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LOCALIZATION OF FATTY ACID HYDROPEROXIDE CLEAVAGE ACTIVITY IN MEMBRANES OF CUCUMBER FRUIT

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Key Word Index—*Cucumis sativus*; Cucurbitaceae; cucumber fruit; lipid hydroperoxide cleavage; subcellular; sucrose gradients; membranes.

Abstract—The subcellular localization of the fatty acid hydroperoxide cleavage enzyme from cucumber fruit has been studied. Activity from the flesh tissue has been located in 3 fractions; plasma and Golgi membranes and endoplasmic reticulum, at equilibrium densities on sucrose gradients of 1.17, 1.15 and 1.12 g/cm^3 respectively. Enzymatic activity and electron microscopy studies were carried out to identify plasma and Golgi membranes. Little activity was associated with microbodies (1.23), plastids (1.21) and mitochondria (1.19 g/cm^3). However, chloroplasts isolated from the peel of the cucumber fruit contained a large amount of hydroperoxide cleavage activity.

INTRODUCTION

Plant tissues contain enzymes which, on physical disruption of the tissue, cause a sequence of lipid-degrading reactions. The subcellular localization of the first two enzymes in this sequence, lipolytic acyl hydrolase and lipoxygenase, were studied in the previous work [1, 2]. Recently, an enzymic sequence has been proposed for the formation of carbonyl fragments from linoleic and linolenic acids in extracts of cucumber fruit [3]. The enzyme responsible for the cleavage of the fatty acid hydroperoxides to form carbonyl fragments, including volatile aldehydes with characteristic cucumber odour, is believed to be third in the sequence of lipid-degrading enzymes. To understand the process of lipid breakdown in disrupted tissue, the subcellular localization of each enzyme is relevant and the present work describe studies on the cleavage enzyme.

RESULTS

Membrane-bound enzyme

Compared with the previous work [1, 2] on lipid-degrading enzymes, the hydroperoxide cleavage enzyme had a very high particulate activity (70–80% of the homogenate activity localised in a 0–150000 *g* pellet). Further evidence for an association between enzyme and membrane came from the effect of Triton X-100, which not only activated the enzyme when used in the assay mixture, but also resulted in its solubilization [3].

Differential centrifugation

Cleavage activity was present in all fractions separated by differential centrifugation (Table 1) and on these results it was impossible to eliminate either plastids (marker enzyme triose phosphate isomerase), present in the 0–4000 *g* fraction, or endoplasmic reticulum (marker enzyme NADH-cytochrome *c* reductase, insensitive to antimycin A) as possible sites for the enzyme.

Linear sucrose density gradients

The resolution of the crude 0–38000*g* pellet was carried out on a linear sucrose gradient. Mitochondria (cytochrome oxidase) equilibrated at a density of 1.19 and endoplasmic reticulum at 1.12 g/cm^3. It should be noted that the medium used lacks Mg^{2+} ions and therefore causes the dissociation of ribosomes from the endoplasmic reticulum [4,5]. The cleavage activity was distributed as a broad peak between 1.18 and 1.12 g/cm^3. Microbodies (catalase) were contaminated with plastids and mito-

Table 1. Localization of enzymes in fractions obtained by differential centrifugation

Enzyme localization	Hydroperoxide cleavage	Catalase	Triose phosphate isomerase	Cytochrome oxidase	ATPase with K^+	IDPase after 3 days	NADH-cytochrome *c* reductase
Fraction	% activity	% activity	% activity	% activity	% activity	% activity	% activity
4000 *g*, 10 min pellet	18	14	8	21	8	9	3
8000 *g*, 20 min pellet	15	11	3	28	13	17	4
38000 *g*, 30 min pellet	31	5	0	12	10	13	19
150000 *g*, 60 min pellet	11	1	0	1	6	18	15

A cucumber homogenate was centrifuged at the stated *g* and the pellet obtained after each centrifugation was resuspended in the extraction buffer. Activity refers to % in initial homogenate.

