JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Kinetic Characterization of the Oxidation of Chlorogenic Acid by Polyphenol Oxidase and Peroxidase. Characteristics of the *o*-Quinone

J. MUÑOZ,† F. GARCIA-MOLINA,† R. VARON,§ J. N. RODRIGUEZ-LOPEZ,† P. A. GARCÍA-RUIZ,# F. GARCÍA-CÁNOVAS,*,† AND J. TUDELA†

GENZ: Grupo de Investigación de Enzimología, Departamento de Bioquímica y Biología Molecular-A, Facultad de Biología, Universidad de Murcia, E-30100 Espinardo, Murcia, Spain; Departamento de Química-Física, Escuela Politécnica Superior, Universidad de Castilla la Mancha, Avenida España s/n, Campus Universitario, E-02071 Albacete, Spain; and Departamento de Quimica Orgánica, Facultad de Quimica, Universidad de Murcia, E-30100 Espinardo, Murcia, Spain

Chlorogenic acid is the major diphenol of many fruits, where it is oxidized enzymatically by polyphenol oxidase (PPO) or peroxidase (POD) to its *o*-quinone. In spectrophotometric studies of chlorogenic acid oxidation with a periodate ratio of $[CGA]_0/[IO_4^-]_0 < 1$ and $[CGA]_0/[IO_4^-]_0 > 1$, the *o*-quinone was characterized as follows: λ_{max} at 400 nm and $\epsilon = 2000$ and 2200 M^{-1} cm^{-1} at pH 4.5 and 7.0, respectively. In studies of *o*-quinone generated by the oxidation of chlorogenic acid using a periodate at ratio of $[CGA]_0/[IO_4^-]_0 > 1$, a reaction with the remaining substrate was detected, showing rate constants of $k = 2.73 \pm 0.17$ M^{-1} s^{-1} and $K' = 0.05 \pm 0.01$ M^{-1} s^{-1} at the above pH values. A chronometric spectrophotometric method is proposed to kinetically characterize the action of the PPO or POD on the basis of measuring the time it takes for a given amount of ascorbic acid to be consumed in the reaction with the *o*-quinone. The kinetic constants of mushroom PPO and horseradish POD are determined.

KEYWORDS: Chlorogenic acid; polyphenol oxidase; peroxidase; ascorbic acid; *o*-quinone; molar absorptivity

INTRODUCTION

Chlorogenic acid (CGA) is the major phenolic compound found in many fruits, including apples, pears, cherries, and apricots (*1*). The product resulting from the enzymatic oxidation of CGA, the *o*-quinone (CGA-Q), has been reported to oxidize other polyphenols, such as flavans, by a coupled oxidation reaction (*1*). The enzymatic oxidation of polyphenols is important because polyphenols are the precursors of several biosynthetic pathways, including those of tannins, lignins, and melanins (*2*).

The browning damage caused to the tissues of fruits and vegetables during postharvest handling and processing is one of the main causes of quality loss (*3*). The main enzymes involved in these processes are polyphenol oxidase (EC 1.14.18.1; PPO) and peroxidase (EC 1.11.1.7; POD) (*4*).

Because CGA is the major component of many fruits, its enzymatic oxidation has attracted much attention. For example, it has been demonstrated that tobacco leaf and mushroom PPOs oxidize CGA at different velocities of catalysis and with different affinities (*5*).

The product formed by the enzymatic oxidation of CGA by PPO and POD is CGA-Q, directly in the case of PPO (*6*) and through the disproportion reaction of the semiquinone in the case of POD (*7*).

The evolution of CGA-Q has been studied by several authors (*6*−*9*). For example, it has been shown that CGA-Q generates H_2O_2 in the medium and CGA (*7*, *8*), which suggests that POD may be involved in the process of enzymatic browning, the enzyme working at the expense of the H_2O_2 generated by PPO in its action on CGA (*7*). The production of H_2O_2 in the melanogenesis pathway associated with L-dopa and dopamine oxidation has also been revealed (*10*).

However, the great instability of the CGA-Q has hindered its characterization. Very low absorptivity coefficients on the order of 970 M^{-1} cm^{-1} have been proposed (*8*), as have several pathways for CGA-Q evolution from the addition of water to generate a trihydroxylated compound, which would be oxidized by another molecule of CGA-Q (*6*, *9*), to the reaction of CGA-Q with molecules of substrate to generate polymers (*9*).

Spectrophotometric measurements of PPO or POD enzymatic activity on CGA may give erroneous results, precisely because of the instability of the CGA-Q, for which reason several authors

* Corresponding author (fax +34 968 363963; e-mail canovasf@um.es).
† Departamento de Bioquímica y Biología Molecular-A, Universidad de Murcia.
§ Departamento de Química-Física, Universidad de Castilla la Mancha.
# Departamento de Quimica Orgánica, Universidad ded Murcia.

10.1021/jf062081+ CCC: $37.00
Published on Web 01/17/2007

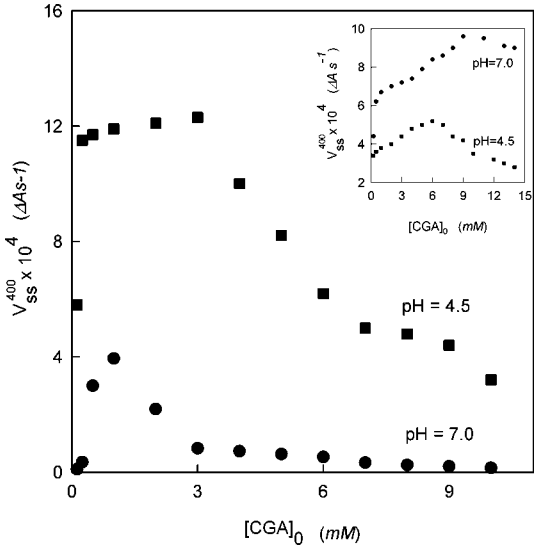


Figure 1. Representation of the rate of oxidation of CGA by PPO and POD. The velocity was monitored by measuring the variation in absorbance at 400 nm. Experimental conditions: phosphate buffer, 30 mM, pH 7.0; acetate buffer, 30 mM, pH 4.5, at 25 °C; [POD] = 2 nM; $[H_2O_2]_0 = 0.25$ mM. (Inset) To measure the rate of oxidation of CGA by PPO at the same pH values, the experimental conditions were the same except $[PPO]_0 = 35$ nM.

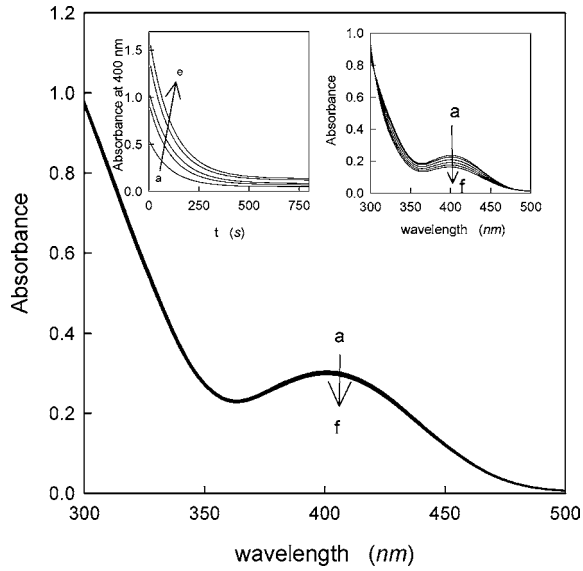


Figure 2. Oxidation of CGA by periodate at ratios of $[CGA]_0/[IO_4^-]_0 < 1$. Experimental conditions: $[CGA]_0 = 0.15$ mM; $[IO_4^-]_0 = 2.25$ mM; acetate buffer, 30 mM, pH 4.5. (Right inset) Oxidation in phosphate buffer, 30 mM, pH 7.0, $[CGA]_0 = 0.11$ mM; $[IO_4^-]_0 = 2.25$ mM. The measurements were made every 60 s. (Left inset) To measure the evolution of the CGA-Q generated by oxidation with periodate at ratios of $[CGA]_0/[IO_4^-]_0 < 1$, the experimental conditions were as follows: phosphate buffer, 30 mM, pH 7.0; $[IO_4^-]_0 = 7.5$ mM; and $[CGA]_0$ was varied [(a) 0.3 mM; (b) 0.45 mM; (c) 0.6 mM; (d) 0.75 mM; (e) 0.9 mM].

