Phytochemistry, 1980, Vol. 19, pp. 1013–1016 © Pergamon Press Ltd. Printed in England. 0031-9422/80/0601-1013 $02.00/0

LIPOXYGENASE FROM CUCUMBER FRUIT: LOCALIZATION AND PROPERTIES

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(*Revised received* 4 *October* 1979)

Key Word Index—*Cucumis sativus*; Cucurbitaceae; cucumber fruit; lipoxygenase; subcellular; sucrose and Ficoll gradients; protoplasts; vacuoles.

Abstract—The subcellular localization of lipoxygenase (LOX) from cucumber fruit has been studied. Two methods have been employed to obtain organelles; (1) maceration of the tissue, followed by separation on a linear sucrose gradient and (2) release from protoplasts by osmotic shock, followed by a discontinuous Ficoll gradient. It was possible to obtain high LOX activity in the intact protoplasts from both peel and flesh tissue. However, fewer intact vacuoles were obtained following osmotic rupture than from macerated tissue. Both methods produced more particulate LOX activity from the peel than from flesh tissue, and both showed that this activity was associated with the vacuoles. The cucumber LOX enzyme was similar to the potato and tomato enzymes, both in pH characteristics and substrate specificity.

INTRODUCTION

A previous paper [1] has described a sequence of lipid-degrading reactions initiated by lipolytic and lipid oxidising enzymes present in cucumbers. These enzymes act very quickly in disrupted tissue to produce the recognized cucumber flavour constituents. The subcellular localization of the enzyme responsible for the cleavage of the fatty-acid hydroperoxides to form carbonyl fragments was studied in an earlier paper [2]. The present paper describes the localization of lipoxygenase (LOX), which converts the unsaturated fatty acids, linoleic and linolenic, to their hydroperoxide derivatives. This enzyme has been ascribed by different workers to a wide range of subcellular fractions in other tissues [3, 4].

Isolation of vacuoles, chloroplasts and other cell organelles from protoplasts has recently been reported by a number of workers. Wagner and Siegelman [5] have described a technique for the isolation of vacuoles and chloroplasts from protoplasts, and Saunders and Conn [6] have combined a short incubation period for the isolation of the protoplasts with a Ficoll gradient to collect intact vacuoles. Isolated protoplasts have also been prepared from cucumber tissue by slicing placenta of fruit in a hypertonic solution [7]. Work on cucumber and potato LOX described in this and a previous paper [8] concerns, in part, the differences between macerating the tissue or using cell degrading enzymes as initial steps in the localization of the enzyme.

RESULTS

Localization

LOX activity in cucumber tissue was consistently low compared to the potato tuber or cauliflower floret, with the peel containing twice as much activity compared to the flesh. It was also observed that fruit imported during the Winter had lower activity than the home crop. Variations in the extraction and resuspension buffers were attempted in order to retain activity for at least 24 hr. Additions to the standard sucrose, bovine serum albumin, HEPES buffer were 2 mM dithiothreitol (DTT), 3 mM Mg^{2+} or 1 mM EDTA. With the standard buffer or with the addition of EDTA alone, only 10% of the activity of a homogenate remained after 24 hr. Combinations of the 3 additives produced a loss of between 30 and 40%, but all activity was retained with the addition of DTT alone. When 0–38 000 g pellets were resuspended in the same buffer as the original extraction medium, all the particulates suspended in buffers containing Mg^{2+} had lower activities than controls in which Mg^{2+} was absent. They also lost further activity over the next 24 hr. Again, the addition of DTT alone gave a particulate preparation which retained LOX activity on storage.

When peel was used in the blender for the normal extraction period, only part of the tissue was disintegrated. Longer extraction periods produced a higher homogenate activity but gave less particulate—13% against 23% for the short extraction.

Differential centrifugation showed the particulate LOX activity to be present mainly in a fraction collected between 4000 and 38 000 g (Table 1), whereas most of the plastids were collected in the fraction sedimenting between 0 and 4000 g. About 20% of the total LOX activity was recovered in pellet form. The relationship between LOX and plastids was exploited in several sucrose density experiments. An initial slow-speed centrifugation step (500 g) on homogenates prepared from peel or flesh was used to remove 2% of the microbodies, 5% mitochondria, 4% LOX and 75% of the chloroplasts.

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

Enzyme localization Fraction	Lipoxygenase % activity	Catalase % activity	Cytochrome oxidase % activity	Triose-phosphate isomerase % activity	Chlorophyll % activity
4000 g 10 min pellet	3	16	11	4	75
38 000 g 30 min pellet	20	15	28	2	5

A cucumber peel 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.

