PERGAMON

Phytochemistry 55 (2000) 59–66

PHYTOCHEMISTRY

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Triacontanol inhibits both enzymatic and nonenzymatic lipid peroxidation

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Received 26 August 1999; received in revised form 31 May 2000

Abstract

The effect of the plant growth regulator, triacontanol (TRIA) on lipid peroxidation was studied in three different systems: (i) isolated chloroplasts of spinach (*Spinacea oleracea* L.) leaves; (ii) egg lecithin liposomes; and (iii) soybean lipoxygenase (LOX) system. The nonenzymatic lipid peroxidation in isolated chloroplasts and egg lecithin liposomes was measured as the amount of thiobarbituric acid reactive substances (TBARS) formed. Inhibition of Fe^{2+} and/or light-induced lipid peroxidation by TRIA was observed in both isolated chloroplasts and egg lecithin liposomes. The kinetics of soybean lipoxygenase-I (LOX-1) was studied using linoleic acid as the substrate. The enzyme was competitively inhibited by TRIA. The K_i for TRIA inhibition of the enzyme was estimated to be 3.2–5.0 μM according to different methods of estimation. TRIA has been known to exhibit anti-inflammatory action in animals and this anti-inflammatory effect of TRIA might be mediated through inhibition of lipid peroxidation. Since LOX inhibitors have been extensively used as therapeutic agents, TRIA, being a natural compound has been suggested to be an effective anti-inflammatory drug.

Keywords: Anti-inflammatory action; Chloroplast lipids; Lipid peroxidation; Liposomes; Lipoxygenase; Photoperoxidation; *Spinacea oleracea* L; Triacontanol

1. Introduction

Triacontanol (TRIA), a long chain primary alcohol ($C_{30}H_{61}OH$) has been known to be a potent plant growth promoting substance of many agricultural and horticultural crops (Ries, 1985). In fact, TRIA occurs in nature as a constituent of cuticular waxes (Kolattukudy and Walton, 1973). Growth promoting effects of TRIA have been observed in various plants and it has been shown that TRIA increases dry weight, carbon dioxide fixation, reducing sugars, soluble proteins and free amino acids leading to the enhancement of plant growth and crop yield (Ries, 1985, 1991; Shripathi, 1996). We have shown that TRIA promotes vegetative growth in cotton (*Gossypium hirsutum* L.) and enhances the level of monogalactosyldiacylglycerol (MGDG) (Shripathi and Swamy, 1994), a galactolipid which appears to be involved in packaging of photosystem-I proteins (Dominy and Williams, 1987). In addition to this, we have also observed that TRIA decreases microviscosity of cucumber (*Cucumis sativus* L.) fruit protoplast membranes (Shripathi et al., 1997).

Apart from this, the effect of TRIA has also been studied on animal system (McBride et al., 1987; Warren et al., 1992). An anti-inflammatory effect of TRIA has been demonstrated in mice with the finding of reduction in thymus weight, number of thymus cells, number of splenocytes and reduced amount of interleukin-1 (Warren et al., 1992). Considering the direct involvement of TRIA in changing the chemical compo-

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0031-9422/00/$ - see front matter
PII: S0031-9422(00)00201-6

sition and physical status of membrane lipids (Shripathi and Swamy, 1994; Shripathi et al., 1997) and its role as an anti-inflammatory compound in animals (McBride et al., 1987; Warren et al., 1992), a study on the effect of TRIA on peroxidative break down of lipids has been undertaken.

Lipid peroxidation results in the formation of hydroperoxides which are converted into a range of secondary products such as active oxygen species, free radicals, aldehydes, alkenes, ketols, oxoacids, jasmonic acid and methyl jasmonates (Vick and Zimmerman, 1987; Halliwell and Gutteridge, 1985). Oxygen and free radicals damage the membrane structure and organisation whereby altering the function of membrane bound enzymes and receptors (Vick and Zimmerman, 1987). However, the other breakdown products of lipid peroxidation have been implicated in triggering defence reactions (Blee, 1998), antipathogenic action (Stallaert et al., 1995) and stress-related responses (Dionisio-Sese and Tobita, 1998). The causes, mechanism and consequences of lipid peroxidation in biological system have been recently reviewed by Girotti (1998).

