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Purification, product characterization and kinetic properties of soluble tomato lipoxygenase

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Abstract – Soluble lipoxygenase (EC 1.13.11.12) from tomato fruits (*Lycopersicon esculentum*, var. Trust) was purified to apparent homogeneity as judged by SDS-PAGE, and the products and kinetics of the enzyme were studied in order to clarify the contradictory results that were obtained with a less purified enzyme. The specific activity of the enzyme, 668 nkat·mg^{-1} protein, was determined spectrophotometrically at pH 6.8 with a 150 mM linoleic acid solution containing 0.5 % methanol. Product analysis revealed that the enzyme mainly formed 9-hydroperoxy-(10*E*,12*Z*)-octadecadienoic acid (96 %) from linoleic acid with an enantiomeric excess of 82 % *S*. The 13-hydroperoxy-(9*Z*,11*E*)-octadecadienoic acid formed (1 %) was racemic. Hydroxides and oxodienoic acids were negligible. Because these products were not observed under anaerobic conditions either, it was concluded that the pigment bleaching reported for tomato lipoxygenase does not result from release of intermediate free radicals to initiate the process of carbonyl formation. The influence of Tween-20 on the tomato enzyme was comparable to that of detergents on soybean lipoxygenase-1. A study of the variation of linoleic acid concentration between 0 and 20 mM in the absence of any detergent resulted in a typical Michaelis-Menten curve with a K_m of 4.1 mM and a V_{max} of 7.4 mM·min^{-1}·mg^{-1} protein. The induction period of the progress curve of soluble tomato lipoxygenase was abolished by incubating the enzyme with 9-hydroperoxy-(10*E*,12*Z*)-octadecadienoic acid or with 13-hydroperoxy-(9*Z*,11*E*)-octadecadienoic acid. © Elsevier, Paris

Detergents /K_m / lipoxygenase / *Lycopersicon esculentum* / product specificity / purification

EDTA, ethylene diamine tetraacetic acid / HOAc, acetic acid / HOD, hydroxy-octadecadienoic acid / HPOD, hydroperoxy-octadecadienoic acid / MeOH, methanol / sTomlox, soluble tomato lipoxygenase / THF, tetrahydrofuran

1. INTRODUCTION

Lipoxygenases (EC 1.13.11.12) catalyse the dioxygenation of fatty acids which contain one or more 1(*Z*), 4(*Z*)-pentadiene systems, yielding chiral (*E,Z*) conjugated hydroperoxy fatty acids [31]. These enzymes are ubiquitous in plants, have been found in mammals and have further been reported to occur in fungi [14], algae [2] and other micro-organisms. The functional role of lipoxygenases in plants is still largely unknown although metabolites of unsaturated fatty acids have been implicated in growth and development, plant senescence and in response to diseases and wounding [23]. Furthermore, in fruits and other plant food products, lipoxygenases play a role in the formation of volatile flavour compounds [10, 11, 19].

Most data concerning the structure and the mechanism of lipoxygenase originate from soybean lipoxygenase-1, because it is abundant in the seeds, easy to purify and its pH optimum (9.0) facilitates preparation of aqueous solutions of substrates. Besides the dioxygenase activity of the enzyme, soybean lipoxygenase-1 shows an interesting hydroperoxidase activity under anaerobic conditions. Feiters et al. [9] described the mechanism of this reaction in which the product hydroperoxide serves as the second substrate instead of oxygen and is converted into an oxodienoic acid. For soybean lipoxygenase-1, only 13-hydroperoxy-octadecadienoic acid (13-HPOD) could serve as the second substrate [32]. The same mechanism may also hold for the co-oxidation of plant pigments by lipoxygenase-1 (bleaching activity) under anaerobic

conditions [9]. The free radicals that dissociate from the enzyme in the anaerobic cycle, or the enzyme-radical complex itself, are thought to be scavenged by these pigments, e.g. β-carotene. The pigments are then cleaved in a radical reaction [16].

Type-2 lipoxygenase from soybeans (pH optimum 6.5) was reported to show little hydroperoxidase activity under anaerobic conditions [26]. However, in the presence of oxygen, this iso-enzyme forms oxodienoic acids and also shows a pigment bleaching activity [7]. The difference between the hydroperoxidase activities of lipoxygenase-1 and lipoxygenase-2 was ascribed to a varying stability of the enzyme-fatty acid free radical complex for the different iso-enzymes, by Veldink et al. [32]. Furthermore, a lower rate of dioxygenation at the active center could result in a more prominent release of fatty acid radicals.

