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The Decrease of Virgin Olive Oil Flavor Produced by High Malaxation Temperature Is Due to Inactivation of Hydroperoxide Lyase

Keywords: *Aroma; hydroperoxide lyase; lipoxygenase; malaxation temperature; olive oil*

INTRODUCTION

Virgin olive oil is obtained by using mild physical methods. Unlike other vegetable oils, it does not require refining and hence preserves the natural flavor compounds that confer its characteristic and valued aroma, which consists of a complex mixture of >100 volatile compounds (Morales et al., 1994). Among them, the most abundant are aldehydes, alcohols, and esters of alcohols of six-carbon atoms (Morales et al., 1995; Ranalli and De Mattia, 1997).

It is well established that those volatile compounds, which are the constituents of the aroma of many fruits and vegetables, are produced from polyunsaturated fatty acids through a cascade of enzymatic reactions known as the lipoxygenase pathway (Hatanaka, 1993). This pathway, which is triggered by tissue disruption, starts with the oxidation of polyunsaturated fatty acids by lipoxygenase (Siedow, 1991) followed by the cleavage of the resulting fatty acid hydroperoxides catalyzed by hydroperoxide lyase (Vick and Zimmerman, 1976). The sequential action of these two enzymes results in the formation of volatile aldehydes of six or nine carbon atoms, depending on the substrate specificities of the enzymes involved. Recent investigations have demonstrated that in olives this enzyme system is highly specific in producing six-carbon aldehydes only (Olías et al., 1993; Salas and Sánchez, 1998a).

The industrial process of olive oil elaboration includes three defined steps: crushing of fruits, malaxation of the resulting paste in the presence of added water, and separation of the oil phase (Kiritsakis, 1990). In the traditional pressure procedure of oil extraction, the second step, malaxation, is usually carried out at 30 °C for 30 min, although these parameters may be changed depending on the rheological properties of the paste. On the other hand, the nowadays more common continuous centrifugation procedure of oil extraction involves longer malaxation times.

The lipoxygenase pathway is triggered by milling of olives and is active during the malaxation step. The volatiles produced are incorporated into the oil phase to confer its characteristic aroma. Therefore, the aroma of a given oil is determined by the activity and properties of the enzymes involved in the lipoxygenase pathway, which can change depending on the cultivar variety and stage of maturation (Aparicio and Morales, 1998; Ranalli and De Mattia, 1997; Williams et al., 1998). On the other hand, malaxation conditions also affect the flavor of the resulting oil (Servili et al., 1998); in particular, a positive correlation between malaxation time and volatile content of the resulting oil has been reported (Kiritsakis, 1998).

Legal regulations dictate that only physical procedures are allowed in the industrial extraction of virgin olive oil. Because malaxation temperature is one of the few, and perhaps the most important, parameter that can be manipulated in the process, the objective of this investigation was to check its effect on the biogenesis of the most prevalent constituents of olive oil aroma. Experiments have been conducted to evaluate the effect of temperature on the enzymes involved in the formation of six-carbon aldehydes by enzyme extracts from olive pulp. The results obtained, which are presented in this paper, might be relevant to future improvements of olive oil manufacturing.

MATERIALS AND METHODS

Olives were harvested from 30-year-old trees growing in a grove near Seville, Spain. The trees were supplied with drop

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irrigation and fertirrigation during the whole reproductive period (April−November).

Preparation of Enzyme Extracts. An acetone powder was prepared from the pulp tissues of olives, harvested 20 weeks after flowering, as described before (Salas and Sánchez, 1998b). Briefly, the preparation involved grinding of the olive pulp in 3 volumes of cold (−20 °C) acetone, filtration under reduced pressure, and washing of the solid residue with diethyl ether. The whitish powder, free of pigments and phenolic compounds, was sieved to remove coarse particles and stored at −20 °C until use. A mass of 10 mg of acetone powder was resuspended in 3 mL of a buffer containing 50 mM MES, pH 6.0, 2 mM 2-mercaptoethanol, and 0.5% Triton X-100, using a glass homogenizer. This suspension was immediately used in labeling experiments with ^{14}C-labeled linoleate.

