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LIPOXYGENASE-MEDIATED CLEAVAGE OF FATTY ACIDS TO CARBONYL FRAGMENTS IN TOMATO FRUITS

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Abstract—Homogenates of tomato fruits catalysed the enzymic conversion of linoleic and linolenic acids (but not oleic acid) to C_6 aldehydes in low (3–5%) molar yield. Hexanal was formed from linoleic acid; *cis*-3-hexenal and smaller amounts of *trans*-2-hexenal were formed from linolenic acid. With the fatty acids as substrates, the major products were fatty acid hydroperoxides (50–80% yield) and the ratio of 9- to 13-hydroperoxides as isolated from an incubation with linoleic acid was at least 95:5 in favour of the 9-hydroperoxide isomer. When the 9- and 13-hydroperoxides of linoleic acid were used as substrates with tomato homogenates, the 13-hydroperoxide was readily cleaved to hexanal in high molar yield (60%) but the 9-hydroperoxide isomer was not converted to cleavage products. Properties of the hydroperoxide cleavage system are described. The results indicate that the C_6 aldehydes are formed from C_{18} polyunsaturated fatty acids in a sequential enzyme system involving lipoxygenase (which preferentially oxygenates at the 9-position) followed by a hydroperoxide cleavage system which is, however, specific for the 13-hydroperoxy isomers.

INTRODUCTION

The enzymic formation of volatile flavour compounds on disruption of plant tissues is widespread [1]. Volatile carbonyl compounds are formed by fatty acid breakdown in a range of tissues; e.g. fruits of cucumber [1–8] tomato [9–11] and banana [12] or leaves of tea [13], ginkgo [14] and other plants [15]. However, the mechanisms for the biogenesis of these compounds are not at all well established. In particular, conflicting views are held on the role of lipoxygenase enzymes in these processes; some workers have specifically excluded lipoxygenases as possible agents in the formation of fatty acid cleavage products [4, 16].

Recently, we have demonstrated in cucumber fruits, a lipoxygenase-mediated pathway by which the polyunsaturated fatty acids of endogenous lipids were cleaved to volatile aldehyde C_9 fragments [6–8]. We wished to compare the cucumber system with that from tomato in which the major volatile products from fatty acid breakdown were known to have a C_6 chain-length, i.e. hexanal and *cis*-3-hexenal [9–11].

RESULTS

C_6 *Volatile aldehyde formation from* C_{18} *fatty acids*

Incubation of 0.3 mM linoleic acid with homogenates of tomato fruits resulted in the formation of hexanal (Table 1). Preliminary experiments established the following optimal conditions for aldehyde formation. The reaction was active at acid pH with an optimum between pH 5.5 and 6.5 and with little activity above pH 7.5; the amount of hexanal produced was proportional to the concentration of enzyme extract; optimal [S] was around 0.3 mM (above which concentration linoleic acid was slightly inhibitory) and an apparent K_m was approx 0.1 mM as determined from 1/v *vs* 1/[S] relation-

Table 1. Formation of fatty acid hydroperoxides and C_6 aldehydes from linoleic and linolenic acids in homogenates of tomato fruits

Substrate	Substrate reacted (μmol)	Products formed (μmol)			
		fatty acid hydroperoxide	hexanal	*cis*-3-hexenal	*trans*-2-hexenal
Linoleic acid + homogenate	2.5	1.5	0.08	—	—
Linoleic acid + boiled homogenate	0.6	0.3	0.02	—	—
Linolenic acid + homogenate	2.4	1.3		0.05	0.02
Linolenic acid + boiled homogenate	0.8	0.3	—	<0.01	—
Oleic acid + homogenate	<0.1	—		—	—

Ten ml incubation mixtures contained ^{14}C-1-labelled fatty acid substrate (2.9 μmol), tomato homogenate (containing 0.7 g fr. wt of tissue) in 0.1 M sodium acetate buffer, pH 5.5. Incubation was at 25° for 30 min. Substrate loss and hydroperoxide formation were determined by radioscanning of TLC separations; hexanal and hexenal were analysed by GLC. Values represent means of duplicates.

ships with linoleic acid. That hexanal was derived from linoleic acid was established using U-^{14}C-linoleic acid as substrate.

