Phytochemistry, 1976, Vol. 15, pp. 1647–1650. Pergamon Press. Printed in England.

THE ENZYMIC DEGRADATION OF LIPIDS RESULTING FROM PHYSICAL DISRUPTION OF CUCUMBER (*CUCUMIS SATIVUS*) FRUIT

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(*Received* 27 *March* 1976)

Key Word Index—*Cucumis sativus*; Cucurbitaceae; cucumber fruit; lipid breakdown; acyl hydrolases; phospholipase D.

Abstract—Homogenization of fresh tissue from cucumber fruits results in a loss of endogenous lipid catalysed by acyl hydrolase enzymes. Deacylation of lipids is not accompanied by accumulation of free fatty acids. The levels of both saturated (mainly palmitic) and polyunsaturated (linoleic and linolenic) fatty acids in the lipids are reduced. Losses of the major acyl lipid constituents of cucumber (triacylglycerols and phospholipids) are mainly responsible for the observed hydrolysis. Triacylglycerol acyl hydrolase (lipase), phospholipase D and polar lipid acyl hydrolase enzyme activities were demonstrated. It is suggested that hydrolytic attack on endogenous lipids is the initial event on disruption of cucumber tissue, in the formation of lipid degradation products, amongst which are the volatile carbonyl compounds responsible for the characteristic flavour of cucumber.

INTRODUCTION

Physical disruption of many plant tissues initiates autolytic changes, catalysed by endogenous enzymes amongst which are those causing hydrolytic and oxidative breakdown of lipids. Oxidation of polyunsaturated fatty acids may produce volatile carbonyl compounds with desirable flavours or undesirable (off-flavour) properties [1–3].

The characteristic flavour of cucumber fruits is mainly due to C_9 unsaturated carbonyl compounds released on disruption of the tissue [4–8]; these compounds are not present in the intact tissue and are not formed if the endogenous enzymes are inactivated before the material is homogenized. The major components of cucumber flavour, mono- and di- unsaturated C_9 aldehydes, are formed from linoleic and linolenic acids respectively when these unsaturated fatty acids are added to cucumber homogenates [7–8]. An enzyme system that converts linoleic acid via hydroperoxide intermediates to C_6 and C_9 carbonyl fragments has recently been isolated from cucumbers [9–10]. Long chain (C_{12}–C_{18}) aldehydes are also present in the volatile fraction of cucumber homogenates [6, 11] and an α-oxidation enzyme system which converts saturated and unsaturated fatty acids to long chain aldehydes has been isolated from cucumbers [12].

The above processes have been observed with free fatty acids as substrates. However, free fatty acids rarely occur in significant amounts in healthy plants and their concentration in cucumbers is low [8, 13]. We have therefore considered the possibility that lipid-bound fatty acids are released by acyl hydrolase action as the initial step in the formation of fatty acid degradation products in cucumber homogenates. This paper demonstrates the disappearance of cucumber lipids by enzymic action in homogenates. Recently, Hatanaka *et al.* [8] published results that demonstrate the loss of fatty acids from cucumber lipids in blended tissue. The present work supports their observations and provides additional information on the lipids concerned and on the enzymic nature of the process.

RESULTS AND DISCUSSION

Tissue from peeled cucumber fruits was homogenized with water or buffer and the mixture was incubated. Enzyme activity was then killed and $CHCl_3$-soluble extracts were analysed for lipid ester content, fatty acid and lipid composition. In addition the presence of lipolytic enzymes in the homogenates was investigated.

Lipid ester content

The concentrations of total lipid esters in homogenates from fresh and enzyme-inactivated (heated) cucumber tissue are given in Table 1. It is reasonable to assume,

Table 1. Acyl ester content of homogenates from fresh and heated tissue from cucumber fruits

	Acyl ester content (μequivalents of ester/g fr. wt)
Fresh tissue	1.12 ± 0.04
Control	1.50 ± 0.04

Cucumber tissue (12 g) was homogenized with 0.1M potassium phosphate buffer pH 6.4 (12 ml) and the mixture was incubated at 25° for 30 min before heating at 100° for 15 min to kill enzyme activity. In the control, the tissue was heated at 100° for 15 min before homogenization. The lipids were extracted into $CHCl_3$ and analysed for acyl ester content. The values given in the table represent means and standard deviations from duplicate analyses on three extracts.

from the composition of cucumber lipids shown here and elsewhere [8, 13, 14], that the ester values represent fatty acid esters of acyl lipids. In comparison with the control extracts, 27% of the lipid ester content was lost during homogenization and subsequent incubation in the experiment quoted in Table 1.

