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ENZYMIC FORMATION OF CARBONYLS FROM LINOLEIC ACID IN LEAVES OF *PHASEOLUS VULGARIS*

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Key Word Index—*Phaseolus vulgaris*; french bean; Leguminosae; hexanal; hexenal; linoleic acid; hydroperoxide; hydroperoxide cleavage.

Abstract—Homogenization of *Phaseolus vulgaris* leaves at acid pH results in the evolution of hexanal, *cis*-3- and *trans*-2-hexenal. With cell-free extracts of leaves, linoleic and linolenic acids are enzymically converted to their hydroperoxides (predominantly the 13-hydroperoxide isomers) and to hexanal or hexenal respectively. Activity was highest in young, dark-green leaves and was stimulated by Triton X-100. Oleic acid is not a substrate for these reactions. Both 9- and 13-hydroperoxides were cleaved to carbonyl fragments and are proposed as intermediates in the formation of volatile aldehydes and non-volatile ω-oxoacids in *P. vulgaris* leaves. Properties of the enzyme systems are described.

INTRODUCTION

The enzymic formation of volatile carbonyl compounds on disruption of plant tissues is widespread [1]. Included amongst these are hexanal and the so-called 'leaf aldehyde' (*trans*-2-hexenal). Recent work in this laboratory has demonstrated an enzymic sequence which leads from endogenous lipids to various volatile flavour components (C_6 and/or C_9 aldehydes) in both cucumber and tomato extracts following tissue disruption [2–5]. In each of these systems fatty acid hydroperoxides are an intermediate product.

Although linoleic and linolenic acids have been identified as the precursors of the C_6 aldehydes and alcohols in leaves [6–8] the biogenetic pathways have not been fully determined and recent papers on C_6 volatile formation in leaves of *Ginkgo biloba* [6], *Thea sinensis* [9] and *Farfuguim japonicum* [10] have implied that they arise by direct cleavage of polyunsaturated fatty acids rather than through a hydroperoxide intermediate. The work presented in this paper provides good evidence for the involvement of hydroperoxide intermediates in the formation of C_6 aldehydes in *Phaseolus vulgaris* leaves.

RESULTS AND DISCUSSIONS

In autolysis experiments using bean leaf tissue a very rapid loss of polar lipid followed cell disruption, as was previously demonstrated by Sastry and Kates [11]. Loss of lipid was maximal at pH 3.5. Production of hexanal and hexenal in bean leaf homogenates was also maximal at acid pH.

In experiments using linoleic acid-[1-^{14}C] as substrate with bean leaf extracts, the fatty acid was converted to different products depending on enzyme concentration as shown in Fig. 1. At low enzyme concentration the major product was the 13-hydroperoxide of linoleic acid whilst at high enzyme concentration the major products were carbonyl fragments, i.e. the non-volatile C_{12} ω-oxo acid (almost equal amounts of 12-oxo-dodec-*cis*-9-enoic acid and its *trans*-10 isomer) and the volatile C_6 aldehyde (hexanal). At intermediate enzyme concentrations a mixture of the above products was found. Very similar results were obtained earlier with cucumber extracts [2].

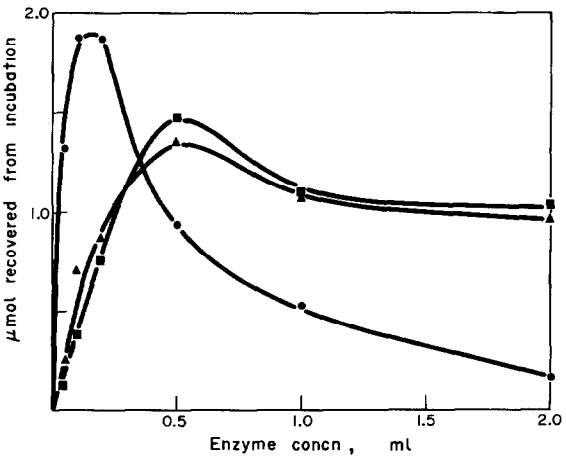

Fig. 1. Incubation mixtures (10 ml) contained linoleic acid-[1-^{14}C] acid (5.3 μmol), bean leaf homogenate (containing 0.33 g fr. wt of tissue/ml) in 0.1 M potassium phosphate buffer, pH 6.5. Incubation was at 25° for 15 min. Formation of hydroperoxide and oxo-acid were determined by radio scanning of TLC separations; hexanal was assayed by GLC. The figure shows μmol recovered from the incubation of linoleic acid hydroperoxide (●—●), oxo-acid (▲—▲) and hexanal (■—■).