Plants possess a number of antioxidant enzymes that protect them against the damaging effects of activated oxygen species (Halliwell and Gutteridge, 1985). Superoxide dismutase is a major scavenger of O_2^- and its enzymatic action results in the formation of H_2O_2 and O_2. The hydrogen peroxide produced is then scavenged by catalase and a variety of peroxidases. Catalase dismutates H_2O_2 into water and molecular oxygen, whereas peroxidase decomposes H_2O_2 by oxidation of cosubstrates such as phenolic compounds and/or antioxidants (Halliwell and Gutteridge, 1985). Apart from this, membrane lipids are protected against peroxidation in vivo by antioxidants such as α-tocopherol, ascorbic acid, β-carotene and other carotenoids (Halliwell and Gutteridge, 1985). In the present investigation we have demonstrated that TRIA, a naturally occurring aliphatic alcohol could also act as an inhibitor of lipid peroxidation. Soybean lipoxygenase (LOX) has been used in the present investigation since it is homologous to that of animals and has been routinely used as a model for studies on animal system (Lominitski et al., 1993).

2. Results and discussion

Lipid peroxidation may be enzymatic-LOX mediated (Stallaert et al., 1995; Croft et al., 1993; Peever and Higgins, 1989) or nonenzymatic-active oxygen species mediated (Stallaert et al., 1995). The peroxidation can be initiated by different compounds like azo initiaters (O'Donnel et al., 1997), photosensitisers (Merchat et al., 1996), transition metal ions like Fe^{2+} (Ohyashiki et al., 1998) and light (Halliwell and Gutteridge, 1985). These initiators generate singlet oxygen or free radicals which would in turn trigger peroxidation chain reaction. Lipid peroxidation is inhibited by naturally occurring α-tocopherol, β-carotene and other carotenoids (Halliwell and Gutteridge, 1985). These compounds facilitate scavenging singlet oxygen or quench excess energy to inhibit singlet oxygen production.

The thylakoid membranes contain a high percentage of polyunsaturated fatty acids and are thus very susceptible to peroxidation (Halliwell and Gutteridge, 1985). A slight perturbation of thylakoid membrane would lead to alteration in photosystem II which in turn affects the photosynthetic process (Chakraborthy and Tripathy, 1992). Considering all these, inhibition of lipid peroxidation by TRIA has been studied in three different systems: (1) light and/or Fe^{2+}-induced lipid peroxidation in isolated chloroplasts; (2) Fe^{2+}-induced lipid peroxidation in egg lecithin liposomes, and (3) soybean LOX system.

Lipid peroxidation in isolated chloroplasts was studied at different time intervals. The amount of thiobarbituric acid reactive substances (TBARS) formed was dependent on the time of incubation. Significant increase in TBARS was noticed in both control and TRIA-treated chloroplasts. However, there was a significant decrease in the amount of TBARS in TRIA-

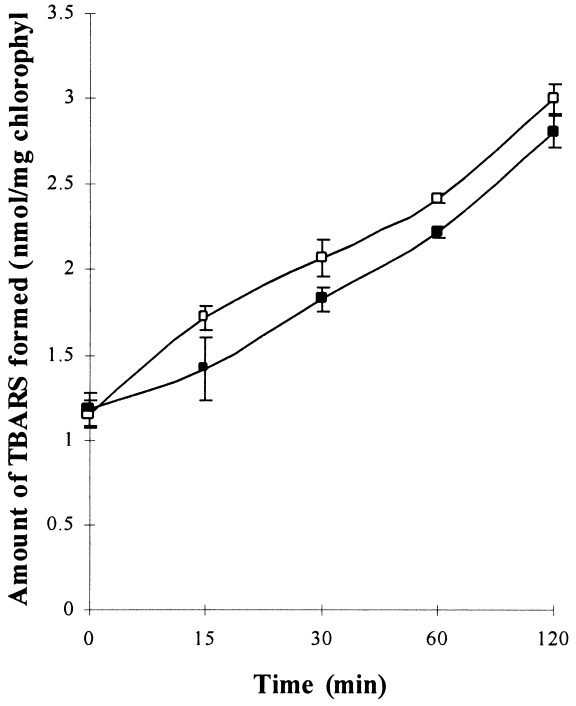

Fig. 1. Effect of TRIA on lipid peroxidation in isolated chloroplasts of spinach (*Spinacea oleracea* L.) leaves in light. (□) Control and (■) TRIA-treated chloroplasts. Each point represents mean value of nine experiments and vertical bars indicate ±SE. Values are significant at $t = 0.05$.

treated chloroplast suspension compared to control, irrespective of incubation time (Fig. 1).

