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INACTIVATION OF SOYBEAN LIPOXYGENASE BY LIPOXYGENASE INHIBITORS IN THE PRESENCE OF 15-HYDROPEROXYEICOSATETRAENOIC ACID

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Abstract—Soybean lipoxygenase is rapidly inactivated when incubated with arachidonic acid and any of several lipoxygenase inhibitors, including NDGA, the aminopyrazolines BW 755C and BW 540C, and the acetohydroxamic acid derivatives BW A4C and BW A137C. Little or no inactivation was found when the enzyme was incubated with substrate or with inhibitors alone. 15-HPETE was as effective as arachidonic acid in promoting inactivation, but linoleic acid and 13-HPOD were much less effective.

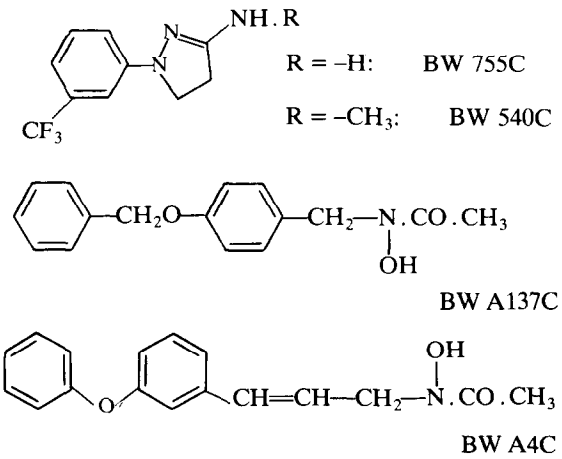
The UV absorption at 235 nm, due to the conjugated diene in 15-HPETE or 13-HPOD, was rapidly destroyed in the presence of soybean lipoxygenase and inhibitor in a presumed pseudoperoxidase reaction. The products of the reaction between linoleic acid, BW A137C and soybean lipoxygenase have been partially characterized. A derivative of arachidonic acid is postulated to be the inactivating agent.

Lipoxygenases have become subject to intensive studies in recent years following the discovery of the potentially pathogenic roles of leukotrienes and other products [1], and a variety of lipoxygenase inhibitors have been described [reviewed in Refs 2–4]. Many lipoxygenase inhibitors have been recognised to be redox-active compounds [2] and anti-oxidant activity has been correlated with lipoxygenase-inhibiting potency [5a]. Some lipoxygenase inhibitors, such as hydroxamic acid derivatives [5b, 6–8], have been postulated to chelate iron which is present in lipoxygenases isolated from soybean [9], pea [10] and reticulocytes from rabbits [11]. Other lipoxygenases including mammalian 5-lipoxygenase and 12-lipoxygenase share similar catalytic features including activation by organic peroxides [12, 13] and potent inhibition by many of the above-mentioned inhibitors [2–8]. It is possible that all plant and animal lipoxygenases contain non-haem iron and act by similar mechanisms.

The anti-oxidant lipoxygenase inhibitors may act as co-substrates in a pseudoperoxidase reaction catalysed by lipoxygenase, removing organic peroxide and lengthening the lag (induction-period) before the rate becomes maximal. Soybean lipoxygenase co-oxidises NDGA* [14, 15a, 15b], α-tocopherol [16], *N*-octylhydroxylamine [17] and quercetin [18], this being accompanied by lag-lengthening [15a, 17] and inhibition. Various plant lipoxygenases can co-oxidise a variety of other compounds [19] but the relationship to inhibition of lipoxygenation has often not been investigated.

Irreversible inactivation of lipoxygenases is produced by their own hydroperoxide products [20, 21], acetylenic fatty acids including ETYA [22–24], other substrate analogues including thia-arachidonic acids [25, 26] and a methylidine derivative [27], phenyldiazine [28] and 10-butyryl-1,8-dihydroxy-9-anthrone [29]. Inactivation by fatty-acid derivatives generally requires molecular oxygen; phenyldiazine, however, reacts only with the Fe(III) form of soybean lipoxygenase, which can be generated by addition of linoleic hydroperoxide (13-HPOD).

