Lipoxygenase Activity in Olive *(Olea europaea)* Fruit

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ABSTRACT: The present work was designed to characterize lipoxygenase activity in olive fruit pulp, in order to determine its significance in the biosynthesis of virgin olive oil aroma. Lipoxygenase activity has been detected in particulate fractions of enzyme extracts from olive pulp subjected to differential centrifugation. The activity in different membrane fractions showed similar properties, with optimal pH in the range of 5.0–5.5 and a clear specificity for linolenic acid, which was oxidized at a rate double that of linoleic acid under the same reaction conditions. The enzyme preparations displayed very low activity with dilinoleoyl phosphatidylcholine, suggesting that olive lipoxygenase acts on nonesterified fatty acids. The enzyme showed regiospecificity for the Δ-13-position of both linoleic and linolenic acid, yielding 75–90% of Δ-13-fatty acid hydroperoxides. Olives showed the highest lipoxygenase activity about 15 wk after anthesis, with a steady decrease during the developmental and ripening periods. Olive lipoxygenase displayed properties that support its involvement in the biogenesis of six-carbon volatile aldehydes, which are major constituents of the aroma of virgin olive oil, during the process of oil extraction.

Paper no. J9118 in *JAOCS 76,* 1163–1168 (October 1999).

KEY WORDS: Aroma, lipoxygenase, *Olea europaea,* olive, virgin olive oil, volatile biosynthesis.

Lipoxygenase (LOX; linoleate:oxygen oxidoreductase, EC 1.13.11.12) is a non-heme iron-containing enzyme that catalyzes the oxygenation of the 1,4-pentadiene sequence of polyunsaturated fatty acids to produce their corresponding hydroperoxides (1). This enzyme, which is widespread in the plant kingdom, catalyzes the first reaction of the so-called lipoxygenase pathway, which mediates the biosynthesis of several regulatory molecules involved in plant responses to stress and wounding, such as traumatin, jasmonic acid, and abscisic acid (2). More recently, products from the lipoxygenase pathway have been found to participate in other cellular processes such as the synthesis of cutin monomers (3) and the regulation of β-oxidation in germinating oil seeds (4).

Plant LOX are also involved in the biosynthesis of volatile compounds of six- and nine-carbon atoms produced upon disruption of plant tissue (5). These compounds are formed from polyunsaturated fatty acids by the sequential action of LOX and hydroperoxide lyase, resulting in the formation of aldehydes of six or nine carbon atoms. Enzymatic reduction of aldehydes, catalyzed by alcohol dehydrogenase, gives rise to their corresponding alcohols, from which ester derivatives can be formed. The volatile aldehydes synthesized through this pathway have been reported to be active against plant pathogen microorganisms (6) as well as to be insect repellents (7). In addition, these compounds are present in the volatile fractions of fruits and vegetables, conferring on them their characteristic "green" odor.

The experiments described here were undertaken within a project designed to investigate the biogenesis of the major volatile compounds constituting the aroma of virgin olive oil. Since this oil is elaborated from fresh fruits by using mild physical procedures (milling, malaxing, centrifugation) only, the natural volatile compounds produced during the crushing and malaxation of these fruits are incorporated into the resulting oil, giving rise to its prized aroma. The six-carbon volatile aldehydes and alcohols produced by the lipoxygenase pathway are the main components of the virgin olive oil aroma (8). Among these, 2(*E*)-hexenal is, by far, the most abundant in oils from all the cultivars analyzed so far, accounting for 50 to 70% of the total volatiles in the headspace of such oils (8,9), and it contributes positively to the typical and appreciated "green note" of olive oils. The LOX activity present in olive fruit has been measured and characterized in the present work. The putative participation of this enzyme in the biogenesis of important volatile compounds, which are constituents of the aroma of olive oil, is discussed.

MATERIAL AND METHODS.