The effect of TRIA on Fe^{2+}-induced lipid peroxidation in isolated chloroplasts was studied in light as well as dark conditions (Figs. 2 and 3). Two different concentrations (1 and 10 mM) of $FeSO_4$ were used to induce lipid peroxidation. Induction of lipid peroxidation by Fe^{2+} was enhanced by light (Figs. 2 and 3). About 50% reduction in the amount of TBARS formed was observed when the reaction was carried out in dark (Fig. 3). In spite of the changes in the extent of lipid peroxidation due to light and/or Fe^{2+}, the inhibition of Fe^{2+}-induced lipid peroxidation by TRIA was observed in both light and dark conditions (Figs. 2 and 3). Fig. 4 represents the effect of TRIA concentration on chloroplast lipid peroxidation under light in the presence of $FeSO_4$. The basal level of lipid peroxidation in Fig. 4 is slightly higher than that of Fig. 2 as these experiments were conducted with different batches of leaves and in different seasons. However, it is evident in both the cases that lipid peroxidation decreases with increase in the concentration of TRIA. In addition, it is also evident that TRIA reduces TBARS formation in the chloroplasts even in the absence of $FeSO_4$ which indicates that TRIA inhibits light-induced lipid peroxidation.

It has been a well established fact that TRIA increases photosynthesis and accumulation of photosynthates (Ries, 1985). Apart from this, we have observed that TRIA increases MGDG, a galactolipid possessing a high degree of polyunsaturated fatty acids (Shripathi and Swamy, 1994) and MGDG appears to be involved in packaging of photosystem-I proteins (Dominy and Williams, 1987). In this connection, it is interesting to note that the role of TRIA in enhancing photosynthesis might lie with its ability to enhance MGDG levels (Shripathi and Swamy, 1994) and protect the thylakoid membranes from peroxidative damage (Figs. 1–3). Since intact chloroplasts were used as experimental material, both LOX and singlet oxygen-mediated lipid peroxidation could be expected

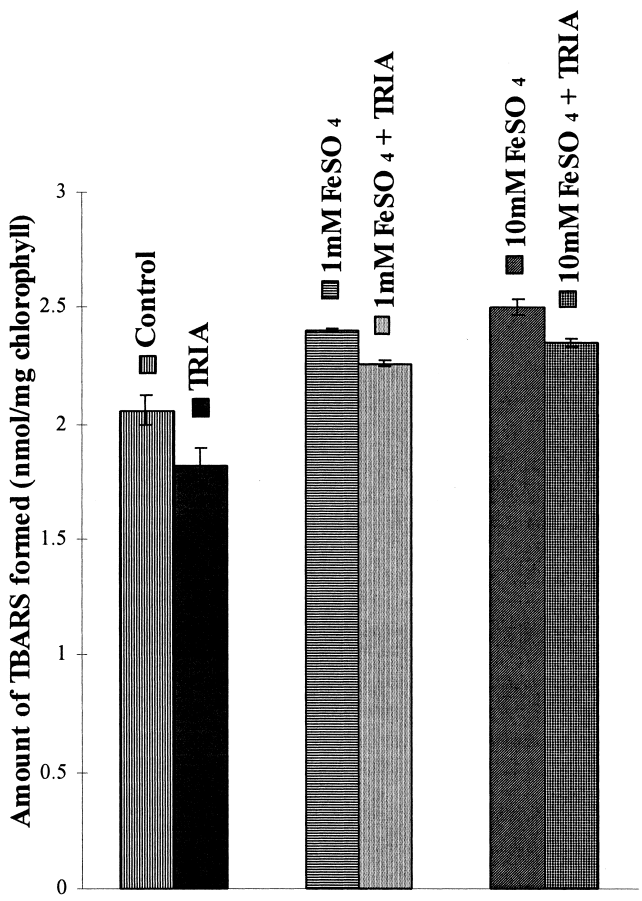

Fig. 2. Effect of TRIA on Fe^{2+}-induced lipid peroxidation in isolated chloroplasts of spinach (*Spinacea oleracea* L.) leaves in light. Results are represented as mean (3) ± SE. Amount of TBARS was estimated at 60 min after incubation. Treatments are indicated above the error bars. Values are significant at $t = 0.05$.

