Studies on the Substrate Specificity of Soybean Lipoxygenase-1 Using an Entire Series of (ω3*Z*,ω6*Z*,ω9*Z*)-C_{12}~C_{22}-Trienoic Acids and -Trienols

Akikazu Hatanaka, Tadahiko Kajiwara, Kenji Matsui, and Miharu Ogura

Department of Agricultural Chemistry, Faculty of Agriculture,
Yamaguchi University, Yamaguchi 753, Japan

Z. Naturforsch. **45c,** 1161–1164 (1990); received June 5, 1990

Fatty Acid, Fatty Alcohol, Soybean Lipoxygenase-1, Substrate Specificity, Hydrophobic Interaction

Substrate recognition mechanism of soybean lipoxygenase-1 (L-1) was studied by using an entire series of (ω3*Z*,ω6*Z*,ω9*Z*)-C_{12}~C_{22}-trienoic acids and -trienols. The maximum activity of L-1 was obtained with C_{20}-trienoic acid of all the substrates used. Among trienols, the maximum activity was obtained with C_{15}-trienol. Analysis of the products obtained by the reaction of these substrates with L-1 revealed that trienoic acids were oxygenated mostly at ω6 position independently on the chain length. The hydroperoxy group at ω6 was introduced at (*S*)-configuration with over 98% enantioselectivity. Although the products formed from most trienols were also selectively oxygenated at ω6 position, only C_{15}-trienol was oxygenated at both ω6 and ω10 positions in the ratio of ω6/ω10 = 48/52.

Introduction

A non-heme iron enzyme, lipoxygenase (EC 1.13.11.12) catalyzes the stereospecific dioxygenation of unsaturated fatty acids containing a methylene-interrupted (*Z*,*Z*)-pentadiene system such as linoleic and linolenic acids. The reaction mechanism of lipoxygenase-1 (L-1) isolated from soybean seeds has been extensively studied [1, 2]. Pentadienyl radical is formed when the pro-(*S*) hydrogen of an active methylene of the pentadiene system is abstracted concomitantly with the conversion of Fe(II) to Fe(III) in the enzyme. The formed radical reacts with oxygen to produce a hydroperoxide, and the Fe(III) is reduced to the native state, Fe(II) [3, 4].

In our previous paper, we have examined substrate and product specificity of soybean L-1 using synthetic (ω6*Z*,ω9*Z*)-C_{12}~C_{20}-dienoic acids and -dienols [5]. It was showed that linoleic acid and (ω6*Z*,ω9*Z*)-pentadecadienol ($C_{15:2}$OH) was the best substrate among the dienoic acids and the dienols, respectively. Although mainly the ω6 position of the acids were oxygenated by L-1 independently on the chain length, the specificity of oxygenation was affected by the chain length when alcoholic substrates were used. The most pronounced result was obtained with $C_{15:2}$OH which was selectively oxygenated at ω10 rather than at ω6. These results suggested that the ionic interaction of carboxylic anion in a substrate with reaction center of L-1 was important but hydrophobic interaction or recognition of the pentadiene system was preferred. To study the substrate recognition mechanism of L-1 more precisely, in this investigation, substrate and product specificity of L-1 were examined with a synthetic entire series of (ω3*Z*,ω6*Z*,ω9*Z*)-C_{12}~C_{22}-trienoic acids and -trienols, all of which have one more hydrogen abstraction site with L-1.

Abbreviations: $C_{n:3}$COOH, (ω3*Z*,ω6*Z*,ω9*Z*)-trienoic acid (n, total carbon number); $C_{n:3}$OH, (ω3*Z*,ω6*Z*,ω9*Z*)-trienol; HPLC, high performance liquid chromatography; L-1, soybean lipoxygenase-1.

Reprint requests to Prof. Dr. Akikazu Hatanaka.

Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/90/1100–1161 $ 01.30/0

Materials and Methods

Materials

Soybean lipoxygenase purchased from Sigma Chemical Co. (type I) was further purified by chromatography on DEAE-Cellulofine A-500 (Seikagaku-Kogyo Co., Tokyo, Japan) to give almost homogenous enzyme [6]. Linoleic and linolenic acids were purchased from Sigma Chemical Co. and (11*Z*,14*Z*,17*Z*)-icosatrienoic and (13*Z*,16*Z*,19*Z*)-docosatrienoic acids were purchased from Nu Chek Prep., Inc., Elysian, Minnesota. The corresponding alcohols were prepared by reduction of the acids with lithium aluminium hydride.