Plant material. Olive (*Olea europaea* cv. Picual) fruits were harvested early in the morning from 30-yr-old trees growing in a grove near Sevilla (Spain), which had been given drop irrigation and fertirrigation (irrigation with suitable fertilizers in the solution) from the time of full bloom to fruit maturation (April to November).

Enzyme extract preparation. Attempts to measure LOX activity in extracts prepared from pulp tissues by using detergent solubilization (10–12) were unsuccessful. This could have been due to the high amount of phenolic compounds present in olive extracts, since these compounds inhibit LOX activity (13,14). In addition, the high levels of storage lipids

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present in the tissue often caused turbidity of the resulting extracts, which hampered the spectrophotometric determination of LOX activity. Therefore, the extraction protocol was modified by exclusion of detergent in the grinding buffer.

Olives were rinsed with tap water and then distilled water. After removing the endocarp (stone), a 1–2-mm-thick ring was cut transversely at the middle of each fruit. These pulp rings were immediately dipped in a 50-mM solution of potassium phosphate, pH 7.0, containing 0.1% ascorbate to prevent tissue browning. For experiments where the epicarp and mesocarp fractions were analyzed, the epicarp (outer, dense green layer) was physically separated from the mesocarp and the two tissue samples processed independently. The pulp tissue was extracted with 50 mL of a solution of 50 mM HEPES buffer, pH 7.5, containing 330 mM sorbitol, 20 mM KCl, 2 mM $MgCl_2$, 5 mM EDTA, 7 mM 2-mercaptoethanol, 3 mM dithioerythritol, 0.1% ascorbate and 10% glycerol (grinding buffer) in the presence of 5 g of acid-washed polyvinylpolypyrrolidone (insoluble). The ground, extracted pulp tissue was filtered through Miracloth tissue (Calbiochem, La Jolla, CA) and submitted to differential centrifugation at 1000 $\times$ *g* for 5 min, 40,000 $\times$ *g* (40 K) for 20 min, and 150,000 $\times$ *g* (150 K) for 80 min. The 1000 $\times$ *g* precipitate was discarded. The 40 K (high-density membrane fraction) and 150 K (microsomal fraction) pellets were resuspended in 25 mM HEPES buffer, pH 7.5, containing 10% glycerol (buffer A), using a manual glass tissue grinder.

Subcellular distribution. To determine the subcellular location of LOX activity the pulp tissue was ground in grinding buffer in the presence of polyvinylpolypyrrolidone as described above, but excluding grinding in liquid nitrogen. The resulting homogenate was fractionated by successive centrifugation at 1,000 $\times$ *g* for 5 min, 3 K for 5 min, 15 K for 10 min, 40 K for 20 min, and 150 K for 80 min. The resulting pellets, except the 1,000 $\times$ *g* pellet, were resuspended in buffer A.

Enzyme assay. LOX activity was routinely assayed by the spectrophotometric method of Axelrod *et al*. (15), based on the increase of absorbance at 234 nm due to the formation of a conjugated double bond system in the fatty acid hydroperoxides, formed in the first minute of reaction. Alternatively, the endpoint method of Jiang *et al*. (16), based on the colorimetric determination of fatty acid hydroperoxides formed in the reaction, was used. In this case, the incubation time was fixed at 5 min. In both methods the assay mixture consisted of 50 mM sodium acetate buffer, pH 5.0, 0.5 mM potassium linoleate or linolenate, 0.4% Tween-20, and enzyme extract equivalent to 40–100 µg of protein, in a final volume of 1 mL. Tween-20 was included to avoid the cloudiness that occurred when the substrates were dispersed in its absence. When dilinoleoylphosphatidylcholine was used as substrate, it was added as an aqueous suspension, prepared by sonication, to a final concentration of 0.25 mM. Assays were carried out at room temperature. When the effect of pH was investigated, sodium acetate, 2-(*N*-morpholino) ethanesulfonic acid (MES) and potassium phosphate buffers were used in the same conditions to cover a pH range from 4.5 to 7.5.