The extent of ester loss varied widely from less than 10% to over 40% under similar conditions for different experiments. The reasons for the variability are not known; the experimental material was purchased from local stores and the cultivars (which varied seasonally) were not identified.

The effect of pH on the enzymic deacylation was examined by preparing homogenates in buffered aqueous media. The pH response curve showed a broad optimum between pH 4.5 and 6 and 50% of maximum activity around pH 4 and 7. The measured pH of water homogenates of cucumbers used was between pH 5.5 and 6.0.

Fatty acid content

Fatty acid methyl esters were prepared by transmethylation of the total lipid extracts with an added internal standard of heptadecanoic acid. The concentrations of individual fatty acids in lipid extracts from homogenates of fresh and boiled tissue are listed in Table 2. The three major components, palmitic (16 : 0), linoleic (18 : 2) and linolenic (18 : 3) acids, were present at significantly lower levels in the homogenate of the fresh tissue than in the control. Reductions of 18, 30 and 37% were recorded in the levels of 16 : 0, 18 : 2 and 18 : 3 acids respectively. The overall reduction in fatty acid content was approximately 27%.

The differences in fatty acid composition (expressed as % of the total fatty acid content of each extract) between homogenates of fresh and boiled tissue were less marked (Table 2) because the three major acids, together representing approx 90% of the total fatty acids in both extracts, were all reduced in concentration in the homogenates of fresh tissue.

The results shown in Tables 1 and 2 refer to the same experiment in which both acyl ester content and fatty acid content both fell by approx 27% during homogenization and subsequent incubation. These results of lipid ester and fatty acid analyses indicate that the free fatty acids, released by hydrolytic action, do not accumulate in the homogenates but they are further converted to other products, and this is substantiated by the lipid analyses described below and by the results of Hatanaka *et al.* [8] who demonstrated even greater losses of fatty acids on homogenization of cucumber fruits.

Table 2. Fatty acid content of homogenates from fresh and heated tissue from cucumber fruits

	Fatty acid composition			
	% Total fatty acids		Concentration relative to internal standard (17 : 0 = 100)	
Fatty acid	Fresh tissue	Control	Fresh tissue	Control
Lauric (12 : 0)	1.0	0.8	2.1 (0.8)	2.5 (0.3)
Myristic (14 : 0)	1.4	1.1	3.2 (0.5)	3.5 (0.2)
Palmitic (16 : 0)	29.5	26.0	65.1 (0.9)	79.3 (0.5)
Stearic (18 : 0)	5.2	3.5	11.5 (3.0)	10.8 (0.6)
Oleic (18 : 1)	3.0	2.5	6.8 (1.0)	7.6 (4.5)
Linoleic (18 : 2)	25.2	26.3	55.8 (3.7)	80.2 (3.4)
Linolenic (18 : 3)	34.6	39.5	76.5 (7.1)	121.2 (5.5)

Reaction and extraction conditions were as described in Table 1. An internal standard of 1mg of heptadecanoic acid (17 : 0) was added during the lipid extraction. Figures in parentheses represent standard deviations of triplicate analyses.

Enzyme systems that attack free fatty acids are present in cucumber homogenates; an α-oxidation system in cucumber oxidizes both saturated (e.g. 16 : 0) and unsaturated fatty acids to long-chain aldehydes [12] and a cleavage reaction, probably mediated by lipoxygenase, leads to the formation of C_9 carbonyl fragments from the polyunsaturated acids, 18 : 2 and 18 : 3 [9, 10]. It is assumed that these enzyme activities were responsible for the observed absence of accumulated free fatty acid as deacylation products.

Lipid analysis

TLC analyses of the lipids from homogenates of fresh and boiled tissue had demonstrated marked reductions in the levels of certain lipids in the enzyme-active homogenates. The results of a quantitative lipid analysis on these extracts are given in Table 3.

Lipid analysis of control (enzyme-inactivated) tissue showed that phospholipids (mainly phosphatidylcholine and phosphatidylethanolamine) were the predominant class; di- and tri- acylglycerols, galactosyldiglycerides cerebrosides and acylated sterol glycosides were also major constituents. These results are qualitatively similar to those of Kinsella [14] although quantitative differences are apparent; a more detailed analysis of cucumber fruit lipids is presented elsewhere [13].