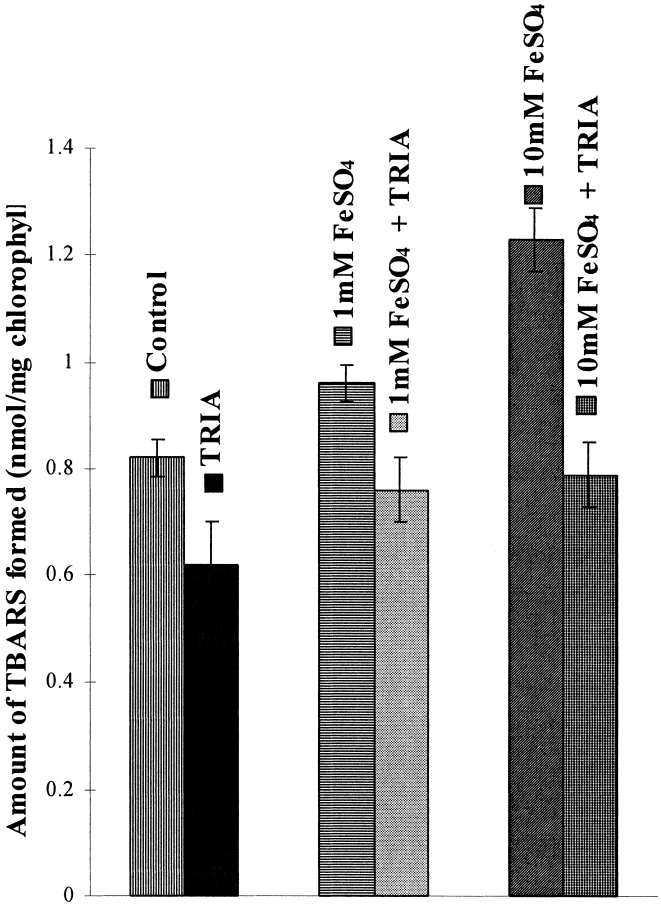

Fig. 3. Effect of TRIA on Fe^{2+}-induced lipid peroxidation in isolated chloroplasts of spinach (*Spinacea oleracea* L.) leaves in dark. Results are represented as mean (6) ± SE. Amount of TBARS was estimated at 60 min after incubation. Treatments are indicated above the error bars. Values are significant at $t = 0.05$.

in the system (Halliwell and Gutteridge, 1985; Blee, 1998). Inhibition of lipid peroxidation by TRIA in chloroplasts under different conditions i.e., without Fe^{2+} in light (Fig. 1) and with Fe^{2+} in light (Fig. 2) or dark (Fig. 3) indicates that TRIA acts as an inhibitor of lipid peroxidation in general. This is evident from our observation of its inhibitory effect on peroxidation in egg lecithin liposomes (Fig. 5) and soybean LOX system (Figs. 6 and 7). Further, the overall enhanced level of lipid peroxidation in chloroplasts in the presence of light (Fig. 2) indicates that the process may be due to the cumulative effect of both light-induced and Fe^{2+}-induced lipid peroxidation.

The amount of TBARS formed was significantly less in the liposomes containing TRIA (Fig. 5). There was about two-and-a-half times increase in the amount of TBARS formed with increase in concentration of Fe^{2+} from 1 to 10 mM (Fig. 5). However, the inhibition of lipid peroxidation by TRIA was observed irrespective of Fe^{2+} concentration (Fig. 5). $FeSO_4$-induced lipid peroxidation in egg lecithin liposomes was also found to decrease with increase in the concentrations of TRIA (results not shown). Although Fe^{2+} could induce lipid peroxidation even in TRIA-containing liposomes, significant difference in the amount of TBARS formed in control liposomes (liposomes made of solely egg lecithin) and TRIA-containing liposomes indicate that TRIA could inhibit Fe^{2+}-induced free radical-mediated lipid peroxidation (Fig. 5).

Enzyme rate measurements of soybean lipoxygenase (LOX-1) were made as a function of substrate concentration in the absence and presence of TRIA (3 μM), and Lineweaver–Burk plot was drawn (Fig. 6). The K_m was estimated to be 22.3 μM in the absence of TRIA and the inhibition of soybean LOX-1 by TRIA has been proved to be competitive (Fig. 6). Dixon plot was constructed with enzyme rate measurements at 25 and 50 μM linoleic acid concentration and 0–10 μM of TRIA (Fig. 7). The K_i value for TRIA with Dixon plot was estimated to be 5.0 μM and it was 3.2 μM from Lineweaver–Burk plot. The K_m and K_i values obtained with Eadie–Hofstee plot were 22.4 and 3.6 μM, respectively (plot not shown).

