ELSEVIER

Food Chemistry 66 (1999) 173–180

Food Chemistry

www.elsevier.com/locate/foodchem

Purification of hydroperoxide lyase from cucumbers

A.R. Hornostaj*, D.S. Robinson

Procter Department of Food Science, University of Leeds, Leeds LS2 9JT, UK

Received 2 August 1998; received in revised form and accepted 5 November 1998

Abstract

Hydroperoxide lyase (HPL) was extracted from cucumber fruit (*Cucumis sativus*) and purified by centrifugation, solubilization with detergent, ion-exchange chromatography and hydroxyapatite chromatography. 9-Hydroperoxy-linoleic acid and 13-hydroperoxy-linoleic acid lysing activities were purified 88-fold and 82-fold, respectively. The purified HPL preparation consisted of a single major band following SDS-electrophoresis with a molecular weight of about 55 000 Da; pH 6 was optimum for the lysis of both 9-hydroperoxy-linoleic acid and 13-hydroperoxy-linoleic acid substrates. The enzyme was relatively stable and retained more than two thirds of original activity after 3 weeks at 4°C, but lost half of its activity after 2 min at 50°C. Apparent K_m values for 9-hydroperoxy-linoleic acid, 9-hydroperoxy-linolenic acid, 13-hydroperoxy-linoleic acid and 13-hydroperoxy-linolenic acid were 6.76, 6.02, 5.46 and 12.4 μM respectively. Corresponding V_{max} values were 19.3, 12.0, 7.58 and 11.4 μmol min. The $V_{max}{}^{app}/K_m{}^{app}$ values for 9-hydroperoxy-linoleic acid, 9-hydroperoxy-linolenic acid, 13-hydroperoxy-linoleic acid and 13-hydroperoxy-linolenic acid were 2.86, 1.99, 1.39 and 0.92, respectively. It is suggested that cucumber mesocarp contains only one type of HPL which is able to more efficiently catalyse the lysis of the 9-acyl hydroperoxides and especially 9-hydroperoxy-linoleic acid.

1. Introduction

Plants have the ability to produce various volatile aldehydes and alcohols which give rise to characteristic flavours and odours. Damage to plants, wounding, cutting, etc., initiates a cascade of enzymic reactions, ending with the production of carbonyl compounds and their derivatives, which are known as the lipoxygenase (LOX) pathway. Enzymes and substrates, usually separated within intact cells, mix under these conditions causing the formation of volatile compounds due to sequential enzymic reactions.

Lipases and hydrolases liberate free linoleic and linolenic acids from lipids. These polyunsaturated fatty acids (PUFAs) are converted by LOX into hydroperoxides which, in plants, are cleaved by hydroperoxide lyase (HPL) to give C_6- and C_9-volatile flavour compounds together with ω-oxoacids. HPL lysis of 13-hydroperoxides yields C_6-volatile compounds: hexanal and (3Z)-hexenal, whereas HPL lysis of 9-hydroperoxides yields C_9-volatile compounds: (3Z)-nonenal and (3Z,6Z)- nonadienal. The flavour profiles of the aldehydes are further modified by isomerisation of the (3Z)-enals into (2E)-enals by isomerases which have been identified in cucumber fruit (Phillips, Matthew, Reynolds, & Fenwick, 1979), pear fruit (Kim & Grosch, 1981), tea leaves (Hatanaka & Harada, 1973), cabbage leaves (Macleod & Pikk, 1979), soybean seeds (Takamura & Gardner, 1996) and seedlings (Olias, Rios, Valle, Zamora, Sartz, & Axelrod, 1990), seedlings of alfalfa (Sekiya, Kajiwara, & Hatanaka, 1979) and cucumber (Sekiya et al., 1979). Aldehyde dehydrogenase may convert the aldehydes into their corresponding alcohols.

The products of the LOX/HPL reaction in plants were known for many years prior to the identification of the enzyme in watermelon seedlings (Vick & Zimmerman, 1976) and cucumber fruit (Galliard, Phillips, & Reynolds, 1976; Galliard & Phillips, 1976). It is likely that HPL is distributed widely throughout the plant kingdom, having been identified in a wide variety of plants and also in certain algae and fungi (reviewed by Gardner, 1989, 1991; Hatanaka, 1996). In higher plants two types of HPL activity exist, and are classified according to their substrate specificity. The first is 9-hydroperoxide specific (9-HPL) as found in pear (Kim & Grosch, 1981), the second is 13-hydroperoxide specific (13-HPL) as found in tomato leaves (Fauconnier, Perez, Sanz, & Marlier, 1997) and bell pepper fruit

* Corresponding author.

0308-8146/99/$ - see front matter
PII: S0308-8146(98)00260-X

(Shibata, Matsui, Kajiwara, & Hatanaka, 1995). Results from earlier studies support the presence of a third non-specific HPL type cleaving both 9- and 13-hydroperoxides as found in cucumber fruit (Galliard & Phillips, 1976, 1977) and seedlings (Sekiya et al., 1979), soybean seeds and seedlings (Gardner, Weisleder, & Plattner, 1991). However, Matsui, Shibata, Kajiwara, & Hatanaka (1989) have claimed the separation 9- and 13-HPL from cucumber seedling cotyledons, suggesting the possible presence of two individual enzymes.