Because of the low solubility of the substrates of lipoxygenase, detergents are often used in assays [1, 18, 29]. In a recent study, Schilstra et al. [25] showed that the inhibition or stimulation of the soybean lipoxygenase-1 and -2 reactions by detergents was the result of a physico-chemical interaction of these compounds with the substrate rather than a direct interaction with the enzyme itself. Thus, detergents in the assay affect the concentration of substrate available for lipoxygenase.

The presence of lipoxygenase in tomatoes was first reported by Matthew et al. [17]. So far, only a membrane-associated tomato lipoxygenase has been completely purified [4]. Soluble tomato lipoxygenase (sTomlox) has been investigated by several groups [3, 5, 8, 21, 22, 28, 35]. Although different purification methods were designed, the enzyme was never completely purified. It was reported [22] that anion-exchange chromatography was more appropriate for purifying tomato lipoxygenase than hydrophobic-interaction chromatography both in yield and purification factor. With a Mono-Q column sTomlox was purified 311-fold to a specific activity of 1 298 nkat·mg^{-1}, although these results could only be obtained when the elution was performed with a discontinuous NaCl gradient. More recently, Smith et al. [28] reported that 0.1 % Triton X-100 is required for effective chromatography of sTomlox; however, the procedure described did not result in a higher purification factor than that obtained by Regdel et al. [22].

The molecular mass of sTomlox was estimated by SDS-PAGE combined with western blotting and reported to be either 87 kDa [3], 92 kDa [8] or 95 kDa [22]. Smith et al. [28] estimated the molecular mass of 4 isoforms of tomato lipoxygenase to be 97 kDa by SDS-PAGE using in-gel lipoxygenase activity staining. The pH optimum of sTomlox is 6.8. At this pH, sTomlox converts both linoleic and α-linolenic acid at similar rates [21, 35] and forms specifically the 9*S*-hydroperoxide. A yield of 96 % 9*S*-HPOD was reported by Matthew et al. [17] and Zamora et al. [35].

Like soybean lipoxygenase-2, sTomlox was reported to bleach β-carotene and other carotenoids under aerobic conditions [5, 34]. However, little is known about the products formed from β-carotene during this process.

K_m values reported for sTomlox range from 15 nM to 1.42 mM [3, 4, 5, 13, 22, 34], but these studies were conducted with various reaction mixtures, most containing a detergent [25].

Here, an improvement of the purification of sTomlox, based on anion-exchange chromatography, is reported. The enzyme products from linoleic acid are analysed with respect to regio- and stereospecificity, and the co-oxidation reaction is discussed in relation to these products. To clarify the broad range of K_m values reported for the enzyme, the influence of Tween-20 on the enzyme kinetics is described and the kinetic parameters of the enzyme are determined in the absence of any detergent.

2. RESULTS

2.1. Enzyme purification

Results of the purification of sTomlox are summarized in *table I*. sTomlox was purified 2 000 times over a Resource-Q column (*figure 1*) to a specific activity of 267 nkat·mg^{-1} protein. This column step was highly reproducible.

To obtain an essentially homogeneous enzyme solution, the Resource-Q fractions were further purified by Phenylsepharose-HP chromatography to a specific activity of approximately 668 nkat·mg^{-1} protein. The highest specific activity measured in an electrophoretically homogeneous fraction with linoleic acid as substrate was 802 nkat·mg^{-1} protein. A silver-stained SDS-PAGE gel only showed a lipoxygenase band at 95 kDa in this case. However, in some preparations, a minor band at 65 kDa was found (*figure 2*). The third protein band visible in *figure 2* appeared in protein-free lanes as well and was therefore considered an artefact.

Table I. Purification of lipoxygenase from 2 kg of tomato fruits. Fractions are described in the Methods section.

