Phytochemistry, Vol 28, No 10, pp. 2577–2581, 1989
Printed in Great Britain.

0031-9422/89 $3.00+0.00

INHIBITION OF LIPOXYGENASE ACTIVITY: A CAUTIONARY NOTE

D KRISTIE* and J. E. THOMPSON†‡

Department of Biology, University of Waterloo, Waterloo, Ontario, Canada, N2L 3G1; †Department of Horticultural Science, University of Guelph, Guelph, Ontario, Canada, N1G 2W1

(*Received* 28 *November* 1988)

Key Word Index—Lipoxygenase; inhibitors, chelators; antioxidants.

Abstract—Two methods are commonly used to measure lipoxygenase activity, a spectrophotometric assay in which the formation of hydroperoxide product is followed at 234 nm and a polarographic assay in which oxygen consumption is determined. In previous studies, a number of antioxidants and metal chelators have been identified as inhibitors of lipoxygenase based on data obtained using either the spectrophotometric assay or the polarographic assay. However, there are large inconsistencies among these studies in the degree of inhibition measured that appear to be related to the assay procedure used. In the present study, we report that these inconsistencies can be explained by the fact that the absorption characteristics of many of the inhibitors including EDTA, azide, SHAM, *n*-propylgallate, Tiron and nordihydroguaiaretic acid preclude accurate measurement of the efficacy of inhibition when the enzyme is measured spectrophotometrically. Specifically, these compounds all absorb strongly at 234 nm and greatly reduce the sensitivity with which the hydroperoxide product of the reaction can be detected.

INTRODUCTION

Lipoxygenase (EC 1.13.11.12) is an ubiquitous enzyme in plants that utilizes molecular oxygen in the conversion of polyunsaturated fatty acids containing a *cis,cis*-1,4 pentadiene to conjugated hydroperoxydiene derivatives. Techniques for the quantitation of lipoxygenase have been recently reviewed [1]. The two most commonly used methods involve spectrophotometrically monitoring the increase in conjugated hydroperoxide product at 234 nm, or following oxygen uptake polarographically with a Clarke electrode. However, the spectrophotometric assay is considered to be unsuitable for use with crude plant extracts because of interfering substances and secondary reactions that result in loss of the conjugated diene product [1, 2].

A number of antioxidants and metal chelators are potent inhibitors of lipoxygenase, and these inhibitors have been used in studies designed to elucidate biochemical features of the enzyme as well as its physiological role. For example, evidence indicating that iron is an essential component of lipoxygenase has been obtained by determining the effects of metal chelators on its activity [3]. No specific physiological function for lipoxygenase has been unequivocally identified, although it has been implicated in ethylene biosynthesis [4] and in senescence [5, 6]. In certain instances, conclusions concerning the involvement of lipoxygenase in these physiological processes have been based in part on the effects of inhibitors of the enzyme. For example, inhibitors of lipoxygenase block ethylene formation from 1-aminocyclopropane-1-carboxylic acid in model systems in which the conversion appears to be mediated by lipoxygenase [4, 7].

Of particular concern is the finding that there is a wide disparity among different studies in the effectiveness of some of these inhibitors of lipoxygenase depending upon whether the enzyme was assayed by measuring hydroperoxide formation at 234 nm or from oxygen consumption. These discrepancies are summarized in Table 1 and have been attributed to the fact that enzymes isolated from different plant sources were used [8]. Specifically, azide and EDTA consistently inhibit lipoxygenase activity when measurements were made using the spectrophotometric assay but not when measurements were made using the polarographic assay (Table 1).

In the present study, we have examined the effects of putative lipoxygenase inhibitors using both the spectrophotometric and polarographic assays and have obtained evidence indicating that interfering absorption characteristics of the inhibitors, rather than different sources of the enzyme, account for most of these discrepancies.

