Degradation of Cyanidin 3-Glucoside by Blueberry Polyphenol Oxidase: Kinetic Studies and Mechanisms

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The use of model systems containing purified anthocyanins, chlorogenic acid (CG), and blueberry polyphenol oxidase (PPO) and of different analysis methods (HPLC and polarographic methods) have allowed the study of the mechanism of anthocyanin degradation by PPO. Cyanidin 3-glucoside (Cy 3-glc) was not oxidized by PPO since no molecular oxygen was consumed. However, the presence of CG induced pigment degradation. The quinone of CG, formed enzymatically, was involved in the degradation of Cy 3-glc by coupled oxidation mechanisms with partial regeneration of CG. This means that part of the CG was incorporated into the degradation products of Cy 3-glc. The ratio of degraded Cy 3-glc to oxidized CG was equal to 2. An anthocyanin degradation mechanism is proposed.

Keywords: *Vaccinium corymbosum; polyphenol oxidase; cyanidin 3-glucoside; chlorogenic acid; oxidation mechanism*

INTRODUCTION

Ripe fruit of Highbush blueberries are normally blue due to anthocyanins in the epidermal and hypodermal cells (Ballinger et al., 1972). Hence, Highbush blueberry color is an important quality factor influencing both fresh-market value and the suitability of the berries for processing. The phenolic composition of Highbush blueberry fruits has been studied by Kader et al. (1996). Fifteen anthocyanins were characterized as monoglucosides, monogalactosides, and monoarabinosides of delphinidin, cyanidin, petunidin, peonidin, and malvidin. Chlorogenic acid (CG) has been found to be the major hydroxycinnamic derivative.

Fresh blueberry fruits develop an intense browning after crushing. The implication of CG, anthocyanins, and polyphenol oxidase (PPO) has been demonstrated by Kader et al. (1997b). The blueberry PPO partially purified by Kader et al. (1997a) presented an important activity toward caffeic acid and CG. This enzyme was characterized as having high activity at acidic pH (3 and 5), which is close to the pH of the fruit (pH 3.1) (Kader et al., 1994).

The stability of the anthocyanins is influenced by several factors. Some of them have been discussed by several authors, and it would appear that PPO plays an important role in the degradation of anthocyanins from fruits and vegetables. Wagenknecht et al. (1960) reported an anthocyanin-decolorizing system in cherries that acts as an oxidizing enzyme. Maximum discolorization was observed when catechol was added to the reaction mixture. The effect of phenolase on anthocyanin discolorization was studied by Peng and Markakis (1963). They showed that in the absence of catechol the reaction is very slow but the rate of discolorization increases rapidly with catechol concentration. These authors proposed a scheme of sequential reactions to explain the effect of mediating phenols. The mechanism involved the oxidation of catechol by phenolase to the corresponding *o*-benzoquinone, which oxidized anthocyanin to a colorless product. This mechanism explains the effect of CG on the degradation of anthocyanins by PPO from eggplant (Sakamura and Obata, 1963; Sakamura et al., 1965). These authors showed that the oxidizing system isolated from eggplant has no effect on pelargonidin 3-glucoside, whereas the destruction rate reached 53% when nasunin [delphinidin 3-(*p*-coumaroylrutinoside)-5-glucoside] was used as a substrate. This result shows that PPO can act on anthocyanins when a triphenolic function is present on the B ring of the flavylium structure. The addition of ascorbic acid retards the loss of pigment as long as ascorbic acid is present in the reaction mixture. The authors suggested that the *o*-quinone formed from the pigment is reduced by a coupled reaction, where ascorbic acid played a role of hydrogen donor. In this view, Sakamura et al. (1965) proposed an oxidizing mechanism of anthocyanin by eggplant PPO without the mediating effect of phenolic compound. Sakamura and Obata (1963) also studied the degradation of anthocyanins in the presence of CG. They showed that CG increased the rate of nasunin degradation by PPO (98% instead of 53% in the absence of CG) and that the discolorization of pelargonidin 3-glucoside reached 92% when CG was added to the reaction mixture. Pifferi and Cultrera (1974) isolated two PPOs from sweet cherries that oxidized CG, pyrocatechol, and catechin. The authors observed a display between maximum anthocyanin degradation and maximum PPO activity. According to

