JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Thermal Stability of Lipoxygenase and Hydroperoxide Lyase from Olive Fruit and Repercussion on Olive Oil Aroma Biosynthesis

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Thermal stabilities of main enzymes involved in the biosynthesis of virgin olive oil (VOO) aroma through the lipoxygenase (LOX) pathway were studied in crude enzymatic preparations. Kinetic parameters of thermal inactivation for LOX were determined graphically and were shown to be compatible with the presence of two LOX isoenzymes (LOX_{lab} and LOX_{res}) having different thermal stabilities and displaying relative activities of 88 and 12% each. Data on hydroperoxide lyase (HPL) suggest the existence of just one HPL isoform. Thermal stabilities of LOX and HPL enzymatic activities in crude preparations seem to explain the observed decrease of volatile contents in VOO aroma as a consequence of heat treatments of olive fruit. Moreover, differences in thermal stability of LOX_{lab} and LOX_{res} would justify the distinct pattern of reduction of C6 and C5 compound contents observed in the aroma of these oils.

KEYWORDS: Lipoxygenase; hydroperoxide lyase; thermal inactivation; olive oil; aroma

INTRODUCTION

In the scope of current search for treatments on olive fruits to modulate bitterness intensity in virgin olive oils (VOO), it was found that water−heat treatments prior to processing considerably reduced this attribute in VOO (*1*). Bitterness is a common and desirable attribute in these oils when present at low to moderate intensity, but it is rejected by consumers when present at high intensity. However, water−heat treatments also affect other quality traits such as color (*2*) and aroma (*3*) of the resulting oils.

Six straight-chain carbon (C6) aldehydes and alcohols and the corresponding esters are the most important compounds in the aroma of VOO, from either a qualitative or a quantitative point of view (*4*, *5*). We have previously established the participation of the lipoxygenase (LOX) pathway in the biosynthesis of C6 compounds of olive oil aroma (*6*), and Angerosa et al. (*7*) demonstrated the relevance of the five straight-chain carbon (C5) compounds also present in the aroma of olive oil. C5 compounds would be generated through an additional branch of the LOX pathway that would involve the production of a 13-alcoxyl radical by LOX in a homolytic way as demonstrated to occur in soybean seeds (*8*, *9*).

The LOX pathway is triggered during milling of olive fruit, the first step in the process to obtain VOO, and continues its activity during the malaxation step. The volatiles produced are incorporated into the oil to confer its characteristic aroma. Therefore, the aroma of VOO is determined by the activity level and properties of the enzymes involved in the LOX pathway, which in turn depend on the olive cultivar and ripening stage. Besides, malaxation conditions also affect the final aroma volatile profile, especially the time and temperature of the process (*10*).

Industrial hot-water treatments of olive fruits before processing seem to promote a partial deactivation of the LOX and hydroperoxide lyase (HPL) enzymes in the fruit while other enzymatic activities within the LOX pathway, such as alcohol dehydrogenase and alcohol acyltransferase, remain apparently unaffected. This promotes a reduction of C6 and C5 compounds and an unaltered level of esters in the oil aroma, which makes the oils attain a fruitier aroma (*3*). This phenomenon has been also described for tomato (*11*) and apple fruits (*12*). Experimental data suggest that the temperature of the olive fruit when entering the mill is the only factor responsible for this phenomenon and that increasing the fruit temperature might reduce the activity level of the LOX/HPL system during crushing (*13*). However, blanching of matured-green olive fruits prior to processing changes just slightly the aroma of the resulting oils (*13*); this observation is compatible with a reversible inactivation of the LOX/HPL system due to a protective effect by the cell environment.