*Preparation of (ω3*Z*,ω6*Z*,ω9*Z*)-C_{12}~C_{22}-trienoic acids*

2-Bromo-2,5-octadiyne was prepared by Grignard coupling of 3-(2-tetrahydropyranyloxy)-propyne and 2-bromo-pentyne followed by bromination with PBr_3. Another counterparts, ω-acetylenic 1-(2-tetrahydropyranyloxy) compounds which have various methylene length, were obtained through coupling of acetylene with ω-bromo 1-(2-tetrahydropyranyloxy) compounds having various methylene length. Grignard coupling of the bromide and the ω-acetylenic compounds and subsequent hydrolysis with HCl in MeOH, gave ω3,ω6,ω9-triynols in appropriate yields. The triynols were reduced by (*Z*)-selective hydrogenation under Lindlar catalyst to give (ω3*Z*,ω6*Z*,ω9*Z*)-C_{12}~C_{22}-trienols. The purified triynols were converted to the corresponding carboxylic acid *via* Jones oxidation.

Geometrical purity of the synthetic specimens were determined to be over 98% pure by HPLC analysis (column: Zorbax ODS, 4.6 × 150 mm from DuPont; eluent: acetonitrile/water/tetrahydrofuran = 90/9/1; flow rate: 1 ml/min, detection at 350 nm) in forms of the 2,4-dinitrophenylhydrazone derivatives of the corresponding aldehydes derived from the trienols or the trienoic acids.

Assay of lipoxygenase activity

Lipoxygenase activity was determined spectrophotometrically by monitoring an increase in absorbance at 234 nm at 25 °C. A standard reaction mixture (3 ml) contained 200 mM borate buffer (pH 9.0), diluted enzyme solution (10 µl) and substrate (25 µl of 10 mM substrate solution containing 0.2% Tween 20, final concentration: 82.4 µM).

Analysis of hydroperoxide

L-1 (200 units) was added to a reaction mixture (50 ml) consisting of 200 mM borate buffer (pH 9.0) and 5 ml of substrate solution (10 mM in 0.2% Tween 20). The reaction mixture was stirred for 60 min at 20 °C under O_2 flow and acidified with 2 N HCl (except for trienols) and extracted with 200 ml of ether. The ether layer was washed twice with sat. NaCl solution, and was evaporated *in vacuo*. The residue was fractionated by dry column chromatography on silica gel to give pure hydroperoxides. The hydroperoxides were reduced with $NaBH_4$ in 4 ml of methanol–borate buffer (1:1, v/v, pH 9.0) for 30 min in ice bath. The reduced products were acidified by 2 N HCl and extracted with ether. Evaporation of the extraction solution gave hydroxy acids or hydroxy alcohols. The hydroxy acids were esterified with diazomethane in ether to give the corresponding hydroxy esters. Geometrical isomers in the hydroxymethyl esters were analyzed with HPLC (column: Zorbax SIL 4.6 × 250 mm from DuPont; eluent: *n*-hexane/dry ethanol = 99/1; flow rate: 1 ml/min at 25 °C, detection et 234 nm). Geometrical isomers in the hydroxy alcohols were analyzed under almost the same conditions but eluted with *n*-hexane/dry ethanol (98/2, v/v) with a flow rate of 2 ml/min. Enantiomeric excess of the separated isomer was determined by chiral phase HPLC (column: Chiralcel OB 4.6 × 250 mm from Daicel Chemical Industries, Ltd.; eluent: *n*-hexane/dry ethanol (99/1, v/v); 2 ml/min at 25 °C, detection at 234 nm).

Results and Discussion

Reactivities of synthetic substrates, (ω3*Z*,ω6*Z*,ω9*Z*)-C_{12}~C_{22}-trienoic acids, for L-1 were examined by spectrophotometric assay (Fig. 1 A). Under the standard assay condition (200 mM borate buffer, pH 9.0), the maximum reactivity was obtained with $C_{20:3}$COOH. With longer or

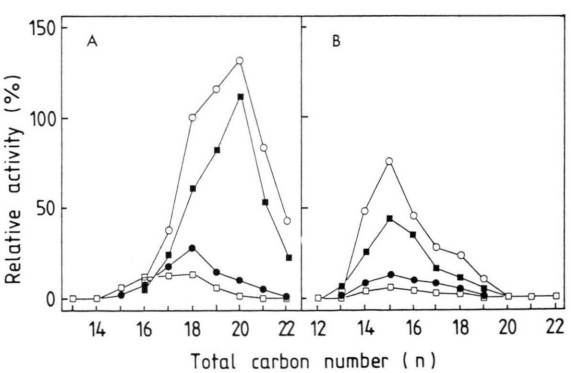

Fig. 1. Substrate specificity of L-1 for (ω3*Z*,ω6*Z*,ω9*Z*)-trienoic acids (A) and -trienols (B). Trienoic acids and trienols were reacted with L-1 at 25 °C in 200 mM phosphate buffer, pH 6.0 (□) or pH 7.0 (●), or in 200 mM borate buffer, pH 8.0 (■) or pH 9.0 (○). The relative activity (%) to the activity obtained with linolenic acid (activity of 160 µmol/mg·min) was plotted against total carbon number (n) of the substrate.