Analysis of the homogenate lipids showed decreases (with respect to the control) in the levels of most of the major lipids, except for cerebrosides and acylated sterol glycosides. The main contributions to the loss of acyl

Table 3. Lipid content of homogenates from fresh and heated tissue from cucumber fruits

	Lipid content (μmol lipid/kg fr. wt)		
Lipid	Fresh tissue	Control	Fresh tissue as % of control
Phosphatidylcholine	86	96	89
Phosphatidylethanolamine	104	122	85
Lysophosphatidylcholine	12.5	7.7	162
Lysophosphatidylethanolamine	12.5	5.8	216
Phosphatidylinositol	1.7	3.6	47
Phosphatidylglycerol	6.8	7.7	87
Phosphatidic acid	1.9	1.2	158
Diphosphatidylglycerol	1.5	1.9	78
Monogalactosyldiglyceride	10.9	14.9	74
Digalactosyldiglyceride	17.8	19.9	89
Triacylglycerol	14.0	26.6	52
1,3-diacylglycerol	5.0	7.6	66
1,2-diacylglycerol	28.6	25.3	112
Monoacylglycerol	6.3	9.4	68
Free fatty acid	3.1	3.9	79
Acylated sterol glycoside	18.2	17.0	107
Cerebroside	29.5	31.0	95

Fresh tissue (100g) homogenate was incubated in 100 ml phosphate buffer, pH 5.7 for 15 min at 22° then mixed with propan-2-ol (500 ml) at 80°. Control tissue was treated with propan-2-ol at 80° before homogenization. Lipids were extracted and analysed as described in the text.

Table 4. Hydrolysis of phosphatidylcholine by cucumber extracts

Experimental conditions	^{14}C-labelled products (as % of total radioactivity) Phosphatidyl-choline	Phosphatidic acid	Non-polar products
Experiment 1			
control	50	15	35
tissue homogenized with substrate	35	31	34
Experiment 2			
control	59	21	20
control + EDTA (5mM)	82	7	11

In control experiments, cucumber homogenates (containing 0.2 g fresh weight of tissue) were incubated with ^{14}C-labelled phosphatidylcholine (25 μg; 10^5 dpm) in 0.1M sodium acetate buffer, pH 5.0, for 15 min at 25° in a total volume of 4.8 ml. The chloroform-soluble ^{14}C-labelled products were analysed by radio-scanning of TLC separations in $CHCl_3$–CH_3OH–7N NH_4OH (63 : 30 : 4).

esters were from triacylglycerol, phosphatidylethanolamine and phosphatidylcholine (equivalent to losses of.38, 36 and 20 μmoles of fatty acyl ester/kg tissue, respectively). The only increases observed were in lipids that may be considered as products of partial enzymic hydrolysis of phospholipids (i.e. lysophospholipids and phosphatidic acid) and triacylglycerols (i.e. 1,2-diacylglycerol); however, these increases were only minor in molar terms and the net result was overall loss of lipid acyl ester of approx 100 μmol/kg of tissue.

The results in Table 3 also show that the free fatty acid content of the control tissue was low and that the content did not increase in the homogenates.

Hydrolytic enzymes

To establish the nature of the enzymes responsible for the loss of the major lipids (phospholipids and triacylglycerols), cucumber extracts were incubated with [^{14}C-acyl]-labelled phosphatidylcholine and trioleylglycerol. As noted above for the acyl ester measurements on cucumber homogenates, the hydrolytic activities of preparations from different batches of cucumbers were also very variable. Nevertheless, it was possible to demonstrate the enzymic hydrolysis of added lipid substrates.

Generally, more efficient hydrolysis was achieved when the tissue was homogenized in the presence of labelled substrate than by adding the substrate to prepared homogenates, (see e.g. phosphatidylcholine hydrolysis results in Table 4). With both substrates hydrolysis was active only at acid pH and was optimal at pH 5.0–5.5.

Phosphatidylcholine was hydrolysed to phosphatidic acid and to non-polar products (fatty acid derivatives) (Table 4). Thus the extracts contained both phospholipase D and phospholipid acyl hydrolase activities. Lysophospholipids (indicative of phospholipase A activities) did not accumulate in these incubations. Addition of EDTA (an inhibitor of phospholipase D activity) reduced the extent of phosphatidylcholine hydrolysis.