The resolution of a homogenate and a 4000–38 000 g pellet, isolated from peel, was carried out on linear sucrose gradients. The results from a homogenate are shown in Fig. 1, where mitochondria (marker enzyme cytochrome oxidase) equilibrated at a density of 1.19, microbodies (catalase) at 1.23 and chloroplasts (chlorophyll) at 1.21 and 1.18 g/cm^3, respectively. These are in agreement with earlier results on cucumber flesh [2]. LOX activity and acid phosphatase activity were observed in two separate peaks at 1.19 and 1.17 g/cm^3, which are similar to the two peaks obtained from LOX in pea roots [3]. Using the brief centrifugation technique with peel homogenate layered on a linear gradient, two peaks of chlorophyll were observed at 1.21 and 1.18 g/cm^3. The intact chloroplasts (triose-phosphate isomerase) contained 0.56 mg chlorophyll per 10 g fr. wt and the broken 0.23 mg. The mitochondria reached a density of 1.16 g/cm^3 whereas the microbodies and LOX had not moved into the gradient.

Protoplasts

Isolated protoplasts have a spherical outline with a central vacuole. When 0.2 M K_2HPO_4–HCl (pH 8) was added or the mannitol concentration was lowered, the plasma membrane ruptured to release a single vacuole. Protoplasts, which ranged in size from 20 to 40 μm and vacuoles, *ca* 40–100 μm, both stained with neutral red as the dye accumulated within the vacuoles.

A comparison was made between the LOX activity in particulate fractions sedimented from homogenates and from protoplasts isolated by the use of cell degrading enzymes. The results (Table 2) showed that more LOX activity was obtained in a degraded extract than in a homogenate from flesh tissue, but less activity was found from degraded peel than after it had been macerated. After centrifugation of the homogenate at 38 000 g, the LOX activity in the particulate fraction from both peel and flesh tissue was *ca* 20% of the homogenate activity, whereas 45% LOX activity was localized in the protoplast fractions isolated from the two degraded extracts. During the first 30 min of incubation, a leakage of soluble enzyme occurred from the damaged cells. Cucumber slices were therefore left in 0.6 M mannitol for a short period before being transferred to the degrading fluid. It was also possible to incubate flesh tissue for only 5 hr and obtain 0.92 units (μmol/min/10 g fr. wt) total activity of which 45% was present in the protoplasts. The addition of 0.5% BSA to the incubation medium did not decrease the activity in the soluble fraction.

In the above experiments, the protoplasts were lysed by the addition of 0.2 M K_2HPO_4–HCl pH 8 [5] and the LOX activity in this suspension was generally higher than from the intact protoplasts. In a typical experiment with flesh tissue, the LOX activity in the protoplast fraction was 0.82 units, acid phosphatase activity 0.9 units (μmol/min/10 g fr. wt) and acyl hydrolase activity 0.06 units (μmol/min/10 g fr. wt).

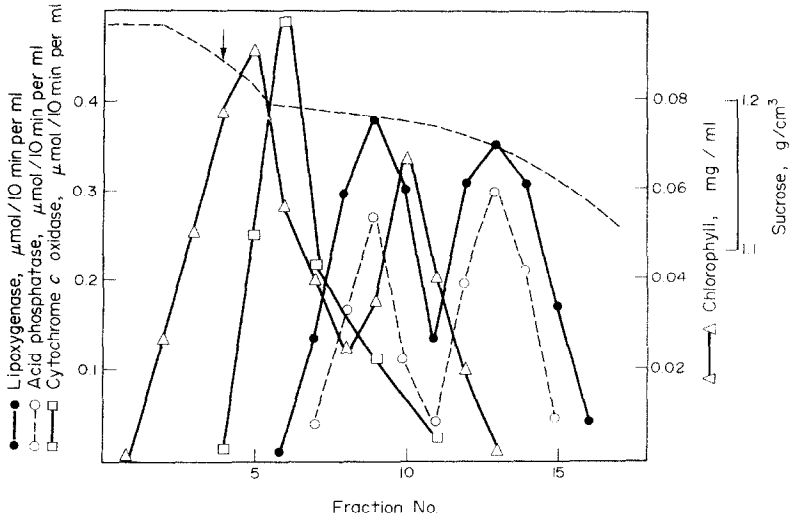

Fig. 1. Distribution of enzymes on a linear sucrose gradient of a homogenate fraction from cucumber peel, after an initial centrifugation for 5 min at 500 g. The gradients were centrifuged for 4 hr at 75 500 g. ↓ denotes catalase peak.

Table 2. Comparison between lipoxygenase activity in homogenates and in degraded extracts from peel and flesh of cucumbers

Enzyme localization	LOX activity			
	Homogenate units*	Particulate %	Degraded extract units*	Protoplasts %
Peel	2.08	22	1.46	44
Flesh	1.06	20	1.44	45

* Enzyme units refer to μmol O_2/min/10 g fr. wt of tissue.