The present paper describes a novel type of irreversible inactivation of soybean lipoxygenase, where both an inhibitor and a fatty acid (or hydroperoxide) are required and appear to interact by a redox-type reaction. Although such reactions can occur with several inhibitors, those of the novel aceto-hydroxamic acid BW A137C [8, 30a] have been investigated in the most detail.



* Abbreviations used: HPETE, hydroperoxyeicosatetraenoic acid; HETE, hydroxyeicosatetraenoic acid; HPOD, hydroperoxyoctadecadienoic acid; HOD, hydroxyoctadecadienoic acid; NDGA, nordihydroguaiaretic acid; ETYA, 5, 8, 11, 14-eicosatetraynoic acid; DMSO, dimethyl sulphoxide.

MATERIALS AND METHODS

Arachidonic acid, linoleic acid, NDGA and soybean lipoxygenase (Type V, prepared by affinity chromatography, stated to be mainly lipoxygenase 1) were obtained from Sigma Chemical Co. 4-Hydroxy-tetramethylpiperidinyloxy was from Aldrich. 15-HPETE was prepared in this laboratory by Mr. D. R. Smith [31]. BW 755C, BW 540C, BW A4C, BW A137C (unlabelled, and also [^{14}C]phenyl) and nafazatrom were prepared as described previously [8, 30b] in the Medicinal Chemistry Department of the Wellcome Research Laboratories. [1-^{14}C]Arachidonic and linoleic acids were obtained from Amersham International, and [U-^{14}C]linoleic acid from New England Nuclear.

Lipoxygenase was assayed by following the absorbance increase at 235 nm in a Gilford 250 spectrophotometer at 30°. The final volume of 0.5 ml contained 0.1 M Tris–HCl pH 9.0, 1 μg (66 μM) arachidonic acid (added in 10 μl ethanol) and (to start the reaction) approximately 50 ng lipoxygenase. Tests for inactivation were carried out at room temperature (approx. 20°) by incubation of lipoxygenase (usually 2.5 μg per 0.5 ml) in buffer (usually 0.1 M Tris–HCl pH 9.0) to which arachidonic acid, linoleic acid or 15-HPETE had been added in ethanol, with or without inhibitor. Inhibitors were usually dissolved in DMSO and diluted in buffer before use. The final concentrations of DMSO and (particularly) ethanol were important because these substances can protect from inactivation (see below). 10 μl samples were withdrawn at intervals for enzyme assays. Conditions similar to those for inactivations were used for observation of spectral changes. Similar incubations were also used for experiments involving conversion of ^{14}C-labelled arachidonic acid, linoleic acid or BW A137C to other compounds followed by TLC: in some cases, reaction mixtures were then treated with 50 μl $NaBH_4$ (10 mg/ml in water) for 2 min on ice. Reactions were stopped by adding 1.25 ml acetone containing 1% (v/v) 5 M HCl; nonpolar solutes were extracted into 1.5 ml $CHCl_3$; after evaporation under N_2 residues were redissolved in $CHCl_3$/MeOH (2:1, v/v) before being applied to silica TLC plates (LK5D, obtained from Whatman). After separation using diethyl ether/hexane/acetic acid (60:40:1, v/v) and drying, the plates were autoradiographed overnight or longer with Kodak DEF2 film.

