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Lipoxygenase from Potato Tubers

PARTIAL PURIFICATION AND PROPERTIES OF AN ENZYME THAT SPECIFICALLY OXYGENATES THE 9-POSITION OF LINOLEIC ACID

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A lipoxygenase (EC 1.13.1.13) was partially purified from potato tubers and was shown to differ from previously characterized soya-bean lipoxygenases in the positional specificity and pH characteristics of the oxygenation reaction. The potato enzyme converted linoleic acid almost exclusively (95%) into 9-D-hydroperoxyoctadeca-*trans*-10,*cis*-12-dienoic acid. The 13-hydroperoxy isomer was only a minor product (5%). Linolenic acid was an equally effective substrate, which was also oxygenated specifically at the 9-position. The enzyme had a pH optimum at 5.5–6.0 and was inactive at pH 9.0. A half-maximal velocity was obtained at a linoleic acid concentration of 0.1 mM. No inhibition was observed with EDTA (1 mM) and cyanide (1 mM) or with *p*-chloromercuribenzoate (0.2 mM). Haemoproteins were not involved in the lipoxygenase activity. The molecular weight of the enzyme was estimated from gel filtration to be approx. 10^5. Preliminary evidence suggested that the enzyme oxygenated the $n-10$ position of fatty acids containing a penta($n-3$, $n-6$)diene structure.

Although lipoxygenase (EC 1.13.1.13) has been known and studied for many years, the physiological role of the enzyme has not been established. Lipoxygenases from seeds of legumes (particularly soya beans) and some cereal- and oil-seeds have been studied most intensively (Holman & Bergström, 1951; Tappel, 1962*a*, 1963) and lipoxygenase was thought to be present only in those tissues (Tappel, 1962*a*). Recently, several laboratories have investigated the positional specificity of lipoxygenase-catalysed oxidation of fatty acids. The ratio of 13- to 9-hydroperoxides formed from linoleic acid with soya-bean lipoxygenase has been variously quoted as 7:3 (Hamberg & Samuelsson, 1965), 4:1 (Zimmerman & Vick, 1970), 23:2 (Hamberg & Samuelsson, 1967) and 100:0 (Dolev, Rohwedder & Dutton, 1967); the ratio obtained with the enzyme from flax seed was 4:1 (Zimmerman & Vick, 1970). Although it has been suggested that the 9-hydroperoxide isomer arises in the flax system by non-enzymic oxidation (Zimmerman & Vick, 1970), recent results with the soya-bean enzyme indicate that the 9-hydroperoxide minor product is optically active (Veldink, Vliegenthart & Boldingh, 1970*a*), as is the 13-hydroperoxide (Hamberg & Samuelsson, 1967). The non-enzymic autoxidation of linoleic acid leads to approximately equal amounts of racemic 9- and 13-hydroperoxides (Shepton & Dutton, 1956; Dolev *et al.* 1967). A partially purified preparation of lipoxygenase from *Zea mays* catalysed the oxidation of linoleic acid mainly to optically active 9-D-hydroperoxyoctadeca-*trans*-10,*cis*-12-dienoic acid (83–88%) although smaller amounts of the 13-hydroperoxy isomer (12–17%) were also formed (Gardner & Weisleder, 1970). Apart from differences in positional specificity, lipoxygenases with different substrate specificities have been described (Koch, Stern & Ferrari, 1958; Koch, 1968) and iso-enzymes have been separated from lipoxygenase preparations from the soya bean (Christopher, Pistorius & Axelrod, 1970; Yamamoto, Yasumoto & Mitsuda, 1970), other legumes (Hale, Richardson, von Elbe & Hagedorn, 1969; Eriksson & Svensson, 1970) and wheat seeds (Hale *et al.* 1969).

The presence of lipoxygenase in plants other than legumes and some cereal- and oil-seeds has not been established unequivocally. Blain (1970) has suggested that the formation of fatty acid hydroperoxides in many plant tissues, including leaves, is catalysed not by lipoxygenase, but by a haematin-mediated autoxidation. On the other hand, Holden (1970) presented evidence that the oxidation of linoleic acid by extracts from leaves is due to a lipoxygenase and not to haematin-containing compounds. Potato tubers contain a relatively high lipid-peroxidizing activity (Rhee & Watts, 1966; Blain, 1970) and previous work from this laboratory indicated that a lipoxygenase enzyme was responsible for this activity (Galliard, 1970).