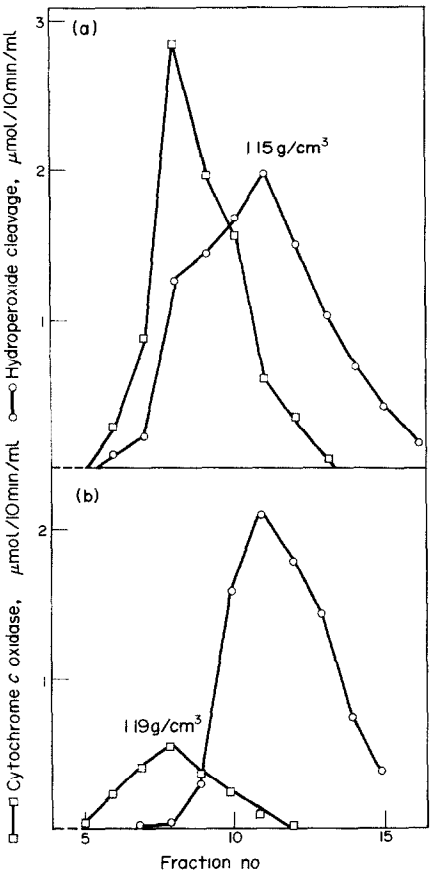

Fig. 1 Distribution of cytochrome *c* oxidase and hydroperoxide cleavage on a linear sucrose gradient of two particulate fractions, isolated from cucumber flesh. The gradient was centrifuged for 5 hr at 75 500 *g*. (a) 0–8000 g for 10 min; (b) 8000–38 000 *g* for 30 min.

chondria and it was necessary to layer an homogenate in order to resolve these fractions. Microbodies equilibrated at 1.23 and plastids at 1.21 g/cm³, both organelles being well separated from the cleavage activity.

In order to resolve whether cleavage activity was coincident with the mitochondria, a 0–8000 *g* fraction and a 8000–38 000 *g* fraction were layered on separate gradients. The results (Fig. 1) showed that both gradients contained cleavage activity with a peak at 1.15 g/cm³, but only in the 0–8000 *g* fraction was there an association between a part of the cleavage activity and mitochondria, which may be due to the large amount of mitochondria present in the fraction, causing other organelles to adhere during the purification.

Identification of membrane fractions with enzyme markers

It therefore appeared that the cleavage activity was localized in fractions lighter than mitochondria, which include plasma membrane vesicles, tonoplast vesicles, Golgi membranes and endoplasmic reticulum. Other workers [6] have found that the effective density of plasma membranes on sucrose gradients overlapped that of the mitochondria and hence it became necessary to remove this organelle before density gradient separation. However, in removing the majority of mitochondria, a

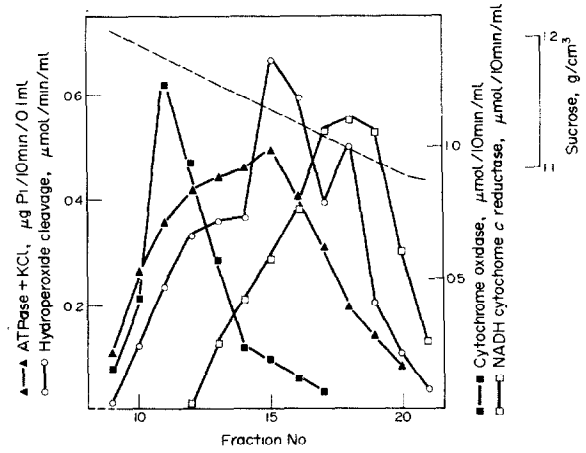

Fig. 2. Distribution of enzymes on a linear sucrose gradient of a 4000–38 000 *g* particulate fraction, isolated from cucumber flesh. The gradient was centrifuged for 5 hr at 75 500 *g*.

sizeable amount of cleavage activity is lost (see Table 1) and a compromise was achieved by layering a 4000–38 000 *g* fraction which contained few plastids and only 60 % of the mitochondria.

