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The Enzymic Conversion of Linoleic Acid Hydroperoxide by Flax-Seed Hydroperoxide Isomerase

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1. The mode of action of flax-seed hydroperoxide isomerase was studied *in vitro* by using as substrates linoleic acid hydroperoxides formed by soya-bean lipoxygenase. 2. The enzyme converts only 13-hydroperoxyoctadeca-*cis*-9-*trans*-11-dienoic acid, whereas the 9-hydroperoxy isomer does not react. 3. The isomerization product was identified by chemical and spectroscopic methods as 13-hydroxy-12-oxo-octadec-*cis*-9-enoic acid. 4. 12,13-Epoxyoleic acid isomers do not act as intermediates in the isomerization reaction. 5. Suggestions for a functional relationship between hydroperoxide isomerase and lipoxygenase are discussed.

Lipoxygenase (EC 1.13.1.13) catalyses *in vitro* the formation of optically active (Privett, Nickell, Lundberg & Boyer, 1955; Veldink, Vliegenthart & Boldingh, 1970*a*) *cis-trans* conjugated hydroperoxides from unsaturated fatty acids containing a penta-*cis*-*cis*-1,4-diene system and molecular oxygen (Dolev, Rohwedder, Mounts & Dutton, 1967).

The enzyme occurs in a large number of plant seeds (Tappel, 1963). Its function in lipid metabolism is still unknown, although some suggestions have been made: e.g. Holman (1948) points to a possible relation between lipoxygenase and catalase during the germination of soya beans, and Heinen & Van den Brand (1963) suggest that lipoxygenase plays a role in the formation of cutine.

It is evident that, if lipoxygenase catalyses the formation of hydroperoxides *in vivo*, the organism must contain enzyme systems to metabolize these products. Indeed, some authors (Schormüller, Weber, Höxer & Grosch, 1969; Täufel & Rothe, 1965; Gini & Koch, 1961; Blain & Barr, 1961) mention the enzymic breakdown of linoleic acid hydroperoxides; however, neither the reaction course nor the structures of the reaction products are known.

Zimmerman (1966) has reported the presence of a hydroperoxide isomerase in flax-seed extracts that converts the hydroperoxide into a mono-unsaturated α-ketol. We have studied in more detail this action of the flax-seed enzyme on the hydroperoxides of linoleic acid formed by soya-bean lipoxygenase. A preliminary account of this work has been published (Veldink, Vliegenthart & Boldingh, 1968).

EXPERIMENTAL

Materials. Linoleic acid and vernolic acid [(+)-12,13-*cis*-epoxyoleic acid] (purity > 98%) were gifts from the Unilever Research Laboratories, Vlaardingen/Duiven, The Netherlands. Soya-bean lipoxygenase (activity 8000 units/mg) was purchased from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. Flax seed was obtained from C.E.B.E.C.O. (National Agricultural Co-operative Wholesale Society), Rotterdam, The Netherlands. All reagents were of analytical-grade quality. The light petroleums were free of carbonyl compounds.

Spectroscopy. U.v. spectra were recorded with a Unicam SP. 800B or a Zeiss PMQ-2 spectrophotometer; i.r. spectra were obtained with a Beckman IR-8 or a Grubb–Parsons Spectromaster instrument; proton-magnetic-resonance (p.m.r.) spectra were registered with a Varian HA-100 spectrometer; mass spectra were recorded with an AEI-MS9 mass spectrometer provided with a direct insertion probe; optical rotation was measured with a Perkin–Elmer 141 polarimeter (10cm tube).

Chromatography. Free fatty acids were chromatographed on glass plates (20cm × 20cm) coated with a layer (0.3mm) of silica gel G (E. Merck A.-G., Darmstadt, Germany) impregnated with oxalic acid. For the preparation of five plates 35g of silica gel was mixed with 70ml of 50mM-oxalic acid. The spots were located by spraying with 5% (w/v) phosphomolybdic acid in 96% (v/v) ethanol followed by heating at 110°C for 10min. Fatty acid methyl esters were chromatographed on glass plates (20cm × 20cm) coated only with silica gel G (layer thickness 0.3mm). Preparative t.l.c. was carried out on 0.5mm thick layers. After spraying with 0.2% 2,7-dichlorofluorescein in 96% (v/v) ethanol the bands were located under u.v. light. The solvent systems consisted of mixtures of *n*-hexane and ether as indicated in the text.

