may be of major importance in this respect.

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Partial Purification and Properties of a Hydroperoxide Lyase from Fruits of Pear

In-Sook Kim and Werner Grosch*

A relatively stable hydroperoxide lyase was partially purified from pears by differential centrifugation, gel chromatography, and isoelectric focusing. The enzyme preparation was optimally active at pH 6.5. It was specific for 9-hydroperoxide isomers of linoleic and linolenic acid. The former substrate was cleaved to *cis*-3-nonenal and 9-oxononanoic acid.

The formation of volatile aldehydes of chain lengths C_6 and C_9 by an enzymatic oxidative cleavage of a C_{18} unsaturated fatty acid containing a *cis*-1,*cis*-4-pentadiene system is widespread in fruits and vegetables (Gardner, 1975; Hatanaka et al., 1978; Tressl and Drawert, 1973). The process proceeds rapidly when plant cells are disrupted in the presence of oxygen. Linoleic and linolenic acids, liberated from endogenous lipids by acyl hydrolases, are converted to their hydroperoxides by lipoxygenase enzyme (Wardale and Galliard, 1975). In watermelon seedlings (Vick and Zimmerman, 1976), tomato fruits (Galliard and Matthew, 1977), tea chloroplasts (Hatanaka et al., 1979), bean leaves (Matthew and Galliard, 1978), and alfalfa seeds (Sekiya et al., 1979), a hydroperoxide lyase has been detected which subsequently cleaves the 13-hydroperoxides in C_6 aldehydes and 12-oxo-*cis*-9-dodecenoic acid. Hexanal is formed from 13-hydroperoxyoctadeca-*cis*-9,*trans*-11-dienoic acid (13-HPOD) and *cis*-3-hexenal from 13-hydroperoxyoctadeca-*cis*-9, *trans*-11,-*cis*-15-trienoic acid (13-HPOT).

Deutsche Forschungsanstalt für Lebensmittelchemie, D-8046 Garching, West Germany.

A hydroperoxide lyase reacting with both 9- and 13-hydroperoxides occurs in fruits of cucumbers (Galliard et al., 1976). As well as 9-oxononanoic acid, *cis*-3-nonenal is released from 9-hydroperoxyoctadeca-*trans*-10,*cis*-12-dienoic acid (9-HPOD) and *cis*-3,*cis*-6-nonadienal from 9-hydroperoxyoctadeca-*trans*-10,*cis*-12,*cis*-15-trienoic acid (9-HPOT). In plant tissues the cis-3 double bond in the enals is often enzymatically isomerized to the conjugated trans-2 derivatives (Phillips et al., 1979).

Hydroperoxide lyases from fruits (Phillips and Galliard, 1978) and seedlings of cucumbers and alfalfa (Vick and Zimmerman, 1976) were partially purified and separated from lipoxygenase. The membrane-bound and extremely heat labile enzyme from cucumber fruits catalyzes the cleavage of hydroperoxides to carbonyl compounds without formation of free intermediates (Phillips and Galliard, 1978).

EXPERIMENTAL SECTION

Materials. Pear fruits (*Pyrus communis* L.) were purchased from local markets; their origin varied seasonably and the cultivars were unidentified. 13-HPOD was prepared from linoleic acid and 13-HPOT from linolenic acid by using purified type I soybean lipoxygenase (Eg-

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mond et al., 1976). 9-HPOD and 9-HPOT were prepared with the aid of a crude potato lipoxygenase (Grosch, 1977). The 9- and 13-hydroperoxides proved to be pure (greater than 95%) according to high-performance liquid chromatography (Chan and Prescott, 1975). The concentration of the hydroperoxides was measured photometrically at 234 nm by using 2.6×10^4 L mol^{-1} cm^{-1} (Matthew et al., 1977) as the extinction coefficient. Methyl 9-oxononanoic acid was prepared by reductive ozonolysis of methyl oleate (Stein and Nicolaides, 1962). *cis*-3-Nonenal was a gift of A. Hatanaka (Yamaguchi, Japan) and *trans*-2,*cis*-6-nonadienal–DNPH was a gift of P. W. Meijboom (Unilever, Vlaardingen, The Netherlands). Linoleic and linolenic acids (greater than 99%) were from Nu Chek Prep (Elysian Minn.), *trans*-2-nonenal was from Atlanta, Heidelberg, and Sepharose CL-2B, Sepharose CL-6B and Blue Dextran 2000 were from Pharmacia. The other chemicals were of analytical grade.

