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Structural Features Required for Inhibition of Soybean Lipoxygenase-2 by Propyl Gallate[1]

EVIDENCE THAT LIPOXYGENASE ACTIVITY IS DISTINCT FROM THE ALTERNATIVE PATHWAY

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ABSTRACT

The ability of 19 structural analogs of propyl gallate to inhibit purified soybean seed (*Glycine max* [L.] Merr. var. Ransom) lipoxygenase-2 (EC 1.13.11.12) was determined. The results indicate that the *o*-dihydroxy and not the ester function of propyl gallate is essential for inhibition of lipoxygenase. Catechol thus represents the minimum inhibitory structure. Among those compounds possessing an *o*-dihydroxy function, the K_i' for inhibition of lipoxygenase is directly related to the lipophilicity of the inhibitor as measured by the octanol-water partition coefficient. The structural features of propyl gallate necessary for inhibition of lipoxygenase were found to differ from those required for inhibition of the plant mitochondrial alternative pathway. This further supports the concept that the alternative oxidase and lipoxygenase are functionally distinct species.

In the 1930s, Hanes and Barker (10) and Van Herk (27) reported that the respiration in some plant tissues was resistant to cyanide, a specific inhibitor of Cyt oxidase. Cyanide-resistant respiration in plants has since been shown to be the result of electron transport through an alternative pathway which branches from the main mitochondrial electron transport chain at the level of ubiquinone (2). Both the biochemical nature and general physiological role of the alternative pathway remain unknown despite almost 50 years of research.

In 1971, Schonbaum *et al.* (19) reported that substituted benzohydroxamic acids specifically inhibited electron transport through the alternative pathway. This finding led to a functional definition of the alternative pathway as that component of O_2 uptake in either tissues or isolated mitochondria which was cyanide (or antimycin)-resistant but inhibited by hydroxamic acids such as SHAM[2] (Fig. 1). A recent report, however, illustrated the confusion that may result when the combination of SHAM sensitivity and cyanide resistance are used as criteria to assess alternative pathway activity. Parrish and Leopold (15) demonstrated that the presence of the enzyme lipoxygenase, a cyanide-resistant dioxygenase found in most plant tissues, can confound measurements of the alternative pathway. They found that the initial burst of cyanide-resistant, SHAM-sensitive O_2 uptake in suspensions of imbibing soybean seed particles was the result of lipoxygenase activity and not due to the alternative pathway. Parrish and

SHAM

n-PROPYL GALLATE

FIG. 1. Chemical structures of propyl gallate and salicylhydroxamic acid.

Leopold (15) suggested that propyl gallate (Fig. 1), a well known inhibitor of lipoxygenase (25), might be used to distinguish between lipoxygenase and the alternative pathway. Siedow and Girvin (21), however, demonstrated that propyl gallate inhibits not only lipoxygenase but also the alternative pathway with a K_i' 5- to 10-fold lower than that found for SHAM.

The similarities in sensitivity to SHAM, propyl gallate, and cyanide make it difficult to distinguish between the activity of lipoxygenase and the alternative pathway in plant tissues and have led to some speculation that the alternative pathway is somehow related to the presence of lipoxygenase (5, 8, 9). In an effort to determine if more subtle differences exist between lipoxygenase and the alternative pathway, studies of the inhibition of these two activities by propyl gallate were undertaken. In an initial study, the functional groups of propyl gallate were varied in a systematic manner and the resulting analogs were tested for their ability to inhibit the alternative pathway in isolated mung bean mitochondria (22). This paper presents the results of a systematic study of the structural features of propyl gallate required to inhibit soybean lipoxygenase-2 activity. A comparison with the results

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[2] Abbreviation: SHAM, salicylhydroxamic acid.

obtained from the earlier mitochondrial study indicates that the structural features of propyl gallate necessary for inhibition of lipoxygenase are different from those required for inhibition of the alternative oxidase.

MATERIALS AND METHODS

Purification and Assay of Lipoxygenase. Lipoxygenase, isozyme-2, was purified from soybean seeds (*Glycine max* [L.] Merr. var. Ransom) according to Christopher *et al.* (3). Lipoxygenase activity was determined polarographically in a 2.0-ml glass reaction vessel fitted with a Clark-type O_2 electrode. The assays were performed in 165 mM sodium phosphate buffer, pH 6.8, and initiated by addition of 1.29 mM linoleic acid, emulsified with Tween 20 according to Surrey (24).