shorter acids than this substrate, the activity of L-1 decreased. It should noticed that $C_{19:3}$COOH and $C_{20:3}$COOH had higher reactivity than a natural substrate, linolenic acid. Another assay procedure using oxygen electrode also confirmed this result. With alcoholic substrate, the maximum activity was obtained with $C_{15:3}$OH (Fig. 1 B).

Although the reactivity of these acids obtained at pH 8.0 was almost the same as that at pH 9.0, lowering the pH to 7.0 drastically decreased the reactivity. Maximum activity of L-1 was observed with the shorter fatty acids than $C_{20:3}$COOH under neutral or acidic assay conditions, *i.e.*, at pH 7.0, $C_{18:3}$COOH showed the maximum reactivity and at pH 6.0, $C_{16:3}$COOH $C_{18:3}$COOH did. Although the reactivity of trienols was also decreased with lowering the pH of the assay mixture, $C_{15:3}$OH always showed the maximum reactivity independently on the pH. Because the terminal carboxyl group of fatty acids was not ionized at neutral or acidic pH, L-1 was thought not to differentiate between fatty acids and fatty alcohols under these assay conditions. This indicates the importance of a terminal anionic group to accelerate the L-1 reaction.

To examine the positional selectivity in the oxygenation, hydroperoxides formed during incubation of C_{15}~C_{22}-trienoic acids and C_{13}~C_{19}-trienols with L-1 were analyzed by straight phase HPLC. All trienoic acids were shown to be selectively oxygenated at ω6 carbon to form ω6-hydroperoxy-(ω3*Z*,ω7*E*,ω9*Z*)-acids (Table I). The position of oxygenation to $C_{20:3}$COOH which showed the highest reactivity among all the acids was ascertained with GC-MS analysis in a form of methyl 15-hydroxy-icosatrienoate: *m/z* 336 (M^+), 318 (M^+-H_2O), 305 (M^+-OCH_3), 267 ($M^+-(CH_3-CH_2-CH=CH-CH_2)$). Next to the ω6 carbon, ω7 carbon was more oxygenated than both ω3 and ω10 carbons were. With most of the trienols, the major lipoxygenase products were ω6-hydroperoxy-(ω3*Z*,ω7*E*,ω9*Z*)-alcohols but $C_{15:3}$OH was oxygenated at ω10 more than at ω6 carbon in the ratio of ω10/ω6 = 52/48 (Table II). The optical purity of the major geometrical isomers formed from trienoic acids was analyzed by chiral phase HPLC. All the ω6-hydroperoxy-(ω3*Z*,ω7*E*,ω9*Z*) isomers were formed to be over 95% *e.e.* with *S*-configuration.

These results indicate that L-1 recognizes hydrophobic region of ω-terminal and/or pentadiene system of fatty acid stronger than carboxylate anion to settle a substrate in proper spatial orientation. This idea was partly confirmed by analyses of the product specificity for trienols which had no functional group to offer ionic interaction with L-1 but still had the hydrophobic moiety and the pentadiene system. But $C_{15:3}$OH was an exception to the hypothesis described above because ω10 carbon of this alcohol was oxygenated almost equally to ω6 carbon was. This result could be explained by a spatially inverse orientation of this fatty alcohol at the active site of the enzyme as compared with linolenic acid. Because the hydrophobic region due to five methylene chains (position 1 to 5) in $C_{15:3}$OH was considered to be almost identical to ω-terminal of linoleic acid, part of this fatty alcohol might be arranged inversely in the

Table I. Product specificity of L-1 with C_{15}~C_{22}-trienoic acid.

