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Purification of hydroperoxide lyase from pea seeds

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Abstract

Hydroperoxide lyase (HPL) development in pea seeds/seedlings was studied during germination. HPL activity in seedlings appeared after 2 days, increased to a maximum at 4 days (3.7 μmol min^{-1} ml^{-1}) and decreased slowly after 8 days (2.56 μmol min^{-1} ml^{-1}). Protein content decreased over 8 days from a maximum of 12.3 to 2.39 mg/ml. This resulted in an approximate 100-fold increase in specific activity of HPL from 0.01 μmol min^{-1} mg^{-1} (day 2) to 1.07 μmol min^{-1} mg^{-1} (day 8). The pI of solubilised pea seedling HPL was found to be 5.23±0.23. HPL was extracted from pea seedlings and purified by centrifugation, solubilisation with detergent, ion-exchange chromatography and hydroxylapatite chromatography. 9-Hydroperoxy-linoleic acid and 13-hydroperoxy-linoleic acid lysing activities were purified similiarly12-fold and 10-fold, respectively. The purified HPL preparation consisted of a single major band, following SDS-electrophoresis, with a molecular weight of about 55 000Da; pH 6.5 was optimum for the lysis of both 9-hydroperoxy-linoleic acid and 13-hydroperoxy-linoleic acid substrates. The enzyme was relatively unstable and lost two thirds of the original activity after 1 week at 4°C. The substrate specificity of the hydroxylapatite-purified HPL was determined by examining the activities of the HPL with various PUFA hydroperoxides. 13-Hydroperoxy-linolenic acid was 1.8, 0.8 and 1.5 times more readily lysed than 9-hydroperoxy-linoleic acid, 9-hydroperoxy-linolenic acid and 13-hydroperoxy-linoleic acid, respectively. Apparent K_m values for 9-hydroperoxy-linoleic acid, 9-hydroperoxy-linolenic acid, 13-hydroperoxy-linoleic acid and 13-hydroperoxy-linolenic acid were 4.5, 14.2, 11.1 and 6.2 μM, respectively. Corresponding V_{max} values were 0.12, 0.12, 0.22 and 0.26 μmol min^{-1}. 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 0.03, 0.01, 0.02 and 0.04, respectively. © 2000 Published by Elsevier Science Ltd.

1. Introduction

The oxidation of lipids by the sequential action of lipoxygenase (LOX) and hydroperoxide lyase (HPL) gives rise to the formation of short chain volatile aldehydes that contribute to the flavour and aroma of plants (Itoh & Vick, 1999; Pérez, Sanz, Olías & Olías, 1999; Rowan, Allen, Fielder & Hunt, 1999; Salas & Sánchez, 1999). Linoleic and linolenic acids from lipids are converted by LOX into 9- or 13-hydroperoxides and these reactive species are then 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 aldehydes can be modified further into alcohols by alcohol dehydrogenase (Fauconnier, Mpambara, Delcarte, Jacques, Thonart & Marlier, 1999; Muller, Gautier, Dean, & Kuhn, 1995).

The aldehydes produced are important additives in the food and perfumery industries. Therefore the use of LOX and HPL as biocatalysts for the production of flavours has attracted the attention of industry and academia alike and is evident by the number of patents applied for in this area (Belin, Dumont & Ropert, 1998; Brunerie & Koziet, 1997; Goers, 1989; Haeusler, Lerch, Muheim & Silke, 1997; Holtz, McCulloch, Garger, Teague & Phillips, 1995; Kanisawa, 1988; Kibler, Kratky & Tandy, 1992; Subbiah, 1997).

Peas are the fourth most important grain legume used worldwide for both human and livestock consumption (Casey, Domoney & Smith, 1993). The presence of lipoxygenase in peas necessitates rapid treatment after harvesting in order to prevent the formation of undesirable flavours which would limit shelf life. Studies, using selective breeding and recombinant technology, have shown that peas contain 2 major and three minor lipoxygenases (Casey et al., 1993) having differing characteristics

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in terms of oxidation product formation (Forster, North, Afzal, Hornostaj, Robinson & Casey, 1999; Hughes et al., 1998) and co-oxidizing properties (Wu, Robinson, Hughes, Casey, Hardy & West, 1999) and how this could be beneficial to the agrifood industry (Busto, Owusu-Apenten, Robinson, Wu, Casey & Hughes, 1999; Hughes, Wu, Hornostaj et al., 1999; Hughes et al., in press).