NADH-cytochrome c reductase (endoplasmic reticulum)

As illustrated in Fig. 2, cleavage activity gave 3 distinct peaks at 1.17, 1.15 and 1.12 g/cm³, which were separated from the mitochondria at 1.19 g/cm³. The lightest peak was coincident with NADH-cytochrome *c* reductase and further evidence for the association of cleavage enzyme and endoplasmic reticulum was obtained by preparing and centrifuging particles in media containing 1 mM Mg^{2+}. This maintains the binding of the ribosomes and changes the isopycnic density of the endoplasmic reticulum membranes. It was confirmed that the peak of NADH-cytochrome *c* reductase activity changed from 1.12 to 1.15 g/cm³ in the presence of Mg^{2+}, with a corresponding decrease in cleavage activity at 1.12 g/cm³.

K^+-stimulated ATPase (plasma membrane)

ATPase activity at a pH of 6.5 and without any KCl gave a broad spread over the gradient, with peaks around 1.165 and 1.15 g/cm³. On the addition of KCl, the activity increased in all fractions (Fig. 2) with the greatest increase at 1.17 g/cm³ (87 %) and 1.15 g/cm³ (84 %).

Acid photophatase

Other workers [7] have reported activity with *p*-nitrophenyl phosphate as substrate, at pH 5, in the plasma membrane fraction, suggesting the presence of an acid phosphatase. We found only 6 % of the acid phosphatase activity of a homogenate was particulate in a 0–150 000 *g* pellet and 2 % in the fraction 4000–38 000 *g* used for linear gradients. After centrifugation of a gradient, acid phosphatase activity was observed at the top of the gradient where cleavage activity was not present. Two small peaks were also found at 1.16 and 1.14 g/cm³. The amount of activity of acid phosphatase recovered from the gradient is consistent with the knowledge of the fragile nature of intact vacuoles noted in other tissues [1, 2].

Table 2. Fatty acid hydroperoxide cleavage, NADH-cytochrome *c* reductase and cytochrome oxidase activities in fractions collected at the interface of a discontinuous gradient

Gradient interface	Cleavage activity		NADH-cytochrome *c* reductase		Cytochrome oxidase activity	
% sucrose	units*	% in interface	units*	% in interface	units*	% in interface
26/34	0.16	47	0.031	72	0.013	11.5
34/37	0.09	26.5	0.006	14	0.007	6
37/40	0.05	15	0.003	7	0.012	10.5
40/48	0.04	11.5	0.003	7	0.082	72

* Enzyme units refer to μmol substrate reacted/min/10 g fr. wt of tissue.
A 4000–38000 *g* particulate fraction was layered on a discontinuous sucrose gradient and centrifuged for 5 hr at 75500 *g*.

Latent IDPase (Golgi membrane)

Using a 4000–38000 *g* pellet layered on a linear gradient, IDPase activity, assayed after 3 days at +1°, was spread throughout the fractions with a peak at 1.15 g/cm^3. When the individual samples were assayed at day 0 and again after 3 days, an increase was noted over the whole gradient with the largest increase occurring in the denser region (1.18–1.15 g/cm^3).

Discontinuous sucrose gradients

It became apparent that individual samples did not contain sufficient activity to show differences between ATPase with and without KCl, or IDPase at zero and 3 day storage. There was also a need to employ further assays to characterize and identify the membranes and therefore a discontinuous gradient was devised to obtain fractions enriched in various enzymes. The distribution of 3 enzymes on fractions collected at the interfaces of a discontinuous gradient after a 4000–38000 *g* pellet had been layered (Table 2), showed that cleavage activity was found in all 4 fractions with the majority from the 26/34% sucrose interface. This fraction also contained the majority of the NADH-cytochrome *c* reductase activity and was therefore rich in endoplasmic reticulum. The fraction collected at the bottom interface, 40/48% sucrose, was enriched in mitochondria.

Although the majority of the cleavage enzyme appeared to be associated with the endoplasmic reticulum, previous results from the linear gradients and differential centrifugation suggested otherwise. Work with mung beans [8] has also shown that evidence based solely on discontinuous gradients must be treated cautiously. The peak of cleavage activity in the linear gradients appeared at 1.15 g/cm^3 and the results from differential centrifugation experiments showed high cleavage activity in the high density fractions where no endoplasmic reticulum was present. Linear gradient results also showed that the mitochondria were unlikely to be associated with cleavage activity.

