An Approach to the Characterization of Betanine Oxidation Catalyzed by Horseradish Peroxidase

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Horseradish peroxidase (HRP, EC 1.1.11.7) catalyzed oxidation of betanine is complex, as judged by the shape of the consecutive absorption spectra of the reaction medium. Graphic analysis of these spectra by a matricial test revealed the presence of at least three kinetically related absorbent species in the reaction mixture. Analysis by HPLC confirmed the presence of several reaction products. During the course of the reaction, a red intermediate and several yellow products, presumably of a polymeric nature, are generated, betalamic acid being one of the final products. Even in the absence of enzyme, the reaction, once initiated, continued again, yielding betalamic acid as a final product, which is formed as both substrate and intermediate product disappear. The chemical nature of the intermediate product and the possible reaction mechanism are discussed.

Keywords: *Beta vulgaris; betanine oxidation; peroxidase; red beet*

INTRODUCTION

Betalains constitute a class of nitrogenous plant pigments characteristic of the *Centrospermae*. They exhibit a wide variety of colors ranging from yellow to deep violet. The color of beet (*Beta vulgaris* L.) roots is due to the presence in the cell vacuoles of red or violet betacyanins and yellow betaxanthins. Betanine (Bt) is the most abundant betacyanin present in red beets.

In the 1970s and 1980s, much attention was paid to the study of the betalain stability, mainly because of their usefulness as natural food colorants instead of artificial dyes (von Elbe, 1975). Thus, the effect of pH, temperature, and light (von Elbe et al., 1974); organic acids, metal cations, antioxidants, and sequestrants (Pasch and von Elbe, 1979); and oxygen (Attoe and von Elbe, 1982) on Bt stability has been reported.

The biosynthetic pathway of betalains has been well-established. Betalamic acid (BA) is derived from tyrosine via DOPA and may condense either with amino acids to give betaxanthins or with cyclo DOPA to produce betacyanins (Mabry, 1980; Piatelli, 1981).

Less is known about the nature of enzymatic betalain degradation, localization of the betalain catabolism, and the products generated during the enzymatic reaction. Pioneering research showed the occurrence of a betacyanine decolorizing enzyme in fresh beet tissue (Soboleva et al., 1976; Lashley and Wiley, 1979). Soboleva et al. (1976) suggested that the decolorizing enzyme might be a peroxidase (EC 1.11.1.7), and Waserman and Guilfoy (1983) reported that the Bt decoloration by a red beet preparation is stimulated by the addition of micromolar concentrations of H_2O_2, thus confirming the involvement of peroxidase in the process.

The subcellular localization of activity decolorizing betacyanin is a subject of controversy. Such activity can be extracted by digestion of tissue slices of beet root with cellulase and pectinase, which suggest that it is located in the cell wall. (Waserman and Guilfoy, 1984). Despite this and other reports that point to an extracellular localization of this activity, the existence of a soluble Bt decolorizing activity in *Amaranthus tricolor* has been described (Elliot et al., 1983). Recent studies carried out in our laboratory indicate that the betacyanin peroxidase activity from beet roots is in part ionic and covalently bound to cell wall and in part soluble in the protoplast, probably in the vacuolar sap (Martínez Parra and Muñoz, unpublished results). The vacuolar localization of betacyanin peroxidase agrees with the well-documented localization of the peroxidase activity involved in polyphenol and alkaloid oxidation (see Pedreño et al. (1993), and literature cited therein).

To date, no reports exist on the nature of the reaction products or on the mechanism of betacyanin oxidation mediated by peroxidase. In the present investigation, we attempted to elucidate these aspects of Bt catabolism by the joint use of spectrophotometry and HPLC technique.

MATERIALS AND METHODS

Plant Material. Roots of a commercial red beet (*Beta vulgaris* L.) cultivar were purchased in a local market and stored at −20 °C until use.

Enzyme. Horseradish peroxidase (HRP, type IX) was supplied by Sigma Chemical Co. (Madrid, Spain).

