A NEW PRODUCT OF LINOLEIC ACID OXIDATION BY A FLAXSEED ENZYME

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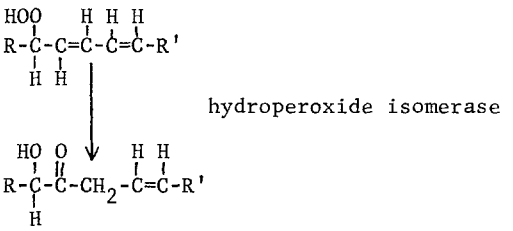
Although the products of lipoxidase activity have been known for some time, the function of this enzyme in plant metabolism is not known (Tappel, 1963). This paper reports the isolation of an enzyme from flaxseed which utilizes the product of lipoxidase as its substrate, and the tentative identification of the resultant product. The reaction catalyzed by the flaxseed enzyme is shown below.



The reaction catalyzed by the flaxseed enzyme is essentially an isomerization of an unsaturated hydroperoxide to a ketohydroxy compound. For this reason the trivial name, hydroperoxide isomerase, is suggested.

Linoleic acid was first incubated with soybean lipoxidase and then with the flaxseed preparation. After acidification and extraction, the product was identified by thin-layer chromatography (TLC), infra-red (IR) and ultraviolet (UV) spectroscopy, nuclear magnetic resonance (NMR) analysis and periodate oxidation.

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EXPERIMENTAL

Crude extracts were prepared by extracting acetone powders of ungerminated flaxseed (CI No. 980) with 0.1 M phosphate buffer, pH 7.4. Cell debris were removed by centrifugation. The crude extract was partially purified by ammonium sulfate fractionation. The fractions precipitating at 42% and 50% saturation were collected by centrifugation and redissolved in buffer. Substrates were prepared according to Surrey (1964). Linoleic acid was purchased from the Hormel Institute and soybean lipoxidase (10,000 units/mg.) from the Sigma Chemical Company.[1]

Lipoxidase activity was determined by conjugated diene absorption at 234 mμ and by peroxide content.

In a typical assay, soybean lipoxidase was incubated with linoleic acid substrate at pH 7.4 for 15 min. at 25° C. Flaxseed extract was added and the incubation continued for 15 min. The mixture was acidified, extracted with diethyl ether, dried over sodium sulfate and concentrated. All operations after the incubation were conducted under nitrogen. Thin-layer chromatography on silica gel H with petroleum ether:diethyl ether:glacial acetic acid (60:40:1) solvent completed the assay.

For preparative purposes, the lipid extract was esterified with diazomethane and then separated on a Florisil column using different amounts of diethyl ether in petroleum ether. Fractions were collected, concentrated, and assayed by TLC.

UV analyses were made with a Beckman DK-2, IR analyses with a Perkin-Elmer 337, and NMR analysis with a Varian A-60A.

Periodate oxidations were conducted on a micro scale, according to the procedure of Dixon and Lipkin (1954).

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[1] The mention of firm names or trade products does not imply that they are endorsed or recommended by the U. S. Department of Agriculture over other firms or similar products not mentioned.

RESULTS AND DISCUSSION

The crude extracts showed considerable lipoxidase activity. When 2,6-dichlorophenolindophenol was added to the reaction mixture, there was a significant decrease in the 234 mμ absorption indicating a loss of conjugated diene. This decrease did not occur with soybean lipoxidase or with a crude extract which had been placed in boiling water for 5 min. Fractionation with ammonium sulfate resulted in the separation of lipoxidase and the hydroperoxide isomerase. That fraction precipitating between 42% and 50% saturated contained isomerase activity but no lipoxidase activity.

Although extensive studies on the enzyme have not been completed, the following observations have been made. The isomerase activity is not inhibited by 2×10^{-4} M nordihydroguaiaretic acid, 10^{-4} M p-chloromercuribenzoate and 10^{-3} M potassium cyanide. The pH optimum is about 7.4. Isomerase activity is lost completely after heating for 1 min. at 70° C. The isomerase is also active with the hydroperoxides formed from linolenic acid.

