PERGAMON

Phytochemistry 53 (2000) 177–185

PHYTOCHEMISTRY

www.elsevier.com/locate/phytochem

Purification, stabilization and characterization of tomato fatty acid hydroperoxide lyase

Catherine N.S.P. Suurmeijer[a], Manuela Pérez-Gilabert[a], Dirk-Jan van Unen[a], Harry T.W.M. van der Hijden[b], Gerrit A. Veldink[a,*], Johannes F.G. Vliegenthart[a]

[a]*Bijvoet Center, Department of Bio-organic Chemistry, Utrecht University, Padualaan 8, NL-3584 CH, Utrecht, The Netherlands*
[b]*Unilever Research Vlaardingen, Olivier van Noortlaan 120, NL-3133 AT, Vlaardingen, The Netherlands*

Received 31 March 1999; received in revised form 28 July 1999; accepted 16 August 1999

Abstract

Fatty acid hydroperoxide lyase (HPO-lyase) was purified 300-fold from tomatoes. The enzymatic activity appeared to be very unstable, but addition of Triton X_{100} and β-mercaptoethanol to the buffer yielded an active enzyme that could be stored for several months at −80°C. The enzyme was inhibited by desferoxamine mesylate (desferal), 2-methyl-1,2-di-3-pyridyl-1-propanone (metyrapone), nordihydroguaiaretic acid (NDGA), *n*-propyl gallate and butylated hydroxyanisole, suggesting the involvement of free radicals in the reaction mechanism and the existence of a prosthetic group in the active center. However, no heme group could be demonstrated with the methods commonly used to identify heme groups in proteins. Only 13-hydroperoxides from linoleic acid (13-HPOD) and α-linolenic acid (α-13-HPOT) were cleaved by the tomato enzyme, with a clear preference for the latter substrate. The pH-optimum was 6.5, and for concentrations lower than 300 μM a typical Michaelis–Menten curve was found with a K_m of 77 μM. At higher α-13-HPOT concentrations inhibition of the enzyme was observed, which could (at least in part) be attributed to 2*E*-hexenal. A curve of the substrate conversion as a function of the enzyme concentration revealed that 1 nkat of enzyme activity converts 0.7 μmol α-13-HPOT before inactivation. Headspace analysis showed that tomato HPO-lyase formed hexanal from 13-HPOD and 3*Z*-hexenal from α-13-HPOT. A trace of the latter compound was isomerized to 2*E*-hexenal. In addition to the aldehydes, 12-oxo-9*Z*-dodecenoic acid was found by GC/MS analysis. To a small extent, isomerization to 12-oxo-10*E*-dodecenoic acid occurred. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Lycopersicon esculentum; Solanaceae; Tomato; Fatty acid hydroperoxide lyase

1. Introduction

Fatty acid hydroperoxide lyases (HPO-lyases) cleave hydroperoxides of unsaturated fatty acids, such as linoleic and linolenic acids, into an aldehyde and an oxo-acid. They are part of the lipoxygenase pathway in plants, which is considered to be the equivalent of the arachidonic acid cascade in animals (Gardner, 1991). The reaction products are important flavours in food products (Hatanaka, 1993).

13-HPO-lyases that specifically cleave 13-hydroperoxy-octadecadienoic acid (13-HPOD) and α-13-hydroperoxy-octadecatrienoic acid (α-13-HPOT), form hexanal and 3*Z*-hexenal, respectively, and 12-oxo-9*Z*-dodecenoic acid. The latter is a precursor of traumatin, a compound involved in wound healing (Gardner, 1991; Hsieh, 1994). Hexanal and 3*Z*-hexenal are flavour compounds with a "green" odour. They can be converted into other aldehydes and alcohols that also belong to the class of green odour notes (Hatanaka, 1993; Phillips, Matthew, Reynolds & Fenwick, 1979; Olías, Pérez, Ríos & Sanz, 1993). The biological function of the aldehydes is unclear but they have been reported to inhibit germination of soybean seeds (Gardner, Dornbos & Desjardins, 1990). Furthermore,

* Corresponding author. Tel.: +31-30-302532661; fax: +31-30-2540980.
E-mail address: veldink@accu.uu.nl (G.A. Veldink).

0031-9422/00/$ - see front matter © 2000 Elsevier Science Ltd. All rights reserved.
PII: S0031-9422(99)00504-X

Table 1
Purification of HPO-lyas from tomato fruits (996 g)

Fraction	Activity (nkat/ml)	Total activity (μkat)	Specific activity (nkat/mg)	Recovery	Purification factor
Filtrate	4.8	12.1	0.2	100%	1
Crude extract	8.4	5.4	7.5	45%	35
Concentrate	16.4	3.9	12.5	32%	58
G-100 pool	12.0	1.9	65.1	16%	126
DEAE pool	21.7	0.9	65.1	8%	300

their application to cotton bolls induced the formation of phytoalexins (Zeringue, 1992), and they have been suggested to be involved in anti-bacterial responses (Croft, Juttner & Slusarenko, 1993). Recently, Koshio, Takahashi and Ota (1995) showed that 2*E*-hexenal, derived from 3*Z*-hexenal, plays an important role in the browning of male flowers of *Cryptomerica japonica*.

