A Simple Method for the Preparation of Pure 9-D-Hydroperoxide of Linoleic Acid and Methyl Linoleate Based on the Positional Specificity of Lipoxygenase in Tomato Fruit

ABSTRACT

Incubation of linoleic acid with crude homogenate of tomato fruit gave a high yield (69%) of linoleic acid hydroperoxides with a ratio of 9- to 13-hydroperoxide isomers of 96:4. After chromatography of the products, as free acids or methyl esters, hydroperoxides with 9- to 13-isomeric ratios of >99:1 were obtained. The major product was characterized as 9-D-hydroperoxy-octadeca-*trans*-10,*cis*-12-dienoic acid. The results demonstrate the positional specificity of lipoxygenase from tomato fruit.

INTRODUCTION

Fatty acid hydroperoxides are important intermediates in fatty acid metabolism and in lipid oxidation processes. Our studies on enzymic degradation of fatty acids and on non-enzymic lipid oxidation demanded separate preparations of pure 9- and 13-hydroperoxides of linoleic and linolenic acids.

13-L-Hydroperoxy-octadeca-*cis*-9,*trans*-11-dienoic acid can now be produced in better than 99% purity using commercial preparations of soybean lipoxygenase at high pH followed by chromatographic purification (1-4). The 9-hydroperoxide is less readily produced; but reasonably pure (95-97% of 9-isomer) preparations in moderate yield are obtained using lipoxygenases from potato (5) or corn germ (2). The present work is based on the high specificity of tomato lipoxygenase for 9-hydroperoxides and the presence of a 13-hydroperoxide-specific cleavage system in tomato fruits (6). We have been able to develop a simple method of producing high yeilds of very pure 9-D-hydroperoxides.

MATERIALS AND METHODS

Firm, red tomato fruits were peeled and, after seeds were removed, the flesh was diced and washed in H_2O at 0 C. Linoleic acid (Sigma Chemical Co., London) and [1-^{14}C] linoleic acid (Radiochemical Centre, Amersham) were mixed (sp. radioactivity: 0.03 μCi/μmole) and converted to the ammonium salt. To the diced tomato tissue (225 g) were added 750 ml of 0.1 M NaOAc buffer (pH 5.5) and ammonium linoleate (1 mM final concentration). The mixture was then homogenized (Ultra-Turrax) and incubated 15-20 min at 25 C in an open vessel. Oxygen was passed through the mixture for ca. 15 sec every 2 min. The products were extracted with chloroform and the extract was dried over Na_2SO_4. Different preparations using from 0.4 mmole to 1 mmole of linoleic acid gave similar conversion efficiencies. Analysis of the products from [^{14}C]-labelled linoleic acid, as determined by radioscanning (5), gave the following approximate thin layer chromatography (TLC) product distribution: fatty acid hydroperoxide, 70%; unreacted fatty acid, 16%; other minor products, 14%. The solvent was removed in vacuo. Subsequent treatment depended upon whether free acids or methyl esters were produced.

Free acid products were dissolved in light petroleum (b.p. 60-80 C)-diethyl ether 97.5:2.5 v/v and were separated on a column (26 cm x 1.5 cm ID) of EDTA-washed silicic acid as previously described (7). Elution was achieved by a gradient of diethyl ether in light petroleum (b.p. 60-80 C) with a mixing volume of 150 ml followed by 80 ml diethyl ether. Fractions (10 ml) were analyzed for ^{14}C-counts, and A_{234}-nm (in ethanol) components were identified by TLC (7) and, after methylation, by high performance liquid chromatography (HPLC) (3) which also determined the ratio of 9 to 13 isomers present in the fractions.

For the preparation of pure methyl hydroperoxydienoates, the chloroform-soluble incubation products were treated with CH_2N_2 and the esters were separated on a column (15 cm x 1.5 cm) of "Hiflosil" (Applied Science Laboratories, Inc, State College, PA) (8).

The column was eluted with light petroleum (b.p. 67-70 C)-diethyl ether mixtures 90:10 v/v (100 ml) followed by 80:20 v/v (100 ml). Fractions (8 ml) were collected and those giving a positive peroxide spot test (8) were analyzed by A_{234nm} (in ethanol) and by HPLC (3).