HPLC Apparatus. The HPLC apparatus consisted of a Model 600 pump, a solvent programmer Model 600 (Waters Associates, Milford, MA), an injector Model 70-10 (Rheodyne, Berkeley, CA) with a 20 μL injector loop, and a column Spherisorb ODS-2, 15 × 0.4 cm, particle size 5 μm (Tracer Analitica, S. A., Barcelona, Spain), fitted with a Model 996 photodiode array detector (Waters Associates, Milford, MA).

Pigment Extraction. Beet roots were sliced and homogenized in a mortar and pestle with cold 80% (v/v) acetone and filtered through filter paper. The residue was successively and alternately washed with 80% (v/v) acetone and pure acetone until no color was observed in the filtrate. The filtrate was dried at reduced pressure and 30 °C and then dissolved in distilled water.

HPLC Analysis. The chromatograms were run with a methanol−water mobile phase (Pourrat et al., 1988). Detailed operating conditions for analysis were as follows: solvent A, methanol/0.05 M KH_2PO_4 (18:82, v/v) adjusted to pH 2.75 with H_3PO_4; solvent B, methanol. A gradient of B in A over 15 min was programmed as follows: 0−3 min, 7% B in A (linear gradient); 3−6 min, 7−12% B in A (linear gradient); 6−9 min, 12−20% B in A (linear gradient); 9−15 min, 20% B in A

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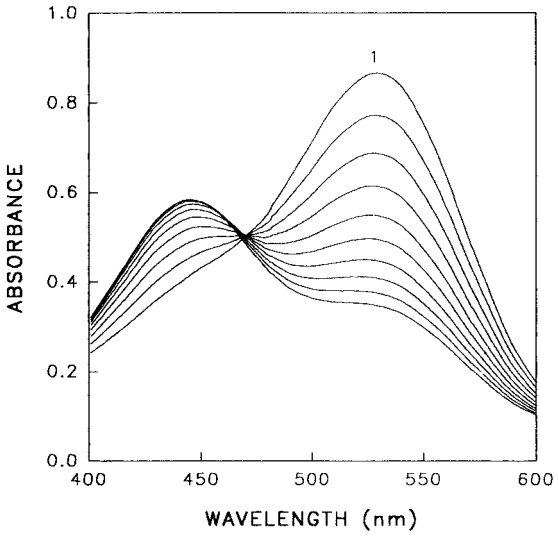

Figure 1. Consecutive spectra of an oxidative medium containing 15 μM betanine, the concentration of which was calculated by using a $\epsilon_{1\%} = 1120$ at 537 nm (Willey and Lee, 1978), 1 mM H_2O_2, and 0.05 UE of HRP in 100 mM Tris [tris-(hydroxymethyl)aminomethane]−acetate (pH 3.0). A scan speed of 400 nm/min was used at 1 min intervals. Line 1, 15 s after enzyme addition.

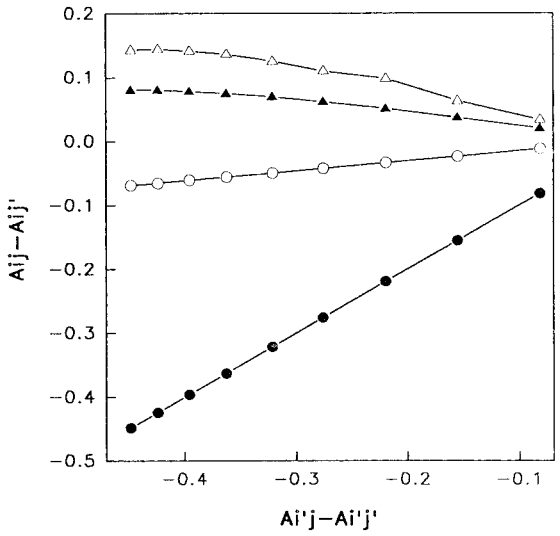

Figure 2. Graphic analysis of the consecutive spectra of the HRP-mediated betanine oxidation shown in Figure 1 according to Coleman et al., 1970. In this analysis, A_{ij} is the absorbance at wavelength i obtained during tracing j, so that A_{12} is the absorbance at 600 nm during the second tracing of the absorption spectrum. The selected wavelengths were: $i_1 = 600$ nm (○), $i_2 = 500$ nm (●), $i_3 = 450$ nm (△), and $i_4 = 400$ nm (▲). $i' = 550$ nm and $j' = 1$ (first tracing), hence the ordinate value ($A_{600,2} - A_{600,1}$) is the difference of absorbances at 600 nm between the second and the first tracing; and the abscissa value ($A_{550,2} - A_{550,1}$) is the difference of absorbances at 550 nm between the second and the first tracing, taking the value $A_{550,1}$ as reference in this graphic analysis.