Fraction	Total activity (nkat)	Total protein (mg)	Specific act. (nkat·mg^{-1})	Purification (fold)	Yield (%)
Starting material	4 910	39 900	0.12	1	100
Supernatant 35 % $(NH_4)_4SO_4$	6 346	32 000	0.20	1.6	129
Pellet 60 % $(NH_4)_2SO_4$	4 442	1 350	0.33	27	90
Resource Q	1 386	5.3	267	2 162	28
Phenylsepharose	701	0.9	668	6 487	14

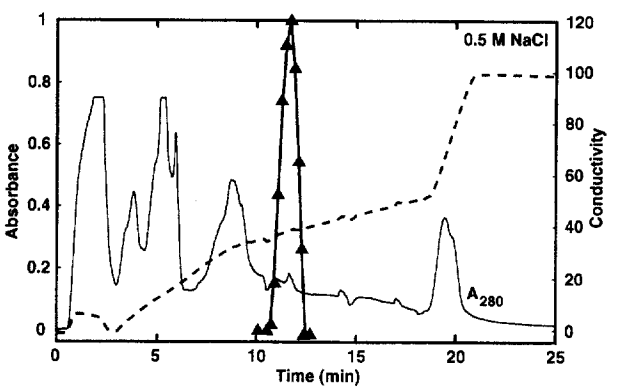

Figure 1. Purification of soluble tomato lipoxygenase over a Resource-Q column. Protein was measured at 285 nm and the NaCl gradient was monitored by a conductivity metre. Triangles represent the lipoxygenase activity as the increase in absorbance at 234 nm·min^{-1} after spectrophotometric screening (maximum value: 2.4 AU·min^{-1}).

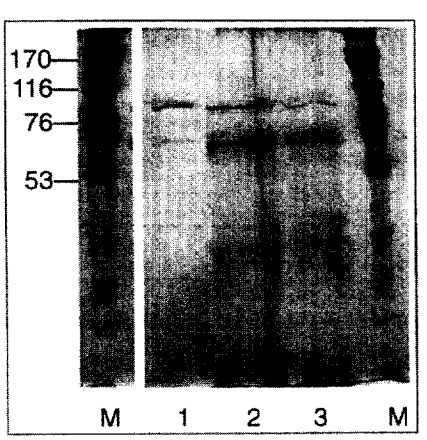

Figure 2. Silver-stained, 7.5 % SDS-PAGE gel of purified sTomlox. Lanes: 1, Phenylsepharose fraction (5 µg protein); 2, Resource-Q, pool 1 (10 µg protein); 3, Resource-Q, pool 2 (10 µg protein); m, molecular mass marker proteins.

The purified enzyme was stored in phosphate buffer pH 6.5 at 4 °C. The activity remained stable for a week, but after two months, it had decreased to about 50 %. At room temperature, the enzyme lost its activity within a day.

2.2. Product specificity of tomato lipoxygenase

The reaction products of tomato lipoxygenase obtained with linoleic acid were analysed by reversed-phase HPLC on a C18-column (*figure 3*). The major product (96 %) was 9-*EZ*-HPOD, while only 1 % 13-*ZE*-HPOD was formed.

Hydroperoxides were reduced with $NaBH_4$, and the hydroxides formed were converted into methyl esters followed by purification on straight-phase HPLC. Then, these compounds were separated into their enantiomers. The enantiomeric excess was 82 % *S* for 9-*EZ*-HPOD and 4 % *R* for 13-*ZE*-HPOD; the amount of 9-*EZ*-HOD formed in the enzymatic reaction (0.7 %) was considered negligible.

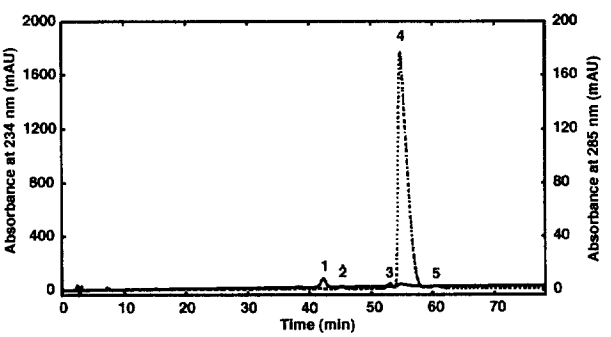

Figure 3. HPLC analysis of the products obtained from linoleic acid with soluble tomato lipoxygenase. After incubation, the products were extracted on an octadecyl solid-phase extraction column and injected on a reversed-phase column eluted with tetrahydrofuran/methanol/water/acetic acid (25/30/45/0.1, v/v/v/v). The chromatogram was recorded at 234 nm to detect hydroperoxides and hydroxides (dotted line) and at 285 nm to detect oxodienoic acids (solid line). Peak identification: 1, oxo-octadecadienoic acid; 2, 9-hydroxy-(10*E*,12*Z*)-octadecadienoic acid; 3, 13-hydroperoxy-9*Z*,11*E*)-octadecadienoic acid; 4, 9-hydroperoxy-(10*E*,12*Z*)-octadecadienoic acid; 5, *E*,*E*-hydroperoxides.

