Product Specificity during Incubation of Methyl Linoleate with Soybean Lipoxygenase-I

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Z. Naturforsch. **39 c**, 171–173 (1984);
received September 13, 1983

Product Specificity, Linoleate, Lipoxygenase-I

The ratios of 13:9 isomers of the hydroperoxides produced during incubation of methyl linoleate with soybean lipoxygenase-I were ca. 50:50 at pH 7.8 and ca. 70:30 at pHs 9.0 and 11.0, respectively. In striking contrast to the lipoxygenase-catalyzed hydroperoxidation of linoleic acid, the hydroperoxidation of methyl linoleate at pH 7.8 gave almost racemic 13-hydroperoxides and that at pHs 9.0 and 11.0 yielded methyl 13-L-hydroperoxylinoleate having low optical purities (L/D, ca. 45/30).

We have been preparing 13-L-(*Z*, *E*)-HPOM to use for a substrate of the hydroperoxide lyase solubilized from tea chloroplasts [1], using soybean lipoxygenase-I. Soybean lipoxygenase (linoleate: oxygen oxidoreductase, E.C. 1.13.1.13) is a non-heme dioxygenase which catalyzes the hydroperoxidation of polyunsaturated fatty acids and their esters containing a (1*Z*, 4*Z*)-pentadiene system [2, 3]. The product specificity of hydroperoxidation of the free fatty acids by soybean lipoxygenase has been investigated in detail [4–6]. With their methyl esters as substrates, however, the product specificity, especially enantiospecificity of the enzymatic hydroperoxidation has been scarcely elucidated so far [7]. On the other hand, Chan *et al.* have reported that regioselectivity alone is no longer a sufficient criterion for enzymatic hydroperoxidation; enantioselectivity appears to be the only valid criterion [8].

In this connection, the product specificity from LM by soybean lipoxygenase-I was characterized by comparison of the proportions of the positional, geometrical and enantiomeric isomers of HPOM and HPOA produced from LM and LA at pH 9.0 with those at pHs 7.8 and 11.0 according to HPLC and GLC analyses, respectively.

Lipoxygenase-I (108 units, Sigma type V which is electrophoretically homogeneous material) was added to a stirred suspension of LM (500 mg, 99%, prepared by methylation of 99% LA) in 200 ml of buffers containing 0.1% Tween 20 or LA (500 mg, 99%, purchased from Wako Pure Chemical Industries Ltd.) in 200 ml of buffers alone (McIlvaine's buffer pH 7.8, 10 mM Borate buffer pH 9.0, 10 mM Ringer buffer pH 11.0). After the complete reaction mixture was incubated at 5 °C for 90 min with bubbling oxygen, the reaction products were extracted with ether. The ether extract of the reaction products of LA was concentrated *in vacuo*

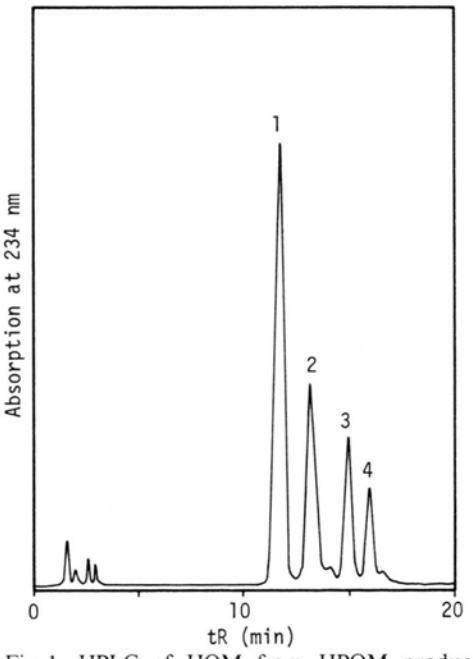

Fig. 1. HPLC of HOM from HPOM produced during incubation of soybean lipoxygenase-I with LM at pH 11.0. **1:** 13-(*Z*, *E*)-HOM; **2:** 13-(*E*, *E*)-HOM; **3:** 9-(*E*, *Z*)-HOM; **4:** 9-(*E*, *E*)-HOM. Analytical conditions: eluting solvent; *n*-hexane:ethanol = 99.5:0.5 (V/V), 2 ml/min on Zorbax SIL column (4.6 mm ∅ × 250 mm) which is packed with silica gel, Shimadzu Dupont Liquid Chromatograph LC-2.

Abbreviations: LA, linoleic acid; LM, methyl linoleate; HPOA, hydroperoxy-octadecadienoic acid; HOA, hydroxy-octadecadienoic acid; 13-HPOA, 13-hydroperoxy-9,11-octadecadienoic acid; 9-HPOA, 9-hydroperoxy-10,12-octadecadienoic acid; 13-(*Z*, *E*)-HPOA, 13-hydroperoxy-(9*Z*, 11*E*)-octadecadienoic acid; 13-(*E*, *E*)-HPOA, 13-hydroperoxy-(9*E*, 11*E*)-octadecadienoic acid; 9-(*E*, *Z*)-HPOA, 9-hydroperoxy-(10*E*, 12*Z*)-octadecadienoic acid; 9-(*E*, *E*)-HPOA, 9-hydroperoxy-(10*E*, 12*E*)-octadecadienoic acid; MTPA, α-methoxy-α-trifluoromethylphenylacetic acid; HOHM, methyl 2-hydroxyheptanoate; HOSM, dimethyl 2-hydroxysebacate; HPLC, high-performance liquid chromatography; GLC, gas liquid chromatography. The methyl esters corresponded to HPOA and HOA refer to HPOM and HOM, respectively.

