Separation, Partial Purification and Characterization of a Fatty Acid Hydroperoxide Cleaving Enzyme from Apple and Tomato Fruits

P. Schreier* and G. Lorenz

Institut für Lebensmitteltechnologie und Analytische Chemie, Technische Universität München, D-8050 Freising-Weihenstephan

Z. Naturforsch. **37 c**, 165–173 (1982), received August 18/November 24, 1981

Apples, Tomatoes, Fatty Acid Hydroperoxides, Cleavage Enzyme, C_6-Aldehydes

A membrane-bound enzyme catalysing the cleavage of 13-hydroperoxy-(*Z*)-9,(*E*)-11-octadecadienoic acid (13-LHPO) and 13-hydroperoxy-(*Z*)-9,(*E*)-11,(*Z*)-15-octadecadienoic acid (13-LnHPO) to C_6-aldehydes was isolated and partially purified from apples and tomatoes. Attempts to employ Ultrogel AcA 34 and AcA 22 in a gel chromatographic purification step were partially frustrated by reaggregation phenomena. However, by using Sepharose CL-4 B an enzyme fraction (MW 200 000 Da) with lipoxygenase and fatty acid hydroperoxide cleaving activity could be separated from a high molecular-weight active eluate. By applying preparative isoelectric focussing to the tomato protein we succeeded in separating the fatty acid cleaving activity from the lipoxygenase, because of their different isoelectric points of pH 5.8–6.1 and pH 5.0, respectively, An 8.4-fold purification of the fatty acid cleaving activity was achieved. A pH-optimum of 5.5 and a K_m-value of 2.6×10^{-5} M/l for the 13-hydroperoxide of linoleic acid were measured. *p*-Chloromercuribenzoic acid (1 mM) showed significant inhibitory effect on the fatty acid hydroperoxide cleaving enzyme, but no evidence of inhibition was found with 1 mM H_2O_2, KCN, DABCO and EDTA or superoxide dismutase (270 U). The maximum amount of fatty acid hydroperoxide decomposition (C_6-aldehyde formation) was determined to be 59%.

Introduction

On disruption of plant tissue, *e.g.* homogenization, large amounts of hexanal, (*Z*)-3-hexenal and (*E*)-2-hexenal are produced [1–3]. These volatile aldehydes occur only at trace levels in intact plant cells, but are the major contributors to the "green" flavour of fruit products [4, 5]. The C_6-components are formed from unsaturated C_{18}-fatty acids after the destruction of the cellular structure and exposure of the cell contents to oxygen [1–3, 6].

Different theories have been published about the reaction mechanism of the C_6-aldehyde formation in disrupted plant tissue. They incorporate nonenzymatic [7–9] as well as enzymatic pathways [1, 2, 10–13]. Some of the authors who discuss the enzymatic reactions suggest that the unsaturated fatty acids undergo a direct fragmentation into C_6-aldehydes without formation of any intermediate compounds [14, 15]. Many authors, however, propose that the fatty acid hydroperoxides (9- and 13-hydroperoxide of linoleic and linolenic acid, respectively [16]) are first produced by lipoxygenase activity, and that these subsequently decompose into the C_6-aldehydes [1–3, 6, 11, 17].

In order to characterize the reaction mechanism of the C_6-aldehyde production in disrupted plant tissue we studied the enzyme system responsible for C_6-aldehyde formation in apples and tomatoes. In both species the high activity of a C_6-aldehyde producing system is well known [1, 2, 6, 11, 18–20].

Materials and Methods

Fruits. Tomatoes (*Hildares, Curabel* and some unspecified cultivars) and apples (*Roter Boskoop*) were kindly provided by Lehrstuhl für Gemüsebau, TU München and Institut für Obstbau und Baumschulwesen, FH, Freising-Weihenstephan.

Substrate preparation. The 9-hydroperoxides of linoleic and linolenic acid (9-LHPO and -LnHPO) were prepared using tomato lipoxygenase [21]; the 13-hydroperoxides of linoleic and linolenic acid (13-LHPO and -LnHPO) were produced with soybean lipoxygenase as previously described [22], but the

* Present address: Lehrstuhl für Lebensmittelchemie, Universität Würzburg, Am Hubland, D-8700 Würzburg.

A part of the results of this work has been presented as poster at the 3rd Weurman Symposium, FLAVOUR '81, Munich, April 1981.

Reprint requests to Prof. Dr. P. Schreier.

0341-0382/82/0300-0165 $ 01.30/0

preparation was run at ±0 °C to produce nearly exclusively the 13-isomer [23]. The yields of fatty acid hydroperoxides were calculated assuming $\varepsilon = 25\,600\ l \cdot mol^{-1} \cdot cm^{-1}$ (234 nm) [24]. For enzymatic assays (see below) ethanol was removed with a rotary vacuum evaporator, and the fatty acid hydroperoxides were emulsified in a 0.25% Tween 20 solution. The isomeric purity of the substrate after thin layer chromatography was checked by HPLC [25]*.