Table 1 compares the volatile products obtained from a fresh homogenate of tomato fruit on incubation with oleic, linoleic and linolenic acids. Hexanal was the only significant product in the C_6–C_9 range when linoleic acid was used as substrate; from linolenic acid, *cis*-3-hexanal was the main product but the isomeric *trans*-2-hexanal was also observed. No volatile C_6–C_9 products were observed when oleic acid was incubated with tomato homogenates and the amounts of the C_6 compounds from linoleic and linolenic acids were greatly reduced when boiled homogenates were used. In contrast to the cucumber system, no C_9 compounds were observed. No major amounts of C_6 alcohols were detected.

Non-volatile products from C_{18} fatty acids

Under the incubation conditions employed most (80–90%) of the linoleic acid or linolenic acid substrates disappeared from the mixtures (Table 1). However, yields of the C_6 aldehydes were low (usually 3–5% on a molar basis). Examination of the ^{14}C-labelled products from [1-^{14}C] fatty acids showed that linoleic and linolenic acids were converted to a range of products but that the major products were fatty acid hydroperoxides (Fig. 1). Relatively little conversion of fatty acid occured with boiled homogenates or when oleic acid was used as substrate (Table 1, Fig. 1).

The fatty acid hydroperoxide products were isolated from incubation mixtures by TLC; identifications were made on methyl esters by comparison with authentic standards and UV spectra, hydroperoxide-positive reactions, TLC and high performance liquid-chromatography; the last technique, which readily separates the methyl 9- and 13-hydroperoxydienoates, demonstrated that the product as isolated contained at least 95% of the 9-hydroperoxide isomer. This result was surprising to us since the incubation systems specifically produced C_6 aldehydes (known to derive from 13-hydroperoxides of C_{18} acids) whereas the major incubation products were 9-hydroperoxides which, by comparison with cucumber systems [7, 8], would be expected to cleave to C_9 aldehyde fragments.

Thus, the formation of C_6 aldehydes in tomato could not be explained in terms of a specific oxygenation of the 13-position of C_{18} acids by lipoxygenase. Two further possibilities existed; either that lipoxygenase and hydroperoxide intermediates are not involved in C_6 aldehyde production in tomato extracts (this would be in line with the suggestion of some other laboratories for