Determination of olive LOX regiospecificity. The combined 3 K and 15 K particulate fractions (containing most of the LOX activity of the olive pulp) prepared as described above were used as the source of enzyme for studies of regiospecificity. The reaction mixture contained 50 mM sodium acetate buffer, pH 5.0, 1 mM of either linoleate or linolenate (potassium salt), and enzyme extract containing 40 mg of protein in a volume of 50 mL. After a 30-min incubation at room temperature, the mixtures were centrifuged at 20,000 $\times$ *g* for 10 min, and the resulting supernatant was submitted to solid-phase lipid extraction in a C18 cartridge (Baker, Phillipsburg, NJ). Lipids were eluted from the column by washing with 3 mL methanol. The resulting lipid extract was fractionated by thin-layer chromatography on silica gel plates using hexane/diethyl ether/formic acid (50:50:1, vol/vol/vol) as the solvent. The bands corresponding to hydroperoxides were scraped from the plates and methylated with diazomethane as described by Schlenk and Gellerman (17). The hydroperoxide methyl esters were analyzed by high-performance liquid chromatography (HPLC)-diode array

Analysis of hydroperoxide methyl esters by HPLC. The hydroperoxy methyl esters resulting from the preceding step were analyzed by HPLC following the method reported by Sanz *et al*. (18). Thus, these compounds, dissolved in hexane, were filtered through 45-µm membranes (Millipore, Bedford, MA) and injected in a HPLC-diode array detector chromatograph (HP 1090; Hewlett-Packard, Wilmington, DE) fitted with a Lichrosorb SiG 60 silica gel column (250 $\times$ 46 mm, 5 µm, Bio-Rad, Hercules, CA). The mobile phase consisted of hexane/ethyl ether (92:8), at a flow of 0.8 mL/min. Under these conditions, four isomers of hydroperoxy methyl esters were separated, Δ-13-*EZ*(*Z*), Δ-13-*EE*(*Z*), Δ-9-*ZE*(*Z*), and Δ-13-9-*EE*(*Z*), which were identified by comparison of their retention times with suitable standards.

Preparation of the standards of hydroperoxide methyl esters. Standard mixtures of hydroperoxides were prepared by incubating the corresponding fatty acids with lipoxygenase from soybean (Sigma, St. Louis, MO) or potato tubers [prepared according Galliard and Phillips (19)]. Volumes of 100 mL of solutions containing 3-mM potassium linoleate or linolenate in 0.2 M potassium borate buffer, pH 9.0 in the case of soybean LOX, or 50 mM MES buffer, pH 6.0 in the case of potato LOX, were incubated at room temperature with 1 µkat (1 µmol of substrate transformed per second under the standard reaction conditions) of LOX activity. When 70–80% of the substrate had been transformed, the hydroperoxides were extracted, isolated, and derivatized to their methyl esters as reported above for olive LOX. The hydroperoxide mixture prepared from soybean LOX contained mainly 13-hydroperoxide isomers (1), whereas the mixture prepared from potato LOX was rich in the 9-isomers (19).

Protein and chlorophyll determination. Protein was determined by a modification of the Folin-Lowry method (20), using bovine serum albumin as standard. Chlorophyll was determined by the method of Bruinsma (21).

RESULTS AND DISCUSSION

Subcellular and histological distribution. Early studies showed that LOX are ubiquitous enzymes present in different cellular compartments of nonphotosynthetic cells (1). This activity has been also reported in chloroplast fractions of photosynthetic cells (22). More recently, LOX has been reported to be associated with the chloroplast envelope membrane (23). Differential centrifugation of ground extracted olive pulp yielded particulate fractions sedimenting at 3, 15, 40, and 150 K that contained LOX activity (Table 1). No activity was detected in the remaining soluble fraction (150 K supernatant). This parallels the case reported for tea leaves (22) and differs from that reported for fruits, in which soluble LOX has been reported to predominate (11,12). Under standard conditions, LOX activity measured in particulate fractions from olive pulp ranged between 0.5 and 5 nkat/g fresh tissue, depending on the developmental stage of the fruit (Fig. 3).

