Interaction between Chloroplast Pigments and Lipoxygenase Enzymatic Extract of Olives

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Lipoxygenase activity in olive fruits increases considerably when the enzyme is extracted using extraction buffer with ethylenediaminetetraacetate, Triton X-100, dithiothreitol, sodium metabisulfite, and hydrated polyvinylpolypyrrolidone. In the absence of these compounds the activity measured of the crude extract is almost negligible, and further effects of the enzymatic reaction, such as bleaching activity on chloroplast pigments, do not appear. Moreover, when the oxidizing level of the medium is increased by the addition of linoleic acid or soybean lipoxygenase with linoleic acid, the crude enzymatic extracts of olives reduce the destructive capacity of soybean lipoxygenase on chlorophylls to one-sixth. These results suggest a double pigment protection against lipoxygenase in the olive crude extract, one inhibiting the enzyme and the other interrupting the chain of oxidative reactions beginning with hydroperoxide formation.

Keywords: *Lipoxygenase; pigments; chlorophylls; carotenoids; bleaching*

INTRODUCTION

The complexity in the composition of the olive fruit has been one of the greatest obstacles to elucidating the transformations undergone by the various components of the fruit during processing (Mínguez-Mosquera et al., 1989). The problems raised are very difficult and sometimes require mutually exclusive solutions. If the lipid content is high, the chlorophyll components cannot be separated, making their individual qualitative and quantitative monitoring impossible. Removal of the lipid fraction by classical methods to enable access to the chlorophyll pigments destroys them. It is possible to escape from this circle only by using specific techniques and methods such as those developed by Mínguez-Mosquera and Garrido-Fernández (1989) for chloroplastic pigments analysis.

The study of the action of the enzyme lipoxygenase in olive probably raises similar problems. Numerous studies assure that the enzyme is present. The increased formation of linoleic acid hydroperoxide when crude enzymatic extract of olive is added and the detection of products such as hexanal or hexenal support the hypothesis that lipoxygenase is present in the olive (Mínguez-Mosquera et al., 1990; Olías et al., 1993). However, the study of this enzyme's activity and the consequences of its action in the olive fruit raise numerous doubts because of the possible presence of interfering agents.

Lipoxygenase catalyzes the hydroperoxidation reaction of fatty acids and some other structures having the *cis,cis*-1,4-pentadiene structure (Axelrod, 1974), and the reaction can be readily monitored by spectrophotometric technique, detecting the increase in absorbance at 234 nm due to the formation of hydroperoxide (Surrey,

1964). A cascade of reactions culminating in the formation of aldehydes having six carbon atoms (Galliard and Matthew, 1977) follows the formation of the hydroperoxide. Each intermediate compound in the reaction sequence has a specific physiological function but also the possibility of oxidizing nonspecifically any compound able to act as antioxidant (Hildebrand, 1989; Farag et al., 1989). Compounds such as chlorophylls and carotenoids, from which electrons can be readily stripped (Terao, 1989), are very quickly affected by the presence of oxidants in the medium and are converted into passive receptors of the oxidizing potential set off by the action of lipoxygenase (Cohen et al., 1985; Hildebrand and Hymowitz, 1982).

From the technological point of view, the enzyme lipoxygenase is worthy of special attention because of its possible repercussion on the final color of vegetable foodstuffs. A medium in which hydroperoxide is formed enzymatically causes the destruction of the chlorophyll and carotenoid pigments present. Strictly, lipoxygenase does not act directly on the chloroplast pigments; nevertheless, the indirect effects of its activity are marked enough to be able to speak of a pigment-bleaching capacity (Stone and Kinsella, 1989). This enzymatic co-oxidation of pigments can be used as a system for indirect measurement of lipoxygenase activity, the rates of pigment destruction correlating with the amount of enzyme (Ikediobi and Snyder, 1977).

Various circumstances suggest that lipoxygenase activity may determine the quality of the main product obtained from the olive fruit by technological processing, olive oil. Olive oil quality depends on the extraction procedure used. The highest quality is given to oils obtained exclusively by physical methods (trituration, pressure, centrifugation, decanting, etc.): these are denominated virgin oils. The oil is separated from solid matter and the aqueous fraction of the fruit, taking with it a large amount of the lipophilic components. Virgin

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oils are subclassed into qualities depending on various parameters, which in general depend on the nature and degree of ripeness of the fruit used (Alba, 1997).

