DOUBLE DIOXYGENATION OF ARACHIDONIC ACID BY SOYBEAN LIPOXYGENASE-1*

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SUMMARY: Soybean lipoxygenase-1, has been found to catalyze the incorporation of two oxygen molecules into arachidonic acid. The product appears to be 8,15 dihydroperoxy-5,9,11,13-eicosatetraenoic acid. This is apparently the first report of the enzymatic production of a conjugated aliphatic triene in vitro.

Lipoxygenase is an enzyme which occurs in many plants and has recently been reported to be in thrombocytes (1). It catalyzes the addition of molecular oxygen to polyunsaturated fatty acids containing the cis-cis-1,4-pentadiene system. Soybeans contain several isoenzymic forms of the enzyme (2). One of these, lipoxygenase-1, catalyzes the formation of the 13-hydroperoxy isomer from linoleic acid. Lipoxygenase has been reported to add oxygen only to the ω-6 position of arachidonic acid to produce 15-hydroperoxy-5,8,11,13-eicosatetraenoic acid (3). This communication reports the identification of a new reaction product of lipoxygenase-1 acting on arachidonic acid. By utilizing lipoxygenase-1 at relatively high enzyme concentrations, arachidonic acid is converted into a conjugated triene in a reaction which consumes two molecules of oxygen.

MATERIALS AND METHODS

Chemicals - All fatty acids (99% purity) were obtained from NuCheck Prep, Inc. N-Methyl-N nitroso-p-toluenesulfonamide was purchased from Aldrich Chemical Company. N,N-Dimethyl-p-phenylenediamine HCl was obtained from

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Sigma Chemical Company. Thin layer chromatography (TLC) plates of silica gel G, were supplied by Analtech Inc.

Enzyme Purification and Assay - Lipoxygenase-1 which was prepared by the method of Christopher et al. (4) was used throughout this work. The enzyme was purified from Amsoy soybeans by ammonium sulfate fractionation, chromatography on DEAE-Sephadex and chromatography on hydroxyapatite. The enzyme preparation, specific activity of 180 units per mg protein, was stored as an ammonium sulfate precipitate. The enzyme was diluted in 0.2 M sodium phosphate buffer pH 6.8 before use. One enzyme unit represents the consumption of 1 μmole of oxygen per minute at 15°C with linoleic acid as substrate at pH 9.0 as defined by Christopher et al. (4). Activities were determined using a Clark oxygen electrode (Yellow Springs) in a Gilson Medical Oxygraph, model KM.

Preparation of Di-dioxygenated Product - A suspension of arachidonic acid was prepared by stirring 400 mg of the fatty acid with a small amount of water and 0.35 ml of Tween 20 and then continuing to mix while adding small increments of water until a volume of 10 ml was obtained. The suspension was made neutral with 2 N NaOH and the volume brought to 100 ml with distilled water. The reaction mixture used to obtain the product contained 180 ml of 0.2 M sodium phosphate buffer, pH 6.8, 1330 enzyme units and 10 ml of the above substrate mixture to give a final concentration of arachidonate of 6.65×10^{-4} M. Oxygen was bubbled through the reaction mixture at room temperature with constant stirring. After 10 minutes, a small aliquot of the reaction mixture was scanned in a spectrophotometer to confirm complete conversion of conjugated diene to conjugated triene.

The incubation mixture was immediately reduced with 200 mg of $SnCl_2$ dissolved in 10 ml of ethanol. The reduction was allowed to proceed for 1 hour at room temperature with constant stirring.

The reaction mixture was extracted twice with 2 volumes of ethyl acetate. The combined extract from 10 runs was evaporated to dryness under vacuum. The residue was taken up in 10% ether in hexane and loaded onto a SilicAR CC-4 column. The column was eluted successively with 200 ml portions of the following ether-hexane mixtures: 10:90, 20:80, 50:50 and finally pure ether. Only the 100% ether eluate contained significant amounts of the product. The fraction was evaporated to dryness under vacuum.

The residue was spotted on 1000μ silica gel G TLC plates and developed in chloroform-methanol-acetic acid-water (90:8:1:0.8, v/v/v/v). Material with an R_f of 0.60, visualized as a dark band under ultraviolet light, was scraped from the plate. This product was extracted in chloroform-methanol (2:1, v/v) and rerun on a second 1000 μ plate.

