Characterization of Betacyanin Oxidation Catalyzed by a Peroxidase from *Beta vulgaris* L. Roots

J. Martínez-Parra and R. Muñoz*

Departamento de Biología Vegetal (Fisiología Vegetal), Facultad de Biología, Universidad de Murcia, Campus de Espinardo, E-30100 Espinardo, Murcia, Spain

A protein fraction with peroxidase (EC 1.11.1.7) activity against guaiacol from *Beta vulgaris* L. roots oxidized both betanidin and betanin (betanidin 5-*O*-β-D-glucoside), the former being the more efficient substrate for the enzyme. The protein fraction contained three strongly basic perxidase isoenzymes. Betanidin quinone was formed as the only product in the course of enzymatic betanidin oxidation, whereas betalamic acid and several oxidized *cyclo*-DOPA 5-*O*-β-D-glucoside polymers were generated during the oxidation of betanin. In accordance with the catalytic properties of peroxidase, a possible mechanism for betanidin oxidation is proposed. This mechanism includes the formation of a betanidin radical, which, by further dismutation, yields betanidin quinone and betanidin. The betanidin oxidation rate showed a Michaelis-type dependence on the substrate concentration. The apparent K_M for the reaction was 0.46 mM. On the basis of the spectral properties of the enzyme responsible for both betanidin and betanin oxidations, its peroxidase nature is suggested.

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

INTRODUCTION

In most plant species, the bright color of flowers and fruits is due to the presence of carotenoids and anthocyanins. However, in Caryophyllales the pigmentation of reproductive and vegetative organs is produced by betalains, a group of nitrogeneous plant pigments. Betalains are classified into two families of compounds: red betacyanins and yellow betaxanthins (*1*).

Although betalain stability has been the subject of numerous studies, mainly because of their use as natural food colorants to replace artificial dyes (*2*), less is known about their catabolism.

Since the pioneering studies of Soboleva et al. (*3*), who described the occurrence of a betacyanin decolorizing enzymatic activity in red beets, a number of papers on the catabolism of betalains in *Beta vulgaris* (*4−6*) and *Amaranthus tricolor* (*7*) have been published. This decolorizing activity might be a peroxidase (EC 1.11.1.7), as can be deduced from the fact that betanin (Bt) decolorization by a red beet preparation is stimulated by H_2O_2 (*6*, *8*). Moreover, horseradish peroxidase (HRP) can oxidize Bt (*8*, *9*). However, to date, the true nature of the enzyme responsible for in vivo betacyanin catabolism has not be established.

Peroxidases are a group of hemoproteins having as their main function the oxidatin of substrates at the expense of H_2O_2. On the basis of their amino acid sequence and metal-binding properties, peroxidases from higher plants, fungi, and bacteria have been categorized into three classes (*10*). Class I includes yeast cytochrome *c* peroxidase, chloroplast and cytosolic ascorbate peroxidases, and bacterial peroxidases, class II comprises the extracellular fungal lignin and manganese peroxidases, and class III contains the classic secretory peroxidases from higher plants.

Basic class III peroxidases of high p*I* are the only isoenzymes located in vacuoles, for which a metabolic role in the oxidation of alkaloids and phenolics has been described (*11*).

Although identification of the reaction products generated in the HRP-catalyzed oxidation of Bt and a possible mechanism of the reaction were recently reported by Martínez-Parra and Muñoz (*9*), to date no study on the enzymatic nature of betanidin (Bd) degradation has been published.

In this study, some aspects of betacyanin oxidation by a protein fraction from *B. vulgaris* are described, and the nature of the enzyme responsible for the reaction as peroxidase is suggested.

MATERIALS AND METHODS

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

Enzyme Extraction. Beet roots were sliced and homogenized in an Omnimixer (Ivan Sorvall Inc., Newtown, CT) at 4 °C with 100 mM tris[hydroxymethyl] aminomethane (Tris)−acetate buffer (pH 7.0) containing 1 M KCl (2:1, w/v), filtered through cheesecloth, and centrifuged at 40000*g* for 20 min. The supernatant was considered as crude enzyme extract.