Cucumber homogenates were prepared from peel or flesh of a cucumber and centrifuged at 38 000 g for 20 min. Degraded peel and flesh extracts were used after 17 hr, after separation of undigested material and protoplasts harvested by centrifugation at 2000 g for 3 min.

After the addition of phosphate, the LOX activity was 1.1 units, of which 12% could be sedimented at 2000 g in 5 min. The remaining activity was soluble due to the release of LOX enzymes from burst vacuoles or present in the cytoplasm. This procedure resulted in the sedimentation of vacuoles and chloroplasts and a recovery of *ca* 8% of the original LOX activity in the extract. This was lower than the percentage of particulate activity obtained when the tissue was macerated.

Difficulty was experienced in the separation of chloroplasts and vacuoles in the 10% sucrose medium. The former were easily centrifuged but contamination with vacuoles could not be avoided, even with 0.5% BSA in the solution. Other workers [6] have successfully used a Ficoll gradient for separation and this was attempted. In order to overcome the large volume of lysed protoplasts and subsequent centrifugation step of the previous method, the concentration of mannitol was lowered to 0.15 M and a 10 ml sample layered directly on a discontinuous Ficoll gradient. After centrifugation, the LOX activity was found in equal concentrations at the 3/9% interface and the 9/12.5% interface. Examination of these bands by light microscopy showed that the former contained mainly vacuoles, and the latter a mixture of vacuoles and protoplasts. The pellet at the bottom of the tube contained cell debris and chloroplasts.

Properties

Enzyme concentration. Initial attempts at fractionation using $(NH_4)_2SO_4$ were abandoned due to gelling of the precipitated protein. This could be avoided by extracting in 0.5 M Pi buffer (pH 7) but a large loss of activity occurred. Concentration was more successful by the use of a membrane filter (Amicon PM 30). A 10-fold reduction in volume was accompanied by an 8-fold increase in enzyme activity.

Substrate specificity. The most active substrates were linoleic acid (100%), linolenic acid (77%), and arachidonic acid (23%). With monolinoleoyl-glycerol as substrate the activity was only 20% of the free linoleic acid. In the cucumber, linoleic acid is cleaved from the glyceride by the enzyme lipolytic acyl hydrolyase [9]. No activity was observed with oleic acid.

Kinetics. As demonstrated by its cloudy appearance, ammonium linoleate exists in the form of large micelles at pH 5.5. The addition of Triton X-100 (0.25%) cleared the solution and doubled the rate of activity. However, at higher Triton levels (0.5%) the rate returned to the original value. Lineweaver–Burk plots were linear and suggested that at the higher level of Triton there was non-competitive inhibition. The apparent K_m value obtained for linoleic acid was 1 mM, but as this depends on pH and critical micelle concentration it probably has little physiological significance.

Optimum pH and temperature. The optimum pH was *ca* 5.5 at a substrate concentration of 1 mM, with moderate activity at 3.5 and no activity at 9. This optimum value was in agreement with those reported for the potato and tomato enzymes. The pH characteristics of the LOX enzyme can be altered by detergents [10]. The potato enzyme in particular [11] showed a broader pH response curve in the presence of Triton X-100. Cucumber LOX showed no change in its pH characteristics when Triton was added, except for a general increase in activity. The optimum temperature for the cucumber LOX was 40°. The enzyme lost half its activity at 50° in 5 min and all its activity at 70° in 2 min and was much less stable than the tomato [12].

Inhibitors. Enzyme activity of the crude extract was not inhibited by calcium, EDTA, dithiothreitol, imidiazole, cysteine, or KCN at 1 mM concentration and was also unaffected by 0.2 mM *p*-chloromercuribenzoic acid. The results of these studies showed that the extract used did not contain an alpha oxidation system, reported to be present in cucumber tissue [13], or a haemprotein [14].

Stability. When the enzyme was extracted in HEPES buffer alone, 85% of the activity was lost in 30 hr. The addition to the supernatant of 2.5 mM DTT or 1 mM EDTA, appeared to have a protecting action (25 and 40% loss, respectively, in 30 hr). Bonnet and Crouzet [12] reported that 1% EDTA was very effective in protecting tomato LOX, but we found 1 mM EDTA to be sufficient. In the extraction of particulate fractions the presence of bovine serum albumin (BSA) was essential to prevent damage to membranes by fatty acids. The addition of 0.1% BSA to the buffer used for these experiments made no difference in the stability of the soluble enzyme.