It would therefore appear that, with the discontinuous gradient, some Golgi and plasma membranes have been trapped on the first interface and that, although we can obtain fractions enriched in enzymes, at the same time they may well be contaminated by other enzymes.

ATPase

All fractions from the discontinuous gradient contained ATPase activity (Table 3) and all increased in activity in the presence of KCl. Only in the fraction collected at the 37/40% sucrose interface, however, was there a large percentage increase due to K^+ and this fact was established in 6 experiments.

IDPase

Similarly, all fractions contained IDPase activity (Table 3), but only the two low density fractions showed increased activity after 3 days storage and the highest percentage increase always occurred in the fraction from the 34/37% sucrose interface. It has been stated [9] that, in the presence of 0.1% sodium deoxycholate, the total IDPase activity could be measured immediately. In our experiments deoxycholate increased the activity in all fractions by over 100% and there was no apparent relationship between Golgi body membranes and IDPase in the presence of deoxycholate.

Table 3. ATPase, IDPase, glucan synthetase and UDPG : sterol glucosyltransferase activities in fractions collected at the interfaces of a discontinuous gradient

Gradient interface	ATPase activity			IDPase activity			Glucan synthetase activity		UDPG: sterol
% sucrose	no K^+	$+K^+$	% increase	0 day	3 day	% increase	1 μM substrate	1 mM substrate	glucosyltransferase activity
		μg Pi			μg Pi		cpm		cpm
26/34	8.9	11.2	26	1.12	2.6	132	243	735	20400
34/37	6.0	7.7	28	0.43	1.9	340	367	495	20500
37/40	0.29	1.04	260	0.29	0.33	14	155	143	7390
40/48	0.71	0.62	0	0.07	0.07	0	139	289	1390

A 4000–38000 *g* particulate fraction was layered on a discontinuous sucrose gradient and centrifuged 5 hr at 75500 *g*. ATPase and IDPase activities refer to μg Pi released/30 min/10 g fr. wt of tissue. Glucan synthetase activity refers to cpm in polymer/30 min/g fr. wt and UDPG: sterol glucosyltransferase refers to cpm/30 min/g fr. wt of tissue.

Fig. 3. Electron microscopy. (a–f). Membrane fractions collected at the following sucrose interfaces from a discontinuous gradient. (a) and (b) 26/34%; (c) and (d) 34/37%; (e) and (f) 37/40%. Sections (a), (c) and (e) were stained with uranyl acetate. Sections (b), (d) and (f) were stained with PTA/CrO_3, which is selective for plasma membranes. (a) and (b) × 37000; (c)–(f) × 74000.

Glucan synthetase

Activity was maximal in fractions from the 34/37% sucrose interface with low UDP-glucose (1 μM) as substrate (Table 3). When the substrate was increased to 1 mM, the highest activity was found in the endoplasmic reticulum fraction.

UDPG: sterol glucosyltransferase

The fractions containing the endoplasmic reticulum and the Golgi bodies were the most active in the synthesis of sterol glucosides (Table 3).

Use of peel tissue

All the previous work had been carried out on the flesh of cucumbers, but when the peel was homogenized, more cleavage activity was found in the 0–4000 *g* pellet than in the 4000–38000 *g* fraction (Table 4). Inner flesh tissue, as well as the flesh taken adjacent to the peel, both had higher activity in the second fraction. Table 4 also shows that chlorophyll, which is a marker for intact and broken chloroplasts, was high in the peel tissue, but fell rapidly in the flesh adjacent to the peel and in the inner flesh. On the other hand, the value for triose phosphate isomerase (marker for intact plastids only) remained high in the 0–4000 *g* pellets from all 3 tissues. One cannot compare the homogenate figures due to the presence of a non-particulate isoenzyme of triose phosphate isomerase. Thus, the 0–4000 *g* fraction from the flesh contained as much plastid material as the 0–4000 *g* fraction from the peel.