DNP-hydrazones of mixtures of aldehydes and aldehyde esters were separated by chromatography on a column (8cm × 1.3cm) of Al_2O_3 containing 8% of water equilibrated with light petroleum (b.p.40–60°C). Aldehyde DNP-hydrazone fractions were eluted with light petroleum (b.p.40–60°C)–diethyl ether (9:1, v/v). Aldehyde ester DNP-hydrazone fractions were eluted with light petroleum (b.p.40–60°C)–diethyl ether (4:1, v/v). Both fractions were further analysed by liquid–liquid partition t.l.c. with Carbowax 400 on kieselguhr G (E. Merck A.-G.) as stationary phase and light petroleum (b.p. 100–120°C) as solvent, as described by Badings & Wassink (1963) and Meijboom (1966).

Preparation of methyl esters. Free fatty acids were esterified with diazomethane in ether solution.

Periodate oxidation. A 5mg portion of the methyl ester of the isolated α-ketol was dissolved in a few drops of 1,4-dioxan. Then 25ml of water (freed from O_2 by boiling and flushing with N_2) and 5ml of a buffer solution, pH2.2, containing 2.1% citric acid and 0.07% Na_2HPO_4 were added. Under continuous flushing with N_2 0.5ml of 0.05 M-KIO_4 and 15ml of light petroleum (b.p.40–60°C) were added. After shaking for 1.5h the light-petroleum layer was isolated. The solvent was then evaporated and the residue dissolved in 1ml of methanol. To the solution was added 150ml of 0.4% 2,4-dinitrophenylhydrazine in 4 M-HCl. After 20h at 4°C the DNP-hydrazones had crystallized out. The crystals were collected by filtration, washed with water, dissolved in light petroleum (b.p. 40–60°C) and transferred to the Al_2O_3 column.

Reductive ozonolysis. A 5 mg portion of the component to be analysed was dissolved in 5ml of *n*-pentane. An O_2–O_3 mixture was passed through the solution until the outcoming gas gave a positive reaction with a 10% KI solution. The excess of O_3 was removed from the solution by flushing with N_2. Then 50mg of a hydrogenation catalyst [5% (w/w) Pd on active carbon] was added and H_2 was passed through the solution until there was no further reaction with KI. The excess of H_2 was removed from the solution by a stream of N_2. The solvent was evaporated and the residue dissolved in 1ml of methanol. The catalyst was then filtered off. To the filtrate was added 150ml of 0.4% 2,4-dinitrophenylhydrazine in 4M-HCl. After 20h at 4°C the crystalline DNP-hydrazones were obtained by filtration, washed with water, dissolved in light petroleum (b.p.40–60°C) and transferred to the Al_2O_3 column.

Gas–liquid chromatography. G.l.c. of methyl hydroxystearates was performed with a F & M Laboratory Chromatograph provided with a hydrogen-flame ionization detector and a stainless-steel column (1.22m × 6.25 mm) filled with 10% SE-30 on Gas-Chrom P. The column was used isothermally at 190°C, with N_2 at a flow rate of 30ml/min. The peak areas were measured with a planimeter.

Preparation of (±)-12,13-trans-*epoxyoleic acid.* Castor oil was dehydrated with $KHSO_4$ by the method of Body & Shorland (1965). Then 50g of the dehydrated oil was converted into the methyl esters by treatment with 10g of methanol in which 0.15g of Na had been dissolved for 45min at 60°C in an N_2 atmosphere. The mixture was poured into cold water and the methyl esters were extracted with light petroleum (b.p.40–60°C). The extract was washed with water and dried over Na_2SO_4. The methyl esters were separated by t.l.c. over silica gel G impregnated with $AgNO_3$ (30%, w/w) in the solvent system *n*-hexane–ether (4:1, v/v). After spraying with 2,7-dichlorofluorescein three bands were detected under u.v. light, namely methyl octadeca-*cis*-9-*cis*-12-dienoate (R_F 0.58), methyl octadeca-*cis*-9-*trans*-12-dienoate (R_F0.68, strong i.r. absorption at 968cm^{-1} corresponding to one isolated *trans*-double bond) and methyl octadeca-*cis*-9-*trans*-11-dienoate (R_F0.85, strong u.v. absorption at 232nm).