The present work presents further information on the enzyme that catalyses hydroperoxidation specifically at the 9-position of linoleic and linolenic acids.

EXPERIMENTAL

Materials

Potato tubers (*Solanum tuberosum* var. Majestic) were obtained at commercial harvest and stored for 3–6 months at 10°C.

Soya-bean lipoxygenase (type I) and linoleic acid and linolenic acid (99% pure) were purchased from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K. Methyl esters of eicosa-5,8,11,14,17-pentaenoic acid and docosa-4,7,10,13,16,19-hexaenoic acid were obtained from the Hormel Institute, Austin, Minn., U.S.A., and were converted into the free acids by saponification.

A preparation containing hexadeca-4,7,10-trienoic acid was obtained by saponification of an acetone-soluble lipid preparation from spinach leaves; the proportion of hexadecatrienoic acid was increased by preparative g.l.c. of the fatty acid methyl esters followed by saponification of the hexadecatrienoic acid fraction. The mixture obtained consisted of palmitic acid (29%), hexadecatrienoic acid (35%), linolenic acid (29%) and small amounts only of other fatty acids.

Methods

Enzyme assays. Lipoxygenase activity was usually determined polarographically with an oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio, U.S.A.). Unless otherwise stated, incubation mixtures (5ml) contained the fatty acid as its ammonium salt (1mM) and sodium acetate, pH5.5 (0.1M). The mixture was stirred continuously and, after attaining equilibrium at 25°C, enzyme solution was injected. Enzyme activities were calculated from initial rates of O_2 uptake, assuming an initial dissolved oxygen concentration of 0.24mmol/l. One unit of lipoxygenase catalysed the uptake of 1μmol of O_2/min. Control assays were run in which enzyme or substrate was omitted. Lipoxygenase activity in large-scale incubations was monitored by removing small samples and measuring the E_{234} in 95% (v/v) ethanol.

Peroxidase (donor–H_2O_2 oxidoreductase, EC 1.11.1.7) was assayed spectrophotometrically at 485nm, with *p*-phenylenediamine as hydrogen donor (Lück, 1963). One unit caused a ΔE^{1cm}_{485} of 1.0/min in a 3ml reaction mixture.

Catalase (H_2O_2–H_2O_2 oxidoreductase, EC 1.11.1.6) was assayed spectrophotometrically by following the decrease in E_{240} (Beers & Sizer, 1952). One unit catalysed the consumption of 1μmol of H_2O_2/min.

Analytical methods. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) with bovine serum albumin as standard. Protein-bound haematin was assayed by the method of Hartree (1955).

Instrumental methods. U.v. spectra were recorded with a Cary 14 spectrophotometer in 1cm path-length cells; i.r. spectra were recorded with a Pye–Unicam SP.200G spectrophotometer in a variable-path-length cell with NaCl windows. Optical-rotation measurements at 546 and 598nm were made with a Bendix model 1100 polarimeter and 2cm path-length cells. Circular-dichroism spectra were obtained with samples in 0.1cm path-length cells of a Cary 61 spectropolarimeter; n.m.r. spectra were recorded with a Varian HA-100 spectrometer; mass spectra were determined for samples injected through the direct-insertion probe of an AEI MS902 mass spectrometer operating at 70eV and a source temperature of 180°C.

Isolation of derivatives of fatty acid hydroperoxides. Large-scale incubation mixtures contained 35–350mg of substrate (5mM) and potato lipoxygenase [approx. 2mg of protein/ml (crude) or 0.7mg of protein/ml (preparation II)]. Incubation conditions were as given in Table 2. The formation of hydroperoxide was monitored by u.v. analysis and, after 15–30min (usually 30–50% oxidation of substrate), the mixtures were cooled to 0°C, acidified with HCl to pH3 and extracted with diethyl ether. The ether extracts were washed with water, dried over anhydrous Na_2SO_4 and concentrated *in vacuo* at ambient temperature. The fatty acid hydroperoxides in the ether extracts were reduced to the hydroxydiene fatty acids by treatment of the mixture under N_2 for 30min at room temperature with excess of $NaBH_4$ in aq. 50% (v/v) methanol buffered at pH9.0 with 0.05M-sodium borate. The mixture was diluted with water (1vol.), then acidified with HCl to pH3, and the fatty acids and their derivatives were extracted with diethyl ether. The fatty acids were esterified with diazomethane in diethyl ether and the methyl esters separated by t.l.c. or silicic acid-column chromatography. Further reduction of hydroxydiene derivatives to the saturated hydroxy acids was performed in methanolic solution with H_2 and Adams catalyst at room temperature for 12h.