The aim of the research described is to purify HPL from cucumber fruits, as this enzyme may have commercial potential as a biocatalyst for the production of C_6- and C_9-aldehydes mainly responsible for characteristic aromas of some fruits and vegetables and especially for members of the cucurbitaceae family. Cucumbers were chosen as they are a good source of 9-hydroperoxide- and 13-hydroperoxide-lysing activity and are readily available. The objectives of the study were: to characterize purified enzymes and determine their activity towards test substrates and to attempt to separate the 9-hydroperoxide- and 13-hydroperoxide-lysing activities and thus to determine whether two separate enzymes are responsible for the lyase reactions.

2. Materials and methods

2.1. Extraction and purification of HPL

Cucumber fruit (*Cucumis sativus*) was purchased from a local supermarket. The origin varied and varieties were not known. The greater HPL activity was obtained in fruits that were slightly under-ripe. The fruit was washed in tap water and the mesocarp (200 g) was homogenized in a Waring blender for 1 min with bis(2-hydroxyethyl)amino-tris(hydroxymethyl) methane (bis-tris) buffer (0.1 M, 200 ml, pH 6.8) containing dithiothreitol (DTT) (4 mM) and polyvinylpyrrolidone K-30 (0.5% w/v) at 4°C. The homogenate was passed through four layers of muslin and the filtrate was centrifuged (25 000 g) at 4°C for 45 min. Bis-tris buffer (20 mM, 25 ml, pH 6.8) containing Triton-X100 (0.5% w/v) and DTT (1 mM) was added to the pellet and stirred gently for 1 h at 4°C. The solution was recentrifuged (25 000 g) at 4°C for 30 min and the resulting supernatant, containing solubilized HPL, was passed through four layers of muslin in order to remove lipids floating on the surface of the supernatant.

2.2. Preparation of hydroperoxides

13-Hydroperoxy-linoleic acid and 13-hydroperoxy-linolenic acid were prepared by incubating linoleic acid and linolenic acid, respectively, with soybean LOX-1 (Sigma-Aldrich Chemical Co. Ltd., Dorset) at pH 10, at 0°C. 9-hydroperoxy-linoleic acid and 9-hydroperoxy-linolenic acid were obtained by using crude tomato LOX at pH 5.5, at 25°C. LOX products were isolated and purified by normal phase HPLC (Wu, Robinson, Domoney, & Casey, 1995). All hydroperoxides were stored as ethanolic solutions (10 mM) at −20°C.

2.3. Spectrophotometric assay of HPL

HPL activity was assayed, at 25°C, by measuring the decrease in absorbance at 234 nm due to the lysis of a C–C bond and the resulting loss of the conjugated structure of 9- or 13-hydroperoxy-linoleic acid substrate. A typical reaction mixture (3 ml) consisted of phosphate buffer (0.1 M, pH 6.5) and 9-hydroperoxy-linoleic acid or 13-hydroperoxy-linoleic acid in ethanol (10 mM, 10 μl). The approximate concentration of hydroperoxide was 33 μM. The mixture was allowed to equilibrate for 2 min, during which time the absorbance was noted in order to determine any non-enzymatic hydroperoxide degradation. The reaction was initiated with the addition of enzyme (5–50 μl) to the mixture followed by stirring and the decrease in absorbance was followed for 2 min. Preliminary experiments showed that the amount of ethanol used in the assay did not affect HPL activity. The value for the extinction coefficient, $\epsilon = 25\,000\ M^{-1}\ cm^{-1}$ was used (Vick, 1991). One unit of HPL activity is defined as the amount of enzyme causing a loss of 1μ mol of substrate/min at 25°C.

2.4. Qualitative determination of HPL using headspace gas chromatography

The presence of HPL activity after each stage of purification was confirmed by analysing the headspace above the reaction mixture, by gas chromatography, for (3Z)-nonenal.

The reaction was performed in enclosed glass vials (7 ml) fitted with an open top screw-cap fitted with a silicone/Teflon septum. A typical reaction mixture (3 ml), consisted of phosphate buffer (0.1 M, pH 6.5, 25°C) to which 9-hydroperoxy-linoleic acid in ethanol (10 mM, 10 μl) was added. The approximate concentration of hydroperoxide was 33 μM. Enzyme solution (5–50 μl) was added to the mixture followed by stirring and the screw cap replaced. The vial was placed in a water bath at 25°C and the reaction allowed to proceed for 10 min after which headspace gas (3 ml) was removed from the vial using a gas-tight syringe and injected onto a Carlo Erba (model 4200) gas chromatograph with a fused silica open tubular column containing BPX 5 (25 m ×0.32 mm ; 0.5 μm) (SGE Ltd, Milton Keynes) with helium as carrier gas at 2 ml/min. Inlet: split 30:1. The temperature programme was 60°C for 3 min followed by a rise at 15°C min^{-1} to 200°C. The retention time of (3Z)-nonenal was compared with that of an authentic

sample prepared by oxidizing (3Z)-nonenol with pyridinium chlorochromate (Corey & Suggs, 1975). Controls were solutions without either enzyme or substrate.