Analysis of reaction mixtures on TLC showed that the major product (about 70% as evidenced by charring) had an R_f .28, and a minor product (10-30%) had an R_f .17. The R_f .28 material produced a yellow color when sprayed with an alcoholic 2,4-dinitrophenylhydrazine solution. The conjugated diene hydroperoxides produced by soybean lipoxidase (Bergstrom, 1946) gave a pink spot (R_f .43) when sprayed with N,N-dimethyl-p-phenylene diamine solution (Vioque and Holman, 1962). Linoleic acid (R_f .69) was observed by spraying with iodine solution or by charring. Esterification with diazomethane produced the corresponding methyl esters and appropriately higher R_f values.

Two experiments used radioactive substrates. The results obtained with linoleic acid-1-C^{14} and linoleic acid-U-C^{14} (Applied Science Laboratories) both showed that the conjugated diene hydroperoxide was converted to two other compounds. These corresponded with spots R_f .28 and .17 observed on TLC using non-radioactive substrates.

IR spectra were determined on the free acid and methyl ester of the keto-compound (R_f .28) in carbon disulfide solution. One of the most significant features of the IR spectra was the absence of any indication of a trans double bond at 952 cm^{-1} or 990 cm^{-1}. Since the products of soybean lipoxidase are 9-hydroperoxyoctadeca-trans-10-cis-12-dienoic acid and 13-hydroperoxyoctadeca-cis-9-trans-11-dienoic acid (Privett et al., 1955), the disappearance of the trans double bond locates the point at which isomerization occurs in the hydroperoxide molecule.

Based on the ratio of absorbance values at 3010 cm^{-1} to 2920 cm^{-1} (Sinclair et al., 1952), there was one cis double bond present. The IR data thus indicated the presence of a secondary hydroxyl group with α-unsaturation, a carbonyl group, and one cis double bond. The significant features of the IR spectra are given in Table 1.

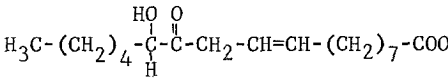
TABLE 1

Major infra-red absorption bands, cm^{-1}

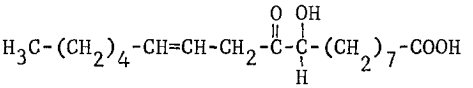
Acid	Methyl ester	Assignment
3480	3500	secondary - OH
3010	3015	C-H of cis double bond
1695	--	carboxyl C=O
--	1745	ester C=O
--	1720	ketone C=O
1070	1075	secondary - OH with α-unsat'n

All of the groupings indicated by the IR data were confirmed by the NMR spectrum. The spectrum indicated two ethylenic protons (one double bond), four α-olefinic protons, two α-carbonyl, α-olefinic protons, and one methine proton. On the basis of these results, and the structure of the products produced by soybean lipoxidase (Hamberg and Samuelsson, 1965), the following structures are proposed as the major products of the flaxseed

hydroperoxide isomerase enzyme:



12-keto-13-hydroxy octadec-cis-9-enoic acid



9-hydroxy-10-keto octadec-cis-12-enoic acid

Oxidation by periodate also suggests that the secondary hydroxy and keto groups are adjacent to each other. A keto-enol isomerism could reverse the hydroxyl and carbonyl positions, however.

The ultraviolet absorption spectrum showed a λ_{max} at 275 mμ and an inflection at 230 mμ. Molar absorptivity has not yet been determined. The IR, UV, and TLC data indicate that the product is not the keto-diene isolated by Vioque and Holman (1962). The positive determination of the location of the hydroxyl, carbonyl, and cis-double bond, as well as the nature of the R_f .17 material, is in progress.

The proposed structures may provide the answer to the function of lipoxidase. The similarity of the keto-hydroxy grouping to that of ascorbic acid, and the fact that both reduce dichlorophenolindophenol, suggest that the unsaturated keto-hydroxy acids may function in an electron transport system. Alternately, it may serve as a reactive intermediate for subsequent cleavage to provide short chain aldehydes and acids. Future work with these compounds should reveal their role in fatty acid metabolism.

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