BBA 66761

FACTORS INFLUENCING THE POSITIONAL SPECIFICITY OF SOYBEAN LIPOXYGENASE*

JOHN P. CHRISTOPHER**, ELFRIEDE K. PISTORIUS, FRED E. REGNIER AND BERNARD AXELROD

Department of Biochemistry, Purdue University, West Lafayette, Ind. 47907 (U.S.A.)

(Received May 2nd, 1972)

SUMMARY

1. The positional specificity of hydroperoxidation of two isoenzymes (lipoxygenases-1 and -2) of the lipoxygenase (linoleate: O_2 oxidoreductase, EC 1.13.1.13) from soybean has been investigated under a number of experimental conditions, *i.e.* temperature, substrate, pH, O_2 tension, and presence or absence of Ca^{2+}.

2. Values for the ratio of 9- and 13-hydroperoxide isomers were calculated after combined gas–liquid chromatographic–mass spectral analysis of the corresponding 9- and 13-hydroxymethyl stearates derived from the hydroperoxides by chemical modification.

3. The results show that not only are the experimental conditions under which the enzyme is incubated important in determining the position oxygenated but also the composition of the lipoxygenase preparation with regard to its isoenzyme content.

4. Results obtained with methyl linoleate as substrate indicate that the variation in values for isomer ratios is not unique to the linoleic acid substrate.

INTRODUCTION

Soybean lipoxigenase (linoleate: O_2 oxidoreductase, EC 1.13.1.13) catalyzes the hydroperoxidation of polyunsaturated fatty acids and esters containing a *cis*, *cis*-1,4-pentadiene system. Several reports have appeared with regard to the positional specificity of hydroperoxidation exhibited by preparations of soybean lipoxygenase. The values reported for the ratio of 13- to 9-hydroperoxides formed from linoleic acid with soybean preparations varied over a significant range. Dolev *et al.*[1] found exclusive formation of the 13-hydroperoxide while other reported values for the ratio of 13- to 9-hydroperoxides are: 95:5 by Eriksson and Leu[2], 92:8 by Hamberg and Samuelsson[3], 90:10 by Veldink *et al.*[4], 80:20 by Zimmerman and Vick[5], and 70:30 by Chang *et al.*[6], Veldink *et al.*[7], and Hamberg and Samuelsson[8]. Zimmerman and Vick[5]

* This is Journal Paper No. 4731 of the Purdue University Agricultural Experiment Station.

** Present address: Biology Department, University of California, San Diego, Calif. 92037.

attributed this wide range of values to various degrees of autoxidation of the linoleic acid substrate.

In their study of lipoxygenase from potato tubers, Galliard and Phillips[9] also examined the positional specificity of the soybean enzyme for comparative purposes. With soybean lipoxygenase, they reported a ratio of 54:46 in favor of the 9-isomer at pH 5.5, whereas, at pH 9.2 the ratio was 26:74 in favor of the 13-isomer. It was suggested that these results could be explained by the existence of two types of lipoxygenase activity: one active at acidic pH and specific for the 9-position and one active at alkaline pH and specific for the 13-position.

HAMBERG[10] has recently developed an ingeneous technique for the complete steric analysis of reaction products. He reported values for the ratio of 9- to 13-hydroperoxide isomers from 4:96 to 10:90 by variations in pH and source of lipoxygenase. He suggested two possibilities to account for the specific formation of the 9-isomer: that reverse orientation of the substrate molecule on the enzyme surface could lead to 9-isomer formation or that preparations of soybean lipoxygenase could be contaminated with another lipoxygenase specific for the 9-position.

We recently made a preliminary report[11] on the positional specificity of two isoenzymes of lipoxygenase. Under the conditions employed, the classical isoenzyme of Theorell *et al.*[12] (referred to as lipoxygenase-1) gave a ratio of 95:5 in favor of the 13-isomer, whereas, lipoxygenase-2 (ref. 13) produced a 50:50 mixture of the isomers. We now report a more extensive, comparative study of the positional specificity of both isoenzymes under a variety of conditions.

MATERIALS

Lipoxygenases-1 and -2 were prepared as previously described[13] and shown to be homogeneous by disc gel electrophoresis at pH 9.5. Linoleic acid and methyl linoleate, having negligible peroxide values, were purchased from Hormel Institute (Univ. of Minn.), $NaBH_4$ from Ventron, Diazald from Aldrich, and platinum oxide from Matheson Coleman and Bell. The 9- and 12-hydroxy isomers of methyl stearate were obtained from NuCheck Prep.

METHODS

Substrate solutions

Linoleic acid was prepared as a 10^{-2} M aqueous solution containing 0.35% Tween-20 (v/v). In addition, both linoleic acid and methyl linoleate were prepared as $8.8 \cdot 10^{-2}$ and $8.5 \cdot 10^{-2}$ M solutions in 95% ethanol, respectively.