Chromatography. Separation of reaction products and derivatives by t.l.c. was performed on 0.25mm layers of silica gel G developed in diethyl ether–light petroleum (b.p. 60–80°C) containing 1% (v/v) acetic acid. Proportions of diethyl ether and light petroleum were varied as required in the range 1:1–3:7 (v/v). Lipids were normally detected with I_2 vapour; fatty acid hydroperoxides were also detected with *NN*-dimethyl-*p*-phenylenediamine (Vioque & Holman, 1962). Preparative t.l.c. was performed similarly by using 0.5mm layers of adsorbent and separated lipids were eluted with diethyl ether–methanol (9:1, v/v).

Larger-scale separations on methyl esters of both saturated and unsaturated fatty acid derivatives were performed on columns of silicic acid under conditions that gave separation of methyl 9- and 13-hydroxystearates (Dolev, Rohwedder & Dutton, 1966).

Enzyme purification. A particle-free (65000**g** for 90min) supernatant preparation from potato tubers was made in 50mM-potassium phosphate(pH7.0)–2mM-sodium metabisulphite (Galliard, 1971). (Addition of sodium metabisulphite prevented the rapid darkening of homogenates caused by phenolic oxidations. Control experiments showed that the reducing agent did not interfere with the lipoxygenase assay under the conditions used.) $(NH_4)_2SO_4$ was added with stirring, to 50% saturation at 0°C. The mixture was left for 2h, then centrifuged to obtain a precipitate, which was resuspended and dialysed against 50mM-potassium phosphate buffer, pH7.0, to give preparation I. A portion of preparation I (450mg of protein) was applied to a column (90cm×4.5cm) of Sephadex

G-150 which was eluted with 50mM-potassium phosphate buffer, pH7.0. Fractions (15ml) containing the peak of lipoxygenase activity were pooled to give preparation II.

RESULTS AND DISCUSSION

Partial purification of lipoxygenase from potato tubers

Although preliminary studies (Galliard, 1970) had indicated that the oxidation of linoleic acid and linolenic acid by extracts of potato was caused by a lipoxygenase and not by haematin-catalysed oxidation, it was desirable to separate an active enzyme from the haemoproteins present in the particle-free supernatant (mainly catalase and peroxidase). This was achieved by using methods similar to those of Eriksson & Svensson (1970) for the purification of pea lipoxygenase. The potato lipoxygenase was present mainly in the particle-free supernatant fraction (Galliard, 1970) and was almost all precipitated by 50%-satd. ammonium sulphate at pH7.0. Table 1 summarizes the purification stages. In the purification achieved at the stage of preparation II very little of the original catalase and peroxidase activity remained. Remaining traces of these haemoproteins were removed from lipoxygenase by subsequent chromatography on DEAE-cellulose but, since the specific activity of the lipoxygenase was not increased after DEAE-cellulose, this stage was usually omitted.

We have been unable to obtain a preparation of lipoxygenase free from lipolytic acylhydrolase activity by the methods listed in Table 1 as well as DEAE-cellulose chromatography, free-flow electrophoresis and acid precipitations. However, it was possible to prepare the lipolytic acylhydrolase enzyme free from lipoxygenase activity by acetone treatment (Galliard, 1971).

The distribution of protein, lipoxygenase, catalase and peroxidase activities obtained in a typical gel filtration on Sephadex G-150 (Fig. 1) is similar to those described by Eriksson & Svensson (1970) in the purification of pea lipoxygenase. The lipoxygenase activity was found in a peak distinct from those of catalase and peroxidase. On a Sephadex G-150 column calibrated for molecular-weight estimations, the lipoxygenase peak was coincident with that of the potato lipolytic acylhydrolase enzyme (Galliard, 1971) and was estimated to have a gel equivalent molecular weight of approx. 100000.

