Effect of Substrate Polarity on the Activity of Soybean Lipoxygenase Isoenzymes

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ABSTRACT

In order to characterize the several isoenzymes of soybeans, they were examined with respect to the effect of the polar nature of the substrate. In general, lipoxygenase-1 was most active when presented with charged substrates such as the anionic form of linoleic acid or of potassium linoleyl sulfate, whereas lipoxygenase-2 and -3 preferred nonpolar substrates such as unionized linoleic acid, methyl linoleate, linoleyl methane sulfonate, 10,13-nonadecadieneamine, or linoleyl acetate. Linoleyl sulfate, which has been advanced as an excellent readily soluble substrate for lipoxygenase, was indeed the best substrate found for lipoxygenase-1. Lipoxygenase-2 and -3 were, by contrast, totally inactive against this substrate. The favorable response of linoleic acid to lipoxygenase-2 and -3 at pH 6.8 was ascribed to the anomalously high pK_a value of linoleic acid compared to that of short chain carboxylic acids. The pH-activity profile obtained with lipoxygenase acting on linoleyl sulfate (which was charged at all pH values examined) was shifted to lower pH values compared to the linoleic acid activity profile. The effect of changing from the charged to the uncharged substrate, when tested against lipoxygenase-1, was to increase the K_m by an order of magnitude.

INTRODUCTION

Lipoxygenase (EC 1.13.11.12), an iron-containing dioxygenase present in many plants, catalyzes the peroxidation of polyunsaturated fatty acids containing the *cis*-1,*cis*-4-pentadiene moiety (1). Three electrophoretically pure isoenzymes of lipoxygenase have been isolated from soybeans (2,3). These differ with respect to amino acid content, pH optima, positional specificity for oxygenation, and secondary product formation (2-5). We have examined the behavior of the three isoenzymes against a variety of linoleic acid derivatives and find that the enzymes differ markedly among each other with respect to their preference for the ionic or polar nature of the linoleic acid derivative.

MATERIALS AND METHODS

Enzyme Purification and Assay

The isoenzymes of soybean lipoxygenase were purified by the procedure of Christopher et al. (2,3) from soybeans of Amsoy variety (1975 crop). Lipoxygenase activities were determined using a Clark oxygen electrode (Yellow Springs) in a Gilson Medical Electronics Oxygraph, Model KM, as previously described (2,3). The reaction vessel had a volume of 1.5 ml. One unit of enzyme activity corresponds to the consumption of 1 μmole of O_2 per min. Assays were conducted at 15 C in 0.2 *M* phosphate buffer, pH 6.8, for lipoxygenase-2 and -3 and in 0.2 *M* borate buffer, pH 9.0, for lipoxygenase-1 unless otherwise indicated.

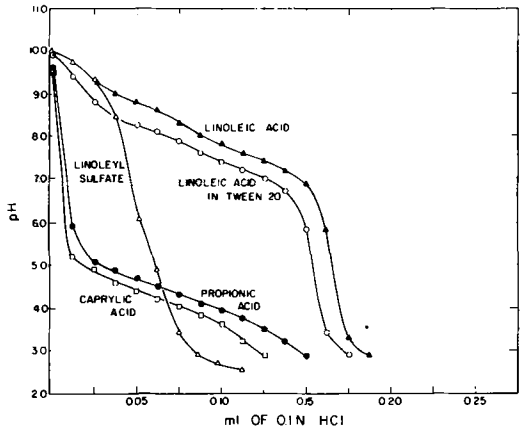

FIG. 1. Titration curves of linoleic acid and the potassium salt of linoleyl sulfate. All acids (0.1 *N*) were made alkaline with NaOH and titrated with 0.1 *N* HCl in a Radiometer Titrigraph. Linoleic acid was prepared in Tween 20 (-○-○-○-) or in 95% ethanol (-▲-▲-▲-). The potassium salt of linoleyl sulfate was prepared as an aqueous solution (-△-△-△-). Propionic acid (-●-●-●-) and caprylic acid (-□-□-□-) are shown for comparison.

