β-Carotene and Capsanthin Co-oxidation by Lipoxygenase. Kinetic and Thermodynamic Aspects of the Reaction

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Carotenoid pigment co-oxidation by lipoxygenase has been studied by factorially combining three pigments: β-carotene, capsanthin, and diesterified capsanthin; two environmental conditions: presence or absence of light; three oxidant conditions in the same reaction medium: presence of both lipoxygenase and linoleic acid, presence of linoleic acid only, and absence of both; and four temperatures: 15, 25, 35, and 45 °C. Independently of the presence or absence of enzyme, diesterified capsanthin and β-carotene were the most unstable pigments, and both exhibited similar lability, although nonesterified capsanthin showed a great stability. This different behavior among pigments could be related with their polarity. The overall reaction clearly follows a first-order kinetic model and the thermodynamic parameters reveal that the co-oxidation reactions of carotenoids are multiple isokinetic forms of just one reaction. In all of the cases studied, enzymatic or nonenzymatic decoloring of carotenoids is a theoretical reaction that can be explained as a loss of conjugation in a sequence of conjugated double bonds. The source of pro-oxidant compounds, the environmental conditions, the reaction medium, and the differences in pigment structure are external factors that modify the kinetic parameters of the reaction and consequently the thermodynamic parameters, but not the reaction pathway.

Keywords: *Carotenoids; lipoxygenase; decoloring; degradation; kinetic*

INTRODUCTION

Lipoxygenase (linoleate:oxygen oxidoreductase, 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 predominant in fruits and seeds of a large number of vegetables (Pinsky et al., 1971). Lipoxygenase (LOX) catalyzes the stereospecific hydroperoxidation of polyunsaturated fatty acids and esters containing a *cis*,*cis*-1,4-pentadiene structure. The generic name "lipoxygenase" includes many different isoenzymes (Hildebrand 1989), each one with its specific substrate and optimum pH (Yoon and Klein, 1979; Hildebrand and Hymowitz, 1982; Ali Asbi et al., 1989).

In 1941, Strain observed that pigments were destroyed in aqueous extracts of soybean in the presence of fatty acids and oxygen. This effect was later observed in peas and seeds of other legumes (Grosch et al., 1976; Kermasha and Metche, 1986; Abbas et al., 1989). The substance which promoted color loss in vegetables was referred to as the "carotene oxidizing factor". Subsequently, this factor was identified as the enzyme lipoxygenase (Kanner and Mendel, 1976; Kanner et al., 1977).

Holden (1965) blanched chloroplastic pigments with soybean extract and found that lipoxygenase promoted more degradation in chlorophyll compounds than in carotenoids, and Hildebrand and Hymowitz (1982) confirmed these results.

Ikediobi and Snyder (1977) specifically studied the capacity of lipoxygenases in the blanching of carotenoids, and found that the mixture of LOX-2 and LOX-3 had a greater blanching effect on β-carotene than the alkaline lipoxygenase alone (LOX-1).

Reynolds and Klein (1982) found low carotenoid blanching capability in LOX-1 from soybeans and peas. Barimalaa and Gordon (1988) studied β-carotene co-oxidation by combining a mixture of natural isoenzymes from soybean. They found that the reactions of β-carotene oxidation and linoleic acid hydroperoxidation had the same temperature dependence because the activation energy of both reactions was within the range of the experimental error. These authors also found that the ability of lipoxygenase to participate in co-oxidation reactions is directly related to the polarity of the compounds being oxidized. There is stronger co-oxidation capacity against β-carotene than against retinol, and the latter is less stable than retinoic acid (Gordon and Barimalaa, 1989).

Various authors have reported different optimum pHs for the blanching ability of lipoxygenase. Holden (1965), Ben-Aziz et al. (1970), and Hildebrand and Hymowitz (1982) reported optimum reaction pHs of 6, 7, and 9, respectively. These values are in accordance with the optimum pH found for the activity of the different isoenzymes (Holden, 1965; Barimalaa and Gordon, 1988).

The co-oxidation mechanism is explained by Richardson (1982) as destruction of carotenoid and chlorophyll molecules by free radicals. The radicals are produced by decomposition of the enzymatically formed hydroperoxide. Similarly, Cohen et al. (1985) have demonstrated that the blanching reaction can occur when the pigments are added to the medium after the enzymatic reaction has taken place. This clearly proves that the pigment oxidation takes place as a second step after the enzymatic reaction.