Methods. *Determination of Hydroperoxide Lyase Activity.* Especially for a rapid location of the enzyme in the eluates of chromatographic systems, the disappearance of 9-HPOD was measured by following the decrease in the absorbance at 234 nm (Vick and Zimmerman, 1976). The breakdown of the HPOD was calculated by using 2.6×10^4 L mol^{-1} cm^{-1} (Matthew et al., 1977) as the extinction coefficient. In addition, the following two procedures were applied: substrate and incubation. Unless otherwise stated the solvent was evaporated from 8 μmol of 9-HPOD in a stream of nitrogen. The 9-HPOD was dissolved in 4 mL of water containing 0.001% Tween 80 and 20 mL of 0.1 M sodium phosphate buffer (pH 6.5) was added. In a vial 0.5 mL of enzyme solution was added to 0.5 mL of substrate, and the mixture was incubated in an atmosphere of nitrogen at 25 °C for a definite time. In method I the formation of carbonyl compounds was measured: The incubation was stopped by addition of 0.2 mL of a saturated solution of 2,4-dinitrophenylhydrazine in 2 M HCl, and the sealed vial was then heated for 30 min at 60 °C. After the vial was cooled, the 2,4-dinitrophenylhydrazones formed were extracted with 1 mL of carbonyl-free benzene. The extract was put in a microcuvette (volume 1.0 mL; pathway 1 cm), and the absorbance was measured at 370 nm against a blank containing all reagents except the enzyme. In method II the breakdown of the hydroperoxy group was determined: 0.1 mL of the incubation mixture was pipetted into 0.3 mL of methanol. After addition of 0.1 mL of water, the ferrous thiocyanate test was carried out with a sample of 0.2 mL (Grosch, 1976). The absorbances measured were referred to a calibration curve obtained with 9-HPOD.

Determination of Lipoxygenase Activity. The enzyme was photometrically assayed by following the diene formation at 234 nm (Kim and Grosch, 1979).

Protein Determination. During chromatography, the concentration of protein was measured by the absorbance at 280 nm. For more accurate measurements, especially when dealing with crude and semipurified preparations, the biuret method as described by Beisenherz et al. (1953) was used.

Isoelectric Focusing. The experiments were carried out with an LKB 8101 electrofocusing column of 110-mL capacity (LKB Producter AB, Bromma, Sweden). Electrofocusing was performed as described in the LKB instruction manual (pH 5–7; 4 °C; 17 h; final potential 500 V).

Partial Purification of the Hydroperoxide Lyase. All steps were performed at 0–2 °C. After the pears were washed, they were cut into small pieces. The pieces (100 g) were soaked in 50 mL of 0.1 M Tris-HCl buffer (pH 7.0) containing 2 mM EDTA, 4 mM dithioerythritol, and 0.2% Triton X-100 and then homogenized with an Ultra-Turrax 4 times for 10 s. The slurry obtained was filtered through two layers of cheesecloth and centrifuged at 10^4g for 30 min. The pellet was discarded and the supernatant was centrifuged at 123000*g* for 1 h. The pellet, suspended in 5 mL of Tris-HCl buffer (pH 7.0), containing 4 mM dithioerythritol and 0.2% Triton X-100, was homogenized with a Potter-Elvehjem homogenizer. After centrifugation at 5000*g* for 20 min, 3.5 mL of the supernatant was chromatographed on a Sepharose CL-2B column (2.0 × 60 cm), which was equilibrated and eluted with a 0.1 M Tris-HCl buffer (pH 7.0). Fractions of 3 mL were collected and assayed for protein, lipoxygenase, and hydroperoxide lyase activities. For convenience the combined eluate containing the hydroperoxide lyase was designated "HL fraction".

Products from 9-HPOD and 9-HPOT. The reaction mixture contained 0.5 mM 9-HPOD or 9-HPOT, HL fraction (5–10 mg of protein) and 20 mL of 0.1 M sodium phosphate buffer (pH 6.5). After 20 min of incubation at 25 °C in an atmosphere of nitrogen the completion of the hydroperoxide breakdown was assessed with the ferrous thiocyanate test (Grosch, 1976) in an aliquot (1 mL) which was pipetted to 3 mL of methanol and then diluted with 1 mL of water. After incubation, the reaction mixture was extracted twice with 25 mL of diethyl ether. The diethyl ether solution was washed twice with 10 mL of water, and dried over Na_2SO_4, and concentrated to 2.7 mL. After addition of 0.3 mL of methanol, the fatty acids were methylated with diazomethane as described by Schlenk and Gellerman (1960).