have proposed kinetic mechanisms of inhibition by excess of substrate (*1*, *2*, *11*−*14*). In the same way, studies involving the inhibition of mushroom PPO using CGA as substrate have concluded that the type and degree of inhibition depend not only on the inhibitor but also, and especially, on the method used to measure the enzymatic activity, which may be spectrophotometric (following the increase in absorbance at 420 nm)

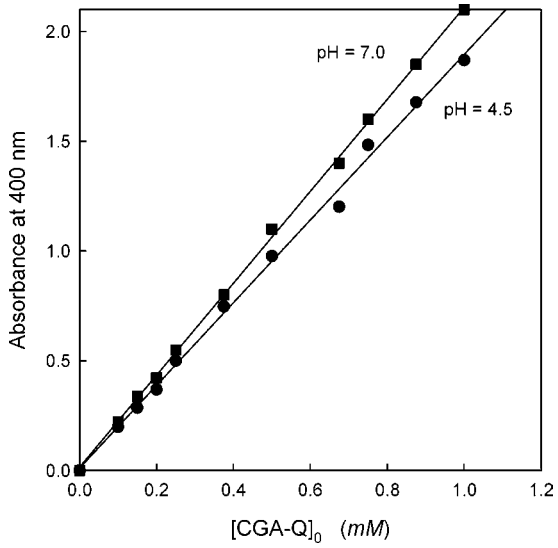


Figure 3. Representation of absorbance versus concentration of CGA-Q. Values of absorbance $\lambda = 400$ nm were obtained by oxidation with periodate at ratios of $[CGA]_0/[IO_4^-]_0 < 1$. Experimental conditions: acetate buffer, 30 mM, pH 4.5; phosphate buffer, 30 mM, pH 7.0. The concentration of $[IO_4^-]_0$ was 1.5 mM, and CGA concentration was the same as CGA-Q.

Scheme 1

$$\text{CGA-Q+CGA} \xrightarrow{k} \text{P}$$

or polarographic (monitoring oxygen consumption using a Wilson oxygraph equipped with a Clark electrode) (*15*). The results described above can be explained by the instability of the CGA-Q.

Because CGA is the major compound in many fruits, several methods have been designed to monitor PPO activity, sometimes termed chlorogenic acid oxidase, measuring the disappearance of chlorogenic acid at 325 nm (*1*). A method based on differential spectrophotometry has been designed for PPO, whereby the reaction is followed in the ultraviolet wavelength range during enzymatic polyphenol oxidation (*16*). Although measurements obtained in this way closely agree with the measurements of oxygen uptake done with the manometric method, the method can be applied only at low concentrations of CGA (*16*).

The study of CGA and its products is important because of the abundance of this compound: for example, 3.4−14 mg/100 mg of fresh weight in potatoes, 12−31 mg/100 mL in apple juice, 89 mg /100 mg in dry tea shorts, and 250 mg per cup of coffee (*17*, *18*). Furthermore, it has been attributed anticarcinogenic (*19*, *20*), antimutagenic (*21*), and antiinflammatory (*22*) properties. Recently, too, the antinitrosating properties of CGA, which serves as an efficient nitrite scavenger and a suppresser of nitrosamine formation in the gastric compartment (*23*−*25*), have also been emphasized. The formation of CGA adducts with glutathiones has also been characterized (*26*).

In this work, we study some of the properties of CGA-Q and some kinetic characteristics of its evolution; we also propose a chronometric spectrophotometric measurement method that kinetically characterizes the enzymes responsible for its oxidation, including PPO and POD.

MATERIALS AND METHODS

Materials. Chlorogenic acid, mushroom tyrosinase (3320 units/mg), and horseradish peroxidase (HRP isoenzyme C type IV, 280 units/mg)

were obtained from Sigma Chemical Co. H_2O_2 was obtained from Scharlau (Madrid, Spain). The substrate was prepared in dilute phosphoric acid.

Other Methods. Protein was determined according to Bradford's method using bovine serum albumin as standard (*27*).

Determination of Enzymatic Activity. Enzymatic activity was determined spectrophotometrically using the method described under Results and Discussion. The only condition is that there must be an absorbance increase (due to product formation) that can be measured once the ascorbic acid is consumed. Preliminary experiments were carried out in the absence of ascorbic acid with the PPO/O_2/CGA and POD/H_2O_2/CGA systems, recording spectrophotometric scans to identify in which zone of the spectrum, particularly the visible zone, the greatest changes in absorbance occurred. The wavelength chosen at both acid and neutral pH was 400 nm. Peroxidase activity was followed in the same way.

Kinetic Data Analysis. Initial rate values (V_0) were calculated from triplicate measurements at each reducing substrate concentration. The reciprocals of the variances of V_0 were used as weighting factors in the nonlinear regression fitting of V_0 versus $[CGA]_0$ data to the Michaelis−Menten equation. The fitting was carried out using Marquardt's algorithm (*28*) implemented in the Sigma Plot 9.0 program for Windows (*29*).

NMR Assays. ^{13}C NMR spectra of chlorogenic acid were recorded at pH 4.5 and 7.0 on a Varian Unity spectrometer (300 MHz) using ^2H_2O as solvent. Chemical shift values (δ) were determined relative to those of tetramethylsilane ($\delta = 0$). The maximum accepted error for each peak was ± 0.1 ppm.

RESULTS AND DISCUSSION

Enzymatic Oxidation of Chlorogenic Acid. CGA is the main acid found in fruits, including apples and pears (*1*). Peroxidase catalyzes the oxidation of the *o*-diphenols to semiquinones, which disproportionate to generate the corresponding *o*-quinone, regenerating substrate at the same time (*30*). The enzymatic oxidation of CGA by POD/H_2O_2 and PPO/O_2 transforms the substrate into one product with a maximum at 400 nm. CGA is oxidized by PPO to its corresponding *o*-quinone (CGA-Q), which shows a maximum at 400 nm (*8*). When the enzymatic reaction is followed at this wavelength, during the oxidation of CGA both with POD/H_2O_2 and with PPO/O_2, the results shown in **Figure 1** and its inset are obtained. The explanation of the results shown in **Figure 1** has been given as an inhibition by excess of substrate (*1*, *2*, *11*−*14*). However, the characteristics of *o*-quinones described in other works (*31*) led us to look at the instability of the CGA-Q and its possible control to enable the kinetic characterization of this important substrate. For this, we carried out the following series of experiments.

Oxidation of CGA by Sodium Periodate. It is known that sodium periodate oxidizes different *o*-diphenols in a way similar to that followed by PPO (*32*). Because our aim was to characterize POD and PPO, which can show pH maxima running from acidic (pH 4.5), in the case of POD, to close to neutrality (pH 7.0) for mushroom PPO (*33*), oxidations were carried out using sodium periodate at both pH values to characterize CGA-Q.