This second step is critical in the blanching process and several factors can modify the oxidant capacity created during the first step (Oszmianski and Lee, 1990). The presence of unoxidized substrate in the reaction medium can modify the oxidative capability of the hydroperoxide formed. The possible oxidation of the

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carotenoids due to the reaction medium and the environmental conditions must also be considered.

In a previous study, the carotenoid decoloration reaction in organic medium was established as a unique thermodynamically compensated reaction, in which the source of energy (light or temperature) is able to remove electrons from the chromophore and consequently destroy the color of the pigment (Mínguez-Mosquera and Jarén-Galán, 1995). Although it is assumed that the enzymatic and nonenzymatic co-oxidation reactions take place via a radical mechanism, the parallelism with other oxidative reactions of carotenoids has not been established. In addition, the extension of the enzymatic co-oxidation reaction has not yet been studied, thus eliminating all of the other factors that can promote concomitant oxidative reactions. The presence of these factors could explain the different results and interpretation found in the literature.

In the present study, the kinetic parameters of the decoloring reaction of various carotenoids (β-carotene, capsanthin, and diesterified capsanthin) in the presence of lipoxygenase were determined. The study was conducted in a manner which allowed determination of the influence of pigment structure in the co-oxidation reaction and the role of the remaining substrate and reaction medium during the enzymatic co-oxidation. Moreover, two environmental conditions (darkness and light) were utilized to elucidate the coupled oxidation. The study was performed at four temperatures in order to establish the thermodynamic parameters of the reaction. A factorial design was used to permit a comprehensive overview of the decoloring carotenoid reaction promoted by lipoxygenase.

MATERIALS AND METHODS

Raw Materials. The pigments used were β-carotene, diesterified capsanthin, and capsanthin. All of the pigments had a purity of 95%. β-Carotene was supplied by Sigma (C-0126), and the diesterified capsanthin and capsanthin were obtained in the laboratory from pepper following methodology described in previous publications (Mínguez-Mosquera et al., 1993b; Mínguez-Mosquera and Hornero-Méndez, 1993a). Each pigment was dissolved in 100 mL of cyclohexane to give stock solutions with a theoretical absorbance of 100. The stock solutions were placed under nitrogen in amber-colored flasks which were hermetically sealed and stored in the dark at −30 °C.

The measurement of enzymatic activity was carried out using commercial soy lipoxygenase (Fluka no. 62340) as biological material. The substrate was linoleic acid (Sigma, of purity greater than 99%, no. L-1376). The enzymatic solution was prepared by dissolving enough enzyme in deionized water to obtain a solution with concentration close to 0.04 mg/mL. The substrate was prepared using a modified form of the procedure of Surrey (Surrey, 1964; Mínguez-Mosquera et al., 1993c). Tween 20 (0.5 mL) was dissolved in 10 mL of tetraborate buffer (0.1 M) and 0.5 mL of linoleic was added dropwise. Next, 1.3 mL of NaOH (1 M) was added and vigorously stirred. The mixture was taken to 200 mL with tetraborate buffer (0.1 M) and the pH adjusted to 9 with concentrated HCl. The linoleic concentration of the mixture was 8.04 mM.

Primary Enzymatic Reaction: Measurement of Hydroperoxide Formation Activity of Lipoxygenase. The mixture in the reaction cuvette comprised 0.05 mL of enzymatic solution, 0.05 mL solubilized substrate, and 1.4 mL phosphate buffer (200 mM pH 8). In the reaction cuvette, the concentrations of linoleic acid and enzyme were 0.268 mM and 0.0013 mg/mL, respectively. The enzymatic activity was quantified spectrophotometrically from the increase in absorbance at 234 nm of hydroperoxide formation between 30 and 90 s of reaction.

Secondary Enzymatic Reaction: Measurement of Co-oxidant Activity of Lipoxygenase on the Carotenoid Pigments. *Environmental Conditions for the Reaction.* A thermostatically controlled chamber was subdivided into two compartments. One was used to study the effects of light and was fitted with fluorescent tubes that produced a white light which was uniformly distributed so that all points of the compartment received an illuminance of 1000 lx. The second compartment was used to study the reactions in darkness. This compartment was lined in black and all joints were sealed to prevent the entry of light; levels of illuminance lower than 5 lx were achieved. The temperatures used in the study were 15, 25, 35, and 45 °C (±3 °C).