The presence of LOX activity in those fractions having both high density and chlorophyll content (Table 1) suggests that the enzyme activity is associated with chloroplasts, as is the case in other photosynthetic tissues (22). These results, together with the finding that olive hydroperoxide lyase is concentrated in the chloroplasts (24), suggest that the synthesis of six-carbon aldehydes, which are major constituents of the aroma of olive oil, takes place in the fruit chloroplast.

On the other hand, two different tissues can be distinguished in the olive pulp, the epicarp, which is rich in chloroplasts and remains green during most of the fruit development, and the mesocarp, where oil accumulation takes place. Since the subcellular distribution of LOX activity, described above, suggested that it could be associated, at least in part, with the chloroplasts, it was of interest to study the distribution of enzyme activity between epicarp and mesocarp. LOX activity was equally distributed between epicarp and mesocarp (Table 1), which is in clear contrast with the case of cucumbers, where the activity is concentrated in the epicarp (11). Furthermore, the distribution of activity among different fractions was similar in both tissues. Because the mesocarp has significantly fewer functional chloroplasts, these results confirm that LOX activity is also present in extrachloroplastic membranes.

Among other factors, substrate concentration is one of the most important parameters affecting the rate of enzyme reactions. In mature olives, the bulk of fatty acids are in the form of triacylglycerols. Polyunsaturated fatty acids, however, are in low proportions in olive triacylglycerols: the oil from the Picual variety used in this investigation contains approximately 5% linoleic acid and about 0.5% linolenic acid. Moreover, the storage of triacylglycerols in oil bodies (25) makes them relatively inaccessible to enzyme action. Therefore, a more likely source of polyunsaturated fatty acids for the lipoxygenase pathway is the polar glycerolipid constituents of cell membranes. Chloroplast membranes are particularly rich in galactolipids, which in olives and other photosynthetic tissues contain high levels of linolenic acid (26). The subcellular studies described in this article indicate that olive LOX is a membrane-bound enzyme that seems to be, at least in part, associated with chloroplasts (Table 1). Such a location could make olive LOX especially efficient in the formation of Δ-13-hydroperoxylinolenate, the precursor of 2(*E*)-hexenal.

Effect of pH. LOX isolated from different sources have been classified according to their optimal pH values (1). On this basis, three different isoenzymes have been isolated from soybean cotyledons. The effect of pH on olive LOX was investigated in two particulate fractions, and the resulting curves were similar: in both cases the activity peaked at pH 5.0–5.5 (Fig. 1), in agreement with the results for other fruits such as tomato (27) and pepper (28). No activity was detected above pH 7.5 (results not shown). Thus, the optimal pH range of olive lipoxygenase is close to that of the olive paste during malaxation (approx. 5), indicating that favorable conditions for enzymatic production of hydroperoxides are present during the process of oil extraction. It is noteworthy that the use of potassium phosphate buffer produced a slight discontinuity in the pH curves, possibly indicating a small inhibitory effect by this buffer.

Substrate specificity and regiospecificity. Six-carbon aldehydes are formed from polyunsaturated fatty acids by the sequential action of two enzymes of the lipoxygenase pathway:

TABLE 1
Distribution of Lipoxygenase Among the Membrane Fractions of the Olive Pulp and Its Different Tissues[a]

	Whole pulp					Epicarp				Mesocarp			
	Lipoxygenase		Protein	Chlorophyll		Lipoxygenase		Chlorophyll		Lipoxygenase		Chlorophyll	
Fraction[b]	(nkat)[c]	(%)	(mg)	(mg)	(%)	(nkat)	(%)	(mg)	(%)	(nkat)	(%)	(mg)	(%)
3 K	2.7 ± 0.0	21.1	6.9	0.08	32	1.6 ± 0.0	12.4	0.06	24	1.1 ± 0.0	8.9	0.02	8
15 K	4.5 ± 0.4	35.2	16.9	0.08	32	2.4 ± 0.2	18.7	0.06	24	2.1 ± 0.3	16.4	0.02	8
40 K	1.4 ± 0.2	10.9	9.6	0.06	24	0.9 ± 0.1	7.0	0.05	20	0.5 ± 0.2	3.9	0.01	4
150 K	4.2 ± 0.5	32.8	13.9	0.03	12	1.8 ± 0.3	14.0	0.02	8	2.4 ± 0.4	18.7	0.01	4
Total	12.8	100	47.3	0.25	100	6.7	52.1	0.19	76	6.1	47.9	0.06	24