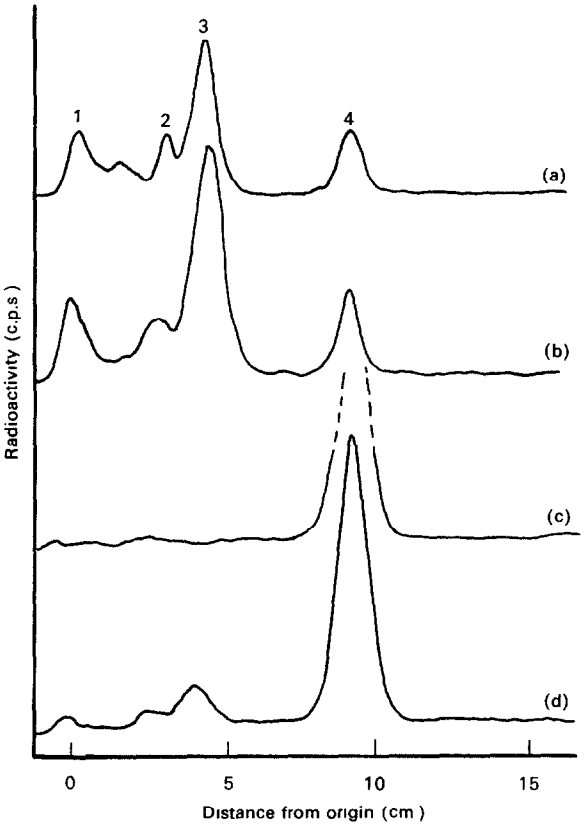


Fig. 1. Radioactivity scan of TLC separations of ^{14}C-labelled products formed from [1-^{14}C] fatty acids in homogenates of tomato fruits. Incubation conditions as in Table 1. Reaction products were separated on Si gel G TLC plates, developed in petrol (bp 60–80°)–Et_2O–HAc (60:40:1). The scans represent results using: (a) linoleic acid; (b) linolenic acid; (c) oleic acid and (c) linoleic acid with boiled enzyme. Peaks 1 and 2 were unidentified; peak 3 = fatty acid hydroperoxide; peak 4 = unreacted fatty acid substrate.

Table 2 Enzymic formation of volatile carbonyl compounds from hydroperoxides of linoleic and linolenic acids in extracts of tomato fruits

Substrate	Additions	Volatile aldehydes produced (nmol)				
		hexanal	*cis*-3-hexenal	*trans*-2-hexenal	*cis*-3-nonenal	*trans*-2-nonenal
Experiment 1						
13-hydroperoxylinoleic acid	none	32	—	—	—	—
13-hydroperoxylinoleic acid	homogenate	161	—	—	—	—
13-hydroperoxylinoleic acid	boiled homogenate	<0.5	—	—	—	—
9-hydroperoxylinoleic acid	homogenate	—	—	—	<0.5	<0.5
Experiment 2						
13-hydroperoxylinolenic acid	none	—	15	<0.5	—	—
13-hydroperoxylinolenic acid	homogenate	—	400	10	—	—
13-hydroperoxylinolenic acid	boiled homogenate	—	25	1	—	—

Incubation conditions as in Table 1. Analysis by GLC.

aldehyde production in cucumber fruits [4] or leaves [16]; or, that the hydroperoxides are intermediates but that the hydroperoxide cleavage process is specific for the 13-isomers (i.e. unlike the system which we have demonstrated in cucumber in which 9- and 13-hydroperoxides are cleaved at equal rates). The following results provide evidence in favour of latter explanation.

Volatile products from fatty acid hydroperoxides

In contrast to the relatively low yields of volatile aldehydes obtained when free fatty acids were added to tomato extracts (Table 1), the 13-hydroperoxides of linoleic and linolenic acid were converted to hexanal and hexenal, respectively, in high molar yields of up to 60% (Table 2). However, unlike the cucumber system [7, 8] no appreciable conversion of linoleic acid 9-hydroperoxide to C_9 or other volatile fragments was observed (Table 2). Parallel checks on the ^{14}C-labelled non-volatile products from the [1-^{14}C]-9-hydroperoxide of linoleic acid confirmed that this isomer was not metabolized by the tomato extracts.

That the aldehydes are formed directly from the fatty acid hydroperoxides (and not from other endogenous substrates by, e.g. a co-oxidation process), is demonstrated by the results (Table 3) in which hexanal and its precursor [U-^{14}C]-linoleic acid-13-hydroperoxide, were shown to have the same specific radioactivities.

Non-volatile products of the hydroperoxide-cleavage system

The ^{14}C-products from 1-^{14}C-labelled linoleic acid-13-hydroperoxide have not yet been fully identified. Preliminary investigations have indicated that a range of cleavage products, as well as C_{18} products, are formed. The product mixture is more complex than that obtained earlier with cucumber extracts [8] in which the oxoacid portion, complementary to the aldehyde fragment, was a major cleavage product.

It may be significant that, from incubations of tomato extracts with linoleic acid 13-hydroperoxide, one of the major non-volatile products was identified (from TLC, infra-red-, nuclear magnetic resonance- and mass-spectra [17–19]) as 12-oxo-13-hydroxy-octadec-*trans*-1-enoic acid (D. R. Phillips and T. Galliard, unpublished observations); this α-ketol derivative has been suggested as a possible intermediate in hexanal formation from linoleic acid in tomato fruits [10]. Further studies are in progress in the hope that identification of the products will give further information on the mechanism of the cleavage system.