Isolation of Mitochondria. Mitochondria were isolated according to Moreland and Boots (14) from 4- to 5-d-old etiolated mung bean (*Vigna radiata* L.) hypocotyls grown in the dark at 28°C and 80% RH.

Calculations. For each inhibitor, lipoxygenase activity was determined at five different inhibitor concentrations centered about the inhibitor concentration which resulted in 50% inhibition. The K_i' was calculated from the x-intercept of a Dixon plot of the data (4). K_i' values represent the mean of at least two separate determinations. If the K_i' of a given compound was greater than 1 mM, it was considered noninhibitory. The $P_{oct:H_2O}$ values, when not available in the literature, were calculated using the Nys and Reckker modification of Hansch's "group contribution approach" (28).

Chemicals. Methyl, ethyl, butyl, and octyl gallate were purchased from K&K Rare and Fine Chemicals (ICN Pharmaceuticals, Inc.) Ethyl resorcylate (3,5-dihydroxybenzoic acid ethyl ester) and ethyl protocatechuate (3,4-dihydroxybenzoic acid ethyl ester) were synthesized according to Thomas and Crowell (26), recrystallized from an ethanol-H_2O mixture, and shown to have melting points and NMR spectra identical with those reported in the literature. Stock solutions of inhibitors in ethanol were prepared fresh daily. All other compounds were purchased from Sigma Chemical Corp.

COMPOUND	NAME	$K_{i\ LOX}'$ (μM)
OH	PHENOL	–
OH OH	CATECHOL	170
OH OH	RESORCINOL	–
OH OH OH	PYROGALLOL	750

FIG. 2. Apparent inhibition constants for the inhibition of soybean lipoxygenase-2 activity by a series of phenolic compounds. K_i' was determined as described in "Materials and Methods."

$R_1-C(=O)-O-R_2$		$K_{i\ LOX}'$ (μM)
R_1	R_2	
	H	—
HO-	H	—
HO HO	H	—
	C_2H_5	—
OH	C_2H_5	—
HO	C_2H_5	—
HO-	C_2H_5	—
HO HO	C_2H_5	68
HO HO	C_2H_5	—

FIG. 3. Apparent inhibition constants for the inhibition of soybean lipoxygenase-2 activity by hydroxy derivatives of benzoic acid and ethyl benzoate. From top to bottom, benzoic acid, *p*-hydroxybenzoic acid, protocatechuic acid, ethyl benzoate, ethyl *o*-hydroxybenzoate, ethyl *m*-hydroxybenzoate, ethyl protocatechuate, ethyl resorcylate. K_i' was determined as described in "Materials and Methods."

RESULTS

Figure 2 shows the inhibition of soybean lipoxygenase by a series of phenolic compounds. Phenol and resorcinol were not inhibitory. Catechol and pyrogallol, however, did inhibit soybean lipoxygenase-2 with K_i' values of 170 and 750 μM, respectively. These data illustrate that at least an *o*-dihydroxy function is essential for inhibition of lipoxygenase. They also indicate that the ester function was not essential for inhibition of lipoxygenase.

Figure 3 shows the effect of varying both the position and the number of hydroxyl groups on benzoic acid and ethyl benzoate on the inhibition of lipoxygenase. Free acids such as benzoic acid, *p*-hydroxybenzoic acid, protocatechuic acid, and resorcylic acid (not shown) did not inhibit. The ethyl ester of benzoic acid did not inhibit, again indicating a requirement of at least some portion of the trihydroxy group of propyl gallate to bring about inhibition of lipoxygenase. None of the monohydroxy ethyl esters of benzoic acid (*o*, *m*, or *p*) inhibited lipoxygenase. Ethyl protocatechuate did inhibit lipoxygenase with a K_i' of 68 μM, while ethyl resorcylate did not. This further demonstrated the requirement of an *o*-dihydroxy function for inhibition.

Figure 4 illustrates the effect of gallic acid and a series of gallate esters of increasing chain length on lipoxygenase activity. Gallic acid did not inhibit lipoxygenase. Among the gallate esters, all were inhibitory with an increase in ester chain length resulting in a decreased K_i'. This decrease in K_i' spanned almost 2 orders of magnitude from 300 μM for methyl gallate to 5 μM for octyl gallate.