Enzyme preparation. The fruits were homogenized for 2 min in a Braun mixer (KM 32) with 2 vols of 0.4 M McIlvain buffer (pH 5.5) containing 0.2% Triton X-100 (buffer A) or with 2 vols of an extraction medium (B) consisting of 0.4 M McIlvain buffer (pH 5.5), 2 mM dithiothreitol, 1% caffeine-sodium benzoate and 0.4 M sucrose. After passing the homogenate through 4 layers of cheesecloth, the crude filtrate was centrifuged at 1000×*g* for 10 min. The supernatant (I) was collected and centrifuged at 15 000×*g* for 20 min. Twohundred milliliters of the resulting supernatant (II) were concentrated under N_2 in an Amicon ultrafiltration cell using a membrane filter (Kalle PA 20). For gel chromatography the supernatant (II) was concentrated twice, for preparative isoelectric focussing 20-fold including a washing step with 300 ml of distilled water. Tests of solubilization were carried out by centrifugation of supernatant II at 100 000×*g* for 60 min using a Beckman L 2-65 B ultracentrifuge. All steps were carried out at 2 °C.

Gel chromatography. Gel chromatography separations were performed using a LKB system consisting of a Varioperpex pump, UV-monitor Uvicord S, fraction collector Ultrorac II and recorder 6520. The following types of columns and elution rates were applied:

1. Ultrogel AcA 34 (LKB), 2.6×95 cm, bed volume 450 ml, 15 ml/h; 2. Ultrogel AcA 34 (LKB), 5×100 cm, bed volume 1760 ml, 40 ml/h; 3. Ultrogel AcA 22 (LKB), 1.6×95 cm, bed volume 170 ml, 5 ml/h; 4. Sepharose CL-4B (Pharmacia), 1.6× 60 cm, bed volume 108 ml, 30 ml/h. Eluent, 0.1 M McIlvain buffer (pH 5.5); sample volumes, 1% of bed volume.

Preparative isoelectric focussing (PIEF). Two milliliters of the 20-fold concentrated supernatant II were focussed in a layer of 100 ml of granulated gel P 60 (Biorad) in a glass trough of 20×20 cm, according to the procedure of Radola [26]. The gel contained 2% (w/v) carrier ampholytes (pH 2-11; Servalyte AG 2-11, Serva). The sample was applied in 2 or more streaks on the surface of the gel layer 3 cm from the cathode, using a Desaga sample applicator. PIEF was carried out in a Desaga Mediphor chamber. Voltage was applied through platinum electrodes which were in contact with the gel layer through pads of MN 866 paper (Macherey & Nagel) soaked with the corresponding electrode solutions (0.5 M sulfuric acid at the anode and 2 M ethylene diamine at the cathode).

The focussing program was 200 V for 15 h, 400 V/1 h, 600 V/1 h, 800 V/1 h and 1000 V/1 h using a Desaga power supply 1200/200. Cooling water at 2 °C was circulated through the apparatus with an LKB 2209 Multitemp. After focussing, the print technique for protein and enzyme detection was used [27]. A 1.5×20 cm strip of Whatman No. 1 paper soaked in 0.1 M McIlvain buffer (pH 5.5) then dried was rolled on the gel layer just after focussing was finished. After contact with the gel layer for a few seconds, the print was removed and dried at 100–120 °C. Carrier ampholytes were removed by washing the print with 10% trichloroacetic acid for 10–15 min. Subsequent staining was carried out with 0.1% Serva Blue R (w/v) (Serva) in methanol-water-glacial acetic acid (25:60:10); the same mixture was used for destaining. After lipoxygenase staining (see below), the pH gradient was measured directly in the layer at regular 1 cm intervals along the focussing track (pH electrode EA 156, Deutsche Metrohm). The gel was divided into 1 cm strips corresponding to the pH measurement points. Each gel strip was removed from the trough and transferred to a small elution column. Proteins were eluted with 10 ml 0.1 M McIlvain buffer to yield solutions (PIEF fractions) suitable for testing.

Enzyme assays. Lipoxygenase staining after preparative isoelectric focussing was carried out according to Heimann et al. [28]. In solution lipoxygenase was assayed using an O_2-electrode (WTW EO 16 B) with a WTW Oxi 39 system (WTW, Weilheim) and Servogor RE 511 (Metrawatt) recorder. The standard reaction mixture (10 ml; pH 5.5) contained 5–8 ml linoleic acid solution according to Surrey [29] and 2–5 ml enzyme solution. After gel chromatography and PIEF, lipoxygenase activity was mea-

* We thank Dr. Westner, Institut für Technologie der Brauerei I, TU München, Freising-Weihenstephan, for the analyses.

sured spectrophotometrically by the increase of absorption at 234 nm in the following reaction mixture: 3 ml containing $2.9-x$ ml 0.1 M McIlvain buffer (pH 5.5), 0.1 ml substrate [29] and x ml enzyme solution.