The increase of the proportion of free unsaturated fatty acids with ripening (Uceda and Hermoso, 1997) creates adequate conditions for lipoxygenase action. At the same time, during ripening and fruit trituration, when all of the fruit components are in contact, pigment loss can be between 60 and 80% in certain varieties with a high content of pigments (Mínguez-Mosquera and Gallardo-Guerrero, 1995). The presence of lipoxygenase in the fruit (Mínguez-Mosquera et al., 1990) and progressive increases in the amount of its substrate during ripening could be related with the decrease in the content of chloroplast pigments (Gandul-Rojas and Mínguez-Mosquera, 1996).

Thus, lipoxygenase could be a factor in oil quality through its effect on color. During olive storage before processing, and during trituration and milling, all of the components of the fruit are in contact. Lipoxygenase is present and with enough substrate to start the enzymatic reaction. However, the simple fact that the enzyme is present and may act does not necessarily make it responsible for pigment destruction. Further consequences of lipoxygenase activity, such as pigment bleaching, would take place only if the enzymatically formed oxidizing species came into contact with the pigments. It might be that olive fruits include compounds that sequester the enzymatically formed products. In such case, even though lipoxygenase is present in the fruit, and acting as postulated in the literature, there will be no destruction of pigments. The present work tests whether a property theoretically attributable to activity of the enzyme lipoxygenase is actually seen in the case of the olive.

MATERIALS AND METHODS

Enzymatic Extract of Olive for Measurement of Lipoxygenase Activity. The system used was that described by Olías et al. (1993). Fresh fruit (10 g) or lyophilized fruit (5 g) is homogenized in an Ultra-Turrax T-25 (Janke & Kunkel, IKA Labortechnik) at 10000 rpm for 2 min with 50 mL of 0.05 M sodium phosphate buffer, pH 6.8, containing 0.2 mM ethylenediaminetetraacetate (EDTA), 0.2% Triton X-100, 0.3 mM dithiothreitol (DTT), 10 mM sodium meta-bisulfite, and 20% hydrated polyvinylpolypyrrolidone (PVPP). The homogenized fruit is vacuum-filtered through S&S Rundfilter 589[1] Schwarzband paper and centrifuged at 11000*g* for 10 min. The intermediate aqueous phase is the enzymatic extract. All operations are carried out at 4° C.

Lipoxygenase Standard. A solution of soybean lipoxygenase type 1 (Fluka 62340) is prepared at a concentration of 0.04 mg/mL in 50 mM phosphate buffer, pH 8.5, and kept at 4° C until used.

Substrate of the Enzymatic Reaction. This is prepared following the method of Surrey (1964). To 0.5 g of Tween 20 is added 0.5 g of linoleic acid and deionized deoxygenated water to a final volume of 25 mL. A solution of 2 M NaOH is added dropwise to remove cloudiness. The solution is kept in 5 mL vials under a nitrogen atmosphere at −35 °C until used.

Measurement of Enzymatic Activity. The enzymatic activity is determined at room temperature according to a spectrophotometric method, using a 3 mL cuvette and measuring the increase in absorbance of the reaction medium at 234 nm (Surrey, 1964). The reaction medium is 0.2 M phosphate buffer adjusted to pH of 6.1, 7, 8, and 8.5. The reaction is started by adding to the medium 20 μL of substrate and enough enzymatic extract to give a measurable increase in absorbance within 1 min.

Expression of Enzymatic Activity. The amount of hydroperoxide formed is quantified from its molar coefficient of extinction at 234 nm ($\epsilon = 25000$ M^{-1} cm^{-1}), and the enzymatic activity *A* (kat) is measured as moles of product formed per second. For comparative purposes, the results are expressed as specific activity A_S (kat/mg), relating katals to milligrams of dry weight of the biological material used.

Crude Extract of Olive for Pigment Bleaching. Fresh, pitted fruit of the variety Arbequina is used. This is homogenized in an Ultra-Turrax T-25 at 10000 rpm for 2 min with 0.05 M sodium phosphate buffer, pH 6.8, with a fruit/buffer ratio of 1:5. The homogenized fruit is centrifuged at 11000*g* for 10 min. The intermediate aqueous layer is used as enzymatic extract. All operations are carried out at 4° C.