Peroxidase Measurement. The peroxidase activity was estimated by the increase in absorbance at 460 nm of a reaction medium (1 mL) containing 5 mM guaiacol, 1 mM H_2O_2, and enzyme extract in 50 mM acetate buffer (pH 5). Enzyme activity was expressed in nanokatals.

Enzyme Purification. Crude enzyme extract was loaded onto a Sephadex G-25 column (2.5 cm diameter, 30 cm length) previously equilibrated with distilled water and eluted (2.5 mL min^{-1}) with distilled water. The fractions containing peroxidase activity checked with guaiacol as substrate were pooled and precipitated with $NH_4(SO_4)_2$ (90% saturation) and centrifuged at 15000*g* for 10 min. The precipitate was suspended in distilled water and desalted by chromatography on a PD-10 Sephadex G-25 column (Amersham Pharmacia Biotech AB, Uppsala, Sweden) equilibrated with 10 mM 2-(*N*-morpholino)-

* Author to whom the correspondence should be addressed (fax +34 968 363963; e-mail rmunoz@um.es).

Peroxidase-Catalyzed Oxidation of Betacyanins

J. Agric. Food Chem., Vol. 49, No. 8, 2001 **4065**

ethanesulfonic acid (Mes)−NaOH buffer (pH 5.5). The eluted peroxidase fractions were pooled and loaded onto a CM-cellulose column (1 cm diameter, 20 cm length) previously equilibrated with 50 mM Mes−NaOH buffer (pH 5.5). The bound peroxidase was eluted with 1 M NaCl in equilibration buffer.

Peroxidase Isoelectrofocusing. The isoenzyme pattern was revealed by isoelectrofocusing on 5% polyacrylamide gels by using a Mini-Protean (Bio-Rad Laboratories, Hercules, CA) system following the instructions of the supplier. The peroxidase activity on the gels was stained by immersion in 1 mM 4-methoxy-α-naphthol and 1 mM H_2O_2 in 50 mM Tris−acetate buffer (pH 5.0) (*12*).

Betanin Extraction. The colored fractions from the Sephadex G-25 chromatography of the crude extract were analyzed by HPLC (see below), and those containing Bt as the main pigment were pooled and concentrated (~10-fold) at reduced pressure. The concentrated pigment extract was then supplemented with pure acetone to 90% (v/v) and centrifuged at 3000*g* for 10 min. The pellet was dried under vacuum, and the acetone-free residue was dissolved in distilled water. The aqueous pigment solution was loaded onto a C-18 Sep-Pak cartridge (Waters Associates, Milford, MA) equilibrated with distilled water, and the retained pigments were eluted with 5% (v/v) methanol in water. The eluted fractions were analyzed by HPLC, and those containing Bt as the sole pigment were pooled and stored at 0−4 °C until use. Bt concentration was estimated by measuring the absorbance at 535 nm using an ϵ of 65000 M^{-1} cm^{-1}(*13*).

Betanidin Preparation. Bd was obtained by enzymatic hydrolysis of 0.6 mM Bt in 50 mM phosphate buffer (pH 3.5) with 3 nkat of a commercial almond β-glucosidase (Sigma Chemical Co., St. Louis, MO). In all of the Bd preparations, total substrate hydrolysis was observed within 50 min.