(isocratic); flow rate 1.5 mL/min; inlet pressure 2000−3000 psi; sample amount 20 μL. The chromatograms were monitored at 537 and 480 nm. Measurement of the peak areas of the eluted substances and their absorption spectra were made with a Millenium software program.

Isolation of Betanine. Betanine was isolated by HPLC from the root extract prepared as described above. For this, the fraction corresponding to Bt was collected at the column outlet, immediately frozen in liquid nitrogen, and lyophilized in a Telstar Model (Lioalfa, Madrid, Spain) lyophilizer. The identification of Bt was carried out by its absorption spectrum and retention time (t_R) (Pourrat et al., 1988; Schwarz and von Elbe, 1980).

RESULTS

Scanning Spectrophotometric Studies on the Oxidation of Betanine by Horseradish Peroxidase (HRP). The time course of the oxidation of Bt by HRP was followed by observing changes in the absorption spectra of the reaction medium (Figure 1). A progressive decrease in the 530−540 nm region and an increase in absorbance in the 430−460 nm region were observed. No spectral changes of the medium in the absence of HRP or H_2O_2 were observed during the time assayed.

The absence of isosbestic point(s) in the set of spectra presented in Figure 1 proves, although not conclusively, that the substrate is transformed into more than one product during the first 9 min of the reaction.

To ascertain the number of species which appear during the reaction, the graphic analysis procedure proposed by Coleman et al. (1970) was applied to the consecutive spectra of the oxidation of Bt for two species. The nonlinearity observed in the tracings corresponding to 400 and 450 nm (Figure 2) demonstrates the presence in the reaction medium of more than two kinetically related absorbent species, Thus, the transformation of Bt into more than one reaction product during the reaction time assayed was confirmed.

HLPC Analysis of the Betanine and Reaction Products. In an attempt to elucidate the actual number of products that appear during enzymatic Bt oxidation and the changes in substrate and product concentration with reaction time, a reaction medium similar to that described in Figure 1 was incubated and the reaction stopped at different times by quickly freezing 50 μL aliquots in liquid nitrogen. The aliquots were then analyzd by HPLC. As a control, a medium without enzyme was also analyzed. HPLC chromatograms of the control medium and of the a medium 15 s after enzyme addition are presented in Figure 3, parts A and B, respectively. The shoulder of the substrate peak (Bt, t_R 3.8 min) and the minor peak (t_R 7.6 min) present in the chromatogram of the control medium

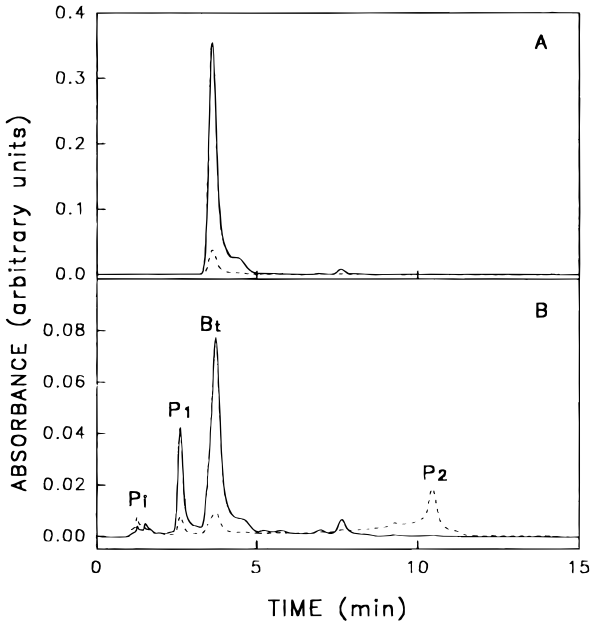

Figure 3. HPLC chromatograms of a medium containing 15 μM betanine and 1 mM H_2O_2 in 100 mM Tris-acetate buffer (pH 3.0) (A) and of a similar medium 15 s after the addition of 0.05 UE of HRP (B). The chromatograms were monitored at 537 nm (—) and at 480 nm (---).