Analysis by GLC. After methylation, the solution was concentrated to 0.2 mL in a stream of nitrogen at 0 °C. GLC was performed on a 3 m × 3.2 mm stainless steel column of 10% FFAP on Chromosorb W (80–100 mesh). The temperature of the oven was programmed from 80 to 230 °C at 2 °C/min; the injector temperature was 200 °C, the detector (FID) temperature was 250 °C; helium was used as the carrier gas (20 mL/min). For identification of the products in the gas chromatogram, the FFAP column was coupled via a Biemann-Watson separator with a Varian CH 7 mass spectrometer; the temperature of the ion source was 200 °C; the energy was 70 eV.

Analysis as 2,4-Dinitrophenylhydrazones (DNPH's). To the methylated and concentrated solution (0.2 mL) of products 2 mL of benzene, 8 mL of a benzene solution saturated with 2,4-dinitrophenylhydrazine and 1 mL of a 12% trichloroacetic acid solution in benzene were subsequently added. After 10 min at room temperature, the reaction mixture was diluted with 10 mL of benzene, 4 times extracted with 10 mL of water, and dried over Na_2SO_4. Celite (0.5 g) was suspended in the solution, and the solvent was removed in vacuum. The DNPH–Celite was applied onto a column (1.5 × 5 cm) packed with neutral aluminum oxide (activity grade III) in the petroleum ether–diethyl ether (1:1 v/v). From the crude DNPH mixture on the top of the column, the monocarbonyl DNPH was separated by elution with the same solvent system.

TLC of DNPH. The DNPH were separated on a preparative scale by TLC on Kieselgur plates impregnated with Carbowax 400 and with cyclohexane as the solvent (Schormüller et al., 1969). The DNPH were scraped off, eluted with chloroform, freed from Carbowax, and rechromatographed by TLC using silica gel plates with CCl_4–hexane–ethyl acetate (10:2:1 v/v/v) as the mobile phase (Grosch et al., 1974). DNPH of unsaturated al-

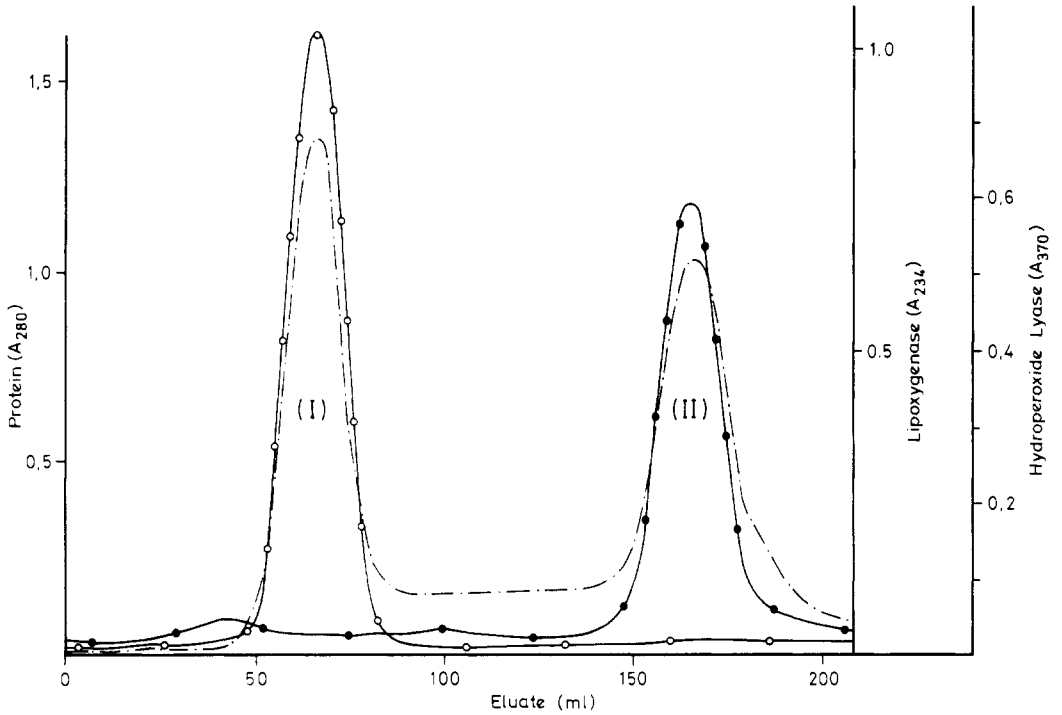

Figure 1. Gel filtration on Sepharose CL-2B. Sepharose column (2.0 × 60 cm) equilibrated with 0.1 M Tris-HCl buffer (pH 7.0); 36 mg of protein dissolved in the same buffer was applied. Elution (15 mL/h) was performed with 0.1 M Tris-HCl buffer (pH 7.0). (---) Protein; (○) lipoxygenase activity at pH 6.0; (●) hydroperoxide lyase activity at pH 6.5.

dehydes were chromatographed on silica gel plates impregnated with 30% $AgNO_3$ with benzene as the solvent (Meijboom, 1968).