HPLC Analysis. HPLC analysis was carried out in an HPLC apparatus consisting of a model 600 pump, a model 600 solvent programmer (Water Associates), an injector model 70-10 (Rheodine, Berkeley, CA) with a 20 μL injector loop, and a Spherisorb ODS-2 column, 15 × 0.4 cm, with a particle size of 5μm (Tracer Analitica S.A., Barcelona, Spain), fitted with a model 996 photodiode array detector (Water Associates). Measurements of the peak areas of the eluted substances and their absorption spectra were made with a Millenium 2010 software program. Detailed elution conditions, based on those used by Pourrat et al. (*13*), were as follows: flux, 1.5 mL min^{-1}; methanol (solvent A), 50 mM phosphate buffer (pH 2.75) (solvent B), and acetonitrile (solvent C), 0−5 min, 15−25% A, 85−75% B, 0% C; 5−10 min, 25−35% A, 75−55% B, 0−10% C; and 10−15 min, 35% A, 55−45% B, 10−20% C.

Assay for Betanin/Betanidin Oxidation. The time course of Bt/Bd oxidations was followed by measuring changes in the peak area of the substrate in HPLC analysis of media containing either Bt or Bd, H_2O_2, and enzyme in 100 mM phosphate buffer (pH 3.5) (optimum pH of the decolorizing enzyme) (*5*). The accumulation of reaction product(s) was estimated by measuring changes in their peak areas in HPLC analysis of reaction media.

RESULTS AND DISCUSSION

Peroxidases are highly polymorphic enzymes, and the functionality of each isoenzyme depends on its nature (acidic or basic) and subcellular localization (*11*). For this reason, the first characteristic to be studied of the enzymatic activity eluted in the CM-cellulose chromatography was the nature and number of isoperoxidases. For this, the protein was analyzed by gel isoelectrofocusing (pH 3.5−9.0). The fact that the staining of peroxidase activity on the gels did not reveal peroxidase bands could signify that the isoenzyme(s) present in the eluted protein have a very high pI (>9.0). To visualize these strongly basic isoperoxidases, the isoelectrofocusing run was stopped before equilibrium was reached.
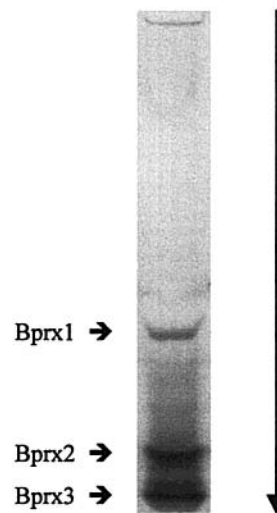

Figure 1. Enzyme staining in nonequilibrium gel isoelectrofocusing of red beet peroxidase (0.5 nkat) isoenzymes stained with 4-methoxy-α-naphthol. The arrow indicates increasing pH.

This nonequilibrium isoelectrofocusing of the enzymatic activity (Figure 1) revealed the presence of three isoenzymes (Bprx1−3).

Previous results obtained in our laboratory indicate that red beet root peroxidase activity is, in part, localized in the protoplast, probably in the vacuolar sap (Martínez-Parra and Muñoz, unpublished results).

From cytochemical and biochemical studies, and their subcellular localization, Pedreño et al. (*11*) classified class III secretory plant peroxidases into three groups: acidic (pI < 7.0), moderately basic (7.0 < pI < 9.0), and strongly basic (pI > 9.0). The reactivity of the last group of isoperoxidases toward alkaloids, such as capsicinoids (*14, 15*), and phenolics, such as flavonols (*16*) and anthocyanins (*17*), and their localization in vacuoles strongly suggest a role for these isoenzymes in the catabolism of secondary metabolites. In view of both the subcellular localization and the reactivity of strongly basic isoperoxidases, the possible involvement of the protein fraction from red beet in both Bt and Bd oxidation was assayed.

The Bd concentration in the different reaction media was calculated by comparing its peak area in the HPLC chromatogram with those shown by the Bd generated in the total enzymatic hydrolysis with almond β-glucosidase of Bt at known concentrations (see Materials and Methods).