RESULTS AND DISCUSSION

The effects of putative inhibitors on the activities of lipoxygenase in tomato cytosol and purified soybean lipoxygenase I are illustrated in Table 2. The enzyme activities were measured both spectrophotometrically (A_{234}) and polarographically (O_2 consumption). For two of the inhibitors tested, sodium cyanide and U37184, comparable levels of inhibition were obtained for each source of enzyme whether the assay was conducted spectrophotometrically or polarographically, although U37184 was clearly a much more effective inhibitor of purified soybean lipoxygenase I than of the impure cytosolic lipoxygenase from tomato (Table 2). By contrast, for Tiron, EDTA and azide, the degree of inhibition

*Present Address: Department of Biology, Acadia University, Wolfville, Nova Scotia, Canada, B0P 1X0.

‡Author to whom correspondance should be addressed.

Table 1 Summary of inhibitory effects of EDTA and azide on lipoxygenase activity from different sources

Sources of enzyme	Inhibitor		% Inhibition: Spectrophotometric assay	% Inhibition: Polarographic assay
Carnation (ref [7])	EDTA	(10 uM)	~80	—
Apple (ref [9])	EDTA	(1 mM)	66	
Broad bean (ref [10])	EDTA	(1 mM)	47	—
		(10 mM)	100	—
	Azide	(1 mM)	26	—
		(10 mM)	100	—
Soybean (ref [3])	EDTA	(1 mM)	—	0
Sunflower (ref [8])	EDTA	(100 mM)	—	0
	Azide	(100 mM)	—	0
Potato (ref [11])	EDTA	(1 mM)	—	0
Eggplant (ref [12])	EDTA	(1 mM)	—	0
	Azide	(1 mM)	—	0
Alfalfa (ref [13])	EDTA	(1 mM)	—	0
	Azide	(1 mM)	—	0

Table 2 Effects of putative inhibitors on lipoxygenase activity

Inhibitors		% Inhibition: *Soybean lipoxygenase I* 234 nm	250 nm	O_2	*Tomato lipoxygenase* 234 nm	O_2
NaCN	(10 mM)	12	ND	24	49	29
U37184	(0 1 mM)	100	ND	100	0	15
Tiron	(10 mM)	100	ND	6	100	0
EDTA	(10 mM)	100	19.5	12	100	0
Azide	(10 mM)	100	24	25	100	0
SHAM	(1 mM)	100	ND	33	100	36
	(5 mM)	100	ND	75	100	71
n-Propyl gallate	(1 mM)	100	ND	ND	100	60
	(10 mM)	100	ND	ND	100	100
NDGA	(1 mM)	100	ND	ND	100	27
	(5 mM)	100	ND	ND	100	72

In each case, the results are from one of three separate experiments all showing the same effect

ND, not determined

n-Propylgallate and nordihydroguaiaretic acid (NDGA) autooxidize at pH. 9 0, and thus polarographic assays of the effects of these inhibitors on soybean lipoxygenase I could not be determined

detected in the spectrophotometric assay was consistently much higher than that detected in the polarographic assay for both the pure soybean lipoxygenase I and the impure tomato enzyme For example, 10 mM Tiron, 10 mM EDTA and 10 mM azide all appear to inhibit lipoxygenase activity completely when measured spectrophotometrically at 234 nm, yet the same compounds had little or no effect on oxygen consumption (Table 2). This was also true for the lower (1 mM) of the two concentrations of SHAM, *n*-propylgallate and nordihydroguaiare-

tic acid tested (Table 2) Nordihydroguaiaretic acid and *n*-propylgallate underwent rapid autooxidation at pH 9.0, and thus reliable measurements of oxygen consumption for soybean lipoxygenase I could not be obtained in the presence of these inhibitors.

In order to determine which of the two assays, polarographic or spectrophotometric, was more accurately detecting inhibition, the inhibiting effects of U37184, which inhibited to a comparable degree in both the spectrophotometric and polarographic assays, and of EDTA and Tiron, which inhibited much more strongly in the spectrophotometric than in the polarographic assay, were compared using a radioisotope assay and purified soybean lipoxygenase 1. U37184 prevented the breakdown of radiolabelled fatty acid, whereas EDTA and Tiron had little or no effect (Fig. 1). These observations indicate that the inhibitory effects of Tiron and EDTA detected spectrophotometrically (Table 2) are artifactual.