By preparative t.l.c. 80mg of the non-conjugated *cis*-*trans*-linoleate was prepared. This component was epoxidized with peracetic acid by the method of Findley, Swern & Scanlan (1945). A mixture of epoxy compounds was obtained, namely 12,13-*trans*-epoxyoctadec-*cis*-9-enoate, 9,10-*cis*-epoxyoctadec-*trans*-12-enoate and a small amount of 12,13-*trans*-9,10-*cis*-diepoxyoctadecanoate. The components were separated by preparative t.l.c. on silica gel G with the solvent system *n*-hexane–diethyl ether (7:3, v/v): 20mg of (±)-12,13-*trans*-epoxyoctadec-*cis*-9-enoate was isolated.

Preparation of (±)-12,13-cis-*epoxyoleic acid.* Pure methyl *cis*-9-*cis*-12-linoleate was epoxidized as described for the *trans*-isomer.

Preparation of linoleic acid hydroperoxides. A 100mg portion of linoleic acid was dissolved in 100ml of 0.04M-NH_3–NH_4Cl buffer, pH9.0, and incubated with 15mg of lipoxygenase for 1h at 0°C under pure O_2. The reaction mixture was acidified with 2M-HCl to pH3.0 and extracted with diethyl ether. The extract was washed with water and dried over Na_2SO_4. After filtration the ether was evaporated and the residue was chromatographed on silica gel G impregnated with oxalic acid with the solvent mixture *n*-hexane–diethyl ether (3:2, v/v). The hydroperoxides were located by spraying the edges of the plate with a KI reagent consisting of 3ml of saturated aqueous KI solution, 30ml of ethanol, 30ml of acetic acid and 20ml of chloroform. The hydroperoxides were scraped off and eluted with ether.

To determine the molar ratio of the 13- and 9-hydroperoxy isomers, part of the material obtained was esterified with diazomethane, reduced with $NaBH_4$ and finally hydrogenated with PtO_2 as catalyst. The mixture of methyl hydroxystearates was chromatographed on silica gel G with the solvent system *n*-hexane–ether (3:2, v/v). The bands of 13- and 9-hydroxystearate were scraped off and eluted with methanol. The two samples were subsequently subjected to g.l.c. on the SE-30 column. The molar ratio of the 13- and 9-isomers was estimated from the measured peak areas.

Preparation of the flax-seed extract containing hydroperoxide isomerase. Flax seed (100g) was ground in a Sorvall mixer under external cooling with ice. The powder was twice extracted with 300ml of acetone for 20min at −20°C. A 5g portion of the acetone-dried powder was extracted with 0.1M-K_2HPO_4–KH_2PO_4 buffer, pH7.4, for 3h at 0°C. The suspension was centrifuged for 30min at 12000*g*. The supernatant was always used immediately.

*Preparation of toluene-*p*-sulphonyl derivatives.* A 10mg portion of the methyl ester of the α-ketol was dissolved in 1ml of pyridine at 0°C and 30mg of toluene-*p*-sulphonyl chloride was added. The reaction mixture was stirred magnetically for 4h at room temperature. The tosylated product was extracted with diethyl ether and purified by

preparative t.l.c. on silica gel G with the solvent system hexane–ether (7:3, v/v).

Borohydride reduction. A 7mg portion of the methyl ester of the α-ketol was treated with a fresh solution of 50mg of $NaBH_4$ in 20ml of methanol for 1h at 0°C. The excess of $NaBH_4$ was destroyed with 2M-HCl. The vicinal diol was isolated by extraction with diethyl ether and purified by preparative t.l.c. on silica gel G with the solvent system hexane–ether (2:3, v/v).

RESULTS

Preparation and structure determination of the product formed by the conversion of linoleic acid hydroperoxide with the flax-seed hydroperoxide isomerase. The reaction was carried out as follows: 25mg of purified linoleic acid hydroperoxides was dissolved in 0.5ml of 1M-ammonia and incubated with 70ml of the isomerase preparation for 20min at room temperature. The reaction was stopped by acidification with 2M-hydrochloric acid to pH3.0. The reaction mixture was then extracted with diethyl ether and the extract was washed, dried, concentrated *in vacuo* and treated with diazomethane. The mixture of methyl esters was separated by preparative t.l.c. on silica gel G with the solvent system *n*-hexane–diethyl ether (3:2, v/v). The α-ketol was located by spraying the edges of the plates with a 2,4-dinitrophenylhydrazine solution.