When cucumber tissue was homogenized in the presence of tracer amounts of tri-[1-^{14}C]oleyl-glycerol, the distribution of ^{14}C-label in the hydrolysis products (Table 5) corresponded to the pattern of breakdown of the endogenous triglycerides as observed by I_2-staining of TLC separations. EDTA did not inhibit this process. The appearance of mono- and 1,2- diacylglycerols in addition to free fatty acid in the hydrolysis products indicates the presence of lipase (triacylglycerol acyl hydrolase, E.C. 3.1.1.3) in the cucumber extracts.

CONCLUSIONS

The studies reported here provide evidence for the initial event in the sequential process by which the endogenous lipids of cucumber fruits are degraded to a range of carbonyl compounds. The two pathways so far demonstrated, i.e. a lipoxygenase-mediated cleavage process [9, 10] and an α-oxidation system [12], both originate from free fatty acids which, it had been presumed, were products of lipid hydrolysis. The present work together with the recent observations of Hatanaka *et al.* [8] has demonstrated that disruption of intact tissue leads to a breakdown of acyl lipids, predominantly of triacylglycerols and phospholipids, with a resultant loss of their fatty acid constituents. It is probable that several lipolytic enzymes are concerned with this hydrolysis.

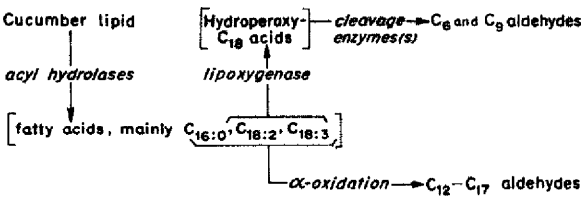
On the basis of work from this and other laboratories

Table 5. Hydrolysis of trioleylglycerol by cucumber extracts

^{14}C-labelled products (as % of total radioactivity) Triacyl-glycerol	1,2-Diacyl-glycerol	Monoacyl-glycerol	Free fatty acid	Unidentified material*
23	7	9	36	25

A mixture containing 3g cucumber tissue, tri-[1-^{14}C]oleyl-glycerol (10^5 dpm) and 0.1M MES buffer, pH 5.5 in a total volume of 4.8 ml was homogenized and the mixture was incubated for 30 min at 25°. The $CHCl_3$-soluble ^{14}C-labelled products were analysed by radio-scanning of TLC separations in petrol–Et_2O–HAc (60 : 40 : 1). * The unidentified material remained at the origin and was assumed to be fatty acid oxidation products.

[4–6, 8–12], a sequence of enzymic reactions, shown in Scheme 1 can be proposed to explain the formation of carbonyl products in disrupted cucumber tissue.



Scheme 1.

EXPERIMENTAL

Materials. Cucumber (*Cucumis sativus*) fruits of unidentified varieties were obtained from local stores. Tri-[1-^{14}C]oleylglycerol (50 μCi/μmole) was purchased from the Radiochemical Centre, Amersham, Bucks, U.K. [^{14}C-acyl]-labelled phosphatidylcholine was prepared from aged disks of potato tuber after incubation with acetate-[u-^{14}C] [15].

Cucumber homogenates. Pieces (approx 1cm^3) from the flesh (peel and seeds removed) of cucumber fruits were homogenized with 1–4 vol of buffered solns (see text) at 0°, using either an Ultra-Turrax homogenizer or an Atomix blender. In control experiments, the tissue–buffer mixture was heated at 100° for 10–15 min before the homogenization. Homogenates were incubated at 25°, with shaking, for 30 min.

Enzyme assays Incubation conditions are given in the text and in Tables 4 and 5. $CHCl_3$-soluble products were isolated and analysed by TLC radio-scanning [16]. Identities of the ^{14}C-labelled hydrolysis products were checked by co-chromatography on TLC in appropriate solvent systems with authentic standards of phosphatidic acid, fatty acids, 2-monopalmitoylglycerol and 1,2- and 1,3- dipalmitoylglycerol. Solvents systems used were $CHCl_3$–MeOH–HAC–H_2O (170 : 30 : 20 : 4), $CHCl_3$–MeOH–7N NH_4OH (65 : 30 : 4), toluene–EtAc–EtOH (2 : 1 : 1), C_6H_6–Et_2O–EtOH–HAC (50 : 40 : 2 : 0.2), petrol (60–80°)-Et_2O–HAC (60 : 40 : 1).

