Lipoxygenase-mediated production of superoxide anion in senescing plant tissue

Daniel V. Lynch and John E. Thompson*

Department of Biology, University of Waterloo, Waterloo, Ontario N2L 3G1, Canada

Received 7 May 1984; revised version received 11 June 1984

Lipoxygenase activity and superoxide ($O_2^{\cdot -}$) production by microsomal membranes and cytosol from bean cotyledons increased in parallel as senescence progressed. Superoxide production was heat denaturable and dependent on the availability of linoleate, the substrate for lipoxygenase. The specific inhibitor of lipoxygenase, U28938, caused a parallel reduction in enzyme activity and the formation of $O_2^{\cdot -}$. These observations demonstrate that lipoxygenase activity mediates the formation of superoxide anion, and support the contention that membrane senescence is attributable to a sequence of reactions in which lipase-derived fatty acids are utilized by lipoxygenase to generate $O_2^{\cdot -}$ and hydroperoxides.

Senescence *Lipoxygenase* *Superoxide anion* *Tiron*

1. INTRODUCTION

Lipoxygenase mediates the conversion of polyunsaturated fatty acids to their conjugated hydroperoxydiene derivatives using molecular oxygen. A specific metabolic role for this enzyme has not been identified, but it is consistently present in senescing and wounded plant tissues [1]. The mechanism of the lipoxygenase reaction has been extensively investigated (review [1]), and it has been proposed that free radical species are formed during the reaction. This is supported by the fact that a number of inhibitors of lipoxygenase (e.g., *n*-propyl gallate) are potent scavengers of free radicals [2].

Enhanced production of free radicals is one of many events associated with senescence. Indeed, free radicals have been implicated in membrane deterioration and loss of important physiological functions in both senescing and stressed plant tissues [3,4]. We have here examined the relationship between lipoxygenase activity and superoxide anion ($O_2^{\cdot -}$) production by isolated microsomal membranes and cytosol from senescing bean cotyledons and, in particular, the prospect that lipoxygenase mediates the formation of $O_2^{\cdot -}$.

* To whom correspondence should be addressed

Abbreviations: U28938, 4-(1-methylethyl)benzynecarbohydrazonoyl chloride *N*-phenyl; CHES, 2-[*N*-cyclohexylamino]ethanesulfonic acid

2. EXPERIMENTAL

Seeds of *Phaseolus vulgaris* L. (c.v. Kinghorn) were germinated under etiolating conditions at 29°C in moist vermiculite. Cotyledons were harvested at intervals following planting, and smooth microsomal membranes were isolated as in [5]. The microsomal supernatant was used as the source of soluble (cytosolic) enzyme.

Lipoxygenase activity was determined spectrophotometrically at 234 nm [6]. The standard assay mixture contained 1.5 mM linoleic acid and 0.5% (v/v) Tween 20 in 50 mM CHES buffer (pH 8.6). An aliquot of membrane suspension or cytosol containing 20–100 μg protein was added to 2 ml reagent in a cuvette.

Levels of $O_2^{\cdot -}$ were determined by ESR using Tiron (1,2-dihydroxybenzene-3,5-disulfonic acid) [3]. The amplitude of the ESR spectrum for Tiron

Published by Elsevier Science Publishers B.V.
00145793/84/$3.00

semiquinone, which is formed when Tiron reacts with $O_2^{\cdot -}$, is a specific and quantitative indicator of $O_2^{\cdot -}$ production [3,7]. For these measurements, samples containing 100–200 $\mu g \cdot ml^{-1}$ protein, 10 mM Tiron, 1.5 mM linoleate and 0.5% (v/v) Tween 20 in 50 mM CHES buffer (pH 8.6) were prepared. Spectra were recorded using a Varian E-12 spectrometer at a field setting of 3393 G, a microwave power setting of 10 mW, a frequency of 9.155 GHz, a time constant of 1 s and a modulation amplitude of 0.8 G. The lipoxygenase inhibitor U28938 [8] was generously provided by Dr D.P. Wallach of the Upjohn Co. All other chemicals and enzymes were obtained from Sigma.

Hydroperoxides of linoleate were obtained by ether extraction of an incubated (20 min) reaction mixture initially containing 1.5 mM linoleate and excess (1–2 $mg \cdot ml^{-1}$) soybean lipoxygenase type-I.