We were unable to detect any haematin-containing protein in the partially purified lipoxygenase preparation. The analysis (Hartree, 1955) would have detected 0.05μg of haematin in 1mg of protein in our enzyme preparation. The possibility that haemoproteins are involved in the lipoxygenase reaction can be discounted. The concentrations of haemoproteins necessary to give the observed rates of fatty acid oxidation would be much higher than the maximum possible in our preparations. For example, Eriksson, Olsson & Svensson (1970) showed that the rate of linoleate oxidation in the presence of undenatured catalase was 20μmol of O_2 consumed/min per μmol of haemoprotein. The partially purified lipoxygenase enzyme from potato contained less than 0.1nmol of haematin/mg of protein. Thus the contribution to linoleate oxidation by any haemoprotein similar to catalase present in the lipoxygenase preparation would be less than 0.002 unit/mg of protein. The specific activity of the lipoxygenase preparation was 6.5 units/mg of protein. Even as heat-denatured or pH-treated haemoproteins, which are more active in catalysing linoleate peroxidation (Eriksson *et al.* 1970), the contribution to hydroperoxide formation would be less than 1% of the rate observed with the lipoxygenase.

Characterization of products formed by potato lipoxygenase

When linoleic acid was normally used as substrate with either crude extract or partially purified enzyme preparation, the diethyl ether-soluble reaction products observed after t.l.c. contained, in

Table 1. *Partial purification of lipoxygenase from potato tubers*

Units of enzyme activity were: lipoxygenase, μmol of O_2 consumed/min; catalase, μmol of H_2O_2 consumed/min; peroxidase, ΔE_{485}^{1cm}/min.

Purification stage	Total protein (mg)	Total lipoxygenase activity (units)	Recovery (%)	Specific activity (units/mg of protein)		
				Lipoxygenase	Peroxidase	Catalase
Particle-free supernatant	2868	3570	100	1.2	0.6	7.9
50%-satd. $(NH_4)_2SO_4$ precipitate (preparation I)	720	2400	67	3.3	0.08	8.5
Sephadex G-150 column pooled fractions (preparation II)	102	670	19	6.5	0.03	<0.5

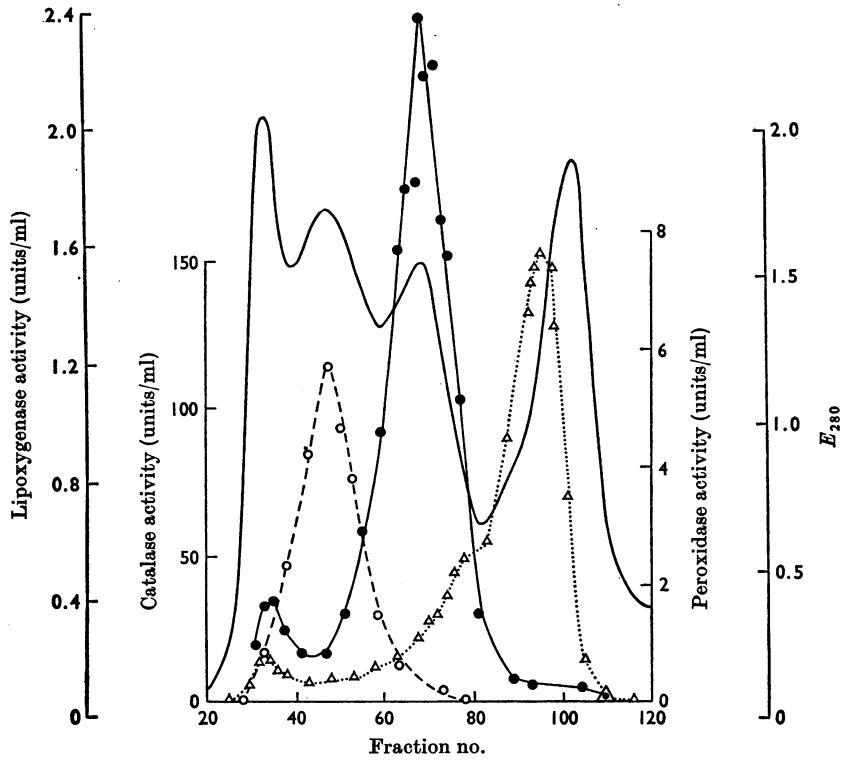

Fig. 1. Gel filtration of 50% satd. $(NH_4)_2SO_4$ ppt. on Sephadex G-150. Fractions (15ml) were collected and assayed for protein (——, E_{280}) and lipoxygenase (●), peroxidase (△) and catalase (○) activities. Fractions 64–75 were combined for further purification.

addition to unchanged fatty acid, one major component, which was identified as a fatty acid hydroperoxide. Ethanolic solutions of the products exhibited u.v. spectra with a single major peak at 234nm.