Research is now aimed at achieving a better control of olive fruit temperature to modulate taste, aroma, and color of VOO to satisfy consumer demands. As far as we know, there is no data reported on the thermal stability of LOX and HPL proteins in olive fruit, which might be of interest for future improvements of VOO production. This paper studies the thermostability of these enzymatic activities in olive fruit and how it is related to

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10.1021/jf070326s CCC: $37.00
Published on Web 06/27/2007

the biosynthesis of VOO aroma. Because of the reversible thermal inactivation of the LOX/HPL system observed in whole fruits (*13*), an approach was therefore utilized by using enzymatic crude preparations from olive fruits for this study.

MATERIALS AND METHODS

Plant Material. Olive fruits (*Olea europaea* L.) cultivar Hojiblanca were harvested in CIFA Cabra-Priego orchards (Cabra, Córdoba, Spain) at green stage, maturity index 1.

Extraction and Measurement of LOX and HPL Activities. LOX extraction was carried out according to Luaces et al. (*2*) by grinding fruit pulp (10 g) in 4 volumes of 100 mM phosphate buffer, pH 6.7, containing 0.1% Triton X-100, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM benzamidine, 5 mM α-aminocaproic acid, and 2 g of PVPP. For HPL extraction, fruit pulp (5 g) was ground in 5 volumes of 50 mM HEPES buffer, pH 7.5, 20 mM KCl, 2 mM $MgCl_2$, 2 mM AEDT, 2 mM $Na_2S_2O_5$, 7 mM DTT, 0.1% ascorbate, and 0.5% Triton X-100. In both cases, grinding was carried out in two 1 min periods with an Ultraturrax homogenizer. The homogenates were filtered under vacuum through Miracloth and centrifuged for 20 min at 27000*g*. The supernatant was centrifuged again for 10 min at 10000*g* and used as crude extract.

LOX and HPL activities were determined spectrophotometrically at 25 °C according to Pérez et al. (*14*). For LOX activity, linolenic acid was used as the substrate and the increase in absorbance at 234 nm was monitored. For HPL activity, 13-hydroperoxide of linolenic acid was used as the substrate and the decrease in absorbance at 234 nm was determined. One unit of enzyme activity is defined as the amount of enzyme producing (LOX) or consuming (HPL) 1 μmol of hydroperoxy-linolenic acid per minute at 25 °C, taking into account a molar extinction coefficient of $2.5 \times 10^4\ M^{-1}\ cm^{-1}$ for this compound.

Thermal Inactivation of Crude Enzymatic Extracts. Thermal inactivation of olive LOX and HPL in the crude enzymatic extracts was studied by isothermal heating in a thermostated water bath (30−60 °C) for up to 60 min carried out in Eppendorf PCR tubes to ensure a high heat transmission rate. At selected heating times, aliquots of the crude extracts (200 μL) were cooled immediately in ice water to avoid a further thermal inactivation and enzymatic activities were measured. Experiments were carried out in duplicate.

Calculation of Kinetic Parameters. The possible kinetic behavior of enzymes during thermal inactivation could be explained through different math models. The first-order kinetic model is described by eq 1

$$A/A_0 = e^{-kt} \quad (1)$$

where A is the activity at time t, A_0 is the activity at initial time, and k is the first-order inactivation rate constant.

According to Ling and Lund (*15*), when more than one enzyme activity exists in a crude enzymatic preparation, each fraction could be inactivated independently following first-order kinetics. Thus, where two isoforms of the activity are proposed, the decrease in total activity vs time was assumed to be the sum of the inactivation of each of the individual isoforms and was described by the two-fraction first-order model or biexponential kinetic model (eq 2)

$$A/A_0 = \alpha e^{-k_1 t} + (1 - \alpha)e^{-k_2 t} \quad (2)$$

where k_1 and k_2 are the inactivation rate constants for the two isoforms and α corresponds to the initial molar fraction of the more thermolabile isoform.