Reaction Media. Aliquots of 0.25 mL of the stock solution of each pigment were taken and placed in respective 25 mL graduated flasks with 0.125 g of Tween 20. The organic solvent was evaporated to dryness. Subsequently, phosphate buffer (200 mM pH 8) was slowly added while the mixture was gently shaken until a final volume of 25 mL was reached. The dilution performed gave solutions in phosphate buffer of each pigment with an absorbance close to unity. All of the procedures were performed under a soft green light at a room temperature of 20 °C.

From each pigment solution, 12 aliquots of 1.4 mL were taken and placed into spectrophotometric cuvettes. The remaining solution was discarded. An aliquot of 0.1 mL deionized water was added to four cuvettes which were subsequently hermetically sealed. The four cuvettes formed the array used to study pigment oxidation resulting from the reaction medium. In another four cuvettes, 0.05 mL of linoleic acid solution and 0.05 mL of deionized water were added to study the activity of enzymatic reaction substrate on the pigments. In the four remaining cuvettes 0.05 mL of linoleic acid solution and 0.05 mL of lipoxygenase solution were added to study pigment degradation promoted by the products resulting from the enzymatic activity on its specific substrate.

Monitoring the Reaction. Two cuvettes of each pigment (three pigments) in each reaction medium (three reaction media) were placed either in darkness or under illumination. Consequently, the experiment permitted the control of 18 reactions occurring simultaneously under the same conditions of reaction temperature and illumination. Each cuvette was periodically removed from the chamber for 3 min for spectrophotometric analysis in order to monitor the progress of the decoloring reaction. In previous controls of the experiment, it was established that in the fastest reactions (β-carotene in presence of lipoxygenase and linoleic acid at 45 °C and under illumination), it was necessary to measure the absorbance each 30 min in order to get at least seven measurements between 1 and 0.1 units of absorbance. In the slowest reactions (capsanthin in phosphate buffer at 15 °C and in darkness) the measurement could be performed at longer time intervals. The measurement of absorbance was always performed in duplicate in each cuvette and the data were expressed as percentage of retention of color. For each temperature the experiment was repeated three times.

Calculus of Kinetic and Thermodynamic Parameters of the Co-oxidation Reaction. For the kinetic study, each reaction had 84 data points at each temperature, reaction medium, and environmental condition. The percentage of retention of color was related to time in hours (t) by the different mathematical fits in accord with a possible zero-order or first-order kinetics. In each of the 72 different reactions, the plot which gave the best correlation was selected, and the slope of the regression line was the rate constant of the degradation reaction (K_v).

For any one pigment and under identical conditions of reaction medium and light, the inverse of the reaction temperature (T) in K was plotted semilogarithmically against the rate constants (K_v) obtained. According to the active complex theory of Arrhenius, the slope of the line corresponds to the energy of activation of the reaction (E_a) divided by the universal gas constant (R), and the ordinate at the origin is the reaction frequency factor (ln A).

Plotting K_v/T against $1/T$ semilogarithmically, the parameters corresponding to the theory of the state of transition—

increment in the enthalpy of activation ($\Delta H^{\ddagger}$) and increment in the entropy of activation ($\Delta S^{\ddagger}$)—were obtained in function of the Planck (h) and Boltzmann (k) constants.

RESULTS

Lipoxygenase is described in the literature as being responsible for pigment co-oxidation, although its real role is restricted to the enzymatic hydroperoxidation of unsaturated fatty acids. The products formed during the enzymatic reaction are exclusively responsible for pigment blanching. To promote the highest co-oxidant capability, it is necessary optimize the enzymatic reaction conditions in order to create the maximum amount of enzymatic reaction product. The procedure followed consists of developing a system to measure the enzymatic activity and later modifing the reaction conditions to enhance the activity.

Measuring the Hydroperoxide Formation Activity of Lipoxygenase. The formation of fatty acid hydroperoxide causes an increase in absorbance at 234 nm. This increase follows the Lambert−Beer law, and allows spectrophotometric quantification of the product formed. Quantification must be made in the optimum region of absorbance, and the reaction must fit the general conditions of enzymatic studies to make the results comparable. With these premises, the reaction is guaranteed to follow zero-order kinetics, the substrate is at saturation concentration, and the activity measured is maximum for the amount of enzyme used under the assay conditions.