The K_m value for soybean LOX-1 obtained here (22.3 μM) falls within the narrow range reported earlier (18.15–25.0 μM) for this enzyme (Galliard and Chan, 1980; Asbi et al., 1989; Gibian and Galaway, 1976). Results obtained with both Lineweaver–Burk plot and Dixon plot confirm that TRIA inhibits the enzyme competitively (Figs. 6 and 7). Since TRIA is a strongly nonpolar molecule, the inhibition of LOX-1 seems to be due to the interaction of TRIA with hydrophobic active site of the enzyme molecule. Inhibition studies on this enzyme with small chain alcohols indicated competitive inhibition with K_i values of 3.6–9.9 mM (Kuninori et al., 1992). However, K_i value for TRIA inhibition of LOX-1 (5.0 μM) is thousand times less than that of shorter chain alcohols. Therefore,

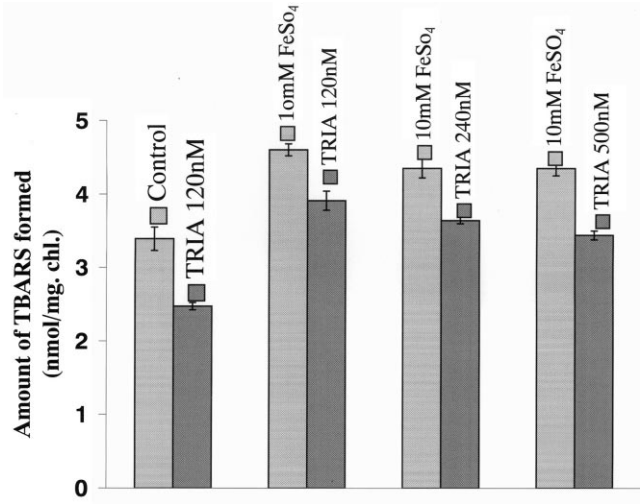

Fig. 4. Effect of different concentrations of TRIA on lipid peroxidation in the isolated spinach chloroplasts in the presence of light and either in the absence or presence of $FeSO_4$. All other conditions were similar to that of Fig. 2. Values are significant at $t = 0.05$.

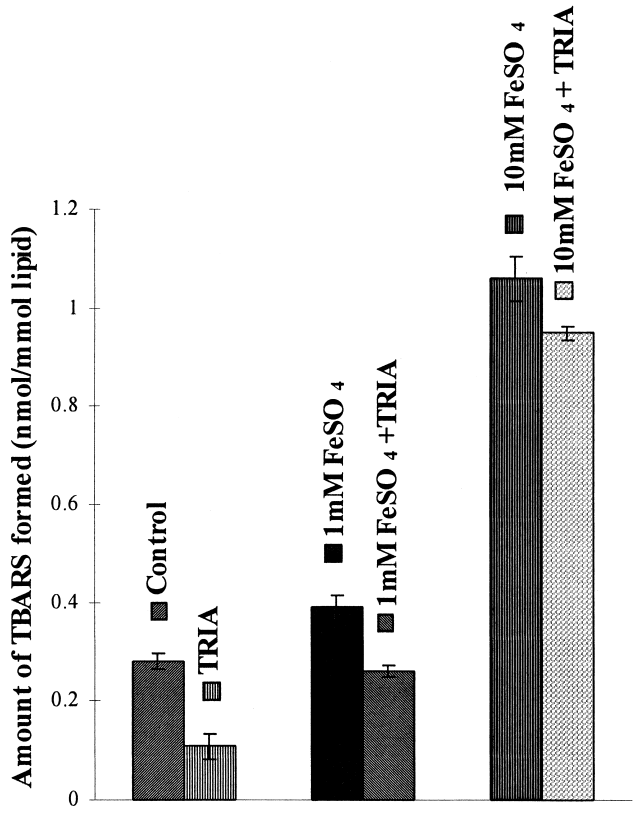

Fig. 5. Effect of TRIA on Fe^{2+}-induced lipid peroxidation in egg lecithin liposomes. Results are represented as mean (3) ± SE. Amount of TBARS was estimated at 60 min after incubation. Treatments are indicated above the error bars. Values are significant at $t = 0.05$.

TRIA happens to be a highly potent inhibitor of the enzyme compared to other aliphatic alcohols.

A large number of synthetic and natural compounds have been tested for their efficacy in inhibiting the LOX activity (Ford-Hutchinson et al., 1994). Inhibitors of LOX are of two main categories: (1) compounds by the virtue of their anti-oxidant capability, their redox potentials favouring them as alternative substrate for LOX as in AA-861, L-656224, NDGA, caffeic acid etc.; and (2) the non-redox competitive inhibitors such as the natural lignan, justicidin-E and synthetic compounds D-3128 and L-697198 (Ford-Hutchinson et al., 1994). Many of these compounds have shown adverse effects in therapeutic use. The antioxidant LOX inhibitors like ascorbic acid ($K_i = 27$ μM), 6-palmitoyl ascorbic acid ($K_i = 3$ μM), trolox ($K_i = 18$ μM), sodium dithionate ($K_i = 49$ μM) (Maccarrone et al., 1995) and caffeic acid phenethyl ester (CAPE) ($K_i = 8$ μM) (Sudina et al., 1993) exhibit sufficiently low K_i values. However, antioxidants are not usually recommended as regular therapeutic agents because of their adverse effects (Ford-Hutchinson et al., 1994). On the other hand, the safer compounds such as sucrose esters of fatty acids exhibit very high K_i values (Nishiama et al., 1993). Therefore, TRIA inhibiting the LOX activity with K_i of about 5 μM appears to be a promising therapeutic agent. In addition, TRIA being a natural component of human diet is not expected to have any adverse effects after use as a therapeutic agent. Furthermore, TRIA has been known to induce anti-inflammatory responses in animals (McBride et al., 1987; Warren et al., 1992), and the oxidative damage of membrane lipids is linked to initiation of inflammatory signal (Girotti, 1998). Thus, the anti-inflammatory effect of TRIA might, at least in part, be mediated through the inhibition of lipid peroxidation.