2. Materials and methods

2.1. 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, for 3 h 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.

2.2. 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 and 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. The extinction coefficient, $\epsilon = 25\ 000\ M^{-1}\ cm^{-1}$ was used (Vick, 1991). One unit of HPL activity was defined as the amount of enzyme causing a loss of 1 μmol of substrate per minute at 25°C.

2.3. Qualitative determination of HPL by GC

The presence of HPL activity was determined by analysing the headspace above the reaction mixture for hexanal.

The enzymic reaction was carried out 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 13-hydroperoxy-linoleic acid in ethanol (10 mM, 10 μl) was added. The final 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) with helium as carrier gas at 2 ml min^{-1}, 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 hexanal was compared with that of an authentic sample. Controls were solutions without either enzyme or substrate.

2.4. Protein determination

Protein was determined colorimetrically using the Pierce Bicinchoninic Acid (BCA) reagent and bovine serum albumin as standard after precipitation with trichloroacetic acid (Pierce BCA Applications note 12, 1990).

2.5. Development of seedlings and HPL activity

Dry mature pea seeds *v.Birte* were soaked by totally immersing in tap water for 24 h. This was taken to be time zero when determining development times. The seeds were then sown on a bed of water-saturated Perlite. The seeds were covered with 1 cm of water-saturated Perlite and placed in a dark cupboard to germinate at room temperature. The seedlings were watered once a day with tap water.

Seedlings (4 g) were removed, cleaned of Perlite, and homogenised in a Waring blender for 30 s with 12 ml bis(2-hydroxyethyl)amino-Tris(hydroxymethyl) methane (bis–Tris) buffer (0.1 M, pH 6.8) containing Triton X-100 (0.5% w/v), dithiothreitol (DTT) (4 mM) and polyvinylpyrrolidone (PVP) K-30 (0.5% w/v) at 4°C. The homogenate was passed through 2 layers of muslin and the filtrate was centrifuged (25 000 g, 4°C) for 45 min. The supernatant was passed through 4 layers of muslin and used for enzyme assays. The extraction was carried out in duplicate.

2.6. Effect of freezing on HPL activity

The effect, on HPL activity, of freezing the root/shoot (RS) part of the seed in liquid N_2 was investigated using 5 day-old germinated seedlings. A portion (2.87 g) was homogenised with 6 ml sodium phosphate buffer (0.1 M, pH 6.8) and filtered through 4 layers of muslin. The filtrate was assayed for enzymic activity. Another portion (2 g) was immersed in liquid N_2 until frozen (5 min), then homogenised with 4 ml sodium phosphate buffer (0.1 M, pH 6.8) and filtered through 4 layers of muslin. The filtrate was assayed for enzymic activity.

The effect, on HPL activity, of freezing the RS in liquid N_2 was confirmed by headspace GC analysis. Freshly removed roots/shoot sections were removed from seeds and incubated for 15 min at 25°C. There was little hexanal produced.

Roots and shoots, that had been frozen in liquid nitrogen and then incubated, showed an 80-fold increase in hexanal formation, indicating that freezing was damaging the cellular structure and activating the HPL pathway. However, the freezing does not decrease enzyme activity during storage.

2.7. Distribution of HPL within pea seedlings

The distribution of HPL within 4 day-old seedlings was investigated by separating the seedling into three sections; the shoot (0.7 g), the root (0.8 g), and the seed with a 1 cm section at the junction of the hypocotyl with the root and shoot (4 g). These sections were homogenised for 30 s with 12 ml bis–Tris buffer (0.1 M, pH 6.8), containing Triton X-100 (0.5% w/v), DTT (4 mM) and PVP K-30 (0.5% w/v) at 4°C. The homogenate was passed through four layers of muslin and centrifuged (25 000 g) at 4°C for 30 min. The supernatant, containing solubilised HPL, was assayed. The extraction was performed in duplicate.