To identify the different phases of thermal inactivation, the range of temperature (30−60 °C) covered the whole region of thermal inactivation from very slow to very fast and sufficient number of temperature values in this interval, so that the influence of different phases of thermal inactivation could be exhibited and consequently identified. The inactivation rate constants (k) were estimated from plots of Ln percent residual activity vs time at different temperatures. The D_T values were calculated from k according to their relation ($D_T = 2.303/k$). Activation energies (E_a) for thermal inactivation were calculated from the slopes of Arrhenius plots according to eq 3

$$\mathrm{Ln}(k) = -E_a/RT + c \quad (3)$$

where R is the gas constant and T is the absolute temperature.

Inactivation rate constants and activation energies for the different enzymatic activities in the crude extracts were estimated through linear regression analysis using the Microsoft Excel 2002 software program.

Heat Treatment of Olive Fruits and Oil Extraction. Fruits were distributed in 3.5 kg batches, dipped in a 90 L thermostatic water bath at different temperatures (30−60 °C) for 3 min, and immediately processed. This treatment time was observed to be enough for the whole fruit (pulp and kernel) to get the desired temperature (*2*). The olive oil extraction was performed maintaining the same operating conditions by using an Abencor analyzer (Comercial Abengoa, S.A., Seville, Spain) that simulates on a lab scale the industrial process of VOO production (*16*). Milling of olive fruits was performed using a stainless steel hammer mill operating at 3000 rpm provided with a 5 mm sieve. Resulting olive pastes were immediately conditioned to 30 °C before malaxation. The malaxation step was carried out always in the same experimental conditions by kneading the olive pastes in a mixer at 50 rpm for 30 min at 30 °C. Centrifugation of the kneaded olive pastes was performed in a basket centrifuge at 3500 rpm for 1 min. After centrifugation, oils were decanted and paper-filtered. Samples for volatile analysis (0,5 g) were taken in 10 mL sealed vials under nitrogen and stored at −18 °C until analysis. Treatments were carried out in triplicate.

Analysis of Volatile Compounds. Olive oil samples were conditioned to room temperature and then placed in a vial heater at 40 °C. After 10 min of equilibrium time, volatile compounds from headspace were adsorbed on a SPME fiber DVB/Carboxen/PDMS 50/30 μm (Supelco Co., Bellefonte, PA). The sampling time was 50 min at 40 °C and was carried out in duplicate. Desorption of volatile compounds trapped in the SPME fiber was done directly into the GC injector. Volatiles were identified and analyzed according to Perez et al. (*3*). Quantification was performed using individual calibration curves for each identified compound by adding known amounts of different compounds to redeodorize high oleic sunflower oil.

For comparison purposes among LOX isoform kinetics, volatiles were divided into two classes: C6 compounds, comprising aldehydes and alcohols of six straight-chain carbon volatiles coming from linoleic and linolenic acids, and C5 compounds, grouping together five straight-chain carbon alcohols and carbonyls, and pentene dimers from either linoleic or linolenic acids.

Chemicals and Reagents. Reagents for enzymatic activity extraction and measurements and reference compounds used for volatile identification were supplied by Sigma−Aldrich (St. Louis, MO) except for (*Z*)-hex-3-enyl acetate purchased from Givaudan Co. (Clifton, NJ) and (*Z*)-hex-3-enal generously supplied by S.A. Perlarom (Louvaine-La-Neuve, Belgium).

RESULTS AND DISCUSSION

The kinetics of thermal inactivation of LOX in the temperature range 30−60 °C and for incubations up to 30 min were measured. The enzymatic activity slowly decreased as the heating time increased at low heating temperatures, but a sharper decrease in LOX activity was observed in the first minutes at higher temperatures. Inactivation of enzymes is frequently assumed to obey a first-order kinetic model describing a simple conversion of native or active enzyme to denatured or inactive enzyme with a specific rate constant k (eq 1). **Figure 1a** shows the semilogarithm of residual activity of olive LOX as a function of heating time at different temperatures. The experimental data only fit to first-order kinetics at temperatures of 30−40 °C. Over 40 °C, the curve consisted of three parts: an initial straight line, an intermediate curved portion, and final straight line with a lower slope. This result might mean that the non-first-order behavior is mainly due to the presence in the crude preparation