2.5. Protein determination

Protein was determined by the Pierce Bicinchoninic Acid (BCA) reagent (Pierce and Warriner, Cheshire) according to the manufacturer, using bovine serum albumin as standard after precipitation with trichloro-acetic acid.

2.6. Ion-exchange chromatography

The solubilized HPL solution was applied to a DEAE-Toyopearl 650 M (Fisher, Leicestershire) column (10 mm × 180 mm) that had been equilibrated with bis-tris buffer (20 mM, 500 ml, pH 6.8) containing Triton X-100 (0.5% w/v) and DTT (1 mM) at a flow rate of 0.5 ml/min^{-1} at 4°C. Cationic proteins were eluted with bis-tris buffer (40 ml). Elution of anionic proteins was carried out using a linear gradient of 0 to 0.5 M NaCl, in bis-tris buffier, to a total volume of 300 ml and 4°C. Fractions (10 ml) were collected.

2.7. Buffer exchange using polyethelene glycol precipitation

Fractions containing the highest activities of HPL were pooled, solid polyethylene glycol (PEG) 6000 (30% w/v) added, followed by gentle stirring for 1 h at 4°C. The solution was centrifuged (25 000 g) for 30 min at 4°C, the supernatant discarded and the pellet resuspended in sodium phosphate buffer (10 mM, 5 ml, pH 6.8) containing Triton X-100 (0.5% w/v), DTT (1 mM) and glycerol (10% v/v).

2.8. Hydroxyapatite chromatography

HPL-active solution was applied to a hydroxyapatite (Bio-Rad Laboratories Ltd., Hertfordshire) column (10 mm i.d. × 140 mm long) that had been equilibrated with sodium phosphate buffer (10 mM, 500 ml, pH 6.8) containing Triton X-100 (0.5% w/v), DTT (1 mM) and glycerol (10% v/v) at a flow rate of 0.5 ml/min at 4°C. The column was washed with phosphate buffer (30 ml) and elution was with a linear gradient of 10–30 mM sodium phosphate buffer containing Triton X-100 (0.5% w/v), DTT (1 mM) and glycerol (10% v/v) to a total volume of 140 ml and fractions (10 ml) were collected.

2.9. Analysis of purity and molecular weight determination by SDS-page

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of samples after each stage of purification was performed in slab gels using the discontinuous buffer system of Laemmli (1970). The separating and stacking gels contained a final acrylamide concentration of 10%(w/v) and 4%(w/v), respectively. Staining for protein was carried out with Coommassie Brilliant Blue. The molecular weight markers were *E. coli* β-galactosidase (116 000 Da), rabbit muscle phosphorylase b (97 000 Da), rabbit muscle fructose-6-phosphate kinase (84 000 Da), bovine serum albumin (66 000 Da), bovine liver glutamic dehydrogenase (55 000 Da), chicken egg ovalbumin (45 000 Da) and rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (36 000 Da).

2.10. Determination of pH optima for purified HPL activity

Three buffers were used for this procedure; sodium phosphate buffers (0.1 M, pH 5–7), tris-HCl buffers (0.1 M, pH 7.5–8.5) and borate buffer (0.1 M, pH 9). assays were at 234 nm using either 9-hydroperoxy-linoleic acid or 13-hydroperoxy-linoleic acid.

2.11. Substrate specificity of purified HPL

HPL activity was determined at 234 nm using either 9-hydroperoxy-linoleic acid, 9-hydroperoxy-linolenic acid, 13-hydroperoxy-linoleic acid or 13-hydroperoxy-linolenic acid in sodium phosphate buffer (0.1 M, pH 6) and purified HPL (0.31 μg).

2.12. Temperature stability of purified HPL

In order to ensure rapid heating, thin-walled glass test tubes (1 × 10 cm) were incubated at the required temperature for 10 min. Enzyme solution (50 μl) containing 1.55 μg of purified HPL was added and heated for 2 min. After heating, the tubes were immediately placed in ice for 5 min and the enzymic activity measured at 234 nm using either 9-hydroperoxy-linoleic acid or 13-hydroperoxy-linoleic acid as substrates in phosphate buffer (0.1 M, pH 6) and heat treated HPL (10 μl).