Oxidation of CGA with Periodate at a Ratio of $[CGA]_0/[IO_4^-]_0 < 1$. When oxidation was carried out at pH 4.5 with $[CGA]_0/[IO_4^-]_0 < 1$, the spectra of CGA-Q showed a maximum at $\lambda = 400$ nm. Successive scans indicated that in these conditions CGA-Q is stable during the measurement time. However, at pH 7.0, the CGA-Q evolves to generate two isosbestic points, indicating stoichiometric evolution (**Figure 2**, right inset). This behavior might be the result of attack by a hydroxyl or by another *o*-quinone (*6*), and the adduct would be freshly oxidized by periodate (**Figure 3**).

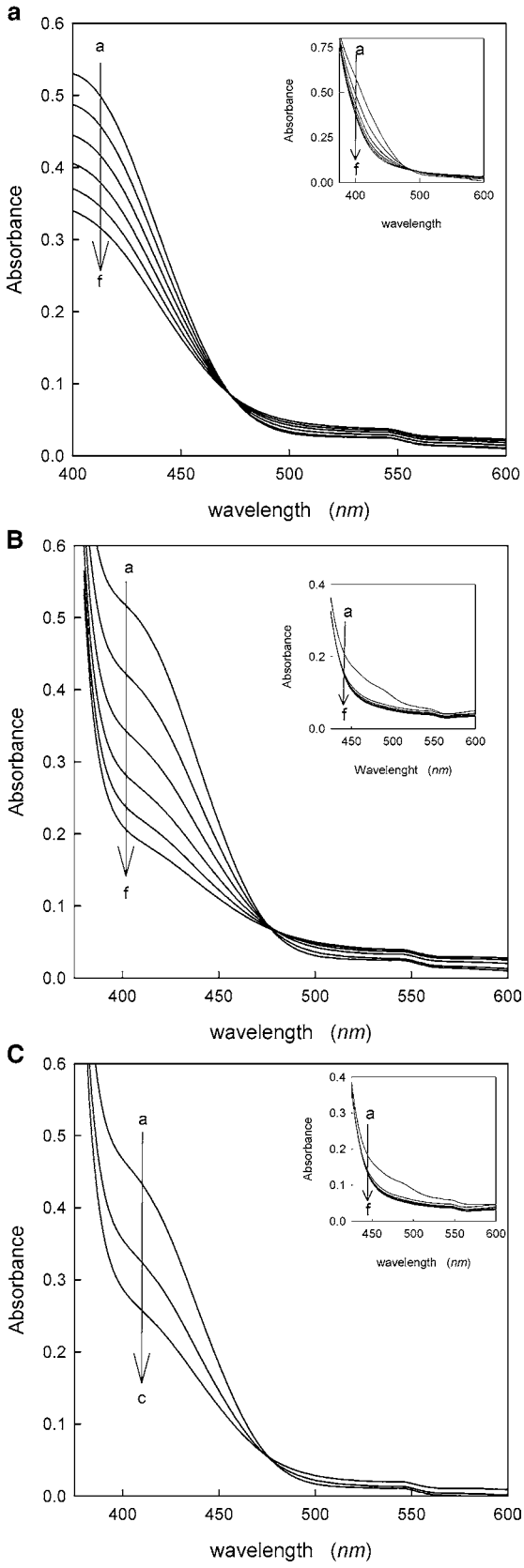


Figure 4. Spectrophotometric scans of CGA-Q generated with periodate at different $[CGA]_0/[IO_4^-]_0$ ratios: (**A**) $[CGA]_0/[IO_4^-]_0$ ratio = 2, $[CGA]_0$ = 0.5 mM, pH 4.5, acetate buffer, 30 mM [(inset) pH 7.0, phosphate buffer, 30 mM]; (**B**) $[CGA]_0/[IO_4^-]_0$ ratio = 6, $[CGA]_0$ = 1.5, pH 4.5 [(inset) pH 7.0]; (**C**) $[CGA]_0/[IO_4^-]_0$ ratio = 8, $[CGA]_0$ = 2 mM, pH 4.5 [(inset) pH 7.0].

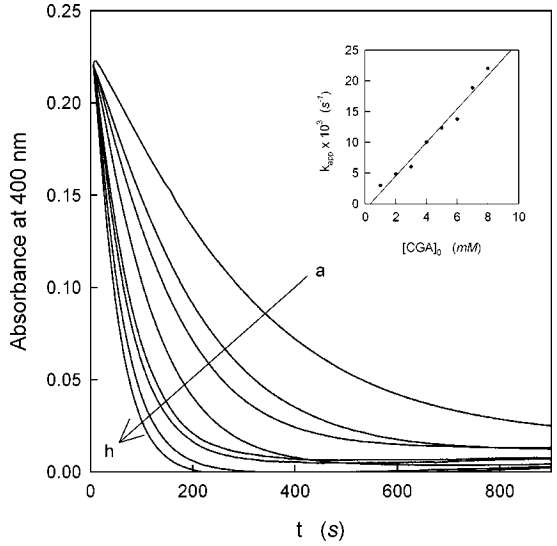


Figure 5. Kinetic of evolution of CGA-Q generated by oxidation with periodate at ratios of $[CGA]_0/[IO_4^-]_0 > 1$. Experimental conditions: acetate buffer, 30 mM, pH 4.5, $[IO_4^-]_0 = 0.12$ mM. Concentrations of $[CGA]_0$: (a) 1 mM; (b) 2 mM; (c) 3 mM; (d) 4 mM; (e) 5 mM; (f) 6 mM; (g) 7 mM; (h) 8 mM. (Inset) Representation of apparent constant with respect to CGA concentration.

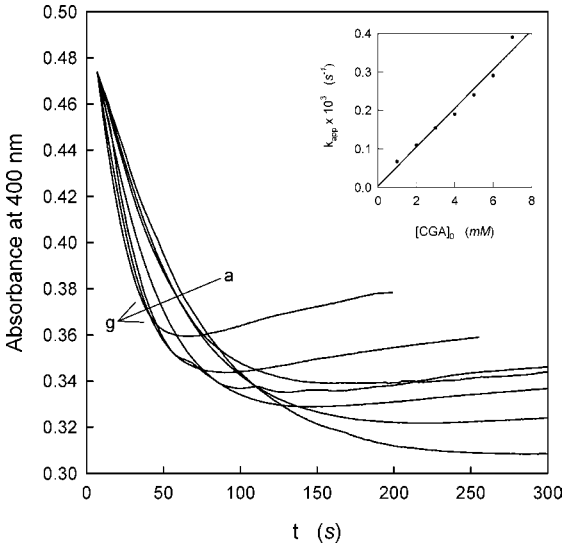


Figure 6. Kinetic of evolution of CGA-Q generated by oxidation with periodate at ratios of $[CGA]_0/[IO_4^-]_0 > 1$. Experimental conditions: phosphate buffer, 30 mM, pH 7.0, $[IO_4^-]_0 = 0.2$ mM. Concentrations of CGA: (a) 0.5 mM; (b) 1 mM; (c) 1.10 mM; (d) 1.5 mM; (e) 2 mM; (f) 2.25 mM; (g) 3 mM. (Inset) Representation of apparent constant with respect to CGA concentration.

Experiments were carried out at different concentrations of CGA at pH 7.0, and the evolution of CGA-Q with time was recorded at $\lambda = 400$ nm. The kinetic of this reaction was complex (**Figure 2**, left Inset).