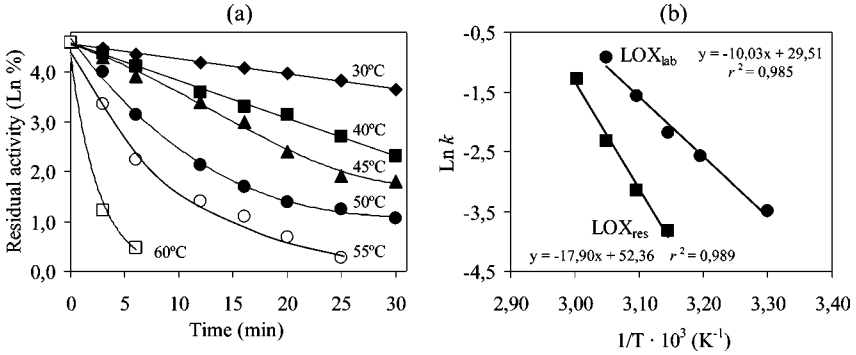

Figure 1. (**a**) Thermal inactivation curves of crude LOX preparations as a function of heating time and temperature and (**b**) Arrhenius plots of inactivation rate constants for LOX activity in crude preparations (data from **Table 1**).

Table 1. Thermal Inactivation Parameters for Olive Fruit LOX Isoforms

	$k \times 10^3$ (min^{-1})		D_T (min)		Q_{10} (45–55°C)		E_a (KJ mol^{-1})	
temp (°C)	LOX_{lab}	LOX_{res}	LOX_{lab}	LOX_{res}	LOX_{lab}	LOX_{res}	LOX_{lab}	LOX_{res}
30	30.33 ($r^2 = 0.995$)		75.93					
40	75.80 ($r^2 = 0.996$)		30.38					
45	111.92 ($r^2 = 0.985$)	21.73 ($r^2 = 0.999$)	20.58	106.00				
50	209.22 ($r^2 = 0.988$)	43.17 ($r^2 = 0.947$)	11.01	53.34	3.54	4.52	83.39	148.82
55	395.86 ($r^2 = 0.999$)	98.20 ($r^2 = 0.996$)	5.82	23.45				
60		277.50 ($r^2 = 0.999$)	3.91[a]	8.30				

[a] Calculated from the equation obtained representing log D_T vs temperature.

Table 2. Thermal Inactivation Parameters for Olive Fruit HPL

temp (°C)	$k \times 10^3$ (min^{-1})	D_T (min)	Q_{10} (45–55°C)	E_a (kJ mol^{-1})
40	4.51 ($r^2 = 0.944$)	511.10		
45	17.26 ($r^2 = 0.996$)	133.46		
50	48.29 ($r^2 = 0.999$)	47.69	6.18	177.53
55	106.73 ($r^2 = 0.999$)	21.58		
60	301.99 ($r^2 = 0.993$)	7.63		

of more than one LOX species with different heat resistances. In this case, the biexponential model better described the experimental inactivation curves and eq 2 fitted the data more closely at each temperature. The nature of these inactivation curves can be explained by assuming the presence of two LOX isoenzymes with different thermal resistance. The presence of LOX isoenzymes has been suggested in olive fruits based on the product specificity (*6*) but never proved due to the difficulties that the purification of this enzyme has in this fruit. Recently, Lorenzi et al. (*17*) reported the purification of an olive LOX isoform producing exclusively 13-hydroperoxides of linoleic and linolenic acids. Besides this isoform, a different one should be present in olive fruit to explain the 9-hydroperoxides found to be produced by olive LOX crude extracts (*6*, *18*).