3. Experimental

Pure TRIA was a gift from B.D.K., Hubli, India. Extra pure reagent grade linoleic acid was obtained from SISCO Research Laboratories, Mumbai, India. Soybean LOX-1 (type V, purified by affinity chromatography; 646,000 units/mg protein) was purchased from Sigma, USA. Other chemicals were of analytical grade.

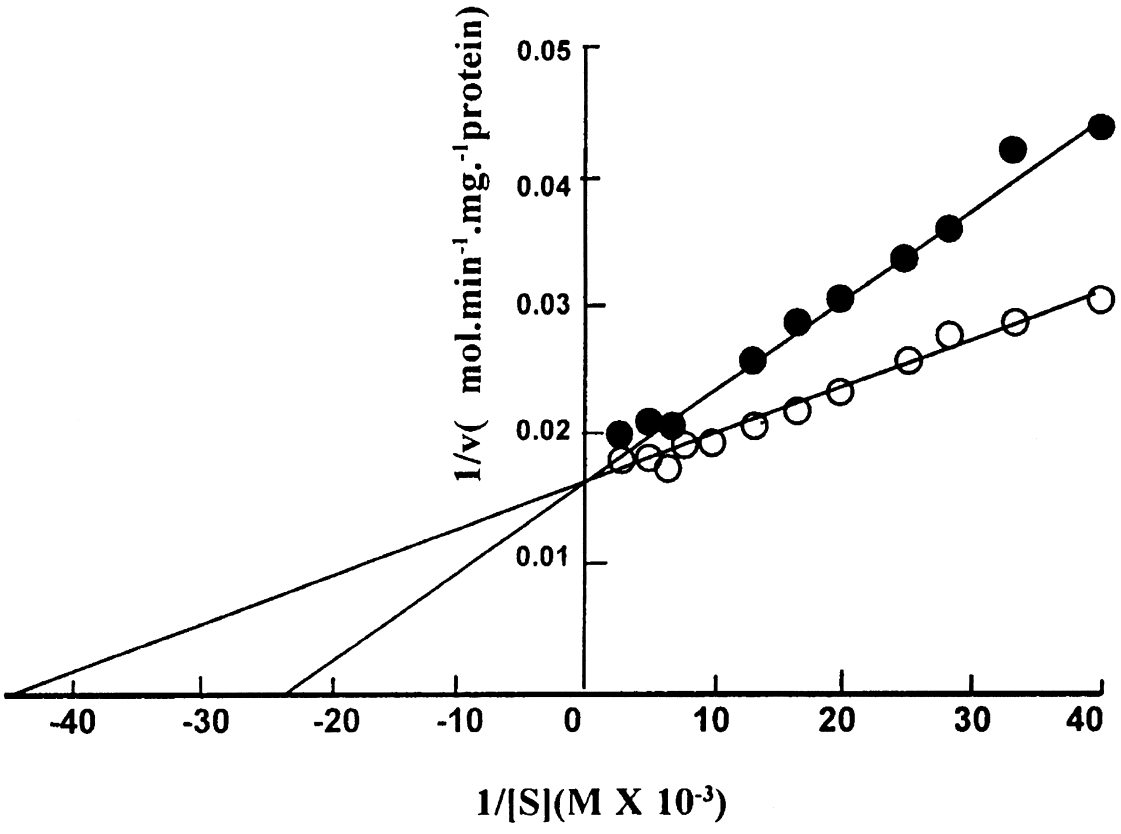

Fig. 6. Lineweaver–Burk plot for soybean LOX-1 activity in the absence (○) and presence (●) of TRIA (3.0 mM). The incubation mixture (3 ml) contained 0.1 M borate buffer at pH 9.0, 25–250 μM linoleic acid, and 0.4 μg of the enzyme.