The material was methylated in the dark with diazomethane in methanol-ether (10:90, v/v) for 1 hour. The methylated material was taken up in chloroform-methanol (2:1, v/v) and spotted on a 250 μ TLC plate and developed as before. One major spot appeared with an R_f value of 0.77. This substance was used for mass spectral analysis.

Analysis of the product - A Perkin-Elmer Model 467 grating infra-red spectrophotometer employing a KBr cell and chloroform as solvent was used for infrared analysis. NMR analysis was carried out on a Varian T-60A spectrometer. Mass spectra were taken at 190°C on a CEC 21-110 double focusing high resolution spectrometer.

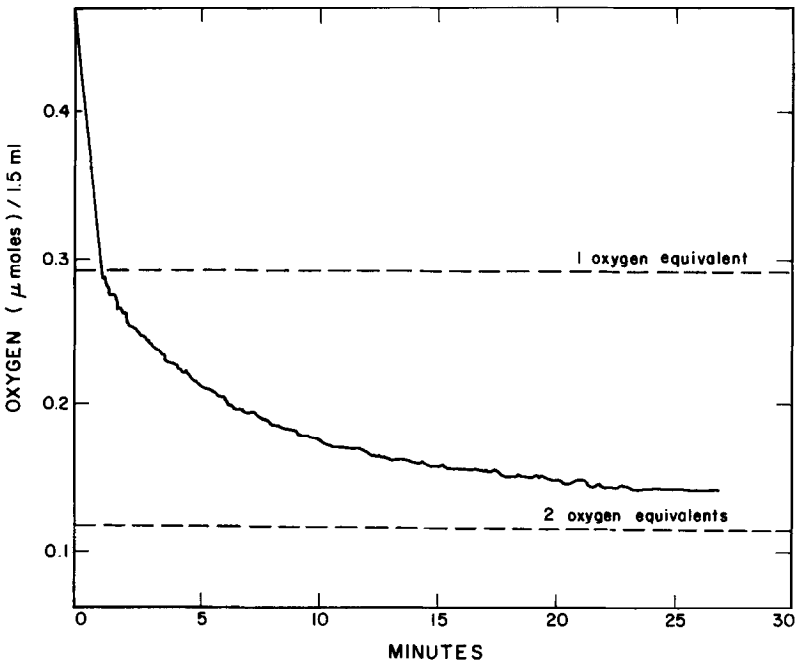


Fig. 1 Oxygraph recording of O_2 consumption in a reaction mixture containing 8.21 units/ml of lipoxygenase and a final concentration of arachidonic acid of 1.18×10^{-4} M in 0.2M sodium phosphate buffer pH 6.8 at 15°C. Total volume of reaction mixture was 1.50 ml.

RESULTS AND DISCUSSION

Measurement of oxygen consumption during the reaction of 12.3 units of lipoxygenase-1 with arachidonic acid at pH 6.8 in the Oxygraph at 15°C indicated that 1 equivalent of oxygen was consumed in less than 1 minute and that another 0.85 equivalent of oxygen was consumed during the next 20 minutes (Figure 1). When the same reaction was carried out in a scanning spectrophotometer an absorbance peak at 238 nm corresponding to conjugated diene appeared rapidly. As the reaction continued, the diene peak (238 nm) slowly disappeared while three new peaks appeared (260, 269, 279 nm) (Figure 2). This triplet corresponds to those obtained with other conjugated trienes, as, for example, that obtained by the alkaline isomerization of arachidonic acid (5), or as seen in α- or β-eleostearic acids (6). Therefore