Enzymic properties of the cell-free system for hydroperoxide cleavage

Linoleic acid-13-hydroperoxide was used as a substrate to study the carbonyl-forming reaction(s). [The UV (Δ 234 nm) method, previously used for the cucumber system [8] was not applicable with crude extracts of tomato because of spectral interference; therefore, the GLC method was employed.] The enzymic nature of the cleavage process was demonstrated (Table 2) by the heat sensitivity and substrate specificity of the process. However, it should be pointed out that, although boiling reduced the cleavage enzyme activity of homogenates, (Table 2), significant amounts of hexanal were usually formed and the process did not appear to be as heat-sensitive as the hydroperoxide cleavage system of cucumber frutis [8]. [S] curves showed increasing activity up to 5×10^{-4}M hydroperoxide; a linear plot of 1/v *vs* 1/[S] was obtained, from which an apparent K_m of 2×10^{-4}M hydroperoxide was calculated. The pH response curve was broad with near optimal activity spread between pH 5.5 and 7.5. Progress curves showed increased hexanal formation with incubation up to at least 45 min. Curves of hexanal *vs* enzyme (i.e. added tomato homogenate) were linear up to 60 mg fresh weight of tomato (approx 1 mg protein)/ml of incubation mixture.

DISCUSSION

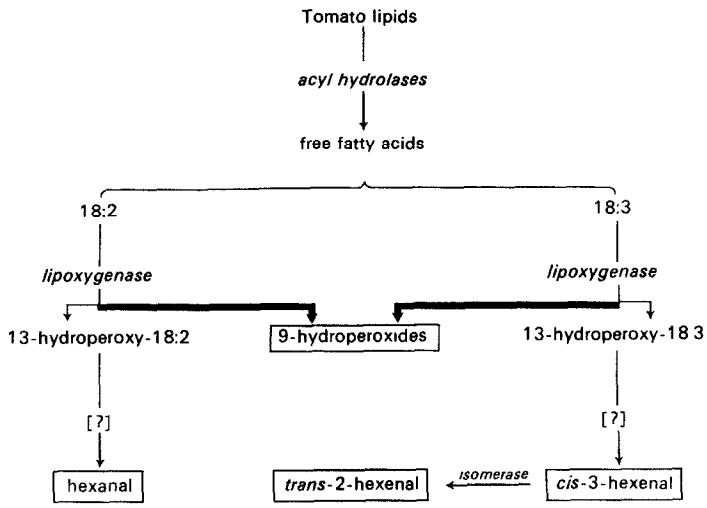
The above results agree with earlier findings of Jadhav *et al.* [10] and Stone *et al.* [11] that the polyunsaturated fatty acids, linoleic and linolenic acids, are converted by enzymes in tomato fruits to volatile carbonyl fragments—hexanal and hexenal—respectively, although we could not confirm the observations [10] that hexanal was also formed from linolenic acid. Furthermore, the present results provide direct experimental evidence to support the previous suggestions [10, 11] that lipoxygenase and fatty acid hydroperoxides are directly involved in the process.

However, the formation of volatile carbonyl components from fatty acids in tomato fruits differs significantly from the process previously demonstrated in cucumber fruits [7–9] and a comparison may be informative: (a) In cucumber, 9- and 13-hydroperoxides of linoleic acid are cleaved with very similar efficacy whereas the hydroperoxide cleavage in tomato appears to be specific for the 13-hydroperoxide isomers. (b) Whereas in cucumber, initial cleavage products, i.e. *cis*-3-enals, are rapidly converted to *trans*-2-isomers by

Table 3. Specific radioactivities of hexanal formed from [U-^{14}C]-linoleic acid by enzymic degradation in tomato homogenates and by chemical cleavage