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the authors, the anhydrobase form of the anthocyanin was more susceptible to reaction with *o*-quinone. The oxidation of phenolic compounds by PPO leads to *o*-quinones, which are responsible for the degradation of anthocyanins. This mechanism has also been confirmed by Raynal and Moutounet (1989). Wesche-Ebeling and Montgomery (1990) showed that the presence of catechin increases the degradation rate of strawberry anthocyanins by PPO. They proposed that the browning reaction was due to copolymerization of anthocyanins into condensation products formed by quinone–phenol reactions. Cheynier et al. (1994) studied the mechanism of anthocyanin degradation in oxidizing grape must, and the authors confirmed the role of the caffeoyltartaric acid *o*-quinone generated by PPO in the degradation of anthocyanins. The rate of anthocyanin degradation was related to their structure. Delphinidin 3-glucoside and petunidin 3-glucoside were rapidly oxidized, whereas non-*o*-diphenolic anthocyanins were also degraded but at a slower rate. Non-*o*-diphenolic anthocyanins should not be susceptible to coupled oxidation but may react with quinones or secondary products of oxidation to form copolymers. More recently, Sarni et al. (1995) studied the mechanism of anthocyanin degradation using model solutions. The authors showed that cyanidin 3-glucoside (Cy 3-glc; *o*-diphenolic anthocyanin) was degraded by a coupled oxidation mechanism, whereas malvidin 3-glucoside (non-*o*-diphenolic anthocyanin) formed an adduct with caffeoyltartaric *o*-quinone. A mechanism of anthocyanin degradation in grape must-like model solutions is proposed by the authors.

The purpose of this work was to study the mechanism of anthocyanin degradation using a purified anthocyanin (Cy 3-glc). The degradation of the different substrates was monitored using HPLC and polarographic methods. The use of model systems and complementary analysis methods should be useful in understanding the mechanism of anthocyanin degradation.

MATERIALS AND METHODS

Fruits. About 3.0 kg of ripe blueberries of the Coville variety were harvested during July 1994 in a commercial plantation in northeastern France (Vosges). Sample ripeness was judged on the basis of skin color of representative berries. The berries were cleaned and then packaged in polyethylene containers (4 °C). The fruits were quickly frozen and stored in the dark at −25 °C under nitrogen.

Chemicals. Cy 3-glc (Kuromanin, HPLC grade) was purchased from Extrasynthèse (Genay, France). CG (HPLC grade) and the bicinchoninic acid (BCA) protein assay reagent were obtained from Sigma Chemicals (St. Quentin Fallavier, France). Methanol (HPLC grade) and formic acid (99% of purity) were obtained from Merck (Darmstadt, Germany). All other chemicals were of reagent grade from Merck. Cy 3-glc (1 mM) and CG (1 mM) were dissolved individually in McIlvaine buffer, pH 3.5.

Enzyme Extraction. The blueberry PPO was extracted according to the procedure (acetone powder plus ultrafiltration) described by Kader et al. (1997a)

Assay for PPO Activity. PPO activity was determined according to a polarographic method using a Gilson Oxygraph equipped with a Clark electrode fitted in a 3.1-mL jacketed cell at 25 °C. The oxygen uptake was measured in the presence of CG at a final concentration of 0.1 mM in McIlvaine buffer, pH 3.5. PPO activity was expressed as the nanomoles of oxygen consumed per second (nanokatals, nkat) in the assay conditions. The reaction was started with 15.5 μL of blueberry PPO (0.194 nkat mL^{-1}, 12.1 nkat mg^{-1} of protein). Before the enzyme extract was added, the solution (CG plus McIlvaine buffer) was stirred for 20 min to saturate the solution with oxygen. The initial oxygen concentration of air-saturated solutions, used to calibrate the Clark electrode, was determined according to the Winkler method as described by Green and Hill (1984) and found at 1 μmol ± 0.05 of O_2 in 3.1 mL (total volume of the cell).

Protein concentration (3.2 mg mL^{-1}) was determined using BCA as a specific reagent (Smith et al., 1985). Bovine serum albumin was used as a standard.

Model System of the Degradation of Cy 3-Glc. Reaction mixture final volumes were 3.1 and 1.0 mL, respectively, for the polarographic and HPLC methods. The assays contained PPO (0.194 nkat mL^{-1}), CG (0.1 mM, final concentration) alone or in combination with Cy 3-glc (0.1 mM, final concentration), and McIlvaine buffer, pH 3.5. The polarographic assays were carried out at 25 °C using a Gilson Oxygraph equipped with a Clark electrode. All of the assays were carried out in air-saturated solutions agitated with a magnetic stirrer. The control solutions (i.e., PPO-free, CG-free) were prepared and incubated under the same conditions of temperature and stirring.