Zimmerman (1966) proposed that the isomerase is capable of converting both linoleic acid hydroperoxides, which would result in a ketol fraction consisting of a mixture of two isomers (Scheme 1).

Structures of these ketols were proposed by Zimmerman (1966) on the basis of i.r. and p.m.r. spectra, but these data are not sufficient to determine the structures unambiguously. The i.r. and p.m.r. results that we obtained for the ketol fraction were in accordance with those published by Zimmerman (1966); however, neither the methyl ester of the ketol nor the ketol itself showed any u.v. absorption in the range 225–300nm (concn. 1mg/ml, 1cm light-path, solvent *n*-hexane), contrary to Zimmerman (1966), who reported an absorption maximum at 275nm. Such an absorption is not in harmony with the structures he proposed.

The specific optical rotation of the methyl ester was $[\alpha]^{20}_{546} -2.3\pm0.2°$ and $[\alpha]^{0}_{436} -6.6\pm0.4°$ (*c* 4.0 in methanol).

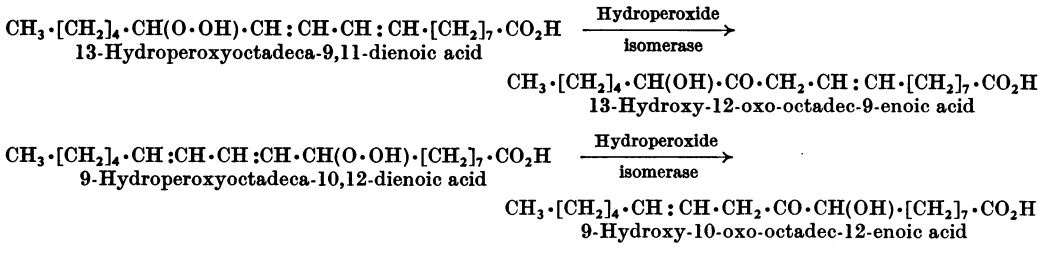
To determine the position of the hydroxyl group, a mass spectrum of the toluene-*p*-sulphonyl derivative was recorded (Fig. 1). A peak at *m/e* 255 (25% of maximum peak height) was found. It corresponds to the fragment $CH_3\cdot[CH_2]_4\cdot CH{:}\overset{+}{O}\cdot SO_2\cdot C_6H_4\cdot CH_3$, as is apparent from the exact mass measurement, which gave 255.1019; the calculated mass for $C_{13}H_{19}O_3S$ is 255.1055. This result confirms that the hydroxyl function was present at C-13 in the original ketol. In the mass spectrum there was no peak indicating the presence of an isomer with the hydroxyl group at C-9.

To prove that the keto and the hydroxyl functions are present at adjacent carbon atoms, the mass spectrum of the saturated diol obtained by reduction of ketol with borohydride followed by catalytic hydrogenation was obtained. It displayed the expected features including peaks at *m/e* 101 and 229, indicating splitting between the oxygen functions.

To obtain further evidence for the structure of the α-ketol, it was oxidized with periodate and the aldehydes formed isolated as DNP-hydrazone derivatives. The aldehyde DNP-hydrazone fraction was exclusively *n*-hexanal DNP-hydrazone, as shown by comparison on partition t.l.c. with reference compounds of the series C_1–C_9 saturated aldehyde DNP-hydrazones.

No aldehyde ester DNP-hydrazone could be detected by chromatography of the oxidation products over alumina such as would be expected if the ketol derived from the 9-hydroperoxide isomer had been present (Scheme 2).

To establish the position of the *cis*-double bond in the α-ketol the substance was subjected to reductive ozonolysis. The mixture of aldehyde DNP-hydrazones and aldehyde ester DNP-hydrazones obtained was fractionated over an alumina column. The aldehyde DNP-hydrazone fraction contained



Scheme 1.