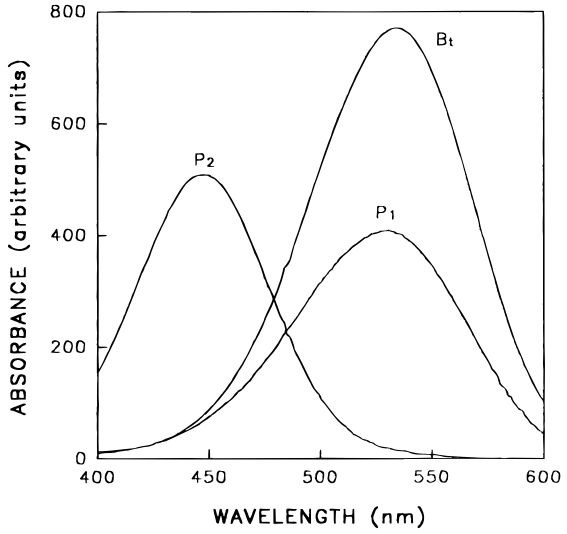

Figure 4. Absorption spectra of the betanine, and of the P_1 and P_2 reaction products.

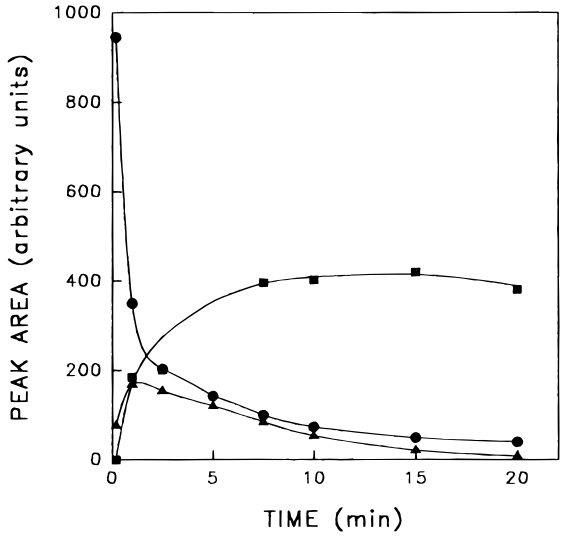

Figure 5. Changes with time of the betanine (●) and P_1 (▲) and P_2 (■) reaction products in a reaction medium similar to that described in Figure 3.

monitored at 537 nm (λ_{max} of the betcyanin spectra) would correspond to two species chemically formed during substrate isolation. The HPLC chromatogram of the reaction medium 15 s after enzyme addition revealed, in addition to the above described peaks, one main peak (P_1, t_R 2.8 min) and a peak formed by the overlapping of minor peaks (P_i, t_R's between 1.2 and 1.5 min). Another peak (P_2, t_R 10.5 min) was observed when the chromatogram was monitored at 480 nm. The similarity of the areas of peaks eluted at 7.6 min (result not showed) and the similarity in size and shape of the shoulder on both HPLC chromatograms prove that the products generated during the substrate isolation are not substrates of the enzyme. The chemical nature of these compounds was not investigated.

The absorption spectra of the Bt, P_1 and P_2 (Figure 4) revealed that the λ_{max} of P_1 (531 nm) was slighty shorter than the Bt (537 nm), and P_2 showed a λ_{max} at 446 nm. It was not possible to record the spectra of the P_i because of poor chromatographic separation.