The above experimental approach with periodate at ratios of $[CGA]_0/[IO_4^-]_0 < 1$ allowed us to calculate the molar absorptivity coefficient of the CGA-Q at the different pH values studied (**Figure 3**), the values at 400 nm being 2000 and 2200 M^{-1} cm^{-1} at pH 4.5 and 7.0, respectively. When CGA was oxidized at a ratio of $[CGA]_0/[IO_4^-]_0 > 1$, similar values were obtained.

Oxidation of CGA with Periodate at a Ratio of $[CGA]_0/[IO_4^-]_0 > 1$. Although the above experimental conditions of oxidation periodate at ratios of $[CGA]_0/[IO_4^-]_0 < 1$ are suitable for calculating the molar absorptivity coefficient, neither PPO nor POD works in these conditions. For any kinetic characterization $[CGA]_0 \gg [E]_0$ must be accomplished, and, because to maintain the steady state the substrate concentration should not vary much, then $[CGA\text{-}Q] \ll [CGA]_0$. These are conditions similar to those obtained by oxidation at ratios of $[CGA]_0/[IO_4^-]_0 > 1$.

Several oxidations of CGA with periodate were carried out with different $[CGA]_0/[IO_4^-]_0$ ratios of 2−8. **Figure 4A** (ratio 2), **Figure 4B** (ratio 6), and **Figure 4C** (ratio 8) show the scans obtained for oxidation by periodate. Note the isosbestic point

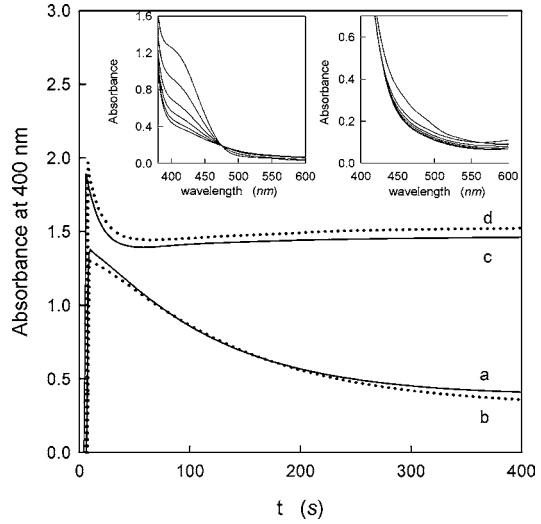


Figure 7. Enzymatic oxidation of CGA: (a) oxidation of CGA with PPO 0.2 μM at pH 4.5;(b) oxidation of CGA with POD 20 nM at pH 4.5 and $[H_2O_2]_0 = 0.5$ mM; (c) oxidation of CGA with PPO 0.2 μM at pH 7.0; (d) oxidation of CGA with POD 20 nM, at pH 7.0 and $[H_2O_2]_0 = 0.5$ mM. In curves a–d, $[CGA]_0 = 3$ mM. (Left inset) Spectrophotometric scans of evolution of CGA-Q generated enzymatically at pH 4.5. (Right inset) Spectrophotometric scans of evolution of CGA-Q generated enzymatically at pH 7.0.

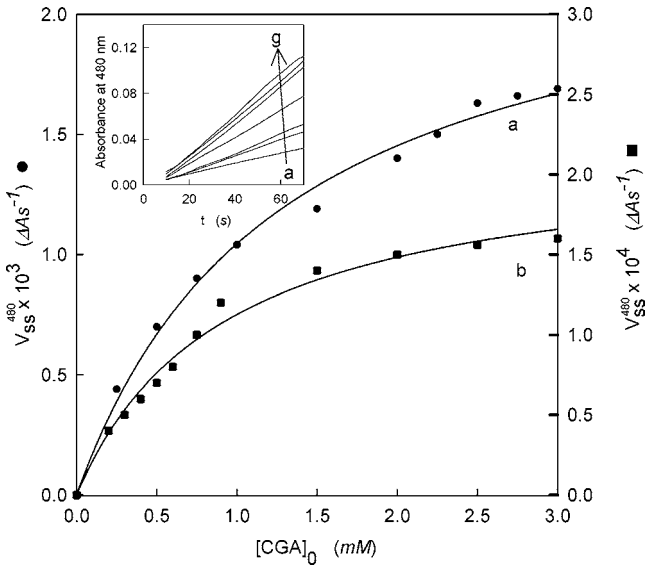


Figure 8. Measurement of PPO and POD activity at pH 4.5; representation of V_0 versus $[CGA]_0$: (a) PPO 25 nM; (b) POD 2 nM at $[H_2O_2]_0 = 0.1$ mM. (Inset) Experimental recordings of isosbestic point $\lambda = 480$ nm, of CGA-Q generation at pH 4.5. Substrate concentration: (a) 0.25 mM; (b) 0.5 mM; (c) 0.75 mM; (d) 1.5 mM; (e) 2.5 mM; (f) 2.75 mM; (g) 3 mM.

Table 1. Kinetic Constants That Characterize the Action of PPO on CGA

substrate[a]	pH	method	k_{cat} (s^{-1})	K_m (mM)	k_6[b] (M^{-1} s^{-1})	$K_m^{O_2}$ (μM)	δ_3	δ_5	reference
CGA	4.5	isosbestic point	135 ± 12	1.30 ± 0.10	$(1.04 \pm 0.8) \times 10^5$	5.8 ± 0.12	145.9	148.8	this paper
	4.5	chronometric	121 ± 7	1.38 ± 0.11	$(8.77 \pm 1.0) \times 10^4$	5.3 ± 0.10			this paper
	7.0	chronometric	176 ± 10	0.87 ± 0.08	$(2.03 \pm 0.5) \times 10^5$	7.6 ± 0.08	146.4	149.3	this paper
MeCAT	7.0	O_2 consumption	842 ± 25	0.12 ± 0.02	$(7.02 \pm 1.5) \times 10^6$	35.8 ± 3.20	146.4	144.1	*34*
TBC	7.0	O_2 consumption	641 ± 20	1.41 ± 0.20	$(4.55 \pm 0.8) \times 10^5$	28.0 ± 3.10	146.2	144.1	*34*
CAT	7.0	O_2 consumption	878 ± 50	0.10 ± 0.01	$(8.78 \pm 1.2) \times 10^6$	40.5 ± 4.00	146.6	146.6	*34*
dopamine	7.0	O_2 consumption	439 ± 25	0.72 ± 0.01	$(6.10 \pm 1.1) \times 10^5$	21.5 ± 3.10	146.9	145.7	*34*

[a] CGA, chlorogenic acid; MeCAT, 1-methylcatechol; TBC, 1-*tert*-butylcatechol; CAT, pyrocatechol. [b] Binding constant of CGA to E_{ox} (**Scheme 3**).