For this first study, it was necessary to fix a series of parameters: the enzymatic solution was prepared with deionized water, the reaction pH was fixed arbitrarily at 7 with phosphate buffer 200 mM, and the assay temperature was fixed at 10 °C. The results indicate that with the linoleic solution used, it was not appropriate to add amounts greater than 0.05 mL to the reaction cuvette. Increased amounts raised the initial absorbance, and hampered the perfect solution of the substrate with the reaction buffer. Thus, lipoxygenase solutions were prepared from initial concentrations of 1−0.04 mg/mL. This latter solution gave perfect linearity for increase of absorbance against time when volumes less than 0.06 mL were used. For convenience, 0.05 mL of this solution was used for later assays.

The increase in absorbance with time resulting from hydroperoxide formation followed a straight line which continued unaltered for more than 3 min. This gives a reasonable period to mix and stir the components, and to place the cuvette into the spectrophotometer for measurement of activity. After 3 min, the reaction continues but the absorbance reached does not allow the reaction to be monitored spectrophotometrically. The time period between 30 and 90 s of the reaction was selected as the region for quantification of the increase in absorbance per minute because it is the period with lowest photometric error.

Optimizing the Enzymatic Reaction Conditions. *Enzyme Solubilization.* Enzymatic solutions were prepared by assaying deionized water and phosphate buffers 200 mM at pH 5, 6, 7, 8, and 9. Activity was measured in phosphate buffer 200 mM at pH 7 and 10 °C as reaction temperature.

The presence of the enzyme predissolved in a buffer does not facilitate the later action of the enzyme. Because enzymatic activity is not modified, it was decided to prepare the soybean lipoxygenase solution simply with deionized water.

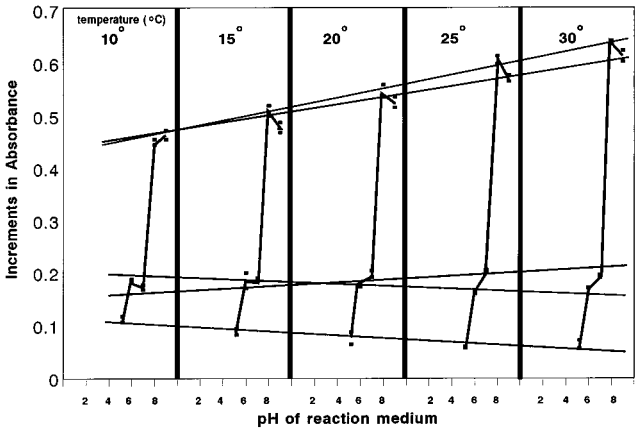

Figure 1. Changes in lipoxygenase activity with pH and temperature.

Joint Influence of pH and Temperature. Throughout the study, the reaction temperature was fixed *a priori* at 10 °C, to prevent possible changes of the substrate and denaturation of the enzyme. Knowing the concentrations to be used and the methodology to be followed for measurement of activity, we widened the temperature range to 15, 20, 25, and 30 °C. Simultaneously, at each temperature the influence of the reaction medium pH using phosphate buffers 200 mM at pH 5, 6, 7, 8, and 9 as reaction medium was checked. In each case, the variation in pH of the medium during the enzymatic reaction was monitored.

At all of the temperatures, the maximum activity was found systematically at pH 8. There was a linear increase in the activity measured at pH 7, 8, and 9 as the temperature increased. At pH 6, temperature had no effect, but at pH 5 the increase in temperature caused a progressive decrease in activity. The statistical treatment of these five lines showed that they were significantly different ($p < 0.05$) and all of them had a correlation coefficient higher than 0.98 (Figure 1).

Because variation of pH during the reaction was practically zero (less than ± 0.01 pH unit), it could be considered that the buffers used have sufficient capacity to buffer the possible variations taking place during the reaction. In contrast, buffers are seen to be ineffective in buffering the variation taking place on addition of substrate. At pH lower than 7, the variation undergone by the reaction medium on addition of substrate was less than 0.2 unit, at pH 8 it was 0.7 unit, and at pH 9 it was 0 units.

At any temperature, the maximum activity of hydroperoxide formation was found using buffer at pH 8 as the reaction medium. This pH was modified to pH 8.7 on addition of the substrate; therefore, the enzyme used should be identified as isoenzyme LOX-1. It is expected that this enzyme will show the highest pigment co-oxidation activity with the described reaction conditions.