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TABLE I

Isoenzyme Reaction Velocities with Linoleic Acid Derivatives

Substrate[a]	Velocity, μmole O_2/min/mg protein					
	Lipoxygenase-1		Lipoxygenase-2		Lipoxygenase-3	
	pH 9.0		pH 6.8		pH 6.8	
Linoleic acid	154		89		4	
Methyl linoleate	16		46		8	
Linoleyl alcohol	34		25		4	
Linoleyl methane sulfonate	27		160		7	
10,13-Nonadecadiene nitrile	11		58		7	
Linoleyl chloride	97		62		5	
Linoleyl acetate	9		35		3	
	pH 6.8	pH 9.0	pH 6.8	pH 9.0	pH 6.8	pH 9.0
Linoleic acid in Tween	4	157	32	<1	57	<1
Potassium linoleyl sulfate (aqueous)	146	216	<1	<1	<1	<1
10,13-Nonadecadieneamine	56	80	21	<1	<1	<1

[a]All assays at 15 C. All substrates prepared at a final concentration of 6.67×10^{-4} *M* in ethanol unless otherwise indicated. pH 9.0 assay buffer was 0.2 *M* borate; pH 6.8 assay buffer was 0.2 *M* phosphate.

Protein concentrations were calculated from absorbance values at 280 nm. Measurement of the dry weight of purified lipoxygenase gave a value of 0.7 mg protein per ml per absorbance unit for all three isoenzymes.

Substrates

Potassium linoleyl sulfate was prepared from linoleyl alcohol by the procedure of Allen (6). The white solid gave a single spot on a thin layer chromatogram (Silica Gel G) developed in hexane-ether-acetic acid (60:40:1). 10,13-Nonadecadieneamine was prepared from 10,13-nonadecadiene nitrile by the method of Yoon and Brown (7) using aluminum hydride as reductant. The formation of 10,13-nonadecadieneamine was confirmed by infrared spectroscopy. All other lipids (purity >99%) were purchased from Nu-Chek Prep (Elysian, MN). Solutions of substrates were prepared in either 95% ethanol or in Tween 20 by a modification of the method of Surrey (8).

pH Profile and Determination of Kinetic Constants

The activity of lipoxygenase-1 towards linoleic acid and linoleyl sulfate as a function of pH was determined at constant ionic strength (0.23). Acetate buffer was employed at pH 6.5 and lower; phosphate, pH 6.5-8.0; borate, pH 8.0 and higher. Experimentally indistinguishable values were obtained where buffers overlapped.

Kinetic constants were calculated on a Hewlett-Packard Model 9825A computor programmed to present Lineweaver-Burke plots based on linear regressions.

pK_a Determination

Apparent pK_a values of fatty acids were determined on a Radiometer Titrigraph, Model SBR3, by adding an excess of 1 *N* NaOH to the acid (0.1 *N*) and back titrating the solution with 0.1 *N* HCl.

RESULTS AND DISCUSSION

Apparent pK_a of Linoleic Acid

The pK_a of fatty acids with chain lengths of C_2-C_9 is reported to be between 4.82-4.95 (9). The apparent pK_a of linoleic acid was determined in a Radiometer Titrigraph by back titrating with 0.1 *N* HCl (Fig. 1). An unexpectedly high apparent pK_a of about 7.9 was observed under these conditions. The small chain fatty acids, propionic acid and caprylic acid, were found to have pK_a's in the expected range when determined in this same system.

Isoenzyme Variation with Derivatives of Linoleic Acid

A wide variation of response of the three isoenzymes for the various derivatives of linoleic acid was found (Table I). Lipoxygenase-1 was most active with linoleic acid and linoleyl sulfate. At the optimum pH reported for lipoxygenase-1 of 9.0, both substrates would be ionized. 10,13-Nonadecadieneamine was about 50% as active as linoleic acid as a substrate. Since the reported pK_a for alkyl amines is 10-11 (10), the 10,13-nonadecadieneamine would be expected to be predominately positively charged at pH 9.0. The nonpolar derivatives, methyl linoleate and linoleyl acetate, were poor substrates. When linoleyl

