

# Oxidation of the Flavonol Quercetin by Polyphenol Oxidase

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Because direct oxidation of flavonols by polyphenol oxidase (PPO) has not previously been reported and, given the importance of flavonols, the ability of broad bean seed PPO to oxidize the flavonol quercetin was studied. The reaction was followed by recording spectral changes with time. Maximal spectral changes were observed at 291 nm (increase) and at 372 nm (decrease). The presence of two isosbestic points (at 272 and 342 nm) suggested the formation of only one absorbent product. These spectral changes were not observed in the absence of PPO. The oxidation rate, which varied with pH, was highest at pH 5.0. The following kinetic parameters were also determined:  $V_m = 11 \mu\text{M}/\text{min}$ ,  $K_m = 646 \mu\text{M}$ ,  $V_m/K_m = 17 \times 10^{-2} \text{ min}^{-1}$ . Flavonol oxidation was efficiently inhibited ( $K_I = 3.5 \mu\text{M}$ ) by specific PPO inhibitors such as 4-hexylresorcinol. The results obtained showed that quercetin oxidation was strictly dependent on the presence of PPO.

**Keywords:** *Polyphenol oxidase; flavonols; quercetin*

## INTRODUCTION

Polyphenol oxidase (PPO, EC 1.14.18.1) is a widely distributed enzyme and is involved in the biosynthesis of melanins in animals and in the browning of plants. The enzyme catalyzes two different reactions involving molecular oxygen: the *o*-hydroxylation of monophenols to *o*-diphenols (cresolase activity) and the subsequent oxidation of *o*-diphenols to *o*-quinones (catecholase activity).

PPO oxidation of monophenols and *o*-diphenols that have simple chemical structures has been extensively studied, although some of these substrates are not even natural compounds. Recent investigations carried out *in vitro* suggest that PPO may participate in the degradation of natural phenols with more complex structures, such as anthocyanins in strawberries (Wesche-Ebeling et al., 1990) and grapes (Sarni-Manchado et al., 1997) and flavanols present in tea leaves (Finger, 1994). These compounds are not directly oxidized by the enzyme but by the quinones formed by PPO from catechol, catechin, or chlorogenic acid.

Much attention is currently focused on polyphenols with complex structures such as flavonoids. These natural polyphenols are widely distributed in fruits and vegetables (Dick et al., 1987; Hollman et al., 1996) and have multiple chemical and biological actions, including antioxidant (Bors, 1990; Lee, 1995), chelating (Gross, 1996), anticarcinogenic (Okuda, 1993; Middleton and Kandaswami, 1994), bacteriostatic (Gross, 1996) and secretory activities (Nguyen and Canada, 1993). For one flavonoid, quercetin, over a dozen biological effects can be listed. Quercetin is particularly interesting because of its anticarcinogenic activities (Deschner et al., 1991) and its significant quantitative presence in human foods (Hertog et al., 1993). The biological activities of quercetin include the inhibition of cell proliferation in animals and cell cultures (Weber et al., 1997) and the

prevention of chemically induced tumors. Recently, much attention has been paid to its antioxidant properties that affect oxygen free radicals and lipid peroxidation, both of which might be involved in several pathological conditions such as atherosclerosis, cancer, and chronic inflammation (Halliwell, 1994).

On the other hand, oxidation is one of the most important processes involved in food deterioration because it may affect food safety, color, flavor, and texture. Antioxidants may help preserve food quality by preventing the oxidative deterioration of lipids (Kinsella, 1993). The antioxidative potential of flavonoids to inhibit lipid peroxidation resides to a great extent in their radical scavenging capacity.

