Phytochemistry, 1978, Vol 17, pp 355–358 Pergamon Press, Printed in England

FLAVOUR BIOGENESIS. PARTIAL PURIFICATION AND PROPERTIES OF A FATTY ACID HYDROPEROXIDE CLEAVING ENZYME FROM FRUITS OF CUCUMBER

DAVID R. PHILLIPS and TERENCE GALLIARD*
Agricultural Research Council, Food Research Institute, Colney Lane, Norwich NR4 7UA, England
* Lord Rank Research Centre, Lincoln Road, High Wycombe, Bucks., HP12 3QR, England

(*Received* 15 *July* 1977)

Key Word Index—*Cucumis sativus*; Cucurbitaceae; cucumber; flavour; volatile aldehydes; fatty acid hydroperoxide; cleavage enzyme.

Abstract—A membrane-bound enzyme, which catalyses the cleavage of fatty acid hydroperoxides to carbonyl fragments, has been partially purified from cucumber fruit. The isomeric 9- and 13-hydroperoxydienes (but not the hydroxydienes) derived from both linoleic and linolenic acids are cleaved by the enzyme but a mixture of 9- and 10-hydroperoxymonoenoic derivatives of oleic acid was not attacked. No evidence was obtained for free intermediates between fatty acid hydroperoxides and the cleavage products. Major volatile products were: *cis*-3-nonenal and hexanal (from 9- and 13-hydroperoxides of linoleic acid respectively) or *cis*-3,*cis*-6-nonadienal and *cis*-3-hexenal (from 9- and 13-hydroperoxides of linolenic acid). The increase in the ratio of *cis*-3- to *trans*-2-enal products with enzyme purification indicated that *cis*-3-enals are the immediate cleavage products and that the *trans*-2- forms are produced by subsequent isomerization.

INTRODUCTION

In previous publications [1–3] we have described the presence, in cucumber, of a sequence of lipolytic and lipid oxidizing enzymes which act very quickly upon disruption of the tissue to produce, via fatty acid hydroperoxides, the volatile aldehydes *cis*-3-nonenal, *cis*-3, *cis*-6-nonadienal, hexanal and *cis*-3-hexenal. A further enzymic isomerization reaction produces *trans*-2-nonenal, *trans*-2,*cis*-6-nonadienal and *trans*-2-hexenal. These compounds are recognized cucumber flavour constituents [4] having very low olefactory thresholds and have been shown to contribute to flavour and odour in other plant tissues [5–8].

In the formation of these volatile aldehydes, the involvement of fatty acid hydroperoxides, produced by lipoxygenase action, has been implicated in most cases [1–3, 5, 9–11] but other workers have suggested a more direct fragmentation of the unsaturated fatty acids without the participation of lipoxygenase [12, 13].

Tressl and Drawert [5] have proposed than an 'aldehyde lyase' is responsible for hydroperoxide cleavage in banana fruits and Vick and Zimmerman [14] recently demonstrated such activity (which they named 'hydroperoxide lyase') in watermelon seedlings and they suggested that the *trans*-2-enals were the primary products of the cleavage activity in watermelon, whereas our earlier work with cucumber [2] and tomato fruits [15] had indicated that the *cis*-3-enals were more immediate products and were subsequently isomerized to the *trans*-2-isomers. No intermediates between hydroperoxides and aldehydes have been demonstrated, although Jadhav *et al.* [11] proposed that α-ketol intermediates could be involved.

RESULTS AND DISCUSSION

Enzyme purification

Our previous work [2] had shown that the hydroperoxide cleavage activity in cucumber was predominantly located in a particulate fraction and complementary studies in this laboratory [16] using density-gradient centrifugation techniques have shown the cleavage activity to be located in several subcellular membranes viz. the endoplasmic reticulum, plasma membrane, Golgi apparatus and also in cucumber skin chloroplasts. Very good recovery of particulate activity was achieved by including 0.5% bovine serum albumin in the 2.5 M sucrose extraction medium. Inclusion of the albumin also enhanced the stability of the particulate preparation which showed less than 10% loss of activity over 3 days storage at 0°, whereas omission of the albumin resulted in 50% loss of cleavage activity in 24 hr [16]. This membrane-bound nature of the cleavage activity was the source of complications during attempted purifications as the solubilized protein tended towards aggregation and gelling on concentration in the absence of protecting agents.

Initial attempts at fractionation and concentration of the cleavage activity using $(NH_4)_2SO_4$ were abandoned as the procedure inactivated a considerable proportion of the enzyme; also, the bulk of the protein precipitated at the same $(NH_4)_2SO_4$ saturation as the activity. Concentration by use of a membrane filter was found to be more successful.