3. Results and discussion

3.1. Purification of HPL

Results for four consecutive HPL purifications are shown in Table 1. Centrifugation of the crude homogenate, yielded a pellet containing membrane-bound HPL. Further purification was achieved using the non-ionic detergent Triton X-100. Approximately 40% of 9- and 13-hydroperoxide-lysing activity was lost during detergent solubilization of HPL: however, the quantity of protein was reduced by over 80%, thus giving a purification of approximately 33-fold. After anion-exchange chromatography with DEAE-Toyopearl 650

M, more than 70% of the applied 9- and 13-hydroperoxide-lysing activity was eluted by a linear salt gradient from approximately 0–0.04 M NaCl (Fig. 1). Only approximately 9% of the protein applied was recovered, thus yielding approximately a 28-fold increase in specific enzymic activity (Table 1). Following buffer exchange by PEG 6000, precipitation and chromatography of the suspended pellet with hydroxyapatite, more than 30% of the applied 9- and 13-hydroperoxide-lysing activity was eluted at a phosphate concentration of approximately 0.010–0.065 M (Fig. 2). Approximately 9.5% of applied protein was recovered yielding approximately an 80-fold increase in specific activity with respect to the crude homogenate (Table 1). HPL activity was confirmed after each stage of purification by headspace gas chromatographic analysis of (3Z)-nonenal formed by lysis of 9-hydroperoxy-linoleic acid. The yields of 9- and 13-hydroperoxide-lysing activity after each stage of purification were nearly the same, suggesting that both enzymic activities may be associated with one protein.

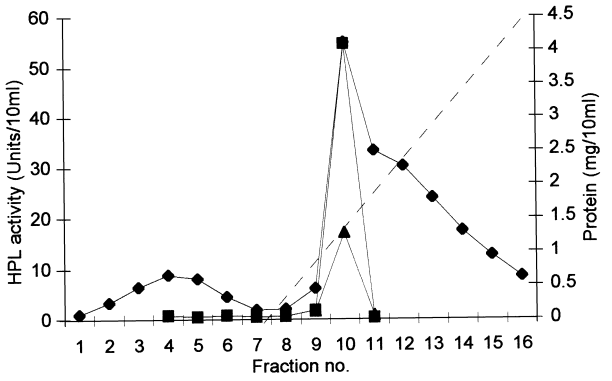

Fig. 1. Ion-exchange chromatography of cucumber HPL on DEAE-Toyopearl, 650 M: ■–■, HPL activity with 9-hydroperoxy-linoleic acid substrate; ▲–▲, HPL activity with 13-hydroperoxy-linoleic acid substrate; ◆–◆, protein measured at 562 nm; - - - NaCl gradient (0-0.15 μM).

HPL, purified by DEAE-Toyopearl and precipitated by PEG 6000, was stored in the hydroxyapatite sample buffer at 4°C. The stabilities at 4°C of 9-hydroperoxy-linoleic acid- and 13-hydroperoxy-linoleic acid lysing activities were similar. Approximately 27% and 23%, respectively, of enzymic activity for both substrates was lost over a three week period. Hydroxyapatite-purified 9- and 13-hydroperoxide-lysing activities, stored in the elution buffer at 4°C, were stable for one week but 30% of enzymic activity for both substrates was lost after three weeks. Similarly Phillips and Galliard (1978) found that partially purified HPL from cucumber mesocarp was stable for up to 10 days at 0°C and that freezing resulted in a 40% loss in enzymic activity and storing the enzyme in ammonium sulphate (2 M) in glycerol (10%) resulted in a 90% loss of activity after dialysis. Similarly, Olias et al. (1990) found that partially purified 13-HPL from soybean seedlings lost all activity after seven days at 4°C in sodium phosphate buffer (50 mM, pH 6.8) containing EDTA (0.2 mM) and DTT (0.3 mM). However, it is possible that a low concentration of DTT and absence of detergent may have contributed to instability of their enzyme.

3.2. Characterization of purified HPL

Electrophoresis of the hydroxyapatite purified HPL preparation in the presence of SDS revealed one

Table 1
Purification of HPL from cucumbers

	Total activity (μmol/min)	Total protein (mg)	Specific activity (μmol/min/mg)	Yield (%)	Purification (-fold)
Crude homogenate					
9-HPOLA	122 ± 18.8	178 ± 78.81	0.69	100	1.00
13-HPOLA	43.9 ± 1.87		0.25	100	1.00
Triton X-100 Solubilized fraction					
9-HPOLA	77.9 ± 17.67	33.7 ± 17.80	2.31	63.0 ± 5.55	3.35
13-HPOLA	28.3 ± 3.91		0.84	64.6 ± 8.72	3.36
DEAE-toyopearl					
9-HPOLA	61.9 ± 11.22	2.93 ± 0.88	21.12	49.9 ± 1.92	30.6
13-HPOLA	20.5 ± 2.9		7.02	46.9 ± 6.78	28.0
PEG 6000 precipitation					
9-HPOLA	42.6 ± 8.77	2.85 ± 1.61	15.00	34.6 ± 2.64	21.7
13-HPOLA	16.5 ± 3.94		5.80	37.3 ± 7.47	23.2
Hydroxyapatite					
9-HPOLA	16.4 ± 7.62	0.27 ± 0.15	60.85	13.0 ± 4.88	88.1
13-HPOLA	5.6 ± 2.34		20.74	12.7 ± 5.47	83.0

