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Proof of the enzymatic formation of 9-hydroperoxy-10-trans, 12-*cis*-octadecadienoic acid from linoleic acid by soya lipoxygenase

Lipoxygenase (EC 1.13.1.13) catalyzes the formation of hydroperoxides of unsaturated fatty acids containing a *cis,cis*-1,4-pentadiene system. Several authors have reported that linoleic acid is converted to a mixture of the two isomeric hydroperoxides, 13-hydroperoxy-9-*cis*,11-*trans*-octadecadienoic acid and 9-hydroperoxy-10-*trans*,12-*cis*-octadecadienoic acid. The molar ratio of the 13-hydroperoxide isomer to the 9-hydroperoxide isomer varies, as reported in the litrature, from 7:3 (ref. 1) to 9:1 (ref. 2), although the exclusive formation of only the 13-hydroperoxide isomer has also been reported[3].

These results, together with our observation that only the 13-hydroperoxide isomer is converted by flaxseed hydroperoxide isomerase[4], prompted us to investigate whether the 9-hydroperoxide isomer is formed enzymatically or non-enzymatically by autoxidation.

To that end we have used the optical activity of the saturated hydroxy acids derived from the two hydroperoxides as a criterion.

Linoleic acid was incubated with soya lipoxygenase in 0.04 M NH_4OH–NH_4Cl buffer (pH 9.0) in an oxygen atmosphere (500 mg substrate, 75 mg enzyme obtained from Nutrional Biochemical Corp., activity 8000 units/mg, total volume 500 ml).

After incubation for 1 h the hydroperoxides were reduced *in situ* by the addition of $NaBH_4$ (4 g in 20 ml of methanol).

The fatty acids were then recovered by acidifying and extracting with ether and were converted into their methyl esters with diazomethane.

Subsequently the mixture of methyl esters was dissolved in methanol and completely hydrogenated with a platinum catalyst.

The hydrogenated compounds were separated by preparative thin-layer chromatography on silica gel G in hexane–ether (60:40, v/v). After development, the edges of the plates were sprayed with a 5% solution of phosphomolybdenic acid in ethanol. The R_F values of 9-hydroxystearate, 13-hydroxystearate and methylstearate were, respectively, 0.54, 0.66 and 0.88. The whole surface was then sprayed with a 0.5% solution of ultraphore B.A.X. in water, and the compounds were localized under ultraviolet light. The regions containing the hydroxy compounds were scraped off and eluted with methanol. Each of the compounds was rechromatographed in the same system to eliminate any possible contamination with its isomer. The molar ratio of 9- and 13-hydroxystearate appeared to be 3:7. The identity of the 9- and 13-hydroxystearates was unambiguously established by mass spectrometry.

The specific optical rotation of the hydroxystearate methyl esters was measured in the microcell of a Perkin–Elmer 141 polarimeter (Table I).

From these results it can be concluded that 9-hydroperoxy-10-*trans*,12-*cis*-octadecadienoic acid is indeed enzymatically formed.

The absolute configuration of 13-hydroxystearate has been derived by com-

Abbreviation: ORD, optical rotatory dispersion.

TABLE I

SPECIFIC OPTICAL ROTATION VALUES OF 9-HYDROXYSTEARATE AND 13-HYDROXYSTEARATE

Solvent: chloroform. Temperature, 20°. The ORD spectrum of methyl-9-hydroxystearate (*c* 2.01 in methanol) shows a plain negative curve. Methyl-13-hydroxystearate obtained after incubation of linoleic acid with lipoxygenase[2] gives a plain positive ORD curve (*c* 5.0 in methanol). Both methyl-13-hydroxystearate derived from corioloc acid[6] and methyl 12-D-hydroxystearate[5,6] show plain negative ORD curves (*c* 2.5 in methanol). Methyl 9-D-hydroxystearate derived from methyl dimorphecolate shows a plain negative ORD curve[7] (*c* 2.51 in methanol).

λ *(nm)*	*9-Hydroxystearate* *c 2.6*	*13-Hydroxystearate* *c 4.0*
578	−0.39 ± 0.03	+0.14 ± 0.01
546	−0.41 ± 0.03	+0.16 ± 0.01
436	−0.61 ± 0.04	+0.29 ± 0.02

parison of its optical rotation with that of 12-hydroxystearate. On this basis HAMBERG AND SAMUELSSON[2] ascribed, the L-configuration to 13-hydroxystearate obtained after incubation of linoleic acid with soya lipoxygenase. They confirmed this by oxidative breakdown of the unsaturated ester and comparison of the resulting α-hydroxyheptanoic acid with a reference substance.

In view of the observed plain negative optical rotatory dispersion (ORD) curve of the 9-hydroxystearate and on the analogy of the reasoning followed by HAMBERG AND SAMUELSSON[2], it seems justified to ascribe the D-configuration to the 9-hydroxystearate.

This tentative conclusion fits with what one would observe when the pentadiene radical moiety is oriented as a flat structure on the enzyme and is attacked by oxygen from one direction at the 9- and 13-position, respectively.

However, the final proof can only be obtained after oxidative cleavage of the 9-hydroxyoctadecadienoic acid and subsequent determination of the absolute configuration of the resulting α-hydroxysebacic acid.

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