Spectroscopy of DNPH. Mass spectroscopy of the DNPH was performed as described (Schieberle and Grosch, 1981).

RESULTS

Partial Purification. A microsomal fraction which was isolated by two centrifugation steps from a homogenate of pears was separated by gel filtration into two fractions of proteins (Figure 1). Peak I contained, as previously reported (Kim and Grosch, 1978), the lipoxygenase activity and peak II, the HL fraction, was active in cleaving 9-HPOD. The protein content of a solution of the HL fraction with known absorption at 280 nm was determined by the biuret reaction. From these data $A^{1\%}_{1cm}$ at 280 nm was calculated as 9.6. The HL fraction was subjected to isoelectric focusing on a preparative scale. One major peak which contained nearly 70% of the protein applied onto the column for isoelectric focusing appeared in the elution diagram Figure 2. It showed hydroperoxide lyase activity with 9-HPOD as the substrate. During purification the hydroperoxide lyase specific activity increased about 12-fold (Table I).

The enzyme was relatively stable. In 0.1 M acetate buffer (pH 6.5) at 50 °C, the HL fraction lost 30% activity in 10 min and was completely inactivated in 60 min. At 0 °C the enzyme lost no activity in 48 h. The molecular weight of the HL fraction was appraised by gel chromatography on a Sepharose CL-6B column (1.5 × 50 cm) calibrated with Blue Dextran. The lower elution volume of the protein fraction (60 mL) in comparison to Blue Dextran (66 mL) indicated a molecular weight of 2×10^6 which was reported by the producer as the average value of the dye.

Optimum pH and Substrate Specificity. The HL fraction had an optimum activity at pH 6.5 (Figure 3). At this pH only the 9- (but not the 13-) HPOD was consumed by the HL fraction (Figure 4). From the disappearance of 9-HPOD in this experiment, a hydroperoxide lyase specific activity of 50 nmol of 9-HPOD min^{-1} mg^{-1} was

Table I. Partial Purification of the Hydroperoxide Lyase from Pears

enzyme preparation	volume, mL	total protein,[b] mg	total act.,[c] A_{370nm}	sp act.,[c] A_{370nm}/mg of protein
(1) supernatant of the centrifugation at 10^4g	115	115	13.9	0.12
(2) residue[a] of the centrifugation at 123000g	5	36	13.4	0.36
(3) peak II from the Sepharose column (HL fraction)	28	14	14.0	1.00
(4) isoelectric focusing	8	9.6	13.5	1.41

[a] After solution of the residue and centrifugation at 5000g (cf. Experimental Section). [b] Protein was measured by the biuret method (steps 1-3) and by the absorbance at 280 nm (step 4). [c] The hydroperoxide lyase activity was measured by method I.

Table II. Substrate Specificity

no.	substrate	ΔA_{505nm} (1-10 min)
1	200 μM 9-HPOD[a]	0.02
2	200 μM 9-HPOD	0.26
3	500 μM 9-HPOD	0.27
4	200 μM 13-HPOD[a]	0.02
5	200 μM 13-HPOD	0.02
6	500 μM 13-HOD	0.02
7	200 μM 9-HPOT	0.24
8	200 μM 13-HPOT	0.03

[a] The HL fraction was omitted. The reaction contained the hydroperoxide isomer, 2 μL/L Tween 80, HL fraction (0.9 mg of protein), and 0.1 M phosphate buffer (pH 6.5) in a total volume of 1 mL. During incubation at 25 °C, the breakdown of the hydroperoxide group was measured by method II.