Kinetics of Carotenoid Pigments Co-oxidation Reaction. Once the enzymatic reaction conditions have been established, the effects of the reaction on the carotenoid pigments can be studied. The use of three reaction media used (phosphate buffer, phosphate buffer with linoleic acid, and phosphate buffer with linoleic acid and lipoxygenase) allows the elucidation of pigment lost when the oxidation is promoted by the reaction medium, the substrate, or the enzymatic reaction. The co-oxidation by combining external and internal oxidative factors can be studied since the reaction takes place in the presence of light or darkness.

Table 1. Rate Parameters of the β-Carotene Decoloring Reaction Fitted by First-Order Kinetic[a]

reaction media and temperatures (°C)	reaction under illumination			reaction in darkness		
	rate constant (h^{-1})	ordinate at the origin	R	rate constant (h^{-1})	ordinate at the origin	R
phosphate buffer						
15	0.014 ± 0.000[b]	4.626 ± 0.034	0.996	0.002 ± 0.000	4.594 ± 0.010	0.967
25	0.020 ± 0.000	4.534 ± 0.053	0.994	0.003 ± 0.000	4.598 ± 0.083	0.931
35	0.021 ± 0.001	4.547 ± 0.035	0.991	0.005 ± 0.000	4.585 ± 0.015	0.974
45	0.060 ± 0.001	4.571 ± 0.017	0.998	0.015 ± 0.001	4.558 ± 0.025	0.980
phosphate buffer with linoleic acid						
15	0.068 ± 0.005	4.836 ± 0.224	0.983	0.002 ± 0.000	4.519 ± 0.034	0.858
25	0.099 ± 0.000	4.577 ± 0.011	0.998	0.009 ± 0.000	4.475 ± 0.130	0.926
35	0.096 ± 0.008	4.570 ± 0.043	0.992	0.011 ± 0.000	4.534 ± 0.067	0.935
45	0.157 ± 0.005	4.583 ± 0.020	0.992	0.035 ± 0.000	4.593 ± 0.009	0.994
phosphate buffer with linoleic and lipoxygenase						
15	0.055 ± 0.002	4.322 ± 0.230	0.974	0.007 ± 0.000	4.515 ± 0.073	0.871
25	0.158 ± 0.003	4.517 ± 0.035	0.989	0.052 ± 0.001	4.546 ± 0.035	0.859
35	0.198 ± 0.003	4.567 ± 0.033	0.998	0.083 ± 0.001	4.451 ± 0.196	0.947
45	0.574 ± 0.004	4.527 ± 0.071	0.992	0.149 ± 0.000	4.510 ± 0.059	0.990

[a] First order: ln % retention = 4.606 − $K_v \times t$(h). [b] Standard deviation.

Table 2. Rate Parameters of the Diesterified Capsanthin Decoloring Reaction Fitted by First-Order Kinetic[a]

reaction media and temperatures (°C)	reaction under illumination			reaction in darkness		
	rate constant (h^{-1})	ordinate at the origin	R	rate constant (h^{-1})	ordinate at the origin	R
phosphate buffer						
15	0.010 ± 0.000[b]	4.678 ± 0.053	0.984	0.001 ± 0.000	4.543 ± 0.028	0.881
25	0.030 ± 0.001	4.607 ± 0.023	0.998	0.006 ± 0.000	4.583 ± 0.023	0.958
35	0.073 ± 0.002	4.586 ± 0.056	0.991	0.012 ± 0.000	4.591 ± 0.009	0.994
45	0.159 ± 0.005	4.551 ± 0.043	0.995	0.038 ± 0.000	4.569 ± 0.024	0.958
phosphate buffer with linoleic acid						
15	0.014 ± 0.000	4.695 ± 0.040	0.994	0.002 ± 0.000	4.537 ± 0.031	0.906
25	0.031 ± 0.001	4.613 ± 0.025	0.997	0.008 ± 0.001	4.565 ± 0.043	0.900
35	0.081 ± 0.003	4.539 ± 0.083	0.993	0.013 ± 0.000	4.571 ± 0.018	0.978
45	0.170 ± 0.004	4.513 ± 0.061	0.994	0.051 ± 0.000	4.570 ± 0.015	0.991
phosphate buffer with linoleic and lipoxygenase						
15	0.012 ± 0.000	4.675 ± 0.037	0.993	0.002 ± 0.000	4.552 ± 0.020	0.955
25	0.037 ± 0.001	4.574 ± 0.010	0.998	0.013 ± 0.001	4.582 ± 0.026	0.976
35	0.087 ± 0.002	4.549 ± 0.057	0.987	0.021 ± 0.000	4.559 ± 0.024	0.979
45	0.458 ± 0.018	4.521 ± 0.065	0.996	0.162 ± 0.011	4.533 ± 0.065	0.989

[a] First order: ln % retention = 4.606 − $K_v \times t$(h). [b] Standard deviation.