The fatty acid hydroperoxide cleaving enzyme concentration was monitored by the disappearance of diene absorption of fatty acid hydroperoxide at 234 nm. Reaction mixture proportions were: $3-x$ ml 0.1 M McIlvain buffer (pH 5.5), $0.1-0.14\times10^{-6}$ M 13-hydroperoxide of linoleic acid (13-LHPO), and x ml enzyme solution. The activity of the hydroperoxide cleaving enzyme was also checked by gas chromatographic C_6-aldehyde determination, including combined gas chromatographic mass spectrometrical (GC-MS) examination [30] after liquid-liquid extraction of the volatiles from the reaction mixture. The incubation mixtures contained 2 ml enzyme solution and 3.2×10^{-6} M 13-LHPO (LnHPO). Reaction time was 10 min at 25 °C; the activity was stopped by addition of 1 ml of a methanolic internal standard solution (1 mg 2-methyl-1-pentanol/ml). After extraction with pentane, 1 µl of the concentrated (0.1 ml) solvent phase was used for GC-MS or GC-analysis. The following conditions were chosen: Varian Aerograph 1400 (FID); 5 m × 1.5 mm i.d. glass column, packed with 5% FFAP on 80/100 mesh Varaport 30; temperature programmed from 70–250 °C at 2°/min; carrier gas flow (N_2) 20 ml/min; injector temperature 210 °C; detector temperature 250 °C. The conditions for coupled gas chromatography-mass spectrometry are described elsewhere [31]. All enzymatic assays were controlled by blank experiments without enzyme and/or substrate contents.

Molecular weight determinations were performed by thin layer gel chromatography [32].

Protein content was determined according to the method of Lowry *et al.* [33].

Results and Discussion

Homogenates

In order to investigate the C_6-aldehyde formation in disrupted tissue of apples and tomatoes we followed the steps of enzyme preparation as they are outlined in Fig. 1. Measuring the C_6-aldehyde formation in homogenates we could confirm the results other authors had described [1–6, 17]. In extension of their investigations we could show that the C_6-aldehydes hexanal, (*Z*)-3- and (*E*)-2-hexenal are formed by the decomposition of linoleic and linolenic acids. These unsaturated fatty acids are constituents of the cell membranes and are hydrolyzed from the membranes by the action of a lipolytic acyl hydrolase [34–36]. This hydrolysis is another result of the plant tissue disruption and is regarded as an initiating step in the C_6-aldehyde formation. By determining the kinetics, the O_2- and pH-dependance (pH-optimum 5.5), heat lability, and methanol inhibition of the specific C_6-aldehyde formation, the process could be characterized as an enzymatic reaction.

Crude filtrates

Studies on substrate specifity, carried out with crude filtrates (buffer A, *cf.* Materials and Methods) from tomatoes showed that the molar C_6-aldehyde production (checked by hexanal formation) clustered around 3–4% after the addition of linoleic acid. After adding the 13-hydroperoxide of linoleic acid (13-LHPO) instead of the fatty acid the value increased to 30%. By these gas chromatographic determinations, which were supported by gas chromatographic-mass spectrometric identifications, it could be shown that only the 13-hydroperoxide was decomposed to hexanal. In accordance with findings of Matthews *et al.* [21], formation of C_6-aldehydes could not be detected after incubation of 9-hydroperoxide.

Nonenzymatic reactions were excluded from involvement in the C_6-aldehyde formation. Hemoglobin, Fe^{3+}, Fe^{2+}, cysteine and ascorbic acid were tested for their ability to cause C_6-aldehyde formation in crude filtrates enriched with the 13-hydroperoxide of linoleic acid. No specific hexanal formation due to the presence of these agents could be detected.

In cereals high-boiling fatty acids or fatty acid hydroperoxide decomposition products such as keto, epoxi, keto-hydroxy and hydroxy compounds may be produced by enzymatic lipid oxidation [10, 37–39]. Investigating crude filtrates of apples and tomatoes we could not find any of these compounds.