3.1. Isolation of chloroplasts

Chloroplasts were isolated from spinach (*Spinacea oleracea* L.) leaves employing the method described by Swamy and Pillay (1985). Freshly harvested 100 g of leaves were blended with 400 ml of extraction medium (50 mM Tris–HCl buffer (pH 8) containing 0.3 M mannitol, 1 mM β-mercaptoethanol and 3 mM EDTA) for 20–30 s. The homogenate was passed through a series of nylon cloths of mesh size 100, 50, 25 and 10 μM, sequentially. The final filtrate was centrifuged at 1000 $\times g$ for 90 s at 4°C. The pellet was resuspended in the extraction medium and again centrifuged at 1000 $\times g$ for 90 s at 4°C. The resultant chloroplast pellet was suspended in incubation medium (0.1 M phosphate buffer (pH 7.5) containing 0.3 M sucrose, 3 mM EDTA and 1 mM β-mercaptoethanol).

3.2. Preparation of liposomes

The liposomes were prepared from egg lecithin which was purified from egg yolk (Shripathi, 1996). A clear suspension of liposomes in 50 mM KCl was obtained following the ether infusion method of liposome preparation (Deamer and Bangham, 1976). In brief, the lipid equivalent to 0.4 mM phosphate for 10 ml liposome suspension was taken in a clean dry test tube and the solvent was evaporated under reduced pressure. The thin film of lipid was then dissolved in 0.5 ml of diethyl ether and this solution was injected into 10 ml 50 mM KCl solution maintained at 55–65°C. The solvent was completely removed from the suspension under reduced pressure at 40°C. TRIA containing liposomes were prepared by cosolubilising the quantity equivalent to 2 mol% of lipids. The TRIA-lipid in diethylether (0.5 ml) was then injected into 10 ml of 50 mM KCl. The solvent was completely evaporated under reduced pressure. The resultant liposomes were claimed to be large unilammellar vesicles (Deamer and Bangham, 1976). The final concentration of phospholipids in the liposome suspension was 0.4 mM as estimated by modified Bartlett's method (Christie, 1982).

3.3. Induction of lipid peroxidation

Chloroplasts were incubated in a medium containing 0.3 M sucrose, 3 mM EDTA and 1 mM β-mercaptoethanol in 0.1 mM phosphate buffer (pH 7.5). The peroxidation was initiated by exposing 2 ml chloroplast suspension (equivalent to 0.1–1.0 mg chlorophyl ml^{-1}) to the light intensity of 120 μmol m^{-2} s^{-1} for