3. RESULTS AND DISCUSSION

The relationship between lipoxygenase activity and $O_2^{\cdot -}$ production for smooth microsomal membranes and cytosol fractions isolated from cotyledons at various stages of senescence is illustrated in fig.1. The specific activity of cytosolic lipoxygenase increased more than 4-fold between days 2 and 9 of senescence, and this was paralleled by an increase in the amplitude of the Tiron ESR signal reflecting enhanced cytosolic production of $O_2^{\cdot -}$ in the presence of exogenous linoleic acid.

The specific activity of lipoxygenase associated with smooth microsomal membranes also increased during the course of senescence (fig.1) although the specific activity of the pelletable enzyme was considerably lower than that of the cytosol. The increase with age in $O_2^{\cdot -}$ production by smooth microsomes closely paralleled the rise in lipoxygenase activity (fig.1). Washing the microsomal membranes did not decrease either $O_2^{\cdot -}$ production or lipoxygenase activity, indicating that the pelletable activities were not attributable to entrapped cytosolic enzyme.

For both cytosol and microsomal membranes, the correlation coefficient for lipoxygenase and $O_2^{\cdot -}$ production was >0.9914, demonstrating that the changes in the two activities are highly correlated. Also, lipoxygenase activity and $O_2^{\cdot -}$ production were both optimal at pH 8.5–8.6 in the

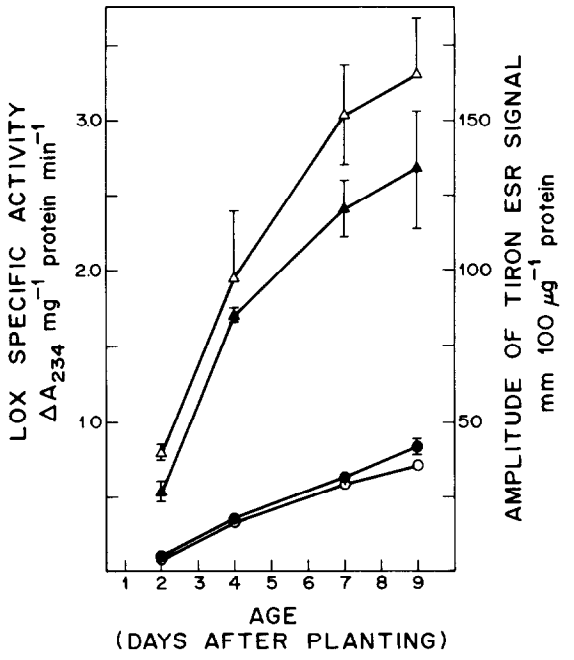

Fig.1. Correlative changes during senescence in lipoxygenase activity (LOX) and $O_2^{\cdot -}$ production for smooth microsomal membranes and cytosol from bean cotyledons. $O_2^{\cdot -}$ production is plotted as the amplitude (mm) of the high-field line of the Tiron semiquinone ESR spectrum. Standard errors of the means are shown except where error bars are smaller than symbols; n = 3–8. (○) Microsomal lipoxygenase, (△) cytosolic lipoxygenase, (●) microsomal $O_2^{\cdot -}$ production, (▲) cytosolic $O_2^{\cdot -}$ production.

cytosolic and membrane fractions, further illustrating the close relationship between the respective activities.

The dependence of $O_2^{\cdot -}$ production on lipoxygenase activity is demonstrated in fig.2. Cytosol in the absence of linoleic acid produced a relatively small Tiron signal reflecting $O_2^{\cdot -}$ production (fig.2A). When cytosol was added to oleate/Tween 20, which is not a substrate for lipoxygenase, the amplitude of the Tiron ESR spectrum remained essentially unchanged (fig.2B). However, addition of linoleate/Tween 20, the standard substrate for lipoxygenase, dramatically increased the amplitude of the Tiron ESR signal (fig.2C). In contrast, $O_2^{\cdot -}$ production by microsomal membranes was unaffected by addition of the linoleate/Tween 20 mixture presumably because free fatty acids present in the membranes were serving as an adequate source