For further analysis the reaction products were reduced with sodium borohydride, converted into methyl esters and separated by t.l.c. A single major spot was observed in the area corresponding to methyl esters of hydroxy fatty acids. Parallel experiments with soya-bean lipoxygenase gave rise to two spots in this area corresponding to methyl 9- and methyl 13-hydroxyoctadecadienoates (Morris, Holman & Fontell, 1960). The major spot in the potato experiment corresponded to the slower-running methyl 9-hydroxy isomer.

From preparative t.l.c. plates areas covering both 9- and 13-hydroxy isomers were removed and the derivatives were reduced to the corresponding methyl hydroxystearates. Analysis by t.l.c. of the reduction products showed two spots of methyl 9- and methyl 13-hydroxystearate from the soya-bean preparation but only one spot corresponding to methyl 9-hydroxystearate from the potato experiment. The same reduction products were analysed by mass spectrometry. Methyl 9- and 13-hydroxystearates give characteristic fragmentation patterns in the mass spectrometer and three major high-molecular-weight fragments are produced by cleavage of the carbon–carbon bonds at both sides of the hydroxyl group (Ryhage & Stenhagen, 1960; Dolev *et al.* 1967). Methyl 9-hydroxystearate produces fragments with *m/e* values of 155, 158 and 187; the 13-isomer has corresponding peaks at *m/e* 211, 214 and 243. In the present work, no evidence of other isomers was obtained and the proportion of 9- and 13-isomers in a sample was determined from the relative intensities of the three fragments from each isomer. Similar experiments were performed with linolenic acid as substrate and with the soya-bean enzyme at different pH values. The results, summarized in Table 2, show that the potato enzyme had a high degree of specificity for the 9-position of both linoleic acid and linolenic acid and that crude extracts of potato tuber were as specific as the partially purified enzyme pre-

parations in this respect. It has been shown that autoxidation of methyl linoleate produces oxygenation to an equal extent on the 9- and 13-positions (Dolev *et al.* 1967). The results with soya-bean lipoxygenase (Table 2) showed that the relative amounts of 9- and 13-hydroperoxide formed depend on the pH of the incubation mixtures. With the soya-bean enzyme at pH 9.2, a ratio of 74% 13-isomer to 26% 9-isomer was obtained, similar to previously published results (Hamberg & Samuelsson, 1965; Zimmerman & Vick, 1970). The potato enzyme was completely inactive at pH 9.2 (Fig. 2). At pH 5.5, however, the 9-hydroperoxy isomer was the major product. The present results with soya bean support the conclusion of previous workers, that more than one enzyme is involved in the lipoxygenase activity from soya beans (Koch *et al.* 1958; Dolev *et al.* 1967; Guss, Richardson & Stahmann, 1967; Christopher *et al.* 1970). It has recently been found that the proportion of 9- and 13-hydroperoxides formed from linoleic acid by soya-bean lipoxygenase varies with environmental conditions, primarily oxygen partial pressure (C. E. Eriksson, personal communication).

Because of the differences in positional specificity of oxygenation between the potato and soya-bean enzymes, the full structures of the reaction products from the potato enzyme were examined.

The methyl 9-hydroxyoctadecadienoate from the product of linoleate oxidation by the potato enzyme was isolated by preparative t.l.c. or by silicic acid column chromatography. The hydroxydiene acid gave a u.v.-absorption maximum at 234nm in ethanol ($\epsilon = 2.44 \times 10^4 l \cdot mol^{-1} \cdot cm^{-1}$). The i.r. spectrum in carbon disulphide solution showed absorption bands at 3590cm^{-1} caused by hydroxyl groups of a conjugated dienol (Badami & Morris, 1965) and a pair at 943cm^{-1} and 980cm^{-1} characteristic of a *cis–trans*-conjugated diene (Chipault & Hawkins, 1959). The heights of the peaks at 943 and 980cm^{-1} were in the ratio 2:3, rather than 1:1, which could indicate the presence of a small amount of *trans–trans*-isomer (Chipault & Hawkins, 1959). However, no evidence of a *trans–trans*-isomer was observed in the n.m.r. spectra in $CDCl_3$ solution which was very similar to that for methyl 13 - hydroxyoctadeca - *cis* - 9,*trans* - 11 - dienoate (Tallent, Harris & Wolff, 1966). Results of spin decoupling at δ2.18 (allylic methylene protons at H-14) and at δ4.15 (carbinol proton at H-9) were identical with those described by Gardner & Weisleder (1970) for methyl 9-hydroxyoctadeca-*trans*-10,*cis*-12-dienoate, the derivative from the major product formed by *Z. mays* lipoxygenase from linoleic acid.