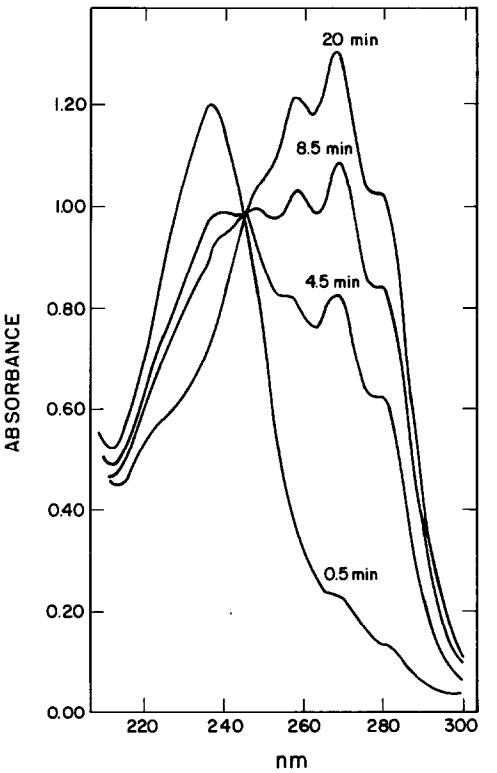


Fig. 2 Spectrophotometric scan of a reaction mixture containing lipoxygenase (5.67 units/ml) and 4.95 x 10^{-5} M arachidonic acid in 0.2M sodium phosphate buffer pH 6.8 at room temperature. Total volume was 3.0 ml.

it appears that under the conditions specified, lipoxygenase-1 rapidly adds one molecule of oxygen to arachidonic acid to produce a conjugated diene, as in the normal lipoxygenase reaction, and adds a second molecule of oxygen at a much slower rate converting the conjugated diene to a conjugated triene.

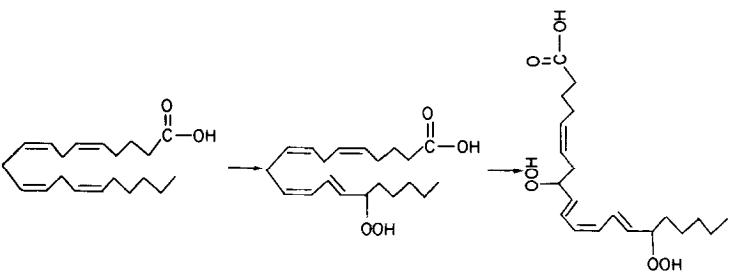
Infrared analysis of the conjugated triene after $SnCl_2$ reduction clearly showed the presence of a trans ethylenic absorption (968 cm^{-1}) and alcoholic absorptions at 1040 cm^{-1} (secondary hydroxyl) and at 3540 cm^{-1}. The NMR spectrum is indicative of an unsaturated fatty acid possessing a secondary hydroxyl group (methine proton, 4.2 ppm; hydroxyl proton, 6.3 ppm). The hydroxyl peak was not seen after the sample was treated with D_2O.

Mass spectral analysis of the methylated material produced from arachi-

donic acid revealed a molecular ion at m/e 350 which corresponds to a C-21 compound containing 4 double bonds and two hydroxyl groups. Other major peaks appeared at m/e 332.235 [$C_{21}H_{32}O_3$, M-18; loss of H_2O], 301 [M-(18+31); loss of H_2O and OCH_3], 261 [M-(18+71); loss of H_2O and C_5H_{11}], 209.157 [$C_{13}H_{21}O_2$, M-141; loss of $C_8H_{13}O_2$] and 191 [M-(141+18); loss of H_2O and $C_8H_{13}O_2$]. Thus the mass spectral analysis is compatible with two hydroxyl groups at the C-8 and C-15 positions.

The final product of the enzymatic reaction gave a positive test on TLC plates with the peroxide-specific reagent, N,N-dimethyl-p-phenylene diamine HCl (7). The somewhat imprecise quantitative test for hydroperoxides based on the oxidation of ferrous ion in the presence of thiocyanate indicated the presence of 2.5 hydroperoxide groups per molecule (8). The insertion of the second molecule of oxygen also occurs at pH 9.0, although at a considerably slower rate. The normal (monohydroperoxidation) reaction by lipoxygenase-1 is well-known to have a pH optimum of about 9.0.

From the data presented we propose that arachidonic acid is converted into 8,15 dihydroperoxy-5,9,11,13 eicosatetraenoic acid by the following series of reactions:



The IR spectrum clearly indicated the presence of <u>trans</u> double bonds. By analogy to the known structure of the product obtained from linoleic acid (9), we tentatively assume that the 9 and 13 bonds are of the <u>trans</u> configuration while the 11 is of the <u>cis</u> configuration, although this remains to be established by direct investigation. The first oxygen molecule is rapidly inserted into the ω-6 position, as has been shown by Hamberg and

Samuelsson (3). With arachidonic acid it appears that a second molecule of oxygen can be inserted in the ω-13 position, thus producing a conjugated triene system. This is the first report of the enzymatic formation of an open conjugated triene and also appears to be the first observation of lipoxygenase acting as a double dioxygenase.

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