Using the brief centrifugation technique on a linear sucrose gradient, where plastids almost reach their equilibrium density whilst other organelles barely move into the gradient, the majority of cleavage activity (78%) from 0–4000 *g* peel particulate was localized at a density of 1.20 g/cm^3. This peak of activity coincided with chlorophyll and with triose phosphate isomerase and was therefore assumed to be localized in the chloroplasts. When evidence of broken chloroplasts has been observed around 1.18 g/cm^3, by a double chlorophyll peak, cleavage activity

Table 4. Comparison between hydroperoxide cleavage activity, triose phosphate isomerase activity and chlorophyll in fractions obtained by differential centrifugation from the peel, flesh adjacent to the peel and inner flesh of the cucumber.

Enzyme localization		Cleavage activity units*	Triose phosphate isomerase activity units*	Chlorophyll mg/20 g
Peel	Homogenate	2.14	78	1.04
	4000 *g*, 10 min pellet	0.78	2.8	0 5
	38000 *g*, 30 min pellet	0 5	1.0	0 04
Flesh adjacent to peel	Homogenate	3.14	76	0.42
	4000 *g*, 10 min pellet	0.9	2.9	0.24
	38000 *g*, 30 min pellet	1.2	1.2	0 04
Inner flesh	Homogenate	3 4	76	0.25
	4000 *g*, 10 min pellet	0.67	2 2	0.08
	38000 *g*, 30 min pellet	1 18	1.3	0.02

* Enzyme units refer to μmol substrate reacted/2 min/20 g fr. wt of tissue.

Cucumber homogenates were prepared from the three different parts of a cucumber and centrifuged at the stated *g*. Pellets obtained after each centrifugation were washed and resuspended in the extraction buffer

has also been associated with them. On the other hand, the 0–4000 *g* fractions from the other 2 samples had the majority of their cleavage activity located at the top of the gradient after a brief centrifugation; 88% from the flesh adjacent to the peel and 90% from the inner flesh. This activity, as we have earlier described, was located in the membranes.

Properties of the hydroperoxide cleavage system from peel

Previous work on the enzymic pathway for the formation of *cis*-3-nonenal, *trans*-2-nonenal and hexanal from linoleic acid hydroperoxide isomers had been carried out on flesh tissue [3]. It was therefore important to ensure in the present work that the assay used to measure cleavage activity (disappearance of the hydroxydiene chromophore at 234 nm) using a particulate preparation from the peel, was valid, because other enzymic transformations of fatty acid hydroperoxidases are known [10].

Similar results of heat inactivation were found using peel as reported previously from the flesh tissue [3]. Heating the 0–4000 *g* and the 4000–38000 *g* pellet fractions at 70° for 2 min resulted in a total loss of activity and 50% of the activity was lost at 50° for 10 min. A comparison was made between the percentage conversion, in a 10 min incubation period, of the disappearance of hydroperoxide chromophore at 234 nm and the major volatile carbonyl compounds formed. Thus, with the 9-hydroperoxide of linoleic acid under the conditions used, there was a 22.5% loss of the 234 nm chromophore with a concomitant production of *cis*-3-nonenal, equivalent to cleavage of 25% of the hydroperoxide substrate. When 13-hydroperoxide was used as substrate, 30% disappeared in 10 min, with a cleavage of 40% of the substrate, measured as hexanal produced. Thus, we assumed that the chromophore activity was indeed due to the same hydroperoxide cleavage system as that in the flesh.

