FORMATION OF α- AND γ-KETOLS FROM ^{18}O-LABELLED LINOLEIC ACID HYDROPEROXIDES BY CORN GERM HYDROPEROXIDE ISOMERASE

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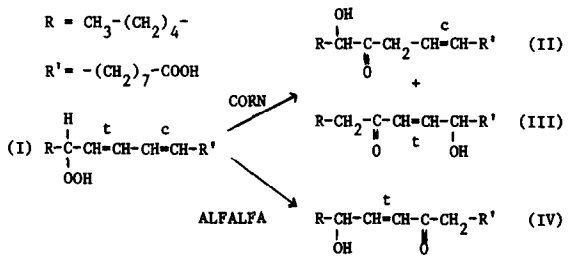
1. Introduction

Enzymes capable of converting unsaturated fatty acid hydroperoxides (e.g. 13-L-hydroperoxy-9 cis, 11 trans-octadecadienoic acid) into ketols have been found in flax seed [1,2], corn germs [3] and alfalfa seed [4]. The modes of formation of α-ketols from 13-L-hydroperoxy linoleic acid by the enzymes from corn and flax seem to be closely related. However, on the basis of product identification, γ-ketol formation seems to proceed along different pathways in corn and alfalfa (Scheme 1).



Scheme 1. Proposed pathways for α- and γ-ketol formation by enzymes from corn germs and alfalfa seeds [3,4].

The main product formed by the corn enzyme is the α-ketol whereas the alfalfa system exclusively yields a γ-ketol. Our previous studies in which ^{18}O-labelled linoleic acid hydroperoxides (I) were used in reactions with the enzyme from flaxseed [5] demonstrated that only the carbonyl–oxygen in the α-ketol stems from the hydroperoxy-group. Similar studies carried out by Esselman and Clagett [4] using the alfalfa enzyme provided evidence for the retention of two ^{18}O-atoms in the γ-ketol. The latter authors suggested that the enzyme from corn germs responsible for the formation of a γ-ketol might be similar to the alfalfa enzyme. This suggestion also implied some doubt as to the correctness of the structure of the γ-ketol formed by the corn enzyme. However, a recent reinvestigation by Gardner [6] of the structure of the γ-ketol produced by the corn enzyme has confirmed structure (III) which he originally proposed [3].

The apparent difference in the positional specificities of γ-ketol formation from the same precursor has led us to use ^{18}O-labelled hydroperoxy-linoleic acid in order to compare the modes of (i) α-ketol formation by enzymes from flax and corn; and (ii) γ-ketol formation by enzymes from corn and alfalfa.

Trivial name: 13-hydroperoxy linoleic acid Enzyme: Hydroperoxide isomerase (EC 5.3.99.1)

2. Materials and methods

^{18}O-labelled linoleic acid hydroperoxides and unlabelled hydroperoxides were prepared as described previously [7]. A crude hydroperoxide isomerase preparation was obtained from acetone powders of corn germs (6 g) by extraction with water (75 ml). After centrifugation the supernatant was filtered through cheese-cloth and the filtrate was then immediately incubated with the hydroperoxide. The hydroperoxide was dissolved in 0.1 M phosphate buffer (pH 6.6) and allowed to react for 30 min

at 0°C with 40 ml of the isomerase preparation. The reaction products were treated in situ with an excess of a freshly prepared solution of $NaBH_4$ (300 mg) in methanol in order to minimize exchange of the carbonyl-oxygens with water. After acidification with 2 M HCl the reaction products were isolated by extraction with diethylether, esterified with CH_2N_2, dissolved in methanol and then hydrogenated in the presence of PtO_2.

Thin-layer chromatography was carried out on pre-coated plates (silica gel 60F-254, E. Merck, A. G., Darmstadt, Germany) with the solvent system hexane: diethylether (30:70, by vol). Bands were located either under u.v. light or by spraying the edges of the plates with phosphomolybdic acid and subsequent heating at 110°C. The methyl esters of α- and γ-dihydroxystearic acid had R_f values of 0.22 and 0.09 respectively. The bands were scraped off, eluted with methanol and then treated with a mixture of hexamethyldisilazane and trimethylchlorosilane (2 : 1) in dry pyridine. After two hours water was added and the trimethyl silylated products were extracted with hexane. The products were analysed via combined gas chromatography-mass spectrometry with JEOL JAC-1100/JHC-07 instruments.

