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Distinction between enzymic and nonenzymic lipid peroxidation

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Abstract

Linoleic, linolenic and arachidonic acid were subjected to nonenzymic lipid peroxidation. After hydrogenation and derivatisation, the mixture of saturated monohydroxy acids was analyzed by gas chromatography–mass spectrometry. Typical α-cleavage fragments enabled quantification of single hydroxy compounds. This investigation revealed that not only oxidation products are found by removal of hydrogen from double allylically activated CH_2 groups, but also that products are generated by removal of hydrogen from monoallylically activated CH_2 groups. This reaction was previously unknown to occur with linolenic and arachidonic acid. The typical patterns of the monohydroxy acids derived after oxidation of polyunsaturated fatty acids allows distinction between an enzymic and a nonenzymic induced lipid peroxidation process. In the first case usually only one hydroxy acid is produced preferentially while in nonenzymic processes two hydroperoxy acids (those with the hydroxy groups at "outer position") are generated in about equal yield.

Keywords: Lipid peroxidation; Linoleic acid; Linolenic acid; Arachidonic acid; Polyunsaturated fatty acids

1. Introduction

Lipid peroxidation (LPO) is assumed to play a crucial role in the genesis of chronic diseases, e.g. atherosclerosis [1,2], psoriasis [3], rheuma [4], Alzheimer disease [5,6], myocardial infarction [7] and multiple sclerosis [8,9]. It is also thought to be connected with the process of aging [10].

It is generally supposed that LPO in tissue is initiated by radicals, e.g. $O_2^{\cdot}$ and $OH^{\cdot}$ [4,11]. The $OH^{\cdot}$ radical is especially considered to initiate LPO. In spite of this, it is well known that generation of eicosanoides is accompanied by activation of lipoxygenases which produce hydroperoxy fatty acids from unsaturated fatty acids (LOOH) [12]. Also LOOH formation in plants occurs enzymically, e.g., as a consequence of injury [13,14]. Therefore, methods are required to determine if a LOOH is produced enzymically or autocatalytically.

Such a distinction is possible since lipoxygenases produce chiral lipid hydroperoxides while $OH^{\cdot}$ is a nonchiral compound. It is therefore expected that lipoxygenase-induced LPO produces preferentially either the *R*- or *S*-LOOH while in a nonenzymic LPO process, both isomers should be obtained in the same yield. LOOHs are reduced enzymically in tissue to corresponding alcohols, without loss of chirality. The obtained hydroxy derivatives of polyunsaturated fatty acids (PUFAs) (LOH) can be subjected after methylation of the carboxylic function to separation on chiral high-performance liquid chromatography (HPLC) columns. Thus Schewe et al. [15] detected that LOOHs obtained from the skin of psoriatic patients contain a remarkable surplus

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of the 9-*R*-hydroxy-10-*trans*-12-*cis*-octadecadienoic acid derived from 9-*R*-hydroperoxy-10-*trans*-12-*cis*-octadecadienoic acid, the LOOH produced by reaction of lipoxygenase with linoleic acid. They therefore deduced a preferential enzymic generation of LOOH in this case. Enzymic production seems to occur also after tissue injury since Gassen was able to show an enrichment of chiral 4-hydroxynonenal [16], a typical LPO product [17] obtained by decomposition of LOOH derived from arachidonic acid, after stimulating liver enzymes.

The investigation of tissue to detect stereoisomers is very difficult due to the great amount of accompanying compounds, e.g., separation of tissue extracts by gas chromatography (GC) reveals the presence of a tremendous number of compounds. The quantification of the tiny peaks corresponding to stereoisomeric hydroxy compounds which show identical mass spectra is impossible in most cases. In contrast, derivatives of regioisomeric hydroxy compounds distinguish in their mass spectra to a great extent, since the main fragments are caused by α-cleavage, depending from the site of the functional group. Therefore structure specific fragments are obtained. Thus the isomers can be detected by registration of their ion currents of these specific fragments even if a compound is buried under large amounts of accompanying material. Also their quantification can be achieved.

To deduce if a LPO process in tissue is caused enzymically or autocatalytically, we used quantification of hydroxy fatty acids of model compounds. We report here on such experiments.

