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Mini Review

Inhibition of plant lipoxygenases by antioxidants, nitric oxide and polyamines

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ABSTRACT

The effects of various antioxidants, nitric oxide (NO)-donor compounds and natural polyamines on the dioxygenation reaction catalyzed by soybean (*Glycine max*) lipoxygenases will be reviewed. Evidence will be presented that chain-breaking antioxidants ascorbic acid, 6-palmitoylascorbic acid and trolox inhibit soybean lipoxygenase-1 in the micromolar range (Ki 27, 3 and 18 μM respectively), whereas nitric oxide (NO)-releasing agents such as sodium nitroprusside and S-nitroso-N-acetylpenicillamine inhibit soybean lipoxygenase-2 with inhibition constants of 525 and 710 μM, respectively. These values correspond to a Ki of NO for lipoxygenase-2 of approximately 0.4 μM. Natural polyamines are shown to inhibit dioxygenase activity of lipoxygenase-1, whereas they are ineffective toward the lipoxygenase-2. The inhibitory power followed the order spermine > spermidine > cadaverine ≥ putrescine. The Ki values of spermine and spermidine for lipoxygenase-1 were 800 and 2700 μM, respectively. The possible physiological significance of these findings will be discussed, in the light of the involvement of the lipoxygenase pathway in biomembrane remodelling and biosynthesis of active biomolecules, such as jasmonic acid and traumatin.

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INTRODUCTION

Lipoxygenases (linoleate:oxygen oxidoreductase, EC 1.13.11.12) are non-heme, non-iron-sulfur dioxygenases which act on fatty acids containing one or more 1,4-*Z*,*Z*-pentadiene moieties to form enantiospecific *Z*,*E*-conjugated hydroperoxides. Lipoxygenases from both animal and plant tissues have been purified and characterized (see [1-3] for reviews). These enzymes play several physiological roles, in particular the product hydroperoxides are precursors of specific regulatory molecules, such as leukotrienes and lipoxins in animals, jasmonic acid and traumatin in plants [1-3]. Since the discovery of the role of lipoxygenases in the biosynthesis of potent effectors critical in animal and plant physiology, extensive research was done to find efficient inhibitors of their activity. Most studies have used soybean (*Glycine max* (L.) Merrill) lipoxygenases as homologues of mammalian lipoxygenases. Soybean contains several lipoxygenases, among which lipoxygenase-1 (LOX-1) and lipoxygenase-2 (LOX-2) are the main isoforms [1, 2]. LOX-1 and LOX-2 are encoded for by different genes, have different molecular mass, substrate specificity, positional selectivity and optimal pH [4, 5]. The three dimensional structure [6, 7] and catalytic mechanism [8-11] of LOX-1 have been characterized in detail.

LOX activity is suppressed by several compounds, which act in different ways on the LOX-catalyzed reaction [12]. In particular, the effect of compounds with antioxidant properties has attracted much attention (reviewed in [13]). Also the possible interaction of nitric oxide (NO) with lipoxygenase has attracted growing interest, because LOX activity gives rise to hydroxyl radicals and superoxide anions [14], which are known to be involved in NO neurotoxicity [15]. It was reported that NO can inhibit lipoxygenase-catalyzed lipid oxidation was reported, but such inhibition was not explained [16]. Furthermore, natural polyamines (putrescine, cadaverine, spermidine and spermine), which play several roles in animals [17] and plants [18], have been shown to inhibit lipid peroxidation by chelating iron, thus impairing the iron-mediated generation of free radicals [19]. Other lines of evidence suggest that the radical scavenging ability [20] and the antioxidant power [21] of polyamines might be important in the inhibition of lipid peroxidation. Remarkably, lipoxygenases play a role in membrane lipid peroxidation by forming hydroperoxides in the lipid bilayer [22].

Here the main results are summarized of studies on the inhibition of LOX-1 activity by antioxidants ascorbic acid [23], 6-palmitoylascorbic acid [24] and trolox (a water-soluble analogue of α-tocopherol) [23], reported in detail in ref. [25]. Also the main findings on the effect of nitric oxide on the activity of LOX-2 is reviewed (details in refs [26, 27]). Finally, the interaction of polyamines with LOX-1 and LOX-2 is presented (see ref. [28] for a detailed discussion).