Table 1 shows estimated thermal inactivation rate constants (k) for each LOX isoform at different temperatures calculated by linear regression of residual LOX activity as a function of time. The relative amounts of LOX activity, calculated in the enzymatic extract, were 88 and 12% for the thermolabile (LOX_{lab}) and thermoresistant (LOX_{res}) isoenzymes, respectively. The D_T values were found to be around five-fold higher for LOX_{res} than for LOX_{lab} at the different heating temperatures assayed. As shown by the inactivation constants and D_T values, both LOX isoforms are quite affected by heat, especially LOX_{lab}, which displays constants of inactivation 1−2 orders of magnitude higher than those reported for LOX from other sources such as potato (*19*), soybean (*20*), or green bean (*21*). However, LOX_{lab} showed inactivation rate constants quite similar to those of tomato LOX2 and higher than the tomato LOX1 isoform (*22*). On the other hand, olive LOX_{res} thermal inactivation properties were quite similar to those displayed by tomato LOX3.

Arrhenius plots of the rate constants for thermal inactivation of LOX (**Figure 1b**) were linear with slopes indicating energies for thermal inactivation (E_a) of 83.39 kJ mol^{-1} for LOX_{lab} and 148.82 kJ mol^{-1} for LOX_{res}. These inactivation energies are lower than those found for green beans (*23*) and potato LOX activities (*19*) but closer to the values found by Rodrigo et al. (*24*) for tomato LOX isoforms, although Anthon and Barrett (*22*) reported higher activation energies for the same LOX. The effect of temperature on reaction kinetics was also given by the Q_{10} value. Calculated Q_{10} values in the range 45−55 °C were 3.5 and 4.5 for LOX_{lab} and LOX_{res}, respectively.

The thermal stability of the second enzyme involved in the biosynthesis of VOO aroma, HPL, was also investigated in olive fruit crude extracts at temperature ranges of 40−60 °C. **Figure 2a** shows semilogarithm plots of HPL enzyme residual activity at different temperatures as a function of time. For all of the temperatures assayed, the graphs were linear. These data would indicate that olive HPL is heat-inactivated in accordance with first-order kinetics and that the simple exponential equation from the first-order model (eq 1) is able to adequately fit the kinetic data at all temperatures with a correlation coefficient (r^2) greater than 0.944. Thus, the crude enzymatic extract from olive fruit responded as if it contained only one HPL enzyme. In this sense, Salas and Sanchez (*25*) reported the presence of two HPL isoforms in olive pulp that displayed quite similar properties in terms of optimum pH and substrate specificity and could, as suggested by the data, also have broadly similar thermal inactivation characteristics.

Table 2 shows thermal inactivation parameters calculated for olive HPL at different temperatures. As shown by the thermal inactivation constants and D_T values, HPL displayed a thermal stability comparable to that of olive LOX_{res} and higher than the thermal stability shown by LOX_{lab}. No other thermal

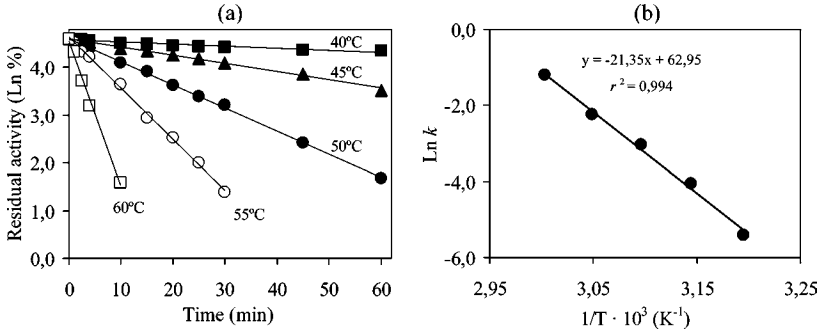

Figure 2. (**a**) Thermal inactivation curves of crude HPL preparations as a function of heating time and temperature and (**b**) Arrhenius plot of inactivation rate constants for HPL activity in crude preparations (data from **Table 2**).