Table 3. Rate Parameters of the Capsanthin Decoloring Reaction Fitted by First-Order Kinetic[a]

reaction media and temperatures (°C)	reaction under illumination			reaction in darkness		
	rate constant (h^{-1})	ordinate at the origin	R	rate constant (h^{-1})	ordinate at the origin	R
phosphate buffer						
15	0.0114 ± 0.0004[b]	4.635 ± 0.029	0.995	0.0003 ± 0.0000	4.602 ± 0.002	0.967
25	0.0142 ± 0.0006	4.566 ± 0.043	0.996	0.0013 ± 0.0001	4.593 ± 0.013	0.985
35	0.0157 ± 0.0007	4.586 ± 0.015	0.997	0.0019 ± 0.0000	4.600 ± 0.008	0.988
45	0.0177 ± 0.0012	4.568 ± 0.037	0.989	0.0033 ± 0.0001	4.588 ± 0.007	0.996
phosphate buffer with linoleic acid						
15	0.0130 ± 0.0003	4.620 ± 0.009	0.999	0.0005 ± 0.0000	4.592 ± 0.005	0.937
25	0.0145 ± 0.0005	4.581 ± 0.020	0.999	0.0027 ± 0.0001	4.583 ± 0.021	0.953
35	0.0168 ± 0.0004	4.591 ± 0.021	0.997	0.0024 ± 0.0003	4.573 ± 0.023	0.969
45	0.0225 ± 0.0010	4.580 ± 0.018	0.995	0.0082 ± 0.0001	4.616 ± 0.013	0.998
phosphate buffer with linoleic and lipoxygenase						
15	0.0244 ± 0.0013	4.570 ± 0.034	0.996	0.0014 ± 0.0000	4.540 ± 0.027	0.815
25	0.0429 ± 0.0018	4.556 ± 0.053	0.998	0.0067 ± 0.0002	4.588 ± 0.016	0.995
35	0.0455 ± 0.0018	4.712 ± 0.151	0.974	0.0059 ± 0.0007	4.566 ± 0.021	0.989
45	0.0463 ± 0.0031	4.589 ± 0.036	0.995	0.0153 ± 0.0006	4.603 ± 0.004	0.999

[a] First order: ln % retention = 4.606 − $K_v \times t$(h). [b] Standard deviation.

Tables 1, 2, and 3 show the rate constant for the reactions studied. In general terms, it can be seen that all of the reactions can be fitted to a first-order kinetic model. As was expected, the rate constant of all of the reactions increases with the temperature. At each temperature, the lowest rate constants for pigment degradation reaction are found in absence of linoleic acid and lipoxygenase. The presence of linoleic acid increases the rate constants for pigment degradation, and the reaction reaches its maximum speed in the presence of linoleic acid and lipoxygenase. Therefore, it can be postulated that in absence of lipoxygenase, linoleic acid autoxidation is responsible for increases of carotenoid oxidation. Perhaps this autoxidation is the same

Table 4. Activation Energy (kcal/mol) of the Enzymatic Co-oxidation Reaction of Carotenoid Pigments

pigment and reaction medium	reaction under illumination			reaction darkness		
	activation energy (kcal/mol)	ln *A*	*R*	activation energy (kcal/mol)	ln *A*	*R*
β-carotene						
buffer	7.82 ± 2.74[a]	9.31 ± 4.56	−0.896	11.66 ± 2.37	14.04 ± 3.96	−0.960
buffer + substrate	4.43 ± 1.25	5.09 ± 2.09	−0.928	15.84 ± 2.88	21.70 ± 4.80	−0.968
buffer + substrate + enzyme	13.04 ± 2.12	20.00 ± 3.54	−0.974	17.55 ± 4.09	26.16 ± 6.82	−0.949
diesterified capsanthin						
buffer	16.55 ± 0.44	24.43 ± 0.74	−0.999	20.95 ± 2.38	29.97 ± 3.80	−0.988
buffer + substrate	15.18 ± 0.59	22.28 ± 0.99	−0.998	18.33 ± 2.26	25.95 ± 3.77	−0.985
buffer + substrate + enzyme	21.28 ± 1.04	32.79 ± 1.74	−0.987	24.52 ± 3.87	36.79 ± 6.46	−0.975
capsanthin						
buffer	2.56 ± 0.27	0.08 ± 0.45	−0.989	13.75 ± 2.70	16.23 ± 4.51	−0.963
buffer + substrate	3.19 ± 0.62	1.27 ± 1.04	−0.963	14.95 ± 4.06	18.82 ± 6.77	−0.933
buffer + substrate + enzyme	3.64 ± 1.56	2.82 ± 2.60	−0.855	12.77 ± 3.75	16.05 ± 6.25	−0.923