Quercetin is a strong antioxidant, as has been demonstrated by several methods to determine its free radical scavenging capacity, such as those using the reaction with the free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Brand-Williams et al., 1995; Sánchez-Moreno et al., 1998) or with the ABTS radical cation. Quercetin scavenges the ABTS radical and also inhibits its formation (Strube et al., 1997). It has also been found that flavonoids in general, and quercetin in particular, are excellent peroxynitrite scavengers (Haenen et al., 1997). The antioxidant potential of this flavonoid has also been tested using the chemiluminescence method (Merfort et al., 1996; Saramet et al., 1996). This is based on the oxidative degradation of luminol by hydrogen peroxide, when reactive species of oxygen (superoxide and hydroxyl) are obtained, which makes it possible to quantify the capacity of substances to inhibit the free radical processes that take place in this test. Quercetin also prevents oxidation of low-density lipoproteins *in vitro* by scavenging free oxygen radicals and may, therefore, contribute to the prevention of atherosclerosis (Steinberg, 1989; Teissedre et al., 1996).

Bearing all of this in mind, our aim was to study the oxidation of a strongly antioxidant flavonol (in this case, quercetin) by PPO. The results show that PPO is capable of oxidizing the flavonol directly, unlike in the case of anthocyanins, which are not degraded directly

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by the enzyme but by the quinones formed by PPO. The spectrophotometric and kinetic characteristics of the oxidation of quercetin by PPO, extracted and partially purified from broad bean seeds, were investigated and the specificity of the reaction was demonstrated by the use of selective PPO inhibitors.

## MATERIALS AND METHODS

**Reagents.** Broad bean seeds were purchased from a local market in the city of Murcia. Quercetin (3,3',4',5,7-pentahydroxyflavone) was obtained from Sigma Química (Madrid, Spain) and 4-hexylresorcinol from Aldrich (Madrid, Spain). TX-114 was purchased from Fluka Química (Madrid, Spain) and condensed three times as described by Bordier (1981) but using 100 mM sodium phosphate buffer, pH 7.3. The detergent phase of the third condensation had a TX-114 concentration of 25% (w/v) and was used as the stock detergent solution for all of the experiments.

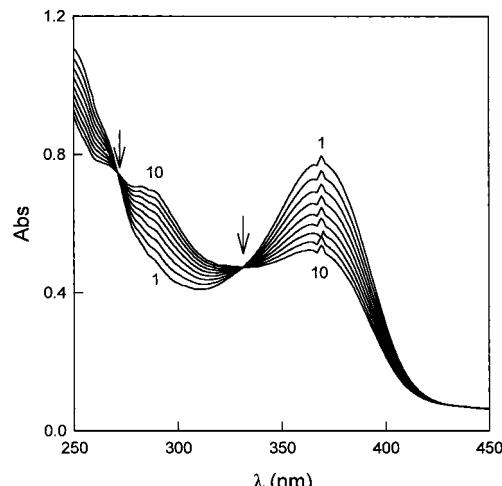
**Enzyme Extraction.** PPO was extracted in its latent state from the chloroplast membranes of broad bean (*Vicia faba*) seeds. All procedures were carried out at 4 °C. Chloroplasts were prepared by homogenizing 50 g of broad bean seeds in 150 mL of sodium phosphate buffer containing 0.33 M sorbitol, 2 mM EDTA, 1 mM MgCl<sub>2</sub>, 1 mM PMSF, and 1 mM benzamidine. The slurry was filtered through eight layers of gauze and centrifuged at 600g for 2 min. The pellet was discarded, and the supernatant was centrifuged at 20000g for 30 min. The resultant pellet was resuspended in a solution containing 50 mL of 10 mM sodium phosphate buffer, pH 7.3, and kept at 4 °C for 20 min. The solution was then centrifuged at 20000g for 20 min, pelleting the chloroplast membranes. These membranes were resuspended with 20 mL of 1.5% (w/v) TX-114 in 100 mM phosphate buffer, pH 7.3, for 30 min at 4 °C. After high-speed centrifugation (60000g for 20 min), this light green extract yielded a clear supernatant with PPO activity. This was subjected to temperature phase partitioning by adding TX-114 at 4 °C to give a final concentration of 8% (w/v). The mixture was kept at 4 °C for 15 min in a Tectron thermostatic bath and then warmed to 37 °C. After 15 min, the solution became spontaneously turbid due to the formation, aggregation, and precipitation of large micelles composed of detergent, hydrophobic proteins, and the remaining chlorophylls. This solution was centrifuged at 5000g for 10 min at room temperature. The clear supernatant was used as latent enzyme source.