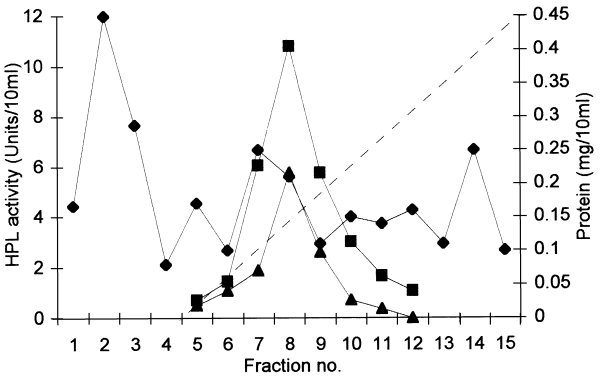

Fig. 2. Hydroxyapatite chromatography of cucumber HPL: ■–■, HPL activity with 9-hydroperoxy-linoleic acid as substrate; ▲–▲, HPL activity with 13-hydroperoxy-linoleic acid as substrate; ◆–◆, protein measured at 562 nm; NaPh gradient (10–230 mM).

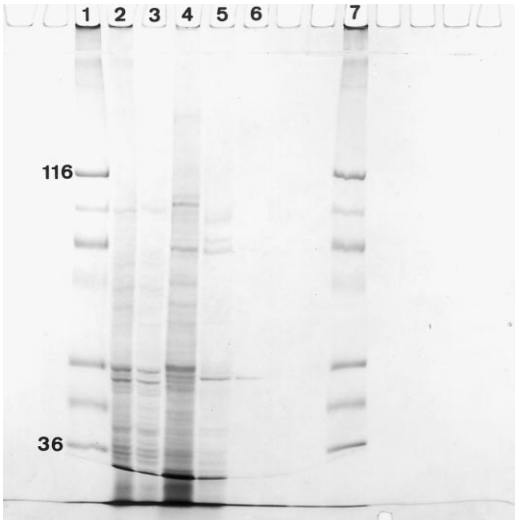

Fig. 3. Samples applied to a SDS-PAGE gel (μg protein/lane): 1, molecular weight markers; 2, crude homogenate (13 μg); 4, Triton X-100 solubilized fraction (12 μg); 5, DEAE-Toyopearl 650 M fraction (11 μg); 6, hydroxyapatite fraction (2 μg); 7, molecular weight markers.

predominant protein-staining band at an approximate molecular weight (MW) 55 000 Da (Fig. 3). This component is thought to be HPL with a MW similar to that attributed to bell pepper fruit HPL (55 000 Da) by Shibata et al. (1995) and tea leaf HPL, (53 000 Da) by Matsui, Toyota, Kajiwara, Kakumo, & Hatanaka (1991). The presence of only one protein-staining band on SDS-PAGE suggests that either the MW of our 9- and 13-hydroperoxide-specific cucumber HPLs were of very similar size or that only one enzyme was present in our purified preparation and was capable of catalysing the lysis of both 13- and 9-hydroperoxides of linoleic and linolenic acids.

Olias et al. (1990) and Shibata et al. (1995) have discussed the possibility of HPL existing as tetramers and trimers by comparing results obtained using gel filtration and SDS-PAGE. However, Olias et al. (1990) did not include detergent in the gel filtration buffer which may have led to aggregation and Shibata et al. (1995) included Triton X-100 in the buffer used for gel filtration which would be expected to complex with HPL, thus increasing the apparent MW by as much as 90 000 Da (Schägger, 1994). Moreover, it is interesting to note that the gel filtration determined MWs of soluble HPLs not requiring detergents for solubilization, from Oscillatoria sp.(56 000 Da) (Andrianarison, Beneytout, & Tixier, 1989) and Chlorella pyrenoidosa (48 000 Da) (Vick, 1989) are similar in size to those determined by SDS-PAGE of higher plants.

Matsui et al. (1989) claim to have separated 9-hydroperoxide- and 13-hydroperoxide-specific HPL from cucumber seedling cotyledons using ion-exchange chromatography: however, their results are not easily understood. The elution of HPL activities was followed using two techniques: 13-hydroperoxide-specific HPL was assayed by measuring hexanal production using headspace gas chromatography and the 9-hydroperoxide-specific HPL was assayed by measuring (2E)-nonenal production using HPLC. However, (2E)-nonenal is not the initial product of 9-hydroperoxy-linoleic acid degradation by HPL but is formed from the isomerization of (3Z)-nonenal. It is known that a soluble (3Z):(2E)-alkenal isomerase exists in cucumber seedlings (Sekiya et al., 1979) and fruits (Phillips et al., 1979) and that it can be separated from HPL (Phillips et al., 1979), therefore Matsui et al. (1989) may have also actually been measuring a separated isomerase. Unfortunately SDS-PAGE results for this separation were not reported.

3.3. Determination of pH optima for purified HPL activity

An optimum pH of 6.5 for crude HPL activity towards both the 9-hydroperoxy- and 13-hydroperoxy-linoleic acids agrees with the results obtained by Galliard, Phillips, and Reynolds (1976). For the hydroxyapatite-purified HPL fraction, the pH optimum was 6 for both the 13-hydroperoxy- and 9-hydroperoxy-linoleic acids (Fig. 4). However, 13-hydroperoxylinoleic acid-lysing activity was more sensitive to increasing pH, as half of the original activity was lost at pH 6.5 and approximately 50% of 9-hydroperoxy-linoleic acid-lyase activity was lost at approximately pH 7.5. This suggests that, at the slightly higher pH value, the enzyme was more able to still catalyse the lysis of the more symmetrical 9-hydroperoxide.