Table 2. Kinetic Constants That Characterize the Action of POD on CGA

substrate	pH	method	k_{cat} (s^{-1})	K_m^S (mM)	$K_m^{H_2O_2}$ (μM)	k_{+5} (M^{-1} s^{-1})	k_{+6}[a] (s^{-1})	k_2[b] (s^{-1})	δ_3	δ_4	reference
CGA	4.5	isosbestic point chronometric	880 ± 40	0.30 ± 0.05	25 ± 1	$(3.00 \pm 1.32) \times 10^4$	1.57×10^3	2000	145.9	148.8	this paper
	4.5	chronometric	910 ± 22	0.52 ± 0.05	26 ± 1	$(3.80 \pm 1.52) \times 10^4$	1.67×10^3	2000			this paper
	7.0		674 ± 13	0.48 ± 0.05	20 ± 1	$(2.60 \pm 1.02) \times 10^4$	1.02×10^3	2000	146.4	149.3	this paper
MeCAT	7.0	chronometric	2155 ± 105	1.30 ± 0.10	128 ± 8	$(1.66 \pm 0.20) \times 10^6$	$>2.00 \times 10^4$	2000	146.4	144.1	*31*
TBC	7.0	chronometric	2146 ± 100	2.60 ± 0.20	125 ± 7	$(8.25 \pm 1.02) \times 10^5$	$>2.00 \times 10^4$	2000	146.2	144.1	*31*
CAT	7.0	chronometric	2200 ± 150	2.30 ± 0.10	129 ± 8	$(9.56 \pm 1.06) \times 10^5$	$>2.00 \times 10^4$	2000	146.6	146.6	*31*
dopamine	7.0	chronometric	447 ± 42	16.80 ± 1.30	27 ± 4	$(2.66 \pm 0.45) \times 10^4$	5.36×10^2	2000	146.9	145.7	*31*

[a] Transformation constant of CGA in CGA-SQ for POD-II (**Scheme 4**). [b] Data taken from ref *31*.

is maintained at 475 nm and pH 4.5, with clear stoichiometry in the evolution of CGA-Q. When oxidation was carried out at pH 7.0, there was an isosbestic point at low concentrations of CGA, but this was broken when concentrations were raised (**Figure 4A−C**, inset). In theory, these experiments show that CGA-Q does not evolve with a clear stoichiometry at pH 7.0.

Kinetic Evolution of the *o*-Quinone. Oxidation of CGA with periodate at a ratio of $[CGA]_0/[IO_4^-]_0 > 1$ at acid pH (4.5) generates CGA-Q, which evolves with a clear stoichiometry (**Figure 4**). The kinetic of this evolution is shown in **Figure 5**. In these experiments, different initial concentrations of CGA were oxidized with the same concentration of sodium periodate and the evolution of the CGA-Q was monitored at $\lambda = 400$ nm. The apparent constant of the evolution of CGA-Q increased with increasing concentrations of CGA, suggesting that the quinone reacted with the substrate according to the mechanism shown in **Scheme 1**, where P may be a dimer.

The rate of disappearance of CGA-Q is

$$\frac{-\mathrm{d[CGA\text{-}Q]}}{\mathrm{d}t} = k_{app}[\mathrm{CGA\text{-}Q}] \quad (1)$$

and so

$$[\mathrm{CGA\text{-}Q}]_t = [\mathrm{CGA\text{-}Q}]_0 \, \mathrm{e}^{-k_{app}t} \quad (2)$$

with

$$k_{app} = k[\mathrm{CGA}]_0 \quad (3)$$

Analysis of the experimental recordings (**Figure 5**) according to eq 2 provides k_{app}, and its dependence on $[CGA]_0$ is shown in the inset of **Figure 5**, giving a second-order rate constant (k) of 2.73 ± 0.17 M^{-1} s^{-1}.

When CGA is oxidized in conditions by periodate at a ratio of $[CGA]_0/[IO_4^-]_0 > 1$ but at pH 7.0, the recordings shown in **Figure 6** are obtained, and the disappearance of CGA-Q can be followed as in **Figure 5**. Analysis of the data using eq 2 gives us the value of k'_{app}, which increases with increasing concentrations of CGA. The inset of **Figure 6** depicts k'_{app} versus $[CGA]_0$, giving a second-order rate constant (k') of 0.05 ± 0.01 M^{-1} s^{-1}. Note that at longer times the absorbance at 400 nm increases, perhaps because the adduct is oxidized by the CGA-Q, to give a new quinone, in agreement with ref *9*. These results would be in agreement with a rupture of the isosbestic point in **Figure 4A−C** (inset), because several reactions would overlap in time.

Enzymatic Oxidation of Chlorogenic Acid. When the CGA is oxidized at pH 4.5 with high enzyme concentrations, the recordings of **Figure 7** are obtained. With PPO, **Figure 7**, trace a, the absorbance at 400 nm increases until all of the oxygen is used up, after which the CGA-Q disappears as a result of the reaction with the substrate, giving a kinetic similar to that shown in **Figure 5**. How the CGA-Q evolves is shown in **Figure 7** (left inset); the isosbestic point is similar to that obtained for the oxidations with sodium periodate at a ratio of $[CGA]_0/[IO_4^-]_0 > 1$ (**Figure 4A−C**). Similar results were obtained with POD [**Figure 7** (trace b and left inset)].

If oxidation is carried out at pH 7.0, we obtain trace c with PPO and trace d with POD, again similar to that obtained with oxidation using sodium periodate at a ratio of $[CGA]_0/[IO_4^-]_0 > 1$. The absence of an isosbestic point (**Figure 7**, right inset) confirms the nonstoichiometric evolution of the CGA-Q during the oxidation with both enzymes at this pH.

In light of the above results, the kinetic characterization of CGA with enzymes such as mushroom PPO at its optimum pH (7.0) is difficult, and the results have been interpreted as an inhibition through excess of substrate (*1*, *2*, *11−14*). Something similar occurs with POD/H_2O_2 (**Figure 1**).

In this work we propose two methods for kinetically characterizing CGA as a substrate for PPO and POD, which have different pH optima. Both methods are based on the monitoring of the evolution of the quinone, but two cases can be distinguished.

(a) Kinetic Characterization at Acid pH. In this case, the isosbestic point obtained for the evolution of CGA-Q is selected as measurement wavelength (**Figure 8**). The wavelength of 480 nm is obtained in **Figure 7** (right inset), and once the molar absorptivity coefficient has been obtained ($\epsilon_i = 360$ M^{-1} cm^{-1}),

the recordings of **Figure 8** (inset) and their analysis give the values of k_{cat} and K_m, which are shown in **Table 1** for PPO and in **Table 2** for POD.

(b) Kinetic Characterization at Acid or Neutral pH. This spectrophotometric method does not involve measuring CGA-Q because of its instability. However, it may be prevented by using ascorbic acid (AH_2), according to **Scheme 2** for PPO.

However, it is not possible to follow the disappearance of AH_2 because the spectra of CGA and AH_2 overlap at the different pH values assayed (results not shown), and so we propose the following chronometric method.

The method uses the antioxidant power of AH_2, which reacts rapidly with the CGA-Q generated by the enzyme (*31*) (**Scheme 2**). The steady-state rate is V_0, which is rapidly reached when $[O_2]_0$ and $[CGA]_0 \gg [E]_0$ in the case of PPO or when $[H_2O_2]_0$ and $[CGA]_0 \gg [E]_0$ in the case of POD, fulfilling the following mass balance:

$$V_0 t - [AH_2] = \text{CGA-Q} \tag{4}$$

The total quantity of material entering the system is $V_0 t$, where t is the time during which the enzyme acts. In the presence of ascorbic acid, the CGA-Q is reduced to CGA, until no AH_2 remains. The straight line absorbance versus t cuts axis X at CGA-Q = 0, and so eq 4 is transformed into

$$V_0 \tau - [AH_2] = 0 \tag{5}$$

Reorganising eq 5 gives

$$V_0 = \frac{[AH_2]_0}{\tau} \tag{6}$$

Taking into account the kinetic mechanisms of PPO and POD described below, some conditions can be established so that this chronometric method provides suitable values for parameter V_0, thus permitting these enzymes to be characterized.