ANTIOXIDANTS

Ascorbic acid was found to be a competitive inhibitor of LOX-1 and showed an inhibition constant, Ki, of 27 μM (Table 1). Inhibition by ascorbic acid was reversible, LOX-1 regaining full activity after 4 min of dialysis of enzyme/inhibitor mixtures (15 nM/100 μM) at 4°C. The ability of ascorbic acid to inhibit linoleic acid oxygenation by LOX-1 was dependent on the pH of the reaction, increasing from pH 5.5 to pH 7.0 and then dropping down between pH 7.0 and 9.0. Interestingly, dehydroascorbic acid inhibited LOX-1 activity to a lesser extent than ascorbic acid. In fact, 150 μM dehydroascorbic acid reduced linoleic acid oxygenation to 70% of the control value, compared to 38% obtained with the same concentration of ascorbic acid. The same inhibition studies performed with ascorbic acid were done with the chain-breaking antioxidants 6-

Table 1

Inhibition of lipoxygenase-1 by chain-breaking antioxidants and sodium dithionite

Inhibitor	Inhibition type	Inhibition constant (Ki, μM)
Ascorbic acid	competitive	27
6-Palmitoylascorbic acid	competitive	3
Trolox	competitive	18
Sodium dithionite	non-competitive	49

palmitoylascorbic acid and trolox. Both compounds were found to be complete, reversible and competitive inhibitors of LOX-1 activity, Ki values being 3 μM and 18 μM, respectively (Table 1). Sodium dithionite, a non-specific reductant, slowed down LOX-catalyzed reaction by acting as a non-competitive inhibitor, with a Ki of 49 μM (Table 1).

Unlike other antioxidant inhibitors of LOX-1 [13], the compounds under study did not reduce Fe(III) to inactive Fe(II) in the active site, in fact the lag phase of the reaction was not prolonged [25]. EPR spectroscopy (not shown) confirmed this finding, clearly indicating the lack of radical formation from the inhibitor molecules during LOX-1 inhibition. At variance with the chain-breaking antioxidants, sodium dithionite acted as non-competitive inhibitor and caused a lengthening of the lag phase of the reaction [25]. This is consistent with sodium dithionite being a non-specific reductant, which turns LOX-1 into the inactive Fe(II) form [29]. It can be suggested that the chain-breaking antioxidants might interact with the interior of the "funnel" leading to the active site [6], because enhancing the lipophilicity of the inhibitor made it more effective (Table 1). This hypothesis is in line with previous work on the competitive inhibition of LOX-1 by *n*-alcohols, where longer alkyl chains were shown to increase the affinity of these compounds for the enzyme [30].

NITRIC OXIDE DONORS

Sodium nitroprusside (SNP) and S-nitroso-*N*-acetylpenicillamine (SNAP) are NO-donors widely used for studying the effect of nitric oxide on cells and enzymes [31]. It was found that dioxygenation of linoleic acid catalyzed by LOX-2 was inhibited by SNP and SNAP in a dose-dependent manner (Fig. 1).

Kinetic analysis showed that both NO-donors acted on the LOX-2 reaction as complete, competitive inhibitors, with inhibition constants (Ki) of 525 μM and 710 μM, for SNP and SNAP respectively [26, 27]. It is

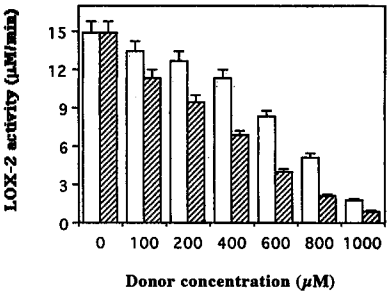

Figure 1

Inhibition of lipoxygenase-2 by nitric oxide donors SNP (hatched bars) and SNAP (white bars)

reasonable to assume that the inhibition of lipoxygenase is due to the binding of NO to iron. In fact, it has been shown by EPR that NO binds to the Fe(II) form of LOX-1 isozyme [32]. It should be taken into account that NO may also reduce Fe(III) LOX-2 to Fe(II). It is possible to calculate from Fig. 1 a Ki of NO for LOX-2 of approximately 400 nM, because micromolar concentrations of SNP or SNAP yielded a linear release of NO in the nanomolar range [26]. On the other hand, physiologically attainable H_2O_2 concentrations were able to stimulate LOX-2, the largest activation being obtained at a H_2O_2:LOX-2 molar ratio of 2:1 (Fig. 2).