The changes in the concentration of substrate, P_1 and P_2, are shown in Figure 5. Bt concentration quickly diminished during the first minute of the reaction, this rapid fall being accompanied by a concomitant increase

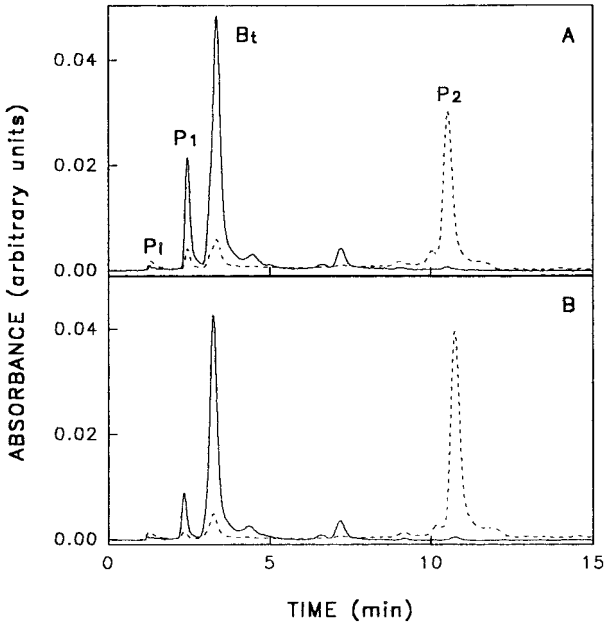

Figure 6. HPLC chromatograms of a reaction mixture immediately (A) and 5 min after filtration through an Amicon filter (B). The intial composition of the reaction medium was as described in Figure 3. The chromatograms were monitored at 537 nm (—) and at 480 nm (- - -).

in P_1 and P_2 concentrations. However, while P_2 accumulation in the reaction medium was parallel to the diminution of substrate concentration, P_1 reached a maximal concentration 1 min after the addition of enzyme and then decreased. The changes in concentration of P_1 demonstrated that this compound is both formed and destroyed during the course of the reaction, the shape of the curve depending on the relative rate of both processes. Quantification of the individual changes in concentration of each of the minor products during the reaction was not possible because of poor chromatographic separation.

To understand better the nature of the reaction, a reaction medium similar to that used in the spectrophometric and HPLC chromatographic studies was prepared. After the reaction had started, the incubation mixture was filtered through an Amicon membrane to remove the enzyme. The HPLC chromatograms of the reaction mixture, immediately (Figure 6A) and 5 min after filtration (Figure 6B), showed that even in the absence of enzyme the substrate and reaction product concentrations changed with time. Thus, besides the enzymatic reaction, a nonenzymatic reaction must be involved in Bt consumption in the conditions assayed. The composition of the mixture was analyzed by HPLC at different times after filtration. For this, as in the case of the changes in composition of the reaction medium in the presence of HRP, 50 μL aliquots of the reaction mixture were taken at different times after filtration and quickly frozen by immersion in liquid nitrogen. The results revealed that while both Bt and P_1 concentrations diminished, P_2 concentration increased with time within the first 5 min after filtration. Afterward, both Bt and P_1 concentrations remained almost constant while P_2 concentration decreased (Figure 7).

DISCUSSION

Plant peroxidases oxidize a wide array of phenolic compounds. According to the well-established peroxi-

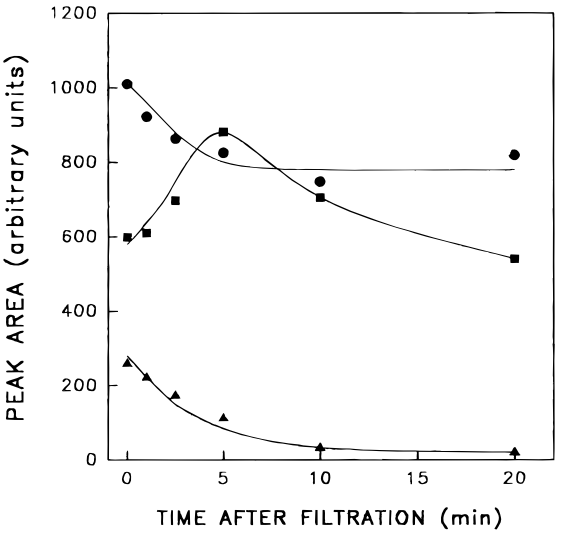

Figure 7. Changes with time after filtration of the medium through an Amicon filter of the betanine (●) and P_1 (▲) and P_2 (■) reaction products.

dase action mechanism (which includes monoelectronic redox reactions between the substrate and the different catalytic forms of the enzyme), the primary phenol oxidation products are their corresponding phenoxy radicals, which are stabilized by delocalization of the unpaired electron at *o*-, *o*′-, and *p*-positions of the aromatic ring. The reaction products of the peroxidase action on monophenols are different polymers which are generated by coupling of the phenoxy radicals in a nonenzymatic reaction, as is the case of lignin formation from its phenolic precursors.