Obtaining the Pigment Concentrate. Fresh leaves of spinach (20 g) are homogenized in an Ultra-Turrax T-25 with 100 mL of acetone. The triturate is vacuum-filtered through S&S Rundfilter 589[1] Schwarzband paper, and the pellet is extracted until the filtrate is colorless. The extracts are combined in a decanting flask with 100 mL of ethyl ether. The solution is homogenized, and 200 mL of 10% aqueous NaCl solution are added. The colorless hypophase is discarded and the ether layer washed repeatedly with NaCl solution. The epiphase is filtered through a bed of anhydrous sodium sulfate and placed in a rotary evaporator flask containing 1 g of Tween 20. The solvent is evaporated until the flask contains only Tween 20 and the pigments. The pigment concentrate is eluted from the flask with small additions of deionized water to a final volume of 100 mL, so obtaining a stock solution of pigments in water, with theoretical absorbances of 100 UA at 418 nm and 50 UA at 670 nm. All of the operations were carried out at room temperature and under soft green light.

System of Reactions To Study Bleaching Activity of Enzymatic Extract of Olive. Twelve reactions are prepared combining the presence or absence of substrate, enzymatic extract of olive, soybean lipoxygenase standard, and two pH values of reaction. Each reaction is prepared in duplicate, dissolving 0.5 mL of the stock solution of pigments in 10 mL measuring flasks to which are added the specific reagents (Table 1) defining each reaction. The solutions are taken to a final reaction volume of 10 mL using 0.2 M phosphate buffer adjusted to the pH fixed for each reaction.

The reactions studied are grouped in four blocks depending on the enzymatic extract used and the pH at which the reaction takes place. The first reaction battery studies bleaching by olive lipoxygenase, at the reaction medium of pH 7. The second battery is similar to the first, but the reaction takes place at pH 8. The third battery studies the bleaching activity of the enzymatic extract of soybean lipoxygenase at pH 8. The last battery studies the joint bleaching activity of the extract of soybean lipoxygenase and the enzymatic extract of olive at pH 8. Each battery comprises four reactions, of which one is the enzymatic reaction—the reaction includes enzymatic extract and substrate besides the pigment extract—and the rest are control reactions: a control allowing measurement of the self-destruction of pigments; a control of destruction induced by the substrate of the enzymatic reaction; and a control of destruction caused by the enzymatic extract in the absence of substrate. This system of reactions and controls reveals what proportion of the bleaching observed is attributable to each reagent taking part in the reaction.

Monitoring the Bleaching Reaction. The 24 flasks containing the 12 different reactions were kept in darkness in a thermostated chamber at 20 °C. During the 100 h established for the study of the reactions, the flasks were removed every 8−10 h to measure in duplicate the absorbances at 670 (specific to the chlorophyll fraction) and 460 nm (common to chlorophylls and carotenoids) (Holden, 1976).

The destruction of chlorophyll pigments in the various reactions assayed was determined by measuring the decrease in absorbance at 670 nm of the sample with time (Abs_i^{670}), expressing the results as percentage of retention in each determination with respect to the initial pigment content (Abs_0^{670}) (eq 1).

Table 1. Volumes of Reagents Used and Conditions of Each Reaction Considered in the Study of Interaction between Chloroplast Pigments and Enzymatic Extract of Olive

reaction code[a]	volume of reagents used (mL)				reaction conditions	
	solution of pigments	solution of linoleic acid	crude extract of olive	solution of soybean lipoxygenase	pH of reaction	final vol
Enzymatic Extract of Olive at pH 7						
7P	0.1	0	0	0	7	10
7PL	0.1	0.06	0	0	7	10
7PE	0.1	0	0.06	0	7	10
7PLE	0.1	0.06	0.06	0	7	10
Enzymatic Extract of Olive at pH 8						
8P	0.1	0	0	0	8	10
8PL	0.1	0.06	0	0	8	10
8PE	0.1	0	0.06	0	8	10
8PLE	0.1	0.06	0.06	0	8	10
Soybean Lipoxygenase at pH 8						
8P	0.1	0	0	0	8	10
8PL	0.1	0.06	0	0	8	10
8PS	0.1	0	0.06	0.48	8	10
8PLS	0.1	0.06	0.06	0.48	8	10
Soybean Lipoxygenase plus Enzymatic Extract of Olive at pH 8						
8P	0.1	0	0	0	8	10
8PL	0.1	0.06	0	0	8	10
8PS	0.1	0	0	0.48	8	10
8PE	0.1	0	0.06	0	8	10
8PES	0.1	0	0.06	0.48	8	10
8PESL	0.1	0.06	0.06	0.48	8	10

[a] Nomenclature of codes: P, pigments; L, linoleic acid; E, enzymatic extract of olive; S, soybean lipoxygenase.

