Presence of Fatty Acid Degrading Enzyme in a Certain Variety of Peas (*Pisum sativum hortense* Cv. Solara)

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Pea (*Pisum sativum*) is the legume seed most used for pig and other animal feeding in Europe. An enzyme with at least dual activities, dioxygenase and fatty acid lyase, has been isolated from *P. sativum hortense* cv. Solara seeds. This enzyme seems to differ from previously characterized lipoxygenase. *P. sativum* lipoxygenase is in many respects quite similar to *Vicia sativa* lipoxygenase, another leguminous family. The enzyme had a pH optimum at 5.8 and two activities that convert linoleic acid into two products: 9-hydroperoxylinoleic acid and 2,4-decadienal. The molecular weight of the enzyme was estimated at 114 000 and its p*I* was 6.09. The dioxygenase and the lyase activities utilize directly linoleic acid as substrate; the existence of a new class of lipoxygenases which expressed two activities termed, respectively, dioxygenase and fatty acid lyase, is suggested.

INTRODUCTION

Leguminous plants are well-known to be used in human feeding. Because of its chemical composition and agronomic properties (Grosjean and Gatel, 1986), pea is the legume seed most used for animal feeding in Europe, not only as green fodder but also as dry seeds. Unfortunately, leguminous plants, as in most cases of cultivation, are attacked by pathogens and pests (Bournoville et al., 1983). On that account, *Pisum sativum hortense*, a variety of pea obtained from genetic engineering, is the most used in France, because of its resistance against pathogens and its nutritional quality (Gatel et al., 1988). It would be of great interest, therefore, to determine whether biochemical plant responses to attack by pathogens occur in cells.

In a previous paper (Andrianarison et al., 1991), we suggested that molecules containing a conjugated unsaturated aldehyde group, due to their reactivity with sulfhydryl and amino groups, could be involved in the response to attack by pathogens. Such molecules can result from action of lyase-like enzyme on polyunsaturated fatty acids or their derivatives in plant cells (Andrianarison et al., 1989, 1991).

The present investigation was undertaken to demonstrate the presence of a fatty acid degradative process in peas for pigs (*P. sativum hortense* cv. Solara), which may be implicated in the biological responses of plant cells to attack by pathogens.

MATERIALS AND METHODS

Plant Materials and Enzyme Extraction. Dry peas (*P. sativum hortense* cv. Solara) were purchased from ITCF Institut Technique des Céréales et des Fourrages de Paris (ITCF). This subspecies of peas gives white flowers and smooth seeds with a high linoleic acid content (Gatel and Grosjean, 1990). White pea seeds are primarily used in animal feeding, whereas the other types are mainly used for human consumption.

All steps of enzyme extraction were performed below 4 °C. About 30 g of *P. sativum hortense* cv. Solara dry seeds were pulverized and homogenized with 160 mL of deoxygenated 66 μM potassium phosphate buffer (pH 7) for 15 min using an Ultra Turrax homogenizer (3000*g*). To ameliorate the purification yield, we tested new detergents, *N*-(alkylamino)-1-deoxylactitol, easy to synthesize and quite inexpensive (Latge et al., 1991). The most effective detergent, named C8, having eight carbons on the alkyl chain, was added to the extraction buffer (0.1%), followed by further stirring for 12 h under anaerobic conditions. After extraction, the mixture was filtered through layered gauze to remove cell debris. The filtrate was clarified by centrifugation at 4700*g* for 60 min. Crystallized ammonium sulfate was added slowly with constant stirring to 60% saturation. After 1 h, the mixture was centrifuged (1000*g* for 1 h) and the precipitate was discarded. The supernatant was dialyzed overnight against 100 volumes of Tris buffer (pH 6.8). After concentration with PEG 6000, the sample was applied to a DEAE-Trisacryl column (2.5 × 40 cm), which was washed with Tris buffer (pH 6.8). Thereafter, a linear gradient of sodium chloride was applied (0–1 M) (Figure 1). Fractions of 3 mL were collected. The active fractions were pooled and dialyzed overnight against 66 μM potassium phosphate (pH 7). After concentration with PEG 6000, the dialysate was subjected to a preparative isoelectric focusing (Catsimpoolas, 1969) in a sucrose gradient column [an LKB 8100 α electrofocusing column of 110-mL capacity (LKB instruments) was used for the