[a] Standard deviation.

Table 5. Thermodynamic Parameters of the Enzymatic Co-oxidation Reaction of Carotenoid Pigments

pigment and reaction medium	reaction under illumination			reaction darkness		
	activation enthalpy (kcal/mol)	activation entropy (cal mol^{-1} K^{-1})	*R*	activation enthalpy (kcal/mol)	activation entropy (cal mol^{-1} K^{-1})	*R*
β-carotene						
buffer	7.31 ± 2.72[a]	−25.30 ± 9.00	−0.885	11.19 ± 2.31	−15.78 ± 7.64	−0.959
buffer + substrate	3.88 ± 1.26	−33.86 ± 4.17	−0.908	15.36 ± 2.96	−0.64 ± 9.81	−0.964
buffer + substrate + enzyme	12.56 ± 2.15	−4.05 ± 7.12	−0.971	17.04 ± 4.27	8.09 ± 14.12	−0.942
diesterified capsanthin						
buffer	16.09 ± 0.58	4.81 ± 1.94	−0.998	20.51 ± 2.43	15.88 ± 8.03	−0.986
buffer + substrate	14.72 ± 0.52	0.55 ± 1.73	−0.998	17.89 ± 2.29	7.90 ± 7.60	−0.983
buffer + substrate + enzyme	20.68 ± 2.40	20.80 ± 7.94	−0.986	24.14 ± 3.90	29.60 ±12.92	−0.974
capsanthin						
buffer	1.98 ± 0.29	−43.94 ± 0.98	−0.978	13.23 ± 2.83	−11.63 ± 9.38	−0.956
buffer + substrate	2.62 ± 0.60	−41.60 ± 1.98	−0.951	14.45 ± 4.15	−6.42 ± 13.74	−0.926
buffer + substrate + enzyme	3.05 ± 1.61	−38.48 ± 5.32	−0.801	12.24 ± 3.85	−12.00 ± 12.73	−0.913

[a] Standard deviation.

reaction that occurs when lipoxygenase is present. However, without the enzymatic catalysis it occurs at much lower speed. This linoleic acid autoxidation should not be underestimated because it clearly increases pigment oxidation in comparison to that occurring in the buffer medium alone. Also, it can be seen that the reactions that take place under illumination conditions have higher degradation rate constants due to degradative synergism.

With respect to the individual pigments, *β*-carotene and diesterified capsanthin are the most unstable pigments in the experimental conditions assayed. This instability is probably related to the nonpolar nature of these pigments. Of these two pigments, it can be observed that *β*-carotene tends to be more unstable at low experimental temperatures in all the conditions and reaction media. At high experimental temperature, diesterified capsanthin surpasses *β*-carotene in lability. Capsanthin is the most stable pigment in this study, having a degradation rate constant around one order of magnitude lower than the other pigments.

Comparing the results obtained in this study with those obtained in a previous publication (Mínguez-Mosquera and Jarén-Galán, 1995), it can be seen that the pigment degradation in water is identical to that in phosphate buffer, either formally or kinetically. This may be due to lack of dependence of the pigment degradation on the pH and ion strength of the reaction medium.

Thermodynamic Aspect of Carotenoid Pigments Co-oxidation Reaction. Tables 4 and 5 show the thermodynamic parameters for the different reactions studied. The parameters have been fitted according to the mathematical models of the activated complex and transition state theories, respectively.

Each reaction is affected in a different way by the temperature increase, without showing any logical sequence or behavior pattern that can make all of the reactions studied comparable. The only general consequence that can be obtained from these results is that for the same reaction, the activation energy or activation entropy is always higher when the reaction is performed under illumination. Mathematically, this can be interpreted that the changes in temperature modify the reaction rate constant less under illumination than in darkness.