3.4. Substrate specificity of purified HPL

Plots of enzymic activity versus enzyme concentration (0.31–1.55 μg) with 9- and 13-hydroperoxy-linoleic acid

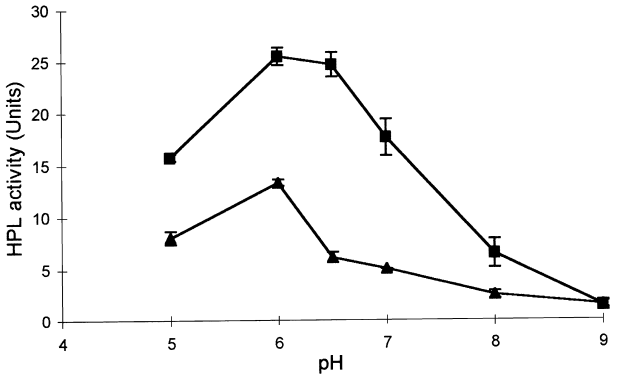

Fig. 4. Activity of purified HPL as a function of pH. ■–■, HPL activity with 9-hydroperoxy-linoleic acid as substrate; ▲–▲, HPL activity with 13-hydroperoxylinoleic acid as substrate.

(33 μM), at optimum pH 6.0, showed linear increases in activity thus demonstrating that the enzymes were saturated with substrate. The activity of the enzyme preparation with the 9-hydroperoxy-linoleic acid and 13-hydroperoxy-linoleic acids ranged from 10.8 to 53.6 μmol/min and 5.23 to 31.5 μmol/min, respectively.

The substrate specificity of the hydroxyapatite-purified HPL determined by examining the activities of the HPL with various PUFA hydroperoxides is shown in Table 2. 9-Hydroperoxy-linoleic acid was 1.6, 2.6 and 2 times more readily lysed than 9-hydroperoxy-linolenic acid, 13-hydroperoxy-linoleic acid and 13-hydroperoxy-linolenic acid, respectively. From double reciprocal plots of HPL activity versus substrate concentrations, apparent K_m^{app} values for 9-hydroperoxy-linoleic acid, 9-hydroperoxy-linolenic acid, 13-hydroperoxy-linoleic acid and 13-hydroperoxy-linolenic acid were 6.76, 6.02, 5.41 and 12.4 μM, respectively (Table 3). This shows that HPL possessed a slightly greater affiffity for 13-hydroperoxy-linoleic acid. The corresponding V_{max}^{app} values were 19.3, 12.0, 7.58 and 11.4 μmol/min. The magnitude of substrate lysis was 9-hydroperoxy-linoleic acid > 9-hydroperoxy-linolenic acid > 13-hydroperoxy-linolenic acid > 13-hydroperoxy-linoleic acid. The V_{max}^{app}/K_m^{app} ratios for 9-hydroperoxy-linoleic acid, 9-hydroperoxy-linolenic acid, 13-hydroperoxy-linoleic acid and 13-hydroperoxy-linolenic acid were 2.86, 1.99, 1.39 and 0.92, respectively, showing that the 9-hydroperoxides were more efficiently lysed than 13-hydroperoxides. Also the hydroperoxides of linoleic acid were more efficiently lysed than the hydroperoxides of linolenic acid. Similarly Phillips and Galliard (1978) found that, for their partially purified cucumber HPL, 9-hydroperoxides were more readily lysed than 13-hydroperoxides. However, Phillips and Galliard (1978) did not supply any information on $K_{m\text{app}}$ values for 9- and 13-hydroperoxy-linolenic acid.

The lipids in cucumber fruit are composed of mainly three fatty acids: 26% palmitic (16:0), 26.3% linoleic (182) and 39.5% linolenic (18:3) (Galliard et al., 1976).

Table 2
Substrate specificity of purified HPLC

Substrate	Relative activity (%)
9-Hydroperoxy-linoleic acid	100 ± 2.09
9-Hydroperoxy-linolenic acid	63.4 ± 1.72
13-Hydroperoxy-linoleic acid	37.9 ± 2.6
13-hydroperoxy-linolenic acid	49.3 ± 3.81

Table 3
Apparent K_m and apparent V_{max} values for substrates with purified HPL

Substrate	K_m^{app} (μM)	V_{max}^{app} (μmol/min)	V_{max}^{app}/K_m^{app}
9-HPOLA[a]	6.76	19.3	2.86
9-HPOLNA[b]	6.02	12.0	1.99
13-HPOLA	5.46	7.58	1.39
13-HPOLNA	12.4	11.4	0.92

[a] HPOLA = hydroperoxy-linoleic acid.
[b] HPOLNA = hydroperoxy-linolenic acid.