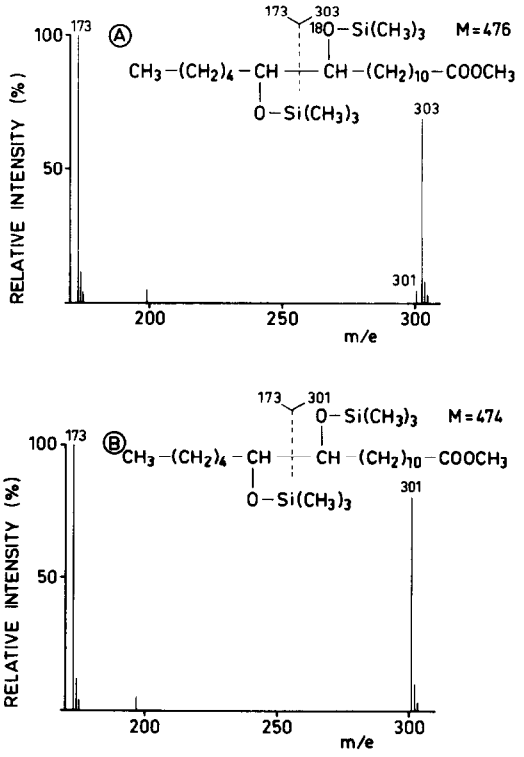


Fig.1. Mass spectrograms of trimethylsilylated dihydroxystearates derived from α (12,13)-ketols formed by enzymic conversion of (A) 13-[$^{18}O_2$] hydroperoxylinoleic acid and (B) 13-hydroperoxylinoleic acid.

3. Results and discussion

3.1. α-ketol information

Partial mass spectrograms of trimethyl silylated α-diols (fig.2, peak 2) derived from unlabelled and ^{18}O-labelled 13-hydroperoxylinoleic acid are shown in figs.1A and 1B respectively. The most prominent fragments arise from splitting of the bond between C-12 and C-13.

From a comparison between the fragments containing the trimethylsilyloxy-moiety attached to C-12 it is immediately apparent that C-12 bears an ^{18}O-atom whereas C-13 does not. Therefore the modes of α-ketol formation by isomerases from flaxseed and corn germs acting upon 13-hydroperoxylinoleic acid are identical.

The starting material also contained minor amounts (about 10%) of 9-hydroperoxylinoleic acid which was not removed prior to incubation with the isomerase. Mass spectra taken just before the main peak (fig.2, peak 1) showed all the characteristics of the methyl ester of 9,10-bis(trimethylsilyloxy)stearic acid containing one ^{18}O-atom. The fragmentation pattern indicated that in this case oxygen had been transferred from C-9 to C-10, quite analogous to the results found with 13-hydroperoxylinoleic acid.

3.2. γ-ketol formation

Partial mass spectrograms of derivatized γ-ketols are given in Figs.3A and 3B. The fragmentation pattern is similar to that described for the trimethyl silylated γ-diols by Esselman and Clagett [4]. Comparison of the unlabelled and the ^{18}O-labelled compound shows a mass shift of 2 daltons for the fragment at *m/e* 187 indicating that also during γ-ketol formation one oxygen is transferred from the hydroperoxy group to the carbonyl group; whereas

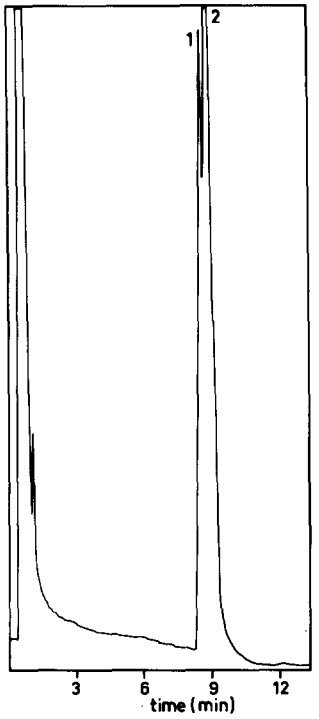


Fig.2. GC-separation of methyl 9,10-bis (trimethylsilyloxy) stearate (peak 1) and methyl 12,13-bis (trimethylsilyloxy) stearate (peak 2) (Column: SE-30; starting temperature 200°C; 4°C/min).