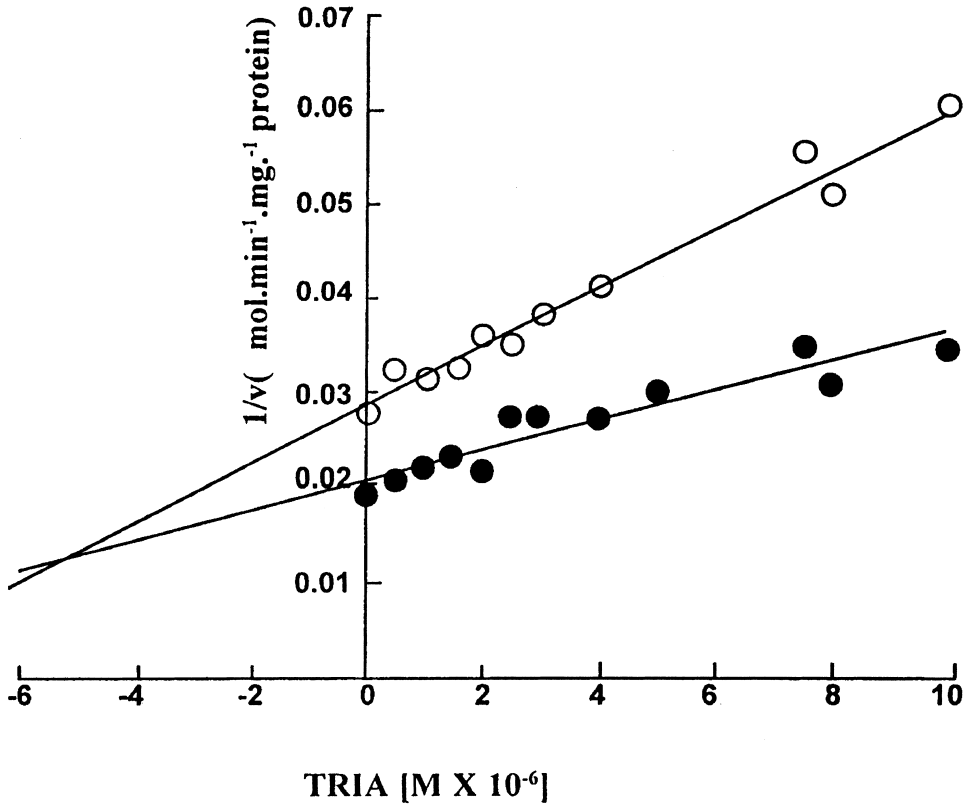

Fig. 7. Dixon plot for soybean LOX-1 activity inhibited by TRIA with substrate concentrations of 25 μM (○) and 50 μM (●) linoleic acid. The incubation mixture (3 ml) also contained 0.1 M borate buffer at pH 9.0, 0–10 μM TRIA and 0.4 μg of the enzyme.

different time intervals. TRIA was used at the concentration of 120 nM in the incubation medium wherever indicated. Peroxidation was assayed as a measure of TBARS formed (Janero and Burghardt, 1988, 1989). The peroxidation was terminated by placing the tubes on ice and adding 0.15 ml of ice cold mixture of 76% TCA in 2.3 N HCl (pH 2.2) ml^{-1} of chloroplast suspension. This was followed by addition of 0.35 ml of freshly prepared mixture of water/67 μM BHT in ethanol/1.5% TBA in 0.2 M Tris–HCl (pH 7.0) in the ratio of 1:1:5 (v/v/v) per ml of chloroplast suspension. The mixture was vortexed thoroughly and incubated at 80°C for 30 min. The TBA test was stopped by plunging the tubes into ice bath and adding 0.5 ml of ice cold 91% TCA followed by 2 ml of chloroform ml^{-1} of sample. The chromophore product of TBA reaction was extracted into chloroform phase by vortexing and the phases were separated by centrifugation at 4°C for 15 min at 2000 $\times g$. The total TBARS were estimated in terms of amount of malondialdehyde (MDA) formed by measuring the absorbance at 532 nm. The extinction coefficient of MDA at 532 nm is taken as 155 mM^{-1} cm^{-1} (Liu et al., 1997).

3.4. Induction of lipid peroxidation in isolated chloroplasts

Ferrous iron-induced lipid peroxidation in isolated chloroplast membrane lipids was studied according to the method described by Janero and Burghardt (1988, 1989) for cardiac membranes, after a convenient modification. The extent of Fe^{2+}-induced chloroplast lipid peroxidation was studied both in light and dark conditions. The peroxidation was initiated adding $FeSO_4$ solution to the final concentration of 1 or 10 mM and immediately exposing the chloroplast suspension (2 ml) to light (120 μmol m^{-2} s^{-1}) or keeping the sample tubes in dark. Estimation of TBARS was done as already described in Section 3.3.

3.5. Fe^{2+}-induced lipid peroxidation in liposomes

Lipid peroxidation in liposomes was initiated by adding $FeSO_4$ solution to the final concentration of 1 or 10 mM Fe^{2+} in 2 ml of liposome suspension. The peroxidation was stopped at 60 min after incubation. The estimation of TBARS was done as described earlier.

3.6. Assay of soybean LOX activity

LOX activity was analysed by continuous monitoring of the absorbance of conjugated diene formed during the enzyme reaction at 234 nm, with ε_{max} of 2.5×10^4 M^{-1} cm^{-1}. The substrate stock was prepared according to the method of Ben-Aziz et al. (1970). The stock consisted of linoleic acid and Tween 20 in the ratio of 1:1. This mixture was diluted with 0.1 M borate buffer (pH 9.0), and clarified by adding a small amount of 1 N NaOH.

The steady state kinetic studies of hydroperoxidation of linoleic acid catalysed by soybean LOX was carried out in 20 mM sodium borate buffer at pH 9.0 using 20 to 250 μM linoleic acid. By measuring the enzyme activity at pH 9.0 the assay will be limited to LOX-1 activity of soybean seeds. The absorbance at 234 nm was continuously monitored. A minimum of two readings of continuous absorbance was made at each time for a sample. Enzyme rate (initial velocity) measurements were made after determining the linear regression of the initial reaction, and the slope of the product formation versus time was used for calculating the reaction rate. Inhibition studies by TRIA was conducted by directly adding the inhibitor to the reaction mixture without preincubation of the enzyme with the inhibitor. The kinetic constants were determined as described by Ritchie and Prvan (1996) using more than one plotting methods.

Acknowledgements

Financial support by the University Grants Commission under Grant No. F-3-6/97 (SR II) to G.S. Swamy is acknowledged.

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