Cucumber fruit LOX oxidized linoleic acid 1.33 times more readily than linolenic acid Wardale & Lambert, 1980), forming predominantly the 9-hydroperoxides (Galliard & Phillips, 1976). If cucumber LOX is more likely to produce 9-hydroperoxy-linoleic acid on injury, then it is possible that cucumber HPL may also have evolved to preferentially lyse these potentially harmful molecules. If plant LOXs have the ability to produce both 9- and 13-hydroperoxides then it seems unlikely that a plant would synthesise two proteins to selectively degrade these acyl hydroperoxides.

K_m^{app} values reported here for the purified HPL with 9-hydroperoxy-linoleic acid (6.7 μM) and 13-hydroperoxy-linoleic acid (5.46 μM) are approx. 0–3.5 times lower than those values obtained for the partially purified cucumber mesocarp HPL (15–20 μM) of Phillips and Galliard (1978). This may be due to the removal during purification of competing enzymes for the substrates and possible inhibitors. A similar observation has been made after the purification of a 13-HPL from tea leaves when 13-hydroperoxy-linoleic acid was used as a substrate. The K_m^{app} value changed from 1.9 mM for solubilized crude enzyine (Hatanaka, Kajiwara, Sekiya, & Inouye, 1982) to 14.6 μM for the more purified enzyme (Matsui et al., 1991). However, it is difficult to compare K_m^{app} values from some sources, because often the purity of the enzyme preparation is unknown. Contaminating enzymes such as peroxygenases, peroxidases and allene oxide synthase competing for the same substrates, may be present together with inhibitors which could be competing for the enzyme active sites thus affecting measured K_m^{app} values. However, a reasonable assumption is that the lower the measured K_m^{app}, then the purer

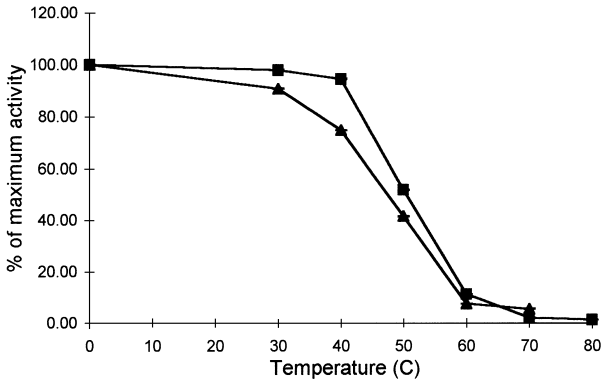

Fig. 5. Temperature stability of purified HPL. ■–■, HPL activity with 9-hydroperoxylinoleic acid as substrate; ▲–▲, HPL activity with 13-hydroperoxy-linoleic acid as substrate.

the enzyme and it may be expected that pure HPLs from different sources might possess K_m^{app} values of similar orders of magnitude. Another feature of the results is the different K_m^{app} values for 13-hydroperoxy-linoleic acid and 13-hydroperoxy-linolenic acid. The K_m^{app} value for 13-hydroperoxy-linolenic acid was 12.4 μM and for 13-hydroperoxy-linoleic acid 5.46 μM. The K_m^{app} values for 9-hydroperoxides of linoleic acid and linolenic acid were found to be similar, 6.76 and 6.02 μM respectively, which might indicate that the more symmetrical 9-hydroperoxy-acids are more easily accommodated by the enzyme.

9-Hydroperoxy-linoleic acid-lysing HPL activity showed no significant loss in activity up to 40°C (Fig. 5). At 50°C, half of initial activity was lost and the enzymic activity was inactivated at 80°C. 13-hydroperoxy-linoleic acid-lysing HPL activity showed a greater sensitivity to heat, where half of initial activity was lost at 45°C. Phillips and Galliard (1978) claimed that partially purified cucumber HIPL was slightly less heat-sensitive; losing 50% activity after 5 min at 50°C but the test substrate was not identified.

In the present study HPL lost activity after short incubation times. This may be due to the presence of Triton X-100 in the storage buffer. As the protein unfolds, on heating, hydrophobic regions are exposed to Triton X-100, which may have bound to the hydrophobic regions leaving them with a detergent coating. Such a "coating" of Triton X-100 could prevent the enzyme from regaining its original conformation on cooling and thus explain the loss of HPL activity.

References

Andrianarison, R. H., Beneytout, J. L., & Tixier, M. (1989). An enzymatic conversion of lipoxygenase products by a hydroperoxide lyase in blue-green algae (*Oscillatoria sp.*). *Plant Physiology, 91*, 1280–1287.

Corey, E. J., & Suggs, J. W. (1975). Pyridinium chlorochromate. An effective reagent for oxidation of primary and secondary alcohols to carbonyl compounds. *Tetrahedron Letters, 31*, 2647–2650.

Fauconnier, M. L., Perez, A. G., Sanz, C., & Marlier, M. (1997). Purification and characterization of tomato leaf (*Lycopersicon esculatum* Mill.) hydroperoxide lyase. *Journal of Agricultural and Food Chemistry, 45*, 4232–4236.