2. Materials and methods

2.1. Materials

N-Methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) was obtained from Machery and Nagel (Düren, Germany). All other chemicals were purchased from Fluka (Neu Ulm, Germany). Solvents, obtained from Merck (Darmstadt, Germany), were distilled before use. Thin-layer chromatography (TLC) was performed with home made TLC plates using 0.75 mm PF_{254} silica gel 60 (Merck).

2.2. Autoxidation of fatty acids

Autoxidation of linoleic, linolenic and arachidonic acid (30 mg) was performed as described previously [18–20] following the LPO method worked out by Esterbauer [21]. The oxidation reactions were stopped after 24 h by addition of 160 μl BHT (2,6-di-*tert.*-butyl-4-methylphenol) (2% in methanol) and 1.2 ml EDTA-Na_2 (ethylenediaminetetraacetic acid disodium salt dihydrate, 1% in bidistilled water).

2.3. Hydrogenation with Pt/H_2 on activated carbon (5% Pt)

After stopping the oxidation and addition of 0.3 mg internal standard 6-monoOH 17:0 (methyl-6-hydroxyheptadecanoate), the aqueous oxidation solutions were extracted with chloroform. Then carboxylic groups were methylated by treatment with an etheric diazomethane solution. The methylates were hydrogenated for 2 h with platinum on activated carbon (5% Pt) in ethyl acetate at room temperature and at 1.5 bar. The fractions containing the monohydroxy fatty acid methylates (R_F=0.54–0.86) were separated from other products by co-chromatography of the standard 6-monoOH 17:0 at the margin of the TLC plates in cyclohexane–ethyl acetate 1:1. Detection was performed with 10% ethanolic $H_3[P(Mo_3O_{10})_4]$ and heating [22]. After TLC separation, monohydroxy fatty acid methylates were eluted with ethyl acetate and trimethylsilylated for 24 h at room temperature by reaction with MSTFA. Identification was carried out by GC–mass spectrometry (MS) using electron impact (EI)-MS [19,20]. All oxidation experiments were repeated three times, all ion current measurement were also three times repeated. Therefore, mean values indicated in Figs. 2–4 are the result of nine determinations.

2.4. Gas chromatography–mass spectrometry

GC was carried out with a Carlo Erba HRGC 5160 Mega Series chromatograph equipped with a flame ionisation detector, using a DB-1 fused-silica glass capillary column (30 m×0.32 mm I.D.), temperature programmed from 80 to 280°C at 3°C/min. The

temperature of the injector and detector was kept at 270 and 290°C, respectively. The carrier gas was hydrogen and the splitting ratio 1:30. Peak-area integration was achieved with a Merck D-2500 integrator.

GC–MS was performed on a Finnigan MAT 312 mass spectrometer connected to a MAT-SS-300 data system. EI mass spectra were recorded at an ionisation energy of 70 eV. A Varian 3700 gas chromatograph with a 30 m×0.3 mm I.D. DB-1 fused-silica column was used for sample separation. The carrier gas was hydrogen and the temperature programme was the same as used for GC.

3. Results

Pure fatty acids (linoleic, linolenic and arachidonic acid) were subjected to oxidation with O_2/ascorbic acid/Fe^{2+}. This process is similar to the so called Fenton reaction [23]. The reaction was stopped by addition of EDTA-Na_2 and BHT [24]. After extraction of the organic acids, diazomethane was added to transform them to their methylates. These were subjected to hydrogenation with platinum on activated carbon (5% Pt). Since it turned out that hydroperoxides were reduced to corresponding hydroxy derivatives, a reduction step could be omitted. After hydrogenation, the obtained mixture of saturated hydroxy fatty acids was separated by TLC in a fraction of nonoxidized compounds (R_F=0.86–1.0), in a fraction of monohydroxy methylates (R_F=0.54–0.86) and in a fraction of dihydroxy derivatives (R_F=0–0.54). The mixture of derivatized saturated monohydroxy compounds was trimethylsilylated and separated by GC. Identification was achieved by MS. The mass spectra of saturated monohydroxy methylates were recognized by the strong α-cleavage fragments [25,26], e.g., the trimethylsilyl derivative of the 9-hydroxystearic acid methylate derived from 9-LOOH produced key fragments of mass 229 and 259, while its 13-isomer derived from 13-LOOH generated key ions of mass 173 and 315. Quantification was achieved by measuring these ion currents. The results of these measurements are reproduced in Fig. 1.