It can be seen from the absorbance at 285 nm (*figure 3*) that hardly any oxodienoic acids were formed by sTomlox. Their formation could not be increased by depletion of oxygen in the reaction mixture nor by varying the pH of the reaction mixture from pH 4.0 to 8.0 (results not shown).

2.3. Kinetics

In spectrophotometric studies of the lipoxygenase-catalysed reaction at neutral pH the low solubility of fatty acids is a common problem. One way of overcoming visual turbidity is to use detergents (e.g. Tween-20) that form micelles which solubilize and optically clear these fatty acid suspensions [29]. Detergents can be added to the substrate [1], or to the buffer [18]. Although both methods effectively prevented turbidity, the results obtained, when the variation of the rate of catalysis with the substrate concentration was studied, were found to depend on the reaction medium (*figure 4*). Furthermore, adding Tween-20 to the cuvette resulted in a sigmoidal response (*figure 4*). The same experiment was carried out dissolving linoleic acid in methanol and using 0.1 M phosphate buffer (pH 6.8) without the addition of detergent. The final concentration of methanol was 1 %, and the concentration of linoleic acid was kept below 20 μM to avoid misinterpretations due to the simultaneous presence of monomers and micelles [25]. A typical Michaelis-Menten curve was obtained while scattering was low. The experimental data were fitted by non-linear regression to the standard Michaelis-Menten equation, yielding a K_m value of 4.1 μM and a V_{max} of 7.4 $\mu M \cdot min^{-1} \cdot mg^{-1}$ protein. Further kinetic assays were performed with the last method because clear solutions were obtained within the range of concentrations used.

The reaction catalysed by sTomlox displayed the characteristic induction period of other lipoxygenases (*figure 5*), but the lag was found to be longer than that observed for lipoxygenase-1 from soybeans [24]. The lag period could be abolished by pre-incubating the enzyme with its reaction product, 9-*EZ*-HPOD, or by 13-*ZE*-HPOD (obtained from the reaction of soybean lipoxygenase-1) (*figure 5*). Furthermore, the enzyme concentration affected the length of the lag period, being shorter at higher enzyme concentrations (*figure 6*), but never completely absent. A plot of 1/lag against the enzyme concentration shows a linear relation for the lipoxygenase concentrations used (*figure 6*). The same relationship between enzyme concentration and 1/lag was observed at low concentrations of soybean lipoxygenase-1 [24].

3. DISCUSSION

3.1. Purification

Reproducible 2 000-fold purification of sTomlox could be achieved giving a 28 % yield and a specific activity of 267 $nkat \cdot mg^{-1}$ protein by Resource-Q chromatography. The tomato lipoxygenase was further purified over a hydrophobic Phenylsepharose-HP column to a specific activity of approximately 668 $nkat \cdot mg^{-1}$ protein (*table I*). The nature of the second protein band at 65 kDa (*figure 2*), which was found in some fractions, is as yet unclear.

The problems experienced by Regdel et al. [22] with mono-Q chromatography did not occur when the 60 % pellet described here was used for anion-exchange

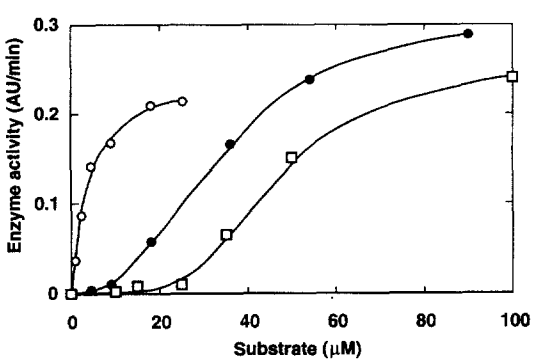

Figure 4. Effect of substrate variation using different reaction media. (□), Sodium linoleate and Tween-20 (1/1, w/w); (●), 0.005 % (w/v) Tween-20 in the buffer; (○), no detergent. All reaction media contained 0.1 M phosphate buffer (pH 6.8), 0.14 nkat tomato lipoxygenase and different concentrations of substrate.