The diphenolase activity of PPO is described in **Scheme 3** (*33*), where E_m, E_d, and E_{oxy} represent the oxygenated and deoxygenated forms and another with a peroxide group, respectively. V_0 is given by (*33*)

$$V_0 = \frac{V_{max}[CGA]_0[O_2]_0}{K_m^{CGA} k_s^{O_2} + K_m^{O_2}[CGA]_0 + K_m^{CGA}[O_2]_0 + [CGA]_0[O_2]_0} \tag{7}$$

where

$$V_{max} = 2k_{cat}[E]_0 = \frac{2k_3 k_7}{k_3 + k_7}[E]_0 \tag{8}$$

$$K_s^{O_2} = \frac{k_{-8}}{k_8} \tag{9}$$

$$K_m^{O_2} = \frac{k_{cat}}{k_8} \tag{10}$$

$$K_m^{CGA} = \frac{k_{cat}}{k_6} \tag{11}$$

POD activity on the reducing substrates (CGA) in the presence of H_2O_2 is described by **Scheme 4** (*30*), where POD stands for the free enzyme, POD-I is compound **I**, POD-II is compound **II**, and CGA-SQ is the semiquinone radical corresponding to the CGA.

The following expression is obtained for the rate of radical formation:

$$V_0 = \frac{2k_{cat}[H_2O_2]_0[CGA]_0[E]_0}{\frac{k_{cat}}{k_5}[H_2O_2]_0 + \frac{k_{cat}}{k_1}[CGA]_0 + [H_2O_2]_0[CGA]} \tag{12}$$

This equation can be rearranged as follows:

$$V_0 = \frac{V_{max}^{app,CGA}[CGA]_0}{K_m^{app,CGA} + [CGA]_0} \tag{13}$$

where

$$V_{max}^{app,CGA} = \frac{2k_{cat}[H_2O_2]_0[E]_0}{\frac{k_{cat}}{k_1} + [H_2O_2]_0} \tag{14}$$

$$K_m^{app,CGA} = \frac{\frac{k_{cat}}{k_5}[H_2O_2]_0}{\frac{k_{cat}}{k_1} + [H_2O_2]_0} \tag{15}$$

and

$$k_{cat} = \frac{k_2 k_6}{k_2 + k_6} \tag{16}$$

$$K_m^{CGA} = \frac{k_{cat}}{k_5} \tag{17}$$

$$K_m^{H_2O_2} = \frac{k_{cat}}{k_1} \tag{18}$$

Characterization of PPO. The experimental conditions in the presence of AH_2 imply that the concentration of reducing substrate (CGA) remains constant during the lag phase, although $[O_2]$ will vary with the stoichiometry $1O_2$:2CGA:$2AH_2$. Thus, the permitted concentration of AH_2 in the experiment will be $\leq 0.1[O_2]$, so that O_2 consumption will be <10% and V_0 will not vary, and the steady-state will be reached.

Kinetic Experiments To Accurately Estimate the Initial Rate. To apply the method, it is necessary to take into account the following points: (a) limitation of the consumption (as mentioned above, the concentration of O_2 determines the suitable concentration of AH_2; thus, $[AH_2]_0 \leq [O_2]_0$ should be used); (b) length of the lag period [the minimum length of the lag period must be greater than the "dead time" of the measurement, τ_d (12 s); we opted for $\tau \geq 3\,\tau_d$, which was achieved by varying $[E]_0$ (see eq 6); however, τ should not be so long that changes in the blank samples (due to the chemical reaction of O_2 with AH_2) become significant]; (c) quantity of AH_2 used in the assays [this quantity is limited by O_2 consumption (see above)].

Validation of Initial Rate Determination. *Effect of Enzyme Concentration.* At constant concentrations of both substrates and AH_2, the lag period diminishes as the enzyme concentration increases, resulting in an increase of V_0 (see eq 6) (results not shown).

Effect of Substrate Concentration. When the concentrations of the other reagents are kept constant and the concentration of substrate (CGA) is increased, the lag period becomes shorter because V_0 increases, according to eq 6 (**Figure 9**).

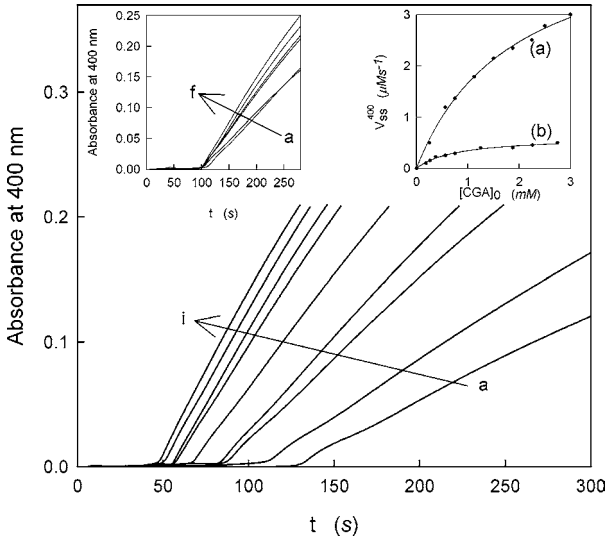
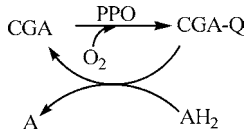
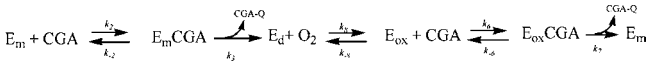


Figure 9. Chronometric method for characterizing enzymatic activity of PPO on CGA; effect of substrate concentration. Experimental conditions: acetate buffer, 30 mM, pH 4.5; $[E]_0 = 13.5$ nM; $[AH_2]_0 = 75$ μM. Substrate concentration: (a) 0.35 mM; (b) 0.55 mM; (c) 0.75 mM; (d) 1.10 mM; (e) 1.85 mM; (f) 2 mM; (g) 2.25 mM; (h) 3 mM; (i) 3.15 mM. (Left inset) Spectrophotometric recordings of chronometric method at constant $[CGA]_0 \times [E]_0$ value. Experimental conditions: acetate buffer, 30 mM, pH 4.5. The value of $[CGA]_0 \times [E]_0 = 6.5 \times 10^{-6}$ mM^2 and the concentrations of $[CGA]_0$ and $[E]_0$ were (a) 0.35 mM, (b) 0.55 mM, (c) 0.75 mM, (d) 0.95 mM, (e) 1.10 mM, (f) 2.25 mM and $[E]_0$ (a) 17.5 nM, (b) 11.5 nM, (c) 8.5 nM, (d) 7 nM, (e) 6 nM, and (f) 3 nM, respectively, and $[AH_2]_0 = 75$ μM. (Right inset) Representation of steady-state rate versus $[CGA]_0$; (a) pH 4.5; (b) pH 7.0.

Scheme 2



Scheme 3



To avoid the effects of V_0 due to the blanks of the assays, we propose that in a kinetic characterization where the substrate varies and therefore V_0, too, the τ may be very different. To make the values of τ as similar as possible so that the oxidation of AH_2 by O_2 or H_2O_2 takes place at the same time, we suggest varying the enzyme and substrate concentrations at the same time in such a way that their product ($[CGA]_0 \times [E]_0$) is constant. In this way τ remains practically constant and the values of V_0 can be normalized in accordance with the corresponding factor (see **Figure 9**, left inset).

Kinetic Characterization of POD. In these experiments, the same restrictions as for PPO are applied, except that, because this enzyme consumes H_2O_2, the concentration of AH_2 must fulfill $[AH_2]_0 \leq 0.1\ [H_2O_2]_0$.