Absorption spectra in the 200–300 nm waveband revealed that Tiron, EDTA, azide, SHAM, nordihydroguaiaretic acid and *n*-propylgallate all absorb strongly at 234 nm at concentrations that gave complete inhibition of lipoxygenase activity in the spectrophotometric assay (Fig. 2). Indeed, with a path length of 1 cm, each of these compounds raised the absorbance of the test solution above the normal operating range of most spectrophotometers. Thus, even though the putative inhibitor was present in both the sample and blank cuvettes, any increase in A_{234} attributable to the formation of hydroperoxides by lipoxygenase was not detected (Fig. 3). After 5 min of reaction time, a substantial peak at 234 nm was evident when soybean lipoxygenase I was allowed to react with linoleic acid in the absence of EDTA (Fig. 3A and B). When 10 mM EDTA was added to both the sample and blank cuvettes, there was no change in A_{234} even after 10 min of reaction time (Fig. 3E, F and G). Similar results were obtained upon the addition of azide, Tiron, SHAM *n*-propylgallate and nordihydroguaiaretic acid when the reaction was scanned at various time intervals between 200 and 300 nm (data not shown). Thus, a major part of the inhibitory effects of these

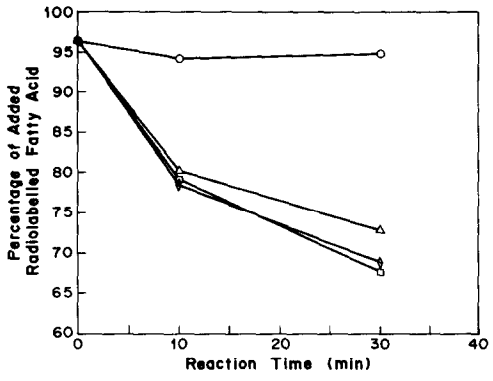

Fig 1. Effects of putative inhibitors of soybean lipoxygenase I measured using a radioisotopic assay. (□), standard reaction mixture plus 500 U of soybean lipoxygenase I; (○), standard reaction mixture plus 500 U of soybean lipoxygenase I plus 0.1 mM U37184; (△), standard reaction mixture plus 500 U of soybean lipoxygenase I plus 10 mM EDTA, (◇), standard reaction mixture plus 500 U of soybean lipoxygenase I plus 10 mM Tiron

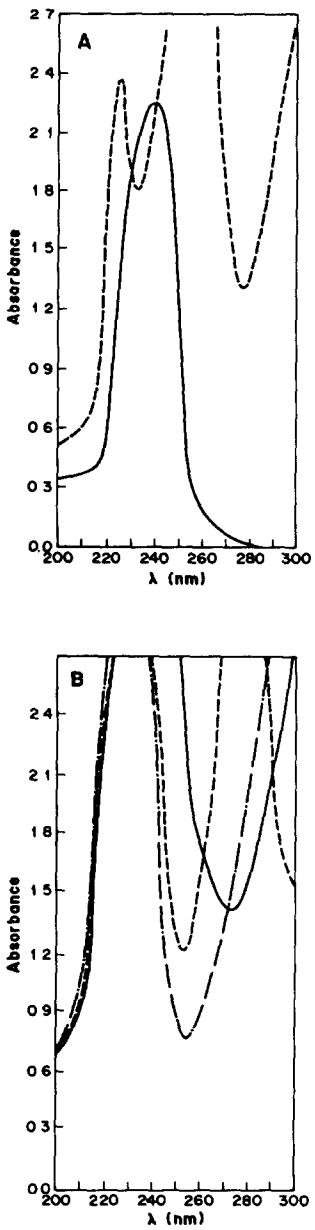

Fig. 2. Absorption spectra for putative inhibitors of lipoxygenase. A· 1 mM Tiron (—), 10 mM EDTA (---) [an essentially identical spectrum (not shown) was obtained for 10 mM azide]. B. 1 mM *n*-propyl gallate (—); 1 mM nordihydroguaiaretic acid (---); 1 mM SHAM (— — —·).

compounds detected spectrophotometrically is artifactual and due to interfering absorbances of the inhibitory compounds.