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MEMBRANE LIPIDS —acyl hydrolase(s)→ FREE FATTY ACIDS —lipoxygenase→ HYDROPEROXY ACIDS —cleavage enzyme→ CARBONYL FRAGMENTS

Scheme 1.

The pH optimum for hexanal production from linoleic acid was 6.5, with half optimal activities at pH 5 and 7.5. Time curves were linear up to 15 min and optimal substrate concentration was *ca* 0.5 mM, higher levels showing marked inhibition.

When linoleic acid and its 13-hydroperoxide were compared as substrates the major products were hexanal and the 12-oxo-acid in each case but production of hexanal (under optimal conditions) was nearly 3 times greater using 13-hydroperoxide as substrate as it was with the free fatty acid (Table 1).

With 13-hydroperoxide as substrate the pH optimum was nearer to 7, time curves were linear up to 15 min, and the optimal substrate concentration was *ca* 0.33 mM. The progression with increasing enzyme concentration was linear up to 0.2 ml of extract (0.7 g fr. wt tissue).

The 9-hydroperoxide isomer of linoleic acid also proved to be a good substrate (Table 1). The volatile product was identified by co-chromatography with authentic material, as *trans*-2-nonenal. Only trace amounts of nonenal were found in incubations with linoleic acid but this was to be expected because the major hydroperoxyacid formed from linoleic acid was the 13-isomer which cleaved to hexanal. HPLC analysis [12] of the hydroperoxides formed from linoleic acid gave a ratio for 13- to 9-hydroperoxide of 9:1. Linolenic acid and its hydroperoxides yielded the analogous products, i.e. *cis*-3- and *trans*-2-hexanal from linolenic acid or its 13-hydroperoxide, *trans*-2, *cis*-6-nonadienal from the 9 hydroperoxide. Oleic acid was not a substrate for this series of enzymes.

It was necessary to use young, dark-green leaves (primary or secondary) in order to obtain active enzyme preparations. The incorproation of Triton X-100 (0.1% w/v) into the extraction medium was necessary. This could be explained if the enzymes involved were concentrated in chloroplasts as suggested by Hatanaka *et al.* [9, 10, 13].

The above results with leaves of *P. vulgaris* appear to support our conclusions that, as in cucumber and tomato fruit, fatty acid hydroperoxide intermediates are directly involved in the conversion of polyunsaturated fatty acids to volatile carbonyl components as indicated in Scheme 1.

Table 1. Volatile products formed from linoleic acid and its hydroperoxides by extracts of *Phaseolus vulgaris* leaves

Substrate	Product (μmol)	
	hexanal	nonenal
Linoleic acid	0.75	trace
9-Hydroperoxy octadecadienoic acid	—	1.47
13-Hydroperoxy octadecadienoic acid	1.86	—
Oleic acid	—	—

Incubation mixtures (10 ml) contained [1-^{14}C]-labelled fatty acid (5.3 μmol) or hydroperoxide (3.3 μmol), 0.2 ml bean leaf extract (0.7 g fr. wt tissue) in 0.1 M phosphate buffer pH 6.5. Incubations were for 15 min at 25°. The volatile products were assayed by GLC.

EXPERIMENTAL

Linoleic acid was obtained from Lipid Supplies, St. Andrews, Scotland, fatty acid hydroperoxides were prepared as described in refs [14, 15] Plants of *P. vulgaris* were grown at the Food Research Institute, in greenhouses during the winter, and outdoors during the summer. Autolysis expts were essentially as described in ref. [16]. Pieces of leaf (*ca* 0.1 g) were homogenized with 100 vol. buffered solns at 0° using an Ultra Turrax homogenizer. In control expts the tissue was heated before homogenization. Homogenates were incubated at 25° for 10 min. The lipids were extracted by the method of ref. [17] and analysed by TLC on Si gel with solvent system $CHCl_3$–MeOH–HoAc–H_2O (34:6:4:1). Enzyme extracts were routinely prepared by homogenizing 10 g of young, dark green bean leaf tissue with 20 ml 0.1 M HEPES buffer pH 7.5 which contained 0.1% Triton X-100. This homogenate was filtered and then centrifuged at 15000 *g* for 30 min. The resultant supernatant was used as the enzyme source. Incubation conditions for expts with linoleic acid and its hydroperoxides are given in Table 1. At the end of the incubation period 5 ml aliquots were taken for TLC, solvent system petrol (bp 60–80°)–Et_2O–HoAc (60:40:1) and the remaining 5 ml was extracted with pentane for GLC anaysis of volatiles, as described in ref. [3]. Hexanal and the hexenals were identified on GLC by comparison with authentic samples. Fatty acid hydroperoxides were identified by HPLC analysis [12]. The 12-oxo acids were characterised on GLC (SE 30) by co-chromatography with authentic samples as described in ref. [5].

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