Solubilization

In contrast with tomateos, in apples the enzymatic activities responsible for C_6-aldehyde production

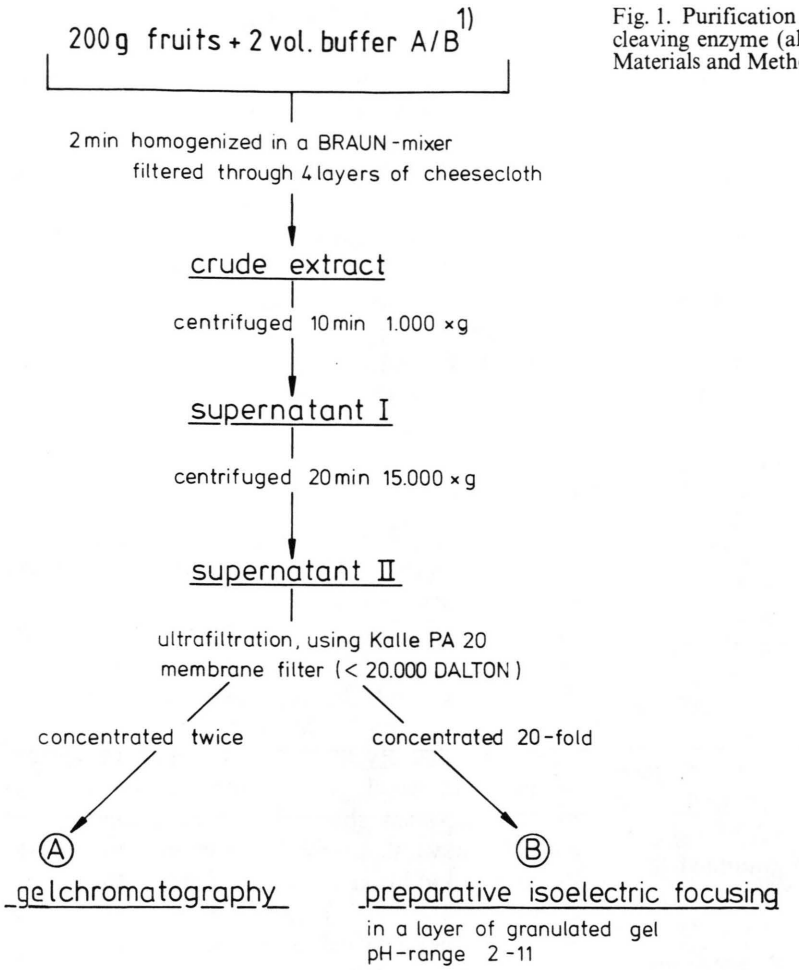

Fig. 1. Purification scheme of fatty acid hydroperoxide cleaving enzyme (all steps were carried out at 2 °C). * See Materials and Methods.

could not easily be solubilized using buffer A (*cf.* Materials and Methods). After centrifugation (Fig. 1) the total activity was found in the sediments, *i. e.* it was located in a membrane-bound form. After an extensive screening effort described in detail elsewhere [40], we were finally successful in partially solubilizing the enzyme by application of buffer B (*cf.* Materials and Methods). For the screening we preferred a rapid measurement of O_2-consumption to the time-consuming gas chromatographic C_6-aldehyde analysis, assuming that lipoxygenase is involved in the C_6-aldehyde formation. The addition of 2 mM dithiothreitol to the buffer was considered to be effective because extensive enzymatic browning of the apple homogenates due to phenoloxidase activity was avoided. Control tests carried out by gas chromatography supported our assumption that lipoxygenase activity is required for C_6-aldehyde formation. During the solubilization of lipoxygenase the fatty acid hydroperoxide cleaving enzyme was also brought into solution, indicating that both exist as membrane-bound enzymes. Recently, lipoxygenase in apples has been described as a membrane-bound enzyme by Kim and Grosch [41]. Thus, the effect of solubilization could be checked by the determination of lipoxygenase activity, applying the measurement of O_2-consumption (Table I). The degree of solubilization was also monitored by ultracentrifugation. 80% of the activity found in supernatant II could be detected in the supernatant, showing that true solubilization had been achieved.

Table II shows the results of parallel gas chromatographic studies indicating that the fatty acid hydroperoxide cleaving activity was solubilized together with lipoxygenase by application of buffer B (*cf.* Materials and Methods).

Table I. Determination of O_2-consumption in enzyme preparations from apples (substrate: 1.75 mM linoleic acid, pH 5.5 [29]).

Enzyme preparation	Volume	Protein		Activity	
	[ml]	[mg/ml]	[mg]	[ppm O_2/min · volume]	[ppm O_2/min · mg]
Homogenate	918	5.4	4938	148	0.03
Crude filtrate	680	5.0	3400	197	0.06
Supernatant I	400	4.0	1588	64	0.04
Sediment I	200	9.7	1940	97	0.05
Supernatant II	343	3.1	1053	86	0.08
Sediment II	22	14.2	312	11	0.03

The purification steps (Fig. 1) and assay methods are given in the experimental part.