Enzymatic oxidation of CG was also monitored spectrophotometrically at 400 nm using a Shimadzu UV-260 spectrophotometer. The CG (0.1 mM) was oxidized by PPO (0.194 nkat mL^{-1}) in McIlvaine buffer, pH 3.5, at 25 °C.

For the HPLC analysis, a reaction mixture was prepared for each incubation time. The reaction was monitored as a function of time (25 °C) and was stopped by adding 0.1 mL of 20% (w/v) trichloroacetic acid. All of the samples were analyzed immediately by HPLC (Merck-Hitachi L-6200 intelligent pump equipped with a diode array detector, Merck-Hitachi L-3000 connected to a Chromojet integrator). The constituents of the reaction mixture were separated on a reaction mixture Lichrosorb 100 RP-18 reversed-phase column (Merck) (250 × 4 mm, 5 μm) using a mobile phase consisting of water/formic acid, 90:10 v/v (solvent A), and methanol (solvent B). The conditions were 10–20% B in A in 5 min followed by 20–50% B in A in 20 min. Elution was performed at a flow rate of 1.0 mL min^{-1}, and 0.1 mL of the reaction mixture was injected using a Basic$^+$ Marathon automatic injector (Spark, Holland). Cy 3-glc and CG were detected at 510 and 325 nm, respectively. All separations were performed at 22 °C. All solvents were of HPLC grade. The results were expressed as a percentage of the initial concentration of each substrate.

For each assay three analyses were conducted on duplicate experimentations. Each data point is the mean of six measurements.

RESULTS AND DISCUSSION

We have previously studied the mechanism of browning that occurs after fresh Highbush blueberry fruits are crushed and demonstrated that PPO, CG, and anthocyanin play an important role in the browning reactions (Kader et al., 1997b). We then investigated the mechanisms of anthocyanin degradation in the presence of PPO and CG using model solutions with pure substrates.

Sakuraba and Ichinose (1982) suggested that the rate of anthocyanin degradation by PPO in the presence of *o*-diphenolic substrates such as CG depends on the hydroxylation pattern of the B-ring. With this in mind, we investigated the degradation of Cy 3-glc (*o*-diphenolic anthocyanin) by PPO in the presence of CG.

Degradation of CG Alone by the Enzymatic Extract (PPO). The degradation of CG by PPO was examined using spectrophtometric, HPLC, and polarographic methods. Figure 1 (curve a) shows that CG is oxidized into the corresponding quinone, which presents maximum absorption at 400 nm. Adding ascorbic acid after 2 min of reaction induced an instantaneous

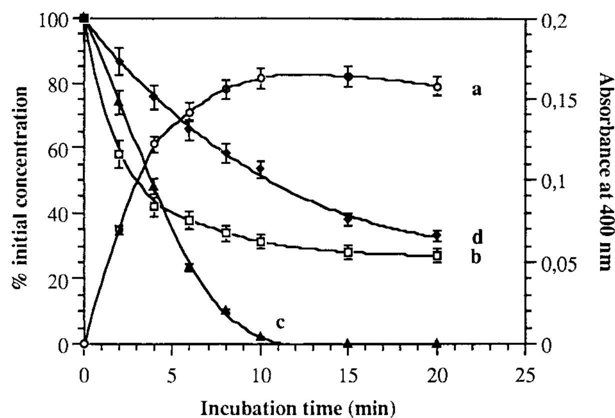


Figure 1. Relative degradation kinetics of (▲) Cy 3-glc (0.1 mM) in the presence of CG (0.1 mM), (◆) CG (0.1 mM) in the presence of Cy 3-glc (0.1 mM), (□) CG alone (0.1 mM), in model solutions containing blueberry PPO (0.194 nkat mL^{-1}) at pH 3.5, and (○) CG *o*-Q formation by spectrophotometric analysis at 400 nm during oxidation of 0.1 mM CG in McIlvaine buffer at pH 3.5 with blueberry PPO (0.194 nkat mL^{-1}). Each data point is the mean of six determinations. Bars indicate standard deviation.