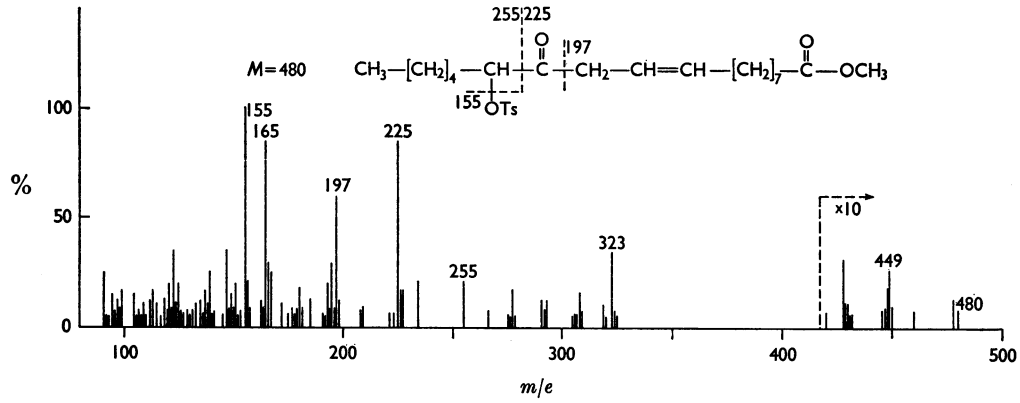
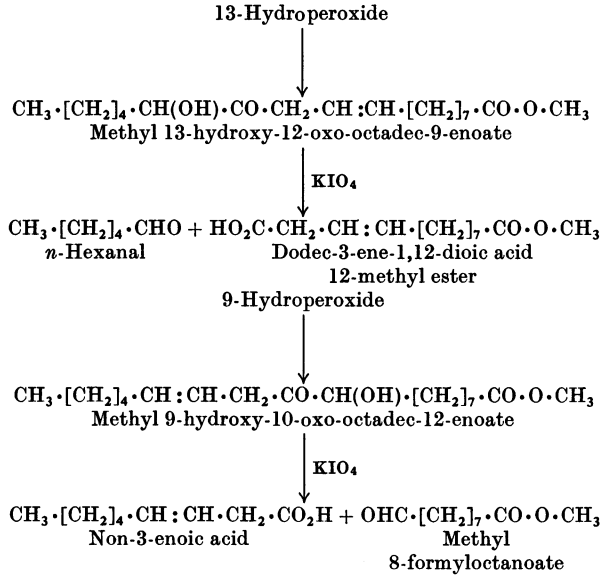


Fig. 1. Mass spectrum of the toluene-*p*-sulphonyl derivative of the α-ketol obtained after incubation of linoleic acid hydroperoxides with a hydroperoxide isomerase preparation from flax seed.



Scheme 2.

only very small amounts of material, which consisted predominantly of *n*-hexanal DNP-hydrazone. The occurrence of this compound must be explained by the tautomerization of the α-ketol to an enediol.

The aldehyde ester DNP-hydrazone fraction consisted mostly of the C_9-aldehyde ester DNP-hydrazone. This compound was identified by comparison with the product obtained after reductive ozonolysis of methyl oleate.

The occurrence of the C_9 fragment proves that the double bond is located between C-9 and C-10. On the basis of the findings mentioned above we can conclude with certainty that the identity of the product of the isomerization reaction of a mixture of linoleic acid hydroperoxides is exclusively 13-hydroxy-12-oxo-octadec-*cis*-9-enoic acid.

Substrate specificity. The absence of 9-hydroxy-10-oxo-octadec-*cis*-12-enoic acid from the reaction product suggests that the isomerase is capable of discriminating between the two linoleic acid hydroperoxides formed by soya-bean lipoxygenase from linoleic acid. To establish definitely this substrate

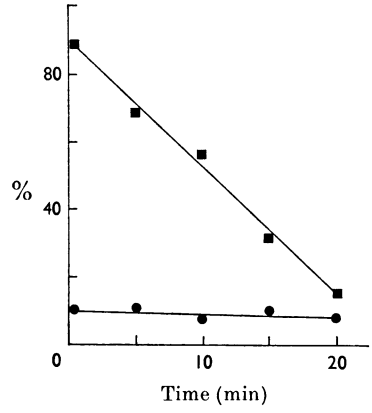


Fig. 2. Relative amounts of 13- and 9-hydroxystearate derived from the unchanged linoleic acid hydroperoxides during the reaction with the flax-seed hydroperoxide isomerase. ■, Methyl 13-hydroxystearate; ●, methyl 9-hydroxystearate.

specificity of the enzyme, the reaction course of the enzyme with a mixture of linoleic acid hydroperoxides was followed by taking samples at regular time-intervals and determining the molar ratio of the unchanged hydroperoxides by g.l.c. of the derived methyl hydroxystearates. The result is shown in Fig. 2.