RESULTS

Inactivation of soybean lipoxygenase

When inhibition by the aminopyrazoline BW 540C was being studied using the direct spectrophotometric assay of soybean lipoxygenase it was noticed that, although the effect on initial rate was slight, the reaction-rate slowed down much more rapidly in the presence of the inhibitor (Fig. 1A). This effect was much more pronounced at pH 9.0 than at pH 7.3 (not shown). Time-dependent inhibition, possibly irreversible, was suspected and so a pre-incubation experiment was set up using the same conditions but with more concentrated enzyme (Fig. 1B), and samples were withdrawn at intervals for spectrophotometric enzyme assay. Pre-incubation with BW 540C alone produced only very slow inactivation; however, when the inhibitor and substrate (arachidonic acid) were both present rapid inactivation occurred, and this could account for the fall-off of activity seen in the assays of Fig. 1A. The rate of inactivation was much less at pH 7.3 than at pH 9.0. Replacing arachidonic acid by 15-HPETE gave equally rapid inactivation in the presence of BW 540C (Fig. 2).

Other inhibitors were found also to give rapid arachidonate-dependent inactivation, including another aminopyrazoline, BW 755C, the phenolic antioxidant NDGA (Fig. 3) and the acetohydroxamic acid derivatives BW A4C (not shown) and BW

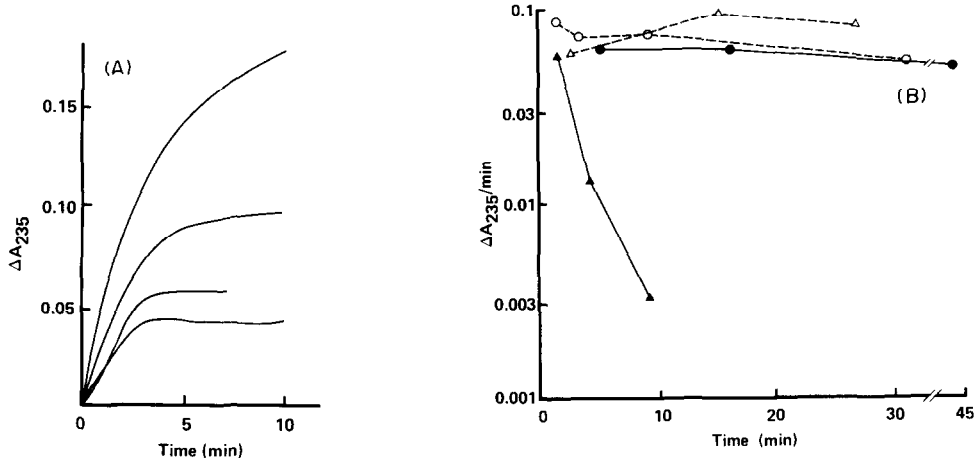


Fig. 1. Effect of BW 540C on activity of soybean lipoxygenase. (A) Spectrophotometric progress curves. In 0.1 M Tris–HCl buffer pH 9.0 at 30° with 66 μM arachidonic acid, 100 ng/ml soybean lipoxygenase and BW 540C concentrations (from top line, downwards), 0, 4 μM, 10 μM, 20 μM. (B) Pre-incubations with samples withdrawn for assay at the indicated times, in 0.1 M Tris–HCl pH 9.0 at 20° with 5 μg/ml enzyme: (▲) + arachidonic acid (66 μM) and BW 540C (20 μM); (●) + BW 540C only; (○) + arachidonic acid only; (△) no substrate or inhibitor. Arachidonic acid was added in ethanol (2% final concentration) and BW 540C in buffer and DMSO (0.4% final concentration).

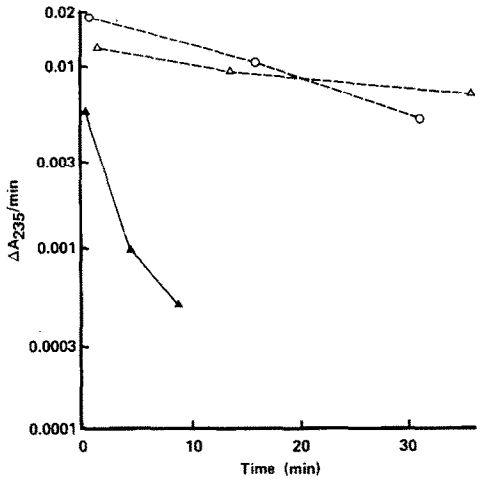


Fig. 2. Effect of pre-incubation of soybean lipoxygenase with BW 540C and 15-HPETE. Conditions as for Fig. 1B. (▲) + 15-HPETE (16 μM) and 20 μM BW 540C; (○) + 15-HPETE only; (△) + BW 540C only.