HO O HO-⟨⟩-C-O-R HO	$K_{i\,LOX}'$ (μM)
R = H	–
= CH_3	300
= C_2H_5	217
= C_3H_7	51
= C_4H_9	37
= C_8H_{17}	5

FIG. 4. Apparent inhibition constants for the inhibition of soybean lipoxygenase-2 activity by gallic acid and a series of gallate esters. K_i' was determined as described in "Materials and Methods."

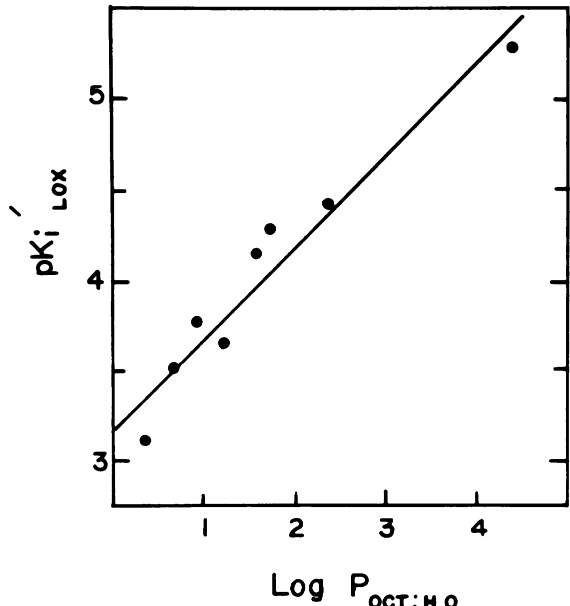

FIG. 5. pK_i' *versus* logarithm ($\log_{10}$) of the octanol-water partition coefficient. pK_i' values and partition coefficients were determined as outlined in "Materials and Methods."

Increasing the chain length of the ester moiety increases its lipophilicity (7). This result suggested a relationship might exist between the lipophilicity of a given inhibitor and its apparent inhibition constant. That such a relationship does exist is shown in Figure 5 where the pK_i' of the inhibitory analogs is plotted as a function of the log of the octanol:water partition coefficient, $P_{oct:H_2O}$. The slope of the line shown in Figure 5 is 0.51.

The importance of inhibitor lipophilicity is also apparent in Figure 2. The higher K_i' observed for pyrogallol (750 μM) compared to that for catechol (170 μM) reflects the approximately 4-fold decrease in lipophilicity ($P_{oct:H_2O}$) associated with the addition of a third hydroxyl group to catechol. Similarly, the difference between the K_i' values of ethyl gallate (Fig. 4) and ethyl protocatechuate (Fig. 3) can be explained by differences in lipophilicity.

DISCUSSION

Propyl gallate proved quite amenable to a systematic study of the structural features required to inhibit soybean lipoxygenase-2 activity. The trihydroxy, and not the ester function of propyl gallate, was necessary for inhibition of lipoxygenase-2 as indicated in Figure 2. Further, within the trihydroxy function, an *ortho*-dihydroxy group represents the minimum structural feature needed to observe inhibition. In fact, addition of the third hydroxyl group results in an increase in K_i' due to the resulting decrease in lipophilicity. Catechol (1,2-dihydroxybenzene), therefore, represents the minimum structure needed to inhibit soybean lipoxygenase-2. While the ester function is not needed to observe inhibition, the presence of the ester chain can have a dramatic effect on K_i' (Fig. 4). Again, this is related to the effect of the ester group on lipophilicity (Fig. 5). Once the required *o*-dihydroxy group is present, the more lipophilic the compound, the better the observed inhibition of lipoxygenase. Protocatechuic acid and gallic acid, however, which do contain an *o*-dihydroxy group, do not inhibit lipoxygenase, probably due to the large decrease in lipophilicity resulting upon deprotonation of the carboxylic acid moiety.

