Effect of Pepper Lipoxygenase Activity and Its Linked Reactions on Pigments of the Pepper Fruit

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The products formed during the enzymatic reaction catalyzed by the lipoxygenase of pepper (variety Agridulce) have in vitro a strong destructive action on the carotenoid pigments of the fruit. When conditions and proportions of enzyme and pigments are similar to those found in the fruit, and at a reaction temperature of 20 °C, almost 30% of the pigments are destroyed after 24 h of reaction. Of this amount, 2.5% is due to autoxidation of pigments, 4.5% to oxidation induced by the presence of linoleic under saturating conditions, and the remaining 22% to the presence in the medium of reaction products of the lipoxygenase-catalyzed reaction. When the enzyme acts under substrate-saturating conditions, the rate of pigment destruction by lipoxygenase can be considered maximal at the experimental temperature. The fact that in vitro pepper lipoxygenase induces a heavy destruction of pigments and that, in vivo, its activity remains almost constant during over-ripening could explain why up to 40% of the pigment content in some varieties is lost during the postharvest period.

Keywords: *Lipoxygenase; carotenoids; pepper; bleaching*

INTRODUCTION

Lipoxygenase (linoleate:oxygen oxide reductase, EC 1.13.11.12), also known as "lipoxidase", is an enzyme widely distributed in plants (Axelrod, 1974; Grosch et al., 1976; Richardson, 1982). It is found mainly in fruits and seeds, as in soy (Holden, 1965), tomato (Bonnet and Crouzet, 1977), rice (Sekhar and Reddy, 1982), lupin (Olías and Valle, 1988), avocado (Marcus et al., 1988), and olive (Mínguez-Mosquera et al., 1990), and in a great variety of vegetables (Pinsky et al., 1971). It catalyzes the stereospecific oxygenation of polyunsaturated fatty acids and their esters and glycerides containing the *cis*,*cis*-1,4-pentadiene double-bond system, originating a hydroperoxide that possesses a chain with a cis,trans conjugated double bond.

From the food technology point of view, the potential effects of the products formed during the enzymatic reaction are much more interesting than the reaction itself. The action of lipoxygenase on its substrate generates highly reactive compounds that are initiators of a cascade reaction in which components playing no part in the enzymatic reaction may be affected secondarily, resulting in indirect losses in nutritive value, organoleptic alterations, and color (Matsushita, 1975). The enzyme lipoxygenase was initially known as an oxidizer of carotene by the color loss it caused in processed vegetable foods. During postharvest and technological treatments, foodstuffs lose structural integrity and come into contact with substances that had been compartmentalized. In such a situation, components that are stable in their natural environment, such as chlorophylls and carotenoids, are transformed and degraded by the oxidizing environment.

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The existence of lipoxygenase in the pepper fruit is well established, although it is not so clear what effect the presence and activity of this enzyme have on the carotenoid content of the fruit. The aqueous extract of pepper pulp was shown to have "carotene-oxidizing" activity when linoleate was present in the medium (Kanner and Mendel, 1976). Later, the protein fraction responsible for this activity was isolated (Kanner et al., 1977), and the pattern of change in activity during ripening, postripening, and drying of the fruits was determined (Mínguez-Mosquera et al., 1993a). However, the quantitative relationship between pepper lipoxygenase activity and pigment-destructive capacity is not known.

The main use of the pepper fruit for paprika is the industrial extraction of natural coloring agents for use in foodstuffs. After picking, the fruit is left to over-ripen before being dried industrially. The dried fruit is milled and used directly as paprika or subjected to solvent extraction to obtain the oleoresin. From ripening, until the process of dehydration stops any metabolic activity, there is an intense reorganization of the carotenoid pigment content. In some varieties, this reorganization is seen as a competition between carotenogenic and carotenolytic processes, with an unforeseeable final balance (Mínguez-Mosquera et al., 1994a). In others, due to the absence of the carotenogenic period of postripening (Mínguez-Mosquera et al., 1994b), the result is plainly destructive, with decreases in product quality and commercial value.