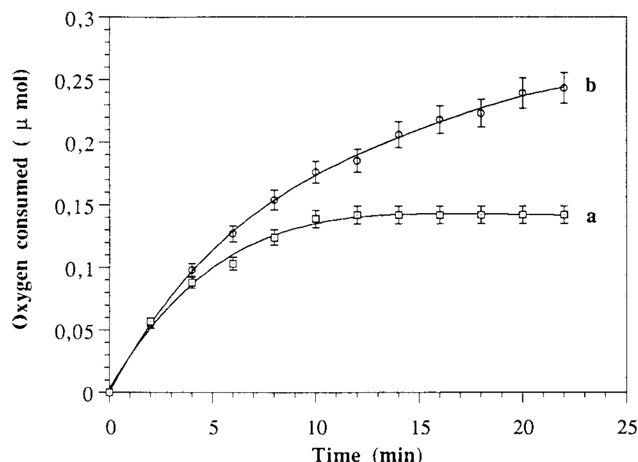


Figure 2. Kinetics of oxygen uptake in the different model systems: (□) CG + PPO (0.194 nkat mL^{-1}); (○) Cy 3-glc + CG + PPO (0.194 nkat mL^{-1}). All of the substrates were at a final concentration of 0.1 mM. Oxygen consumption was monitored using an Oxygraph K-IC (Gilson) equipped with a Clark electrode at 25 °C. Each data point is the mean of six determinations. Bars indicate standard deviation.

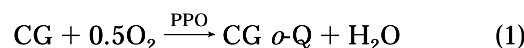
bleaching (Kader et al., 1997b). In the presence of excess reducing agent, *o*-quinones are reduced to their original *o*-diphenols (Rouet-Mayer et al., 1990). In the presence of enzymatic extract, CG decreases rapidly (Figure 1, curve b). The observed decrease does not involve hydrolysis as none of the hydrolysis products (caffeic acid) could be detected in the reaction mixture.

After 20 min of reaction, only 27% of the CG remained in the reaction mixture. It should be emphasized that CG is not completely oxidized by the PPO, and this is probably due to the inhibitory effect of the oxidation products formed by PPO (Sanderson 1965; Raynal and Moutounet 1989). However, Cheynier et al. (1989) have shown that PPO is not inactivated by the quinones or the condensation products. The authors concluded that caftaric acid oxidation was protected by condensation products either because they compete as substrate for the PPO or because caftaric acid is regenerated from its quinone in their coupled oxidation. In our laboratory we observed that CG oxidation by purified blueberry PPO produced a degradation of only 50%, whereas 70% oxidation was obtained when a nonpurified PPO was used. These results indicate that PPO in the nonpurified extract is probably protected by non-PPO proteins, which react with the oxidized polyphenols, thereby sparing the PPO. This aspect is not well-known and still needs clarification.

The degradation of CG was also monitored by measuring oxygen uptake. It can be seen from Figure 2 (curve a) that CG degradation consumed molecular oxygen, which confirms the presence of PPO activity in the enzymatic extract. The decrease in CG is followed by a concomitant increase in oxygen uptake. After ~10 min, the reaction had not consumed any oxygen, while a small part of the CG continued to disappear. CG *o*-quinone (CG *o*-Q) was able to react with the CG by a mechanism analogous to a Michael 1,4 addition (Cheynier and Moutounet, 1992; Richard-Forget et al., 1992). Thus, a small amount of CG was degraded by a nonoxidative mechanism, and this reaction was able to take place as soon as the quinone was formed. Richard-Forget et al. (1992) have observed the same behavior during the oxidation of CG by apple PPO. The importance of this reaction is probably variable from one phenol to another depending on both the instability of the *o*-quinone and the pH of the reaction mixture. Such reactions involving *o*-quinones and their originating phenols have already been described (Cheynier and Moutounet, 1992; Fulcrand et al., 1994).