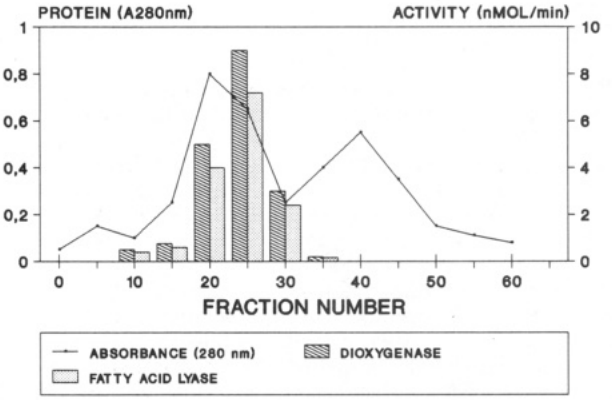

Figure 1. Elution profile of *P. sativum* lipoxygenase from DEAE-Trisacryl column (25 × 400 mm). Column was equilibrated with 0.02 M Tris–acetate buffer (pH 7.2) and elution conducted by 0–1 M NaCl linear gradient at a flow rate of 1 mL/min. Fractions of 3 mL were collected, and proteins were monitored at 280 nm. A molar extinction coefficient of 23 600 M^{-1} cm^{-1} was used to convert absorbance at 235-nm readings to moles of conjugated diene, and a molar extinction coefficient of 22 000 M^{-1} cm^{-1} was used to convert absorbance at 280-nm readings to moles of oxodiene product.

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experiments]. The carrier ampholyte (Pharmacia Fine Chemicals) was selected to give a pH gradient ranging from pH 4 to 8 for analytical studies. The column was operated as described in the LKB manual application sheet. Electrofocusing was performed for 48 h with a final potential of 800 V at 4 °C. Fractions of 1.0 mL were collected. The absorbance at 280 nm and the pH of each fraction were measured. The active fractions were pooled and used for subsequent studies.

Molecular Weight. The molecular weight of the enzyme was estimated by gel filtration. The concentrated enzyme was put on a Sephadex G-150 column (4 × 46 cm) equilibrated with 66 μM potassium phosphate buffer (pH 7). The enzyme was eluted with the same buffer. β-Amylase (200 000), alcohol dehydrogenase (150 000), bovine albumine (66 000), carbonic anhydrase (29 000), and cytochrome c (12 400) were used as marker proteins.

Enzymic Activity Measurements. Dioxygenase activity was determined by measuring the conjugated diene formation at 235 nm using a Perkin-Elmer Lambda 5 UV–vis spectrophotometer. One unit of enzyme was defined as the amount of enzyme that produced 1 nmol of conjugated diene/min at 20 °C. A molar extinction coefficient of 23 600 M^{-1} cm^{-1} (Gibian and Vanderberg, 1987) was used to convert absorbance at 235-nm reading to moles of conjugated diene. The formation of lyase product was monitored at 280 nm (ϵ = 22 000 M^{-1} cm^{-1}) (Mack et al., 1987). A 10 mM stock solution of substrate was prepared by solubilizing linoleic acid in 0.025 M borate buffer (pH 9) with an equimolar concentration of nonionic surfactant.

Oxygen Uptake Measurements. The enzymic activities were also determined by measuring the oxygen uptake with a Clark oxygen electrode at 20 °C (Grossman and Zakut, 1979) using a YSI Model 5300 biological oxygen monitor.

Reversed-Phase High-Pressure Liquid Chromatography (RP-HPLC). To purify the reaction products, RP-HPLC was performed with a Waters Lambda-Max Model 481 equipped with a HPLC pump Model 501 and a LC spectrophotometer Model 481. The organic extract was applied to a Nova-Pak C 18.4-μm column (5 mm × 10 cm) operated with a Radial-Pak cartridge (Waters RCM, 8 × 10). A Waters Guard-Pak precolumn module was inserted into the HPLC system immediately before the Radial-Pak cartridge to remove unwanted particulate and chemical contamination from the mobile-phase stream. The products were eluted with methanol/water/triethylamine/acetic acid (80:20:0.05:0.1 v/v) at flow rate of 1.5 mL/min. Absorbance was monitored at 235 or 280 nm.