Galliard, T., & Phillips, D. R. (1976). The enzymic cleavage of linoleic acid to C9 carbonyl fragments in extracts of cucumber (*Cucumis sativus*) fruit and the possible role of lipoxygenase. *Biochimica et Biophysica Acta, 431*, 278–287.

Galliard, T., Phillips, D. R., & Reynolds, J. (1976). The formation of cis-3-nonenal, trans-2-nonenal and hexanai from linoleic acid hydroperoxide isomers by a hydroperoxide cleavage enzyme system in cucumber (*Cucumis sativus*) fruits. *Biochimica et Biophysica Acta, 441*, 181–192.

Gardner, H. W. (1989). How the lipoxygenase pathway affects the organoleptic properties of fresh fruit and vegetables. In D. B. Min, & T. H. Smouse (Eds.), *Flavour chemistry of lipid foods* (pp. 98–112). Champaign, IL: American Oil Chemists Society.

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

Gardner, H. W., Weisleder, D., & Plattner, R. D. (1991). Hydroperoxide lyase and other hydroperoxide-metabolizing activity in tissues of soybean *Glycine max*. *Plant Physiology, 97*, 1059–1072.

Hatanaka, A. (1996). The fresh green odor emitted by plants. *Food Review International, 12*, 303–350.

Hatanaka, A., & Harada, T. (1973). Formation of cis-3-hexenal,trans-2-hexenal and cis-3-hexenol in macerated *Thea sinensis* leaves. *Phytochemistry, 12*, 2341–2346.

Hatanaka, A., Kajiwara, T., Sekiya, J., & Inouye, S. (1982). Solubilization and properties of the enzyme-cleaving 13-L-hydroperoxylinolenic acid in tea leaves. *Phytochemistry, 21*, 13–17.

Kim, I. S., & Grosch, W. (1981). Partial purification of a hydroperoxide lyase from fruits of pear. *Journal of Agricultural and Food Chemistry, 29*, 1220–1225.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, 227*, 680–685.

Mcleod, A. J., & Pikk, H. E. (1979). Formation of (E)-hex-2-enal and(Z)-hex-3-en-1-o1 by fresh leaves of *Brassica oleracea*. *Journal of Agricultural and Food Chemistry, 27*, 469–473.

Matthew, J., & Galliard, T. (1978). Enzymic formation of carbonyls from linoleic acid in leaves of *phaseolus vulgaris*. *Phytochemistry, 17*, 1043–1044.

Matsui, K., Shibata, Y., Kajiwara, T., & Hatanaka, A. (1989). Separation of 13- and 9-hydroperoxide lyase activities in cotyledons of cucumber seedlings. *Zeitschrift fur Naturforschung, 44C*, 883–885.

Matsui, K., Toyota, H., Kajiwara, T., Kakuno, T., & Hatanaka, A. (1991). Fatty acid hydroperoxide cleaving enzyme, hydroperoxide lyase, from tea leaves. *Phytochemistry, 30*, 2109–2113.

Olias, J. M., Rios, J. L., Valle, M., Zamora, R., Sartz, L. C., & Axelrod, B. (1990). Fatty acid hydroperoxide lyase in germinating soybean seedlings. *Journal of Agricultural and Food Chemistry, 38*, 624–630.

Phillips, D. R., & Galliard, T. (1978). Flavour biogenesis. Partial purification and properties of a fatty acid hydroperoxide cleaving enzyme from fruits of cucumber. *Phytochemistry, 17*, 355–358.

Phillips, D. R., Matthew, J., 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.

Schägger, H. (1994). Chromatographic techniques and basic operations in membrane protein purification. In: G. Von Jagow and H. Schägger (Eds.), *A practical guide to membrane protein purification*, (pp. 23–57). London: Academic Press.

Sekiya, J., Kajiwara, T., & Hatanaka, A. (1979). Volatile C6-aldehyde formation via hydroperoxides from C-18 unsaturated fatty acids in etiolated alfalfa and cucumber seedlings. *Agricultural and Biological Chemistry, 43*, 969–980.

Shibata, Y., Matsui, K., Kajiwara, T., & Hatanaka, A. (1995). Purification and properties of fatty hydroperoxide lyase from green bell pepper fruits. *Plant Cell Physiology, 36*, 147–156.

Takamura, H., & Gardner, H. W. (1996). Oxygenation of (3Z)-alkenal to (2E)-hydroxy-2-alkenal in soybean seed (*Glycine max* L). *Biochimica et Biophysica Acta, 1303*, 83–91.

Vick, B. A. (1989). Metabolism of fatty acid hydroperoxides by *Chlorella pyrenoidosa*. *Plant Physiology, 90*, 125–132.

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.

Wardale, D. A., & Lambert, E. A. (1980). Lipoxygenase from cucumber fruit: localization and properties. *Phytochemistry, 19*, 1013–1016.

Wu, Z., Robinson, D. S., Domoney, C., & Casey, R. (1995). High performance liquid chromatographic analysis of the products of linoleic acid oxidation catalysed by pea (*Pisum sativum*) seed lipoxygenases. *Journal of Agricultural and Food Chemistry, 43*, 337–342.