2.8. Estimation of pI of solubilised HPL using preparative isoelectric focusing: Rotofor

Samples of 7 day-old RS (4 g), stored at −20°C, were homogenised in a Waring blender for 30 s with 12 ml bis–Tris buffer (0.02 M, pH 6.8) containing Triton X-100 (0.5% w/v), DTT (5 mM) and PVP K-30 (4% 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 30 min and the resulting supernatant, containing solubilised HPL, was passed through four layers of muslin in order to remove lipids floating on the surface of the supernatant. A volume (5 ml) of solubilised HPL, equivalent to approximately 8 mg protein, was diluted with bis–Tris buffer (20 mM, pH 6.8) containing Triton X-100 (0.5% w/v), DTT (5 mM) and glycerol (10% v/v), together with Ampholine (1.9 ml, pH 3–10) to a final volume of 55 ml. The focusing chamber was assembled according to the manufacturer's instructions and electrophoresis was carried out for four hours at 4°C and constant power of 12 W. After completion of focusing, the samples were harvested in 20 fractions and assayed for HPL activity. The pH of each fraction was recorded using a glass electrode.

2.9. Extraction and purification of HPL

Seven day-old pea seedlings (25 g), stored at −20°C, were homogenised in a Waring blender for 30 s with 75 ml bis–Tris buffer (0.1 M, pH 6.8), containing DTT (5 mM) and PVP 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. 25 ml bis–Tris (20 mM, pH 6.8), containing Triton X-100 (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 passed through a Whatman GD/X filter (13 mm diameter 0.2 μm PVDF membrane) to remove starch granules and membranes.

2.10. Ion-exchange chromatography

The solubilised HPL solution was applied to a DEAE-Toyopearl 650M column (10 mm i.d.×180 mm long) 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 0–0.3 M NaCl, in bis–Tris buffer, to a total volume of 100 ml and 4°C. Fractions (5 ml) were collected.

2.11. Buffer exchange using PEG precipitation

Fractions containing the highest activities of HPL were pooled; solid PEG 6000 (40% w/v) was 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 10 ml sodium phosphate buffer (10 mM, pH 6.8) containing Triton X-100 (0.5% w/v), DTT (1 mM) and glycerol (10% v/v).

2.12. Hydroxylapatite chromatography

HPL-active solution was applied to a hydroxylapatite 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^{-1} at 4°C. The column was washed with phosphate buffer (30 ml) and elution was with a linear gradient of 10–300 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 100 ml and fractions (2.5 ml) were collected.

2.13. 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 *Escherichia 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).

2.14. Determination of pH optima for purified HPL activity

Four buffers were used for this procedure; citric acid/phosphate buffers (pH 5.0–6.0), sodium phosphate buffers (0.1 M, pH 6.0–8.0), Tris–HCl buffers (0.1 M, pH 8.0–8.5) and borate buffers (0.1 M, pH 8.5–9.0). Assays were at 234 nm, using either 9-hydroperoxy-linoleic acid or 13-hydroperoxy-linoleic acid.

2.15. 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 6.5) and purified HPL (0.27 μg).

3. Results and discussion

Perlite was found to be the most suitable growing medium as it retained water well and could be easily brushed off the seedlings.

3.1. Development of seedlings and HPL activity

Fig. 1 shows the development of HPL activity in germinating pea seedlings. HPL activity appeared after 2 days, increased to a maximum at 4 days (3.7 μmol min^{-1} ml^{-1}) and decreased slowly after 8 days (2.56 μmol min^{-1} ml^{-1}). Protein content decreased over 8 days from a maximum of 12.3–2.39 mg/ml. This resulted in an approximate 100-fold increase in specific activity from 0.01 μmol min^{-1} mg^{-1} (day 2) to 1.07 μmol min^{-1} mg^{-1} (day 8). Similarly, Sekiya, Kajiwara and Hatanaka (1979) showed that HPL activity was high in dry alfalfa seeds and in the early stages of germination (1–2 days). HPL activity was lost completely after 5 days or more. However, for cucumber seeds, HPL activity was not detected until after 5 days' germination. HPL activity was not detected in dry seeds.