DISCUSSION

Work on the localization of fatty acid hydroperoxide cleavage activity [2] showed the advantages of using

cucumber fruit for extraction and location of organelles. In the present work it has been possible to obtain a high recovery of intact protoplasts from both peel and flesh tissue. However, following osmotic rupture the particulate LOX activity was less than that recovered by the usual maceration method. This latter extraction method, followed by sucrose density gradients, localized the LOX activity in two separate peaks, coincident with acid phosphatase and at the same densities as previously observed with pea root LOX [3]. The experiments with isolated protoplasts also indicate that the enzyme activity is located in the vacuoles. It has been reported [15] that several acid hydrolases were present only in the soluble cytoplasmic fraction prepared from *Hippeastrum* flower petal protoplasts, but acid phosphatase, RNase and DNase were also present in the vacuole lysate fraction. Hydrolytic enzymes have also been located in isolated vacuoles from castor bean endosperm [16].

EXPERIMENTAL

Plant material. Cucumber fruits (*Cucumis sativus*) were purchased locally and their origin varied throughout the year.

Tissue extraction. The buffer used in the localization work contained 0.25 M sucrose, 0.1 M HEPES buffer, pH 7.5, 0.5% BSA and 2 mM DTT. Pieces from the flesh (peel and seed removed) of mature cucumber fruit were cut into cubes and homogenized in a blender for 2×5 sec bursts. Peel was shredded with razor before similar treatment. Homogenates were filtered through muslin and centrifuged at the stated *g*. The crude pellet was resuspended in a few ml of the original buffer, minus BSA. Particulates were used directly or layered on to sucrose density gradients. The buffer used for the enzyme properties contained 0.2 M HEPES buffer, pH 7.5, 2 mM DTT, 2 mM EDTA and 0.1% Triton X-100. Cucumber flesh was homogenized for 15 sec. Peel was also used in the substrate and pH characteristic expts. The homogenate was filtered, centrifuged at 30 000 *g* for 20 min and the supernatant used as the enzyme source.

Density gradients. These were sucrose solns made up to 100% with 0.1 M HEPES buffer (pH 7.5). Linear gradients, for the study of homogenates, were composed of 2 ml of a 55% sucrose soln, followed by 8 ml of a linear gradient from 55 to 40%, 6 ml of 40% and a further 9 ml of a linear gradient from 40 to 24%. A homogenate (30 ml) was layered on top. Gradients for the study of particulate fractions (3–4 ml) were 3 ml of a 56% sucrose soln, followed by 40 ml of a linear gradient from 56 to 16%, with a final 5 ml of 16%. The tubes were than placed in a SW 25-2 rotor in a Beckman L2 ultracentrifuge and centrifuged at 1° for 4 hr at 25 000 rpm (75 500 *g*) or 3 min at 5000 rpm followed by 10 min at 11 500 rpm (16 000 *g*). Other details as described previously [3].

Isolation of protoplasts. Thin slices of flesh or peel (30 g) were incubated for 5 and 17 hr, respectively, in 100 ml 0.5 M mannitol, 25 mM K-Pi–citrate buffer (pH 5.5) containing 15 mg/ml cellulysin (Calbiochem) and 5 mg/ml macerase (Calbiochem), at 20°. The digested material was filtered through muslin and rinsed with a small quantity of 0.5 M mannitol in buffer. The protoplasts were harvested by centrifuging for 3 min at 2000 *g*. Vacuoles were released either by ref. [5] or [6]. Gradients for the study of isolated vacuoles were composed of Ficoll soln made up to 100% with 25 mM Tris–HCl buffer (pH 8) and 0.5 M mannitol. Discontinuous gradients were composed of 10 ml fractions of 3, 9, 12.5 and 20% Ficoll and 10 ml lysed protoplasts were layered on the top. The gradient was centrifuged for 2 hr at 25 000 rpm.

Enzyme assays. Catalase, triose-phosphate isomerase, cytochrome *c* oxidase, acid phosphatase, lipolytic acylhydrolase and chlorophyll were assayed as described previously [3, 4]. Linoleic acid (containing <0.5% of conjugated diene inpurities) was converted to its NH_4^+ salt in the presence of Triton X-100 (0.25%) and used as substrate with acetate buffer, pH 5.5, in the polarographic method for LOX determination. Three enzyme concns were used to obtain a linear relationship between O_2 uptake and enzyme concn. One unit of enzyme activity catalysed the consumption of 1 μmol/O_2/min at 25°, assuming an intial dissolved O_2 concn of 0.24 mmol/l. Use of the zero suppression facility on the potentiometric recorder was made in order to obtain a 5× increase of the rate. All buffers were filtered, under vacuum, through a membrane filter (cellulose acetate Grade 0.45 μm) to remove microorganisms and obtain stable base lines. Controls were run in which the substrate or the enzyme were omitted.

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