Source of hexanal	Peak area (units)	Radioactive counts†	Specific radioactivity (counts/unit area)
Incubation of ^{14}C-U-labelled 13-hydroperoxy-linoleic acid with tomato homogenate*	268 ± 16	25 ± 5	0.09 ± 0.02
Reductive ozonolysis of [U-^{14}C]linoleic acid‡	3560	320	0.09

Enzyme incubations as in Table 1 except that ^{14}C-U-labelled 13-hydroperoxylinoleic acid was used as substrate. Analysis was by low background-radio-GLC. *Values represent means and S.D. of four analyses; †Corrected for background (av. 5 counts across peak);‡ The same preparation of [U-^{14}C]linoleic acid as used to make the 13-hydroperoxylinoleic acid substrate.



Scheme 1. Proposed pathway for the formation of C_6 aldehydes and C_{18}-9-hydroperoxides from C_{18} fatty acids by homogenates of tomato fruits.

endogenous isomerases, in tomato extracts, *cis*-3-hexenal is the major product from linolenic acid and its 13-hydroperoxide, indicating lower enal isomerase activity in this tissue. (c) Cucumber extracts gave much higher molar yields of aldehydes (predominantly C_9) from free fatty acid substrates than did tomato homogenates; this can readily be explained by the results in this paper which show clearly that the major products of the tomato lipoxygenase activity on C_{18} substrates are the 9-hydroperoxides which are not subject to the cleavage reaction(s); only the (minor) 13-hydroperoxide products are further converted to the cleavage products.

We suggest that the volatile flavour compounds, hexanal, *cis*-3- and *trans*-2-hexenal are formed in disrupted tomato tissue by the pathway summarized in Scheme 1.

The specific degradation of 13-hydroperoxide isomers by tomato homogenates and the high yield of 9-hydroperoxide from linoleic acid has enabled us to develop a simple procedure for producing good yields of pure (>99%) 9-D-hydroperoxy-octadeca-*trans*-10,*cis*-12-dienoic acid [23].

EXPERIMENTAL

Materials. Tomato fruits (imported; varieties unspecified) were normally used in a firm, red stage of ripeness. Fatty acids were from Sigma (London) Chemical Co. Ltd. and ^{14}C-1 labelled fatty acids from the Radiochemical Centre, Amersham, U.K. [U-^{14}C]-linoleic acid was prepared from the total ^{14}C-lipids obtained by incubating potato tuber disks with [1,2-^{14}C] acetate by a modification of ref. [20]; the proportion of ^{14}C-linoleic acid was increased to 25% of the total fatty acid labelling by increasing the incubation period to 6 hr. Transmethylation of the ^{14}C-lipids followed by separation of the methyl esters on a column of $Si O_2$–$AgNO_3$ gave pure (by GLC and TLC) methyl [U-^{14}C]-linoleate of high specific activity (approx. 2μCi/μmol). [U-^{14}C]-13-hydroperoxylinoleic acid was prepared by mixing [U-^{14}C]- and unlabelled methyl linoleate to a specific radioactivity of 0.05μ Ci/μmol; the free acid was liberated by saponification and converted to the hydroperoxide (95–99% of the 13-hydroperoxide isomer) using soyabean lipoxygenase as previously described [8].

Enzyme preparations. Tomato flesh (skin, seed and locular material removed) was homogenized with 2 vols. of 0.1 M HEPES buffer, pH 7.5 at 0°. After filtration through Miracloth, the extract was used immediately as the enzyme source. Protein content of homogenates was approx. 5.3 mg/ml (i.e. approx. 16 mg of protein/g fr. wt of tissue).

Enzyme assays. Incubation conditions are given in the text; fatty acids and their hydroperoxides were added as NH_4^+ salts. volatile carbonyl compounds were analysed as described elsewhere [8] by extraction into trimethylpentane containing an internal standard of octanal for GLC determination. Non-volatile ^{14}C-labelled products were analysed from radio-TLC scans as in ref. [21]. High performance liquid chromatography was used to separate hydroperoxide isomers [22].

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