For enzyme assays, microsomes were prepared from olive pulp as described in Salas and Sánchez (1998c). Pulp tissues (10 g) were ground to a powder in liquid nitrogen using a mortar and pestle. The resulting powder was extracted in 50 mL of 50 mM Hepes, pH 7.5, 330 mM sorbitol, 20 mM KCl, 5 mM EDTA, 2 mM $MgCl_2$, 7 mM 2-mercaptoethanol, 3 mM DTE, 0.1% ascorbate, and 10% glycerol, in the presence of 5 g of acid-washed polyvinylpolypyrrolidone. The homogenate was filtered and centrifuged at 1000*g* for 5 min and at 40000*g* for 20 min, and the pellets were discarded in both cases. The resulting supernatant was finally centrifuged at 150000*g* for 80 min. The resulting pellet (microsomes) was resuspended in 1.5 mL of 25 mM Hepes, pH 7.5, containing 10% glycerol and used as enzyme extract for lipoxygenase assay. To assay hydroperoxide lyase, the microsomal pellet was resuspended in the same buffer supplemented with 0.5% Triton X-100.

Labeling Experiments. Experiments were carried out by using either [1-^{14}C]linoleate (2.2 GBq/mmol) or [U-^{14}C]linoleate (37 GBq/mmol) purchased from Dupont NEN (Boston, MA). In the first case, the precursor was incubated with 0.25 mL of the acetone powder suspension, in a final volume of 0.3 mL, at a concentration of 1 mM (5.7 kBq/μmol). The mixture was incubated for 30 min. Lipids were extracted according to the method of Hara and Radin (1978). The lipid extract was evaporated under nitrogen, redissolved in 50 μL of chloroform, and chromatographed on silica gel plates using hexane/diethyl ether/formic acid (50:50:1) as the mobile phase. After chromatography, radioactive bands were located by using a Betascope Instant Imager (Packard), scrapped off from the plate, and quantified by liquid scintillation counting.

In the experiments carried out with [U-^{14}C]linoleate the precursor was added at a concentration of 0.5 mM (50 kBq/μmol). Incubations were carried out in 2 mL screw-capped vials endowed with a Teflon-lined septum. After 30 min of incubation, 250 Bq of isoamyl [^{14}C]acetate (0.16 MBq/μmol) was injected in the vial as internal standard for quantification of radiolabeled volatile products of the reaction. The mixture was heated at 70 °C for 30 min. A sample of 1 mL was taken from the headspace, by using a syringe thermostated at 70 °C, and analyzed by radio-gas chromatography on a Carbowax 20M (Supelco) column. Radioactivity in volatile products (hexanal) was calculated by comparison with the internal standard (isoamyl acetate) with the corrections derived from the distribution of the compounds between the liquid and vapor phases under the conditions used. Such coefficients were experimentally determined and found to be 0.0133 and 0.0089 (vapor/liquid) for hexanal and isoamyl acetate, respectively (Salas, 1999).

Enzyme Assays. Lipoxygenase (LOX) activity was determined by using the end-point colorimetric method of Jiang et al. (1991). The assay mixture contained 50 mM sodium acetate, pH 5.0, 0.5 mM linoleic acid, and enzyme extract equivalent to 20 μg of protein in a volume of 0.5 mL. Incubations were carried out for 30 min.

Hydroperoxide lyase (HPL) activity was assayed by determining the hexanal produced from 13-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid (13-ZE-HPODA). The reaction mixture consisted of 50 mM MES, pH 6.0, 1 mM 13-ZE-HPODA, and enzyme extract equivalent to 40 μg of protein, in a volume of 1 mL contained in a screw-capped tube. After 30 min of incubation, 0.25 mL of 2% 2,4-dinitrophenylhydrazine in 5 M HCl was added, and the mixture was heated at 80 °C for 10 min to allow the formation of dinitrophenylhydrazones, which were then extracted three times with 0.5 mL of chloroform, concentrated by evaporation under nitrogen, and chromatographed on silica gel plates with hexane/diethyl ether/formic acid (50:50:1). The band corresponding to 2,4-dinitrophenylhydrazone of hexanal was scrapped from the plate and eluted with 1 mL of ethanol, and the absorbance at 327 nm of the resulting solution was measured. The molar extinction coefficient of the 2,4-dinitrophenylhydrazone of hexanal was determined to be 23000.