A137C (Fig. 4). Of these, BW A137C was chosen for more detailed study. As shown in Fig. 4, even a high concentration of BW A137C (100 μM) gave no inactivation without fatty acid. Furthermore, surprisingly, linoleic acid, although a good substrate for soybean lipoxygenase, was not able to replace arachidonic acid as a co-inducer of inactivation. However, linoleic acid was able to induce inactivation by NDGA and BW 755C although not as rapidly as with arachidonic acid. BW A137C shared with BW 540C the property of slower inactivation at pH 7.3 than pH 9.0, but NDGA inactivated approximately equipotently at each pH. The pH-dependence of inactivation by BW A137C was shown to be independent of buffer type at pH 9.0—Tris with or without phosphate, or borate, all gave rapid inactivation.

The effects of various agents on inactivation by BW A137C and arachidonic acid at pH 9 were examined. The presence of the radical 4-hydroxy

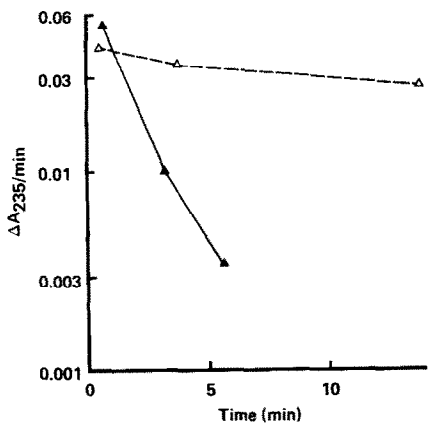


Fig. 3. Effect of pre-incubation of soybean lipoxygenase with NDGA. Conditions as for Fig. 1B. With 10 μM NDGA, 2% DMSO. (▲) with 66 μM arachidonic acid and 2% ethanol; (△) without arachidonic acid or ethanol.

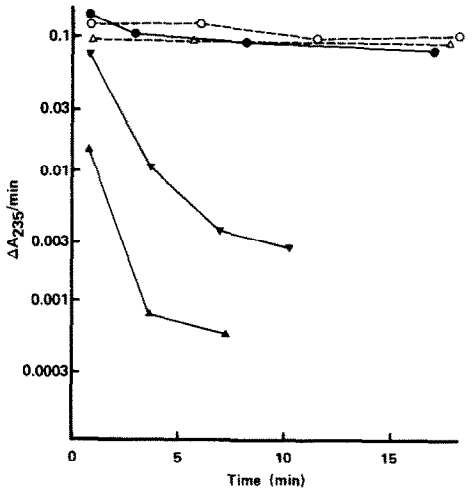


Fig. 4. Effect of pre-incubation of soybean lipoxygenase with BW A137C and arachidonic acid or linoleic acid. Conditions as for Fig. 1B. (▲) + 100 μM BW A137C and 16 μM arachidonic acid; (▼) + 10 μM BW A137C and 16 μM arachidonic acid; (●) + 36 μM linoleic acid and 100 μM BW A137C; (△) + 100 μM BW 137C only; and (○) + 16 μM arachidonic acid only. Fatty acids were added in ethanol (1% final concentration); 2% DMSO was present in all inactivations.

tetramethylpiperidinyloxy did not result in inactivation with arachidonate nor did it protect from inactivation by BW A137C. EDTA (1 mM), superoxide dismutase (25 μM subunits), mannitol (95 mM) and NaCl (1 M) had at best only very slight protective effects. Ethylene glycol and especially ethanol gave considerable protection (Fig. 5); acetone also protected well, but not as well as ethanol. Since ethanol (usually 1% final concentration) was normally used as vehicle to add the fatty acid