To elucidate whether there were formal, kinetic, and thermodynamic differences among the reactions studied, the results obtained were interpreted as a kinetically compensated system. For the isokinetic theory, one single reaction can have different kinetic and thermodynamic parameters depending on the reaction conditions, although in all cases the reaction is the same (Rhim et al., 1989, 1990; Canjura et al., 1991; Mínguez-Mosquera and Jarén-Galán, 1995). The condition required for a kinetically compensated system is that the different thermodynamic parameters obtained for the same reaction in different environments have to define an isokinetic line. This theoretical line includes all of the different kinetic and thermodynamic appearances of a single reaction.

Plotting the pair of thermodynamic parameters of all of the reactions studied, we find that a perfect line ($R = 0.992$) is obtained. This can be interpreted as the isokinetic line (Figure 2). The isokinetic temperature was calculated (304 K) that is placed between the range of temperatures under study (288−318 K). According to Rhim et al. (1990), this happens when there are changes in the reaction conditions but not in the reaction. In our case, the reaction conditions changed,

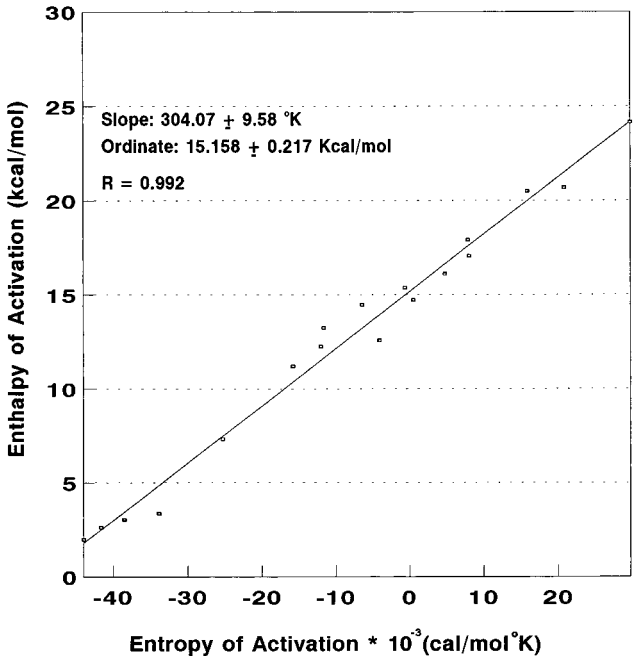

Figure 2. Isokinetic line for enzymatic and nonenzymatic carotenoid pigment degradation in aqueous medium.

even dramatically, from one reaction to another. It could be expected that the reaction in which diesterified capsanthin is oxidized under the presence of lipoxygenase and linoleic acid in darkness would be different with respect to the oxidation of β-carotene under illumination in phosphate buffer. The fact that both reactions define the same isokinetic line shows that both are the same reaction and the environment which surrounds the reaction is responsible for the displacement of the thermodynamic parameters along the same isokinetic line.

In this case the theoretical single reaction is represented by a colored compound that becomes colorless, possibly due to the loss of conjugation in a molecule with a chromophore comprising several conjugated double bonds. The other functional groups of the pigment molecule (not included in the chromophore) can be considered as external factors that can modify the amount of energy required for loss of conjugation. The medium in which the reaction occurs or the environmental conditions are also external factors that do not modify the pattern of the reaction but change its speed and temperature dependence. Also, the nature of the compounds that promote the loss of conjugation (peroxides, light, oxygen, etc.) are external factors affecting the reaction quantitatively but not qualitatively. Therefore, it can be concluded that the carotenoid decoloration reaction mechanism is the same whether it is promoted by enzymatic co-oxidation, co-oxidation by fatty acid autoxidation, or simply pigment oxidation. The different kinetic and thermodynamic values reflect the influence of these external factors on the same reaction.

Comparing these result with those found previously in pigment decoloration in organic solvents, we found that the isokinetic line in the two studies is statistically similar. This is in accordance with the single reaction theory, clearly showing that the decoloration pigment reaction, in all the cases, has the same reaction pattern. The mechanism is independent of the reaction medium, nature of the products that promote the oxidation, or source of energy for the reaction. The system to monitor the reaction does not allow the evaluation of possible intermediate steps in the reaction. However, these intermediate steps will probably be, at least qualitatively, similar in all cases.

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