Preparation of Substrates and Standards. The 13-ZE-HPODA was prepared by enzymatic oxidation of linoleic acid with soybean lipoxygenase (Sigma) according to the method of Phillips and Galliard (1978). The 12-oxo-9(*Z*)-dodecenoic acid was prepared by degradative synthesis from vernolic acid (Olías et al., 1990). Isoamyl [^{14}C]acetate was prepared by reacting 1 μmol of isoamyl alcohol and 5 μmol of [^{14}C]acetic acid (0.16 MBq/μmol) in the presence of anhydrous sodium sulfate during 4 days at 80 °C in a screw-capped tube. Afterward, the remaining acetic acid was neutralized with ammonium hydroxide, and the labeled ester was extracted three times with 1 mL of hexane.

Protein Assay. Protein was determined according to a modification of the Folin−Lowry method (Dulley and Grieve, 1975).

RESULTS AND DISCUSSION

The effect of temperature on the formation of volatile aldehydes by extracts from olive pulp was investigated by measuring the overall activity of the lipoxygenase pathway. In this experimental approach, an acetone powder from olives (20 weeks after flowering) was used to prepare enzyme suspensions capable of catalyzing the formation of hexanal from linoleic acid, a process that involves the sequential action of LOX and HPL. Two radiolabeled substrates were used in these experiments. By using [1-^{14}C]linoleate it was possible to measure the formation of nonvolatile intermediates of the lipoxygenase pathway (fatty acid hydroperoxides and oxoacids), which retained the label in the carboxylic end. To measure the formation of volatile aldehydes, derived from the methylic end of the fatty acid molecule, uniformly labeled linoleic acid, [U-^{14}C]linoleate, was employed. In both cases, as well as in the enzyme assays described later, the incubations were carried out under conditions close to those used in the malaxation step of the industrial process of olive oil extraction: 30 min of incubation at acidic pH. These are also the standard conditions for small scale extraction of olive oil, carried out in the laboratory by using, for example, Abencor equipment (Martínez-Suárez et al., 1975).

When the enzyme suspension from acetone powders of olive pulp was incubated with [1-^{14}C]linoleate, two main labeled products were detected, fatty acid hydroperoxides and ω-oxoacids, as was expected from the action of LOX, which catalyzes the oxidation of linoleate, and HPL, which cleaves the hydroperoxide yielding an oxoacid, which carries the label, and a nonlabeled aldehyde. These two compounds accounted for at least 85% of the radioactivity associated with the metabolism of labeled linoleate. The effect of temperature on the formation of nonvolatile products of [1-^{14}C]linoleate was investigated in a range of 0−35 °C. The highest rates of transformation of the labeled precursor were measured at temperatures <25 °C (Figure 1) with a drastic decrease of activity at 30−35 °C, which are the temperatures commonly employed in the malaxation step

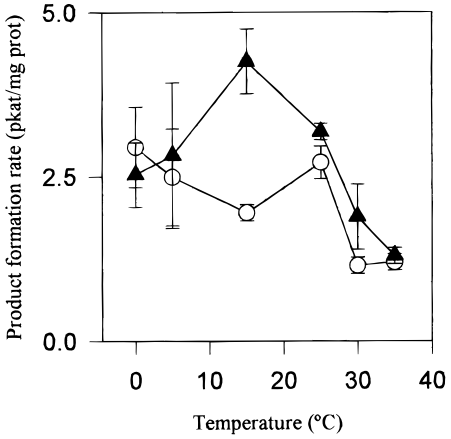

Figure 1. Effect of temperature on the metabolization of 1 mM [1-^{14}C]linoleate through the lipoxygenase pathway by an olive acetone powder prepared from fruits harvested 20 weeks after flowering: (▲) ω-oxoacids; (○) hydroperoxides. Results are means of three independent determinations (± standard deviation).

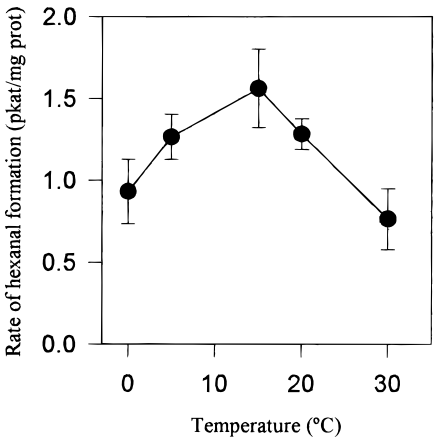

Figure 2. Effect of temperature on the formation of hexanal from 0.5 mM [U-^{14}C]linoleate. An acetone powder from olive fruits harvested 20 weeks after flowering was used. Every point is the mean of two independent determinations (± standard deviation).

in the olive oil industry (Alba, 1997). A closer inspection of the results in Figure 1 shows how the formation of ω-oxoacids, the product of the reaction catalyzed by HPL, peaked at 15 °C, whereas the formation of the intermediate fatty acid hydroperoxide was less affected by temperature.