The variety Agridulce, because of its physical characteristics and high carotenoid content, is very suitable for processing and is being used as a basis of variety diversification and improvement. However, it does show heavy losses during over-ripening and storage (Mínguez-Mosquera et al., 1994b). Even mild drying conditions result in losses, which are usually heavier in the first

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phase of dehydration and lessen once the moisture content of the fruit has decreased to ~70%. From then on, destruction is slow and possibly attributable to the environmental conditions.

In the variety Agridulce, the only possible origin of pigment losses in the initial phases of the drying process is metabolic. Over-ripening causes softening from degradation of the structures responsible for cell and organ integrity. Under such conditions, effects earlier prevented by compartmentation may appear, such as the co-oxidation of pigments by lipoxygenase action on its specific substrate. Knowing the pigment-destructive capacity of the fruit during the stages before treatment would enable the development of strategies to minimize the effect.

The present work attempts to elucidate the pigment-destructive capacity of the products formed during the reaction catalyzed by the enzyme lipoxygenase of pepper of the variety Agridulce. The experiment has been planned as a model simulation of what takes place when compartmentation is lost, with lipoxygenase acting on its substrate and the carotenoid pigments of pepper present in the medium. The study is designed such that all of the possible linked reactions that might take part in pigment destruction can be evaluated independently.

MATERIALS AND METHODS

Preparation of the Enzymatic Extract. Ripe fruits of the pepper plant of the variety Agridulce picked in the zone of La Vera were used. The enzymatic extract of lipoxygenase was prepared from 1 kg of stalk-, seed-, and placenta-free fruits. These were sliced, and four samples of 10.00 g were separated and homogenized in a Polytron Ultra-Turrax T-25 with 30 mL of phosphate buffer (50 mM and pH 7). The suspension was filtered through paper (S&S Rundfilter 589[1] Schwarzband). The filtrate was the crude enzymatic extract, with a concentration of lipoxygenase enzymatic activity equivalent to 0.25 g of fruit/mL. All operations were carried out at ice bath temperature.

Preparation of Substrate. The method used was that of Surrey (1964). Linoleic acid (Sigma L-1376) (0.5 g weighed exactly) of purity >99% and 0.5 g of Tween 20 were solubilized in deionized and deoxygenated water. In the case of turbidity, 2 N NaOH was added dropwise until complete transparency. The final volume of the mixture was taken to 25 mL. Aliquots of 2 mL were placed in flasks, which were closed under nitrogen atmosphere.

Measurement of Activity. The method used was that described by Mínguez-Mosquera et al. (1993a). Based on the spectrophotometric method of Theorell et al. (1946), the reaction is begun and run in the spectrophotometric cuvette. To 3 mL of phosphate buffer (200 mM and pH 6.5) was added an amount of crude enzymatic extract between 0 and 60 μL and a constant volume of linoleic acid solution of 20 μL. The increase in absorption at 234 nm between 10 and 40 s of reaction was used for the quantification of activity per unit of time. The results are expressed as specific enzymatic activity (katals per milligam), defined as moles of product formed per minute and milligram of fruit employed.

Preparation of the Pigment Extract. Fresh fruit (50 g) was homogenized in a Polytron Ultra-Turrax T-25 with 200 mL of acetone. The supernatant was collected in a decanting flask. The extraction operation was repeated with identical volumes of acetone until the extract was colorless. The acetone extracts were combined and transferred to ethyl ether, and the solvent was evaporated to dryness. The dry residue was dissolved with 10 mL of cyclohexane. Next, 5 mL of the solution of pigments in cyclohexane was taken, and 2.5 g of Tween 20 was added. The solvent was evaporated in a current of nitrogen, and then phosphate buffer (200 mM and pH 6.5) was added slowly to a final volume of 500 mL. The solution contained the pigments equivalent to 0.05 g/mL of ripe red pepper, with the natural degree of esterification and in the proportions found in the fruit.