The methyl 9-hydroxyoctadecadienoate from the potato experiments was optically active and was of the same rotation sign but lower value ($[\alpha]_D^{25} = +1.0°$; $[\alpha]_{546}^{25} = +1.7°$: 0.025g/ml in $CHCl_3$) than that given by Gardner & Weisleder (1970) for the same product obtained from the corn lipoxygenase system ($[\alpha]_{546}^{25} = +5.3°$: 0.03g/ml in hexane) or for methyl 9-hydroxyoctadeca-*trans*-10,*cis*-12-dienoate from *Calendula* oil, i.e. $[\alpha]_D^{20} = +3.6°$: 0.19g/ml in $CHCl_3$ (Badami & Morris, 1965). Confirmation of the optical activity of the product formed by the potato enzyme was obtained from circular-dichroism spectra of the methyl 9-hydroxyoctadecadienoate in 2,2,4-trimethylpentane; a peak of positive rotation was observed at the absorption maximum (234nm) of the conjugated dienol function. Both the 9-hydroxyoctadecadienoate and its 9-hydroxystearate reduction product gave circular-dichroism peaks of negative rotation in the absorption region of the carboxyl function (190–200nm). The absolute configuration of the major product formed by the corn lipoxygenase was

Table 2. *Location of hydroperoxide group in fatty acid hydroperoxide formed by lipoxygenase preparations*

Incubation mixtures (25ml) contained fatty acid substrate (5mM), 0.1M-sodium acetate buffer, pH 5.5, or 0.1M-tris–HCl buffer, pH 9.2, and sufficient enzyme to give 30–50% reaction in 15min at 25°C. During incubation, the mixtures were continuously shaken under a stream of oxygen. Reaction products were isolated and the methyl hydroxystearates derived from the fatty acid hydroperoxides were analysed by mass spectrometry.

Substrate	Enzyme preparation	pH	Enzyme added (mg of protein)	Products observed (%): Methyl 9-hydroxystearate	Products observed (%): Methyl 13-hydroxystearate
Linoleic acid	Potato extract (crude)	5.5	50	95	5
	Potato lipoxygenase (preparation II)	5.5	20	94	6
Linolenic acid	Potato lipoxygenase (preparation II)	5.5	50	95	5
Linoleic acid	Soya-bean lipoxygenase	5.5	50	54	46
		9.2	50	26	74

shown to be 9-D-hydroperoxyoctadeca-*trans*-10,*cis*-12-dienoic acid (Gardner & Weisleder, 1970). Since the methyl 9-hydroxyoctadecadienoate derived from the potato product had the same positive rotation as the same derivative from the corn system, the potato lipoxygenase must also form the 9-D-hydroperoxide. Veldink *et al.* (1970*a*) reported that the minor product of soya-bean lipoxygenase oxidation of linoleate was also the 9-D-hydroperoxide, whereas the major product is 13-L-hydroperoxyoctadecadienoic acid (Hamberg & Samuelsson, 1967).

Although the 9-hydroperoxyoctadecadienoic acid is a minor product of linoleic acid oxidation by flax-seed extracts, this isomer remains while another enzyme specifically attacks the 13-hydroperoxide major product (Veldink, Vliegenthart & Boldingh, 1970*b*). The fact that the 9-hydroperoxy isomer is found almost exclusively in potato systems is not due to such selective loss of the 13-hydroperoxy isomer because (*a*) partial purification of the lipoxygenase does not significantly change the proportions of the isomers; (*b*) no isomerase products or other lipid materials are formed in significant amounts; (*c*) reaction rates were similar for O_2 uptake and hydroperoxide formation. Thus we conclude that the high proportion (95%) of the 9-hydroperoxy isomer represents the true specificity of the potato lipoxygenase, at least under the conditions used in the present work. It is possible that the small amount of the 13-isomer formed is due to some autoxidation during incubation and extraction procedures.