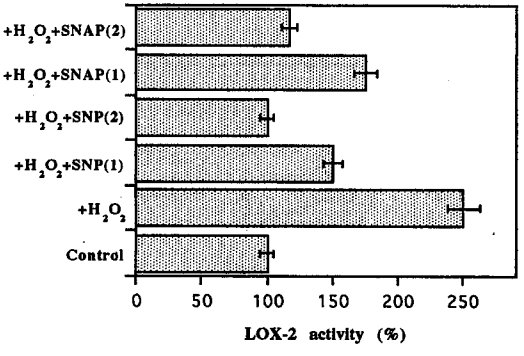

Figure 2

Effect of SNP and SNAP on the stimulation of LOX-2 activity by H_2O_2

Linoleic acid dioxygenation catalyzed by 0.5 nM LOX-2 was stimulated by 1 nM H_2O_2, an effect which was counteracted by SNP or SNAP at 5 μM (1) or 10 μM (2) final concentration. This observation is in keeping with the reported H_2O_2 stimulation of LOX-1 activity [33], and is attributable to the oxidation of Fe(II) to Fe(III) by peroxides [33].

Remarkably, both SNP and SNAP were able to counteract the H_2O_2-mediated activation of LOX-2, in a dose-dependent manner (Fig. 2). This finding corroborates the hypothesis of a direct interaction of NO with the catalytic iron, suggesting a reduction of Fe(III) to Fe(II) by NO, which counteracts the hydrogen peroxide-mediated oxidation of ferrous iron to ferric.

POLYAMINES

The dioxygenase activity of soybean LOX-1 was inhibited by polyamines, in a dose-dependent manner (Fig. 3A).

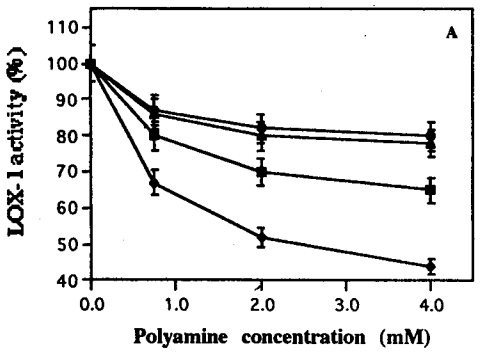

Figure 3

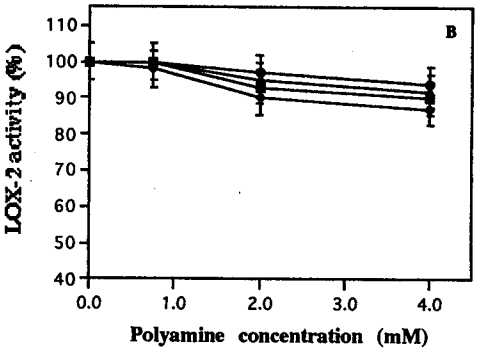

Figure 3

Effect of polyamines on dioxygenase activity of LOX-1 (A) and LOX-2 (B)

Putrescine (circles), cadaverine (triangles), spermidine (squares) and spermine (diamonds) were added to the reaction mixture, containing 90 μM linoleic acid as substrate.

The inhibitory power followed the order: spermine > spermidine > cadaverine ≥ putrescine. Therefore, spermine (Spn) and spermidine (Spd) were chosen to further characterize the inhibition mechanism. Unlike LOX-1, LOX-2 activity was hardly affected by polyamines (Fig. 3B). Lineweaver-Burk plots of the reaction catalyzed by LOX-1, in the presence or absence of Spd or Spn, indicated that both compounds are uncompetitive inhibitors of LOX-1 with respect to linoleic acid, the inhibition constants being 2.70 mM and 0.80 mM, for Spd and Spn respectively [28]. Since cadaverine (1,5-diaminopentane) was approximately as effective as putrescine (1,4-diaminobutane), the spacing between the amino groups seems not to influence the inhibitory ability of polyamines. The uncompetitive inhibition of LOX-1 with respect to linoleic acid indicated that Spd and Spn bind to the enzyme-substrate complex only. The presence of a negative charge on the substrate molecule seems not to be involved in the interaction with polyamines, because dioxygenation of linoleic acid (negatively charged) and methyl-linoleate

(uncharged) is inhibited to the same extent (Table 2).