Reaction Conditions for Pigment Bleaching. The reaction of pigment bleaching by pepper lipoxygenase was studied by distinguishing all of the possible simultaneous subreactions that could affect stability. For each subreaction, 10 flasks of ~10 mL in volume, containing 5 mL of pigment solution in buffer, were used. The first subreaction, to examine pigment autoxidation, was prepared by adding 2 mL of buffer to each of the 10 flasks containing the pigment solution. In the second subreaction, to study the bleaching reaction of the enzymatic extract, 1 mL of buffer and 1 mL of enzymatic extract were added to the flasks containing the pigment solution. The third subreaction was the bleaching induced by the presence of linoleic acid in saturating concentrations: 1 mL of buffer and 1 mL of linoleic acid solution were added to each of the 10 flasks. In the fourth and final subreaction, pigment bleaching was studied using 1 mL of enzymatic extract and 1 mL of linoleic acid solution. Both enzymatic extract and substrate are in proportions in the reaction medium identical to those used to study lipoxygenase activity in fruits of the pepper plant (Mínguez-Mosquera et al., 1993a) to guarantee substrate-saturating conditions.

The flasks containing each reaction were sealed hermetically and placed in darkness at a constant temperature of 23 °C. At 24 h intervals, one flask of each studied reaction was used to monitor pigments.

Separation and Quantification of Pigments. Three 2 mL aliquots were taken from each sampling flask. Each aliquot was placed in a decanting funnel containing 20 mL of ethyl ether and 0.2 mL of internal standard (β-apo-8′-carotenal) solution (70 μg/mL), and 20 mL of aqueous NaCl solution (10%) was added to separate the phases. The colored epiphase was washed repeatedly with the saline solution, and the hypophases from the different washings were either collected and re-extracted, if any color had been retained, or discarded. The ether phase was saponified by adding an equal amount of 20% KOH in methanol. After 2 h, saponification was stopped by adding aqueous NaCl solution (10%), and the carotenoid pigments were again transferred to ethyl ether. The solvent was evaporated, and the pigments were recovered in 1 mL of acetone.

For the separation and quantification of the carotenoid pigments, the system described by Mínguez-Mosquera and Hornero-Méndez (1993) was used. This method uses a reversed phase column (Spherisorb ODS2), 25 cm in length, 0.46 cm i.d., with a particle size of 5 μm. Separation is performed using a binary gradient (acetone−water at a flow rate of 1.5 mL/min). The sample volume injected is 5 μL, and detection is carried out at 450 nm using a UV−visible fixed wave detector. Each separated pigment is quantified by relating the peak area and concentration of internal standard with the response factor and specific peak area of each pigment.

Monitoring of the Bleaching Reaction. Kinetic calculations were made correlating the percentage of each pigment concentration retained (%ret) versus time in hours (t). For reaction time zero, pigment concentration was assigned 100% retention (%ret_0). In successive samples, the percentage retained was calculated by relating the concentration measured at each determined time with the concentration at time zero.

For each subreaction and at each sampling, three replicates were made, giving a final data set for the kinetic calculation of 30 results per pigment and subreaction. The parameters time and percentage retained were correlated in accord with zero-, first-, and second-order kinetic models, and the model showing the highest level of confidence was selected.

RESULTS

Pepper Lipoxygenase Activity. The fruits used in the study show an activity of 14 nkat/mg. This activity remains constant for the concentration range 10−50 μL of enzymatic extract in the reaction cuvette, and the

increase in absorbance is linear during the time the enzymatic reaction is monitored.

Under the conditions of lipoxygenase activity measurement, the enzymatic extract is not subjected to any purification process, nor are coadjutants employed that increase the activity. The enzyme is thus found in the medium as it would be in a situation of cell rupture. Substrate is introduced into the reaction medium in a concentration sufficient to ensure saturation during the first minutes of reaction (Mínguez-Mosquera et al., 1993a) in a medium buffered to a pH close to that of the fruit. It can be assumed that the amount of product formed by enzymatic action is similar to that formed in the fruit, with two qualifications: on the one hand, a dilution factor is introduced that affects all of the components of the fruit and ranges between 1/1200 and 1/240 (10−50 μL of enzymatic extract in a final volume of 3 mL); on the other hand, substrate is introduced in saturating concentration.