Enzyme incubations

All incubations were conducted in a final volume of 18 ml for 45 min with no agitation under various conditions.

Linoleic acid–Tween 20 substrate. Each isoenzyme (0.05 mg) was incubated with 1.8 mM linoleic acid at three pH values: 0.047 M sodium phosphate (pH values 7 and 8) and 0.047 M sodium borate (pH 9). Parallel incubations were carried out under two conditions of oxygen tension and temperature: (a) solutions were gassed with O_2 at 0 °C and (b) solutions were left open to the atmosphere at 25 °C.

Linoleic acid–ethanol substrate. Each isoenzyme (0.05 mg) was incubated with 1 mM linoleic acid at 0 °C in O_2-gassed solutions under the above conditions of pH as well as in the presence (0.55 mM) and the absence of Ca^{2+}.

Methyl linoleate–ethanol substrate. Each isoenzyme (0.05 mg) was incubated with 0.9 mM methyl linoleate at 0 °C in O_2-gassed solutions at 2 pH values: 0.047 M sodium phosphate (pH 7) and 0.047 M sodium borate (pH 9).

The extent of the reaction was followed by measuring the increase in absorption at 234 nm of aliquots of the diluted reaction mixture. Formation of secondary products was similarly followed by absorption at 280 nm.

Hydroxyperoxides

The hydroperoxides were converted to the 9- and 13-hydroxy isomers of methyl stearate. All incubations were treated in a similar manner to minimize artifacts.

Reduction. Hydroperoxides were reduced by addition of 15 mg $NaBH_4$ to each reaction mixture. Reaction mixtures at pH values 7 and 8 were adjusted to pH 9 with 2 M NaOH prior to reduction. Solutions were stirred magnetically for 1 h at room temperature, while maintaining a constant stream of N_2 gas over the surface.

Methyl esterification. The above mixtures were acidified to pH 3 with concentrated HCl and extracted 4 times with equal volumes of diethyl ether. Ether extracts were treated with anhydrous Na_2SO_4 and taken to near dryness under N_2. Esterification was then performed by generating diazomethane from Diazald according to Schlenk and Gellerman[14]. The complete destruction of excess reagent, which was accomplished by addition of a solution of acetic acid–diethyl ether (1:1, v/v), was indicated by disappearance of the yellow color.

Hydrogenation. The solutions from above were evaporated to dryness under N_2 and taken up in 2 ml of absolute ethanol. Platinum oxide (5 mg) was added to each and hydrogenation performed under 40 lb/inch^2 of hydrogen at room temperature for 3 h with shaking.

Quantitative analysis of isomer compositions. The unresolved, chemically-modified, enzymic reaction mixtures were analyzed for the ratios of 9- and 13-hydroxy isomers of methyl stearate by combined gas–liquid chromatography–mass spectroscopy. The analyses were performed on an LKB 9000 gas chromatograph–mass spectrometer. The gas chromatograph was equipped with a 12 ft × 1/16 inch internal diameter column packed with 3% Dexsil 300 on Gas Chrom Q. Chromatography was carried out isothermally at 240 °C with a helium gas flow rate of 15 ml/min. The conditions for mass spectroscopy were: source temperature, 270 °C; ionizing voltage, 70 eV; accelerating voltage, 3.5 kV. Isomer ratios were determined from the relative intensities (M. K. Rohwedder, personal communication) of mass fingerprint peaks, *i.e.* mass peak 155 for the 9-isomer and mass peak 211 for the 13-isomer[15]. The accuracy of the intensities obtained by measuring line heights on the most favorable scale was ± 4%. Operating conditions for gas–liquid chromatography were chosen to insure that both isomers would be resolved from other components in the reaction mixtures, but not from each other.

RESULTS

That the hydroxy isomers of methyl stearate are not separable under the con-

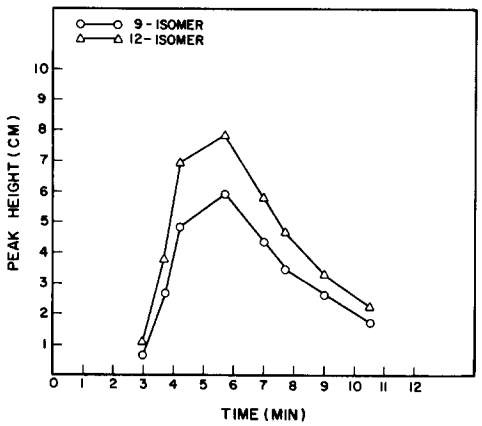

Fig. 1. Peak heights of fingerprint ions of the 9- and 12-hydroxy isomers of methyl stearate as a function of position in effluent. A standard solution containing both isomers was analyzed by combined gas–liquid chromatography–mass spectroscopy (see Methods).