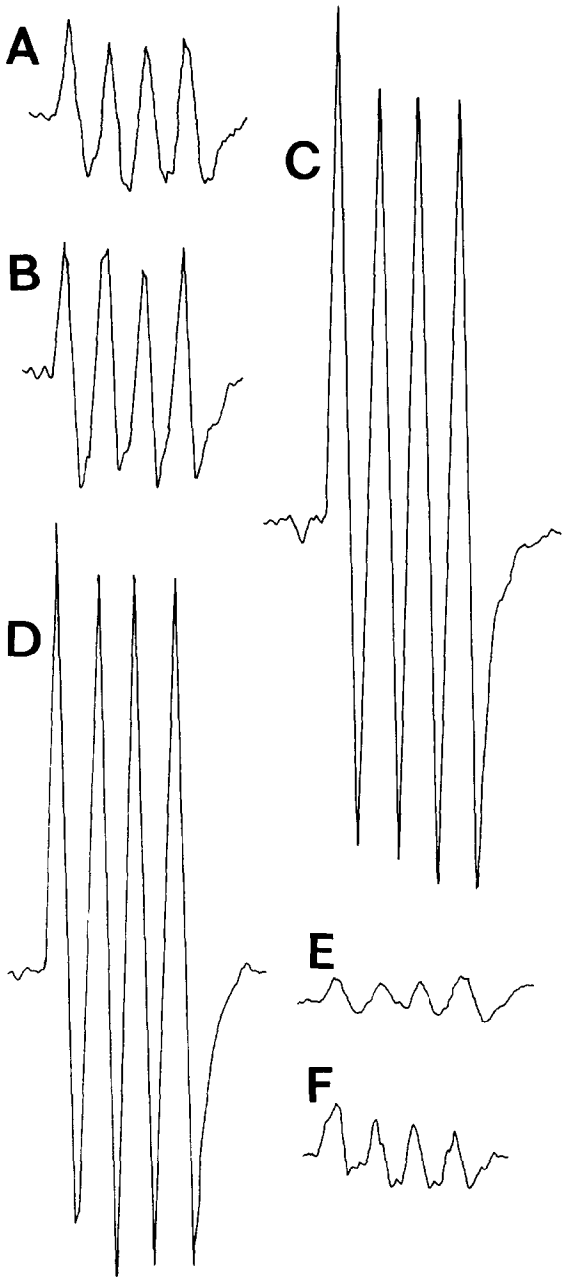

Fig.2. ESR spectra of the Tiron semiquinone radical derived from $O_2^{\cdot -}$. All samples contained 10 mM Tiron. (A) Cytosol from 7-day-old cotyledons (100 μg · protein ml^{-1}), (B) 7-day-old cytosol plus oleate/Tween 20, (C) 7-day-old cytosol plus linoleate/Tween 20, (D) 7-day-old cytosol plus heat-denatured 7-day-old membrane (150 μg · protein ml^{-1}) and 3 units phospholipase A_2. (E) Heat-denatured 7-day-old membrane and 3 units phospholipase A_2. (F) Fatty acid hydroperoxides extracted from a reaction mixture containing linoleate and soybean lipoxygenase.

of substrate for lipoxygenase. Heat-denatured membranes did not produce a Tiron ESR spectrum of significant amplitude even when treated with 3 units phospholipase A_2 (fig.2E). However, when cytosol (fig.2A) was combined with heat-denatured membranes treated with 3 units phospholipase A_2, the amplitude of the Tiron signal increased markedly reflecting enhanced $O_2^{\cdot -}$ production (fig.2D). This suggests that cytosolic lipoxygenase is able to associate with membranes and utilize phospholipase-liberated fatty acids as substrate although the prospect that fatty acid substrate is released from the membrane before being used by the cytosolic enzyme is not precluded.

The possibility that $O_2^{\cdot -}$ formed during the lipoxygenase reaction is simply derived from the hydroperoxy fatty acids was discounted by experiments in which freshly extracted hydroperoxy fatty acids were combined with Tiron, and ESR spectra recorded. Even in excess, these products produced only a small Tiron ESR signal (fig.2F), which presumably reflected a low titer of $O_2^{\cdot -}$ produced by dismutation of the hydroperoxides.

The effect of U28938, a specific inhibitor of lipoxygenase [8], on $O_2^{\cdot -}$ production was also examined. When U28938 was added to cytosol or membrane samples, lipoxygenase activity and $O_2^{\cdot -}$ production were both inhibited to a similar degree (table 1). It is perhaps noteworthy that, unlike most inhibitors of lipoxygenase, U28938 is not a potent free radical scavenger inasmuch as $O_2^{\cdot -}$ production by illuminated chloroplasts, which was detected by ESR as in [3], was diminished by less than 20% in the presence of 20 μM inhibitor. This same concentration of inhibitor reduced the lipoxygenase-associated $O_2^{\cdot -}$ production by 73% (expt.B, table 1).