There is a rapid decrease in the 13-isomer. However, the concentration of the 9-isomer remains virtually constant, so the isomerase acts specifically on the 13-isomer.

Testing of epoxy compounds as possible intermediates in the isomerization reaction. In former experiments with ^{18}O-labelled linoleic acid hydroperoxides we found that only one oxygen atom of the hydroperoxy function is transferred to the ketol in the isomerization reaction, namely from C-13 in the hydroperoxide to C-12 in the ketol (Veldink, Vliegenthart & Boldingh, 1970*b*). This observation led us to postulate that a cyclic intermediate was involved in the reaction, possibly an epoxy compound. To test this hypothesis, vernolic acid [(+)-12,13-*cis*-epoxyoleic acid], (±)-12,13-*cis*-epoxyoleic acid and (±)-12,13-*trans*-epoxyoleic acid respectively were incubated with the isomerase preparation as described for the experiments with linoleic acid hydroperoxides. None of the isomers showed any conversion into the ketol, the epoxy compounds remaining unchanged. Incubations with the epoxy compounds in the presence of linoleic acid hydroperoxides showed that only the linoleic acid hydroperoxide was reacting. Thus we conclude that epoxyoleic acids are not substrates for the isomerase and that these compounds as such are not intermediates in the isomerization reaction.

DISCUSSION

This investigation forms part of studies aimed at the elucidation of the physiological role of lipoxygenase in plant lipid metabolism. Lipoxygenase needs no cofactors or prosthetic group for the oxidation of suitable unsaturated fatty acids by molecular oxygen. It occurs in a great variety of plant seeds. There is no indication that hydroperoxides are end products *in vivo*. Flax-seed hydroperoxide isomerase, discovered by Zimmerman (1966), is the first well-defined enzyme capable of metabolizing hydroperoxides. We have found that this enzyme converts only 13-hydroperoxylinoleic acid into 13-hydroxy-12-oxo-octadec-*cis*-9-enoic acid. This feature raises the question of whether 9-hydroperoxylinoleic acid is formed by flax-seed lipoxygenase, or whether the latter compound has a specific function. The physiological significance of the α-ketol for flax seed is completely unknown.

It may be that the role of the isomerase is to protect the seed against an excessive amount of linoleic acid hydroperoxide, should seed damage occur during germination. For this we advance the following arguments: in seed damage there may be sufficient oxygen available for the rapid formation of hydroperoxides. We found that, *in vitro*, for soya-bean lipoxygenase the incubation medium must be kept saturated with oxygen for the production of hydroperoxides only. At lower oxygen concentrations the combination of linoleic acid, linoleic acid hydroperoxides and lipoxygenase gives rise to the formation of 13-oxotrideca-9-*cis*(*trans*)-11-*trans*-dienoic acid (Vliegenthart, Garssen & Boldingh, 1970). This enzymic reaction proceeds even in the almost complete absence of oxygen. Eriksson (1967) demonstrated that in peas lipoxygenase activity is located for the greater part in the inner part of the cotyledon. It is therefore reasonable to suppose that in peas, and perhaps in other seeds too, lipoxygenase acts at low oxygen concentrations, thus favouring the breakdown reaction.

There is another example showing that the reaction of lipoxygenase with linoleic acid can be modified, yielding products other than hydroperoxides. Graveland (1970) found that wheat lipoxygenase when associated with wheat glutenin converts linoleic acid into 12,13-epoxy-9-hydroxyoctadec-10-*trans*-enoic acid and 9,10-epoxy-13-hydroxyoctadec-11-*trans*-enoic acid, which readily hydrolyse to the corresponding trihydroxy compounds. Soya-bean lipoxygenase in combination with wheat glutenin gives the same results. It is surprising that the action of an enzyme can be so profoundly influenced by association with another protein. We suggest that in other seeds also proteins may occur that are capable of modifying the lipoxygenase reaction.

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