Structural Determination of Reaction Products. The hydroperoxy acids purified by RP-HPLC were reduced to alcohol by $NaBH_4$ and methylated with diazomethane. The hydroxy acid methyl ester derivatives were analyzed by GC/MS after silylation of hydroxyl groups by bis(trimethylsilyl)trifluoroacetamide as previously described (Andrianarison et al., 1989).

Product isolated from RP-HPLC showing a maximum of absorption near 280 nm was analyzed without derivation by GC/MS. Identification of this product was confirmed by coelution with authentic 2,4-decadienal purchased commercially (Fluka). Mass spectra were recorded using a magnetic field mass spectrometer monitored by a computer system (LKB 2091-0-61). The column (25-m Carbowax 20 M) was held isothermal at 60 °C for 1 min and then programmed at 2 °C/min to 120 °C.

RESULTS AND DISCUSSION

Evidence for Dioxygenase Activity in *P. sativum hortense* Cv. Solara. Oxygen uptake values (data not shown) showed that the crude extract possesses activity toward linoleic acid. Oxygen uptake was strongly inhibited after addition of NDGA, a lipoxygenase inhibitor (Kemal et al., 1987). Thus, oxygen uptake can be assumed to be of an enzymatic origin and a lipoxygenase-like enzyme may be probably involved in this process.

Enzyme Extraction in Relation to Detergent Utilization. Enzyme has been extracted using different detergents (0.1%). The protein measurement was performed according to the method of Lowry et al. (1951) using bovine serum albumin as standard. For the same concentration, it appeared clearly that Brij 99 and C8 were the best detergents to optimize the protein extraction from pea seeds. Detergents are known to interact with protein, especially by the fixation of their hydrophobic moiety on the protein fragment normally in contact with lipids in cell membrane. No structural evidence on interaction between protein and surfactants has yet been discovered, but it has been hypothesized that proteins are encapsulated in a micellar system formed by detergents.

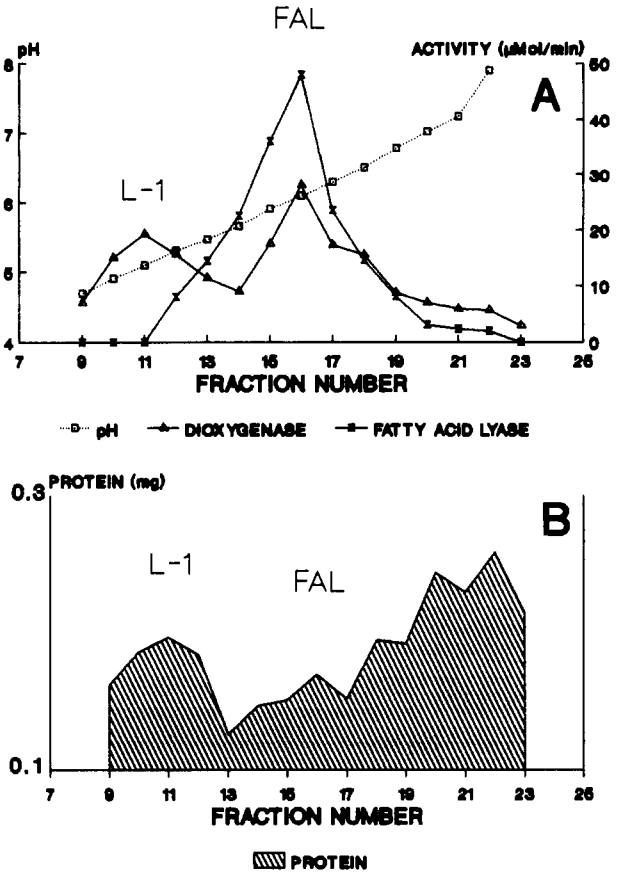

Figure 2. Isoelectric focusing of *P. sativum hortense* cv. Solara fatty acid lyase between pH 4 and 8. Fractions of 1 mL were collected, and the pH of each fraction was measured (A). The protein measurement was performed according to the method of Lowry et al. (1951) using bovine serum albumin as standard (B). Dioxygenase and lyase activities were assayed spectrophotometrically at pH 6 in 0.07 M sodium phosphate buffer. L-1, lipoxygenase type 1; FAL, fatty acid lyase.