The destruction of carotenoid pigments was determined by measuring the decrease in absorbance at 460 nm (Abs_i^{460}), with respect to the initial pigment content (Abs_0^{460}). Because the latter is a wavelength common to both carotenoids and chlorophylls, the absorbance due to the latter compounds must be subtracted.

The absorbance at 460 nm due to the chlorophyll compounds in this specific sample is calculated from the constancy in the ratio between the absorbance of the chlorophyll compounds at 460 and 670 nm. This ratio has been calculated by taking eight aliquots of 0.5 mL from the pigment stock solution. Four of them were saponified and redissolved up to 10 mL using 0.2 M phosphate buffer, pH 8, with 0.05 g of Tween 20 to obtain the carotenoid extract free of chlorophyll compounds (Mínguez-Mosquera and Garrido-Fernández, 1989), and the absorbance at 460 nm of the extracts were measured. The other four aliquots containing all of the pigments were dissolved directly in buffer to a volume of 10 mL, and their absorbance at 460 nm was measured. Subtracting the absorbance of the carotenoid extract from the absorbance of the pigment extract at that wavelength, one obtains the absorbance due to the chlorophyll pigments at 460 nm. The ratio between the absorbance of the chlorophyll pigments at 460 and 670 nm (Abs_{460}/Abs_{670}) in buffer was established as 0.52 ± 0.012 for the specific chlorophyll pigment composition of this sample. Equation 2 enables the empirical calculation of the overall carotenoid content, which is deduced simply from the ratio of absorbance of total chlorophyll pigments at 670 and 460 nm.

$$\text{\% of retention of chlorophylls} = \frac{Abs_i^{670}}{Abs_0^{670}} \times 100 \quad (1)$$

% of retention of carotenoids =
$$\frac{Abs_i^{460} - (0.52 \times Abs_i^{670})}{Abs_0^{460} - (0.52 \times Abs_o^{670})} \times 100 \quad (2)$$

Kinetic Calculations. The calculations of degradation reaction rate (K_v) are based on the percentage of color retained against time in hours (t). The mathematical fit is made by assuming that the reaction is zero- or first-order. The integrated equations of rate expressed as percentage of retention in function of time are shown as eqs 3 and 4.

reaction order 0: $\text{\% of retention} = 100 - k_v t$ (3)

reaction order 1: $\text{\% of retention} = 100/e^{k_v t}$ (4)

RESULTS

Lipoxygenase Activity. The measured activity of the enzymatic extract of olive containing all of the coadjutants at pH 6.1 is ~6.8 nkat/mg (Table 2), perfectly fulfilling all of the requirements for a correct enzymatic determination. At this pH, the activity of soybean lipoxygenase is much higher, ~90 nkat/mg. In principle, this suggests that lipoxygenase activity in olive is low and that all of the consequences attributable to the reaction initially catalyzed by lipoxygenase, such as pigment bleaching, will be minor.

The extraction system used in the measurement of olive lipoxygenase activity involves numerous compounds to enable the measurement, none of which is present naturally. When cell and organ compartmentation is lost, the fruit components come into contact. This allows lipoxygenase to act on its specific substrate, setting off pigment destruction without the involvement of external components. Such conditions, free of activity promoters, are ideal for the study of chloroplast pigments bleaching by olive lipoxygenase. They simulate what could be found in an olive paste obtained by trituration, although the experiment will have certain limitations: a single olive variety; uniform samples regarding degree of ripeness; and treatment under conditions less drastic than those of industrial processing.

When all of the additives are not present in the extraction medium, activity falls to levels of 0.02 nkat/mg. This low activity could suggest that there is little pigment destruction capacity, although the oxidizing potential for a lesser or greater effect on the chloroplast pigments is not known.

