxidation. This assumption is in agreement with the findings of other groups (Chan, 1973; Pistorius and Axelrod, 1974).

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The following paper, presented at the Symposium on Lipoxygenase: Its Biochemistry, Products, and Role in Plant and Animal Chemistry, will appear in the next issue: "Recent Progress in the Study of Soybean Lipoxygenase", by J. F. G. Vliegenthart, G. A. Veldink, and J. Boldingh. Other papers presented at the Symposium but not printed in this issue are as follows: "Characterization and Occurrence of Cyclic Fatty Acids Produced by Plant Extracts", by D. Zimmerman, P. Feng, and B. Vick; "Lipoxygenase-Like Enzyme from Rat Testes Microsomes—Purification and Partial Characterization", by S. Grossman and I. Shahin; "Analysis of Lipid Oxidation Products by High-Performance Liquid Chromatography", by J. A. Singleton and H. E. Pattee.

REVIEW

Review of Quantitative Analyses of Citrus Essential Oils

Philip E. Shaw

A compilation of reported quantitative values for individual components of cold-pressed oils of sweet orange, grapefruit, mandarin, lemon, lime, bitter orange, bergamot, certain hybrid oils, and of distilled lime oil is presented. Different analytical methods used to determine these values are compared. Reasons for differences in quantitative values determined by gas chromatography (GLC) are ascribed to method of preliminary separation, method of calculating relative percent composition, type of column or detector used, decomposition during GLC separation, and sample history. Valid conclusions regarding chemotaxonomy of hybrids cannot be made from the available data because of variable sample histories and analytical techniques.

Quantitative values on a few individual components of citrus essential oils have been reported over many years, but it was not until the widespread use of gas chromatography that meaningful quantitative values for many components of each citrus essential oil became available. Attempts to correlate the presence of single components with the characteristic flavor of each fruit have been partly successful, but we now realize that several components are blended together in a specific proportion to create the unique full flavor of oil from each citrus species or hybrid (Braddock and Kesterson, 1976; Shaw, 1977, and references therein). Thus, the accuracy of the quantitative values that have been reported for each component becomes critical when we determine the mixture of components necessary for full citrus flavors, create synthetic or partially synthetic blends with flavor properties characteristic of each particular fruit, and study the chemotaxonomy of citrus hybrids.

Several reviews on quantitative analyses of citrus essential oils have been reported. Kefford and Chandler (1970) summarized the quantitative data available to them, but included data on terpeneless oils and individual fractions as well as total oil without relating quantities in various fractions to the level present in the total peel oil. Shaw (1977) supplemented the data of Kefford and Chandler by reporting quantitative values determined on a single sample of whole oil for each species of citrus so that the relative properties of components present would be more meaningful. In neither of these studies were all reported values for each component tabulated so that on a comparable basis the most meaningful values might be selected. No attempt to critically review the analytical methods to aid in selection of best probable quantitative values was made in either study.

The present review tabulates quantitative values for individual compounds as percent of total oil and compares

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