For two of the putative inhibitors, EDTA and azide, a sharp peak centred at *ca* 250 nm became increasingly apparent as the reaction progressed (Fig. 3F and G). The possibility that this peak was attributable to a reaction between EDTA (or azide) and a component of the enzyme reaction was tested by following the lipoxygenase reaction at 250 nm in the presence and absence of the putative inhibitors. The addition of 10 mM azide or

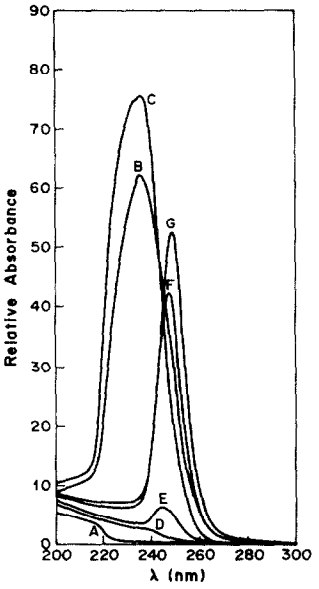

Fig 3 Absorption spectra illustrating the reaction of soybean lipoxygenase I in the presence and absence of EDTA. (A) Buffer and substrate in the sample and blank cuvettes, (B) same as A except spectrum recorded 5 min after the addition of 500 U of soybean lipoxygenase I to the sample cuvette, (C) same as A except 10 mM EDTA added to the sample cuvette, (D) same as C except 10 mM EDTA added to the blank cuvette, (E) same as D except 500 U of soybean lipoxygenase I added to the sample cuvette and the spectrum recorded immediately, (F) same as E expect spectrum recorded 5 min after the addition of enzyme, (G) same as E except spectrum recorded 10 min after the addition of enzyme

10 mM EDTA actually reduced the absorbance at 250 nm indicating that the reaction had been inhibited to a degree comparable to that measured by oxygen consumption (Table 2). Thus, the ghost peak at 250 nm appears to be a function of the relatively narrow absorption peaks of EDTA and azide (Fig 2A) combined with a broadening of the hydroperoxide peak into wavebands beyond those obscured by EDTA or azide as the reaction proceeds.

Previous studies utilizing the spectrophotometric assay have indicated that EDTA and azide in the range 1–10 mM strongly inhibit lipoxygenase activity in extracts of apple and broad bean [9, 10] By contrast, polarographic studies have indicated that 1 mM EDTA had no effect on soybean lipoxygenase I [3] and that even higher concentrations (100 mM) of azide or EDTA had no effect on sunflower lipoxygenase [8]. Such quantitative differences in the efficacy of putative lipoxygenase inhibitors between different studies could be due to the use of different sources of enzyme, but they may also reflect peculiar absorption characteristics of the inhibitory compounds that confound the spectrophotometric assay Berkeley and Galliard (1976) have argued previously that the spectrophotometric method for measuring lipoxygenase should not be used for crude extracts of the enzyme because of the possibility that contaminants in the crude extract might have interfering absorption characteristics In the present study we have shown using both a crude enzyme preparation and a purified enzyme that major errors can arise from interfering absorbances of a large number of documented inhibitors of lipoxygenase when the spectrophotometric assay is used. For example, although nordihydroguaiaretic acid, SHAM and *n*-propylgallate are all well documented inhibitors of lipoxygenase, quantitative errors in the effectiveness of these compounds as inhibitors can arise when the reaction is assayed spectrophotometrically (Table 2).

It is noteworthy that the metal chelators, NaCN, azide, Tiron and EDTA, were all found to be comparatively poor inhibitors of tomato and soybean lipoxygenase in the present study This may be related to the fact that the efficacy of metal chelators as inhibitors of lipoxygenase depends upon the extent to which the enzyme is in its native state Metal chelators inhibit lipoxygenase by complexing with the iron that is associated with the enzyme, and the efficacy with which chelators complex the iron is greatly increased if the enzyme is partially denatured [3]

EXPERIMENTAL

Chemicals and enzymes U37184 was obtained from Upjohn Limited, Tiron from Fisher Scientific Company, NaEDTA from J T Baker, NaAzide from Aldrich, *n*-propylgallate, salicylhydroxamic acid (SHAM), nordihydroguaiaretic acid (NDGA) and soybean lipoxygenase I from Sigma