Furthermore, in agreement with the stoichiometry of **Scheme 4**, the expression of V_0 is

$$V_0 = \frac{2[AH_2]_0}{\tau} \qquad (19)$$

When the concentrations of the other reagents are kept constant and the concentration of substrate (CGA) is increased,

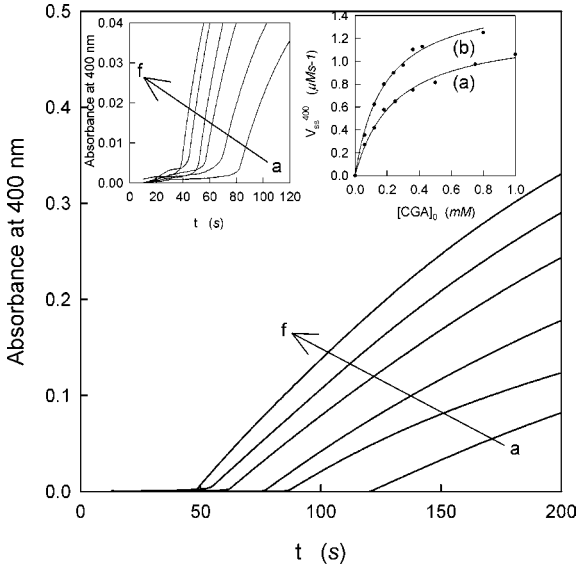
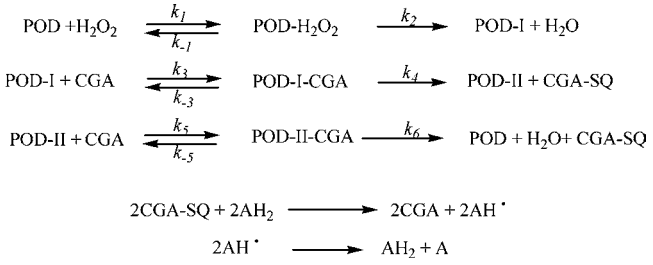


Figure 10. Chronometric method used for characterizing enzymatic activity of POD on CGA. The effect of the substrate concentration is presented. Experimental conditions: acetate buffer, 30 mM, pH 4.5; $[AH_2]_0 = 50$ μM; $[H_2O_2]_0 = 0.5$ mM; $[E]_0 = 2$ nM. Substrate concentrations: (a) 0.12 mM; (b) 0.20 mM; (c) 0.25 mM; (d) 0.5 mM; (e) 0.75 mM; (f) 1 mM. (Left inset) Results obtained in phosphate buffer, 30 mM, pH 7.0, $[AH_2]_0 = 50$ μM, $[H_2O_2]_0 = 0.50$ mM, $[E]_0 = 2$ nM. Substrate concentrations: (a) 0.05 mM; (b) 0.10 mM; (c) 0.20 mM; (d) 0.25 mM; (e) 0.30 mM; (f) 0.35 mM; (g) 0.40 mM. (Right inset) Representation of steady-state rate versus $[CGA]_0$: (a) pH 4.5; (b) pH 7.0.

Scheme 4



the lag period becomes shorter because V_0 increased, according to eq 19 (**Figure 10** and its left inset) at pH 4.5 and 7.0, respectively.

Kinetic Constants. When the methods described above are applied to the kinetic characterization of the substrate CGA, a hyperbolic dependence is obtained for V_0 versus $[CGA]_0$ (**Figure 9**, right inset, and **Figure 10**, right inset). Analysis of the data, as described under Materials and Methods, provided the information described in **Table 1** for PPO and in **Table 2** for POD.

The kinetic constants determined for the action of PPO on CGA are compared with the values obtained for other *o*-diphenols in **Table 1** (*33*). The CGA, with its higher δ_4, has a lower V_0, which reflects electronic effects. With regard to the values of k_{cat}, the presence of a voluminous group in C-1 (in the case of TBC) provokes a great increase in K_m, as discussed in other studies (*33*). However, in the case of CGA, although the volume of substituent is greater in C-1, perhaps the length of the acid chain does not hinder the attack of the OH of C-4 on the copper of the active center. Note that the K_m increases as the pH falls, as in the case of mushroom PPO, with other substrates (*34*), whereas K_m hardly varies.

With regard to the other PPO substrate, O_2, its k_{cat} in the presence of CGA depends on the nature of the substrate and, according to eq 10 and the value determined for $K_m^{O_2}$ (*34*), its value is very small, which facilitates the enzyme's action at low concentrations of O_2.

In **Table 2**, the values of the constants determined for the action of POD on CGA are compared with those obtained for other *o*-diphenols (*30*).

The value of the δ_4 of CGA compared with the other *o*-diphenols considered in **Table 2** is very high, which implies that the electron density of the oxygen of the OH in C-4 is less and therefore more difficult to oxidize by POD, meaning that the value of k_{+8} will be less than that of MeCat, CAT, and TBC. The carbonyl group of CGA, with its ester bond, may favor the union in the active center of the enzyme, as occurs with benzhydroxamic acid (BHA), whose NH−CO group means that this compound reacts with a constant of 7.8×10^5 M^{-1} s^{-1} (*35*). The Michaelis constant, k_{cat}, is lower than in the case of dopamine, reflecting the effect of a positive charge near the ring.

The Michaelis constant for H_2O_2, calculated according to eq 18, reflects the great affinity for the H_2O_2. The value of K_m^S, the constant of oxidation by compound **II**, is low, in accordance with the high value of δ_4.

In conclusion, this work has characterized the CGA-Q corresponding to the CGA (k_6 and λ_{max}) and studied the kinetics of its evolution, describing its reaction with the substrate. A chronometric spectrophotometric method for measuring the enzymatic activities of PPO and POD is established, enabling the characterization of their action on CGA. The values of the δ_3 and δ_4 obtained by NMR are discussed in relation with other *o*-diphenolic substrates of these enzymes.

LITERATURE CITED

(1) Murata, M.; Kurokami, C.; Homma, S. Purification and some properties of chlorogenic acid oxidase from apple (*Malus pumila*). *Biosci., Biotechnol., Biochem.* **1992**, *56*, 1705−1710.

(2) Zawistowski, J.; Biliaderis, C. G.; Eskin, N. A. M. Polyphenol oxidase. In *Oxidative Enzymes in Foods*; Robinson, D. S., Eskin, N. M. A., Eds.; Elsevier Science: London, U.K., 1991.

(3) Mathew, A. G.; Parpia, H. A. B. Food browning as a polyphenol reaction. *Adv. Food Res.* **1971**, *19*, 74−145.

(4) Nicholas, J. J.; Richard-Forget, F.; Goupy, P.; Amito, M. J.; Aubert, S. Enzymatic browning reactions in apple and apple products. *CRC Crit. Rev. Food Sci. Nutr.* **1994**, *34*, 109−157.

(5) Sisler, F. C.; Evans, H. J. A comparison of chlorogenic acid and catechol as substrates for the polyphenol oxidase from tobacco and mushroom. *Plant Physiol.* **1958**, 255−257.

(6) Pierpoint, W. S. The enzymic oxidation of chlorogenic acid and some reactions of the quinone produced. *Biochem. J.* **1966**, *98*, 567−580.

(7) Richard-Forget, F.; Gaulliard, F. Oxidation of chlorogenic acid, catechins, and 4-methylcatechol in model solutions by combinations of pear (*Pyrus communis* Cv. Williams) polyphenol oxidase and peroxidase: a posible involvement of peroxidase in enzymatic browning. *J. Agric. Food Chem.* **1997**, *45*, 2472−2476.