**Enzyme Assays.** Unless otherwise noted, the spectrophotometric assays were performed at 25 °C in a reaction medium (1 mL) containing 75 μM quercetin and 2 mM SDS in 0.1 M sodium acetate buffer, pH 5.0. The reaction was initiated by the addition of PPO. Quercetin oxidation was monitored by measuring the increase in absorbance at 291 nm or the decrease at 372 nm.

Differences between extinction coefficients ( $\Delta\epsilon$ ), corresponding to the difference between absorption due to substrates and absorption due to reaction products, were calculated for the oxidation process. The  $\Delta\epsilon$  values were calculated from a calibration curve of quercetin (0–10 μM) quantitatively oxidized in the presence of an excess of sodium periodate at different pH values, by plotting the increase in absorbance at 291 nm or the decrease in absorbance at 372 nm, from  $t = 0$  to constant absorbance values against quercetin concentration. The values found, at pH 5.0, were  $\Delta\epsilon_{291} = 12\ 114\ M^{-1}\ cm^{-1}$  ( $r = 0.9952$ ) and  $\Delta\epsilon_{372} = 19\ 487\ M^{-1}\ cm^{-1}$  ( $r = 0.9971$ ).

**Optimum pH.** pH studies were carried out using 0.1 M sodium acetate and sodium phosphate buffers from pH 3.0 to pH 7.0 in the presence of 2 mM SDS. The pH of the assay solution was determined at room temperature, using a Crison micro pH 2002 meter. After catalysis, the pH of the assay solution was again measured.

**Optimal Activation by SDS.** Activation studies were carried out in a reaction medium (1 mL) containing 75 μM quercetin and different detergent concentrations in 0.1 M



**Figure 1.** Consecutive spectra, at 25 °C, obtained in the oxidation of quercetin by broad bean seed PPO. The assay medium (1.0 mL) contained 40 μM quercetin and 2 mM SDS in 0.1 M sodium acetate buffer, pH 5.0. The reaction was started by addition of the enzyme (71 μg/mL). Scan speed was at 1 min intervals for 10 min.

sodium acetate buffer, pH 5.0. The concentration of SDS varied from 0 to 8.7 mM.

**Inhibition Studies.** Inhibition of quercetin oxidation by PPO was followed at 372 nm and 25 °C by using 4-hexylresorcinol and cinnamic acid as specific inhibitors of the enzyme. Unless otherwise stated, the reaction medium (1.0 mL) contained quercetin (24–90 μM), 4-hexylresorcinol (0–41 μM), and 2 mM SDS in sodium acetate buffer 0.1 M, pH 5.0. The reaction was started by addition of the enzyme.

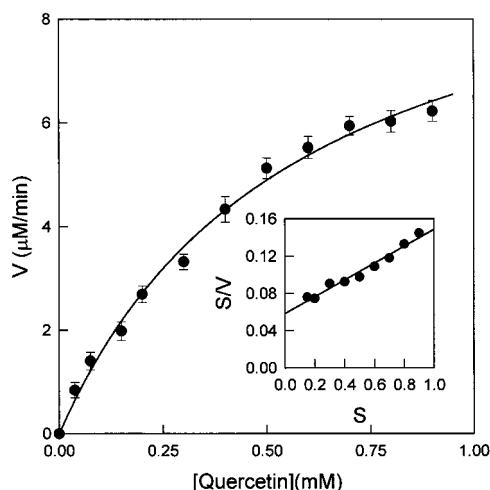
**Other Methods.** The protein content was determined according to the Bradford Bio-Rad protein assay using bovine serum albumin as a standard (Bradford, 1976).

## RESULTS AND DISCUSSION

Quercetin oxidation by PPO extracted from broad bean seeds was followed by observing changes in the UV-visible spectrum with time (Figure 1). Maximal spectral changes in the oxidation medium were observed at 372 nm (decreases in absorbance) and at 291 nm (increases in absorbance). The presence of two isosbestic points at 272 and 342 nm (arrowheads) suggests that only one absorbant product is formed during the course of quercetin oxidation by PPO. Because these changes were not observed in the absence of the enzyme, they were considered to be the result of PPO activity.