Phenolic antioxidants have previously been shown to inhibit, to varying degrees, lipoxygenases from a number of plant sources (6, 25). Among the commonly tested phenolic antioxidants, nordihydroguaiaretic acid was generally found to be the most potent inhibitor of lipoxygenase (25, 29). This finding is consistent with our results inasmuch as nordihydroguaiaretic acid contains two *o*-dihydroxy groups and is very lipophilic, having a $P_{oct:H_2O}$ comparable to that of octyl gallate. Yasumoto *et al.* (29) tested a number of conventional antioxidants on crystalline soybean lipoxygenase, including several propyl gallate analogs, and obtained results qualitatively similar to those presented here, *i.e.* *o*-diphenols were better inhibitors of lipoxygenase than *m*- and *p*-diphenols. Mitsuda *et al.* (13) previously demonstrated the importance of lipoxygenase inhibitor lipophilicity when they reported a linear relationship between the pK_i for inhibition of lipoxygenase by a series of monohydric alcohols and the effective carbon chain length of the alcohols. Whereas these qualitative comparisons indicate that our results are consistent with those found in earlier studies, quantitative comparisons are difficult due to differences in enzyme preparation, reaction pH, substrate preparation, and assay methods. Furthermore, this paper presents a more systematic attempt to quantify the exact structural features necessary for inhibition of lipoxygenase by phenolic compounds.

When the present results are compared with those of a similar study of the inhibition of the mitochondrial alternative pathway (22), it is found that the structural features required for inhibition of soybean lipoxygenase-2 activity differ from those required for inhibition of the alternative pathway. Catechol is the minimum structure which will inhibit lipoxygenase activity while a single phenolic hydroxyl group represents the minimum structure which will inhibit the alternative pathway (22). For inhibition of lipoxygenase, the K_i' is directly related to the lipophilicity of the inhibitor, with the more lipophilic molecules being the most inhibitory. For the alternative pathway, no significant relationship was observed between K_i' and $P_{oct:H_2O}$. There was instead a relationship between the K_i' and the pK_a of the essential hydroxyl group; the lower the pK_a of the hydroxyl group, the lower the K_i' for inhibition of the alternative pathway. There was no relationship between the K_i' for inhibition of lipoxygenase and the pK_a of the *p*-hydroxyl group.

Differences in the structural features of propyl gallate necessary for inhibition of soybean seed lipoxygenase and the alternative pathway in mung bean mitochondria indicate that the two activities are distinct. However, several workers have speculated that

the alternative pathway in some plant mitochondria is due to the presence of lipoxygenase. Goldstein *et al.* (8, 9) concluded in two similar reports that the alternative pathway in wheat mitochondria was due to the presence of lipoxygenase. Dupont (5) also suggested that lipoxygenase may account for some of the cyanide-resistant, SHAM-sensitive O_2 uptake by isolated cauliflower mitochondria. The oxidation of tricarboxylic acid cycle intermediates by the alternative pathway, however, is known to be stoichiometric with respect to O_2 reduction (18) yet neither Goldstein nor Dupont explain how oxidation of α-ketoglutarate or succinate is linked to a lipoxygenase-mediated lipid peroxidation.

To ensure that the observed differences between the structural features necessary for inhibition of soybean lipoxygenase-2 and the alternative pathway were not due to differences between soybean seed and mung bean hypocotyl lipoxygenases, the same analogs were tested for their ability to inhibit the lipoxygenase activity which contaminates washed mung bean mitochondrial preparations (21). The results with mung bean lipoxygenase activity were comparable to those found for soybean seed lipoxygenase-2. This further supports the argument that, since the structural features of propyl gallate essential for inhibition of lipoxygenase and the alternative pathway differ, the two activities are distinct, *i.e.* the alternative pathway is not due to lipoxygenase activity.

These results support other reports which indicate that the mitochondrial alternative pathway is distinct from lipoxygenase. Miller and Obendorf (12) observed that levels of disulfiram which completely inhibited the alternative pathway in isolated soybean mitochondria had no effect on the lipoxygenase activity present. Also mitochondria isolated from skunk cabbage and *Peltandra virginica* spadices showed significant levels of alternative pathway, but no measurable lipoxygenase activity (23). Similarly, most of the lipoxygenase activity present in aged potato tuber mitochondria prepared by differential centrifugation is removed when the organelles are purified by sucrose gradient centrifugation while the alternative pathway is usually unaffected (20). Clearly, the experimental evidence which demonstrates a distinction between lipoxygenase and the alternative oxidase is substantial.

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