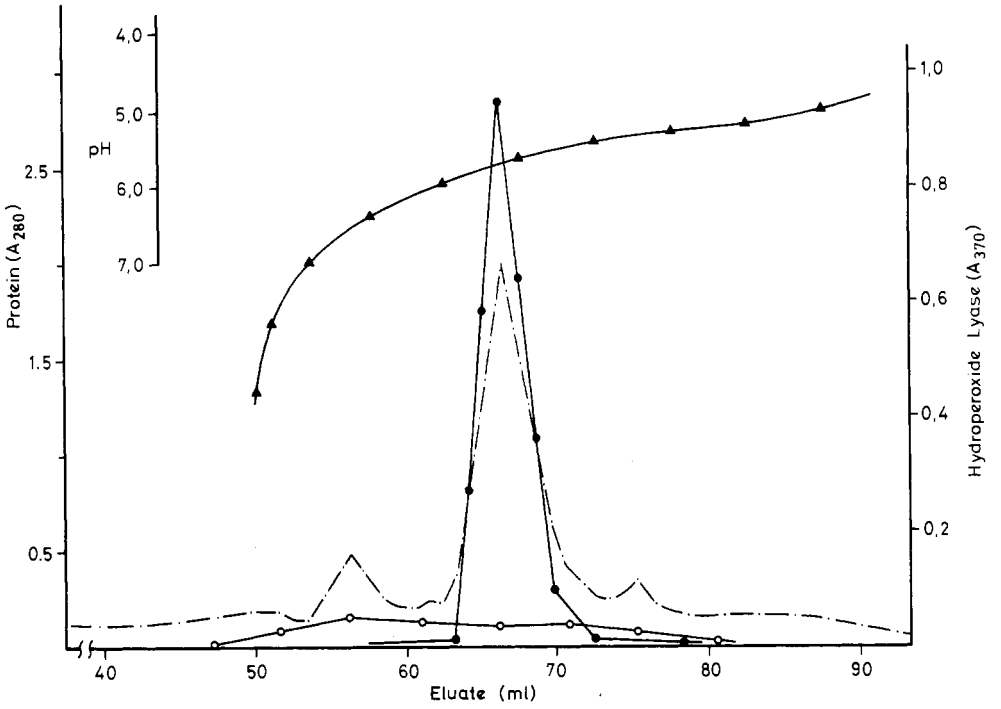

Figure 2. Isoelectric focusing of peak II from the Sepharose column between pH 5 and pH 7 in a sucrose density gradient. Column loaded with 14 mg of protein. (---)Protein; assay for hydroperoxide lyase activity with (●) 9-HPOD or with (○) 13-HPOD as the substrate; (▲) pH.

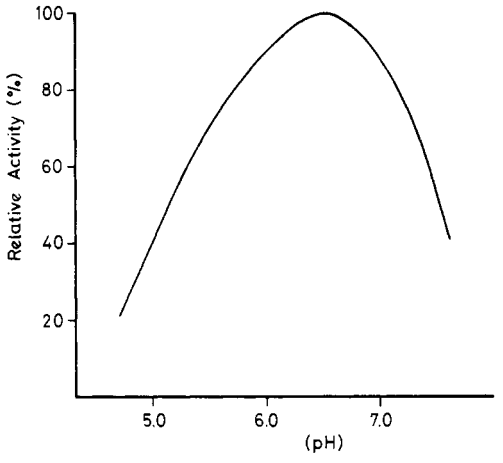

Figure 3. pH response curve for hydroperoxide lyase activity. The hydroperoxide lyase assay was performed by method I. The following 0.1 M buffer solutions were used: sodium acetate (pH 3.5–6.5) and triethanolamine hydrochloride–NaOH (pH 6.5–8.0).

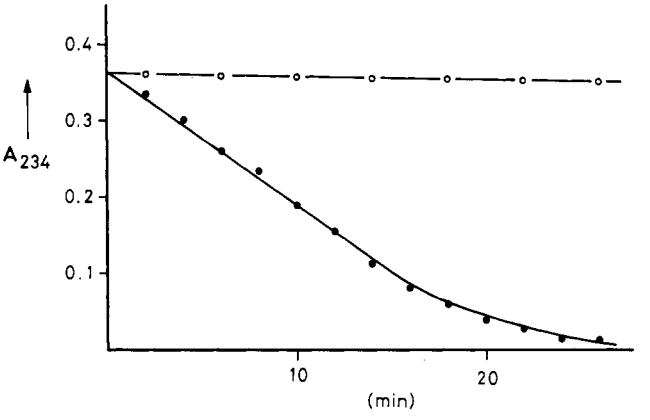

Figure 4. Breakdown of hydroperoxides by the HL fraction. The reaction mixture contained 25 μM HPOD, 2 μL/L Tween 80, HL fraction (24 μg of protein), and 0.1 M phosphate buffer (pH 6.5) in a total volume of 2 mL. During incubation at 25 °C, the decrease in the absorbance at 234 nm was measured with (●) 9-HPOD or with (○) 13-HPOD as the substrate.

calculated. This is in good agreement with the value of 44 nmol of 9-HPOD min^{-1} mg^{-1} found in experiment no. 2 (Table II) in which the breakdown of the hydroperoxide group was determined by the ferrous thiocyanate test.