HPO-lyase from green bell pepper fruits was identified as a cytochrome P-450 protein (Matsui, Shibutani, Hase & Kajiwara, 1996), which makes the involvement of a substrate-derived free radical in the cleavage reaction likely. However, for HPO-lyase from tea leaves a reaction mechanism was proposed in which fatty acid hydroperoxides are heterolytically cleaved by an amino acid in the active site that acts as a Lewis acid. Oxygen isotope labeling experiments corroborate the latter mechanism (Gardner & Plattner, 1984; Hatanaka, Kajiwara, Sekiya & Toyota, 1986). Support for a role for heme instead of an acidic amino acid may be found in the observation that inactivated heme proteins convert linolenic acid into 2*E*-hexenal (Coggon, Romanczyk & Sanderson, 1977). Moreover, it was found that hydrophobic radical scavengers protect HPO-lyase from inactivation when it is incubated with its substrate. This may indicate the involvement of a substrate-derived free radical species in the inactivation of HPO-lyase (Matsui, Kajiwara & Hatanaka, 1992).

A few studies on HPO-lyase from tomato fruit have appeared thus far. The enzyme specifically cleaves 13-hydroperoxides (Jadhav, Singh & Salunkhe, 1972; Galliard & Matthew, 1977; Galliard, Matthew, Wright & Fishwick, 1977), although in crude enzyme preparations some formation of 2*E*-nonenal from 9-HPOD was observed (Hatanaka, Kajiwara, Matsui & Kitamura, 1992). Schreier and Lorenz (1982) achieved a 5-fold purification of the enzyme but further enrichment was difficult because the activity was rapidly lost. Although this partially purified HPO-lyase had a preference for α-13-HPOT over 13-HPOD as substrate, the pH optimum of the enzyme (5.5) and the K_m (26 μM) were determined with 13-HPOD.

In this paper a 300-fold purification of HPO-lyase from tomatoes is described, and the enzyme is characterized with α-13-HPOT as substrate. Furthermore, the volatile and non-volatile products of the enzyme were identified.

2. Results and discussion

2.1. Enzyme stability and purification

HPO-lyase was partially purified from "Trust" tomatoes to a specific activity of 65 nkat/mg (Table 1). The same procedure was carried out with several other tomato varieties with no significant difference in the resulting specific activity.

The enzyme activity appeared to be very sensitive to pH: when the crude extract was stored at 4°C at pH 5.5, 50% of the activity was lost within 1 day, whereas at pH 8.5, the activity decreased by only 40% in 12 days. Upon inclusion of 0.1% (w/v) Triton X-100 and 0.01 M β-mercaptoethanol in the buffer (pH 8.5), the crude extract showed no activity loss over 20 days storage. However, after DEAE-chromatography HPO-lyase was inactivated within 24 h at 4°C. Dialysis of the crude extract against buffer A also caused a decrease in activity, although less dramatic. Since the enzyme precipitated together with pectins upon addition of calcium chloride to the filtrate, it could be that calcium ions or the increased viscosity caused by the high carbohydrate concentration were additional stabilizing factors in the crude extract. However, the addition of divalent cations after dialysis in concentrations between 10 μM and 1 mM, did not protect the enzyme activity at 4°C. To test the influence of viscosity, sucrose (0.4 M) or glycerol (10% v/v) was added to the DEAE-pool. Because the enzyme was still inactivated within 1 day when stored at 4°C, an interaction more specific than viscosity seemed to be the stabilizing factor in the crude extract. At −20°C, the residual activity is higher in the samples with sucrose or glycerol than without it. This can probably be explained by the influence of these compounds on freezing and thawing. At −80°C, the enzyme activity was best retained, also in the absence of the additives. Therefore, it was decided to store the enzyme without additives at −80°C after DEAE-chromatography. Under these storage conditions, the enzyme activity

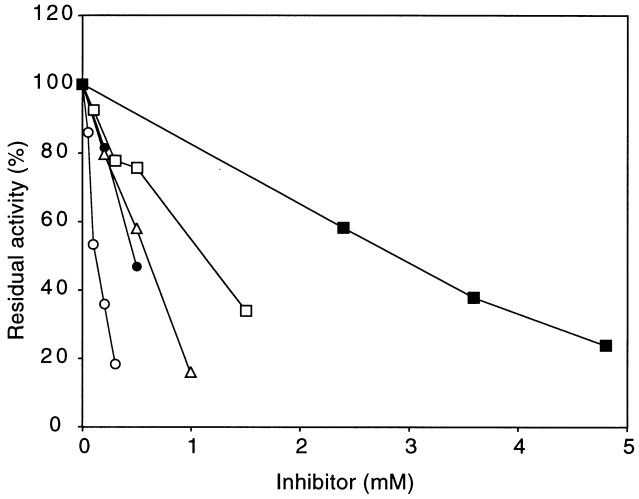

Fig. 1. The inhibition of tomato HPO-lyase by several agents; ○, NDGA; ●, metyrapone; △, butylated hydroxyanisole; □, *n*-propyl gallate; ■, desferal. The residual activity was monitored at 234 nm with an α-13-HPOT concentration of 80 μM.

remained stable for several months. A minor loss of activity (±10%) occurred, due to freeze-thawing.

Although gel electrophoresis still showed three bands after silver staining, at 65, 47 and 35 kDa, the purity of the enzyme significantly improved by this procedure.

Moreover, the enzyme preparation showed no activity in lipoxygenase assays. Therefore, the method presented here yields a highly purified enzyme preparation that can be used to study HPO-lyase in more detail.

2.2. Prosthetic group

Several compounds were tested as possible inhibitors of HPO-lyase activity: desferoxamine mesylate (desferal), 2-methyl-1,2-di-3-pyridyl-1-propanone (metyrapone), nordihydroguaiaretic acid (NDGA), *n*-propyl gallate and butylated hydroxyanisole, significantly reduced the activity of the enzyme (Fig. 1).