Evidence for the Involvement of a Single Protein in the Synthesis of 9-Hydroperoxylinoleic Acid (9-HPOD) and 2,4-Decadienal. Figure 2 indicates clearly that the dioxygenase and lyase activities are cochromatographed on the electrofocusing column, and the p*I* of the protein was 6.09. Electrophoresis of the final purified protein on 10% polyacrylamide gel showed one major band, suggesting that a single enzyme isolated from *P. sativum hortense* cv. Solara seeds possesses both dioxygenase and lyase activities. It is interesting to notice that a second peak (L-1) with a lipoxygenase activity was isolated from the electrofocusing column (p*I* was 4.90). RP-HPLC and GC/MS studies showed that this second enzyme catalyzes the oxidation of linoleic acid specifically into 13-HPOD and consequently corresponds to the classical lipoxygenase type 1 (Hamberg and Samuelsson, 1967).

Molecular Weight. The active fractions from preparative isoelectrofocusing column were pooled, concentrated, dialyzed, and subjected to a Sephadex G-150

Table I. Effects of Ethanol and Detergents on Dioxygenase Activity

nonionic surfactant	sp act., units/mg	nonionic surfactant	sp act., units/mg
C8	381.0	Triton X-100	331.0
Tween 20	70.5	ethanol	293.6
Brij 99	109.0		

column. The molecular weight of the enzyme, estimated at 114 000, was quite similar to the result of Hardy et al. (1991). The enzyme has been found to be larger than most lipoxygenases, which are generally less than 100 000 (Mack et al., 1987).

Effect of Detergents on Substrate Dispersion. We have studied the effect of ethanol and various detergents on linoleic acid dispersion. The specific activities measured at pH 5.8 in potassium phosphate buffer are summarized in Table I. These results showed clearly that Tween 20 inhibits the dioxygenase activity considerably, while C8 has no inhibitory effect on the dioxygenase and lyase activities. The specific activity for substrate dispersed in Brij 99 was about 4-fold lower than that of substrate dispersed in C8.

Reaction Products. The enzymic conversion of linoleic acid led to two compounds corresponding to the two major peaks on the RP-HPLC profile which have, respectively, the same retention times as authentic 2,4-decadienal product from *Vicia sativa* seed lipoxygenase (Andrianarison et al., 1991) and 9-HPOD product from potato tuber lipoxygenase (Mulliez et al., 1987). The first peak showed an absorption band at 280 nm in methanol, while the second one showed an absorption band at 234 nm in the same solvent. Absorption near 280 nm was also characteristic of a conjugated dienone chromophore (Vioque and Holman, 1962). The mass spectrum of the first product absorbing at 280 nm was characterized by a molecular ion (M^+) at m/z 152 and by diagnostic ions at m/z 123 [152 − 29 (C_2H_5)] and 81 [152 − 71 (C_5H_{11})], in good agreement with the fragmentation pattern of 2,4-decadienal. The second peak was collected and analyzed by GC/MS as described under Materials and Methods. The mass spectrum of the methyl ester TMS derivative of this second peak was characterized by a molecular ion (M^+) at m/z 382 and by diagnostic ions at m/z 225 and 311. From these results, the molecule absorbing at 234 nm corresponds to 9-hydroperoxy-10,12-octadecadienoic acid.

Effects of pH on Product Specificity. To study the effect of pH on enzyme activities, the reactions were initiated by addition of 30 μM of linoleic acid dispersed with an equimolar quantity of the detergent C8 in 3 mL of buffer at 20 °C. Only one buffer was used: citrate–phosphate–borate/HCl for pH 4–8.5. The pH dependency of the two enzyme activities showed symetric curves with the same pH optima at 5.8 (data not shown). Hardy et al. (1991) have reported an investigation of lipoxygenase-like activity from *P. sativum*. Unfortunately, they did not give any information on the species and subspecies of the pea seeds used. Hardy et al. (1991) have indicated that linoleic acid oxidation by enzyme preparation from pea seeds was found to be low up to pH 7, beyond which it increased with rising pH to the highest level at pH 9. The pH optimum of pea lipoxygenase was found to be 5.9 by Regdel et al. (1985). Arens et al. (1973) and Chen and Whitaker (1986) reported that pea seeds contain lipoxygenase with pH optimum close to 6.5. In all cases, these authors did not mention the formation of products having maxima of absorption near 280 nm.