It can be seen from the data in Table II that all the 9-hydroperoxide isomers derived from linoleic and linolenic acid were cleaved with similar rates but the 13-hydroperoxide isomers are clearly unsuitable substrates since the rate of their breakdown was almost identical in experiments both with and without the HL fraction. The specificity of the HL fraction for 9-hydroperoxide isomers was further demonstrated in the experiments with varying concentrations of 9- and 13-HPOD (Figure 4; Table II) and also in the isoelectric focusing experiment (Figure 2).

Identification of Products. After incubation of the HL fraction with 9-HPOD and 9-HPOT, respectively, the products were separated by GLC (Figure 5). The cleavage of each substrate resulted in two major products. Compounds Ia and III and the side product Ib were obtained from the 9-HPOD and compounds II and III from the 9-HPOT (Figure 5).

Products Ia and Ib were identified as *cis*-3-nonenal and *trans*-2-nonenal, respectively, on the basis of the following data: Both compounds were coincident with authentic samples on GLC. The mass spectra of Ia and Ib were similar (parts a and b of Figure 6) up to the difference in the intensities of the fragment ions m/e 69 and 70 which possibly reflect the different position of the double bond in the molecule of both nonenals. In contrast to Ia, the m/e 70 ion predominated in intensity relative to the m/e 69 ion in the spectrum of Ib, since as proposed in Figure 6b a cleavage of this molecule into two halves of equal molecular weight is favored by a MacLafferty rearrangement.

For confirmation of the proposed structures, both Ia and Ib were converted to DNPH derivatives. On Kieselgur/Carbowax TLC both DNPH's were slightly more mobile than octanal–DNPH. However, on the basis of the dif-

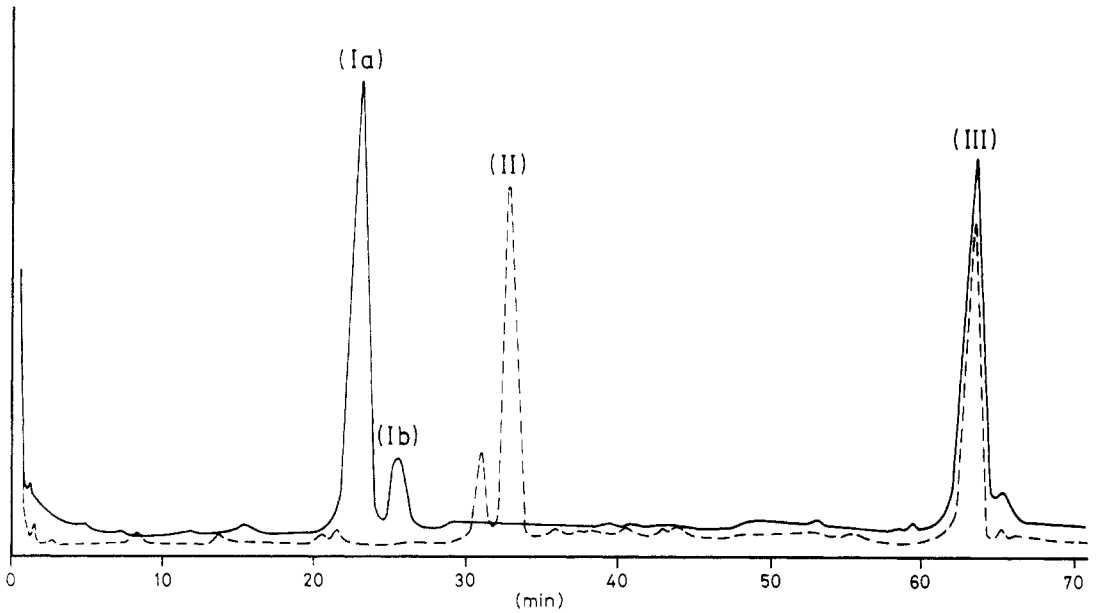

Figure 5. Gas–liquid chromatography of the products obtained from incubation of the HL fraction with (—) 9-HPOD and (- - -) 9-HPOT. The oxo fatty acids were chromatographed as methyl esters.