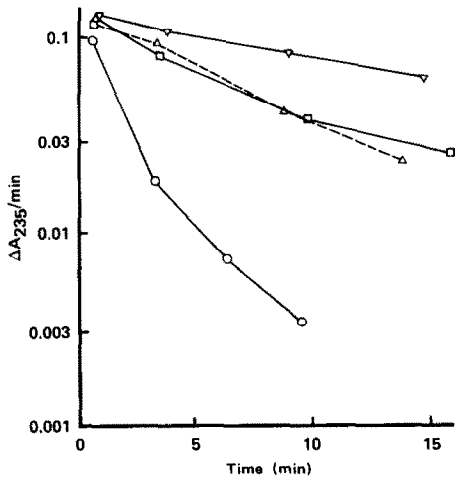


Fig. 5. Protection by ethanol and ethylene glycol of soybean lipoxygenase from inactivation by BW A137C (10 μM) and arachidonic acid (16 μM). Conditions as in Fig. 1B. All inactivations in the presence of 2% DMSO. (○) 1% ethanol; (△) 3% ethanol; (▽) 5% ethanol; (□) 1% ethanol and 20% ethylene glycol.

substrate, the ethanol concentration must be taken into account when interpreting rates of inactivation. Ethanol and ethylene glycol were also found to inhibit the rates of arachidonate lipoxygenation and of pseudoperoxidase (see below), but not as dramatically as they protected from the inactivation.

Attempts to reactivate the enzyme by dialysis were not successful. Soybean lipoxygenase (2.5 μg), 16 μM arachidonic acid, 1% ethanol, 2% DMSO and 100 μM BW A137C were incubated for 12 min at room temperature, during which activity fell to 0.3% of that in a control sample (identical but with omission of BW A137C). After dialysis for 20 hr against 3 changes of 250 ml 0.1 M Tris–HCl pH 9.0, no activity was detected in the BW A137C treated sample while 21% (uncorrected for dilution during dialysis) remained in the control sample.

Inactivation was found to vary with enzyme concentration. Very dilute enzyme (125 ng per 0.5 ml, i.e. a 20-fold dilution when compared with the standard incubations for inactivation) was inactivated considerably more slowly than normal. However, when much more concentrated enzyme was used (100 μg per 0.5 ml), only a little inactivation (25%) was found—hence relatively dilute enzyme (5 μg/ml) was used in the reactivation attempts described above.

Not all lipoxygenase inhibitors were associated with arachidonate-dependent inactivation. Diphenyldisulphide and nafazatrom gave essentially no inactivation, and 4-nitrocatechol very little. Quercetin also appeared to give very little, but a firm conclusion is precluded because of the poor solubility of this compound.

Pseudoperoxidase reactions: metabolism of hydroperoxides and inhibitors

Under the conditions used for inactivations with BW A137C, rapid absorbance changes could be observed at 240 nm due to the formation and then inhibitor-dependent destruction of conjugated-diene chromophore (Fig. 6A). This was accompanied by an increase in absorbance at 280 nm (Fig. 6B). Such changes were larger with linoleic acid than with arachidonic acid—with the latter substrate the reaction appeared to slow down rapidly, which may be attributed to enzyme inactivation. The trough in absorbance at 240 nm with linoleic acid in Fig. 6A (at which time the 280 nm absorbance has essentially reached its plateau) followed by the small rise suggests that sequential reactions are taking place with a transient intermediate. With arachidonic acid, changes after more than 30 sec are clearly much slower than with linoleic acid, and no trough is seen at 240 nm. This is probably attributable to the very rapid inactivation of the enzyme under these conditions. Similarly-shaped curves (apart from the first few seconds) were produced when BW A137C was added last, suggesting that reaction involves the peroxide product and the inhibitor. Use of authentic 15-HPETE demonstrated that enzyme was required for the subsequent reaction.