The nature of the spectral changes that took place at 291 and 372 nm during quercetin oxidation indicated that the formation of the reaction product was proportional to time during the first 10 min of the reaction. This observation and the dependence of these spectral changes on the presence of enzyme suggest that such changes are a reliable measure of quercetin oxidation. Therefore, flavonol oxidation by broad bean PPO was routinely assayed by measuring either the increase in the absorbance at 291 nm or the decrease at 372 nm versus reaction time.

To further characterize quercetin oxidation by PPO, the influence of the pH was analyzed after the  $\Delta\epsilon$  values were determined, as described under Materials and Methods, for all of the different pH values assayed. These  $\Delta\epsilon$  values were seen to be pH dependent, and this variation was taken into account to calculate the quercetin oxidation rate at the different pH values. The oxidation rate varied with pH, the optimal pH being 5.0



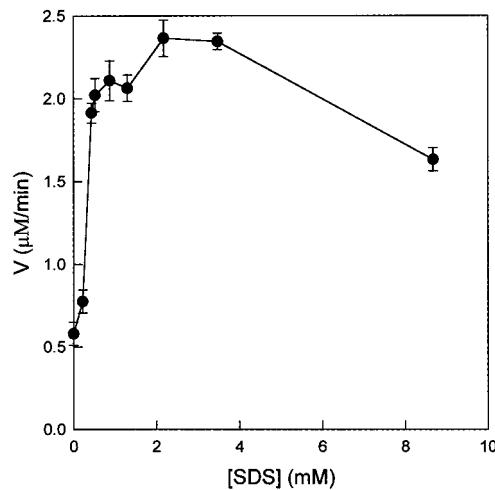
**Figure 2.** Dependence of quercetin oxidation on its concentration. The reaction medium contained quercetin, at the indicated concentration, 2 mM SDS, and 34  $\mu$ g/mL enzyme in 0.1 M sodium acetate buffer, pH 5.0. The quercetin oxidation rate was monitored by measuring the increase in absorbance at 291 nm. Inset: Hanes-Woolf plot of the kinetics results.

(figure not shown). Quercetin oxidation was monitored by measuring the increase in the absorbance at 291 nm. Similar results were obtained when the activity was monitored by following the decrease in absorbance at 372 nm.

The dependence of the quercetin oxidation rate on flavonol concentration is depicted in Figure 2, in which the enzyme shows Michaelis-Menten type kinetics. Enzyme saturation by the substrate was not reached in the concentration range assayed because, due to the high absorbance of the substrate at the measurement wavelength, it was not possible to further increase its concentration, despite the fact that spectrophotometric cells with a path length of 5 mm (half of the standard length) were used to decrease the initial absorbance values. Hanes-Woolf plots for the kinetic data of quercetin oxidation resulted in linear relationships (inset in Figure 2) from which the kinetic parameters were evaluated ( $V_m = 11 \mu\text{M}/\text{min}$ ,  $K_m = 646 \mu\text{M}$ ,  $V_m/K_m = 17 \times 10^{-2} \text{ min}^{-1}$ ).

One unusual and intriguing characteristic of PPO is its ability to exist in an inactive or latent state. For the enzyme to be extracted in its latent form, the extraction method must be very mild to prevent its modification or oxidation, as occurs with acetone powders (Golbeck and Cammarata, 1981). The method used to extract and partially purify PPO has been described previously and used to purify PPO in latent state from different sources (Sánchez-Ferrer et al., 1990; Chazarra et al., 1996; Jiménez and García-Carmona, 1996a). PPO can be activated by various treatments including proteolytic attack (King and Flurkey, 1987), changes in pH (Ketten, 1957), phospholipid release (Hutcheson and Buchanan, 1980), and anionic detergents (Moore and Flurkey, 1990; Jiménez and García-Carmona, 1996b). SDS activation is particularly interesting because few enzymes are known to be activated by this anionic detergent. Thus, PPO is activated at high SDS concentrations (Moore and Flurkey, 1990) that would denature many other enzymes.