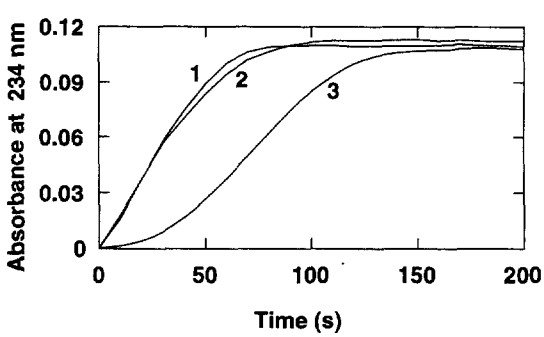

Figure 5. Effect of 13- and 9-HPOD on the induction period of tomato lipoxygenase. The enzyme (0.14 nkat) was incubated for 3 min with 2 mM 9-HPOD (1) or 5 mM 13-HPOD (2) prepared using soybean lipoxygenase-1. Curve (3) corresponds to the same reaction without the addition of HPOD. The reaction was started by the addition of 9 mM linoleic acid dissolved in methanol.

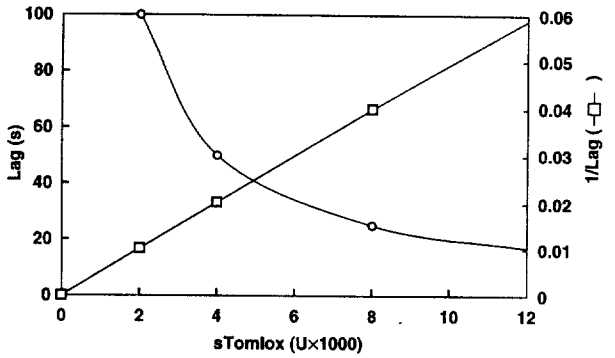

Figure 6. Effect of enzyme concentration on the duration of the lag period. (○), Lag; (○), 1/lag. The reaction medium contained 18 mM linoleic acid (dissolved in methanol) and different amounts of soluble tomato lipoxygenase in 0.1 M phosphate buffer (pH 6.8).

chromatography (preliminary results, not shown). A possible explanation for the different behaviour observed here might be that the pectins were removed because they interfered with the ammonium sulfate precipitation [5]. The higher specific activity found for sTomlox is most probably caused by differences in the lipoxygenase assays used [22].

The higher total activity in the 35 % ammonium sulfate supernatant compared to the starting material (*table I*) could be caused by removal of a lipoxygenase-inhibiting compound in this step. However, this compound was not identified in this study.

3.2. Product specificity

Analysis of the products of sTomlox showed that 9*S*-*EZ*-HPOD was the major product, with only small amounts of 9*R*-*EZ*-HPOD, 13-*ZE*-HPOD (racemic), *EE*- compounds and hydroxides formed. These results agree with earlier findings that 9*S*-*EZ*-HPOD is the main product of tomato lipoxygenase [21]. Furthermore, the enantiomeric composition of the 13-*ZE*-HPOD supports the suggestion that this product is not enzymatically formed [17].

The absence of oxodienoic acids as reaction products under both aerobic and anaerobic circumstances is remarkable since tomato lipoxygenase was reported to show a carotene bleaching activity [5, 34]. For soybean lipoxygenases-1 and -2 this pigment bleaching activity was related to carbonyl formation [7, 31]. The results presented in this paper suggest that intermediate fatty acid radicals do not appreciably dissociate from sTomlox. This mechanism, by which carbonyls are formed [9], leaves the enzyme in the inactive Fe(II) state. Recently, Pérez-Gilabert et al. [20] reported that the enzyme in the Fe(III) state did not lose activity in the presence of H_2O_2, but was significantly inactivated in the Fe(II) state, supporting the suggestion that sTomlox does not exhibit significant radical leakage.

3.3. Kinetics

The data presented in *figure 4* confirm the observation that the influence of detergents on the lipoxygenase reaction is due to their interaction with substrates [25]. Both the addition of a constant amount of Tween-20 to the buffer and its use to prepare the substrate solution gave rise to a sigmoidal curve. The difference between the two curves can be explained by the fact that in the first case, the concentration of detergent is kept constant during the assay, while if the method of Axelrod et al. [1] is used, the amount of Tween-20 increases with the substrate concentration. Besides, a higher affinity of the enzyme for linoleic acid than for sodium linoleate cannot be excluded. Upon varying the substrate concentration without the addition of detergent to the reaction medium, a typical Michaelis-Menten curve was obtained. Bearing in mind that the presence of detergent decreases the free fatty acid concentration, these results could indicate that tomato lipoxygenase has a higher affinity for the monomeric form of linoleic acid than for fatty acids incorporated in micelles.