Absorbance changes were also investigated with NDGA (Figs 7A and 7B). As well as indicating pseudoperoxidase activity, most clearly shown by the increase in absorbance at 280 nm with linoleic acid, the sigmoid shape of the progress curves shows that the lipoxygenase reaction (measured as net formation of chromophore at 235 nm, i.e. the hydroperoxide product) is strongly inhibited in its early stages.

Products were examined and partially characterized for the reaction between soybean lipoxygenase, BW A137C and linoleic acid with ^{14}C-labelled substrate or inhibitor, followed by TLC and autoradiography (Fig. 8) and in preliminary experi-

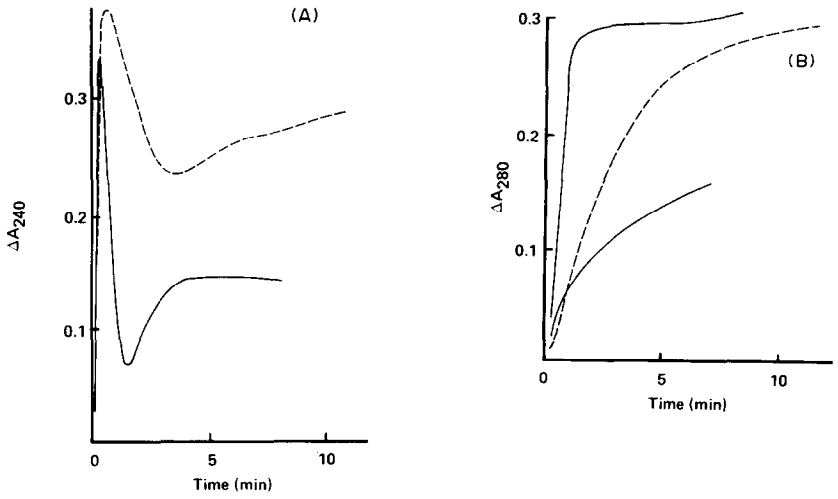


Fig. 6. Absorbance changes at 240 nm (A) and 280 nm (B) in the soybean-lipoxygenase-catalysed reaction between BW A137C and linoleic acid or arachidonic acid. Dashed lines are in 0.1 M K^+ phosphate buffer pH 7.3, and continuous lines in 0.1 M Tris–HCl pH 9.0. BW A137C (100 μM), DMSO (2%) and 5 μg/ml enzyme in all inactivations. In (A), linoleic acid (36 μM) and 1% ethanol were present in both reactions. In (B), linoleic acid (36 μM) and 1% ethanol were present for the upper continuous trace and the dashed trace; in the lower continuous trace arachidonic acid (33 μM) was present.