The pattern of obtained hydroxy acids is characterized by the high abundance of the 9- and 13-isomer [27–29], the latter (more distant from the carboxylic group) is produced in an excess of about 5–10%. Besides these typical LPO products, derived by hydrogen abstraction from the double allylically activated CH_2 groups in position 11 [27–29], isomers are found which are produced by removal of hydrogen from CH_2-groups activated by one double bond only (monoallylically induced oxidation, see later). Products [30,31] which were generated by this "monoallylically activation" carrying the OH-function at an "outer" position (8 and 14) are much more abundant than those with the OH function at an inner position (10 and 12, see Fig. 2 and Scheme 1).

The photosensitized oxidation of linolenic acid, investigated earlier [27,32,33], revealed that the main oxidation products, initiated by removal of hydrogen from the two double allylically activated CH_2 groups in position 11 and 14, produce mainly the isomers carrying the functional group at an outer position (9 or 16). The products resulting from removal of hydrogen from the double allylically activated CH_2 groups by formation of hydroperoxyl groups at inner positions (the 12- and 13-isomers) are produced in much lower yield [27,34]. Hydroxystearic acid derivatives are also detected which originate by direct addition of O_2 after removal of activated hydrogens in position 11 and 14.

Fig. 3 reveals that we found in this investigation also products which are generated by hydrogen removal from the only monoallylically activated CH_2 groups in positions 8 and 17. Again, the isomers resulting from this reaction predominate which carry the functional groups at the outmost positions (8 or 17). Those located at the inner positions (10 or 15) are obtained in much lower yield (Scheme 2, Fig. 3).

The oxidation of arachidonic acid is in agreement with the above outlined oxidation rules for unsaturated acids. The LPO products, generated by oxidation after hydrogen removal from double allylically activated CH_2 groups (7, 10 or 13) at the "outer" positions-the 5-substituted and 15-isomers, are found in high abundance [35], in agreement with other results [26,34]. Again, oxidation at the alkyl end acid is more pronounced than at the carboxylic end, therefore the 15-isomer is more abundant than the 5-substituted one. LPO products derived by oxidation at the inner positions 8, 9, 11 and 12 are produced in much lower amounts [26,34]. Oxidation