Like soybean lipoxygenase-1, sTomlox showed a kinetic lag phase. This lag was longer than that of soybean lipoxygenase-1 and therefore, it could be studied without the use of stopped-flow techniques. The duration of the lag was inversely proportional to the enzyme concentration suggesting that the enzyme follows the steady state approximation proven valid for low concentrations of soybean lipoxygenase-1 [24]. Furthermore, the lag of sTomlox was not only abolished by the product of its reaction but also by 13-*ZE*-HPOD.

4. METHODS

4.1. Enzyme purification. Breaker stage tomatoes (*Lycopersicon esculentum*, variety 'Trust') were bought from a local greenhouse and homogenized as described by Bonnet and Crouzet [3] with a slight modification. Two kg tomatoes were homogenized in 0.5 M Tris-HCl (pH 8.0) containing 1 % (w/v) ascorbic acid, 1 % (w/v) EDTA and 1 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 30 000 × *g* for 10 min. The supernatant was brought to 20 mM $CaCl_2$ by addition of a 1 M stock solution and after stirring for 2 h, the suspension was centrifuged at

30 000 × *g* for 20 min. The supernatant (referred further as starting material) could be stored at −20 °C for several months without significant loss of enzyme activity. For further purification, solid ammonium sulfate was added to the supernatant and the fraction which precipitated between 35 % and 60 % saturation at 4 °C (referred to as 60 % pellet) was collected and dialysed against 10 mM MOPS-KOH, pH 6.8. The dialysed fraction was clarified by centrifugation at 10 000 × *g* for 10 min. In addition, the protein was filtered through a folded paper filter just before FPLC-chromatography (Pharmacia LCC-501 plus). Aliquots of 150 mg protein were loaded onto a 6 mL Resource-Q column (Pharmacia) equilibrated with 10 mM MOPS-KOH (pH 6.8) at 6 mL·min^{-1}. Absorbed proteins were eluted with a NaCl gradient from 0 to 500 mM NaCl. Active fractions were combined and concentrated to a volume of 7.5 mL in a Centriprep-30 (Amicon, 15 mL). Before applying the sample to a 1-mL Phenylsepharose-HP column (Pharmacia), 2.5 mL of 50 mM phosphate buffer (pH 6.5) saturated with ammonium sulfate was added. The lipoxygenase activity was eluted with a linear gradient from 25 % ammonium sulfate saturation to 0 %. During purification, the protein was kept at 4 °C or on ice except while it was eluting from the columns.

4.2. Lipoxygenase assay. Column fractions were assayed routinely as follows: 5 µL of a 30 mM linoleic acid solution in methanol was mixed in a cuvette with 970 µL of 100 mM phosphate buffer (pH 6.8). The reaction was started by adding 25 µL of the enzyme solution and the increase in absorbance at 234 nm was measured at room temperature. Enzyme activities (in nkatal) were calculated from the linear part of the curve. Protein concentrations were determined by measuring the absorbance at 280 nm and multiplying by 0.71 to give the protein concentration in mg·mL^{-1} [6]. Before column chromatography, a correction was made for contaminating DNA and RNA as described by Kalckar [15]. Samples without ammonium sulfate yielded the same protein concentrations with the bicinchoninic acid method [27]. For the kinetic measurements, different reaction media were tested to avoid light scattering problems due to the low solubility of fatty acids at pH 6.8: (i) mixing equal amounts (w/w) of Tween-20 and linoleic acid and adding NaOH until the solution is clear [1]; (ii) adding Tween-20 (0.005 % w/v) to the buffer and dissolving linoleic acid in methanol; (iii) dissolving linoleic acid in methanol without the addition of detergent to the buffer. The third method was chosen to perform the rest of the kinetic experiments because the low K_m displayed by the enzyme under these conditions made the use of detergents to clarify the solution unnecessary. The linoleic acid concentration was kept below 20 µM and the concentration of methanol in the assay was 1 % (v/v). The induction period was defined as the graphical extrapolation of the linear part of the product accumulation curve to the time axis. All optical measurements were performed on a HP 8452A diode array spectrophotometer (Hewlett-Packard).