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Table 2. Specific Activity of Soybean Lipoxygenase Solution, Olive Lipoxygenase Extract, and Crude Enzymatic Extract of Olives (Ripe Var. Arbequina) at Various pH Values of Reaction[a]

pH	olive lipoxy- genase extract[b]	crude enzymatic extract of olives[c]	solution of soybean lipoxygenase
6.1	6.83 ± 0.03	0.028 ± 0.001	91.4 ± 0.46
7		0.190 ± 0.013	146.7 ± 0.68
8		0.738 ± 0.045	197.5 ± 1.02
8.5		0.072 ± 0.002	204.3 ± 0.98

[a] In nkat/mg of dry wt. Mean of eight determinations ± confidence interval 95%. [b] Extraction buffer: 0.0.5 M sodium phosphate, pH 6.8, containing 0.2 mM ethylenediaminetetraacetate (EDTA), 0.2% Triton X-100, 0.3 mM dithiothreitol (DTT), 10 mM sodium meta-bisulfite, and 20% hydrated polyvinylpolypyrrolidone (PVPP). [c] Extraction buffer: 0.05 M sodium phosphate, pH 6.8.

The reaction conditions for pigment bleaching by the crude enzymatic extract of olive and soybean lipoxygenase were established from the profiles of enzymatic activity against pH of the reaction medium (Table 2). It can be seen that, in contrast to what is postulated in the literature, maximum olive lipoxygenase activity in the crude extract is found in the zone of pH 7−8. Rather than as a contradiction of the bibliographic data, this should be understood as the comparison of two different aspects. Lipoxygenase in the crude extract is in contact with all of the inhibitors and modulators present in the fruit. At each pH, the measured activity is the result of the actual activity of the enzyme at that pH less the decrease in activity caused at that pH by the presence of inhibitors. The two variables do not have to change in parallel, and the maximum activity will be at the pH at which the difference between intrinsic enzymatic activity and inhibitory capacity is greatest. Logically, this does not have to coincide with the pH of maximum activity of the enzyme. The study is thus restricted to pH 7−8, at which the enzymatic extract of olive seems to show greatest lipoxygenase activity, and can be assumed to have the greatest destructive effect on pigments. Moreover, this zone is one of high activity for the solution of soybean lipoxygenase.

Degradation Kinetics of Chloroplast Pigments in the Presence of Enzymatic Extract of Olive and Soybean Lipoxygenase. Tables 3 and 4 detail, respectively, the kinetic parameters found for the destruction of chlorophylls and carotenoids in each reaction studied, calculated from the two kinetic models considered.

The first noteworthy result is that there are theoretical reactions of destruction of chlorophyll and carotenoid pigments with very low correlation coefficients, for both zero- and first-order kinetic models. The cause of the low correlation is strictly mathematical: there is no correlation because there is no dependence between variables during the time of reaction studied, as shown by the slopes. In the chlorophyll bleaching reaction at pH 8 in the absence of enzyme and linoleic, the correlation coefficient is 0.164. The rate constant of this reaction calculated for a theoretical zero-order reaction is 0.007, which means that in 100 h of reaction, 0.7% of chlorophyll pigments is lost. It can be said that the self-destruction of pigments during the 100 h of reaction is practically nil and that the concentration of chlorophyll pigments remains constant during the experiment.

When the same reaction is carried out with the introduction of soybean lipoxygenase and linoleic acid, the slope obtained is 0.652, with a correlation coefficient of 0.903. This implies that in 100 h, 65.2% of the initial

Table 3. Kinetic Constants for the Degradation of Chlorophyll Pigments in the Presence of Lipoxygenases of Olive and Soybean under Various Conditions of Reaction[a]