To investigate the product(s) generated during Bd oxidation, a reaction medium containing H_2O_2, and Bd was analyzed by HPLC at *t* = 0 and 15 s after the reaction had been initiated by the addition of beet root partially purified peroxidase (Figure 2). A sole reaction product (peak 1) was produced during the reaction. The two minor peaks (2 and 3) present in the medium before the start of the reaction were also oxidized by the enzyme. The chemical nature of compounds eluted as peaks 2 and 3 was not investigated but possibly corresponded to isobetanidin and neobetanidin. No change in Bd concentration was observed in the absence of H_2O_2.

The absorption spectra of both the substrate and the reaction product are shown in the insert of Figure 2. As can be seen, Bd showed one absorption band centered at 541 nm, whereas the reaction product presented two bands centered at 403 and 555 nm, respectively.
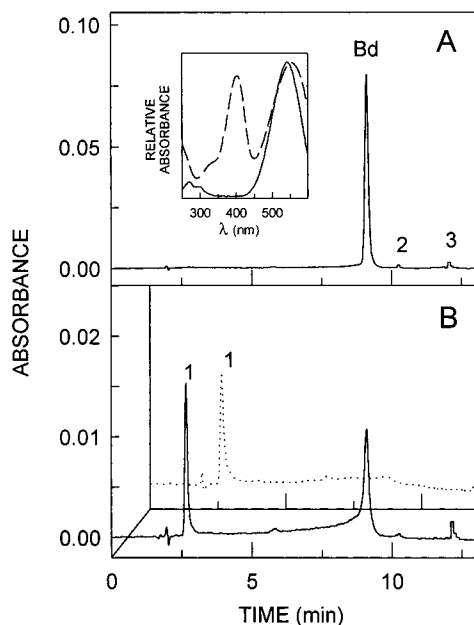
Figure 2. HPLC chromatograms of a medium containing 25 μM Bd and 1 mM H_2O_2 in 100 mM phosphate buffer (pH 3.5) before (A) and 15 s after the addition of 0.1 nkat of purified red beet peroxidase (B). In (B), the HPLC chromatogram (···) of the product was generated in the oxidation of 0.01 mM Bd in 100 mM phosphate buffer (pH 3.5) by 0.01 mM sodium periodate. (Insert) Absorption spectra of Bd (—) and reaction product (- - -) generated in both enzymatic and chemical oxidations of Bd obtained from the HPLC chromatograms. The chromatograms were monitored at 537 nm.

The time course in a 0–60 s interval of Bd consumption and product accumulation in a medium containing 30 μM Bd, 1 mM H_2O_2, and 0.03 nkat of enzyme in 100 mM phosphate buffer (pH 3.5) showed a rapid substrate consumption accompanied by concomitant product generation (results not shown).

For comparative purposes, the products formed during Bt oxidation catalyzed by red beet peroxidase and any changes with reaction time were also analyzed. The nature of the reaction products, as well as their evolu-

tion, was identical to that observed during the Bt oxidation catalyzed by HRP (results not shown) (*9*).

Martínez-Parra and Muñoz (*9*) reported a possible mechanism for Bt oxidation catalyzed by HRP, based on the monophenol nature of the substrate and the well-known action mechanism of peroxidase. The proposed mechanism described the formation of Bt radical as the first reaction product followed by its polymerization and simultaneous hydrolysis to yield an oxidized *cyclo*-DOPA 5-*O*-β-D-glucoside dimer as an intermediate product and several oxidized *cyclo*-DOPA 5-*O*-β-D-glucoside polymers and betalamic acid as final products. The similarity of the behavior shown by red beet peroxidase to that of HRP points to the existence of a general reaction mechanism for peroxidase acting on Bt.