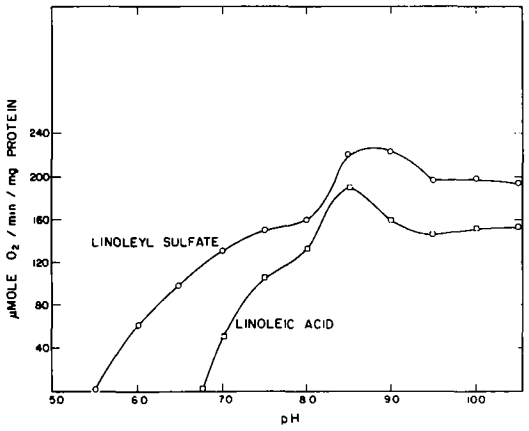

FIG. 2. pH profile of lipoxygenase-1 with linoleyl sulfate and linoleic acid. Reaction velocities were determined as μmoles of oxygen consumed per min per mg protein in the oxygraph. Buffers were prepared at constant ionic strength. Acetate buffer was used at pH 6.5 and below, phosphate pH 6.5-8.0 and borate pH 8.0 and above.

chloride was tested, the reaction rate decreased before either oxygen or substrate was completely consumed, suggesting that the enzyme was being inactivated.

Lipoxygenase-2, on the other hand, was most active with nonpolar substrates. At pH 6.8, the optimum for the enzyme, linoleic acid is predominately in the unionized form. Lipoxygenase-2 was totally inactive against linoleyl sulfate. The methane sulfonate was the best among the substrates tested.

Lipoxygenase-3 was significantly active only against linoleic acid at pH 6.8, apparently favoring the unionized state of the substrate. Like lipoxygenase-2, the enzyme was inactive against the sulfate.

The possibility that isoenzymes of lipoxygenase differ in their response to the polarity of substrates was first proposed by Koch et al. in 1958 (11). Employing two partially purified fractions of soybean extract, they proposed the existence of two isoenzymes based on the variation of their activities against linoleic acid and trilinolein. In 1970, Christopher et al. (2) reported that a second isoenzyme of soybean lipoxygenase differed from the original Theorell lipoxygenase (12) (lipoxygenase-1) in exhibiting a preference for methyl linoleate or trilinolein, as opposed to linoleic acid. Any physiological significance of these differences remains to be established.

The three isoenzymes also differed in their behavior with respect to the nature of the dispersal of the linoleic acid. Lipoxygenase-1 had nearly identical activity with linoleic acid prepared in ethanol and Tween 20, whereas lipoxygenase-2 was nearly 3 times as active with linoleic acid prepared in ethanol as that prepared in Tween 20. Lipoxygenase-3, on the other hand, was over 10 times more active when the substrate was prepared in Tween 20.

pH Profile of Lipoxygenase-1

The ratio of the activity of lipoxygenase-1 with linoleic acid at pH 9.0 and pH 6.8 was ca. 40 whereas with linoleyl sulfate the ratio was closer to 1.5. This difference may be accounted for by the ionization state of the substrates. Linoleyl sulfate is ionized throughout the pH range tested while linoleic acid is predominately nonionized below its apparent pK_a of 7.9. For similar reasons, the pH profile of lipoxygenase-1 with linoleic acid and linoleyl sulfate should differ, reflecting the preference of the enzyme for the charged substrate. Indeed, as Figure 2 shows, the pH profile of the enzyme with linoleyl sulfate was broadened and extended to lower pH values as compared to the profile obtained with linoleic acid. The optimum pH value was nearly the same with both substrates.

Kinetic Constants of Lipoxygenase-1 with the Free Acid and the Sulfate

The K_m's of lipoxygenase-1 for linoleic acid and linoleyl sulfate were determined at pH 6.8 and pH 9.0 (Table II). At pH 9.0, where both substrates are negatively charged, the K_m's were similar. However, at pH 6.8, where the linoleic acid is predominately nonionized and the linoleyl sulfate ionized, the K_m for linoleic acid was nearly 10 times higher than that for linoleyl sulfate. The V_{max} with linoleyl sulfate at pH 9.0 was only 1.5 times greater than at pH

TABLE II

Comparison of Kinetic Parameters of Lipoxygenase-1 with Linoleic Acid and Linoleyl Sulfate

	K_m		V_{max} (oxygen μmole/min/mg protein)	
	pH 6.8	pH 9.0	pH 6.8	pH 9.0
Linoleic acid	4.9×10^{-4} M	8.5×10^{-5} M	55	169
Linoleyl sulfate	5.5×10^{-5} M	8.0×10^{-5} M	121	181

6.8, while with linoleic acid the ratio was closer to 3.0.

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