Gel chromatography

After differential centrifugation and subsequent ultrafiltration, gel chromatography was applied for further purification of the fatty acid hydroperoxide cleaving activity (Fig. 1, route A). Buffer solution B was used for both tomatoes and apples. The enzymatic activities were determined by spectrophotometric tests measuring the increase (lipoxygenase) and the decrease (fatty acid hydroperoxide cleaving enzyme), respectively, of diene absorption at 234 nm. The spectrophotometric method proved to be more suitable for testing the large number of elution fractions obtained by gel chromatography. It was less time-consuming than gas chromatographic determination of C_6-aldehyde formation. When the volatile products were concentrated and the C_6-aldehydes were examined by gas chromatography, results were obtained which correlated well with those from the spectrophotometric method.

Attempts to separate the fatty acid hydroperoxide cleaving factor from the lipoxygenase activity by using Ultrogel AcA 34 and AcA 22 (LKB) were unsuccessful. Both activities were eluted in the void volume of the gels, which would normally indicate a high molecular weight. This was thought to be due to reaggregation phenomena which have been described for various membrane-bound enzymes [42]. Better results were obtained with Sepharose CL-4B (Pharmacia). Fig. 2 shows the separation of tomato enzymes as an example. With both the Ultrogel and the Sepharose, the bulk of the total protein material was eluted in the later fractions, indicating relatively low molecular weight. With Sepharose two common maxima were detected for both lipoxygenase and fatty acid hydroperoxide cleaving activity, the first one in the void volume of the gel (2 mill. Da), and a second at 200 000 Da. Thus, portions of the enzymatic activities were again found in an aggregated form (appearing in the void volume); relative to the

Table II. Formation of hexanal from the 13-hydroperoxide of linoleic acid (3.2 μM) in enzyme preparations from apples after solubilization.

Enzyme preparation	Volume[a]	Protein		Hexanal formed	
	[ml]	[mg/ml]	[mg]	[μmol/volume]	[μmol/mg] · 10^{-3}
Supernatant I	95	5.1	484	0.58	1.2
Supernatant II	100	2.8	288	0.45	1.6
UF	8	3.7	30	0.48	16

[a] Aliquote portions of the total volumes given in Table I.
UF = Ultrafiltered concentrate.
The purification steps (Fig. 1) and assay methods are given in the experimental part.

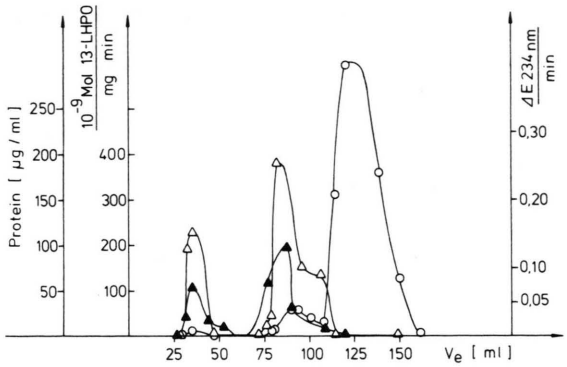

Fig. 2. Gel chromatographic separation of 3 ml concentrated supernatant II, prepared by buffer B, on Sepharose CL-4B using column 4 (*cf.* Materials and Methods). △–△ Fatty acid hydroperoxide cleavage activity [10^{-9} M 13-LHPO · mg^{-1} · min^{-1}]; ▲–▲ lipoxygenase activity [$\Delta E_{234\,nm}$ · min^{-1}]; ○–○ protein.

total fatty acid hydroperoxide cleaving activity eluted, it amounted to 36%.

Analogous results were obtained for the enzymes from apples. However, in these gel chromatographic studies, much lower activities were determined [40].

Preparative isoelectric focussing (PIEF)

The enzymatic activities were obtained in very diluted form (Fig. 2). For subsequent studies, *e.g.* separation by isoelectric focussing, a concentration of 0.5% should be available in order to permit visualization of the separation. Thus, the active fractions of many gel chromatographic separations were combined, stored at ±0 °C and again concentrated. Unfortunately, the fatty acid hydroperoxide cleaving enzyme in particular was very unstable. Due to marked losses during the different purification steps (ultrafiltration, gel chromatography, storage, ultrafiltration) we had to change the purification sequence, employing isoelectric focussing immediately after differential centrifugation and the first ultrafiltration (Fig. 1, B). Isoelectric focussing in granulated gel was selected. This technique can be carried out on a preparative scale (PIEF) and is known to achieve good results even with only partially purified protein or enzyme solutions [26]. Furthermore, another advantage is that enzyme activity may be measured spectrophotometrically, using the eluates from the gel after PIEF. Attempts to develop a visualization method for the fatty acid hydroperoxide cleaving enzyme after PIEF on the basis of the 5-amino-5-hydrazino-1,2,4,triazol-3-thiol (AHTT) -aldehyde test [43] were unsuccessful. The PIEF was carried out only with tomato proteins (buffer solution B). At this stage of the investigation only stored apples were available, which are known to exhibit very low activities of C_6-aldehyde formation [4]. As shown in Fig. 3 we were successful in separating the hydroperoxide cleaving activity from lipoxygenase on the basis of their different isoelectric points. The hydroperoxide cleaving enzyme focussed between pH 5.8–6.1, where at least two protein bands could be detected, whereas lipoxygenase focussed at pH 5.0. After fractionation of the gel and elution with buffer, in the pH-range 5.8–6.1 two active fractions were obtained (10–11 cm and 11–12 cm in Fig. 3). Table III shows the degree of purification of the hydroperoxide cleaving enzyme achieved by PIEF. The values in Table III, on the basis of the spectrophotometric test at 234 nm, show 8.4- and 5.0-fold purification, as well as a recovery rate of 24% in the PIEF-fractions. The increase of total activity in the crude filtrate in comparison to the homogenate (Table III) may be due to the fact that possible inhibitors were separated by filtration.