RESULTS AND DISCUSSION

Free 9-hydroperoxyoctadecadienoic Acid Preparation

When the products formed from 140 mg (13.5 μCi) of [1-^{14}C]linoleic acid were separated on a silicic acid column, early fractions from the gradient elution (30-120 ml) con-

tained unreacted linoleic acid (16% of total radioactivity) and traces (<1%) of a component, tentatively identified as a conjugated ketodienoic acid. Later fractions of the gradient (120-150 ml) and early fractions of the diethyl ether eluate (0-40 ml) contained only hydroperoxydienoic acid (69%). Subsequent fractions contained traces (1%) of hydroperoxide mixed with hydroxydienoic acid (5%) followed by unidentified polar products (9%). When uncontaminated fractions were combined, 99.7 mg of pure hydroperoxydienoic acid were obtained with a 9-:13-isomeric ratio of 96:4. However, as expected (2), fractions on the front of the peak contained relatively higher proportions of the 13-hydroperoxide isomer. Successive 10 ml fractions across the peak gave the following 9-:13-isomeric ratios and yields of hydroperoxide: 78:22 (6 mg), 95:5 (33 mg), 98:2 (22.2 mg), 99:1 (22.0 mg), >99:1 (9.0, 3.8, and 1.5 mg). Hydroperoxydienoic acid with a very high (>99:1) proportion of the 9-isomer was obtained at the expense of reduced yield (36.3 mg) by combining only those fractions with 9-:13- isomeric ratios ≥99:1.

Methyl 9-hydroperoxyoctadecadienoate Preparation

The methylated products from 50 mg linoleic acid gave an analogous isomeric ratio distribution across the hydroperoxide peak eluted from a silicic acid column. Successive 8 ml fractions gave the following 9-:13- isomeric ratios and yields: 60:40 (0.4 mg), 84:16 (2.1 mg), 94:6 (2.8 mg), 97:3 (5.5 mg), 98:2 (4.7 mg), 99:1 (2.6 mg), and >99:1 (1.4 mg). Combination of fractions with isomeric ratios ≥ 94:6 gave 17 mg (30% yield) of methyl hydroperoxydienoate containing 98% of the 9-hydroperoxy isomer.

Identification of the Hydroperoxydienoic Acid Product

The major product cochromatographed in HPLC with an authentic sample of the methyl ester of '9-D-hydroperoxide' (5). The 9-D-hydroperoxyoctadeca-*trans*-10,*cis*-12-dienoic acid obtained gave the following analyses as methyl ester. The molar ratio of peroxide (9) to conjugated diene (ϵ_{234} = 26,000) was 1:1.03; the infrared spectrum (CCL_4) had absorption bands at 951 and 989 cm^{-1} (*cis, trans*-conjugated diene); and the nuclear magnetic resonance spectrum was essentially identical with that of methyl 13-hydroperoxyoctadeca-*cis*-9-*trans*-11-dienoate (10) (*trans* double bond adjacent to the hydroperoxide group). The optical rotation of the hydroxy derivative obtained by $NaBH_4$ reduction gave $[\alpha]_D^{25}$ = +4.7° (3.1% in hexane), similar to published values (11-13). The methyl hydroxystearate, prepared and analyzed as previously (3), gave major fragment ions at m/e values of 155, 158, and 187 (characteristic of methyl 9-hydroxystearate). The corresponding characteristic ions of the 13- hydroxy isomer at m/e = 211, 214 and 243 were present in only minor amounts (<5% of the analogous 9-isomer fragments), confirming the HPLC data on isomeric distribution.

This work demonstrated that good yields of high purity 9-D-hydroperoxide of linoleic acid can be obtained from readily available materials and without prior enzyme purifications. The high proportion of 9-hydroperoxide isomer produced in this system is due to the previously unrecognized positional specificity of tomato lipoxygenase and to the presence in the tomato fruit of a hydroperoxide cleavage enzyme that is highly specific for the 13-hydroperoxide, thus selectively removing this isomer from the incubation system (6). A further advantage of the present method is that high yields of hydroperoxide product are obtained. It appears that the product inhibition usually experienced with lipoxygenases (14) is less marked in the crude tomato extracts.

Preliminary results with linolenic acid have shown that the method is also applicable to the preparation of the analogous 9-hydroperoxytrienoic acid. We do not know to what extent the small amount of 13-hydroperoxide in our preparations are due to residual products of enzyme activity or to autoxidation and isomerization (8) processes during the incubation and extraction procedures. Preparations of hydroperoxy acids and of their methyl esters are stable for several days in the dark at -20 C as dilute solutions in hexane-diethyl ether. A slow isomerization of 9- to 13-isomers occurs over a period of weeks. Isomerization is accelerated at higher concentration and temperature (8).

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