With regard to enzymatic activity, the dilution introduced is obviated by expressing the results as specific activity. The activity measured per milligram of fruit is a reflection of the amount of reaction product that this amount of fruit is able to form. However, the saturation with substrate means that the enzyme acts at its maximum catalyzing capacity, forming as much hydroperoxide as is available from the substrate—an extreme situation that is not found in the fruit. Therefore, saturation makes the reaction that of maximal formation of products reachable in the fruit. The opposite situation is the nonaddition of substrate to the reaction medium, meaning an enzymatic activity below that actually existing in the fruit, as the substrate supplied by the fruit also undergoes dilution. In the pepper fruit, the hydroperoxide-forming capacity, and thus the pigment-destructive capacity, of lipoxygenase is intermediate between that in the experimental extremes of substrate saturation and that in the absence of substrate.

Pigment-Destructive Activity of Pepper Lipoxygenase. The aim of the study is to reveal the pigment-destructive capacity of the products of the lipoxygenase-catalyzed reaction. This main reaction is separated into four subreactions, each of which gives information about the incidence of possible linked reactions of pigment oxidation and reveals the contribution of each oxidation promoter. In all of the reactions studied, the best correlation coefficients are obtained with the first-order kinetic model. Table 1 shows the kinetic parameters for the degradation reactions of the main pigments of pepper under the different experimental conditions assayed. The rate constants are calculated with time expressed in hours. The same table gives the correlation coefficients of the straight lines from which have been calculated the slope, the rate constant (k_v), and the ordinate at the origin (%ret$_0$).

Subreaction 1 is the control for the other reactions, being the pigment auto-decomposition reaction. It is a reaction that, in principle, takes place whether there are parallel oxidation pathways or not. The degradation rate constants of all the pigments, apart from violaxanthin, are very low, so that the total pigment concentration is practically unchanged during the reaction time. As the experimental reaction temperature is relatively low, pigment degradation is not very marked. For a reaction time of ~100 h, the pigment autoxidation reaction can be considered to contribute only minimally to bleaching of the aqueous solution of pigments.

Table 1. Kinetic Parameters (Mean ± Standard Error) for the Degradation Reaction of Carotenoid Pigments in Peppers of the Variety Agridulce (Mathematical Fit from First-Order Kinetic Model)

pigment	rate constant $k_v \times 100$ (h^{-1})	ordinate at the origin (ln %ret$_0$)	R
Subreaction 1, Spontaneous Oxidation of Pigments			
β-carotene	0.06 ± 0.006	4.60 ± 0.003	0.988
β-cryptoxanthin	0.04 ± 0.002	4.60 ± 0.001	0.996
zeaxanthin	0.04 ± 0.004	4.60 ± 0.002	0.979
capsanthin	0.15 ± 0.008	4.59 ± 0.004	0.995
violaxanthin	0.25 ± 0.003	4.60 ± 0.002	0.999
capsorubin	0.04 ± 0.004	4.60 ± 0.002	0.983
total	0.10 ± 0.004	4.60 ± 0.002	0.997
Subreaction 2, Pigment Oxidation Induced by Enzymatic Extract			
β-carotene	0.52 ± 0.004	4.60 ± 0.002	0.999
β-cryptoxanthin	0.16 ± 0.002	4.60 ± 0.001	0.999
zeaxanthin	0.14 ± 0.004	4.60 ± 0.002	0.998
capsanthin	0.33 ± 0.005	4.60 ± 0.003	0.999
violaxanthin	0.10 ± 0.002	4.60 ± 0.001	0.999
capsorubin	0.11 ± 0.003	4.60 ± 0.002	0.998
total	0.19 ± 0.003	4.60 ± 0.001	0.999
Subreaction 3, Pigment Oxidation Induced by Spontaneous Peroxidation of Linoleic Acid			
β-carotene	0.75 ± 0.016	4.61 ± 0.009	0.999
β-cryptoxanthin	0.34 ± 0.005	4.60 ± 0.003	0.999
zeaxanthin	0.29 ± 0.036	4.60 ± 0.021	0.976
capsanthin	0.35 ± 0.005	4.60 ± 0.003	0.999
violaxanthin	0.12 ± 0.003	4.60 ± 0.002	0.998
capsorubin	0.13 ± 0.001	4.60 ± 0.000	0.999
total	0.29 ± 0.006	4.60 ± 0.003	0.999
Subreaction 4,[a] Pigment Oxidation Induced by Enzymatic Peroxidation of Linoleic Acid			
β-carotene	2.35 ± 0.10	4.54 ± 0.01	0.997
β-cryptoxanthin	5.38 ± 2.07	4.64 ± 0.06	0.995
zeaxanthin	1.96 ± 0.10	4.64 ± 0.05	0.996
capsanthin	1.91 ± 0.06	4.65 ± 0.03	0.998
violaxanthin	1.77 ± 0.46	4.44 ± 0.16	0.966
capsorubin	0.79 ± 0.09	4.67 ± 0.05	0.978
total	1.39 ± 0.17	4.54 ± 0.10	0.977