Enzyme properties

Using the PIEF-fractions from tomato proteins the following properties of the hydroperoxide cleaving enzyme were determined. The optimum of pH 5.5,

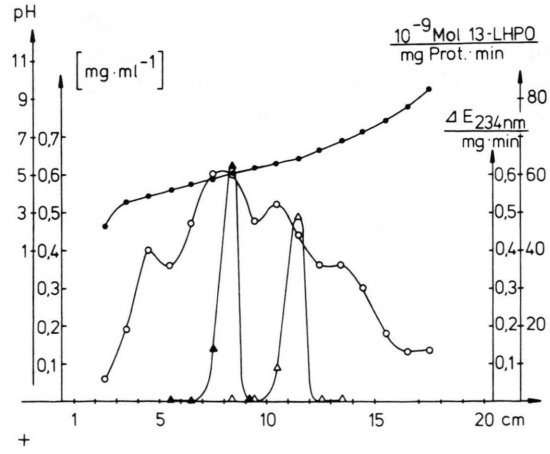

Fig. 3. Preparative isoelectric focussing (PIEF) of tomato protein in a layer of granulated gel. Experimental conditions in Materials and Methods. △–△ Fatty acid hydroperoxide cleaving enzyme; ▲–▲ lipoxygenase; ○–○ protein; ●–● pH gradient.

Table III. Purification of the fatty acid hydroperoxide cleaving enzyme from tomatoes.

Enzyme preparation	Volume	Protein		Cleavage activity		Recovery [%]	Purification
	[ml]	[mg/ml]	[mg]	[nmol 13-LHPO/min · ml]	[nmol 13-LHPO/min · mg]		
Homogenate	600	4.0	2400	36	9.0	100	1
Crude filtrate	460	4.5	2070	60	13.3	128	1.5
Supernatant I	360	4.3	1548	78	18.1	130	2.0
Supernatant II	330	4.4	1452	81	18.4	124	2.0
UF	11	61.8	680	880	14.2	74[a]	1.6
PIEF[b]	10	0.5	5	38	76.0		8.4
PIEF[c]	10	0.4	4	18	45.0	24[d]	5.0

13-LHPO = 13-Hydroperoxy-(*Z*)-9, (*E*)-11-octadecadienoic acid.
PIEF = Fractions of preparative isoelectric focussing.
The purification steps (Fig. 1) and assay methods are given in the experimental part. UF = Ultrafiltered concentrate from 200 ml supernatant II. [a]Calculation related to the total volume (330 ml) of supernatant II. [b]Fraction 11 – 12 cm, and [c]fraction 10 – 11 cm, respectively (Fig. 3); use of 2 ml UF for PIEF. [d]Calculated from[b] plus[c] in relation to the total volume (11 ml) of UF.

already found by the studies of homogenate, was confirmed. The activity was totally destroyed in alkaline medium (pH > 8.5).

A plot of enzyme concentration *versus* product formed was linear in the range of 0.02 – 0.35 mg protein.

A K_m-value of 2.6×10^{-5} M/l for the 13-hydroperoxide of linoleic acid was determined. Phillips and Galliard [44] calculated a K_m between 1.5 and 2.0×10^{-5} M/l for a hydroperoxide cleaving enzyme in cucumber. These authors also found their enzyme to be extremely heat-labile, with 100% inactivation after 1 min at 70 °C. In our studies, the activity of the hydroperoxide cleaving enzyme of tomatoes was totally destroyed after being heated at 90 °C for 1 min.

At 25 °C the decomposition of the 13-hydroperoxide of linoleic acid was finished after 40 – 50 min; using 13-hydroperoxide of linolenic acid as substrate the initial rate was very high, nearly ten times higher than with the hydroperoxide of linoleic acid, but the reaction was finished after 5 min of incubation. Hatanaka *et al.* [45] described similar kinetics for the activity of a C_6-aldehyde producing factor in tea chloroplasts.