Because ω-oxoacids and aldehydes are formed in stoichiometric amounts from fatty acid hydroperoxides, it was of interest to check how the temperature affects the rate of volatile aldehydes formation by the same enzyme system. In so doing, [U-^{14}C]linoleate was used as the labeled substrate in incubations carried out at temperatures ranging from 0 to 30 °C. As shown in Figure 2, hexanal was the only product detected in the headspace of the incubation vial, as was to be expected due to the absence in the incubation mixture of suitable reductant (NADPH) to carry out the reduction of hexanal to hexanol catalyzed by alcohol dehydrogenase (Salas and Sánchez, 1998b). These results show that the olive enzyme system is highly specific for the formation of six-carbon aldehydes, which fully agrees with the profile of volatile compounds in the aroma of olive oil (Morales et al., 1995; Ranalli and De Mattia, 1997) as well as with the positional and substrate specificities of olive LOX and HPL (Salas and Sánchez, 1998a,c). On

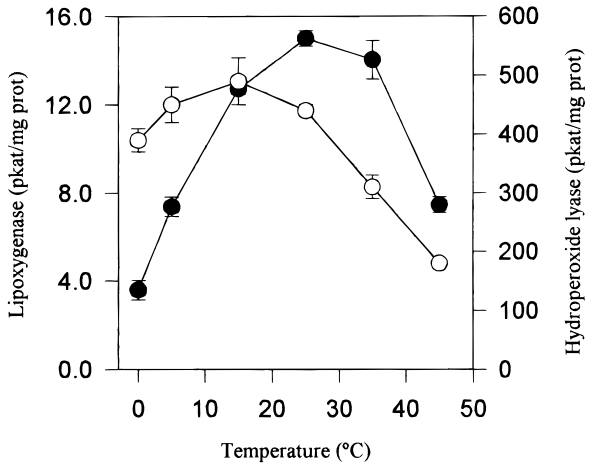

Figure 3. Temperature curve of the lipoxygenase (●) and hydroperoxide lyase (○) from olive fruit pulp prepared from fruits harvested 34 weeks after flowering. Every point is the mean of three independent determinations (± standard deviation).

the other hand, results in Figure 2 showed that formation of [^{14}C]hexanal had a maximum at 15 °C, in full agreement with the results obtained for the nonvolatile product of the HPL reaction, which confirmed the results in Figure 1.

The results of the labeling experiments described above show that the LOX/HPL enzyme system present in olive pulp specifically catalyzes the formation of hexanal from linoleic acid. The highest rates of hexanal formation were measured at temperatures below those typically used in olive oil extraction. To further characterize the behavior of the system, the effect of temperature on both enzymes was studied. In so doing, LOX and HPL activities were assayed in microsomes from ripening olives (34 weeks after flowering), because these subcellular fractions have been demonstrated to possess high levels of both activities (Salas and Sánchez, 1998a,c). As shown in Figure 3, the effects of temperature on these two enzyme activities were different. LOX, when assayed with linoleic acid as the substrate, displayed a rather broad optimum temperature around 25 °C and maintained a high activity at temperatures as high as 35 °C. HPL activity, on the other hand, peaked at 15 °C and showed a clear decrease at 35 °C in assays carried out using 13-hyroperoxylinoleic acid as the substrate.

These results fit well with those of Figures 1 and 2 and indicate that the reduction in the production of volatile aldehydes from linoleic acid through the lipoxygenase pathway by olive extracts can be ascribed to the unusual behavior of HPL against temperature. Moreover, these results explain, at the biochemical level, the observation that olive oils extracted at low malaxation temperature (20 °C) possess higher flavor than those obtained under standard conditions (J. Alba, personal communication).

ABBREVIATIONS USED

EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid; DTE, dithioerythritol; HPL, hydroperoxide lyase; 13-ZE-HPODA, 13-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid; LOX, lipoxygenase; MES, 2-(*N*-morpholino)ethanesulfonic acid; NADPH, reduced nicotinamide adenine dinucleotide phosphate.

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