(8) Murata, M.; Sugiera, M.; Sonokawa, Y.; Shimamura, T.; Homma, S. Properties of chlorogenic acid quinone: relationship between browning and the formation of hydrogen peroxide from a quinone solution. *Biosci., Biotechnol., Biochem.* **2002**, *66*, 2525−2530.

(9) Richard-Forget, F.; Amiot, M. J.; Goupy, P.; Nicolas, J. Evolution of chlorogenic acid *o*-quinones in model solutions. In *Enzymatic Browning and Its Prevention*; Lee, C. Y., Whitaker, J. R., Eds.; American Chemical Society: Washington, DC, 1995; pp 145−158.

(10) Mastore, M.; Kolher, L.; Nappi, A. J. Production and utilization of hydrogen peroxide associated with melanogenesis and tyrosinase-mediated oxidations of DOPA and dopamine. *FEBS J.* **2005**, *272*, 2407−2415.

(11) Abukharma, D. A.; Woolhouse, H. W. The preparation and properties of o-diphenol:oxygen oxidoreductase from potatoes tubers. *New Phytol.* **1966**, *65*, 477−478.

(12) Zawistowski, J.; Biliaderis, C. G.; Murria, E. D. Isolation and some properties of an acidic fraction of polyphenol oxidase from Jerusalem artichoke (*Helianthus tuberosus* L.). *J. Food Biochem.* **1988**, *12*, 23−25.

(13) Interesse, F. S.; Ruggiero, P.; D'Avella, G.; Lamparelli, F. Characterization of wheat o-diphenolase isoenzyme. *Phytochemistry* **1983**, *22*, 1885−1889.

(14) Koller, A.; Neukom, H. Untersuchungen ubre den abbaumechanismus einer gereinigten polygalakturonase aus *Aspergillus niger*. *Eur. J. Biochem.* **1969**, *7*, 485−489.

(15) Kermasha, S.; Goetghebeur, M.; Monfette, A. Studies on inhibition of mushroom polyphenol oxidase using chlorogenic acid as substrate. *J. Agric. Food Chem.* **1993**, *41*, 526−531.

(16) Tono, T.; Fujita, S. Difference spectra spectrophotometry for polyphenol oxidase assay. In *Enzymatic Browning and Its Prevention*; Lee, C. Y., Whitaker, J. R., Eds.; American Chemical Society: Washington, DC, 1995; pp 188−199.

(17) Kono, Y.; Shibata, H.; Kodama, Y.; Sawa, Y. The suppression of the N-nitrosating reaction by chlorogenic acid. *Biochem. J.* **1995**, *312*, 947−953.

(18) Clifford, M. N. Chlorogenic acid and the other cinnamates—nature, occurrence and dietary burden. *J. Sci. Food Agric.* **1999**, *79*, 362−372.

(19) Yagasaki, K.; Miura, Y.; Okauchi, R.; Furuse, T. Inhibitory effects of chlorogenic acid and its related compounds on the invasion of the hepatoma cells in culture. *Cytotechnology* **2000**, *33*, 229−235.

(20) Shimizu, M.; Yoshimi, N.; Yamada, Y.; Matsunaga, K.; Kawabata, K.; Hara, A.; Morikawa, H.; Mori, H. Suppressive effects of chlorogenic acid on *N*-methyl-*N*-nitrosourea-induced glandular stomach carcinogenesis in male F344 rats. *J. Toxicol. Sci.* **1999**, *24*, 433−439.

(21) Yamada, J.; Tomita, Y. Antimutagenic activity of caffeic acid and related compounds. *Biosci., Biotechnol., Biochem.* **1996**, *60*, 328−329.

(22) Krakauer, T. The polyphenol chlorogenic acid inhibits staphylococcal exotoxin-induced inflammatory cytokines and chemokines. *Inmunopharmacol. Inmunotoxicol.* **2002**, *24*, 113−119.

(23) Stich, H. F. Teas and tea components as inhibitors of carcinogen formation in model systems and man. *Prev. Med.* **1992**, *21*, 377−384.

(24) Li, P.; Wang, H. Z.; Wang, X. Q.; Zu, Y. N. The blocking effect of phenolic acid on *N*-nitrosomorpholine formation in vitro. *Biomed. Environ. Sci.* **1994**, *7*, 68−78.

(25) Torres y Torres, J. L.; Rosazza, J. P. N. Reactions of *p*-coumaric acid with nitrite: product isolation and mechanism studies. *J. Agric. Food Chem.* **2001**, *49*, 1486−1492.

(26) Panzella, L.; Napolitano, A.; d'Ischia, M. Oxidative conjugation of chlorogenic acid with glutathione: structural characterization of addition products and a new nitrite-promoted pathway. *Bioorg. Med. Chem.* **2003**, *11*, 4797−4805.

(27) Bradford, M. M. A rapid and sensitive method for the quantification of microgram quantities of proteins utilising the principle of protein−dye binding. *Anal. Biochem.* **1976**, *72*, 248−254.

(28) Marquart, D. W. An algorithm least-squares estimation of nonlinear parameters. *J. Soc. Ind. Appl. Math.* **1963**, *11*, 431−441.

(29) Jandel Scientific. *Sigma Plot 9.0 for Windows*; Jandel Scientific: Core Madera, CA, 2006.

(30) Rodriguez-Lopez, J. N.; Gilabert, M. A.; Tudela, J.; Thorneley, R. N.; Garcia-Canovas, F. Reactivity of horseradish peroxidase compound II toward substrates: kinetic evidence for a two-step mechanism. *Biochemistry* **2000**, *39*, 13201−13209.

(31) Muñoz, J. L.; Garcia-Molina, F.; Varon, R.; Rodriguez-Lopez, J. N.; Garcia-Canovas, F.; Tudela, J. Calculating molar absorptivities for quinones: application to the measurement of tyrosinase activity. *Anal. Biochem.* **2006**, *351*, 128−138.

(32) Weidman, S. W.; Kaiser, E. T. The mechanism of the periodate oxidation of aromatic systems. A kinetic study of the periodate oxidation of catechol. *J. Am. Chem. Soc.* **1966**, *88*, 5820−5826.

(33) Rodríguez-López, J. N.; Fenoll, L. G.; García-Ruiz, P. A.; Varón, R.; Tudela, J.; Thorneley, R. N.; García-Canovas, F. Stopped-flow and steady-state study of the diphenolase activity of mushroom tyrosinase. *Biochemistry* **2000**, *39*, 10497−10506.

(34) García-Cánovas, F.; Tudela, J.; Martínez Madrid, C.; Varón, R.; García-Carmona, F.; Lozano, J. A. Kinetic study on the suicide inactivation of tyrosinase induced by catechol. *Biochim. Biophys. Acta* **1987**, *912*, 417−423.

(35) Aviram, I. The interaction of benzhydroxamic acid with horseradish peroxidase and its fluorescent analogs. *Arch. Biochem. Biophys.* **1981**, *212*, 483−490.

Received for review July 24, 2006. Revised manuscript received November 28, 2006. Accepted December 1, 2006. This work was supported in part by grants from the MCYT (Spain) Project AGL 2002-01255 ALI, from the Fundación Séneca, and from the Fundación Séneca/Consejería de Educación y Universidades (Murcia) Project 00672/PI/04. F.G.-M. has a fellowship from the MEC (Spain), Reference AP2003-0891. J.M. has a fellowship from the MEC (Spain) Reference AP2005-4721.

JF062081+