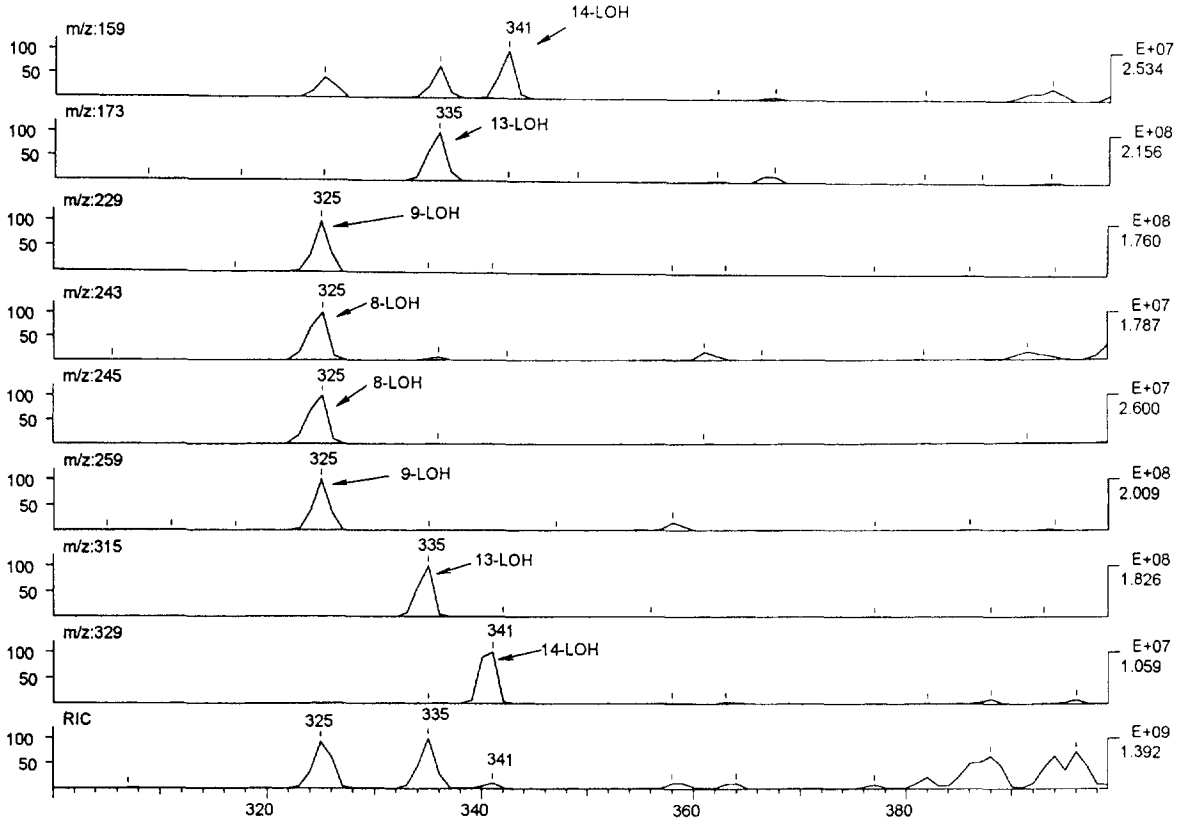


Fig. 1. Ion current measurements of typical ions due to α-cleavage of trimethylsilylated derivatives of 13-LOH (methyl-13-hydroxyoctadecanoate, α-cleavage products m/z=173, 315); 8-LOH (methyl-8-hydroxyoctadecanoate, α-cleavage products m/z=243, 245); 9-LOH (methyl-9-hydroxyoctadecanoate, α-cleavage products m/z=229, 259) and 14-LOH (methyl-14-hydroxyoctadecanoate, α-cleavage products m/z=159, 329).

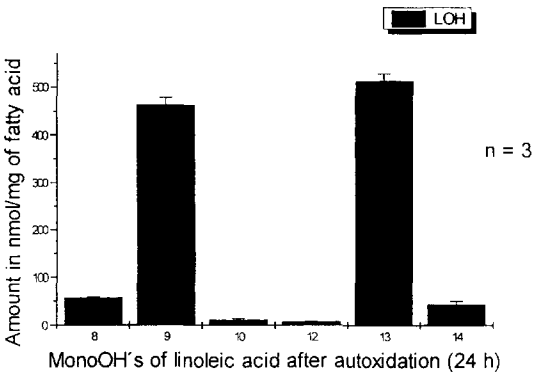
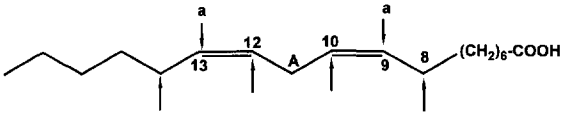


Fig. 2. Pattern of trimethylsilylated hydroxystearic acid methylates obtained after oxidation of linoleic acid followed by hydrogenation of the double bonds.



Scheme 1. Sites of preferential hydrogen removal in linoleic acid: the double allylically activated CH_2 group is indicated by A, corresponding hydroperoxidized carbons are indicated with letter a; monoallylically activated positions are indicated by arrows only.

directly at the sites of hydrogen removal (at position 7, 10, 13) is hardly detected (Scheme 3, Fig. 4).

We again observed products which are derived by hydrogen abstraction from the monoallylically activated CH_2 groups in position 4 and 16. Remarkably, these products occur in higher abundance than the isomers with the OH-function in position 7, 10 or 13.