Preparation of tomato cytosol A known wt of ripe red tomato (*Lycopersicon esculentum* cv Caruso) pericarp tissue was homogenized in 100 mM MOPS–KOH, 0 5 mM sorbitol, pH 7 8 (1 1 w/v) The homogenate was filtered through 4 layers of cheesecloth and the filtrate centrifuged at 10000 *g* for 20 min The supernatant was centrifuged at 50000 *g* for 1 hr and the resulting supernatant used for lipoxygenase activity assays

Spectrophotometric and polarographic assays of lipoxygenase activity Two sources of lipoxygenase were used, tomato cytosol and purified soybean lipoxygenase I Lipoxygenase activity was determined spectrophotometrically at 234 nm or polarographically with an O_2 electrode For both methods, the reaction mixt (3 ml final vol) contained 0 5 mM linoleic acid solubilized with 0 5% Tween 20 in 50 mM phosphate buffer, pH 7 1 for tomato lipoxygenase or 50 mM Ches buffer, pH 9 0 for soybean lipoxygenase I The reaction was initiated by the addition of an aliquot of soybean lipoxygenase I suspension or tomato cytosol adjusted to give a final activity of 150 units/ml [1 unit of enzyme oxidizes 0 12 nmol substrate per min at 25°] Inhibitors were added at concns specified in the Tables and Fig legends

Radioisotopic assay of lipoxygenase activity Uniformly labelled ^{14}C-linoleic acid (0 68 μCi) was added to the standard reaction mixt and the enzyme reaction initiated by the addition of 500 units of soybean lipoxygenase 1 At 0, 10 and 30 min intervals after the reaction was initiated, 1 ml aliquots were removed from the reaction mixt and the lipids extracted using the method of ref [14] Lipid reaction products were sepd by TLC on silica gel using petrol–Et_2O–HOAc (70 30 1) Sepd lipids were visualized with I_2 vapour, identified using authentic standards, scraped from the plates and counted by liquid scintillation as described previously [15]

Acknowledgements—The authors gratefully acknowledge financial assistance in the form of a grant-in-aid from the Natural Sciences and Engineering Research Council of Canada D Kristie was supported by a Killam Post-doctoral Fellowship

REFERENCES

1. Gallıard, T. and Chan, H (1980) ın *The Biochemıstry of Plants* Vol 4 (Stumph, P. K and Conn, E. E, eds), pp. 131–161 Academıc Press, New York.
2. Berkeley, H D and Gallıard, T (1976) *Phytochemıstry* **15**, 1475
3. Pıstorıus, E and Axelrod, B (1974) *J Biol Chem.* **249**, 3183.
4. Bousquet, J F and Thımann, K V (1984) *Proc Natl Acad. Scı U.S A.* **81**, 1724
5. Grossman, S and Leshem, Y Y. (1978) *Physıol. Plant.* **43**, 359.
6. Pauls, K P and Thompson, J. E. (1984) *Plant Physıol* **75**, 1152.
7. Lynch, D. V., Srıdhara, S and Thompson, J E. (1985) *Planta* **164**, 121
8. Leonı, O , Iorı, R and Patmıeri, S. (1985) *J. Food Scı.* **50**, 88.
9. Kim, I. and Grosch, W. (1979) *J. Agrıc Food Chem* **27**, 243
10. Al-Obaıdy, H. M and Siddıqı, A. M. (1981) *J. Food Scı* **46**, 597.
11. Gallıard, T and Phıllıps, D R (1971) *Bıochem J.* **124**, 431
12. Grossman, S , Trop, M., Autalıon, R. and Pınsky, A. (1972) *Lipıds* **7**, 467.
13. Sıddıqi, A M. and Tappel, A. L. (1956) *Plant Physıol.* **31**, 320
14. Bligh, E G and Dyer, W J (1959) *Can J. Bıochem. Physıol.* **37**, 911
15. Brown, J H., Lynch, D. V and Thompson, J E. (1987) *Plant Physıol.* **85**, 679