We have established the stoichiometry of the reaction. The oxidation of CG by the PPO involved the consumption of 0.63 µmol of O_2/µmol of CG oxidized. Richard-Forget et al. (1992) studied the oxidation of CG by apple PPO. During the initial period of oxidation (0−3 min), the stoichiometry was found at 0.5 µmol of O_2/µmol of CG (at pH 4.0), which is close to our value of 0.45. According to the authors, the stoichiometry depends on many factors such as the nature of the phenol oxidized, the chemical conditions of the reaction, and the ratio of enzyme to substrate, which influence the relative rates among enzymatic and nonenzymatic reactions. Pierpoint (1966) studied the oxidation of CG by tobacco PPO. This author determined a stoichiometry of 0.80 µmol of O_2/µmol of CG oxidized. The observed differences in the stoichiometry could be attributed to the method used. Moreover, it is important to emphasize that Pierpoint (1966) determined the amount of CG remaining in the oxidized solutions by thin-layer chromatograpy (TLC), and in these conditions no CG has been detected in the reaction mixture after oxidation. However, HPLC is more sensitive and also more accurate than TLC, which could explain the observed difference in stoichiometry. In the case of caftaric acid, 0.7 mol of oxygen was consumed per mole of caftaric acid oxidized (Cheynier and Van Hulst, 1988).

According to reaction 1, the oxidation of CG into the corresponding quinone should consume 0.5 µmol of O_2 per micromole of CG.



$$CG + 0.5O_2 \xrightarrow{PPO} CG\ o\text{-}Q + H_2O \qquad (1)$$

The oxidation of CG consumed 0.142 µmol of oxygen (Figure 2, curve a) instead of 0.113 µmol (27% of the initial CG remained in the reaction mixture, and a total of 0.31 µmol of CG was used for the oxidation). The additional oxygen uptake (0.029 µmol = 0.142−0.113) measured in the case of CG oxidation was probably necessary to oxidize intermediate products.

Oszmianski and Lee (1990) studied the oxidation of CG by PPO (tyrosinase). The reactions were monitored by HPLC, and the authors have detected six oxidation products that have elution times longer than that of CG. However, no information is provided on the structures of these oxidation products.

Degradation of Cy 3-Glc by PPO in Model Solutions. The degradation of Cy 3-glc was monitored by HPLC analysis, and polarographic assays were carried out to measure concomitant O_2 consumption. Cy 3-glc alone is not oxidized by PPO since no molecular oxygen was consumed. It is well-known that anthocyanins are poor substrates of PPO, and this is probably due to the presence of the sugar moiety causing steric hindrance, since the aglycon forms are often oxidized by PPO (Mathew and Parpia, 1971). Raynal and Moutounet (1989) reported that PPO extract from plum exocarp has no effect on the degradation of cyanidin 3-rutinoside (the main pigment of plum exocarp). Furthermore, Sakamura and Obata (1963) showed that eggplant PPO could oxidize delphinidin 3-(*p*-coumaroylrutinoside)-5-glucoside (nasunin) but is unable to oxidize pelargonidin 3-glucoside. On the other hand, Wesche-Ebeling and Montgomery (1990) observed the degradation of Cy 3-glc (20% rate of degradation) and pelargonidin 3-glucoside (5%) in model systems containing PPO plus anthocyanins. The low degradation rate is probably due to the presence of contaminating aglycon forms in the pigment solutions. More recently, Sarni et al. (1995) showed that grape anthocyanins are not oxidized directly by PPO.

The degradation of Cy 3-glc was studied by incubating equimolar concentrations of CG and Cy 3-glc (0.1 mM, final concentration) with blueberry PPO extract. In the presence of CG and PPO, Cy 3-glc was very rapidly degraded. After ~10 min, the pigment could not be detected in the reaction mixture (Figure 1, curve c), and after 20 min, the reaction mixture turned brown. The presence of CG induced the oxidation of the pigment. If it is considered that Cy 3-glc degradation proceeds by a coupled oxidation mechanism (Sarni et al., 1995), we can assume that the rate of pigment degradation depends on the rate of CG *o*-Q formed from the CG oxidation by PPO. This suggests that the Cy 3-glc oxidation curve is very similar to the CG oxidation curve.

After 4 min of reaction, half the Cy 3-glc is degraded. At the same time the CG *o*-Q is reduced to give half the expected amount of CG (Figure 1, curve d). Consequently, the ratio of degraded Cy 3-glc to oxidized CG is equal to 2. The remaining CG is higher than that observed during the oxidation of CG alone by PPO. Between 4 and 10 min the reaction continues with the same reaction pattern as described before. However, we can observe that the ratio of degraded Cy 3-glc to oxidized CG increased slightly, meaning that secondary reactions which consume oxygen can take place.