ditions of gas–liquid chromatography was indicated by the result (Fig. 1) obtained with a mixture of standard 9- and 12-hydroxy isomers. A slow injection of this mixture was purposely made. The ratio of intensities of mass peaks 155 and 197 remained essentially constant throughout the chromatographic curve, which exhibited a retention time of 5.5 min. It was also shown that the 9- and 13-isomers were unresolved by determining the ratios of the intensities of their respective fingerprint mass peaks, 155 and 211, at different positions throughout the peak. Several unresolved reaction mixtures were randomly checked in this fashion.

When linoleic acid dispersed in Tween-20 was employed as substrate in O_2-gassed solutions at 0 °C, pH 7, lipoxygenase-2 favored the 9-position while lipoxygenase-1 favored the 13-position (Table I). A dependence on pH was observed with lipoxygenase-1. In addition, it was shown that the ratios obtained for lipoxygenase-1 were also dependent on O_2 tension and/or temperature. When solutions were left

TABLE I

CONTENT OF HYDROPEROXIDE ISOMERS IN INCUBATIONS OF EACH ISOENZYME WITH LINOLEIC ACID DISPERSED IN TWEEN 20 AS SUBSTRATE

L-1, lipoxygenase-1; L-2, lipoxygenase-2.

		Conditions			
		Solutions gassed with O_2 at 0 °C		*Solutions open to atmosphere at 25 °C*	
		L-1	*L-2*	*L-1*	*L-2*
pH 7	9-isomer (%)	24	60	52	70
	13-isomer (%)	76	40	48	30
pH 8	9-isomer (%)	15	ND*	46	67
	13-isomer (%)	85	ND	54	33
pH 9	9-isomer (%)	10	ND	52	55
	13-isomer (%)	90	ND	48	45

* Isomers were not detected.

TABLE II

CONTENT OF HYDROPEROXIDE ISOMERS IN INCUBATIONS OF EACH ISOENZYME WITH LINOLEIC ACID DISPERSED IN ETHANOL AS SUBSTRATE

Solutions were gassed with O_2 at 0 °C. Ca^{2+} was either present (0.55 mM) or absent.

	pH 7				*pH 8*				*pH 9*			
	9-isomer (%)		*13-isomer (%)*		*9-isomer (%)*		*13-isomer (%)*		*9-isomer (%)*		*13-isomer (%)*	
	$+Ca^{2+}$	$-Ca^{2+}$	$+Ca^{2+}$	$-Ca^{2+}$	$+Ca^{2+}$	$-Ca^{2+}$	$+Ca^{2+}$	$-Ca^{2+}$	$+Ca^{2+}$	$-Ca^{2+}$	$+Ca^{2+}$	$-Ca^{2+}$
Lipoxygenase-1	32	28	68	72	18	10	82	90	11	8	89	92
Lipoxygenase-2	48	38	52	62	48	59	52	41	53	60	47	40

open to the atmosphere at 25 °C, the enzyme exhibited nearly the same positional specificity at three pH values. However, lipoxygenase-2 again showed an apparent preference for the 9-position at all three pH values, although the ratios were more in favor of the 9-isomer at lower pH.

The results obtained for incubations employing linoleic acid dispersed in ethanol as substrate are summarized in Table II. Lipoxygenase-1 favored the 13-position under all conditions, *i.e.* pH and presence and absence of Ca^{2+}. However, lipoxygenase-2 in the presence of Ca^{2+} gave a 50:50 mixture of the isomers at all three pH values. In the absence of Ca^{2+}, the ratio was 38:62 for the 13-isomer at pH 7 and 60:40 for the 9-isomer at pH 9.

TABLE III

CONTENT OF HYDROPEROXIDE ISOMERS IN INCUBATIONS OF EACH ISOENZYME WITH METHYL LINOLEATE DISPERSED IN ETHANOL AS SUBSTRATE

Solutions were gassed with O_2 at 0 °C.

	pH 7		*pH 9*	
	9-isomer (%)	*13-isomer (%)*	*9-isomer (%)*	*13-isomer (%)*
Lipoxygenase-1	24	76	19	81
Lipoxygenase-2	42	58	57	43

TABLE IV

YIELD OF HYDROPEROXIDES IN INCUBATION OF EACH ISOENZYME WITH LINOLEIC ACID OR METHYL LINOLEATE AS SUBSTRATE UNDER VARIOUS CONDITIONS

Yield is expressed in %. L-1, lipoxygenase-1; L-2, lipoxygenase-2.