	kinetic model of order 0			kinetic model of order 1		
reaction code[b]	rate constant k_v (h^{-1})	ordinate at origin	R	rate constant k_v (h^{-1}) $\times$ 100	ordinate at origin	R
Enzymatic Extract of Olive at pH 7						
7P	0.011	96.1	0.245	0.011	4.56	0.238
7PL	0.093	98.3	0.911	0.102	4.58	0.922
7PE	0.133	99.8	0.870	0.156	4.67	0.871
7PLE	0.063	96.9	0.756	0.067	4.57	0.766
Enzymatic Extract of Olive at pH 8						
8P	0.007	97.9	0.164	0.000	4.58	0.160
8PL	0.017	93.6	0.277	0.017	4.53	0.267
8PE	0.013	96.8	0.374	0.013	4.57	0.371
8PLE	0.018	93.4	0.227	0.018	4.53	0.216
Soybean Lipoxygenase at pH 8						
8P	0.007	97.9	0.164	0.000	4.58	0.160
8PL	0.017	93.6	0.277	0.017	4.53	0.267
8PS	0.004	91.2	0.043	0.000	4.51	0.024
8PLS	0.652	100.0	0.903	1.476	4.60	0.983
Soybean Lipoxygenase plus Enzymatic Extract of Olive at pH 8						
8P	0.007	97.9	0.164	0.000	4.58	0.160
8PL	0.017	93.6	0.277	0.017	4.53	0.267
8PS	0.004	91.2	0.043	0.000	4.51	0.024
8PE	0.013	96.8	0.374	0.013	4.57	0.371
8PES	0.019	93.2	0.302	0.020	4.53	0.296
8PESL	0.092	89.1	0.731	0.110	4.48	0.763

[a] Mathematical fits from kinetic models of order 0 and 1. Calculation carried out using 10 determinations in duplicate in each one of the two flasks used for the study of each reaction. Reaction time = 100 h. Sampling time = 10 ± 2 h. [b] Nomenclature of codes: P, pigments; L, linoleic acid; E, enzymatic extract of olive; S, soybean lipoxygenase.

chlorophyll content has been destroyed. In this case there is an obvious dependence between reaction time and pigment destruction, and the destruction is attributable only to enzyme activity on its specific substrate, because the other possible cause (self-destruction of pigments) is completely ruled out for the reaction time studied.

The reactions with low correlation generally coincide with controls of the actual enzymatic reactions, which helps in the interpretation of the results, because they can be considered of zero slope. The precise establishment of the kinetic parameters of these slow reactions is outside the aim of this study.

From the kinetic results set out in Tables 3 and 4, two general conclusions can be drawn. First, and obvious from only the sign of the slopes, under all of the conditions assayed there is pigment loss, although sometimes in amounts that are practically zero. Second, all of the reactions of pigment degradation seem to fit better a first-order kinetic model, although the values of the correlation coefficients are not sufficiently different to enable a definitive order to be attributed.

Going on to the study of each of the reaction blocks, at pH 7 a low self-destruction of chlorophyll pigments is observed, which increases when linoleic acid is present in the medium and increases even more in the presence of the enzymatic extract of olive. However, when enzymatic extract and linoleic acid are present together, degradation is less than with either. With carotenoid pigments, the order of individual destructive capacity is reversed. Linoleic acid greatly increases the

Table 4. Kinetic Constants for the Degradation of Carotenoid Pigments in the Presence of Lipoxygenases of Olive and Soybean under Various Conditions of Reaction[a]

reaction code[b]	kinetic model of order 0			kinetic model of order 1		
	rate constant k_v (h^{-1})	ordinate at origin	R	rate constant k_v (h^{-1}) × 100	ordinate at origin	R
Enzymatic Extract of Olive at pH 7						
7P	0.033	103.8	0.684	0.033	4.64	0.688
7PL	0.301	97.1	0.985	0.417	4.58	0.995
7PE	0.078	103.6	0.905	0.071	4.64	0.899
7PLE	0.122	100.7	0.980	0.135	4.61	0.984
Enzymatic Extract of Olive at pH 8						
8P	0.217	104.5	0.977	0.252	4.65	0.982
8PL	0.217	100.4	0.973	0.262	4.61	0.984
8PE	0.102	103.4	0.941	0.109	4.64	0.946
8PLE	0.183	102.4	0.978	0.211	4.63	0.982
Soybean Lipoxygenase at pH 8						
8P	0.217	104.5	0.977	0.252	4.65	0.982
8PL	0.217	100.4	0.973	0.262	4.61	0.984
8PS	0.258	96.8	0.982	0.342	4.58	0.990
8PLS	0.760	100.0	0.783	1.538	4.60	0.896
Soybean Lipoxygenase plus Enzymatic Extract of Olive at pH 8						
8P	0.217	104.5	0.977	0.252	4.65	0.982
8PL	0.217	100.4	0.973	0.262	4.61	0.984
8PS	0.258	96.8	0.982	0.342	4.58	0.990
8PE	0.102	103.4	0.941	0.109	4.64	0.946
8PES	0.122	99.5	0.993	0.137	4.60	0.991
8PESL	0.382	86.9	0.956	0.701	4.48	0.987