[a] Reaction conditions: reaction medium, phosphate buffer 200 mM, pH 7; equivalent concentration of pigments, 0.035 g of ripe fruit/mL; equivalent concentration of enzymatic activity, 0.035 g of ripe fruit/mL; concentration of linoleic acid, 0.02 mM.

Subreaction 2 quantifies the destruction induced by the enzymatic extract when its specific substrate is not added. The rate constants of pigment degradation for the reaction conditions assayed are slightly higher than those for pigment autoxidation. This increase in pigment destruction is probably due to a minimum action of the enzyme, using linoleic coextracted with the pigments from the pulp of the fruit (Mínguez-Mosquera and Hornero-Méndez, 1994). The concentration of substrate under such conditions will be very low—even lower than in the fruit, as a result of dilution—and the pro-oxidant activity of the enzyme means only a small increase with respect to auto-decomposition.

For the enzymatic reaction to take place fully, the substrate must be saturating the reaction medium. The direct effect of this excess is that two parallel and simultaneous pathways of linoleic decomposition are created—one spontaneous and thus slow, and the other enzymatic and logically faster—and both contribute to

an increase in pigment-oxidizing levels in the medium. The pigment-oxidizing process induced by auto-decomposition of linoleic acid can be distinguished from that induced by enzymatic action using the third subreaction, in which both pigment solution and linoleic acid are introduced into the reaction medium. The resulting destruction is a consequence of the oxidant levels created by auto-decomposition of the linoleic acid.

The presence of linoleic acid in the absence of the enzyme (subreaction 3) increases the oxidizing nature of the medium and causes a greater destruction of pigments, as shown by the rate constants for this subreaction. The substrate tends to autoxidize naturally and form hydroperoxide, similar to what takes place during the enzymatic reaction, although logically more slowly as the reaction is not catalyzed.

The enzymatic reaction itself is that of the fourth subreaction, in which pigment solution, enzymatic extract, and linoleic solution are put into the same reaction medium. Under these conditions, the rate constants are high and pigment degradation is considerable. The rate constants range between 0.007 and 0.053. Of all the pigments, β-cryptoxanthin is the most affected by the oxidizing environment created by the enzymatic extract, and capsorubin is the least affected.

Under these experimental conditions, the pigment destruction induced by pepper enzymatic extract is not inappreciable. Deducting both the control reaction and the control destruction induced by linoleic acid, the rate constants of degradation attributable directly to the enzymatic extract action are reduced somewhat. In the case of β-carotene, the degradation rate constant is 0.0235, but deducting the two linked subreactions, this is reduced to 0.0154, which, although lower, is still 65% of the directly measured rate constant. For the other pigments, the effect of these parallel reactions is not so great, and the contribution of lipoxygenase to the measured rate constant ranges between 73 and 92%.