Using a Millipore 142 mm Hi-flux Cell with a PSED Pellicon Molecular filter, an aliquot of crude supernatant was concentrated 10-fold. A further 5-fold concentration was effected by using an Amicon Minicon B15

Table 1. Purification of the hydroperoxide cleavage enzyme from cucumber

Enzyme preparation	Total protein (mg)	Total activity (μmol min ml)	Specific activity (μmol min mg)	Recovery (%)
Crude filtrate	85	156.	1.8	100
Crude supernatant	65	156	2.4	100
Sephadex-G-200—1st run (pooled fractions)	3	62	10.6	40
Sephadex-G-200—2nd run	1.6	32.5	20.3	21

The values quoted were obtained during purification of 500 g of cucumber tissue. The purification steps and assay methods are given in the text.

cell and this small volume sample was loaded onto a column of Sephadex G-200 (2 × 90 cm). The cleavage activity was eluted slowly by a buffer system comprising 0.05 M Tris–HCl pH 7.0, 1 mM EDTA, 2 mM dithiothreitol and 0.1% Triton X-100. The main cleavage activity was eluted in a peak well separated from the void volume. The greatest proportion of the protein was eluted after the active protein. The void volume peak contained a small amount of activity, possibly in an aggregated form.

Active fractions from this first G-200 fractionation were combined and again concentrated, using the Minicon B15 cell, before reloading onto the G-200 column for a second fractionation. Table 1 shows the degree of purification achieved by this procedure. Extensive attempts to obtain further purification using ion-exchange Sephadex and cellulose media or by iso-electric focussing resulted in a marked loss of activity and effective reduction in specific activities. The difficulties in purification of membrane-bound enzymes are well known and we appear to have encountered very similar problems to those described recently by van der Oudoraa *et al.* [17] in the purification of the prostaglandin endoperoxide synthetase enzyme.

The main intention of this partial purification was to determine the nature of the cleavage reaction. Thus, if a free intermediate between the hydroperoxide and aldehyde existed, separation of the enzyme catalysing the first step might have been expected during purification and this would have been detected by comparing the decrease in A_{234} with the quantity of volatile aldehydes produced. However, at no stage in this purification procedure was any indication of an intermediate product noted. The non-volatile products from the reaction of ^{14}C-labelled hydroperoxide with enzyme from the various stages of purification were screened by radio-TLC of the ether extract of the acidified incubation system as described previously [1]. The only significant product from 9-hydroperoxylinoleic acid was the 9-oxo-nonanoic acid and, to a small extent, its reduction product, 9-hydroxynonanoic acid. Identities were confirmed by GC–MS of the methyl esters and by co-chromatography with authentic samples [1].

Table 2. Volatile carbonyl products formed from 9-hydroperoxy linoleic acid by purified enzyme preparations from cucumber

Enzyme preparation	*cis*-3-Nonenal %	*trans*-2-Nonenal %
Crude supernatant	2	98
Sephadex-G-200—1st run	69	31
Sephadex-G-200—2nd run	92	8

Incubation mixtures contained substrate, 9-hydroperoxy linoleic acid (33.10^{-6} M), and enzyme preparations as indicated, equivalent in amount to 1 g fr. wt of tissue in a total volume of 5 ml of 0.1 M phosphate buffer pH 6.5. The incubations were run at 25° for 10 min. The carbonyl products were extracted with 1 ml pentane containing 20 μg methyl hexanoate as internal standard and analysed by GLC.

In a recent publication describing a similar cleavage activity in watermelon seedlings [14], it was stated that the cleavage enzyme from that source was also responsible for the isomerization of the 12-oxo-*cis*-9-dodecenoic acid product, from 13-hydroperoxylinoleic acid to the *trans*-10-isomer. The possibility that the isomerization in cucumber is catalysed by a separate enzyme was investigated using 9-hydroperoxylinoleic acid as substrate and monitoring, by GLC, the amounts and identities of the volatile aldehydes formed. The results in Table 2 show that, in the stages of purification, as the specific activity of the cleavage enzyme increases so the percentage conversion of the *cis*-3-nonenal product to the *trans*-2-nonenal isomer is reduced. This indicates that the isomerase activity is separate from the cleavage activity. A study of the enzymic conversion of *cis*-3-enals to *trans*-2-enals is in progress in this laboratory.

Substrate specificity

The partially purified cleavage activity was incubated with the different hydroperoxide isomers from linoleic and linolenic acids and the reaction rate measured by loss of the A_{234} and by assay of the volatile products. It was shown (Table 3) that the enzyme had very similar specificities for the 9- and 13-hydroperoxy isomers and almost identical activities with the same positional hydroperoxide isomers of linoleic and linolenic acids. In incubations containing a mixture of 9- and 10-hydroperoxyoleic acid, no change was observed in the substrate on radio-TLC of the extracted incubation products.