Desferal is a transition metal chelator which has been used as an inhibitor of various heme proteins. Recently, it has been reported that the chelating properties of desferal are not responsible for the inhibition of phenol-oxidizing enzymes, such as laccase and peroxidase. The inhibitory effect on these enzymes is rather due to a reaction of desferal with phenoxy radicals (De Pinto & Barceló, 1996). Metyrapone is a pyridine derivative with a strong affinity for both oxidized and reduced forms of cytochrome P-450 proteins, and it is used as a potent inhibitor of these enzymes (Testa & Jenner, 1981). HPO-lyase is also inhibited by *n*-propylgallate, butylated hydroxyanisole and NDGA, radical scavengers often used as lipoxygenase inhibitors

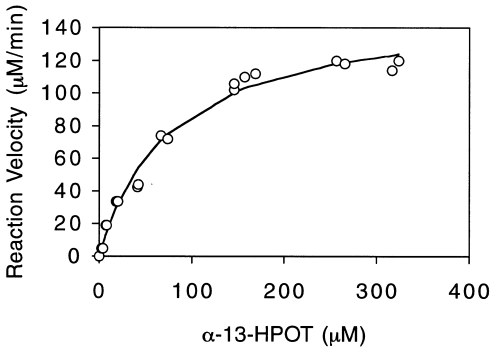

Fig. 2. Reaction velocity of HPO-lyase versus α-13-HPOT concentration; ○, points measured; line, calculated Michaelis–Menten curve with $K_m = 77 \pm 6$ μM and $V_{max} = 153 \pm 9$ μM/min.

(Vick & Zimmerman, 1987). In addition to its radical scavenging properties, NDGA converts the active Fe(III)-form of lipoxygenase into its inactive Fe(II)-form (Veldink & Vliegenthart, 1991). The results of the inhibition experiments suggest the involvement of radicals in the HPO-lyase reaction mechanism and the existence of an iron-containing prosthetic group in the active center of this enzyme.

However, the detection methods commonly used to identify a heme group in proteins, gave negative results for tomato HPO-lyase. First, the absorption maxima between 400 and 500 nm in the UV-spectrum of the enzyme preparation did not shift upon addition of dithionite, and no absorbance was observed in the CO-difference spectrum. Furthermore, staining with 3,3′,5,5′-tetramethylbenzidine (TMBZ) did not provide evidence for a heme group. Finally, atomic absorption spectroscopy (AAS), performed to detect iron in the sample, gave a negative result as well.

A possible explanation for these results might be that the concentration of β-mercaptoethanol, used during the enzyme purification, caused the heme group to dissociate from the protein (Thomas, Ryan & Levin, 1976). Therefore, the resuspended calcium chloride pellet was filtered through a 30 kDa filter. However, the filtrate did not stain with TMBZ. Therefore, the results could only be explained by a very low heme concentration in the sample which was too low to be detected.

2.3. Kinetics

In line with the observation of Schreier and Lorenz (1982), it was found that the purified enzyme converted α-13-HPOT 8 times faster than 13-HPOD. For the reaction with α-13-HPOT, HPO-lyase showed a pH-optimum around pH 6.5. In Fig. 2 the reaction velocity, measured at this pH with 3 fkat enzyme, is plotted as a function of substrate concentration. Up to 300 μM the data could be fitted by non-linear re-

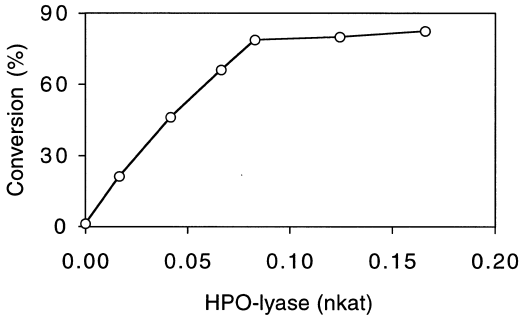

Fig. 3. Substrate conversion as a function of the amount of HPO-lyase, measured spectrophotometrically. The reaction was allowed to proceed until no further decrease in absorbance at 234 nm was observed. The percent conversion was calculated from the decrease in absorbance, assuming $\varepsilon = 25{,}000$ for α-13-HPOT.

gression to the standard Michaelis–Menten equation, yielding a K_m of 77 μM (±6 μM) and a V_{max} of 153 μM/min (±9 μM/min), however, at higher concentrations the reaction rate decreased. Both the pH-optimum and the K_m differ significantly from the values reported for tomato HPO-lyase with 13-HPOD as substrate (Schreier & Lorenz, 1982). This may either be explained by the use of α-13-HPOT as substrate instead of 13-HPOD, or by the differences in use of detergents. In the experiments described here the amount of detergent was constant during the assay while it increases with substrate concentration with the method of Schreier and Lorenz (1982). For tomato lipoxygenase, it was found that this can lead to different progress curves (Suurmeijer, Pérez-Gilabert, van der Hijden, Veldink & Vliegenthart, 1998).

The decline in reaction rate for concentrations higher than 300 μM is possibly caused by product inhibition. This is in agreement with the observation that the reaction velocity of α-13-HPOT cleavage increased when it was measured indirectly, by converting the aldehydes formed immediately into the corresponding alcohols (Vick, 1991). Furthermore, it was found that the presence of 400 μM 2*E*-hexenal in the direct spectrophotometric assay lowered the reaction rate to 66%, while 1 mM 2*E*-hexenal inhibited HPO-lyase completely. At 0.4 and 1 mM 3*Z*-hexenal the enzyme was not affected. A partial inhibition was observed at 2 mM 3*Z*-hexenal, but this was probably an effect of the partial 3*Z* : 2*E*-isomerization of the compound. Hexanal concentrations of 400 μM and 4 mM did not influence the reaction velocity. From this experiment it may be concluded that the inhibiting effect is caused by 2*E*-hexenal. α, β-Unsaturated aldehydes are known to react with proteins and particularly sulfhydryl