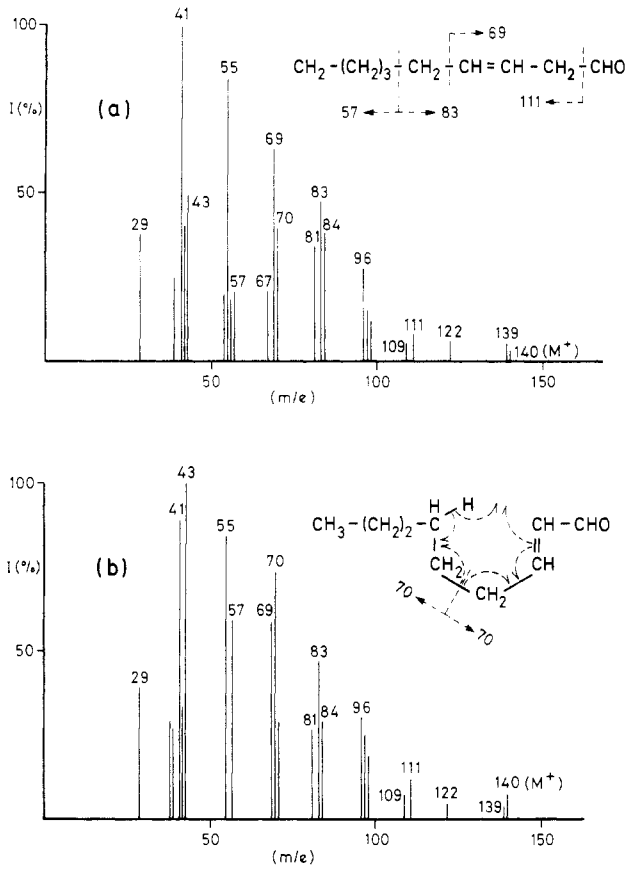

Figure 6. Mass spectra of products Ia (a) and Ib (b).

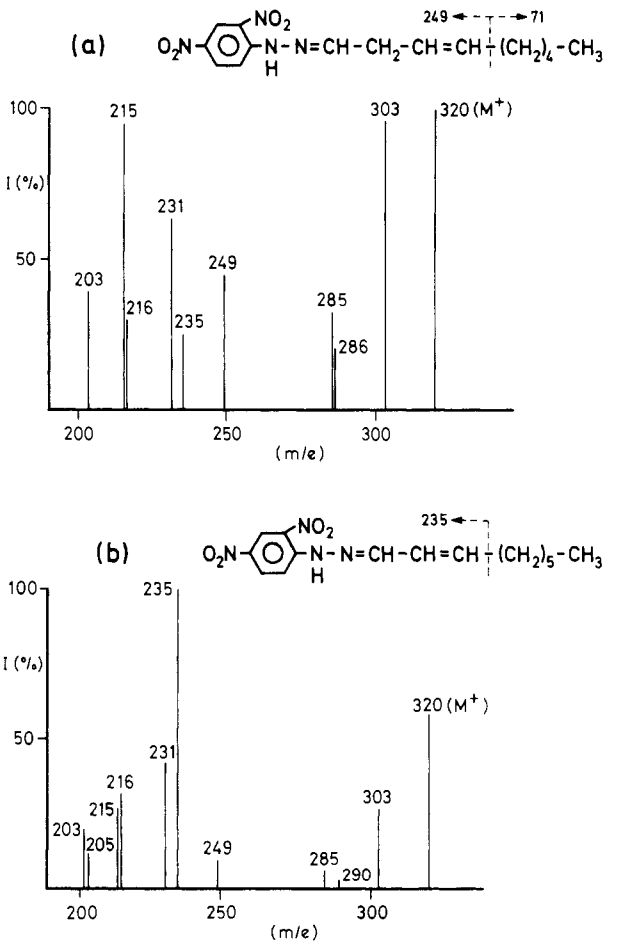

Figure 7. Mass spectra (ions m/e 200) of the 2,4-dinitrophenylhydrazones of products Ia (a) and Ib (b).

ference in geometry of the double bond, they could be separated on silica gel–$AgNO_3$ plates. Cochromatography with authentic samples showed that the DNPH of Ia was as mobile as *cis*-3-nonenal–DNPH (R_f 0.61) and the DNPH of Ib was coincident with *trans*-2-nonenal–DNPH (R_f 0.83). The difference in the UV spectra of the DNPH (λ_{max} in $CHCl_3$ = 335 nm for Ia and 370 nm for Ib) is caused by the conjugation of the double bond to the chromophore in the derivative of Ib. The mass spectra of both DNPH's which shows an abundant molecule ion at m/e 320 (Figure 7) and the intensities of the ions m/e 249 and 235 indicated the position of the double bond in Ia and Ib, respectively (comparison of parts a and b of Figure 7).