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0341-0382/84/0100-0171 $ 01.30/0

Table I. Compositions of positional, geometrical and enantiomeric isomers of HPOM and HPOA formed by soybean lipoxygenase-I from LM and LA.

pH	Substrates	Geometrical isomers[a]				Optical isomers[b]				Positional[a] isomers		Conversion ratios [%]
		13-HPO[c]		9-HPO[c]		13-HPO[c]		9-HPO[c]		HPO[c]		
		(*Z*, *E*)	: (*E*, *E*)	: (*E*, *Z*)	: (*E*, *E*)	D	: L :	D	: L	13	: 9	
7.8	LM	35	19	29	17	30	24	16	30	54	46	3.5
	LA	83	3	11	3	11	75	10	4	86	14	54.0
9.0	LM	49	19	18	14	23	45	14	18	68	32	5.3
	LA	83	5	7	5	8	80	6	6	88	12	64.0
11.0	LM	53	20	17	10	31	42	11	16	73	27	4.7
	LA	88	4	4	4	7	85	4	4	92	8	64.0

[a] Analyzed as HOM by HPLC.
[b] Analyzed as HOHM and HOSM by GLC [glass column 3 mm ∅ × 3 m, 15% Silicone DC-QF-1 on Chromosorb W (AW-DMCS), 80–100 mesh; column temperature, 190–230 °C (1 °C/min); injector temperature, 250 °C; detector temperature, 250 °C; flow rate, 60 ml/min].
[c] HPO refers to HPOM when LM was used as a substrate; HPOA for LA substrate.

to leave crude products which were purified by column chromatography (SiO_2 for dry column chromatography, Woelm Pharma, W. Germany; eluent, pet. ether/diethyl ether = 9/1 ~ 5/1) to give HPOM. The HPOA was separated from the reaction mixture of LA as previously reported [9, 10].

The separated HPOM was reduced to the corresponding HOM by reduction with $NaBH_4$ in methanol-10 mM Borate buffer (1/1). The HPOA was converted to the hydroxy-ester (HOM) by $NaBH_4$ reduction followed by esterification with CH_2N_2. By HPLC analysis of aliquots of the HOM thus obtained, the proportions of positional and geometrical isomers of the parent HPOM and HPOA were analyzed according to Chan's method as shown in Fig. 1 [11]. On the other hand, the remainders of the HOM were converted to diastereomeric MTPA esters using (R) − (+)-MTPA according to Mosher's method [12], which were led to a mixture of the diastereomeric MTPA esters of HOHM (from the 13-isomers) and HOSM (from the 9-isomers) by oxidative ozonolysis of the MTPA esters of HOM and subsequent methylation with CH_2N_2. The enantiomeric compositions of the parent HPOM and HPOA were determined by GLC analysis of the mixture of the diastereomeric MTPA esters of HOHM and HOSM according to the previously reported method [9, 10].

The percentage compositions of the positional, geometrical and optical isomers of the hydroperoxides were obtained by measuring the area of the components and the results are summarized in Table I. The lipoxygenase-catalyzed conversion ratios of LM to HPOM were ca. ~ 10% of those of LA. When LM was incubated with heat-denatured lipoxygenase under the same condition as the enzymatic reaction, ~ 0.9% of LM used was converted to HPOM. As seen in Table I, the ratios of 13-HPOM : 9-HPOM from LM were ca. 50 : 50 at pH 7.8 and ca. 70 : 30 at pHs 9.0 and 11.0, respectively. In contrast to the 13-HPOA from LA, the 13-HPOM formed from LM at pH 7.8 was almost racemic, whereas the 13-L-(*Z*, *E*)-HPOM produced at pHs 9.0 and 11.0 were low in optical purities (L/D, ca. 45/30). Also the racemic 9-HPOM were produced as minor components during the incubation with LM at pHs 9.0 and 11.0. The regioselectivity of the lipoxygenase-catalyzed hydroperoxidation of LM at pHs 9.0 and 11.0 under conditions used or usual conditions was high as compared with that of LA, whereas the enantioselectivity of the reaction of LM was low.

As the lipoxygenase-I-catalyzed hydroperoxidation of LM has been known to be regioselective so far, the enzymatic reaction of LM has been deduced to proceed enantiospecifically like in the case of LA. Thus, it should be noted that the structural change of carboxyl group of LA to methyl ester group causes not only decrease of the hydroperoxidation rate but also depression of the enantiospecificity.

Acknowledgements

The authors would like to express their thanks to Miss K. Mizoguchi for her skilled technical assistance. Financial support from the Ministry of Education Science and Culture (Research No. 58560205) is gratefully acknowledged.

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