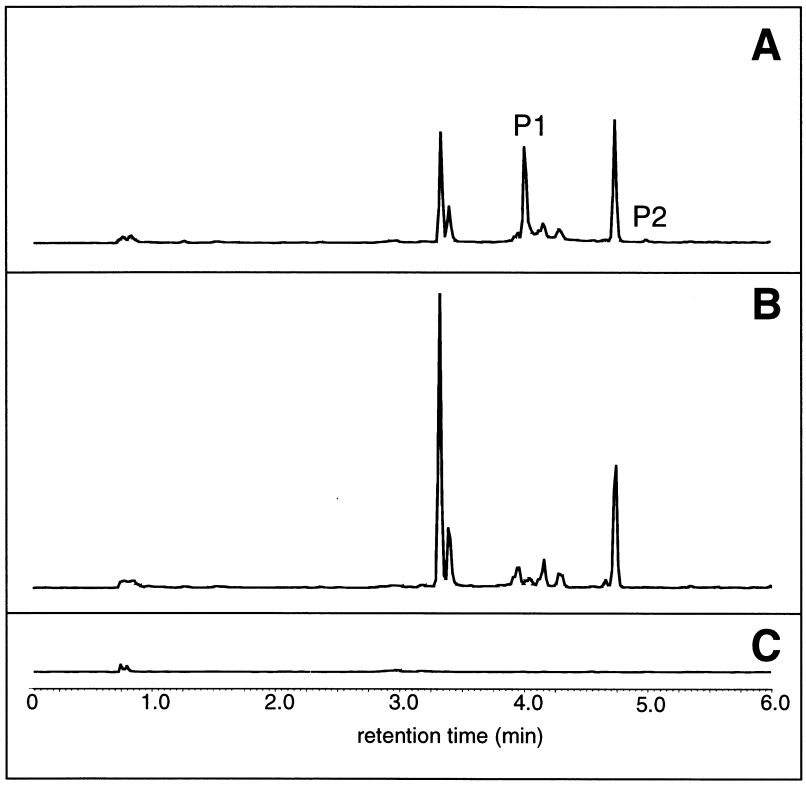

Fig. 4. Headspace analysis of the incubation with 100 μM α-13-HPOT at pH 6.5, volatiles were, immediately after collection in the headspace, desorbed at 200°C in the injection port of the GC/MS. Panel A: incubation, panel B: control without enzyme, and panel C: control without substrate. P1 was identified as 3*Z*-hexenal, P2 as 2*E*-hexenal.

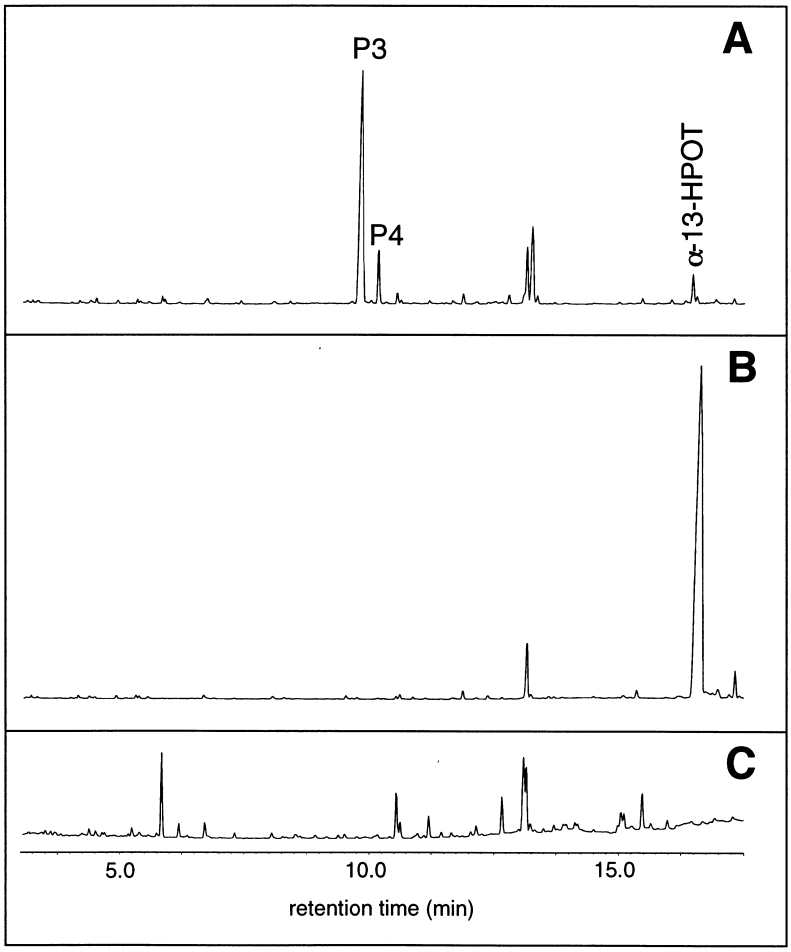

Fig. 5. GC/MS-chromatogram of the non-volatile products from α-13-HPOT. After extraction, the compounds were reduced, methylated and silylated before injection. Panel A: incubation, panel B: control without enzyme, and panel C: control without substrate. P3 was identified as 12-oxo-9*Z*-dodecenoic acid and P4 as 12-oxo-10*E*-dodecenoic acid.

enzymes are inactivated by this process (Schauenstein, Esterbauer & Zollner, 1977). Therefore, 12-oxo-10*E*-dodecenoic acid, one of the other products of 13-HPO-lyases could have an inhibiting effect as well. These results are in agreement with the finding reported by Matsui, Kajiwara and Hatanaka (1992) that HPO-lyase from tea has a sulfhydryl group in its active centre.