Total carbon number (C_n)	C_{15}	C_{16}	C_{17}	C_{18}	C_{19}	C_{20}	C_{21}	C_{22}
Structure								
ω3(ω4*E*,ω6*Z*,ω9*Z*)*[1]	2.2	1.0	2.1	3.0	2.7	0.1	3.0	2.8
ω3(ω4*E*,ω6*E*,ω9*Z*)	2.1	1.2	0.3	1.5	0.5	0.2	0.7	1.0
ω6(ω3*Z*,ω7*E*,ω9*Z*)	81.1	80.2	86.3	86.7	86.7	85.2	81.2	85.8
ω6(ω3*Z*,ω7*E*,ω9*E*)	13.5	6.7	1.4	1.3	1.3	2.0	2.7	0.7
ω7(ω3*Z*,ω5*E*,ω9*Z*)	5.0	1.1	4.4	3.6	3.6	8.0	4.9	3.2
ω7(ω3*E*,ω5*E*,ω9*Z*)	4.8	5.4	2.1	2.2	2.2	4.0	2.4	3.8
ω10(ω3*Z*,ω6*Z*,ω8*E*)	0.8	2.4	0.1	2.5	2.5	0.3	2.1	1.3
ω10(ω3*Z*,ω6*E*,ω8*E*)	2.4	2.1	3.2	0.5	0.5	0.2	1.6	1.0

*[1], ω3(ω4*E*,ω6*Z*,ω9*Z*); ω3-hydroperoxy-(ω4*E*,ω6*Z*,ω9*Z*)-trienoic acid. All the other positional and geometrical isomers are symbolized in the same manner.

Table II. Product specificity of L-1 with C_{13}~C_{19}-trienols.

Total carbon number (C_n)	C_{13}	C_{14}	C_{15}	C_{16}	C_{17}	C_{18}	C_{19}
Structure							
ω3(ω4*E*,ω6*Z*,ω9*Z*)*[1]	1.6	0.8	3.4	5.2	2.8	1.3	3.3
ω3(ω4*E*,ω6*E*,ω9*Z*)	8.8	2.9	2.4	2.1	0.3	0.5	0.8
ω6(ω3*Z*,ω7*E*,ω9*Z*)	78.6	84.2	38.9	86.8	91.2	92.6	84.3
ω6(ω3*Z*,ω7*E*,ω9*E*)	1.1	4.0	5.0	1.0	0.3	0.4	1.6
ω7(ω3*Z*,ω5*E*,ω9*Z*)	4.1	1.4	0.6	2.6	1.6	2.1	1.6
ω7(ω3*E*,ω5*E*,ω9*Z*)	4.2	4.0	1.6	1.6	1.3	0.7	5.1
ω10(ω3*Z*,ω6*Z*,ω8*E*)	1.0	0.1	47.6	0.2	2.0	0.9	0.7
ω10(ω3*Z*,ω6*E*,ω8*E*)	0.6	0.7	0.6	0.6	0.6	1.4	1.1

*[1], ω3(ω4*E*,ω6*Z*,ω9*Z*); ω3-hydroperoxy-(ω4*E*,ω6*Z*,ω9*Z*)-trienol. All the other positional and geometrical isomers are symbolized in the same manner.

reaction center of L-1. Recognition of a substrate in spatially inverse orientation at the active site of the enzyme as compared with conventional orientation caused by linolenic acid was reported in case of double dioxygenation of arachidonic acid by soybean L-1 [7]. The high reactivity of $C_{15:3}$OH is thought to be due to the existence of two oxygenative sites within this alcoholic substrate.

In summary, soybean L-1 recognize both (ω6*Z*,ω9*Z*)-pentadiene and hydrophobic region consisting of ω-alkyl chain extending to the (ω6*Z*)-double bond first of all and the terminal carboxyl group is important to accelerate the activity of L-1.

Acknowledgements

This work was financially supported by the Grant-in-Aid for Scientific Research on Priority Areas No. 02250228 from the Ministry of Education, Science and Culture, Japan and Nihon Seimei Foundation.

[1] D. Shibata, J. Steczko, J. E. Dixon, M. Hermodson, R. Yazdanparast, and B. Axelrod, J. Biol. Chem. **262,** 10080 (1987).
[2] D. Shibata, J. E. Dixon, P. C. Andrews, M. Hermodson, and B. Axelrod, J. Biol. Chem. **263,** 6816 (1988).
[3] J. F. G. Vliegenthart and G. A. Veldink, in: Free Radicals in Biology (W. A. Pryor, ed.), **Vol. 5,** p. 29, Academic Press, New York.
[4] M. R. Egmond, P. M. Fasella, G. A. Veldink, J. F. G. Vliegenthart, and J. Boldingh, Eur. J. Biochem. **76,** 469 (1977).
[5] A. Hatanaka, T. Kajiwara, K. Matsui, and M. Yamaguchi, Z. Naturforsch. **44c,** 64 (1989).
[6] B. Axelrod, T. M. Cheesbrough, and S. Laakso, Methods Enzymol. **71,** 441 (1981).
[7] C. P. A. Van Os, G. P. M. Rijke-Schilder, H. V. Halbeek, J. Verhagen, and J. F. G. Vliegenthart, Biochim. Biophys. Acta **663,** 177 (1981).