Substrate specificity of potato lipoxygenase

Previous work with soya-bean lipoxygenase established that a penta-*cis*–*cis*-1,4-diene structure in the substrate was essential for enzyme activity (Holman, 1949), that the double bonds should be ($n-6$, $n-9$) (i.e. from the methyl-terminal end) and that the hydroperoxide group was inserted mainly at the ($n-6$) position irrespective of the fatty acid chain length (Hamberg & Samuelsson, 1965, 1967; Holman, Egwin & Christie, 1969). Since the potato enzyme specifically converts linoleic acid into the 9-hydroperoxide ($n-10$), it was decided to compare substrate specificities. Crude potato extracts oxidized both linoleic acid and linolenic acid to hydroperoxides at similar rates (Galliard, 1970). Table 3 shows that, with the partially purified lipoxygenase also, linoleic acid ($n-6$, $n-9$) and linolenic acid ($n-3$, $n-6$, $n-9$) were equally effective substrates; the monoenoic oleic acid and the saturated stearic acid were inactive. Other substrates with the ($n-3$, $n-6$, $n-9$) triene structure were studied; eicosa-5, 8, 11, 14, 17 - pentaenoic acid and docosa-4,7,10,13,16,19-hexaenoic acid, which both have the ($n-3$, $n-6$, $n-9$) triene systems were oxidized more slowly by the potato enzyme (Table 3) and specific identifications of the products were not made. When a mixture containing similar amounts of linolenic acid and hexadeca-4,7,10-trienoic acid was used as substrate, the hexadecatrienoic acid ($n-3$, $n-6$, $n-9$) was oxidized at about half the rate for linolenic acid, as indicated by g.l.c. analysis of the reaction products. When the methyl esters of the reaction products were reduced and analysed by g.l.c.–mass spectrometry, only two hydroxy acid derivates were observed; methyl 9-hydroxystearate (derived from linolenic acid) and methyl 7-hydroxypalmitate (derived from the hexadecatrienoic acid). This preliminary result indicated that the potato enzyme catalysed insertion of the hydroperoxy group at the ($n-10$) position of both C_{16} and C_{18} fatty acids with the ($n-6$, $n-9$)-pentadiene system. We have not yet examined a sufficient range of possible substrates to make

Table 3. *Substrate specificity of potato lipoxygenase*

Incubation mixtures (5 ml) contained substrate (1 mM), 0.1 M-sodium acetate buffer, pH 5.5, and either lipoxygenase preparation I (120 μg of protein) or preparation II (64 μg of protein). Initial rates of O_2 uptake were measured at 25°C.

	Relative lipoxygenase activity (rate with linolenic acid = 100)	
Substrate	Preparation I	Preparation II
Linolenic acid	100	100
Linoleic acid	100	98
Oleic acid	—	0.6
Stearic acid	—	<0.1
Eicosa-5,8,11,14,17-pentaenoic acid	19	—
Docosa-4,7,10,13,16,19-hexaenoic acid	16 (approx.)	—
Hexadeca-4,7,10-trienoic acid	50 (see the text)	—

definitive conclusions but it appears that the $n-10$ positional specificity of the hydroperoxidation by the potato lipoxygenase is governed by the methyl-terminal end of the molecule, as is the $n-6$ oxidation by soya-bean lipoxygenase. The low reaction rates with C_{20} and C_{22} substrates suggests that the potato enzyme possesses more specificity for substrate chain length than does the soya-bean lipoxygenase, which oxidized linoleic acid, linolenic acid and arachidonic acid at similar rates (Holman & Elmer, 1947).

Properties of potato lipoxygenase

pH *optimum*. The pH–response curve (Fig. 2) shows an optimum at pH 5.5–6.0 and little activity at alkaline pH values. The corn lipoxygenase, which also forms predominantly the 9-hydroperoxide from linoleic acid, had an optimum at pH 6.5 (Gardner & Weisleder, 1970) whereas studies on the positional specificity of the soya-bean lipoxygenase were performed at pH 9.0 (Hamberg & Samuelsson, 1965; Dolev *et al.* 1967). In the present work we found that the proportion of 9- and 13-hydroperoxides formed from linoleic acid by the soya-bean lipoxygenase was dependent on pH (Table 2). The pH characteristics of lipoxygenase preparations from several plants can be modified by the presence of detergents (Surrey, 1964), a phenomenon exhibited by several enzymes that act on hydrophobic substrates (Borgström, 1954; Quarles & Dawson, 1969). However, Triton X-100 at concentrations that produced marked changes in the pH characteristics of the lipolytic acylhydrolase enzyme of potato tubers (Galliard, 1971), had no effect on the pH optimum or activity of the lipoxygenase from the same tissue.