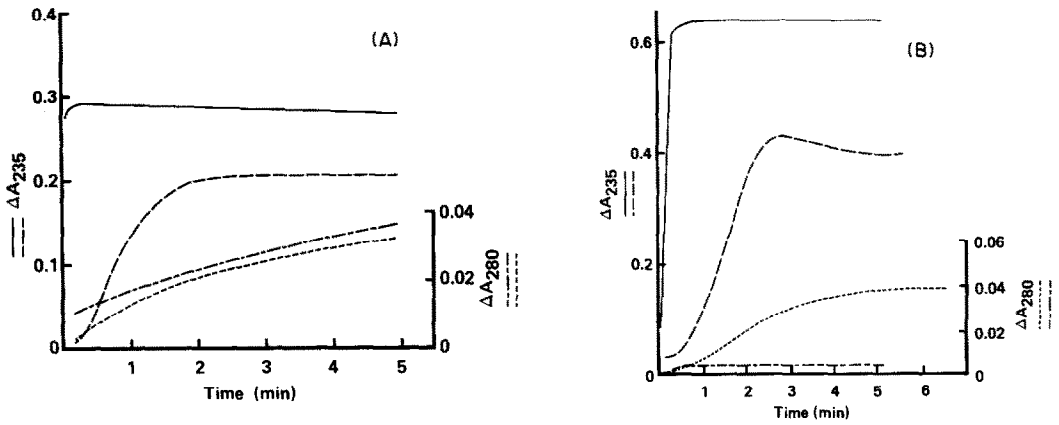


Fig. 7. Absorbance changes in the soybean-lipoxygenase-catalysed reaction between NDGA (10 μM) and arachidonic acid (A, 16 μM) or linoleic acid (B, 36 μM). Continuous lines show absorbance changes at 235 nm with fatty-acid only, and the dash-dot lines show the corresponding changes at 280 nm. The dashed lines show the absorbance changes at 235 nm with fatty-acid + NDGA, and the dotted lines show the corresponding changes at 280 nm. 1% ethanol was present throughout, and NDGA was added in DMSO (2% final concentration).

ments by HPLC (not shown). [1-^{14}C]Linoleic acid (band I), on reaction with soybean lipoxygenase, gave two close bands (IIa and IIb), probably 13- and 9-HPOD. When BW A137C was also present, a compound migrating in the position of IIb was the major product but, after reduction with borohydride, the major product (band V) was much more polar and remained very near the origin. [Phenyl-^{14}C] BW A137C gave a streaked band (band C) near the origin and was not affected by lipoxygenase in the absence of linoleic acid. However, in the presence of both lipoxygenase and linoleic acid two new, more polar bands appeared; band A migrated just ahead of linoleic acid and band B migrated just ahead of band IIa. Borohydride did not affect significantly these products from BW A137C, and no product containing radioactivity from both linoleic acid and BW A137C could be detected. Band A has been identified by co-chromatography, IR, GS-MS and NMR as the oxime (R. D. Farrant and A. S. Gilbert, personal communication); the reaction products in bands IIb, V and B have not yet been fully characterised. Bands III and IV were relatively minor and were not always seen. The oxime of BW A137C did not inactivate soybean lipoxygenase, nor did it act as a substrate in the pseudoperoxidase reaction.

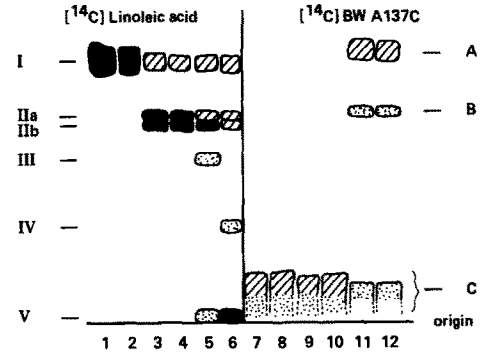


Fig. 8. Autoradiograph of thin-layer chromatogram of products of [1-^{14}C]linoleic acid and [phenyl-^{14}C]BW A137C given by soybean-lipoxygenase. Reaction mixtures for lanes 1–6 contained 1 μg (190 nCi) [1-^{14}C]linoleic acid + 4 μg nonradioactive linoleic acid (giving 36 μM), and those for lanes 7–12 contained 50 nCi [phenyl-^{14}C]BW A137C plus nonradioactive BW A137C to give 10 μM. 36 μM nonradioactive linoleic acid was also present for lanes 11 and 12, and 100 μM nonradioactive BW A137C for lanes 5 and 6. Soybean lipoxygenase (2.5 μg) was present in all reactions except those for lanes 1, 2, 7 and 8. Incubations (0.5 ml, in 0.1 M Tris–HCl pH 9.0) were for 10 min at 30°; even-numbered samples were treated with $NaBH_4$. All reactions contained 2% ethanol and 2% DMSO.

DISCUSSION

The work described above has demonstrated a time-dependent inactivation of soybean lipoxygenase (primarily lipoxygenase-1) given by several inhibitors and dependent upon arachidonic acid or, more specifically, its 15-hydroperoxide. When linoleic acid replaced arachidonic acid inactivation was much decreased, although linoleic acid (or rather, HPOD) was also able to take part in a pseudoperoxidase reaction. Presumably a derivative of arachidonic acid is responsible for the inactivation, and linoleic acid is not able to produce an analogous reactive derivative. However, the lack of inactivation given by linoleic acid and BW A137C may be due in part to the rapid exhaustion of either linoleic acid (peroxide) or BW A137C, depending which was in excess.