In fact, the amount of oxygen consumed after 4 min of reaction (3.7 min) in the model system CG + Cy 3-glc + PPO is the same as that observed in the enzymatic oxidation of CG alone (Figure 2, curves a and b). Under these conditions, the amount of oxygen calculated from the CG *o*-Q formed in the presence of Cy 3-glc is $(0.310 \times 0.5) \times 0.5 = 0.078$ μmol of oxygen, whereas the amount of oxygen consumed is 0.090 μmol (experimental data). This shows that the additional oxygen uptake measured, $0.090 - 0.078 = 0.012$ μmol in the case of CG + Cy 3-glc + PPO, is in fact necessary to oxidize products from secondary reactions. The same phenomenon can be observed between 4 and 10 min. The amount of O_2 consumed is 0.177 μmol instead of 0.155 μmol, as expected from a predominant mechanism of coupled oxidation.

From 10 to 20 min, this step is characterized by two observations: (a) The initial Cy 3-glc is totally transformed into degradation derivates that can be oxidized either enzymatically (PPO + oxygen) or by CG *o*-Q involving coupled oxidation with regeneration of CG. (b) After 10 min, 53% of CG remained in the reaction mixture (CG + Cy 3-glc + PPO). In these conditions two paths can be considered. First, if the Cy 3-glc/Cy 3-glc degradation derivative couple has a lower redox potential than the CG/CG *o*-Q couple, the *o*-diphenolic derivatives of Cy 3-glc can be subjected to coupled oxidation with CG *o*-Q that delays the oxidation of CG. Second, if the *o*-diphenolic derivatives of Cy 3-glc formed from the degradation of the Cy 3-glc did not react with the CG *o*-Q, then the enzymatic oxidation of the remaining CG could proceed as observed in the case of CG alone. To confirm one of these hypotheses, we compared the amount of oxygen consumed by the model system CG + Cy 3-glc + PPO between 10 and 20 min and the amount of oxygen consumed by the enzymatic oxidation of CG alone at a concentration close to that observed in the CG + Cy 3-glc + PPO model system after 10 min (in this model system 50% of the CG remained after 10 min of reaction). Between 3 and 20 min, in the case of CG alone, 0.07 μmol of oxygen was consumed and 23% of CG was oxidized. Between 10 and 20 min, in the case of the CG + Cy 3-glc + PPO model system 0.06 μmol of oxygen was consumed and 18% of CG was oxidized. These results show that the dominant oxidative process involves the enzymatic oxidation of the remaining CG when all of the pigment has disappeared.

The results indicate that Cy 3-glc degradation is possible only in the presence of CG. The degradation rate of CG was apparently slower in the presence of Cy 3-glc than in its absence (Figure 1, curves b and d). This sparing effect does not mean that the oxidation of CG in the presence of Cy 3-glc is slowed but, on the contrary, is globally facilitated. The same behavior was observed by Raynal and Moutounet (1989), Oszmianski and Lee (1990), and Sarni et al. (1995). According to these authors, this effect is attributed to coupled oxidation mechanisms, which are not observed with non-*o*-diphenolic anthocyanins such as malvidin 3-glucoside (Cheynier et al., 1994; Sarni et al., 1995).

The Cy 3-glc degradation proceeded to a great extent by coupled oxidation mechanisms involving the CG *o*-Q formed by oxidation of CG by PPO (reaction 2). This reaction led to a complete discolorization of the reaction mixture.

$$\text{CG } o\text{-Q} + \text{Cy 3-glc} \rightarrow \text{CG} + \text{Cy 3-glc } o\text{-quinone} \qquad (2)$$

During the first step of the reaction (0–10 min) the ratio of degraded Cy 3-glc to oxidized CG is relatively constant and equal to 2, which means that for 1 μmol of CG oxidized into quinone 2 μmol of Cy 3-glc are degraded. To improve these experimental data, it is necessary to consider that the oxidation of 1 μmol of CG into 1 μmol of CG *o*-Q is accompanied by the reaction of 0.5 μmol of CG *o*-Q with 0.5 μmol of Cy 3-glc, which leads to the regeneration of 0.5 μmol of CG. In these conditions, the curve of Cy 3-glc degradation (Figure 1, curve c) shows a complex reaction process of condensa-

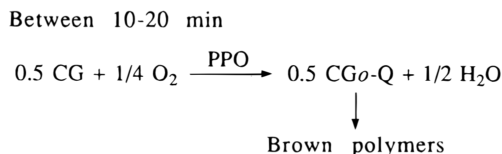
tion. This was accompanied by the disappearance of the Cy 3-glc after 10 min of reaction into colorless products as well as half of the CG.