The conversion of α-13-HPOT was determined spectrophotometrically as a function of the enzyme concentration (see Fig. 3). When less than 0.08 nkat of enzyme was added to the reaction mixture containing 200 μM α-13-HPOT, the substrate was not completely consumed, suggesting that the enzyme is irreversibly inactivated. Because the conversion was linearly proportional to the amount of enzyme ($r^2 = 0.993$) it could be derived that 1 nkat of enzyme can convert around 0.7 μmol of α-13-HPOT before it is inactivated.

2.4. Product identification

The volatile products formed by HPO-lyase, were identified by headspace analysis with GC/MS. The enzyme was incubated with 9- or 13-hydroperoxides of linoleic, α-linolenic and γ-linolenic acid respectively. Only 13-HPOD and α-13-HPOT yielded aldehydes, reflecting cleavage by the enzyme. The C_9-aldehydes that Hatanaka et al. (1992) found in incubations of linoleic acid and 9-HPOD with crude tomato extract are thus formed by another enzyme. Two peaks appear in the incubation with α-13-HPOT, that are absent in the controls in which either enzyme or substrate is left out (see Fig. 4). The retention time and mass spectrum of the major product, P1, correspond to those of the reference aldehyde 3*Z*-hexenal, EIMS, 70 eV, *m*/*z* (rel. int.): 98 $[M]^+$ (10), 97 $[M-H]^+$ (7), 83 $[M-CH_3]^+$ (23), 69 $[M-CH_2CH_3]^+$ (58), 55 $[M-CH_2CH_2CH_3]^+$ (44), 41 $[M-CH_2CH_2CH_2CH_3]^+$ (100), 39 (38). The

minor compound, P2, could be identified as 2*E*-hexenal, although the background signals could not be completely eliminated from the mass spectrum. Isomerization of 3*Z*-hexenal to 2*E*-hexenal occurs either spontaneously (Whitehead, Muller & Dean, 1995), or enzymatically (Phillips, Matthew, Reynolds & Fenwick, 1979). When α-13-HPOT was incubated with HPO-lyase in the presence of alcohol dehydrogenase and NADH (Vick, 1991; Whitehead, Muller & Dean, 1995), only 3*Z*-hexenol was formed which demonstrates that the tomato HPO-lyase produces 3*Z*-hexenal and that the 2*E*-hexenal observed is due to subsequent isomerization.

Upon incubation of the enzyme with 200 μM 13-HPOD, a small amount of hexanal was formed, which is in line with the lower affinity of the enzyme for 13-HPOD.

To identify the non-volatile products of HPO-lyase, they were collected by C18-extraction and then reduced, methylated and silylated before GC/MS analysis. In the GC-chromatograms in Fig. 5 it can be seen that the enzyme has reduced the amount of α-13-HPOT considerably and produced P3-EIMS, 70 eV, *m*/*z* (rel. int.): 300 $[M]^+$ (0), 285 $[M - CH_3]^+$ (6), 270 (21), 253 $[M - 47]^+$ (19), 210 (22), 178 (47), 159 (35), 136 (25), 103 $[(CH_3)_3SiOCH_2]^+$ (100), 73 $[(CH_3)_3Si]^+$ (98)- and P4-EIMS, 70 eV, *m*/*z* (rel. int.): 300 $[M]^+$ (0), 285 $[M - CH_3]^+$ (5), 253 $[M - 47]^+$ (22), 143 (20), 129 $[(CH_3)_3SiOCHCHCH_2]^+$ (100), 73 $[(CH_3)_3Si]^+$ (88).

The mass spectra of the products are typical for ω-hydroxy fatty acids in that they contain peaks at M-15 and M-47 (Eglinton, Hunneman & McCormick, 1968). The molecular mass of 300 corresponds to compounds with 12 carbon atoms and 1 double bond. The spectrum of the minor product, P4, could readily be assigned to 12-oxo-10*E*-dodecenoic acid (Vick & Zimmerman, 1976).

Compound P3 is most likely 12-oxo-9*Z*-dodecenoic acid, since it is generally regarded as the product of HPO-lyase, and the precursor of the conjugated 12-oxo-10*E*-dodecenoic acid (Gardner, 1991; Hatanaka, 1993; Vick & Zimmerman, 1987). Moreover, the fragmentation pattern of the product corresponded to that of the TMSi- ether of 3*Z*-hexenol, a compound with the same structural element. Therefore, P3 was identified as the derivative of 12-oxo-9*Z*-dodecenoic acid. The analysis was repeated for an incubation with 200 μM 13-HPOD, and, as expected, both P3 and P4 were found in the GC/MS chromatogram. In accordance with the lower turnover of HPO-lyase for this substrate, the amount of 12-oxo-dodecenoic acids formed was much lower.

The product profiles demonstrate that HPO-lyase from tomatoes converts 13-hydroperoxides of linoleic and linolenic acids into C_6-aldehydes and 12-oxo-9*Z*-dodecenoic acid. 9-Hydroperoxides are not a substrate for this enzyme. The low level of *Z*/*E*-isomerization of 3*Z*-hexenal and 12-oxo-9*Z*-dodecenoic acid should probably be attributed to non-enzymatic processes. Furthermore, the GC-chromatograms do not show compounds generated by other fatty acid hydroperoxide converting enzymes (e.g. HPO-isomerase or HPO-cyclase).