Table 3. Contents of C6 and C5 Compounds (ng/g Oil) in Olive Oils from Hojiblanca Fruits Heat-Treated at Different Temperatures

volatile compound	30 °C	40 °C	50 °C	60 °C
(*E*)-hex-3-enal	293.4 ± 11.6	308.3 ± 25.7	201.3 ± 16.8	114.0 ± 15.1
(*Z*)-hex-3-enal	5112.1 ± 244.1	3757.9 ± 210.9	2577.6 ± 142.6	1408.6 ± 92.8
(*Z*)-hex-2-enal	585.6 ± 20.2	464.2 ± 8.6	296.5 ± 5.5	206.0 ± 11.5
(*E*)-hex-2-enal	4888.5 ± 238.7	3200.5 ± 128.7	2151.7 ± 85.2	1182.4 ± 7.1
(*E*)-hex-3-enol	2.0 ± 0.2	1.0 ± 0.0	0.6 ± 0.0	1.0 ± 0.0
(*Z*)-hex-3-enol	178.9 ± 4.1	68.5 ± 0.6	46.5 ± 0.4	33.2 ± 0.3
(*E*)-hex-2-enol	4.6 ± 0.2	8.9 ± 1.2	5.6 ± 0.7	8.6 ± 0.3
hexanal	721.6 ± 25.7	667.9 ± 1.4	454.3 ± 1.0	590.3 ± 1.4
hexan-1-ol	26.8 ± 1.4	21.5 ± 0.8	15.1 ± 0.6	20.0 ± 0.2
Σ C6 compounds	**11813.4 ± 546.3**	**8498.8 ± 377.8**	**5749.1 ± 252.8**	**3564.2 ± 128.7**
pent-1-en-3-one	436.6 ± 5.2	398.3 ± 21.7	170.3 ± 9.3	55.9 ± 0.4
(*Z*)-pent-2-enal	68.2 ± 3.0	53.9 ± 2.2	23.9 ± 1.0	6.7 ± 0.4
(*E*)-pent-2-enal	71.6 ± 6.0	121.0 ± 2.9	52.3 ± 1.2	70.1 ± 1.6
pent-1-en-3-ol	360.1 ± 11.4	563.3 ± 10.1	241.6 ± 4.3	144.3 ± 2.5
(*E*)-pent-2-en-1-ol	24.4 ± 0.7	38.4 ± 0.4	15.8 ± 0.2	8.5 ± 0.4
(*Z*)-pent-2-en-1-ol	275.8 ± 9.8	323.3 ± 3.2	133.0 ± 1.3	76.5 ± 0.8
pentene dimers	4681.0 ± 146.8	1653.0 ± 28.7	720.5 ± 24.4	169.8 ± 8.1
Σ C5 compounds	**5917.8 ± 182.8**	**3151.2 ± 69.1**	**1357.4 ± 41.7**	**531.6 ± 14.2**

inactivation data have been reported for HPL in olive or other plant product except for tomato (*22*, *24*). Thermal inactivation of tomato HPL displays two phases, a rapid phase resulting in the loss of about 50% activity at temperatures below 50 °C, and a second phase responsible for the inactivation of the rest, suggesting the presence of more than one HPL isoform with different thermal stabilities.

The thermal dependence of the inactivation rate constants was expressed by the Q_{10} and E_a values (**Table 2**). The HPL activity has a Q_{10} value of 6.18 in the temperature range 45−55 °C, higher than those shown by LOX isoforms in the same temperature interval. This greater effect of temperature on inactivation rate constants for HPL seems to agree with the reduction of volatiles occurring during the malaxation process to obtain VOO and which is ascribed to an unusual behavior of HPL against temperature (*26*). E_a was calculated from the slopes of the regression lines in **Figure 2b**. An Arrhenius plot was constructed, and E_a was found to be 177.53 kJ mol^{-1} (**Table 2**). This value is quite close to the only one reported, so far, for this enzymatic activity in a plant product, 197 kJ mol^{-1} for tomato HPL (*22*).