[a] Mathematical fits from kinetic models of order 0 and 1. Calculation carried out using 10 determinations in duplicate in each one of the two flasks used for the study of each reaction. Reaction time = 100 h. Sampling time = 10 ± 2 h. [b] Nomenclature of codes: P, pigments; L, linoleic acid; E, enzymatic extract of olive; S, soybean lipoxygenase.

destruction of pigments, and the enzymatic extract does so only slightly. With both together, pigment destruction is intermediate.

The increased degradation of both chlorophyll and carotenoid pigments when linoleic acid is present in the reaction medium at pH 7 is normal and expected. The autoxidation of an unsaturated fatty acid creates an oxidizing environment resulting in pigment destruction. The fact that pigment degradation increases when enzymatic extract is introduced is also not strange. A great number of compounds may exist that act as pro-oxidants. Nevertheless, the effect seen when the enzymatic extract of olive is in the presence of linoleic acid is surprising. In such a situation, a ready reaction might be expected because of the action of lipoxygenase on its specific substrate. However, not only is there no potentiating effect but there is not even an additive effect on the individual oxidizing capacities. What is observed is a mutual cancellation of the pigment-destructive capacity shown by linoleic acid and the enzymatic extract of olive when these act separately.

At pH 8, the self-destruction of chlorophyll pigments is even less than at pH 7. With linoleic acid and enzymatic extract of olive in the medium, both individually and together, the destruction increases, though to levels that are still extremely low, varying from 0.7 to 1.8% for 100 h of reaction. Given the extremely low rate constants and the low correlation of the reactions, it is sensible to assume that none of the reactions has actually occurred. The case of the carotenoid pigments is different. Pigment self-destruction is very intense. With linoleic acid in the reaction medium, the degrada-

tion rate constant hardly alters; thus, self-destruction can be considered so high that the rate constant is not changed by the oxidizing contribution of the linoleic acid. However, this latent destruction of carotenoid pigments is reduced to almost half when the medium contains enzymatic extract of olive; the enzymatic extract has protected the carotenoid pigments from self-destruction. Under reaction conditions in which linoleic acid and enzymatic extract of olive are present, the destruction of carotenoid pigments is slightly greater than when there is only enzymatic extract. This is in principle logical, given that an additional pro-oxidant has been introduced into the medium.

In brief, at pH 8, the chlorophyll pigments are more stable under every circumstance and are practically unaffected because the medium also contains linoleic acid or enzymatic extract of olive. However, the carotenoids are highly unstable, but the presence of enzymatic extract of olive in the medium gives protection against self-destruction, even when the medium also contains linoleic acid.

As a summary of what takes place with the enzymatic extract of olive at pH 7 and 8, for both types of pigments, linoleic acid has been found to accelerate the reaction of pigment destruction. When enzymatic extract of olive is introduced, the contrary effect is found: pigment destruction induced by linoleic is checked.

With soybean lipoxygenase, the results found are completely different from those seen for the enzymatic extract of olive. With the individual presence of enzymatic extract, pigment self-destruction levels are similar to those found in its absence; however, when both linoleic acid and soybean lipoxygenase are present in the medium, chlorophyll destruction increases drastically. The increase in carotenoid destruction is also significant, although proportionally less. Soybean lipoxygenase acts as expected. In accord with the kinetic parameters found for zero-order reaction, the self-destruction of chlorophylls at pH 8 is 0.007; with linoleic acid in the medium this increases to 0.017, due to linoleic autoxidation, and with soybean lipoxygenase reaches 0.652. With soybean lipoxygenase, linoleic acid oxidation is not spontaneous but catalyzed, increasing at the same rate as the collateral destruction of chlorophyll pigments. The case of the carotenoid pigments is similar. There is pigment self-degradation, and with soybean lipoxygenase, the degree of destruction is similar or even somewhat greater. The individual presence of linoleic acid practically does not alter the destruction of pigments, but as enzyme and substrate are present together in the reaction medium, the rates of pigment destruction practically triplicate.