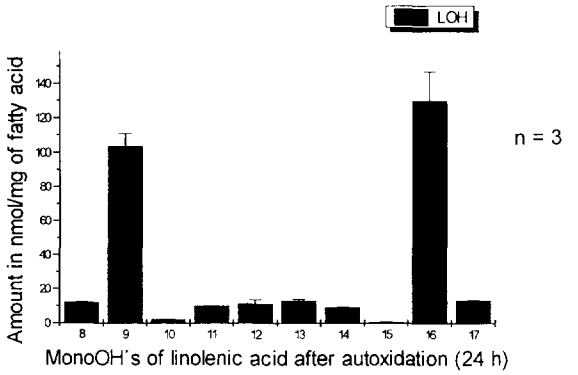


Fig. 3. Pattern of trimethylsilylated hydroxystearic acid methylates obtained after oxidation of linolenic acid followed by hydrogenation of the double bonds.

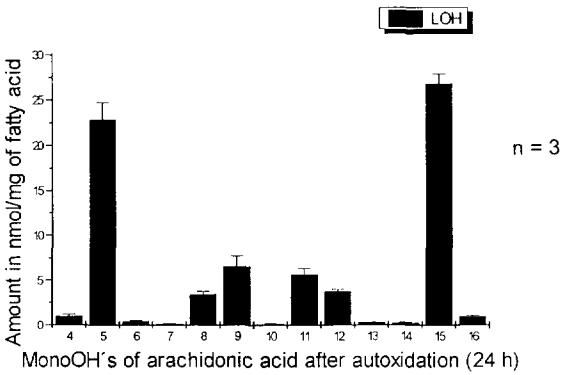
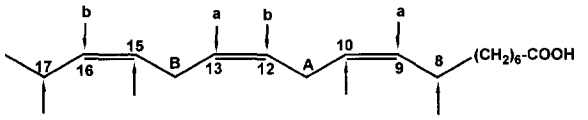


Fig. 4. Pattern of trimethylsilylated hydroxystearic acid methylates obtained after oxidation of arachidonic acid followed by hydrogenation of the double bonds.

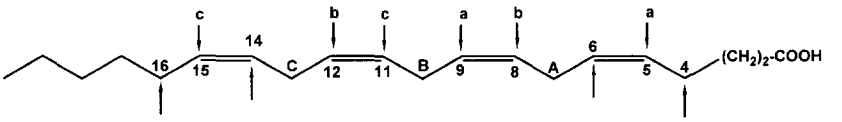


Scheme 2. Sites of preferential hydrogen removal in linolenic acid: the double allylically activated CH_2 groups are indicated by A and B respectively, corresponding hydroperoxidized carbons are indicated with letters a and b respectively; monoallylically activated positions are indicated by arrows only.

Monoallylically induced hydrogen abstraction in position 4 or 16 also produces isomers with the oxygen function in position 6 and 14 (at an inner position), but these are generated only with low abundance compared to the isomers with the oxygen function in position 4 and 16 (Scheme 3, Fig. 4).

4. Discussion

Methods for analysis of oxygenated LPO products of PUFAs by GC–MS had been developed by several groups [25,36–39]. In principle, produced unsaturated hydroperoxy- and hydroxy acids were hydrogenated and subjected after appropriate derivatisation to GC–MS. These investigations revealed that unsaturated acids containing the 1,3-pentadienyl system (–HC=CH–CH_2–CH=CH–) suffer easy abstraction of hydrogen from the double allylically activated CH_2 group [26,30,31,33]. The resulting mesomeric radical reacts with oxygen and then with another molecule by hydrogen abstraction to form in nearly equal yield two monohydroperoxy compounds containing a conjugated dienoic system. Thus a 9- and 13-hydroperoxide is obtained from linoleic acid [40–42]. Linolenic and arachidonic acid contain 2 and 3 respectively, equally double allylically activated CH_2 groups and therefore are principally able to produce 4 and 6 isomers respectively [32,43]. In fact, the isomers with the functional group at "outer" carbon atoms (the 9- and 16-isomer in the case of linolenic acid [32], and the 5- and 15-isomer in the case of arachidonic acid [26]), predominate over the isomers with hydroperoxy groups at "inner" positions (the 12- and 13-isomer in the case of linolenic and the 8, 9, 11 and 12-isomers in the case of arachidonic acid). In addition it was detected that the two main products (e.g. the



Scheme 3. Sites of preferential hydrogen removal in arachidonic acid: the double allylically activated CH_2 groups are indicated by A, B and C respectively, corresponding hydroperoxidized carbons are indicated with letters a, b and c respectively; monoallylically activated positions are indicated by arrows only.

