OXYGEN TRANSFER IN THE ENZYMATIC CONVERSION OF ^{18}O-LABELLED LINOLEIC ACID HYDROPEROXIDE INTO THE 12-KETO-13-HYDROXY-OCTADEC-*CIS*-9-ENOIC ACID

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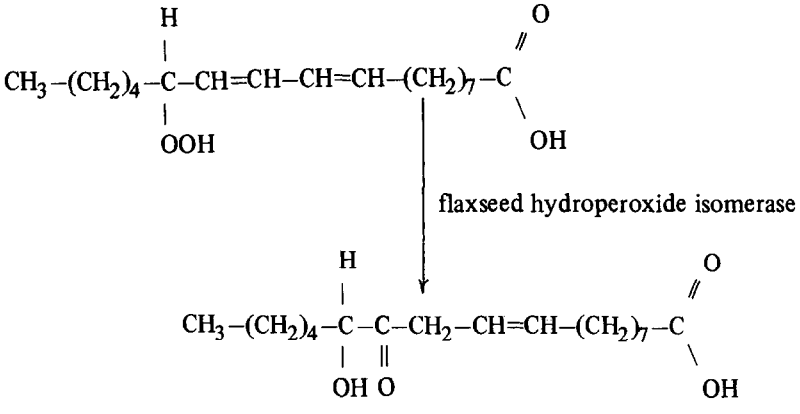
1. Introduction

Lipoxygenase (E.C.1.13.1.13) catalyses the formation of hydroperoxides of unsaturated fatty acids containing a *cis, cis*-1,4-pentadiene system. Zimmerman [1] demonstrated, that flaxseed contains an enzyme which is able to convert, *in vitro*, such hydroperoxides into mono-unsaturated α-ketols. Veldink et al. [2] found that of the two hydroperoxides formed by lipoxygenase from linoleic acid, only 13-hydroperoxy-*cis*-9-*trans*-11-octadecadienoic acid is converted into an unsaturated α-ketol (see scheme 1).

9-Hydroperoxy-*trans*-10-*cis*-12-octadecadienoic acid remains unchanged, although *in vitro* this substance is enzymatically formed by lipoxygenase [3].

To gain insight into the mechanism of the isomerisation reaction, we studied the conversion of linoleic hydroperoxides labelled with ^{18}O in the peroxy group. Holman [4] and Dolev [5] have shown that during the reaction of lipoxygenase with a suitable unsaturated fatty acid, one mole of atmospheric oxygen is consumed per mole fatty acid. In our experiments, ^{18}O-labelled linoleic acid hydroperoxides could easily be obtained by performing the lipoxygenase reaction in an $^{18}O_2$-atmosphere. We used the labelled hydroperoxide mixture as a substrate for the flaxseed enzyme. The saturated diol fraction which could be obtained after reduction, esterification and hydrogenation of the reaction product, was investigated by mass spectrometry. It became clear that, in the mono-unsaturated α-ketol, surprisingly only the oxygen atom of the carbonyl group stems from the hydroperoxy group.



Scheme 1

2. Materials

Lipoxygenase was obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio, U.S.A. (activity 8000 units/mg). Linoleic acid (purity > 98%) was a gift of the Unilever Research Laboratory, Vlaardingen/Duiven, The Netherlands.
$^{18}O_2$ (^{18}O: 92.48 atm %; ^{17}O: 0.417 atm %) was obtained from N.V. Philips-Duphar Cyclotrone and Isotope Laboratories, Petten, the Netherlands.

3. Methods

Preparative thin-layer chromatography (TLC) was performed on 20 × 20 cm glass plates covered with silica gel G (E.Merck). Solvent system: hexane-ether = 60 : 40 v/v. The spots were made visible by spraying with phosphomolybdic acid. Mass spectra were recorded on an AEI-MS 9 mass spectrometer. The samples were introduced via a direct insertion lock.

3.1. *Preparation of ^{18}O-labelled linoleic acid hydroperoxides*

In a reaction vessel attached to a gas burette, linoleic acid was incubated with lipoxygenase in an $^{18}O_2$-atmosphere at pH 9.0 in a 0.04 M NH_4Cl/NH_4OH buffer for 60 min. After acidification and extraction with ether, the hydroperoxides were isolated by preparative TLC on silica gel G impregnated with oxalic acid.

3.2. *Preparation of the hydroperoxide isomerizing enzyme*

A crude enzyme fraction was obtained by the extraction of acetone powder of flaxseed with 0.1 M phosphate buffer pH 7.4 for 3 hr. The extract was centrifuged at 12,000 *g* for 30 min.

3.3. *Isomerization reaction*

The mixture of ^{18}O-labelled linoleic acid hydroperoxides was incubated with the 12,000 *g* supernatant at room temperature. Before the reaction had come to completion, the ketol was reduced *in situ* with an excess of $NaBH_4$ in methanol, in order to restrict the exchange of the carbonyl oxygen as far as possible [6]. After acidification the reaction products were extracted with ether and esterified with diazomethane. The mixture of esters was separated via preparative TLC. The diol fraction was hydrogenated with PtO_2 as a catalyst. The saturated vic. diol was purified by preparative TLC and subsequently investigated by mass spectrometry.