Quercetin oxidation by PPO extracted from broad bean seeds was also increased by the presence of SDS (Figure 3). Scans of the SDS concentration carried out at pH 5.0 showed the optimum SDS concentration to



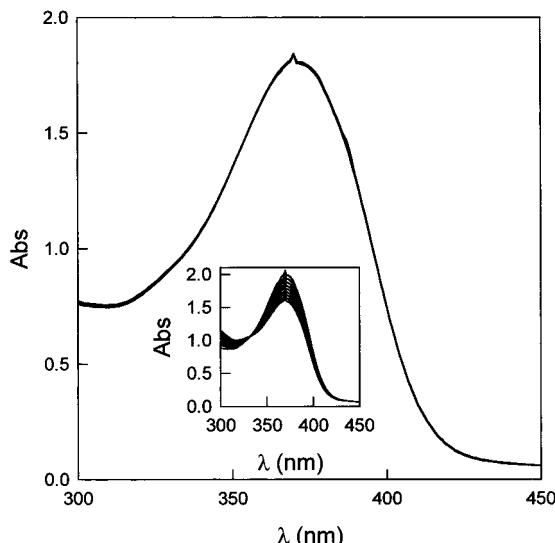
**Figure 3.** Effect of SDS on the rate of quercetin oxidation catalyzed by PPO. The reaction medium, at 25 °C, contained 75  $\mu\text{M}$  quercetin and 39  $\mu\text{g}/\text{mL}$  enzyme in 0.1 M sodium acetate buffer, pH 5.0.

be 2 mM, because higher detergent concentrations led to a decrease in enzymatic activity. The activation degree ( $R$ ) at 2 mM SDS, expressed as the ratio between the activity of the enzyme in the presence and in the absence of SDS, was 4. This value was much lower than the activation degree obtained during the oxidation of an *o*-diphenol such as 4-*tert*-butylcatechol ( $R = 40$ ) (data not shown). It has been reported that activation by anionic detergents depends on the substrate used to follow the enzymatic activity. This substrate-dependent activation phenomenon has been reported for potato leaf PPO with SDS and dodecanesulfonic acid (Sánchez-Ferrer et al., 1993) and also with other enzymes such as pyruvate oxidase (Schrock and Gennis, 1997).

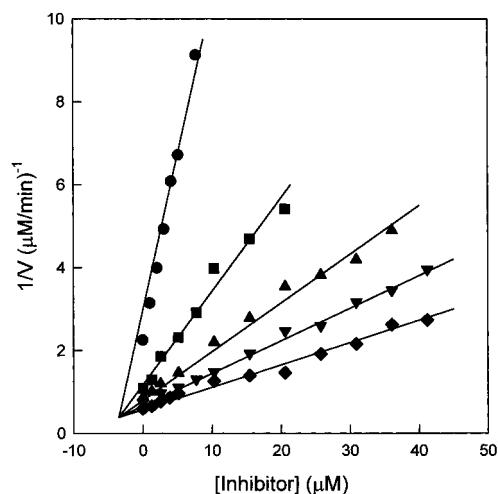
The spectral changes for quercetin oxidation depicted in Figure 1 were completely dependent on the presence of active enzyme. To further prove the specificity of the flavonol oxidation by PPO, the effect of selective inhibitors was also examined. A considerable number of PPO inhibitors are known, one large group of which is constituted by compounds such as benzoic acids and their derivatives, which are structurally analogous to phenolic substrates and which generally show competitive inhibition with respect to these substrates. Other recently recognized PPO inhibitors include 4-substituted resorcinols, which are also structurally related to phenolic substrates. 4-Hexylresorcinol has been shown to be effective in preventing black spot formation in shrimps (Iyengar et al., 1991) and for browning control in different fruits (Frankos et al., 1991). 4-Hexylresorcinol and other 4-substituted resorcinols are also very effective inhibitors of mushroom tyrosinase (McEvily et al., 1992; Jiménez and García-Carmona, 1997), inhibiting the enzyme in a nonclassical manner and classified as slow competitive inhibitors (Jiménez and García-Carmona, 1997).