Acyl ester and fatty acid analyses. Incubation mixtures were heated at 100° for 10 min to stop enzyme activity. $CHCl_3$-soluble extracts were prepared as previously described [17]. The acyl ester content was determined by a slight modification [18] of the hydroxamate method [19]. For fatty acid analyses, heptadecanoic acid was added as an internal standard before the $CHCl_3$ extraction. Preparation of fatty acid methyl esters by transmethylation and their analysis by GLC was as previously described [20].

Lipid analysis. Lipid samples prepared from fresh and heat-treated tissues were isolated by hot *iso*-PrOH and $CHCl_3$ extraction [13] and then purified from nonlipid contaminants by passage through a Sephadex G-25 column [21]. Neutral lipid and acidic phospholipid fractions were obtained by chromatography on DEAE cellulose columns [22]. The neutral lipid fraction was further divided into a neutral plus glycolipid and a phospholipid fraction by chromatography on Si gel columns [23]. Individual lipids in these fractions were estimated by quantitative TLC on Si gel with the solvent systems $CHCl_3$–MeOH–HAC–H_2O (170 : 30 : 20 : 4) for polar lipids and C_6H_6–Et_2O–HAC (50 : 40 : 2 : 0.2) for non polar lipids. Phospholipids were estimated as inorganic phosphorus [24], glycolipids by GLC of the TMS-derivatives of methylated glucose or galactose [25] and simpler glycerides by GLC of fatty acid methyl esters prepared by transmethylation in 5% H_2SO_4 in MeOH at 60° in the presence of an internal standard, methyl behenate.

REFERENCES

1. Tressl, R. and Drawet, F. (1973) *J. Ag. Food Chem.* **21**, 560.
2. Gardner, H. W. (1975) *J. Agr. Food Chem.* **23**, 129.
3. Galliard, T. (1975) in *Recent Advances in the Chemistry and Biochemistry of Plant Lipids* (Galliard, T. and Mercer, E. I. eds.), pp. 319. Academic Press, London.
4. Forss, D. A., Dunstone, E. A., Ramshaw, E. H. and Stark, W. (1962) *J. Food Sci.* **27**, 90.
5. Fleming, H. P., Cobb, W. Y., Etchells, J. C. and Bell, T. A. (1968) *J. Food Sci.* **33**, 572.
6. Kemp, T. R., Knavel, D. E. and Stoltz, L. P. (1974) *J. Agr. Food Chem.* **22**, 717.
7. Grosch, W. and Schwartz, J. M. (1971) *Lipids* **6**, 351.
8. Hatanaka, A., Kajiwara, T. and Harada, T. (1975) *Phytochemistry* **14**, 2589.
9. Galliard, T. and Phillips, D. R. (1976) *Biochim. Biophys. Acta* **431**, 278.
10. Galliard, T. Phillips, D. R. and Reynolds, J. (1976) *Biochim. Biophys. Acta* (in press).
11. Kemp, T. R. (1975) *J. Am. Oil Chem. Soc.* **52**, 300.
12. Galliard, T. and Matthew, J. A. (1976) *Biochim. Biophys. Acta* **424**, 26.
13. Fishwick, M. J., Wright, A. J. and Galliard, T. unpublished observations.
14. Kinsella, J. E. (1971) *J. Food Sci.* **36**, 865.
15. Galliard, T. (1972) *Biochim. Biophys. Acta* **260**, 541.
16. Galliard, T. and Dennis, S. (1974) *Phytochemistry* 13, 1731.
17. Galliard, T. (1970) *Phytochemistry* **9**, 1725.
18. Renkonen, O. (1961) *Biochim. Biophys. Acta* **54**, 361.
19. Snyder, F. and Stevens, N. (1959) *Biochim. Biophys. Acta* **34**, 244.
20. Galliard, T. (1972) *Phytochemistry* **11**, 1899.
21. Wuthier, R. E. (1966) *J. Lipid Res.* **7**, 558.
22. Roughan, P. G. and Batt, R. D. (1968) *Anal. Biochem.* **22**, 74.
23. Rouser, G., Kritchevsky, G., Simon. G. and Nelson, G. J. (1967) *Lipids* **2**, 37.
24. Parker, F. and Peterson, N. F. (1965) *J. Lipid Res.* **6**, 455.
25. Christie, W. W. (1973) in *Lipid Analysis* pp. 223–226. Pergamon Press, Oxford.