4. Results and discussion

The mass spectrum of ^{16}O-12,13-dihydroxy-octadecanoic acid methyl ester is presented in fig. 1. This ester was obtained from linoleic acid hydroperoxides prepared with atmospheric oxygen ($^{16}O_2$). In this spetrum the $m + 1$ peak greatly predominates over the

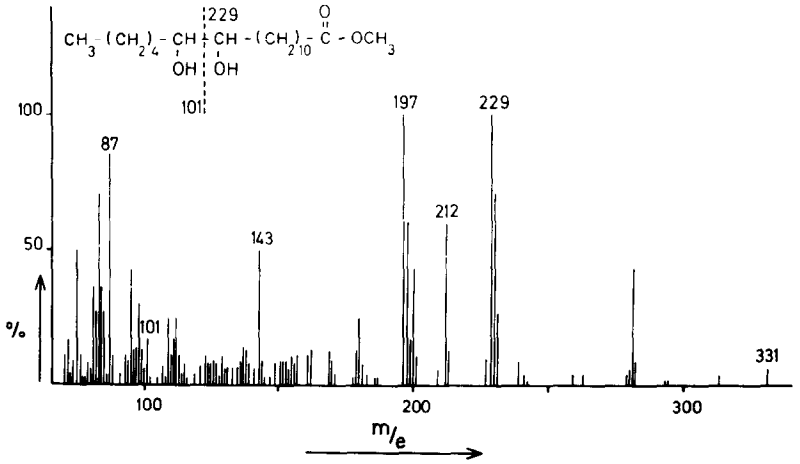


Fig. 1. Mass spectrum of ^{16}O-12,13-dihydroxyoctadecanoic acid methyl ester.

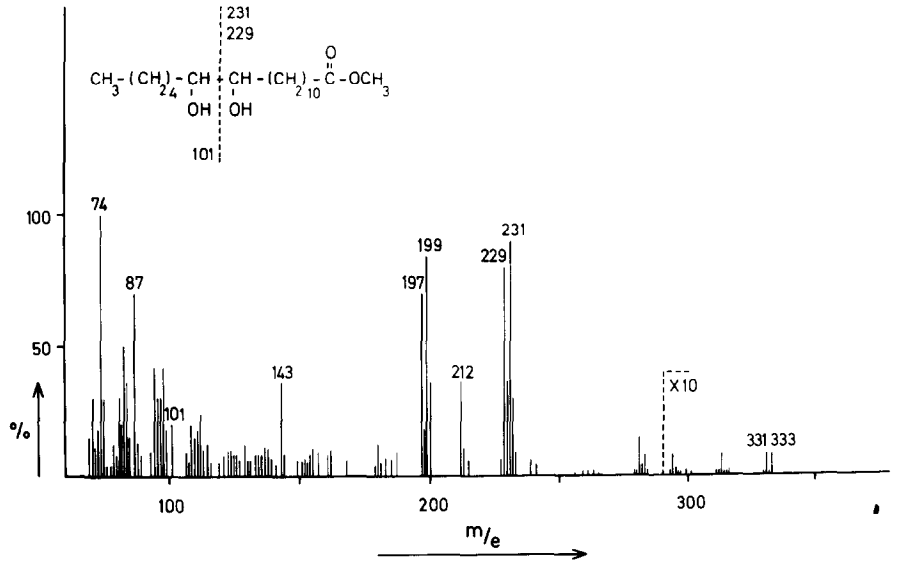


Fig. 2. Mass spectrum of the diol fraction obtained from ^{18}O-labelled linoleic acid hydroperoxides after incubation with the flaxseed hydroperoxide isomerase preparation.

parent peak. The fragmentation pattern closely resembles that of 9,10-dihydroxyoctadecanoic acid methyl ester [6]. Characteristic is the cleavage between the hydroxy groups resulting in a peak at *m/e* value 229. The mass spectrum of the diol fraction obtained from ^{18}O-labelled linoleic acid hydroperoxides is presented in fig. 2. Evidently, this fraction consists of two components which differ 2 mass units in molecular weight. The first component is identical to ^{16}O-12,13-dihydroxyoctadecanoic acid methyl ester. The second component is a similar ester containing one ^{18}O oxygen atom. The peak at *m/e* value 231 originates from the cleavage between the hydroxyl groups in the latter molecule and this fragment still contains a ^{18}O atom. This leads to the conclusion that the ^{18}OH group must be localized at position 12 in the molecule, corresponding to the carbonyl group in the α-ketol. The presence of the ^{16}O-analogue in the diol fraction is due to rapid exchange of the carbonyl with water during the enzymatic formation of the ketol. It is noteworthy that the fragment at *m/e* value 212 is formed from the ions 231 and 229 by the loss of ^{18}OH and ^{16}OH respectively. This interpretation is confirmed by the occurrence of the metastable peaks at *m/e* values 194.6 and 196.2 respectively. The presence of the ^{18}O atom at position 12 in the α-ketol, in contradistinction to the hydroperoxide in which the peroxy group is attached to carbon atom 13, may indicate that a cyclic intermediate is involved in the isomerization reaction, e.g. a cyclic peroxide or an epoxy compound.

Acknowledgements

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