Electron microscopy

Membrane material sedimenting at the interfaces after discontinuous gradient centrifugation was negatively stained using uranyl acetate. Little information was gained from the electron micrographs after negative staining, save that the fraction from the 26/34% sucrose interface was different in appearance from those at the 34/37% and 37/40% interfaces. It was also noticeable that the vesicles from the latter fraction were larger than from the 34/37% interface. However, electron microscopy of fixed stained sections from the sucrose interfaces was more informative and showed the 3 fractions mentioned above to be in the form of membrane vesicles, devoid of other cellular contaminants (Fig. 3) with the dense fraction from the 37/40% interface containing a few flattened membranes. The fraction collected at the 40/48% interface was mainly composed of mitochondria. It has been observed [11] that the size of vesicles increased with increasing density and this was confirmed. The same workers were critical of the phosphotungstic acid–chromic acid (PTA/CrO_3) stain, whereas others [12] showed clear evidence that the plasma membranes were selectively stained. The micrographs in Fig. 3 demonstrate clearly that the endoplasmic reticulum does not stain with PTA/CrO_3, whereas some membranes in the 37/40% interface stain heavily. Other membranes present in the same fraction that do not stain so readily may be from the tonoplast. The results of the fraction from the 34/37% interface are not clear cut. They do not stain as heavily as the plasma membranes but they have stained to some degree.

Cassagne *et al.* [13] reported that each type of membrane had a constant thickness and that it was easy to see these variations in the membrane vesicles of the pellet. We observed variations of thickness between the endoplasmic reticulum (6 nm) and the Golgi and plasma membranes (8 nm) but could not identify tonoplast membranes by this method. Staining by PTA/CrO_3 appeared to increase the size of the plasma membranes.

DISCUSSION

Hydroperoxide cleavage activity, from the cucumber flesh, has been located in 3 separate membranes, plasma Golgi and endoplasmic reticulum. They have been separated by linear and discontinuous sucrose gradients and identified by marker enzymes and electron microscopy.

However, the present work has illustrated the problems of positive identification of subcellular membranes in non-meristematic tissue.

Recent work has been published on the isolation and identification of plasma membrane fractions in higher plant tissues which include corn roots [6], kidney bean abscission zone [12] and leek epidermal cells [13]. These are meristematic tissues and plasma membranes were identified in these tissues at an isopycnic density around 1.16–1.17 g/cm^3, by the use of a marker enzyme, K^+-stimulated ATPase, in the presence of Mg^{2+}, at a pH of 6.5. The association between ATPase (K^+-stimulated) and the plasma membrane has been based largely on the use of PTA/CrO_3 stain. Work on ATPase activity in a membrane fraction from soybean callus and root cells [14] located the plasma membranes between 1.13 1.155 g/cm^3 density, but other research [11], using shoots from *Suaeda maritima*, found the plasma membranes not easily identifiable.

IDPase activity is considered to be associated with Golgi body membranes and is latent (i.e. activity increases with storage at +1°). Work with pea seedlings [5, 15, 16] showed a single peak of IDPase activity at 1.15 g/cm^3, whereas in soybean root and suspension cultures [14, 17] peak activity occurred at a lower density of 1.12 g/cm^3. Other workers on mung bean root tissue, onion stem and corn and oat roots [8, 9, 6, 18] found two IDPase peaks, one around 1.09–1.12 g/cm^3 and the other at 1.15 g/cm^3. IDPase has also been reported in the plasma membrane fraction at 1.65 g/cm^3 and was not latent [12] and in all gradient fractions from the shoots of *S. maritima* [11] with the highest activity at 1.15 g/cm^3. This was the only fraction to increase on storage.

Cytochemical studies showed ATPase activity associated with a number of membranes in *S. maritima* [19], especially plasma membrane and tonoplast. IDPase activity was also located in both these membranes by the same workers and in plasma, Golgi and endoplasmic reticulum from onion root tips [20].

From our results on the activity of ATPase and IDPase we would agree that these enzymes could well be present in a number of membranes. However, it appears that a high percentage increase of (a) IDPase activity during storage and (b) ATPase activity due to K^+, is a valid test for the presence of Golgi and plasma membranes respectively, especially when backed up by other enzyme tests and EM evidence.

Glucan synthetase, at the low substrate level, is reported to be a marker for Golgi body membranes [5, 14–16, 21] and our results are in agreement with this. At the high level of substrate the enzyme is reported to be associated with the plasma membrane [8, 14, 21] and the synthesis of glucan increases markedly when the substrate concentration is raised. Our results, however, showed that the incorporation of radioactivity was only slightly enhanced at the 1 mM substrate level and the most active of the 4 fractions were the endoplasmic reticulum and Golgi body membranes.