4.4. Synthesis and analysis of lipoxygenase products. For product analysis 50 mL of a 100 µM solution of linoleic acid in 100 mM phosphate (pH 6.8) containing 1 % methanol was incubated with 5 nkat purified tomato lipoxygenase. The reaction was allowed to continue for 1 h at 0 °C in a 100 % oxygen atmosphere. Then, it was stopped by acidification to pH 3.0 with 2 N HCl, and the products were extracted with an octadecyl solid-phase extraction column eluted with methanol. The solvent was evaporated, the residue was dissolved in 0.5 mL methanol and the products were analysed with a HP 1090 Liquid Chromatograph on a Cosmosil 5 C18 AR-column (Nacalai Tesque, 4.6 × 250 mm). Compounds were detected with a HP 1040A diode array UV detector (Hewlett-Packard) and data was processed on a HP 7994A analytical work station (Hewlett-Packard). Products were eluted isocratically with tetrahydrofuran/methanol/H_2O/acetic acid (25/30/45/0.1, v/v/v/v) at a flow rate of 1 mL·min^{-1}. This method allows separation of hydroperoxides, hydroxides and oxodienoic acids in a single run without derivatization. Relative amounts of the different products were calculated from the peak areas, assuming a molar absorbance of 25 000 M^{-1}·cm^{-1} at 234 nm for hydroperoxides and hydroxides and of 22 000 M^{-1}·cm^{-1} at 285 nm for oxodienoic acids [33]. For the enantiomeric analysis, the products were reduced with an excess of $NaBH_4$ (in methanol, 0 °C). After 20 min, 50 mL water was added, the reaction mixture was acidified with 2 N HCl until a precipitate appeared and the reduced products were extracted with an octadecyl solid-phase extraction column. The solvent was evaporated and the residue was dissolved in 2 mL ether. The products were esterified with ethereal diazomethane and purified over a straight-phase CP-sphere Si column (Chrompack, 5 µm particles, 4.6 × 250 mm) eluted with n-hexane/diethylether/HOAc (90/10/0.1, v/v/v) [30] at a flow of 2 mL·min^{-1}. The absolute configurations of the resulting methylesters of 9-hydroxy-(10*E*,12*Z*)-octadecadienoic acid (9-*EZ*-HOD) and 13-*ZE*-HOD were determined by chiral-phase HPLC on a Chiralcel OD-R column (J.T. Baker). Compounds were eluted with methanol/water/acetic acid (85/15/0.1 v/v/v) [12].

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REFERENCES

[1] Axelrod B., Cheesbrough T., Laakso S., Lipoxygenase from soybeans, Method Enzymol. 71 (1981) 441–451.
[2] Beneytout J.-L., Andrianarison R.-H., Rakotoarisoa Z., Tixier M., Properties of a lipoxygenase in green algae (*Oscillatoria* sp.), Plant Physiol. 91 (1989) 367–372.
[3] Bonnet J.-L., Crouzet J., Lipoxygenase from tomato fruit: partial purification and study of some properties, J. Food Sci. 42 (1977) 625–628.
[4] Bowsher C.G., Ferrie B.J.M., Ghosh S., Todd J., Thompson J.E., Rothstein S.J., Purification and partial

characterization of a membrane-associated lipoxygenase in tomato fruit, Plant Physiol. 100 (1992) 1802–1807.

[5] Cabibel M., Nicolas J., Lipoxygenase from tomato fruit (*Lycopersicon esculentum* L.) partial purification, some properties and in vitro cooxidation of some carotenoid pigments, Sci. Aliment. 11 (1991) 277–290.

[6] Christopher J.P., Pistorius E.K., Axelrod B., Isolation of a third isoenzyme of soybean lipoxygenase, Biochim. Biophys. Acta 284 (1972) 54–62.

[7] Cohen B., Grossman S., Klein B.P., Pinsky A., Pigment bleaching by soybean lipoxygenase type-2 and the effect of specific chemical modifications, Biochim. Biophys. Acta 837 (1985) 279–287.

[8] Droillard M.-J., Rouet-Mayer M.-A., Bureau J.-M., Laurière C., Membrane-associated and soluble lipoxygenase isoforms in tomato pericarp, Plant Physiol. 103 (1993) 1211–1219.

[9] Feiters M.C., Boelens H., Veldink G.A., Vliegenthart J.F.G., Navaratman S., Allen J.C., Nolting H.-F., Hermes C., X-ray absorption spectroscopic studies on iron in soybean lipoxygenase: a model for mammalian lipoxygenases, Recl. Trav. Chim. Pays-Bas 109 (1990) 133–146.

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

[11] Hatanaka A., Kajiwara T., Matsui K., The biogeneration of green odour by green leaves and its physiological functions - past, present and future, Z. Naturforsch. 50c (1995) 467–472.