[a]These data correspond to 10 g of pulp prepared from olives harvested 28 wk after anthesis. Activity was measured using linoleic acid as the substrate. Results are means of three determinations ± SD.
[b]K, meaning 1000 × *g*.
[c]nkat, nmoles of substrate transformed per second under standard reaction conditions.

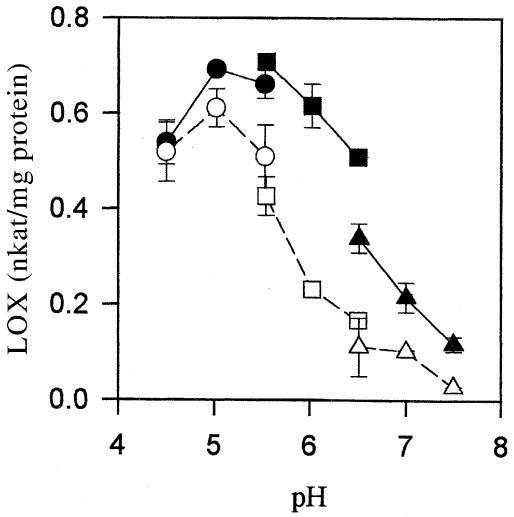

FIG. 1. Effects of pH on lipoxygenase (LOX) activity of particulate fractions from olive fruit pulp: 3 (——) and 150 K (----) fractions from olives harvested 28 wk after anthesis. Results are means of three determinations ± SD, carried out using linoleic acid as the substrate. Sodium acetate (●, ○), MES (■, □), and potassium phosphate (▲, △) were used as the assay buffers. Abbreviations: K, 1000 × *g*; nkat, nmoles of substrate transformed per second under reaction conditions; MES, 2-(*N*-morpholino) ethanesulfonic acid.

lipoxygenase and hydroperoxide lyase. In industrial olive oil extraction, the lipoxygenase pathway is triggered upon crushing of olives and operates during malaxation of the olive paste, which has an average pH of approximately 5. The products formed are incorporated into the oil, which is subsequently separated from the aqueous phase by centrifugation. The nature of the volatile products formed through the lipoxygenase pathway is determined by the properties of the enzymes involved.

The physiologically relevant substrates of plant LOX are linoleic and linolenic acid, the most abundant polyunsaturated fatty acids in plant tissues. Depending on the fatty acid used in the LOX reaction and the position at which the hydroperoxide group is inserted (Δ-9 or Δ-13 carbon of the acyl chain), four different products, which are substrates of the subsequent reactions of the lipoxygenase pathway catalyzed by hydroperoxide lyase, can be formed. Thus, substrate specificity and regiospecificity of LOX determines the chain length and degree of unsaturation of the volatile products formed through the lipoxygenase pathway. LOX from different plant sources, in general, show the highest activity with the most abundant polyunsaturated fatty acid constituent of the tissue from which they have been isolated. Thus, LOX from photosynthetic tissues are usually more active with linolenic acid, whereas those isolated from nonphotosynthetic tissues show higher activity with linoleic acid (29).

The substrate specificity of olive LOX was determined in two particulate fractions from the pulp tissue. Both fractions displayed higher activity with linolenic acid, which was oxidized at a rate twice that measured for linoleic acid. Similar results have been reported for photosynthetic tissues (Table 2) (29).