UV spectra analysis (Figure 3) showed that the profile of spectrophotometer scanning response is related to the pH of the incubation mixture. The peak with maximum at 280 nm is more important at pH 5.8 than at pH below or above this value. These results show that above pH 7.4 the proportion of oxodiene product was very weak, suggesting that the pH may influence product specificity. It seems that the lyase activity is only expressed by the enzyme at the optimum pH, which is 5.8.

Effects of Inhibitors. When purified enzyme was preincubated with 100 μM NDGA for 10 min, the dioxygenase and the lyase activities were both strongly inhibited (Figure 4A). On the contrary, the use of 100 μM caffeic acid leads to slight inhibition of dioxygenase activity and moderate activation of lyase activity (Figure 4B). Taking into account the simultaneous formation of 9-HPOD and 2,4-decadienal, as judged from these results we suggest that a first step leads to the formation of a common intermediate (Figure 5). It is believed that peroxyl radicals are important intermediates in the oxidation of fatty acids (Corey and Nagata, 1987). Then it seems conceivable that the intermediate formed from the first step of linoleic oxidation by *P. sativum* lipoxygenase is a peroxyl radical. This first step is strongly inhibited by NDGA, suggesting the implication of an organoiron-mediated pathway (Kemal et al., 1987). This peroxyl radical is converted into 2,4-decadienal probably by an internal homolitic cleavage of the intermediate and also into 9-HPOD by addition of a hydrogen radical. This last step is inhibited by caffeic acid inducing more conversion of the intermediate peroxyl radical into 2,4-decadienal. The 2,4-decadienal synthesis is inhibited only by NDGA, not by caffeic acid, confirming the formation of a common intermediate for 9-HPOD and 2,4-decadienal formations.

Conclusion. Though many investigations have been made on the study of lipoxygenase in peas, information regarding the exact species or subspecies used was not given. This could explain the marked differences among the results of different authors.

In a previous paper (Andrianarison et al., 1991), we also established the presence of a single protein with dual activities, viz. dioxygenase and fatty acid lyase in *V. sativa* seeds. The lipoxygenase activity of the enzyme utilizes linoleic acid to form 9-HPOD, while the lyase activity cleaves linoleic acid to form *trans*-2,*cis*-4-decadienal. It is well-known that the hydroperoxy fatty acid product from the dioxygenation of unsaturated fatty acids by lipoxygenase may be enzymatically transformed to secondary products (Gardner, 1991). A membrane-bound hydroperoxide lyase, found in higher plants, catalyzes the cleavage of the fatty acid chain between the hydroperoxide group and the α-olefinic carbon, affording an aldehyde and an ω-oxo acid (Hatanaka et al., 1986). On the other hand, a soluble enzyme from mushroom (Wurzenberger and Grosch, 1984) and two species of algae (Andrianarison et al., 1989; Vick and Zimmerman, 1989) was found to cleave the fatty chain on the opposite side of the hydroperoxide-bearing carbon, that is, between the hydroperoxide carbon and the α-methylene, resulting in an alcohol (or hydrocarbon) and an ω-oxo acid. These enzymes, termed hydroperoxide lyase, were separable from lipoxygenase. In the case of *P. sativum* and *V. sativa* seeds, the two enzyme activities, viz. dioxygenase and lyase, are contained in the same protein. On the other hand, we noticed that neither 13-HPOD nor 9-HPOD is a substrate for the lyase activity, and so we suggest the existence of a new class of lipoxygenases which express two activities termed, respectively, dioxygenase and fatty acid lyase. The fatty acid lyase activity does not act on hydroperoxide derivatives of linoleic acid but directly on the unoxidized