no ^{18}O is found in the fragment at *m/e* 259. Further evidence for the position of the ^{18}O-atom is found in the occurrence of peaks at *m/e* 299 and 301 (loss of $(CH_3)_3$ SiOH from fragments at *m/e* 391 and 389 respectively).

GC-separation of the isomeric γ-ketols was less complete than of the α-ketols. Therefore, in the γ-ketol spectra significant peaks are present which must be attributed to the γ(10,13)-ketol originating from 9-hydroperoxy linoleic acid in the starting material. Analogous peak shifts (*m/e* 273→275) again point to retention of the carbonyl oxygen, whereas no shifts are observed for fragments containing the oxygen atom of the hydroxyl group. These results demonstrate that the mechanism of γ-ketol formation by the corn enzyme is entirely different from that of the alfalfa enzyme [4].

The loss of one ^{18}O-atom during both α- and γ-ketol formation and the transfer of an oxygen atom to a carbonyl-group next to the C-atom originally bearing the hydroperoxy function suggest that these two products are formed through a closely related mechanism and probably by one enzyme.

Previously, we proposed [8] a mechanism for the formation of α-ketols involving nucleophilic displacement of the hydroperoxy–oxygen either by OH^- or other nucleophiles. This suggestion is supported by the results described in this paper and by those reported by Christianson and Gardner [9].

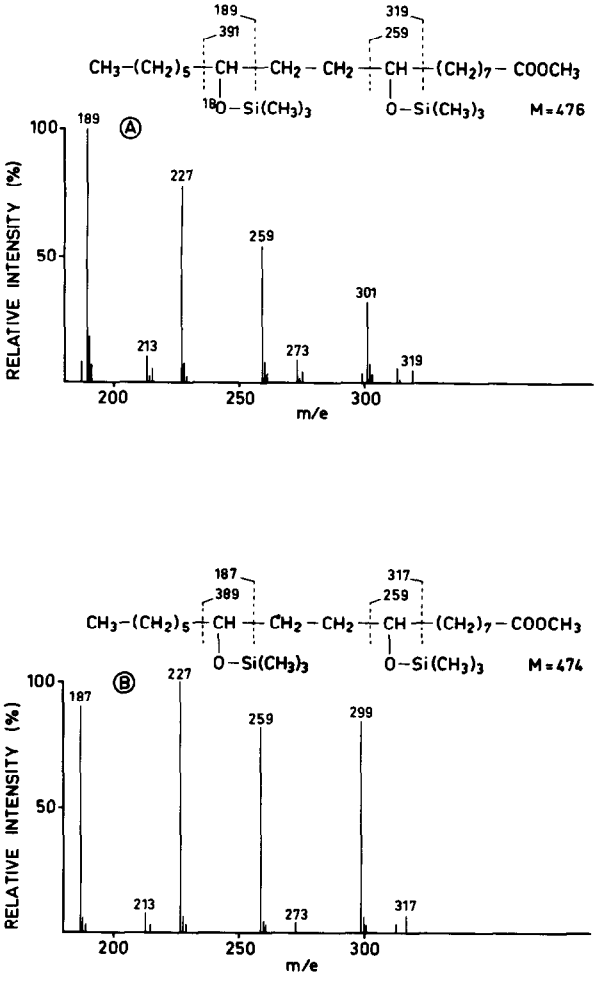


Fig.3. Mass spectrograms of trimethylsilylated dihydroxy-stearates derived from γ (9,12)-ketols formed by enzymic conversion of (A) 13-[$^{18}O_2$] hydroperoxylinoleic acid and (B) 13-hydroperoxylinoleic acid.

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