With 13-hydroperoxide of linoleic acid as substrate, 47% of this compound was decomposed by the hydroperoxide cleaving enzyme from tomatoes after 10 min. These results obtained by spectrophotometric tests at 234 nm were confirmed by gas chromatographic determinations of hexanal formation. The highest hydroperoxide decomposition (C_6-aldehyde formation) rate was 59% after 50 min.

Hatanaka *et al.* [12, 46] proposed a decomposition mechanism in tea and *Farfugium japonicum* chloroplasts involving singlet oxygen ($^1O_2$) during photoperoxidation reactions. They found an inhibition with DABCO, known to be a $^1O_2$ quencher [47], and supposed $^1O_2$ as being important for the reaction. Presuming that superoxide anion is the precursor of $^1O_2$ the enzymatic hydroperoxide decomposition should also be inhibited by superoxide dismutase, which removes the superoxide anion from the reaction mixture. Hatanaka *et al.* [12, 46] could not detect any inhibition of the hydroperoxide cleaving activity after addition of superoxide dismutase. Thus, the assumption that $^1O_2$ takes part in the reaction remained uncertain. In our studies, the hydroperoxide cleaving enzyme of tomatoes was not inhibited either by DABCO or superoxide dismutase, so we can exclude any role for $^1O_2$ in the hydroperoxide cleaving reaction.

The effects of further inhibitors were examined with the 13-hydroperoxide of linoleic acid and the PIEF-fraction. Significant inhibition (70%) was obtained with 1 mM *p*-chloromercuribenzoic acid. Phillips and Galliard [44], studying cucumber activity, and Vick and Zimmerman [48], with watermelon enzyme, found a 20% and a 82% inhibition, respectively, using *p*-chloromercuribenzoic acid. This indicates that certain sulfhydryl groups have to be intact to preserve enzyme activity.

In the present study, there was no evidence of inhibition with H_2O_2, KCN or EDTA (0.3 and 1 mM). Phillips and Galliard [44] reported contrary

results with KCN. With lipoxygenase we found the same inhibition pattern Bonnet and Crouzet [49] described for the tomato enzyme: 1 mM H_2O_2 inhibited 80% of activity, superoxide dismutase (270 U) inhibited 11%, whereas EDTA and KCN seemed to activate or at least stabilize the enzyme activity.

By means of thin layer gel chromatography of the concentrated (again by ultrafiltration) PIEF-fraction a molecular weight of 200 000 Da could be determined for the hydroperoxide cleaving enzyme. This result is similar to those of Phillips and Galliard [44] and Vick and Zimmerman [48].

Further tests concerning the substrate and product specifity of the hydroperoxide cleaving enzyme from tomatoes showed that linoleic acid was not decomposed. On the other hand, lipoxygenase did not react with the 13-hydroperoxide of linoleic acid either. These results demonstrate that in tomatoes there are *two* separable enzymes responsible for the formation of C_6-aldehydes by decomposition of hydroperoxides. In tea and *Farfugium japonicum* Hatanaka *et al.* [12, 46] assume an enzyme system, splitting unsaturated fatty acids to hexanal and (*Z*)-3-hexanal. According to these authors, this enzyme system decomposes linoleic acid and its 13-hydroperoxide as well to hexanal.

Acknowledgement

This work was supported by the Deutsche Forschungsgemeinschaft.