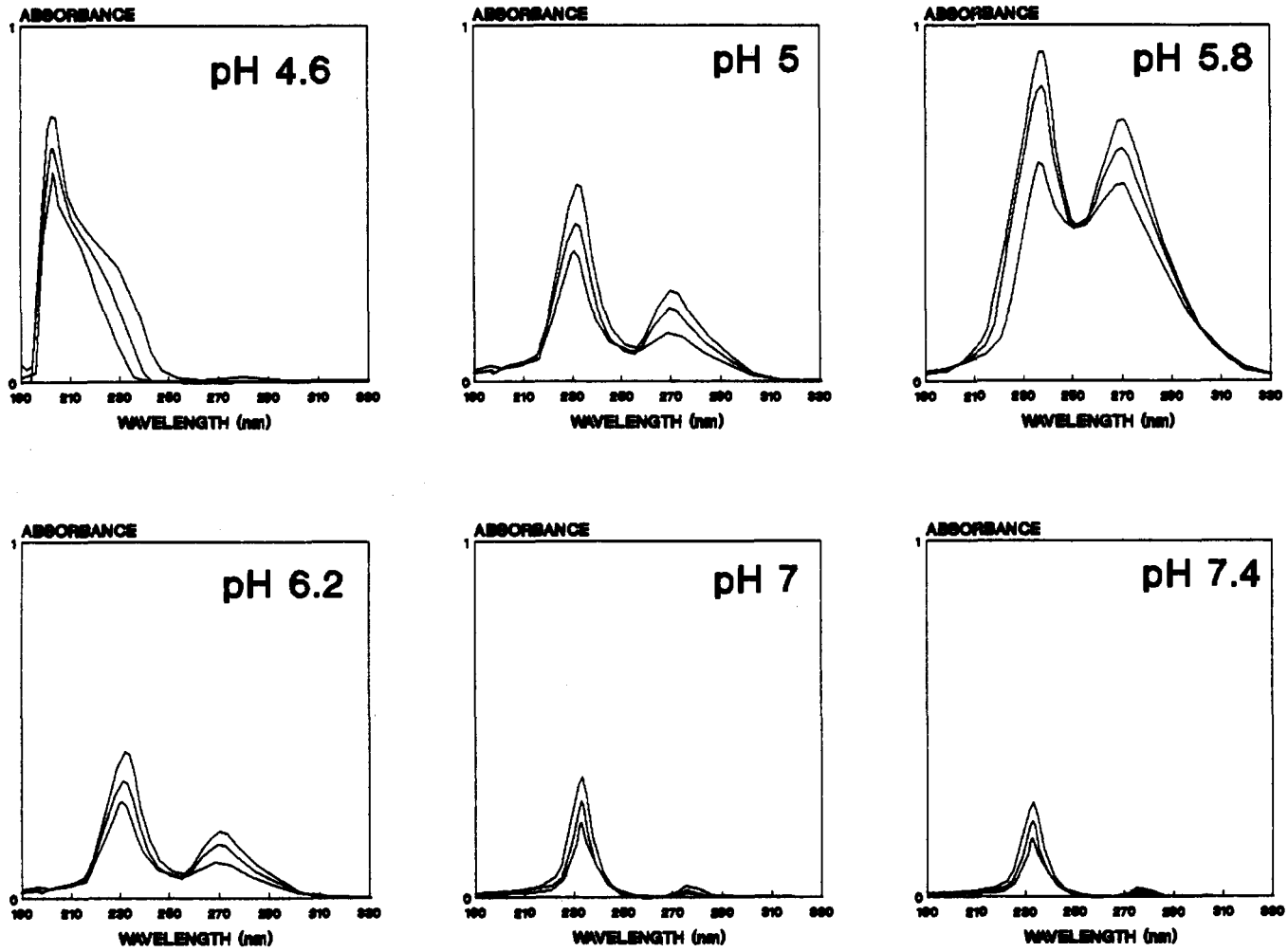

Figure 3. UV spectra of linoleic acid degradation in relation to the pH of the incubation mixture. The figures show the UV spectra from 0 to 21 s. The cycle time was 7 s.