UDPG : sterol glucosyltransferase has been described as a valuable marker enzyme for Golgi body membranes [22], but other workers have associated it with plasma membranes [23], chloroplasts, mitochondria [24] and microsomes [25]. Our results showed most of the activity was associated with the Golgi body membranes and endoplasmic reticulum.

The material used in this work contains large vacuoles and it is possible that cleavage activity is also localized in the tonoplast. ATPase activity is thought to be associated with the vacuole membrane and tonoplast vesicles will equilibrate in the sucrose gradients at densities very near to that of the plasma membrane [6]. The K^+-stimulated ATPase activity located at 1.15 g/cm^3, in the same peak as the Golgi membranes, could well be present in tonoplast vesicles. The membrane fraction rich in plasma membranes contained large vesicles which did not stain heavily with PTA/CrO_3. These may well be from the tonoplast which, on the same electron micrograph evidence, could also be present in the Golgi-rich fraction. We cannot be positive until a specific marker is developed.

The cleavage activity found in the peel of cucumbers is localized in a different organelle from that observed from the flesh tissue. A 0–4000 *g* pellet prepared from peel has the majority of enzyme associated with the chloroplasts, whereas the plastids, present in the same fraction from the flesh, contain very little activity.

Other workers [26] have described an enzyme, hydroperoxide lyase from watermelon seedlings, which catalyzes the conversion of 13 hydroperoxide to 12-oxo-*trans*-10-deodecenoic acid and hexanal. On the basis of recent results [27] we believe that the cleavage enzyme (lyase) from water melon is the same as that from cucumber described in this paper.

EXPERIMENTAL

Plant materials. Cucumber fruits (*Cucumis sativus*), cv Fem Dam, were grown at the Institute, or purchased locally and used when mature.

Tissue extraction. The grinding medium contained 0.25 M sucrose, 0.1 M HEPES buffer, pH 7.6, 0.5% BSA and 1 mM EDTA. 1 mM $MgCl_2$ was added to the extraction and suspension buffers when Mg^{2+} ions were used in gradients. With the full medium, activity was maintained for several days. Lack of EDTA made a slight difference but, without BSA in the extraction buffer, 50% activity was lost in 24 hr. Pieces from the flesh (peel and seeds removed) of cucumber fruit were cut into small cubes and homogenized in a blender for two 5 sec bursts. Peel was treated in a similar manner, but was shredded with a razor prior to homogenization. All operations were carried out at 0–2°. The homogenate was then filtered through several layers of muslin and centrifuged at the stated *g*. The crude particulate pellet was resuspended in a few ml of the original medium, but not containing BSA. BSA was added to an aliquot for the glucan synthetase assay. Particulates were used directly or layered on to linear or discontinuous sucrose gradients.

Density gradients. These were w/w sucrose solns made up to 100% with 0.1 M HEPES buffer, pH 7.6 and 1 mM EDTA. Linear gradients, for the study of flesh tissue, were composed of 3 ml of a 56% sucrose soln, followed by 40 ml of a linear gradient from 56–16%, with a final 5 ml of 16%. Discontinuous gradients were composed of 5 ml of 48%, followed by 9 ml 40%, 10 ml of 37% and 12 ml each of 34 and 26%. The particulate fraction (3–4 ml) was layered on the top of these gradients. Linear gradients for brief centrifugations were formed from 42 ml of 34–60% sucrose and 5 ml of 34%. 1 mM $MgCl_2$ was added where appropriate. All linear gradients were prepared 24 hr in advance to allow for equilibration. The tubes were then placed in a SW 25-2 rotor in a Beckman Spinco L2 ultracentrifuge and centrifuged at 1° for 5 hr at 25000 rpm (75500 *g*), or 15 min at 15000 rpm (27000 *g*). Other details were as previously described [1]. With discontinuous gradients, where the bands of material were clearly visible, the entire band was collected. This fraction was then concd by initially diluting with buffer, followed by centrifuging at 50000 *g* for 30 min. The pellet was resuspended as previously described.