The *o*- and *p*-diphenols are also peroxidase substrates, but instead of yielding oxidative polymers as monophenols do, they normally generate their corresponding *o*- and *p*-benzoquinones (Zapata et al., 1992).

Unlike other glycosides, such as the flavonols quercetin-3-arabinoside, quercetin-3-rhamnoside, and myricetin-3-rhamnoside, and the anthocyanin cyanin, which are not substrates (Morales et al., 1993; Calderón et al., 1992), Bt (betanidin 5-*O*-*β*-glucoside) is a substrate of HRP (Figure 1). Thus, while flavonols and anthocyanidins have to be hydrolyzed by a glycosidase before they can be oxidized by peroxidase (Morales et al., 1993; Calderón et al., 1992), Bt does not have such a requirement.

According to its structure, Bt should react as a monophenol in the peroxidase-mediated reaction, its correspondening phenoxi radical being the initial reaction product (reaction A in Figure 8).

The proposed mechanism for Bt losses during the processing of red beets involves a nucleophilic attack by water at the C adjacent to the indolic N. This hydrolysis causes the formation of cyclo DOPA 5-*O*-*β*-D-glucoside (CDG) and betalamic acid (BA) as products (von Elbe et al., 1981). By analogy with this mechanism, BA must be produced during the reaction by hydrolysis of the Bt radical while it is being generated (reaction B in Figure 8). The formation of BA is additionally supported by the similarity of the absorption spectra of P_2 (Figure 4) to that reported for BA (von Elbe et al., 1981). The other product of the Bt radical hydrolysis would be cyclo DOPA 5-*O*-*β*-D-glucoside radical (CDG•), which is stabilized by delocalization of the unpaired electron along its aromatic ring. This stabilization would be responsible for the higher rate of hydrolysis shown by Bt in the presence of peroxidase

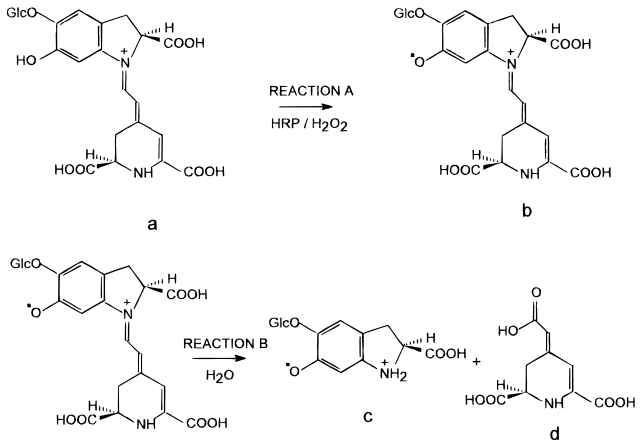

Figure 8. Proposed reactions involved in the first steps of the degradation of betanine: (a) betanine, (b) betanine radical, (c) cyclo DOPA 5-*O*-*β*-D-glucoside radical, (d) betalamic acid.

than that reported in the absence of enzyme (von Elbe et al., 1981).