[1] F. Drawert, W. Heimann, R. Emberger, and R. Tressl, Liebigs Ann. Chem. **694,** 200–208 (1966).
[2] F. Drawert, R. Tressl, W. Heimann, R. Emberger, and M. Speck, Chem. Mikrobiol. Technol. Lebensm. **2,** 10–22 (1973).
[3] F. Drawert, Aroma Research (H. Maarse, P. J. Groenen, eds.), pp. 13–39, Pudoc, Wageningen 1975.
[4] P. Schreier, Quality in stored and processed vegetables and fruit (P. W. Goodenough, C. Atkin, eds.), pp. 355–371, Academic Press, London 1981.
[5] H. E. Nursten and A. A. Williams, Chem. Ind. **1967,** 486–497.
[6] S. Jadhav, B. Singh, and D. R. Salunkhe, Plant Cell Physiol. **13,** 449–459 (1972).
[7] W. Grosch, G. Laskawy, and R. H. Fischer, Lebensm. Wiss. u. Technol. **7,** 335–338 (1974).
[8] W. Grosch, Z. Lebensm. Unters.-Forsch. **160,** 371–376 (1976).
[9] P. Coggon, L. J. Romanczyk jr., and G. W. Sanderson, J. Agric. Food Chem. **25,** 278–283 (1977).
[10] H. W. Gardner, J. Agric. Food Chem. **23,** 129–136 (1975).
[11] T. Galliard, G. A. Matthew, A. J. Wright, and M. J. Fishwick, J. Sci. Food Agric. **28,** 863–868 (1977).
[12] A. Hatanaka, J. Sekiya, and T. Kajiwara, Plant Cell Physiol. **18,** 107–116 (1977).
[13] B. O. de Lumen, E. J. Stone, S. J. Kaceniak, and R. H. Forsythe, J. Food Sci. **43,** 698–702 (1978).
[14] W. Grosch and J. M. Schwarz, Lipids **6,** 351–352 (1971).
[15] R. T. Major and M. Thomas, Phytochemistry **11,** 611–617 (1972).
[16] B. Axelrod, Food Related Enzymes (J. R. Whitaker, ed.), pp. 324–348, ACS, Washington 1974.
[17] T. Galliard, Recent Advances in the Chemistry and Biochemistry of Plant Lipids (T. Galliard, E. J. Mercer, eds.), pp. 335–370, Academic Press, London 1975.
[18] F. Drawert, W. Heimann, R. Emberger, and R. Tressl, Z. Naturforsch. **20 b,** 497–498 (1965).
[19] E. J. Stone, R. M. Hall, and S. J. Kaceniak, J. Food Sci. **40,** 1138–1141 (1975).
[20] P. Schreier, F. Drawert, and A. Junker, Z. Lebensm. Unters.-Forsch. **165,** 23–27 (1977).
[21] J. A. Matthew, H. W. S. Chan, and T. Galliard, Lipids **12,** 324–326 (1977).
[22] W. Heimann and P. Schreier, Helv. Chim. Acta **54,** 2794–2803 (1971).
[23] K. Leu, Lebensm. Wiss. u. Technol. **7,** 82–85 (1974).
[24] A. E. Johnston, K. T. Zileh, E. Selke, and H. J. Dutton, J. Amer. Oil Chemist's Soc. **38,** 367–371 (1961).
[25] H. W. S. Chan and F. A. A. Prescott, Biochim. Biophys. Acta **380,** 141–144 (1975).
[26] B. J. Radola, Biochim. Biophys. Acta **386,** 181–195 (1975).
[27] B. J. Radola, Isoelectric focussing (J. P. Arbuthnott, J. A. Beeley, eds.), pp. 182–197, Butterworth, London 1975.
[28] W. Heimann, P. Dresen, and P. Schreier, Z. Lebensm. Unters.-Forsch. **152,** 147–151 (1973).
[29] K. Surrey, Plant Physiol. **39,** 65–70 (1964).
[30] P. Schreier and F. Drawert, Z. Analyt. Chemie **279,** 141–142 (1976).
[31] P. Schreier, J. Agric. Food Chem. **28,** 926–928 (1980).
[32] H. Determann, Gelchromatographie, Springer, Berlin, Heidelberg, New York, 1976.
[33] O. H. Lowry, N. J. Roseborough, A. L. Furr, and R. J. Randall, J. Biol. Chem. **193,** 265–275 (1951).
[34] T. Galliard, Biochem. J. **121,** 379–390 (1971).
[35] D. A. Wardale and T. Galliard, Phytochemistry **14,** 2323–2329 (1975).
[36] O. Hirayama, H. Matsuda, H. Takeda, K. Maenaka, and H. Takatsuka, Biochim. Biophys. Acta **384,** 127–137 (1975).
[37] D. C. Zimmerman and B. A. Vick, Plant Physiol. **46,** 445–453 (1970).
[38] W. Heimann and P. Dresen, Helv. Chim. Acta **56,** 463–469 (1973).
[39] W. Heimann and V. Klaiber, Z. Lebensm. Unters.-Forsch. **165,** 144–146 (1977).
[40] G. Lorenz, Über den Reaktionsmechanismus der C_6-Aldehyd-Bildung in zerkleinertem pflanzlichen Gewebe, Dissertation TU München 1981.

[41] J. S. Kim and W. Grosch, J. Agric. Food Chem. **27,** 243–246 (1979).
[42] L. Roden and N. B. Schwartz, MTP Int. Rev. Sci., Biochem. Ser. One, Vol. 5 (W. J. Whelan, J. William, eds.), pp. 95–152, Butterwoth, London 1975.
[43] C. H. Rahn and W. Schlenk, Lipids **8,** 612–616 (1973).
[44] D. R. Phillips and T. Galliard, Phytochemistry **17,** 355–358 (1978).
[45] A. Hatanaka, T. Kajiwara, J. Sekiya, and K. Fujimara, Agr. Biol. Chem. **43,** 175–176 (1979).
[46] A. Hatanaka, J. Sekiya, T. Kajiwara, and T. Miura, Agr. Biol. Chem. **43,** 735–740 (1979).
[47] C. Ouannès and Th. Wilson, J. Amer. Chem. Soc. **90,** 6527–6528 (1968).
[48] B. A. Vick and D. C. Zimmerman, Plant Physiol. **57,** 780–785 (1976).
[49] J. L. Bonnet and J. Crouzet, J. Food Sci. **42,** 625–628 (1977).