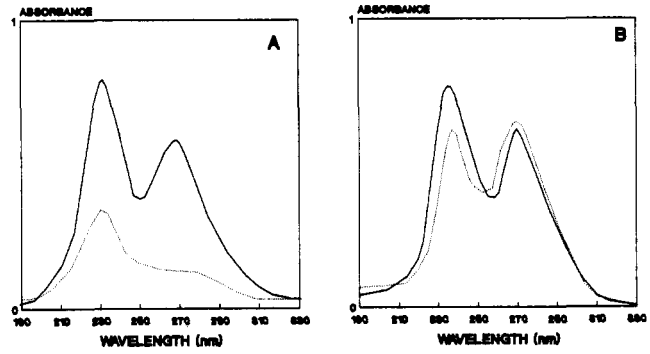

Figure 4. Effects of inhibitors. Enzyme (30 units) was preincubated for 10 min with 100 μM inhibitor in a potassium phosphate buffer (pH 5.8) (3 mL), and the reaction was started up by addition of 30 μM of linoleic acid (dispersed by equimolar quantity of C8). UV spectra were taken after 15 min of incubation. (A) With NDGA; (B) with caffeic acid; (—) standard; (- - -) after incubation with inhibitors.

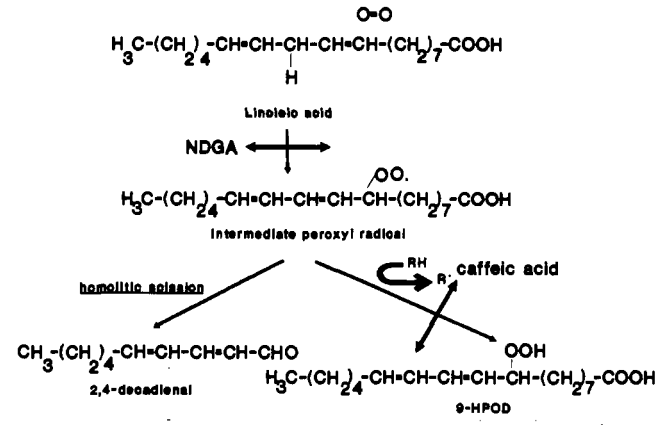

Figure 5. Proposed schematic pathway for the degradative process of linoleic acid by fatty acid lyase from *P. sativum hortense* cv. Solara.

fatty acid itself to yield carbonyl. Lipoxygenases from *P. sativum* and *V. sativa* seeds both metabolize linoleic acid into 9-HPOD and 2,4-decadienal and have the same pH optima at 5.8. Nevertheless, the size of the lipoxygenase from *P. sativum* seeds (114 000) is smaller than the one of *V. sativa* (237 000). It is of special interest that this class of enzyme has smaller pH optima (5.8) than the lipoxygenases classified by Galliard and Chan (1980).

Hardy et al. (1991) have reported that purification of lipoxygenase-like enzyme from pea seeds using Sephadex G200 yielded a single peak (M_r 114 000). Molecular weight, thus, resembles that from lipoxygenase-like enzyme isolated by our laboratory, but they did not mention the formation of products which have maxima of absorption around 280 nm. Hardy et al. (1991) have found that dark-germinated seedlings contained at least three isoforms of lipoxygenase. A single isoform was found in the quiescent seeds. According to Domoney et al. (1990), peas had at least seven isozymes of lipoxygenases, five of which were minor. Domoney et al. did not report if these isozymes are versatile in catalyzing the formation of products other than monohydroperoxides.

In spite of the large amount of information available on lipoxygenase and its isozymes in higher plants, the role of this enzyme in plants remains rather speculative. The more important fact in our results is the ability of *P. sativum* lipoxygenase to synthesize an aldehyde, 2,4-decadienal, which posseses a conjugated aldehyde group that would likely have high toxicity, due to its reactivity with sulfhydryl and amino groups, suggesting to us the possibility that the lipoxygenase from *P. sativum hortense* cv. Solara could be involved in the response to attack by a pathogen. This hypothesis could explain the ability of this new variety of peas to resist pests and pathogens. A number of studies have shown an increase in lipoxygenase activity in various plant tissues following pest attack, and it has been hypothesized that the role of lipoxygenase in pest and pathogen resistance may be either direct or indirect in plants (Bhatt et al., 1991; Mohri et al., 1990; Farmer and Ryan, 1992). Further work is being done in our laboratories to test these possibilities.

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