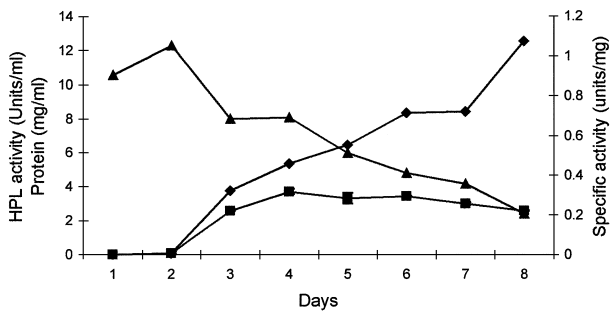

Fig. 1. Development of HPL activity in germinating pea seedlings: ■—■, HPL activity with 13-hydroperoxy-linoleic acid substrate; ▲—▲, protein measured at 562 nm; ◆—◆, specific activity.

The effect of freezing on RS of pea seedlings is shown in Table 1. Approximately 1.7 times more activity was extracted from frozen sections compared with fresh sections. A higher quantity of protein was also extracted, resulting in a 1.5-fold increase in the specific activity of the frozen section compared to the non-frozen section. Freezing may have damaged the cellular structure and, therefore, allowed more cellular material to be extracted. This was confirmed by measuring the formation of hexanal from intact fresh and frozen shoot/roots sections. There was an approximate 80-fold increase of hexanal formation in frozen sections compared to fresh sections.

For HPL purification, whole seedlings were used. Freezing of the seedlings in liquid nitrogen and storage at −20°C did not affect the stability of HPL. The specific activity of HPL remained relatively constant for 35 days at −20°C.

The distribution of HPL within 4 days old seedlings is shown in Table 2. Over 90% of HPL activity was located in the seed + junction. The seed + junction contained 20 and 40 times more activity than the shoots and roots, respectively, although it also contained approximately 30 times more protein than the shoots and roots. It was therefore decided to use the whole seedling in the purification of HPL, even though the quantity of protein was increased dramatically The specific activity of the shoot section was 1.5 and 2.0 times higher than the seed + junction and roots, respectively. In watermelon seedlings, HPL was found primarily in the hypocotyl-root junction where cell division occurred rapidly (Vick & Zimmerman, 1976) whereas, for cucumber seedlings, the roots were the richest source of HPL (Hatanaka, Kajiwara & Matsui, 1988). Matsui, Kaji, Kajiwara & Hatanaka (1996) have suggested that HPL activity is highest when rapid cell division occurs.

The pH and HPL activity of each fraction, after preparative isoelectric focusing, are shown in Fig. 2. The mean pH of fractions containing the highest HPL activities for 9-hydroperoxy-linoleic acid and 13-hydroperoxy-linoleic acid as substrates was found to be 5.23±0.23. This value is close to the pI of 5.5–5.8, reported for pear HPL (Kim & Grosch, 1981), and the pI of 5.8–6.1, reported for tomato HPL (Schreier & Lorenz, 1982).

Table 1
Effect of freezing on HPL activity

	Root/shoot section fresh weight (g)	Activity (μmol min^{-1} ml^{-1})	Protein (mg/ml)	Specific activity (μmol min^{-1} mg^{-1})
Fresh root/shoot	2.87	0.53	3.66	0.14
Frozen root/shoot	2.00	0.94	4.14	0.22

Table 2
Distribution of HPL within pea seedlings

	Fresh weight (g)	Activity (μmol min^{-1} ml^{-1})	Protein (mg/ml)	Specific activity (μmol min^{-1} mg^{-1})
Shoot	0.67±0.05	0.24±0.010	0.33±0.007	0.71±0.015
Root	0.77±0.06	0.10±0.007	0.27±0.019	0.36±0.003
Seed + junction	4.03±0.18	4.24±0.225	9.07±0.742	0.47±0.013

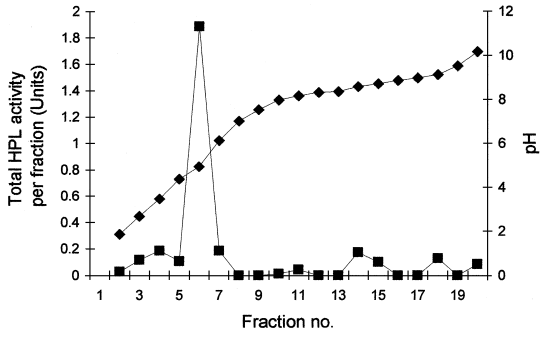

Fig. 2. Preparative isoelectric focusing of hydroperoxide lyase activity: ■—■, HPL activity with 13-hydroperoxy-linoleic acid substrate; ▲—▲, ◆—◆, pH.