3. Materials and methods

3.1. Materials

Tomatoes (variety "Trust") were bought in a local green-house. EDTA (99%), calcium chloride (p.a.), 2*E*-hexenal (99%), hexanal (96%), 2*E*-hexenol (96%), hexanol, 3*Z*-hexenol (98%) and potassium chloride were from Acros Chimica, Geel, Belgium. 3*Z*-hexenal in triacetin (1 : 1) was a gift from Unilever Research, Vlaardingen, The Netherlands. Hydrogenperoxide, 30% (w/v), pyridine, 1,1,1,3,3,3-hexamethyldisilazane, chlorotrimethylsilane, *n*-propylgallate and 2-methyl-1,2-di-3-pyridyl-1-propanone (metyrapone) were obtained from Aldrich, Milwaukee, WI, USA. Bicinchoninic acid, bovine serum albumin (essentially fatty acid free), alcohol dehydrogenase from bakers yeast (5678 nkat/mg, solid), β-NADH 3,3′,5,5′-tetramethylbenzidine (TMBZ), desferoxamine mesylate (desferal), nordihydroguaiaretic acid (NDGA), butylated hydroxyanisole and (*N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]) HEPES were from Sigma, St. Louis, USA. Octadecyl solid-phase extraction columns were from J.T. Baker B.V., Deventer, The Netherlands. Linoleic acid (~99%), α-linolenic acid (~99%), α-linolenic acid and γ-mercaptoethanol were purchased from Fluka, Buchs, Switzerland. Triton X-100 was from Serva, Heidelberg, Germany. Methanol from Rathburn Chemicals, Walkerburn, Scotland, was of HPLC grade. Water was of Milli-Q quality, and diethyl ether was distilled before use. 13-Hydroperoxides of linoleic, α-linolenic and γ-linolenic acids were each prepared by incubation of the fatty acids with soybean lipoxygenase-1 at pH 9.0 under an oxygen-flow at 4°C (Elshof, Janssen, Veldink & Vliegenthart, 1997). Hydroperoxides were extracted from the incubation mixture with ether and their purity was determined by analysis with GC/MS as described in the Section 2.4. For 9-hydroperoxides a crude preparation of soluble tomato lipoxygenase was used at pH 6.8 (Suurmeijer et al., 1998).

3.2. Enzyme purification

One kg of ripe tomatoes was homogenized in a Waring blender with 2 l, 0.1 M HEPES buffer, pH 7.5.

The homogenate was filtered through four layers of cheesecloth and to the filtrate 2% (v/v) of a 1 M $CaCl_2$-solution was added. After stirring for 2 h the suspension was centrifuged at 30,000 g for 20 min and the pellet was resuspended in buffer A (0.02 M Tris/HCl buffer, pH 8.5, containing 0.1% (w/v) Triton X-100 and 0.01 M β-mercaptoethanol). Maximum solubilization of HPO-lyase activity was achieved with 4 ml buffer A per g pellet. The suspension was clarified by centrifugation at 15,000 g for 30 min and the HPO-lyase solution (further referred to as crude extract) was concentrated by ultrafiltration (PM30-membrane, Amicon). The concentrate was purified over a G-100-Sephadex column (35 cm × 5.0 cm i.d.), eluted with buffer A (0.5 ml/min), and then the active fractions were combined and loaded onto a DEAE-Sephadex (A-50) column (30 cm × 2.5 cm i.d.). Activity was eluted at 0.75 ml/min with a gradient of 0–300 mM KCl in buffer A. The fractions were assayed immediately after elution and HPO-lyase was stored at −80°C. During the entire procedure the protein was kept at 4°C or on ice.

3.3. Heme analyses

The CO-difference spectrum was recorded as described by Rutten et al. (1987). A lyase sample was diluted to a concentration of 5.0 nkat/ml with 0.1 M phosphate buffer, pH 7.4, containing 1 mM EDTA and 20% (v/v) glycerol. The sample was bubbled with CO for 1 min at a flow of 5 bubbles/s and then divided over sample and reference cuvette. Dithionite (10 μl, 1.25 M) was added to the sample cuvette and the difference spectrum was recorded after 7 min on a Shimadzu UV-160 A double beam spectrophotometer. As a positive reference a cytochrome P-450 from rat liver was used.

Heme staining was performed by a procedure adapted from Thomas et al. (1976). For this purpose three volumes of a 6.3 mM TMBZ-solution in methanol were mixed with seven volumes 0.25 M sodium acetate buffer, pH 5.0. One ml of this freshly prepared reagent was added to 50 μl protein solution and 35 μl 0.3% H_2O_2 was added. Presence of heme was indicated in the reference proteins hemoglobin and myoglobin by appearance of a blue colour after 1 min which further intensified during 30 min.

3.4. HPO-lyase activity assay

During the purification, activities were assessed routinely by the indirect assay described by Vick (1991), in order to distinguish between HPO-lyase and other hydroperoxide-converting enzymes. The reaction was started by adding 20 μl 5 mM α-13-HPOT to a mixture of 100 μM NADH, 835 nkat alcohol dehydrogenase, 100 μl enzyme sample and 50 mM phosphate buffer, pH 6.5, to a total volume of 980 μl. The decrease of NADH during the conversion of aldehydes to alcohols was followed at 340 nm as a measure of the rate of aldehyde formation.

Protein concentrations were measured with the bicinchoninic acid method (Smith et al., 1985). Because β-mercaptoethanol disturbs this assay, it was removed by lyophilizing the samples and redissolving them in water.

For the kinetic measurements HPO-lyase activity was determined spectrophotometrically by measuring the decrease in absorbance at 234 nm due to the cleavage of α-13-HPOT. For final substrate concentrations lower than 40 μM, 20 μl of an α-13-HPOT solution in methanol was mixed with 880 μl, 50 mM phosphate buffer, pH 6.5 and 100 μl of tomato HPO-lyase in a 1 cm cuvette. Higher substrate concentrations (up to 350 μM) were measured in a 2 mm cuvette. Because of the smaller volume of this cuvette, the reaction mixture was halved leaving the ratio methanol/buffer/enzyme unchanged.