Figure 4 clearly shows the inhibition of quercetin oxidation by PPO in the presence of 4-hexylresorcinol. Spectral changes in the 300–450 nm region were completely abolished when the inhibitor was added to the reaction medium. Spectral changes at 250–300 nm were not registered due to the absorbance shown by the inhibitor in that wavelength range.

A kinetic analysis of the inhibition of quercetin oxidation by PPO was carried out by Dixon plots of 1/V



**Figure 4.** Consecutive spectra obtained in the oxidation of quercetin by PPO in the presence of 4-hexylresorcinol. The reaction medium, at 25 °C, contained 75  $\mu$ M quercetin, 2 mM SDS, 119  $\mu$ g/mL enzyme, and 0.5 mM 4-hexylresorcinol. Inset: Consecutive spectra obtained for quercetin oxidation by PPO without 4-hexylresorcinol in the reaction medium. The composition of the assay medium was exactly the same but without the inhibitor.



**Figure 5.** Dixon plots for the inhibitory effect of 4-hexylresorcinol on quercetin oxidation catalyzed by PPO. The reaction medium contained flavonol and inhibitor at the indicated concentrations, 2 mM SDS, and 123  $\mu$ g/mL enzyme. The quercetin concentrations used were (♦) 90, (▼) 75, (▲) 60, (■) 45, and (●) 24  $\mu$ M. The correlation coefficient for all the straight lines was  $>0.99$ .

versus  $I$  at five different substrate concentrations. The results obtained (Figure 5) showed that 4-hexylresorcinol behaved as a classical competitive inhibitor. The value of the inhibition constant ( $K_I = 3.5 \mu\text{M}$ ) was determined by extrapolating the interception of the straight lines to the abscissa axis. This  $K_I$  value was similar to that obtained for the inhibition of mushroom tyrosinase by 4-hexylresorcinol (2.4  $\mu\text{M}$ ), although, as previously mentioned, this inhibitor has been described as a competitive slow-binding inhibitor of mushroom tyrosinase (Jiménez and García-Carmona, 1997).

An inhibition study of quercetin oxidation was also carried out with cinnamic acid, a commonly used tyrosinase inhibitor that has been identified as a competitive inhibitor for PPO from different sources

(Vámos-Vigyázó, 1981). Flavonol oxidation was also inhibited by the presence of cinnamic acid in the reaction medium, although this inhibitor was less effective than 4-hexylresorcinol, as shown by the  $K_I$  value obtained ( $K_I = 2.5 \text{ mM}$ ) from the Dixon plots (not shown).

We can conclude, therefore, from the inhibition studies that quercetin oxidation is completely dependent on the presence of active PPO and that the oxidation is more effectively inhibited by 4-hexylresorcinol, reputedly one of the most effective inhibitors for browning control in the food industry (McEvily et al., 1992).

This paper, therefore, shows that PPO can directly oxidize quercetin in vitro, such a direct involvement of PPO in flavonol oxidation not having been described previously. Other authors have suggested that PPO could play a role in the degradation of anthocyanin pigments from grapes and strawberries (Wesche-Ebeling et al., 1990; Sarni-Manchado et al., 1997) and in the oxidation of flavonols present in tea leaves (Finger, 1994) and apple (Nicolas and Potus). However, direct oxidation by PPO did not appear to be the main decolorization route. Quinones and the intermediary compounds formed during the oxidation by PPO of other phenols with a "simpler" structure might be responsible for the destruction of anthocyanin through either oxidation or copolymerization.

The fact that PPO oxidizes quercetin in vitro poses questions about the enzyme's potential involvement in the oxidation of natural polyphenols in vivo. Future studies should be aimed at ascertaining whether PPO is involved in the loss of antioxidant capacity due to the oxidation of phenolic compounds, particularly during the improper handling or storage of fruits and vegetables.

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