TABLE 2
Substrate Specificity of the Lipoxygenase Present in Different Membrane Fractions of Olive Fruit Pulp[a]

	High-density membrane fraction		Microsomal fraction	
Substrate	Lipoxygenase (nkat/mg protein)	(%)	Lipoxygenase (nkat/mg protein)	(%)
Linoleic acid	0.53 ± 0.04	54 ± 4	0.8 ± 0.04	53 ± 3
Linolenic acid	0.98 ± 0.02	100 ± 2	1.5 ± 0.05	100 ± 3

[a]Data recorded 27 wk after anthesis. Results are the mean of three determinations ± SD. For abbreviation see Table 1.

On the other hand, although it is well established that nonesterified fatty acids are physiological substrates of plant LOX, the oxidation of polyunsaturated fatty acids esterified to glycerophospholipids has been described recently (30). Therefore, the capability of olive LOX to oxidize esterified fatty acids was assayed by using dilinoleoyl phosphatidylcholine (DLPC) as a model substrate. Microsomal fractions from olive pulp were capable of oxidizing DLPC, with the activity being related to the developmental stage of the fruit (Fig. 2). However, when LOX activity using different substrates was compared, it was found that DLPC was oxidized at a much lower rate than either nonesterified linoleic or linolenic acids (Table 3), as reported for soybean LOX-1 (30). These data support the theory generally accepted that nonesterified fatty acids are the physiological substrates of plant LOX. However, more experiments, using other molecular species of phospholipids and galactolipids as the substrates, should be carried out to confirm this point in olive fruit.

Olive LOX present in the high-density fraction showed a clear specificity for the position Δ-13 of the linoleic and linolenic acid, yielding preferentially the Δ-13-*ZE*(*Z*) isomer (Table 4). This isomer is the precursor of six-carbon alde-

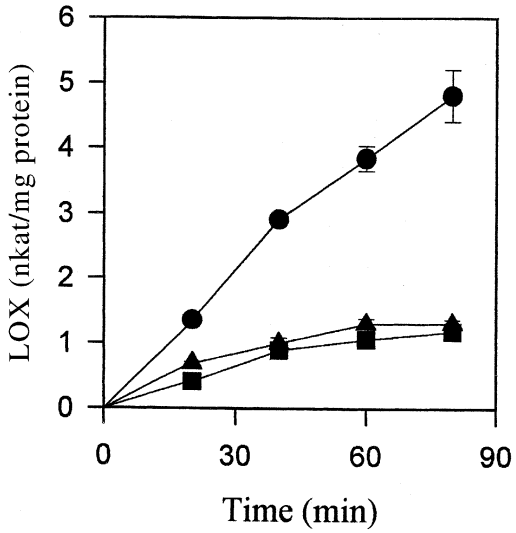

FIG. 2. Oxidation of dilinoleoyl phosphatidylcholine by the LOX present in the microsomal fraction of olive fruits harvested 14 (●), 25 (▲), and 34 (■) wk after anthesis. Every point represents the mean of three determinations ± SD. For abbreviations see Figure 1.

TABLE 3
Comparison of the Oxidation Rates of Free and Esterified Fatty Acids by Olive Fruit Pulp Lipoxygenase[a]

Substrate	Lipoxygenase (nkat/mg protein)	%
Linolenic acid	1.67 ± 0.06	100
Linoleic acid	0.77 ± 0.04	46
DLPC	0.01 ± 0.00	<1

[a]Microsomes prepared from olives harvested 20 wk after anthesis were used as the source of enzyme. Results are the mean of three determinations ± SD. The concentration of fatty acid in the assay mixtures was 0.5 mM. Dilinoleoyl phosphatidylcholine (DLPC) was added as an aqueous suspension, prepared by sonication, to a final concentration of 0.25 mM. For abbreviation see Table 1.

hydes *via* the hydroperoxide lyase reaction. Since it has not been demonstrated that the Δ-13 *EE*(*Z*) isomer is a product of the LOX reaction, its presence among the reaction products could be due to spontaneous isomerization of the *ZE*(*Z*) isomer. The Δ-13/Δ-9 ratio of products formed in the reaction was 3:1 for linoleic acid, and 7:1 for linolenic acid.