pH	*Linoleic acid–Tween 20*				*Linoleic acid–ethanol*				*Methyl linoleate–ethanol*	
	0 °C		*25 °C*		$+Ca^{2+}$		$-Ca^{2+}$			
	L-1	*L-2*	*L-1*	*L-2*	*L-1*	*L-2*	*L-1*	*L-2*	*L-1*	*L-2*
7	47	29	21	17	82	60	82	33	11	11
8	47	7	20	7	80	62	82	6	—	—
9	49	2	23	7	64	11	78	6	18	10

When methyl linoleate was employed as substrate, lipoxygenase-1 produced more 13-isomer at both pH values whereas lipoxygenase-2 gave values of 42:58 at pH 7 and 57:43 at pH 9 for the ratio of 9- to 13-isomer (Table III).

Since the enzymatic reactions did not progress to equal extents the yield of hydroperoxide attained under the diverse conditions tested is shown in Table IV.

DISCUSSION

With regard to the ratio of the 9-isomer to the 13-isomer when linoleic acid was the substrate, both isoenzymes exhibited variability depending on the reaction conditions. Lipoxygenase-1 showed a wide range of ratios: from 92:8 in favor of the 13-isomer to 52:48 in favor of the 9-isomer. Lipoxygenase-2 also varied but not to such an extent, from 70:30 in favor of 9-isomer to 62:38 in favor of the 13-isomer. Thus, it appears that at least two factors must be considered when interpreting data concerning the positional specificity of soybean lipoxygenases: not only are conditions such as temperature, pH, dispersing agent, possibly Ca^{2+} concentration, and O_2 tension under which the enzyme is incubated important, but also the composition of the lipoxygenase preparation with regard to its isoenzyme content.

In addition, data obtained with the fatty acid ester, methyl linoleate, indicate that the variation in values for isomer ratios is not unique to the linoleic acid substrate. A pH dependence was observed with both isoenzymes as well as a characteristic difference between the two under comparable conditions.

Another consideration in any examination of isomer ratios is the formation of products other than hydroperoxides during the enzymic reaction. Several workers have noted the accumulation of secondary reaction products[16–18] which are carbonyls, and absorb strongly in the vicinity of 280 nm. In most of the mixtures analyzed above, only a negligible amount of material absorbing at $A_{280\ nm}$ was present, although dimers were found to a significant degree in the linoleic acid–ethanol systems. It is conceivable that only one of the hydroperoxide isomers is utilized in the formation of these compounds, thus, indirectly biasing the ratio in favor of the unaltered isomer.

REFERENCES

1 A. Dolev, W. K. Rohwedder and H. J. Dutton, *Lipids*, 2 (1967) 28.
2 C. E. Eriksson and K. Leu, *Lipids*, 6 (1971) 144.
3 M. Hamberg and B. Samuelsson, *J. Biol. Chem.*, 242 (1967) 5329.
4 G. A. Veldink, J. F. G. Vliegenthart and J. Boldingh, *Biochem. J.*, 120 (1970) 55.
5 D. C. Zimmerman and B. A. Vick, *Lipids*, 5 (1970) 392.
6 C. C. Chang, W. J. Esselman and C. O. Clagett, *Lipids*, 6 (1971) 100.
7 G. A. Veldink, J. F. G. Vliegenthart and J. Boldingh, *Biochim. Biophys. Acta*, 202 (1970) 198.
8 M. Hamberg and B. Samuelsson, *Biochem. Biophys. Res. Commun.*, 21 (1965) 531.
9 T. Galliard and D. R. Phillips, *Biochem. J.*, 124 (1971) 431.
10 M. Hamberg, *Anal. Biochem.*, 43 (1971) 515.
11 J. Christopher and B. Axelrod, *Biochem. Biophys. Res. Commun.*, 44 (1971) 731.
12 H. Theorell, R. T. Holman and Å. Åkeson, *Acta Chem. Scand.*, 1 (1947) 571.
13 J. Christopher, E. Pistorius and B. Axelrod, *Biochim. Biophys. Acta*, 198 (1970) 12.
14 H. Schlenk and J. L. Gellerman, *Anal. Chem.*, 32 (1960) 1412.
15 R. Ryhage and E. Stenhagen, *Mass Spectrometry of Organic Ions*, Academic Press, New York, 1963, p. 435.
16 O. S. Privett, C. Nickell, W. O. Lundberg and P. D. Boyer, *J. Am. Oil Chem. Soc.*, 32 (1955) 505.
17 E. Vioque and R. T. Holman, *Arch. Biochem. Biophys.*, 99 (1962) 522.
18 G. J. Garssen, J. F. G. Vliegenthart and J. Boldingh, *Biochem. J.*, 122 (1971) 327.