Further attempts to purify HPL, using chromatofocusing techniques, were unsuccessful as no lyase was eluted, although it was possible to separate soluble proteins having a pI difference of 0.1 (β-lactoglobulin A + B).

3.2. Purification of HPL

After anion-exchange chromatography with DEAE-Toyopearl 650 M, approximately 40% of the applied 9- and 13-hydroperoxide-lysing activity was eluted by a linear salt gradient from approximately 0.04–0.07 M NaCl, whereas only approximately 20% of the protein applied was recovered, thus yielding approximately a 2-fold increase in specific enzymic activity (Table 3). Following buffer exchange by PEG 6000 precipitation and chromatography of the resuspended pellet with hydroxylapatite, approximately 8% of the applied 9- and 13-hydroperoxide-lysing activity was eluted at a phosphate concentration of approximately 0.065–0.14 M. Approximately 3% of applied protein was recovered, thus giving (approximately) a 10-fold increase in specific activity with respect to the solubilised fraction. During storage, absence of DDT resulted in inactivation of HPL and an optimum concentration (1 mM) was necessary to stabilise the Triton X-100-solubilised fraction. Rehbock and Berger (1998) have suggested that HPL contains sensitive sulfhydryl groups at the active site which require a reducing agent to retain stability. Hydroxylapatite-purified 9- and 13-hydroperoxide-lysing activities, stored in the elution buffer at 4°C, lost approximately 60% of enzymic activity for both substrates after one week.

Electrophoresis of the hydroxylapatite-purified HPL preparation in the presence of SDS revealed one predominant protein- staining band with (approximately) MW 55 000Da (Fig. 3), which is similar to HPL claimed for cucumber fruit (55 000 Da) by Hornostaj and Robinson (1999), bell pepper fruit HPL (55 000 Da), by Shibata, Matsui, Kajiwara and Hatanaka (1995) and tea leaf HPL, (53 000 Da), by Matsui, Toyota, Kajiwara, Kakuno and Hatanaka (1991). The presence of only one protein-staining band on SDS-PAGE suggests that either the molecular mass 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 which was able to catalyse the lysis of both 13- and 9-hydroperoxides of linoleic and linolenic acids. Individual plant lipoxygenases have the ability to produce both 9- and 13-hydroperoxides (Gardner, 1991); therefore, it is not entirely surprising that the pea HPL seems able to degrade both hydroperoxides.

For crude HPL activity, towards both 9-hydroperoxy- and 13-hydroperoxy-linoleic acids, the optimum pH was 6.0. However, for the hydroxylapatite-purified HPL fraction, the pH optimum was 6.5 for lysis of both the 13-hydroperoxy- and 9-hydroperoxy-linoleic acids. However, the 13-hydroperoxy-linoleic acid-lysing activity was less sensitive to increases in pH, compared with the 9-hydroperoxy-linoleic acid-lyase activity, for which only half of the original activity was observed at (approximately) pH 7.0. Similar ranges of optimum values for HPL from alfalfa and cucumber seedlings (Sekiya et al., 1979) and watermelon seedlings (Vick & Zimmerman, 1976) have been found.

Table 3
Purification of HPL from pea seedlings

	Total activity (μmol min^{-1})	Total protein (mg)	Specific activity (μmol min^{-1} mg^{-1})	Yield (%)	Purification (-fold)
Triton X-100 Solubilised fraction					
13-HPOLA	25.9	34.1	0.76	100	1.00
9-HPOLA	14.2		0.42	100	1.00
DEAE-Toyopearl					
13-HPOLA	10.3	6.99	1.47	39.6	1.93
9-HPOLA	6.84		0.98	48.2	2.33
PEG 6000 precipitation					
13-HPOLA	7.38	2.39	3.09	28.5	4.07
9-HPOLA	4.5		1.88	31.7	4.48
Hydroxylapatite					
13-HPOLA	0.54	0.07	7.71	2.1	10.1
9-HPOLA	0.36		5.14	2.5	12.2