It has been suggested that an α-ketol could act as an intermediate in the cleavage reaction [11] but when incubations were conducted which contained an authentic sample of 9-hydroxy-10-oxo-octadeca-*cis*-12-enoic acid the α-ketol did not act as substrate for the cleavage enzyme. 9-Hydroxylinoleic acid produced no volatile products and the hydroxydiene remained unchanged in the incubation. Thus the cleavage activity was specific for hydroperoxydienes as opposed to hydroxydienes or hydroperoxy-monoenes but (in contrast to a related

Table 3. Substrate specificity of a partially purified cleavage enzyme activity from cucumber

Substrate	Volatile products	
	μmol formed	Identity
9-hydroperoxylinoleic acid	0.07	*cis*-3-nonenal
9-hydroperoxylinolenic acid	0.09	*cis*-3,*cis*-6-nonadienal
13-hydroperoxylinoleic acid	0.04	hexanal
13-hydroperoxylinolenic acid	0.06	*cis*-3-hexenal

Incubations contained substrate (33.10^{-6} M) and an amount of enzyme from the Sephadex-G-200 2nd run equivalent to 1 g fr. wt of tissue in a total volume of 5 ml of 0.1 M phosphate buffer (pH 6.5). Incubations were run for 10 min at 25° The carbonyl products were extracted with 1 ml pentane containing methyl hexanoate as internal standard and analysed by GLC.

process in tomato fruits which was specific for 13-hydroperoxides [15] both 9- and 13-hydroperoxy isomers of linoleic and linolenic acids were effective substrates. The failure to detect ketol intermediates and the lack of activity on a potential ketol substrate provided evidence against the involvement of such derivatives (at least as free intermediates) in the cleavage process.

Enzyme properties

Using partially purified enzyme preparations, Lineweaver–Burke plots of V^{-1} against $[S]^{-1}$ were linear and the apparent K_m obtained for both 9-hydroperoxylinoleic acid and 13-hydroperoxylinoleic acid was between 1.5×10^{-5} and 2.0×10^{-5} M, similar to the values previously obtained with crude enzyme extracts [2]. A plot of enzyme concentration versus product formed was linear.

The G-200-2 fraction was stable when stored at 0° for up to 10 days in the elution buffer system. Attempts to extend the storage period using 2M $(NH_4)_2SO_4$ in 10% glycerol were unsuccessful and resulted in only *ca* 10% recovery of the original activity after dialysis. Freezing the eluted activity was also unsuccessful as a means of storing the enzyme and resulted in a 40% loss of activity in the purified sample.

The purified enzyme activity was extremely heat labile. Loss of 50% of activity was incurred by heating at 50° for 5 min. Heating at 70° for 1 min resulted in 100% inactivation of the cleavage enzyme. Similar results were found with the crude extract of cucumber [2].

The effects of inhibitors were examined with both 9- and 13-hydroperoxy linoleic acid as substrate using the partially purified enzyme. Significant inhibition was obtained with 10^{-4} M *p*-chloromercuribenzoic acid (−20%) and 10^{-3} M KCN (−40%). There was no evidence of inhibition with 10^{-2} M iodoacetamide or with 10^{-3} M EDTA. Vick and Zimmerman [14] found, with the watermelon enzyme activity, that a partially purified enzyme was not inhibited by 10^{-2} M KCN or 10^{-2} M iodoacetamide but its activity was inhibited to 83% by 10^{-4} M *p*-chloromercuribenzoic acid.

No activation was found when transition metals (10^{-2} M) were added to incubations containing the partially purified enzyme except for Cu^+ and Cu^{2+} which even in the absence of the enzyme, very rapidly cleaved the hydroperoxide substrate indicating the possible involvement of a free radical reaction.

EXPERIMENTAL

Materials. Cucumber fruits (*Cucumis sativus*) were purchased from a local supplier, their origin varied throughout the season and the cultivars were not known. The ^{14}C-labelled linoleic and linolenic acids were obtained from The Radiochemical Centre, Amersham, Bucks.

Substrate preparation. The 9-hydroperoxides of linoleic and linolenic acids were prepared using tomato lipoxygenase [18] and 13-hydroperoxides of linoleic and linolenic acids were prepared using soyabean lipoxygenase as previously described [2]. The isomeric purity of the substrate after column chromatography was checked by HPLC [19], the ratio of 9:13 hydroperoxides in the 9-hydroperoxy linoleic and linolenic acids was 49:1, and in the 13-hydroperoxy linoleic and linolenic acids it was 2:23 and 1:19 respectively. The mixed sample of 9- and 10-hydroperoxy oleic acids, a gift from Dr. H. W.-S. Chan, was prepared by photochemical oxidation of ^{14}C-1-oleic acid by a similar procedure to that described for the methyl ester [19]. The 9-hydroxy-10-oxo-octadeca-*cis*-12-enoic acid was prepared by the method of ref. [20] by incubation of 9-hydroperoxy linoleic acid with an aq. extract from hexane defatted corn germ flour, a gift from Dr. H. W. Gardner. The 9-hydroxylinoleic acid was prepared by $NaBH_4$ reduction of 9-hydroperoxy linoleic acid.