Next, the CDG• might react with either the substrate to yield cyclo DOPA 5-*O*-*β*-D-glucoside dimer radical [$(CDG)^{\bullet}_2$] and BA

$$CDG^{\bullet} + Bt \rightarrow (CDG)^{\bullet}_2 + BA$$

or with cyclo DOPA 5-*O*-*β*-D-glucoside dimer [$(CDG)_2$] to generate the trimer radical [$(CDG)^{\bullet}_3$]

$$CDG^{\bullet} + (CDG)_2 \rightarrow (CDG)^{\bullet}_3{}' \qquad (1)$$

The formation of $(CDG)_2$ may be the result of the coupling of two units of CDG•:

$$CDG^{\bullet} + CDG^{\bullet} \rightarrow (CDG)_2 \qquad (2)$$

The polymerization chain of the reactions leading to the formation of cyclo DOPA 5-*O*-*β*-D-glucoside polymer radicals [$(CDG)^{\bullet}_i$] could be formulated as follows:

$$(CDG)^{\bullet}_n + Bt \rightarrow (CDG)^{\bullet}_{n+1} + BA \qquad (3)$$

and

$$(CDG)^{\bullet}_n + (CDG)_2 \rightarrow (CDG)^{\bullet}_{n+2} \qquad (4)$$

According to reactions 1 and 2, $(CDG)_2$ is simultaneously consumed and generated in the peroxidase-mediated oxidation of Bt. Thus, the dimer would be the red intermediate product P_1.

Reactions 3 and 4 would explain why both Bt and $(CDG)_2$ are comsumed while BA is generated during the non-enzyme-dependent reactions (Figures 6 and 7). The progressive decrease of the consumption rate of both Bt and $(CDG)_2$ (Figure 7) would be due to the reduction of the pool of radicals during the reaction. The nature of further degradation of BA (Figure 7) was not investigated, although it is possibly due to oxidative processes caused by the free radicals.

The most probable resonant structures of CDG• and structures of the $(CDG)_2$ are given in Figure 9. Dimer II has a structure similar to that of the isodityrosine and diferuloyl bridges present in extensin and hemicelluloses, respectively, which are also generated in reactions catalyzed by peroxidase (Fry, 1987; Biggs and

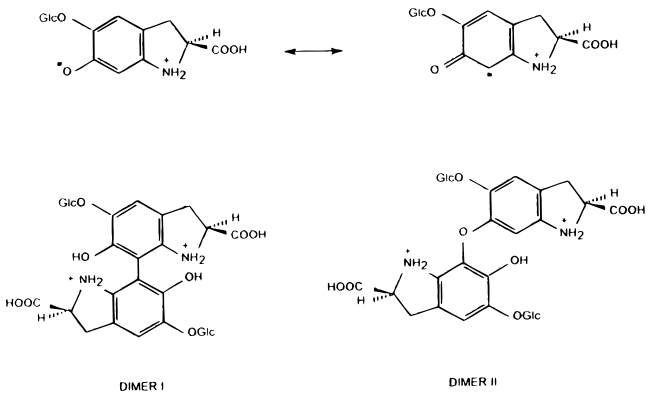

Figure 9. Resonant structures of cyclo DOPA 6-*O*-*β*-D-glucoside radical (top). Possible structures of cyclo DOPA 5-*O*-*β*-D-glucoside dimer (bottom).

Fry, 1987). The λ_{max} of P_1 (Figure 4) suggests that the dimer II is not formed in reaction 2 because this compound would present maximum absorbance at a shorter wavelength. The chromophore of dimer I would be responsible for an absorbance spectrum as the shown by P_1.

Finally, the polymerization chain ceases when polymer radicals couple at any step

$$(CDG)^{\bullet}_{l} + (CDG)^{\bullet}_{m} \rightarrow (CDG)_{l+m}$$

the different $(CDG)_{l+m}$ being the P_i which appear in the HPLC chromatograms shown in Figures 3 and 6.

In conclusion, peroxidase-catalyzed Bt oxidation produces Bt radicals, which generate different polymers of CDG through a chain of polymerization reactions and BA.

The results mean that the spectrophotometrc estimation of the rate of peroxidase-catalyzed Bt oxidation measured by changes in absorbance at 537 nm should not be considered correct, because at this wavelength both substrate and intermediate $(CDG)_2$ are absorbent species and, consequently, any changes in absorbance do not reflect modifications of substrate concentration during the enzymatic reaction.

ABBREVIATIONS USED

BA, betalamic acid; Bt, betanine; CDG, cyclo DOPA, 5-*O*-*β*-D-glucoside; DOPA, dihydroxyphenylalanine; HRP, horseradish peroxidase; t_R, retention time.

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