Product II was tentatively identified as *cis*-3,*cis*-6-nonadienal from the following properties of the DNPH derivative: The mass spectrum showed an intense molecular ion at m/e 318. On silica gel–$AgNO_3$ the DNPH derivative of II (R_f 0.17) was not as mobile as *trans*-2,*cis*-6-nonadienal–DNPH (R_f 0.29) which is indicative for the occurrence of two double bonds with cis configuration in product II.

The UV spectrum (λ_{max} = 335 nm in $CHCl_3$) showed that the double bonds are not in conjugation with the carbonyl group.

Product III was identified as 9-oxononanoic acid from the following results: The methyl ester was coincident with the authentic methyl 9-oxononanoate on GLC and in the features of the mass spectrum. Product III formed a DNPH derivative (λ_{max} = 335 nm in $CHCl_3$). After methylation the DNPH derivative of III gave a mass spectrum with an intense molecular ion at m/e 366. The spectrum was identical with that of the corresponding reference substance.

DISCUSSION

A relatively stable hydroperoxide lyase showing optimal activity at pH 6.5 was partially purified from pears. The enzyme is the first example of a hydroperoxide lyase which cleaves exclusively the 9-hydroperoxides of linoleic and linolenic acid. Its substrate specificity contrasts with that of the specific hydroperoxide lyases mentioned in the introduction which are only active against the corresponding 13-hydroperoxides.

Incubation of the HL fraction with 9-HPOD yields *cis*-3-nonenal and 9-oxononanoic acid as major products and relatively small amounts of *trans*-2-nonenal. The trans isomer may be formed from *cis*-3-nonenal either by a nonenzymatic rearrangement or by the action of *cis*-3,-*trans*-2-enal isomerase which has been found, e.g., in cucumber fruit (Phillips et al., 1979) and tea leaves (Hatanaka and Harada, 1973). From the difference in the amounts of both nonenals formed during incubation of the HL fraction with 9-HPOD, we conclude that the isolated hydroperoxide lyase might be possibly contaminated with a small amount of isomerase activity.

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Sterols, Esterified Sterols, and Glycosylated Sterols of Cow Pea Lipids (*Vigna uguiculata*)

Vhundi G. Mahadevappa and Piyara L. Raina*

Four sterol-containing lipid fractions, viz., free sterol, esterified sterol, sterol glycoside, and esterified sterol glycoside, were isolated from the chloroform–methanol extracted lipids of cow pea by preparative column and thin-layer chromatography. On a total lipid basis, these comprised 0.13%, 0.024%, 0.036%, and 0.029%. The major fatty acids in both the esterified fractions were linoleic, linolenic, and palmitic acids. Esterified sterol was more unsaturated (calculated iodine value of 139) than esterified sterol glycoside (calculated iodine value of 93). All the four sterol lipids contained high proportions of β-sitosterol and stigmasterol. About 3% campesterol has also been reported. The sugar identified in both the glycosylated sterols was only D-glucose. On the basis of the findings, the major representative species deduced are as follows: esterified sterols, β-sitosterol/stigmasterol linoleate, and to a lesser extent linolenate and palmitate; sterol glycoside, β-D-glucopyranosyl-(1→3)-S where S represents either β-sitosterol or stigmasterol; esterified sterol glycoside, 6-*O*-acyl-β-D-glucopyranosyl-(1→3)-S where S again represents either β-sitosterol or stigmasterol.

Cow pea (*Vigna uguiculata*) is one of the very important legumes grown and consumed as a source of protein in the southern part of India. Lipid composition of this legume has been reported earlier (Mahadevappa and Raina, 1978a). These workers have further shown that the lipids extracted from the legume had a hypocholesterolemic effect in rats and rabbits maintained on atherogenic diets which may stem from the considerable levels of polyunsaturated fatty acids (Mahadevappa, 1980). This legume also carries high proportions of sterol-containing lipids.

Discipline of Biochemistry and Applied Nutrition, Central Food Technological Research Institute, Mysore 570 013, India (P.L.R.), and Department of Biochemistry, Karnatak Medical College, Hubli 580 022, India (V.G.M.).

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