Taken together, these results indicate that olive lipoxygenase can efficiently form the Δ-13 hydroperoxide of linolenic acid, which is the precursor of unsaturated six-carbon aldehydes, including 2(*E*)-hexenal, the most prevalent constituent of the aroma of olive oil (8,9).

Developmental profile. Changes in LOX activity with growth and development have been reported for some species when trying to assess the physiological role of the enzyme (1). Growth and development of olive fruit is a long process that lasts 6 to 8 mon, from anthesis to ripening, depending on the cultivar and other factors. Oil accumulation in the pulp starts 12–13 wk after anthesis and continues for some 20 wk until fruit ripening. LOX activity was measured in olives harvested at different developmental stages from 13 to 34 wk after anthesis, thus covering the whole period of oil synthesis and accumulation. LOX activity measured with two different substrates yielded, as expected, comparable results: LOX activity was high at early stages of development and underwent a steady decrease after 14 wk throughout the period tested (Fig. 3). Similar results have been reported for other fruits like tomato and pepper (28,31).

The high levels of LOX activity detected at early stages of fruit development suggest that in olives, as in other plants, the enzyme is important in the physiological response to stress (2). A steady decrease of LOX activity was observed at more advanced stages of maturation, from 25 to 35 wk after anthesis, when the fruits are normally harvested for oil extraction. Indeed, a decrease in the amount of six-carbon volatiles in oils prepared from olives harvested at advanced stages of ripening has been reported (32). This decrease might be ascribed, at least in part, to a decrease in LOX activity in the pulp tissue.

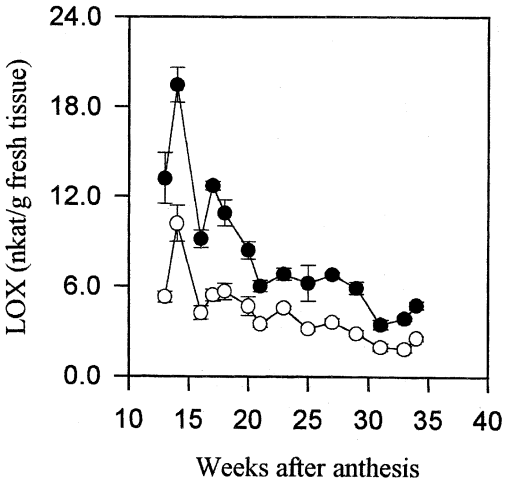

FIG. 3. LOX changes during the development of the olive fruits. The activity was measured using both linoleic (○) and linolenic (●) acids as the substrates. Every point represents the mean of three determinations ± SD. For abbreviations see Figure 1.

Taken together, the results described here show the existence, in the pulp tissue of developing olives, of a LOX activity whose characteristics and properties strongly suggest a vital role in the formation of the six-carbon volatile constituents of the aroma of olive oil. A deeper knowledge of this enzyme, as well as the other components of the lipoxygenase pathway, would help to improve the manufacturing practices of olive oil extraction. Investigations into such characteristics are underway in our laboratories.

TABLE 4
Regiospecificity of the Olive Fruit Lipoxygenase[a]

Precursor	Hydroperoxide isomer (%)			
	13-*ZE*(*Z*)	13-*EE*(*Z*)	9-*EZ*(*Z*)	9-*EE*(*Z*)
Linoleic acid	67	11	17	7
Linolenic acid	73	15	12	N.D.

[a]The 15 K membrane fraction isolated from fruits harvested 28 wk after anthesis was used as the source of enzyme. The concentration of fatty acid substrates was 1 mM. N.D., not detected.

ACKNOWLEDGMENTS

The financial support from the European Commission (AIR 3-93-1967) is gratefully acknowledged.

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[Received January 11, 1999; accepted June 8, 1999]