For measurements that were done at a constant substrate concentration, 200 μM α-13-HPOT was used, with the exception of the inhibition measurements with compounds that also absorb at 234 nm. The optical limitations of the spectrophotometer (HP 8452A, diode array) required a substrate concentration $\leq$ 100 μM when measuring the effect of 2*E*-hexenal, and $\leq$80 μM during the experiments with desferal, metyrapone, NDGA, *n*-propyl gallate and butylated hydroxyanisole.

3.5. Product identification

For the identification of volatiles 7.5 ml of a 100 μM solution of α-13-HPOT or 13-HPOD in 50 mM phosphate buffer, pH 6.5, was prepared. Immediately after starting the reaction by adding 500 μl of HPO-lyase (4 nkat), the reaction vessel (20 ml) was closed with a septum, and a solid phase micro extraction (SPME) fiber, coated with 100 μm polydimethylsiloxane (Supelco Inc., Bellefonte, USA), was placed above the reaction mixture. Volatiles were collected in the headspace for 30 min, whereupon they were desorbed from the fiber at 200°C for 1 min in the injection port of a GC/MS (Fisons GC 8000 series and Fisons Instruments MD 800 MassLab spectrometer). The temperature program was started at the same time as the desorption: it was kept at 35°C for 2 min and then raised at 10°C/min to 200°C. The column used was an HP-Innowax, (0.25 μm film thickness; 30 m × 0.32 mm i.d.; Hewlett-Packard). Eluted compounds were compared to reference aldehydes or alcohols which were collected on the SPME fiber (1 μl in 8 ml buffer) and analyzed with the same method.

Non-volatiles were analyzed from identical incubations which were now stopped after 30 min by acidifying with HCl to pH 4.0. The products were extracted with an octadecyl solid-phase extraction column, rinsed with water to remove salts and then eluted with methanol to obtain the products. The solvent was evaporated to remove traces of water. The residue was redissolved in methanol and the compounds were reduced by addition of an excess of $NaBH_4$ at 0°C. After 30 min, 50 ml water was added, the reaction mixture was acidified until a precipitate appeared and the reduced products were extracted with an octadecyl solid-phase extraction column as described above. The solvent was evaporated and the fatty acids were esterified with ethereal diazomethane. After 30 min, ether and diazomethane were removed with a nitrogen flow and replaced by a few drops of silylating reagent (pyridine/1,1,1,3,3,3-hexamethyldisilazane/chlorotrimethylsilane 5:1:1 v/v/v).

GC/MS analysis of the compounds was performed on the same apparatus as the volatiles, for this purpose equipped with a DB-1 column (0.25 μm film thickness; 30 m × 0.32 mm i.d.; J and W Scientific) using a temperature program from 140°C (2 min), rising at 6°C/min to 280°C (2 min). All mass spectra were recorded under electron impact with an ionization energy of 70 eV.

Acknowledgements

This work was supported by Unilever N.V. and a postdoctoral grant from the European Union to Manuela Pérez-Gilabert: No FAIR-CT 96-5010. The authors thank G. van Zadelhoff for his support with the headspace analyses, I. van Holsteijn for her help with recording the CO-difference spectrum and R. van Veen for conducting the AAS-measurements.

References

Coggon, P., Romanczyk, L. J., & Sanderson, G. W. (1977). Extraction, purification, and partial characterization of a tea metalloprotein and its role in the formation of black tea aroma constituents. *Journal of Agricultural and Food Chemistry*, *25*, 278–283.

Croft, K. P. C., Juttner, F., & Slusarenko, A. J. (1993). Volatile products of the lipoxygenase pathway evolved from *Phaseolus vulgaris* (L) leaves inoculated with *Pseudomonas syringae pv-Phaseolicola*. *Plant Physiology*, *101*, 13–24.

De Pinto, M. C., & Barceló, A. R. (1996). Inhibition of both peroxidase and laccase by desferal (desferrioxamine mesylate). *Phytochemistry*, *42*, 283.

Eglinton, G., Hunneman, D. H., & McCormick, A. (1968). Gas chromatographic-mass spectrometric studies of long chain hydroxy acids. *Organic Mass Spectrometry*, *1*, 593–611.

Elshof, M. B. W., Janssen, M., Veldink, G. A., & Vliegenthart, J. F. G. (1997). Biocatalytic large-scale production of 13(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid from hydrolysed safflower oil by a crude soybean flour extract as lipoxygenase source. *Recueil des Travaux Chimiques des Pays-Bas*, *115*, 499–504.

Galliard, T., & Matthew, J. A. (1977). Lipoxygenase-mediated cleavage of fatty acids to carbonyl fragments in tomato fruits. *Phytochemistry*, *16*, 339–343.

Galliard, T., Matthew, J. A., Wright, A. J., & Fishwick, M. J. (1977). The enzymic breakdown of lipids to volatile and non-volatile carbonyl fragments in disrupted tomato fruits. *Journal of Science of Food and Agriculture*, *28*, 863–868.

Gardner, H. W. (1991). Recent investigations into the lipoxygenase pathway of plants. *Biochimica et Biophysica Acta*, *1084*, 221–239.

Gardner, H. W., & Plattner, R. D. (1984). Linoleate hydroperoxides are cleaved heterolytically into aldehydes by a Lewis acid in aprotic solvent. *Lipids*, *19*, 294–299.