The curve of Cy 3-glc degradation corresponds to the curve of CG oxidation into CG *o*-Q, which assumes that the oxidative reaction CG + Cy 3-glc + PPO is accompanied by the consumption of 0.5 mol of oxygen followed by the complete degradation of the Cy 3-glc and the regeneration of all the CG (reaction 2). In fact, only half of the CG is regenerated; therefore, it has to be accepted that the reaction process (reaction 2) is accompanied by secondary reactions of condensation between Cy 3-glc, Cy 3-glc *o*-quinone, and CG *o*-Q, which lead to a number of combinations with a global stoichiometry that corresponds to 1CG *o*-Q/1Cy 3-glc/1Cy 3-glc *o*-Q. This reaction process is accompanied by secondary reactions of oxidation that consume oxygen. These reactions could well explain the additional oxygen uptake by the reaction.

In summary, in a model system containing equimolar concentrations of CG and Cy 3-glc, the degradation of Cy 3-glc is faster than the oxidation of CG into CG *o*-Q, because half of the latter is regenerated during the first step of the reaction (0−10 min), meaning that part of the CG (50%) is incorporated into condensation products of the pigment. During this step (0−10 min) no brown product could be detected in the reaction mixture.

Sarni et al. (1995) studied the degradation of Cy 3-glc in the presence of both caftaric acid and grape PPO (*Vitis vinifera* var. Grenache Blanc) at pH 3.6. These authors analyzed the oxidation product of Cy 3-glc by HPLC. The HPLC profiles showed that the new products formed were of intermediate polarity. The UV−Visible spectra of the oxidation products from Cy 3-glc showed that they contained both anthocyanin and caftaric acid moieties. The ratio of degraded Cy 3-glc to caftaric acid incorporated in the condensation products was approximately 2.5, which is not that different from our value (2.0). The degradation of Cy 3-glc in the presence of caftaric acid involves the formation of anthocyanin−caftaric acid adducts and anthocyanin−anthocyanin products.

When all of the Cy 3-glc has disappeared from the reaction mixture, the degradation of the remaining CG (50%) continues as observed in the case of the oxidation of CG alone. Between 10 and 20 min the oxidation



degradation of the remaining CG by PPO continues at the same rate as that observed in the model system containing CG plus PPO for the same concentration of CG. This process of oxidation is accompanied by the formation of brown products. The PPO activity is slowed by the formation of condensation products CG *o*-Q/CG, whereas PPO in the model system Cy 3-glc + CG is not inhibited by the products of the reaction, which are different from the one formed during the oxidation of CG alone. However, after 10 min (second step of the reaction), these products can be formed by oxidation of the remaining CG, which leads to the inhibition of the PPO.

The browning reaction is mainly due to the formation of polycondensation products resulting from the oxidation of the CG and probably not to the degradation products of Cy 3-glc.

In conclusion, the degradation of Cy 3-glc did not occur in the presence of PPO alone, but the addition of CG (the main hydroxycinnamic acid derivative of blueberry fruit) led to the degradation of the Cy 3-glc. Cy 3-glc is oxidized by a coupled oxidation mechanism involving CG *o*-Q generated by PPO in the presence of CG. The ratio of degraded Cy 3-glc to oxidized CG is relatively constant and equal to 2, which means that part of the CG is incorporated into degradation products of Cy 3-glc. A mechanism is proposed for the oxidative degradation of Cy 3-glc in the presence of CG and blueberry PPO. Actually, we have investigated the synthesis and the purification of the quinone. The use of a pure solution of quinone may be useful to determine the exact mechanism of degradation. The preparation of pure quinone by chemical oxidation should allow simplification of the model reactions. It will be easier to understand the mechanism of Cy 3-glc degradation and to specify the contribution of CG *o*-Q respectively to the oxidation of Cy 3-glc and to the process of condensation with the flavylium structure. The characterization of the reaction products should be another way of investigation.

ABBREVIATIONS USED

Cy 3-glc, cyanidin 3-glucoside; Cy 3-glc *o*-Q, cyanidin 3-glucoside *o*-quinone; CG, chlorogenic acid; CG *o*-Q, chlorogenoquinone; PPO, polyphenol oxidase.

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