9- and 16-isomer of linolenic acid) are not produced in exactly the same amount, but that the isomer with the functional group more remote to the carboxylic group is always obtained in slightly increased amounts.

In any case, two isomers (those with the functional groups at an "outer" position) are produced preferentially in a nonenzymic LPO. In enzymic induced LPO, hydrogen is removed exclusively from a double allylically activated CH_2 group in a regiospecific and stereospecific reaction [40,41]. Although stereoisomers can not be distinguished by GC–MS, the pattern of hydroxy acids obtained after processing of biological material should contain, at least theoretically, only one single peak, since regioisomers are not produced. This should enable a unambiguous distinction of an enzymic and a nonenzymic LPO.

In fact the distinction is more complicated. Firstly, biological material sometimes contains several lipoxygenases, resulting in several products, but these are usually not produced in equal amounts [25].

Secondly, oxidation of PUFAs is not achieved by lipoxygenases only, but also by the enzyme complex cytochrome P450 [44,45]. P450 is able to epoxidize double bonds of unsaturated fatty acids [46,47] producing isomeric epoxides. These are obviously transformed further to monohydroxy acids by reductive ring opening [48], generating always from one epoxide two regioisomeric hydroxy derivatives. Therefore, profiles are obtained which show pairs of hydroxy derivatives in about equal amounts at adjacent carbon atoms (e.g. the 5,6- or 8,9- or 11,12- or 14,15-isomeric pairs in the case arachidonic acid) [7,49]. These can be easily distinguished from patterns obtained from lipoxygenase induced or a nonenzymic oxidation process.

Thirdly, in many cases a nonenzymic LPO is superimposed on an enzymic one. A distinction is possible in most of these cases, due to the prevalence of one LPO product in a lipoxygenase induced LPO.

In addition, a careful analysis of LPO products provides further information. As already detected by Grosch in an investigation of LPO products of linoleic acid, the main oxidation products, the 9- and 13-isomer, are accompanied by additional LPO products. Although hydrogen removal occurs with highest probability from CH_2 groups in a pentadienyl system of PUFAs, there is also a hydrogen abstraction possible from only monoallylically activated CH_2 groups, e.g., in linoleic acid from the CH_2 groups at position 8 and 14. Thus mesomeric radicals are produced. Oxygen addition occurs mainly at the outer carbons (4 and 16) and not at the inner carbons (6 and 14) of the mesomeric system, in analogy to preferential generation of the 5- and 15-isomers from arachidonic acid, induced by removal of double allylically activated hydrogens (Scheme 3). Similar reactions produce from linolenic acid the 8- and 17-hydroperoxide and the 6- and 15-isomers respectively (Scheme 2). The monoallylic induced oxidation products of linoleic acid are obtained in higher yield compared to those of linolenic acid and arachidonic acid, because the latter contain more double allylically activated CH_2 groups.

These isomers resulting from monoallylically induced LPO can not be generated in an enzymic induced LPO of mammalian tissue (but such isomers have been detected in high yield as oxidation products of fungi [50,51]). Nevertheless, such products have been detected in tiny amount in enzymic LPO processes [52]. It might well be that in these cases, the enzymic LPO was superimposed by a nonenzymic LPO process.

In any case, the careful investigation of the pattern of monohydroxy acid derivatives obtained by LPO of tissue gives valuable hints, if a LPO had been induced enzymically or nonenzymically, if nonenzymic processes contribute to formation of products and in some cases even to which extent processes are overlapped.

5. Notation

LPO,	lipid peroxidation
MSTFA,	N-methyl-N-trimethylsilyltrifluoroacetamide
BHT,	2,6-di-*tert.*-butyl-4-methylphenol
EDTA-Na_2,	ethylenediaminetetraacetic acid disodium salt dihydrate
GC,	gas chromatography
GC–MS,	gas chromatography–mass spectrometry
TMS,	trimethylsilyl

MonoOH, Methyl ester of saturated monohydroxy fatty acid
6-MonoOH 17:0, methyl 6-hydroxyheptadecanoate
LOOH, hydroperoxy fatty acids derived from unsaturated fatty acids
LOH, hydroxy fatty acids derived from hydroperoxy fatty acids

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