Table 3 shows the differences found in the contents of C6 and C5 compounds in the aroma of VOO obtained from fruits heated at different temperatures. As expected, increasing fruit temperature gave rise to a decrease of total volatiles in VOO aroma as already observed in different olive cultivars (*3*, *13*). This decrease of volatile contents followed a different pattern depending on the class of volatile compound considered. Contents of C6 compounds decreased almost linearly by increasing heating temperature while contents of C5 compounds displayed a higher decrease rate, especially at low temperatures, and fit better to a second-order equation. Thus, the ratio C6/C5 compound went from a value of 2 at 30 °C to 6.7 at 60 °C. These data are compatible with the suggested presence of two LOX isoforms in the crude preparations that are involved in the biosynthesis of VOO aroma during processing of olive fruits: LOX_{lab}, responsible for the production of C6 and C5 compounds, and LOX_{res}, producing mainly, or exclusively, C6 compounds.

The D_T value at 60 °C for LOX_{res} was 8.30 min, and the calculated D_T value for LOX_{lab} was found to be 3.91 min (**Table 1**). These data might explain the C6/C5 compound ratio found at this temperature as compared to the ratio found at lower heating temperatures. From the above D_T values, the theoretical LOX residual activity in the crude preparation at 60 °C was calculated to be around 20%. This level of LOX activity might explain the amount of total volatiles (23%) found in the oil aroma from olive fruits treated at 60 °C with respect to volatile contents quantified in oils after fruit heating at 30 °C (**Table 3**). The similarities between the remaining LOX activity in the fruit and the content of volatiles in the oil aroma might suggest that the thermal stabilities of olive LOX proteins seem to be quite similar in the whole fruit and in crude preparations. In this sense, literature shows that this is not uncommon among plant products; even in the same product, LOX isoforms can coexist with the same thermal characteristics in intact products and crude preparations and isoforms that differ completely as the case of pea seed LOX (*27*). On the other hand, the D_T value at 60 °C for HPL was found to be 7.63 min (**Table 2**), indicating that HPL is not probably the limiting factor and that there is still enough activity in the fruit after 3 min of heating (calculated 40% residual activity) to metabolize the hydroperoxides pro-

duced by LOX. Similarly, it is possible that olive HPL activity shows quite the same thermal stability in crude extracts and whole fruit.

In conclusion, experimental data on thermal stabilities of main enzymes involved in the biosynthesis of VOO aroma are compatible with the existence in olive fruit crude preparations of two LOX isoforms (LOX_{lab} and LOX_{res}) having different thermal stabilities and one HPL species or more than one with similar thermal properties. Both LOX_{res} and HPL seem to display quite similar thermal stabilities, and they are higher than that shown by LOX_{lab}. These thermal characteristics of LOX and HPL enzymatic activities in crude preparations seem to explain the observed decrease of volatile contents in VOO aroma as a consequence of heat treatments of olive fruit. Moreover, differences in thermal stability of LOX_{lab} and LOX_{res} might justify the distinct pattern of reduction in the contents of C6 and C5 compounds. Data indicate that LOX_{lab} activity in olive fruits might be the origin of C6 and C5 compounds, while LOX_{res} activity would give rise mainly, or exclusively, to C6 compounds.

ACKNOWLEDGMENT

We are grateful to Brígida Giménez (CIFA Cabra-Priego) for providing the fruits and to Mar Pascual for her excellent technical assistance.

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Received for review February 5, 2007. Revised manuscript received May 14, 2007. Accepted May 22, 2007. This work was supported by research projects AGL2002-02307 and AGL2005-03959 from Programa Nacional de Recursos y Tecnologías Alimentarias funded by the Spanish Government.

JF070326S