The inactivation would appear to be due either to the formation on the enzyme active site of a derivative of 15-HPETE, or to the release of a short-lived reactive species which then inactivates the enzyme. On balance the evidence to date perhaps favours the former possibility. Inactivation seems not to involve release of superoxide ion, since superoxide dis-

mutase does not protect; likewise other oxygen radicals which should react with mannitol or 4-hydroxytetramethylpiperidinyloxy appear not to be involved; EDTA did not affect inactivation, suggesting that a metal-ion-catalysed reaction is not involved. When yeast alcohol dehydrogenase was included in a solution in which BW A137C and arachidonic acid were inactivating soybean lipoxygenase, the alcohol dehydrogenase was completely unaffected, suggesting that a generally reactive species had not been released (unpublished data). Yeast alcohol dehydrogenase is known to have reactive sulphydryl groups [32]. Also in favour of an active-site-bound inactivating substance is the failure to obtain substantial inactivation when very high enzyme concentrations were used; a released compound might be expected to react with any enzyme molecule it encountered regardless of the latter's concentration, while conversely most enzyme molecules would survive if the peroxide or inhibitor (BW A137C) had all been used up in a pseudoperoxidase reaction at high enzyme concentrations. The slower inactivation at very low enzyme concentrations, although apparently at variance with this, could be accounted for by a requirement for hydroperoxide concentrations to build up. Finally, protecting agents such as ethanol and ethylene glycol also inhibited the lipoxygenase reaction and the pseudoperoxidase reaction, suggesting that catalysis and inactivation are linked. Ethanol and other alcohols are known to bind to soybean lipoxygenase [33].

The products of the lipoxygenase-catalysed reaction between linoleic acid and BW A137C have been partially characterized. Band A (Fig. 8) has been identified as the oxime of BW A137C. This would be formed by hydrolysis with liberation of acetate and by two-electron oxidation. The other product detected from BW A137C (band B in Fig. 8) has not yet been identified. The BW A137C-dependent linoleic acid product (migrating as band IIb in Fig. 8 (lane 5)) appears from NMR to be an oxo derivative with only one double-bond which has a *trans* configuration (R. D. Farrant, personal communication). The products seem to be different, therefore, from those formed in the anaerobic reaction of lipoxygenase with HPOD and linoleic acid [34, 35], or HPOD alone [36]. Further structural elucidation may allow prediction of the products that arachidonic acid might form. When [1-^{14}C]arachidonic acid was used, there was a greater variety of products than with linoleic acid; the yields were smaller, possibly because of enzyme inactivation. [^{14}C]Linoleic acid was apparently converted by BW A4C to the same products as by BW A137C, but BW 755C and NDGA gave a greater variety of products with no one band predominating.

It is possible that hydroxamic acids may inactivate the enzyme by chelating to iron in the enzyme [5b, 6–8] and removing it from the enzyme. However, this explanation cannot readily account for the much greater effectiveness of arachidonate at promoting inactivation compared to linoleate.

Arachidonate-dependent inactivation may occur with other lipoxygenases; this is difficult to test experimentally because of the ready inactivation of these enzymes by hydroperoxides [20, 21, 37], and must await further investigation. However, it is clearly not the only mechanism of inhibition by such compounds since NDGA [Ref. 15a and Fig. 7] and *N*-octylhydroxylamine [17] lengthen the lag in the enzyme assay. For example, inhibition by hydroxamate derivatives is highly structure-specific [7, 8, 30a] consistent with specificity for the active-site of 5-lipoxygenase.

The inactivation described in this paper appears to be a novel type of irreversible inhibition. The inhibitor does not inhibit on its own, but appears to subvert a normally harmless substrate (or product) and cause it to inactivate the enzyme.

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