Individualized monitoring of the degradation reaction for each pigment enables the effect of lipoxygenase activity on the chromatic and nutritional quality of the fruit to be evaluated. By their contribution to the chromatic quality, pepper pigments are divided into the yellow fraction, comprising β-carotene, β-cryptoxanthin, and zeaxanthin, and the red fraction, comprising capsanthin and capsorubin (Mínguez-Mosquera et al., 1993b). β-Carotene and β-cryptoxanthin are also nutritionally important for their provitamin A activity. Both properties are altered negatively when oxidative reactions are set off in the medium by the hydroperoxidation of fatty acids. During subreaction 3, in which the linoleic acid undergoes spontaneous hydroperoxidation, the rate constants of red and yellow pigment destruction are markedly different. The reaction results in a proportionally greater loss of yellow pigments, as their rate constants range between 0.0075 h^{-1} for β-carotene and 0.0029 h^{-1} for zeaxanthin, against the rate constants of red pigment destruction, ranging between 0.0035 h^{-1} for capsanthin and 0.0013 h^{-1} for capsorubin. Under these conditions of pigment oxidation caused by spontaneous hydroperoxidation of linoleic acid, the loss in yellow pigmentation and provitamin A value is proportionally greater than that of total pigments.

When the hydroperoxidation of linoleic acid is catalyzed by the intervention of the enzyme lipoxygenase (subreaction 4), the difference in the rates of destruction of red and yellow pigments becomes much greater. Therefore, the action of the enzyme causes not only a net destruction of pigments but also an increase in the destruction observed in the spontaneous hydroperoxidation reaction. Enzyme action displaces the ratio between red and yellow pigments toward a dominance of the red fraction, with much greater losses in carotenoids having provitamin A activity than in total pigments.

The greater liability of the yellow xanthophylls under conditions of unsaturated fatty acid hydroperoxidation could be related with the fact that these pigments are >50% esterified by linoleic acid (Mínguez-Mosquera and Hornero-Méndez, 1994). In contrast, the red xanthophylls are mostly esterified by short-chain, saturated fatty acids. Possibly mediated by a more unstable type of fatty acid that naturally esterifies the yellow pigments or by the polarity that these give the carotenoid molecule, there is a preferential loss of yellow pigments, affecting the pigment content, the color, and the nutritional value of the fruit.

From the kinetic results, it can be concluded that the exclusive action of lipoxygenase of pepper fruits of the variety Agridulce is to destroy in 24 h almost 22% of the initial pigment content. When the set of simultaneous parallel reactions is taken into account, pigment destruction reaches 30%. These reactions can be eliminated from the reaction medium mathematically but not physically, as there must be both a working temperature and substrate in the medium for lipoxygenase activity to exist and, thus, the possibility of parallel co-oxidation, independent of the enzymatic reaction.

In a previous work (Mínguez-Mosquera et al., 1994b), it was observed that during drying of fruits of the variety Agridulce at 40 °C and in darkness, in the first 24 h, when the moisture content fell from 87 to 72%, pigment concentration decreased ~35−40%. Within this range of moisture, lipoxygenase activity decreased only 15% (Mínguez-Mosquera et al., 1993a). The coherence between the facts that lipoxygenase remains active in this stage and that there is a pigment degradation of ~35−40% is in accord with the results obtained in the model making pepper lipoxygenase act against the pigment extract. The hypothesis that lipoxygenase is responsible for the loss of pigmentation during the stages of over-ripening, storage, and beginning of drying of pepper fruit for paprika could be accepted as probable.

The effect of lipoxygenase action is intimately linked with the variety of pepper fruit used. In varieties such as Agridulce, with high pigment content and in which pigment synthesis culminates with ripening, postharvest lipoxygenase activity is obviously undesirable, and fast drying must be used to reduce pigment losses. In other varieties in which pigment synthesis continues even after fruit softening, as in the variety Bola (Mínguez-Mosquera et al., 1994a), lipoxygenase activity can be palliated by the biosynthetic replacement of carotenoids. In these varieties, it is not necessary to accelerate the processes prior to drying.

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