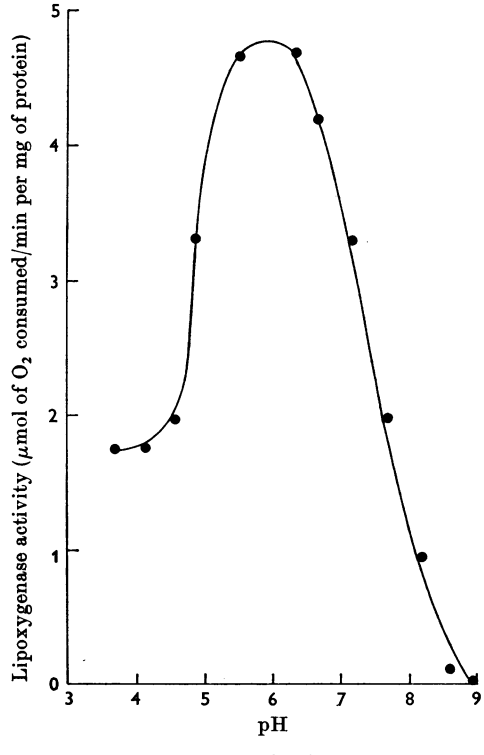

Fig. 2. pH–response curve for lipoxygenase activity. Incubation mixtures contained 1 mM-linoleic acid, and 0.1 M buffer in 5 ml at 25°C. The following buffer solutions were used: sodium acetate (pH 3–5.5); potassium phosphate (pH 6.0–7.0) and tris–HCl (pH 7.5–9.5).

Kinetics. With the partially purified lipoxygenase enzyme and linoleic acid as substrate, a linear relationship was obtained between maximal velocity and enzyme concentration. Substrate concentration curves showed saturation at [linoleic acid] 0.5 mM and a half-maximal velocity at 0.1 mM substrate. The K_m value for lipoxygenase depends on pH and critical micelle concentration (Allen, 1968) and probably has little physiological significance.

Inhibitors. Previous work had shown that the formation of fatty acid hydroperoxides by potato extracts was not inhibited by Ca^{2+}, F^-, EDTA or cyanide, all at 1 mM (Galliard, 1970). The activity of the partially purified enzyme was also unaffected by 0.2 mM-*p*-chloromercuribenzoate, at which concentration there was complete inhibition of mung-bean lipoxygenase (Siddiqi & Tappel, 1957), although other lipoxygenases studied are not inhibited by thiol inhibitors (Tappel, 1963). The potato enzyme was inhibited 66% when 0.2 mM-nordihydroguaiaretic acid was mixed with the substrate before addition of enzyme, thus showing with this competitive inhibitor a sensitivity similar to that of lipoxygenase preparations from legumes and cereal seeds (Siddiqi & Tappel, 1957).

Stability. The partially purified enzyme preparation was unstable in dilute solution at pH 7, but when stored at 0°C as a concentrated suspension in 2.2 M-ammonium sulphate, most activity was retained for up to 1 week. The enzyme in 0.1 M-tris–0.05 M-acetic acid, pH 8.3, was completely inactivated by treatment at 75°C for 2 min; a 50% loss of activity occurred in 2 min at 57°C.

Conclusions

The lipoxygenase from potato tubers is similar in some respects to previously characterized lipoxygenase enzymes from other plants, e.g. with respect to molecular weight and insensitivity to various inhibitors. However, it differs from the classical soya-bean lipoxygenase in two major respects, i.e. in the specificity of the enzyme for the position on

the substrate at which the hydroperoxy group is inserted and in the pH response of the enzyme. A standard method for the assay of lipoxygenase activity is performed at pH 9.0 (Tappel, 1962*b*) at which non-physiological pH the potato enzyme is completely inactive. Thus, at high pH only lipoxygenase of the soya-bean type would be detected. The results with the soya-bean enzyme described in the present work also show that at pH 9.0 the 13-hydroperoxy derivative of linoleic acid is the major product, whereas at pH 5.5 more of the 9-hydroperoxy isomer is formed. It seems likely that two major types of lipoxygenase activity exist in plants, one active at acidic pH and specific for the 9-position of linoleic acid and one active at alkaline pH, favouring the 13-position. [This has possibly now been confirmed with isoenzymes of soya-bean lipoxygenase (J. Boldingh, personal communication).] Some plants presumably contain both types of enzyme, e.g. soya bean, whereas others, e.g. potato, contain mainly one type. The occurrence of isoenzymes of lipoxygenase and of enzymes with different substrate specificities, as mentioned above, as well as lipoxygenases with different sensitivities to thiol reagents (Siddiqi & Tappel, 1957) suggests a complex range of enzymes under the general name of lipoxygenase.

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