Enzyme assays. Catalase, triose-phosphate isomerase, phosphatase, cytochrome *c* oxidase, NADH-cytochrome *c* reductase (in the presence of antimycin A) and chlorophyll were assayed as described previously [1, 2]. Latent IDPase was assayed initially and after 3 days storage at 0–2°, by the method of ref. [15]. ATPase was assayed at 37° with and without K^+ ions. The reaction contained 3 mM ATP, 0.1 M HEPES buffer, pH 6.5, 3 mM $MgSO_4$ and 50 mM KCl where appropriate. With both ATPase and IDPase the final vol. was 1.3 ml, excluding 0.2 ml of 30% TCA used to stop the reaction after 30 min Inorganic P_i was measured from a 0.5 ml sample, containing less than 1 μmol of ATP or IDP, by the method of ref. [28] and activities expressed, after the removal of enzyme blanks, as μg of phosphate. Hydroperoxide cleavage was assayed by the disappearance of 9 or 13-hydroperoxy-octadecadienoic acids in a recording spectrophotometer at 234 nm [3]. The 13 hydroperoxide was prepared from linoleic acid by the method of ref. [29] using sodium borate buffer, pH 10.5 at 0°. The reaction mixture for assaying cleavage activity contained 33 μg of substrate in 0.1 M Pi buffer, pH 6.5, in a total vol. of 3 ml. UDPG : sterol glucosyltransferase assay was a modification of the method of ref. [22]. The reaction mixture contained 0.3 ml of 0.1 M Tris–HCl buffer, pH 7.5, 0.1 ml of 0.5 mM DTE, 0.1 ml of 0.5 mM β sitosterol, 4 μl of UDP [U-^{14}C] glucose ammonium salt (306 mCi/mmol) and 0.1 ml of enzyme prepn. The sterol was prepared as an emulsion (0.5 mM sterol, 1 mM lecithin and 2 mg/ml Triton X 100) after sonication. The reaction was run for 15 min at 30°, subsequent extraction of the incorporated labelled glucose was by the method of ref. [30]. The $CHCl_3$ layer (1 ml) was evapd to dryness and 10 ml of scintillation fluid added, 0.4% 2,5-diphenyloxazole (PPO) in toluene, Triton X-100 and H_2O (6:3:1) and the radioactivity counted. Glucan synthetase was assayed in the presence of high and low substrate concns, in a total vol. of 0.1 ml. The reaction contained 10 μl of UDP glucose at 10 mM or 10 μM, 2 μl UDP [U-^{14}C] glucose, 20 μl of 0.25 M Tris/acetate pH 8, containing 0.1 M $MgCl_2$, 20 μl of 0.1 M cellobiose and 50 μl of particulate enzyme fraction. The reaction was carried out at 25° for 45 min and was stopped by addition of 1 ml of 75% EtOH. The insoluble products containing the labelled glucose were extracted by filtering under vacuum through a millipore membrane filter (Type HA 0.45 μm). The membrane was thoroughly washed with 10 ml of 75% EtOH, dried and dissolved in 1 ml of 2-methoxyethanol. A further 9 ml of scintillation fluid (6:3, toluene : Triton X-100) was added and the radioactivity counted as previously described. Volatile carbonyl products were analysed as described previously [3]. 25 g skin or flesh were extracted with 20 ml (skin), 12 ml (flesh) of 0.05 M Tris–HCl buffer, pH 7, 2 mM DTT, 1 mM EDTA and 0.1% Triton X-100. The incubation mixture contained 50 μg hydroperoxide in 5 ml buffer + 200 μl enzyme for 10 min.

Electron microscopy. Samples from the discontinuous gradient were treated as in ref. [1] The PTA/CrO_3 stain for plasma membranes were prepared by the method of ref [31]. Sections were cut with a diamond knife and stained by the PTA/CrO_3 or uranyl/lead acetate method. Negative stains were prepared by placing fractions from the discontinuous density gradient on carbon coated copper grids after fixation with glutaraldehyde, removal of sucrose by the method of ref. [32] and then staining with uranyl acetate

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