[12] Hilbers M.P., Kerkhoff B., Finazzi-Agrò A., Veldink G.A., Vliegenthart J.F.G., Heterogeneity and developmental changes of lipoxygenase in etiolated lentil seedlings, Plant Sci. 111 (1995) 169–180.

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

[14] Jensen E.C., Ogg C., Nickerson K.W., Lipoxygenase inhibitors shift the yeast/mycelium dimorphism in *ceratocystis ulmi*, Appl. Environ. Microbiol. 58 (1992) 2505–2508.

[15] Kalckar H.M., Differential spectrophotometry of purine compounds by means of specific enzymes, J. Biol. Chem. 167 (1947) 461–475.

[16] Klein B.P., Grossman S., King D., Cohen B.-S., Pinsky A., Pigment bleaching, carbonyl production and antioxidant effects during the anaerobic lipoxygenase reaction, Biochim. Biophys. Acta 793 (1984) 72–79.

[17] Matthew J.A., Chan H.W.-S., Galliard T., A simple method for the preparation of pure 9-D-hydroperoxides of linoleic acid and methyl linoleate based on the positional specificity of lipoxygenase in tomato fruit, Lipids 12 (1977) 324–326.

[18] Mulliez E., Leblanc J.P., Girerd J.J., Rigaud M., Chottard J.C., 5-Lipoxygenases from potato tubers, Biochim. Biophys. Acta 916 (1987) 13–23.

[19] Olías J.M., Pérez A.G., Ríos J.J., Sanz L.C., Aroma of virgin olive oil: biogenesis of the 'green' odor notes, J. Agric. Food Chem. 41 (1993) 2368–2373.

[20] Pérez-Gilabert M., Veldink G.A., Vliegenthart J.F.G., Protection by different agents against inactivation of lipoxygenase by hydrogen peroxide, Lipids 31 (1996) 1245–1250.

[21] Regdel D., Kühn H., Schewe T., On the reaction specificity of the lipoxygenase from tomato fruits, Biochim. Biophys. Acta 1210 (1994) 297–302.

[22] Regdel D., Schewe T., Kühn H., Tomato fruit lipoxygenase: isolation, partial purification, and interaction with biological membranes, Biochemistry (Moscow) 59 (1994) 581–586.

[23] Rosahl S., Lipoxygenases in plants - Their role in development and stress response, Z. Naturforsch. 51c (1996) 123–138.

[24] Schilstra M.J., Veldink G.A., Vliegenthart J.F.G., Kinetic analysis of the induction period in lipoxygenase catalysis, Biochemistry 32 (1993) 7686–7691.

[25] Schilstra M.J., Veldink G.A., Vliegenthart J.F.G., Effect of nonionic detergents on lipoxygenase catalysis, Lipids 29 (1994) 225–231.

[26] Siedow J.N., Plant lipoxygenase: structure and function, Annu. Rev. Plant Physiol. Plant Mol. Biol. 42 (1991) 145–188.

[27] 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., Measurement of protein using bicinchoninic acid, Anal. Biochem. 150 (1985) 76–85.

[28] Smith J.J., Linford R., Tucker G.A., Soluble lipoxygenase isoforms from tomato fruit, Phytochemistry 45 (1997) 453–458.

[29] Surrey K., Spectrophotometric method for determination of lipoxidase activity, Plant Physiol. 39 (1964) 65–69.

[30] Teng J.I., Smith L.L., High performance liquid chromatography of linoleic acid hydroperoxides and their corresponding alcohol derivatives, J. Chromatogr. 350 (1985) 445–451.

[31] Veldink G.A., Vliegenthart J.F.G., Lipoxygenases, Free Radical Biol. 5 (1982) 29–64.

[32] Veldink G.A., Vliegenthart J.F.G., Boldingh J., Plant lipoxygenases, Prog. Chem. Fats Other Lipids 15 (1977) 131–166.

[33] Verhagen J., Bouman A.A., Vliegenthart J.F.G., Boldingh J., Conversion of 9D- and 13-L-hydroperoxylinoleic acids by soybean lipoxygenase-1 under anaerobic conditions, Biochim. Biophys. Acta 486 (1977) 114–120.

[34] Zamora R., Macias P., Mesias J.L., Carotene cooxidation activity of tomato lipoxygenase-2, Die Nahrung 32 (1988) 965–969.

[35] Zamora R., Olías J.M., Mesias J.L., Purification and characterization of tomato lipoxygenase, Phytochemistry 26 (1987) 345–347.