The substrate specificity of the hydroxylapatite-purified HPL was determined by examining the activities of the HPL with various PUFA hydroperoxides, as shown in Table 4. 13-Hydroperoxy-linolenic acid was 1.8, 0.8 and 1.5 times more readily lysed than 9-hydroperoxy-linoleic acid, 9-hydroperoxy-linolenic acid and 13-hydroperoxy-linoleic acid, respectively. Pea seedlings produce both C_6– and C_9– aldehydes when reacted with 13-hydroperoxy-linoleic and 9-hydroperoxy-linoleic acids, respectively, as determined by headspace GC. Cucumber (Sekiya et al., 1979), soybean seedlings (Gardner, Weisleder & Plattner, 1991) and alfalfa seedlings (Noordermeer, Veldink & Vliegenthart, 1999) also lysed both substrates, whereas mung bean seedlings (Rehbock & Berger, 1998) and watermelon seedlings (Vick & Zimmerman, 1976) have been claimed to lyse only 13-hydroperoxy-linoleic acid.

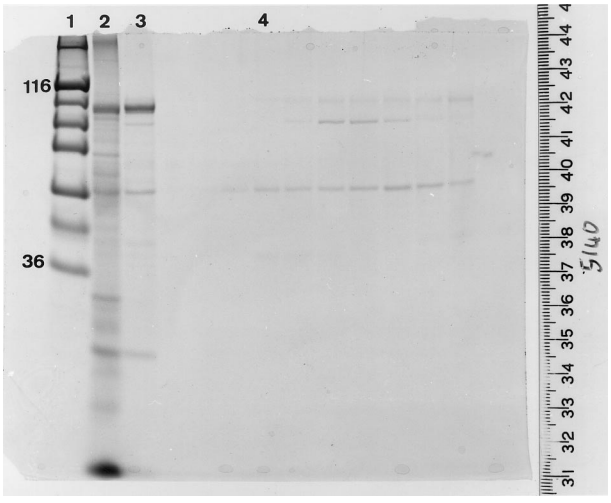

Fig. 3. Samples applied to a SDS-PAGE gel (μg protein/lane): (1) molecular weight markers; (2) Triton X-100 solubilised fraction (75 μg); (3) DEAE-Toyopearl 650 M fraction (35 μg); (4) hydroxylapatite fraction (2 μg).

From double reciprocal plots of HPL activity versus substrate concentration apparent K_m^{app} values for 9-hydroperoxy-linoleic acid, 9-hydroperoxy-linolenic acid, 13-hydroperoxy-linoleic acid and 13-hydroperoxy-linolenic acid were 4.5, 14.2, 11.1 and 6.2 μM, respectively (Table 5). This shows that HPL possessed a slightly greater affinity for 9-hydroperoxy-linoleic acid, followed by 13-hydroperoxy-linoleic acid. The corresponding V_{max}^{app} values were 0.12, 0.12, 0.22 and 0.26 μmol min^{-1}. The magnitude of substrate lysis was 13-hydroperoxy-linoleic acid > 13-hydroperoxy-linoleic acid > 9-hydroperoxy-linolenic acid and 9-hydroperoxy-linoleic acid. It has been reported that pea seeds *v. Birte* produce approximately 2.5 times more 13-hydroperoxy-linoleic acid than 9-hydroperoxy-linoleic acid (Forster et al., 1999). This may account for the preferential lysis by HPL of 13-hydroperoxides over 9-hydroperoxides. The

Table 4
Substrate specificity of purified HPL

Substrate	% Relative activity
9-Hydroperoxy-linoleic acid	56.5
9-Hydroperoxy-linolenic acid	78.3
13-Hydroperoxy-linoleic acid	65.2
13-Hydroperoxy-linolenic acid	100

Table 5
Apparent K_m and apparent V_{max} values for substrates with purified HPL

Substrate	K_m^{app} (μM)	V_{max}^{app} (μmol min^{-1})	V_{max}^{app}/K_m^{app}
9-HPOLA[a]	4.5	0.12	0.03
9-HPOLNA[b]	14.2	0.12	0.01
13-HPOLA	11.1	0.22	0.02
13-HPOLNA	6.2	0.26	0.04

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

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 0.03, 0.01, 0.02 and 0.04, respectively.

Acknowledgements

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