Finally, in the joint action of the enzymatic extract of olive and soybean lipoxygenase, it is observed that the two extracts together and in the absence of linoleic acid lead to a level of chlorophyll degradation that is practically the sum of those that each gives individually and, consequently, of very low degradative capacity. This does not happen in the case of carotenoids. Individually, the enzymatic extract of olive decreases carotenoid self-destruction at pH 8, whereas soybean lipoxygenase slightly increases the rates of self-destruction. With both extracts together, the protective effect of the enzymatic extract of olive prevails, although not so effectively as when it acts alone.

When linoleic acid is included in the reaction medium that contains both enzymatic extracts, the chlorophyll-

destructive activity practically disappears. This contrasts strongly with previous findings. In the experiment at pH 8, the destruction of chlorophyll pigments caused by the enzymatic extract of olive in the presence of linoleic acid is practically nil. At the same time, soybean lipoxygenase in the presence of linoleic acid very considerably increases the destruction of chlorophyll pigments. The expected result of the joint action of the two extracts is that the activity of soybean lipoxygenase prevails; nevertheless, all of the pigment-destructive capacity shown by this extract when it acts individually is annulled when the enzymatic extract of olive is present in the medium.

The enzymatic extract of olive not only does not take part in the destruction of chlorophyll pigments, it strongly protects them against the degradation induced by the extract of soybean lipoxygenase. This activity is also suggested by the results of the experiment at pH 7. The destruction induced by linoleic acid decreased if the medium included enzymatic extract of olive, but the level of protection was low: the rate constant of the reaction decreased to practically half. When the reaction that includes linoleic acid and soybean lipoxygenase is compared with the same reaction introducing the enzymatic extract of olive, the rate constant decreases to one-seventh.

With carotenoid pigments, the situation is similar, although the protective effect appears from the start. At pH 7, the enzymatic extract of olive reduced the destruction induced by linoleic acid; at pH 8, the effect was the same, but less marked. With soybean lipoxygenase and linoleic acid, high rates of pigment destruction were reached, but if the enzymatic extract of olive was included in this medium, the rate constant of the reaction of pigment destruction was reduced to half.

The set of results obtained enables the following hypothesis to be established. Linoleic acid increases pigment destruction. If the medium includes soybean lipoxygenase, the destruction is more intense. When enzymatic extract of olive is introduced, both the destruction induced by linoleic acid and that resulting from the enzymatic reaction of soybean lipoxygenase decrease. Thus, it seems that, rather than promoting pigment oxidation by its possible content in lipoxygenase, the enzymatic extract of olive is an efficient protector of pigments against oxidizing activity.

The protection given to pigments by the enzymatic extract of olive against the reaction catalyzed by endogenous and exogenous lipoxygenases can be explained by the joint, simultaneous action of two mechanisms. The first is purely enzymatic. The enzymatic extract of olive may include an inhibitor that acts directly on the enzyme, whether endogenous or exogenous, completely paralyzing its action; thus, it is not possible to create sufficient oxidizing capacity in the medium to destroy the pigments. This hypothesis could be in accord with the fact that to measure lipoxygenase activity using classical methods, it is necessary to include additives such as DTT, EDTA, bisulfite, PVPP, and Triton X-100 in the enzyme extraction medium.

The second possible mechanism of action of the enzymatic extract of olive is less specific. The oxidation of linoleic acid may be spontaneous or through enzymatic action. In both cases, the reaction is identical, although logically much faster in the presence of enzyme. Compounds present in the enzymatic extract of olive check the flow of reactive compounds toward the pigments, interrupting the sequence of reactions starting from linoleic peroxide and ending with pigment oxidation. This second mechanism would explain how the enzymatic extract of olive is able to reduce the pigment-destructive capacity of linoleic acid.

Although lipoxygenase is present in the olive fruit, its activity is strongly reduced when all of the components of the fruit are in contact. Two different interpretations can be used for these results. The decrease in the activity is real and some compounds present in the fruit inhibit the activity of the enzyme, or this decrease is false and the low activity measure is due to a quick consumption of the enzymatically formed hydroperoxide, not reflecting the measure the actual activity of the enzyme. The fact that the further effects of the reaction, the bleaching of the chloroplastic pigments, do not appear proves that the enzyme is not acting. The pigment protection is not just due to an enzymatic inhibition because the crude enzymatic extract of olives is able to protect even against a pro-oxidant introduced in the medium.

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