Thus, 3 lines of evidence – the correlation between lipoxygenase activity and $O_2^{\cdot -}$ production during senescence, the dependence of $O_2^{\cdot -}$ production on lipoxygenase activity, and the common effects of a specific inhibitor of lipoxygenase – demonstrate that the enhanced production of superoxide anion by microsomal membranes and cytosol of senescing cotyledon tissue is attributable to lipoxygenase. Moreover, it seems reasonable to propose that the sequence of reactions whereby lipases generate free fatty acids which are used by lipoxygenase to produce $O_2^{\cdot -}$ and hydroperoxides, is a fundamental feature of membrane deteriora-

Table 1

Effects of U28938 on lipoxygenase activity and Tiron signal amplitude reflecting $O_2^{\cdot -}$ production

Expt.	Components in test reaction mixture	Lipoxygenase	$O_2^{\cdot -}$
A	control, containing cytosol plus methanol	100	100
B	cytosol plus U28938 (30 $\mu g \cdot mg^{-1}$ protein)	35.3	27.0
C	cytosol plus U28938 (300 $\mu g \cdot mg^{-1}$ protein)	4.9	<1
D	control, containing membrane plus methanol	100	100
E	membrane plus U28938 (300 $\mu g \cdot mg^{-1}$ protein)	2.0	<1

Values are expressed as percent of control. Inhibitor was supplied as a methanolic solution. All samples for ESR measurements of $O_2^{\cdot -}$ contained 10 mM Tiron. Inhibitor concentration is expressed as mg^{-1} sample protein since the protein concentration of the ESR assay was 10-fold higher than that of the lipoxygenase assay. Thus, for expt.B, the inhibitor concentration was 2 and 20 μM in the lipoxygenase assay and ESR assay, respectively

tion during senescence, for it explains such key symptoms of declining membrane integrity as the loss of membrane fatty acids reflected by a rising sterol/fatty acid ratio [4,10], increased production of superoxide anion by senescing membranes [11], and membrane rigidification attributable to free radical mediated lipid peroxidation [4,12]. The observation linking lipase activity with free radical production through lipoxygenase may also have broader implications. It has been demonstrated, for example, that enhanced phospholipase activity and the production of superoxide radical in human neutrophils are stimulated by phorbol myristate acetate [13], and it is conceivable that the formation of $O_2^{\cdot -}$ is attributable to lipoxygenase in this system as well.

REFERENCES

[1] Galliard, T. and Chan, H.W.S. (1980) in: The Biochemistry of Plants (Stumpf, P.K. and Conn, E.E. eds) vol.4, pp.131–161, Academic Press, New York.

[2] Baker, J.E., Lieberman, M. and Anderson, J.D. (1978) Plant Physiol. 61, 886–888.

[3] McRae, D.G. and Thompson, J.E. (1983) Planta 158, 185–193.

[4] Chia, L.S., Thompson, J.E. and Dumbroff, E.B. (1981) Plant Physiol. 67, 415–420.

[5] McKersie, B.D. and Thompson, J.E. (1977) Plant Physiol. 59, 803–807.

[6] Tappel, A.L. (1962) Methods Enzymol. 5, 539–542.

[7] Miller, R.W. and MacDowall, F.D.H. (1975) Biochim. Biophys. Acta 387, 176–187.

[8] Wallach, D.P. and Brown, V.R. (1981) Biochim. Biophys. Acta 663, 361–372.

[9] McKersie, B.D. and Thompson, J.E. (1979) Biochim. Biophys. Acta 550, 48–58.

[10] McKersie, B.D., Lepock, J.R., Kruuv, J. and Thompson, J.E. (1978) Biochim. Biophys. Acta 508, 197–212.

[11] Mayak, S., Legge, R.L. and Thompson, J.E. (1983) Phytochemistry 22, 1375–1380.

[12] Pauls, K.P. and Thompson, J.E. (1980) Nature 283, 504–506.

[13] Stocker, R. and Richter, C. (1982) FEBS Lett. 147, 243–246.