Enzyme preparation. An aq. homogenate of cucumber was prepared in a Moulinex juice extractor from 2 vols of diced cucumber tissue and 1 vol. of extraction medium consisting 0.1 M Tris–HCl buffer pH 7, 2 mM EDTA, 4 mM dithiothreitol and 0.2% Triton X-100. After passing this homogenate through Miracloth the filtrate was centrifuged at 15000 *g* for 30 min. The supernatant was decanted off and this prepn was termed the crude supernatant enzyme.

Enzyme assays. Incubation details are to be found in the tables. Volatile carbonyl products were analysed by GLC as in [2] using octanal or methyl hexanoate as int. stand. Disappearance of 9- and 13-hydroperoxides during incubations was measured by following A_{234} in a recording spectrophotometer. Enzyme activities were determined from initial velocities assuming $\varepsilon = 24000$ for the conjugated *cis–trans* diene chromophore in the substrate. For inhibition studies the incubation system containing the inhibitor and enzyme was made up to 90% of the final vol. and pre-incubated for 5 min before the substrate was added to start the main incubation. The ^{14}C-labelled non-volatile products were assayed by radio-TLC of Et_2O extracts of the acidified incubation system as previously described [1].

Chromatographic methods. Thin layer, column and gas chromatographic separations of fatty acid derivatives and their volatile and non-volatile fragmentation products have all been described previously [1, 21, 22]. Protein was assayed as in ref. [23] using BSA as reference.

REFERENCES

1. Galliard, T. and Phillips, D. R. (1976) *Biochim. Biophys. Acta* **431**, 278.
2. Galliard, T., Phillips, D. R. and Reynolds, J. (1976) *Biochim. Biophys. Acta* **441**, 181.
3. Galliard, T., Matthew, J. A., Fishwick, M. J. and Wright, A. J. (1976) *Phytochemistry* **15**, 1647.
4. Forss, D. A., Dunstone, E. A., Ramshaw, E. H. and Stark, W. (1962) *J. Food Sci.* **27**, 90.
5. Tressl, R. and Drawert, F. (1972) *J. Agric. Fd Chem.* **21**, 560.
6. Gardner, H. W. (1975) *J. Agric. Fd Chem.* **23**, 129.
7. Hatanaka, A., Sekiya, J. and Kajiwara, T. (1977) *Plant Cell Physiol.* **18**, 107.
8. Hatanaka, A. and Harada, T. (1973) *Phytochemistry* **12**, 2341.
9. Eriksson, C. (1975) *J. Agric. Fd Chem.* **23**, 126.
10. Stone, E. J., Hall, R. M and Kazeniac, S. J. (1975) *J Food Sci* **40**, 1138.
11. Jadhav, S., Singh, B. and Salunke, D. K. (1972) *Plant Cell Physiol.* **13**, 449.
12. Grosch, W. and Schwartz, J. M. (1971) *Lipids* **6**, 351.
13. Major, R. T. and Thomas, M. (1972) *Phytochemistry* **11**, 611.
14. Vick, B. A. and Zimmerman, D. C. (1976) *Plant Physiol.* **57**, 780.
15. Galliard, T. and Matthew, J. A. (1977) *Phytochemistry* **16**, 339.
16. Wardale, D. A., Lambert, E. A. and Galliard, T. (1978) *Phytochemistry* **17**, 205.
17. Van der Ouderaa, F. J., Buytenhek, M., Nugteren, D. H. and Van Dorp, D. A. (1977) *Biochim. Biophys. Acta* **487**, 315.
18. Matthew, J. A., Chan, H. W.-S. and Galliard, T. (1977) *Lipids* **12**, 324.
19. Chan, H. W.-S. and Prescott, F. A. A. (1975) *Biochim. Biophys. Acta* **380**, 141.
20. Gardner, H. W., Kleiman, R., Christianson, D. D. and Weisleder, D. (1975) *Lipids* **10**, 602.
21. Chan, H. W.-S. (1977) *J. Am. Oil Chem. Soc.* **54**, 100.
22. Galliard, T. and Phillips, D. R. (1972) *Biochem. J.* **129**, 743.
23. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265.