LOX-1 can dioxygenate also triacylglycerols such as trilinolein [34]. This activity, though considerably lower than the activity with free linoleic acid (Table 2), is essential in energy mobilization from lipid bodies [11, 35]. Interestingly, Spd and Spn inhibited to a similar extent the dioxygenation of trilinolein and that of linoleic acid (Table 2). Neither Spd nor Spn inhibited LOX-1 by reducing Fe(III) to inactive Fe(II) in the catalytic site, because the duration of the lag phase of the reaction, which is a function of the iron redox state, was not affected [28]. It seems noteworthy that the radical trapping ability of polyamines increases from putrescine to spermine [19, 36-38], in keeping with their inhibitory power towards LOX-1 (Fig. 3A). Interestingly, Spd and Spn significantly inhibited also the co-oxidase and peroxidase activities of LOX-1, which are both related to the formation of radical intermediates and play a role in the antioxidant defence of cells [38, 39]. It is also tempting to suggest that the lack of inhibition of LOX-2 by polyamines might reside in the absence of a (negatively charged) polyamine binding site on the isozyme surface. This suggestion would be in keeping with the known differences in isoelectric point among soybean lipoxygenases [1-5]. The existence of a polyamine binding site might at least partially explain the reported pH dependency of LOX-1 inhibition by polyamines [28], to which also a conformational change in the enzyme molecule may contribute [40]. It is noteworthy that specific polyamine binding sites have been found in other relevant classes of enzymes, *e.g.* protein kinases [41], although further structural data on lipoxygenase protein are needed to make more conclusive statements [6, 7].

The evidence reported here suggests that polyamines, in a concentration range close to that found in cells [20,

Table 2

Inhibition of the LOX-1-catalyzed dioxygenation of linoleic acid, methyl-linoleate and trilinolein by natural polyamines

Substrate (90 μM)	LOX-1 activity (nmol hydroperoxide.min^{-1}.mg protein^{-1})		
	No Inhibitor	Spermidine (2 mM)	Spermine (2 mM)
Linoleic acid	19330±970 (100)	13000±650 (67)	8830±440 (46)
Methyl-linoleate	2410±240 (100)	1720±170 (71)	1400±210 (58)
Trilinolein	650±65 (100)	490±50 (75)	390±40 (60)

Values in brackets represent percentage of the untreated controls, arbitrarily set to 100.

42- 44], efficiently inhibit LOX-1 activity, while they are ineffective toward LOX-2. Remarkably, lipoxygenase activity is inhibited as well inside the cell [28]. Thus, it can be proposed that polyamines can rapidly cross the cell membranes but, once inside the cell, they cannot diffuse back easily. Such a polyamine influx into the cell cytosol might be regulated by specific polyamine channels, which have indeed been discovered in *Arabidopsis thaliana* outer membranes [45].

CONCLUDING REMARKS

In this mini-review, evidence has been presented that the chain-breaking antioxidants ascorbic acid, 6-palmitoylascorbic acid and trolox act as competitive inhibitors of soybean LOX-1. It has been shown also that NO and peroxides have opposite effects on the iron oxidation state of soybean LOX-2, and that NO competes with the substrate (linoleate) for the binding site. Furthermore, natural polyamines have been shown to bind to the enzyme-substrate complex only, leading to uncompetitive inhibition of LOX-1 but not of LOX-2.

Taken together, these findings support the hypothesis that lipid peroxidation might be prevented by antioxidants, nitric oxide or polyamines through a direct inhibition of LOX-1 and/or LOX-2. These results might have physiological significance in the light of lipoxygenase involvement in biomembrane alteration, both in animals [46] and plants [47, 48]. Moreover, they might be relevant also in those processes where antioxidants and/or polyamines are suggested to protect from lipid peroxidation, *e.g.* plant senescence [49], response to ozone [22] or osmotic stress [18, 50].

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