Gardner, H. W., Dornbos, D. L., & Desjardins, A. E. (1990). Hexanal, trans-2-hexenal, and trans-2-nonenal inhibit soybean, *Glycine-max*, seed germination. *Journal of Agricultural and Food Chemistry*, *38*, 1316–1320.

Hatanaka, A. (1993). The biogeneration of green odour by green leaves. *Phytochemistry*, *34*, 1201–1218.

Hatanaka, A., Kajiwara, T., Matsui, K., & Kitamura, A. (1992). Expression of lipoxygenase and hydroperoxide lyase activities in tomato fruits. *Zeitschrift für Naturforschung*, *47c*, 369–374.

Hatanaka, A., Kajiwara, T., Sekiya, J., & Toyota, H. (1986). Oxygen incorporation in cleavage of ^{18}O-labeled 13-hydroperoxylinoleyl alcohol into 12-hydroxy-3(*Z*)-dodecenal in tea chloroplasts. *Zeitschrift für Naturforschung*, *41C*, 359–362.

Hsieh, R. J. (1994). Contribution of lipoxygenase pathway to food flavors. In C.-T. Ho, & T. G. Hartman, *Lipids in food flavors* (558 ed) (pp. 30–48). Washington, DC: American Chemical Society.

Jadhav, S., Singh, B., & Salunkhe, D. K. (1972). Metabolism of unsaturated fatty acids in tomato fruit: linoleic and linolenic acid as precursors of hexanal. *Plant Cell Physiology*, *13*, 449–459.

Koshio, K., Takahashi, H., & Ota, Y. (1995). Induction of browning of male flowers of Cryptomeria japonica by treatment with fatty acids: mechanism and the role of trans-2-hexenal. *Plant Cell Physiology*, *36*, 1511–1517.

Matsui, K., Kajiwara, T., & Hatanaka, A. (1992). Inactivation of tea leaf hydroperoxide lyase by fatty acid hydroperoxide. *Journal of Agricultural and Food Chemistry*, *40*, 175–178.

Matsui, K., Shibutani, M., Hase, T., & Kajiwara, T. (1996). Bell pepper fruit fatty acid hydroperoxide lyase is a cytochrome P450 (CYP74B). *FEBS Letters*, *394*, 21–24.

Olías, J. M., Pérez, A. G., Ríos, J. J., & Sanz, L. C. (1993). Aroma of virgin olive oil-biogenesis of the green odor notes. *Journal of Agricultural and Food Chemistry*, *41*, 2368–2373.

Phillips, D. R., Matthew, J. A., Reynolds, J., & Fenwick, G. R. (1979). Partial purification and properties of a *cis*-3:*trans*-2-enal isomerase from cucumber fruit. *Phytochemistry*, *18*, 401–404.

Rutten, A. A. J. J. L., Falke, H. E., Catsburg, J. F., Topp, R., Blaauboer, B. J., van Holsteijn, I., Doorn, L., & van Leeuwen, R. F. X. (1987). Interlaboratory comparison of total cytochrome P-450 and protein determinations in rat liver chromosomes. *Archives of Toxicology*, *61*, 27–33.

Schauenstein, E., Esterbauer, H., & Zollner, H. (1977). α,β-Unsaturated aldehydes. In J. R. Lagnado, *Aldehydes in biological systems: their natural occurrence and biological activities* (pp. 25–102). London: Pion.

Schreier, P., & Lorenz, G. (1982). Separation, partial purification and characterization of a fatty acid hydroperoxide cleaving enzyme from apple and tomato fruits. *Zeitschrift für Naturforschung*, *37c*, 165–173.

Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K.,

Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., & Klenk, D. C. (1985). Measurement of protein using bicinchoninic acid. *Analytical Biochemistry*, *150*, 76.

Suurmeijer, C. N. S. P., Pérez-Gilabert, M., van der Hijden, H. T. W. M., Veldink, G. A., & Vliegenthart, J. F. G. (1998). Purification, product characterization and kinetic properties of soluble tomato lipoxygenase. *Plant Physiology and Biochemistry*, *36*, 657–663.

Testa, B., & Jenner, P. (1981). Inhibitors of cytochrome P-450s and their mechanism of action. *Drug Metabolism Reviews*, *12*, 1–117.

Thomas, P. E., Ryan, D., & Levin, W. (1976). An improved staining procedure for the detection of the peroxidase activity of cytochrome P-450 on sodium dodecyl sulfate polyacrylamide gels. *Analytical Biochemistry*, *75*, 168–176.

Veldink, G. A., & Vliegenthart, J. F. G. (1989). Substrates and products of lipoxygenase catalysis. In Atta-ur-Rahman, *Studies in natural products chemistry*, *vol. 9* (pp. 559–589). Amsterdam: Elsevier Science Publishers BV.

Vick, B. A., & Zimmerman, D. C. (1987). Oxidative systems for modification of fatty acids: the lipoxygenase pathway. In P. K. Stumpf, *The biochemistry of plants — A comprehensive treatise*, *vol. 9* (pp. 53–90). New York: Academic Press.

Vick, B. A. (1991). A spectrophotometric assay for hydroperoxide lyase. *Lipids*, *26*, 315–320.

Vick, B. A., & Zimmerman, D. C. (1976). Lipoxygenase and hydroperoxide lyase in germinating watermelon seedlings. *Plant Physiology*, *57*, 780–788.

Whitehead, I. M., Muller, B. L., & Dean, C. (1995). Industrial use of soybean lipoxygenase for the production of natural green note flavor compounds. *Cereal Foods World*, *40*, 193–197.

Zeringue, H. J. (1992). Effects of C6–C10 alkenals and alkanals on eliciting a defence response in the developing cotton boll. *Phytochemistry*, *31*, 2305–2308.