Bd is an *o*-diphenol and, consequently, its peroxidase-mediated oxidation would produce the corresponding *o*-quinone (peak 1 in Figure 2), as is the case of the peroxidase-catalyzed oxidation of *o*- and *p*-diphenols (*18*). The proposed reaction mechanism is shown in Figure 3. In a first step (reaction I), the peroxidase-mediated oxidation of the substrate generates the Bd radical (as in Bt oxidation), which suffers a further nonenzymatic dismutation to produce Bd quinone (peak 1 in Figure 2) and Bd (reaction II). The longer λ_{max} (555 nm) shown by the absorption spectrum of the reaction product than that of Bd (541 nm) (Figure 2A insert), which indicates higher conjugation of the reaction product, suggests the formation of Bd quinone in the enzymatic oxidation of Bd. Furthermore, the generation of Bd quinone in the oxidation of Bd could also be supported by the appearance of an absorption maximun at 403 nm (characteristic of *o*-quinones) and the disappearance of the maximum of absorption at 270 nm (characteristic of phenols). To ascertain the formation of Bd quinone, a sample of Bd prepared as described under Materials and Methods was oxidized with sodium periodate, an oxidant that unequivocally produces the *o*-quinones in the oxidation of *o*-diphenols. The absorption spectrum of the oxidation product and its retention time in an HPLC analysis were identical to that shown
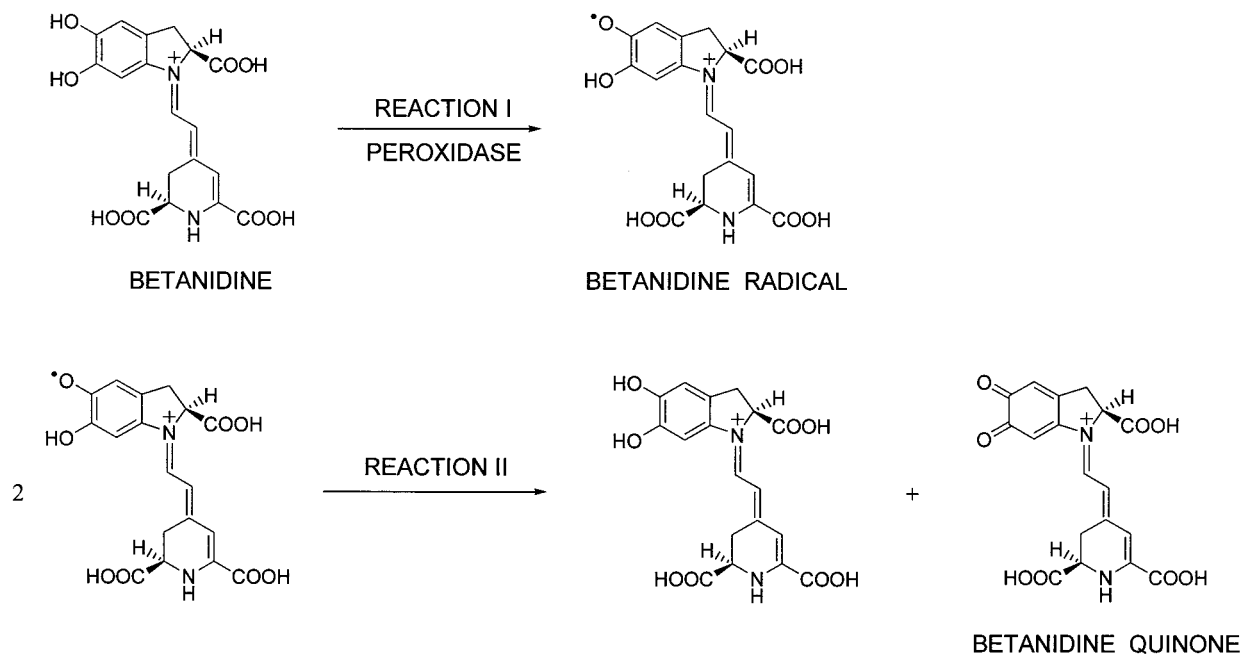

Figure 3. Proposed reactions involved in the enzymatic degradation of Bd.

Peroxidase-Catalyzed Oxidation of Betacyanins
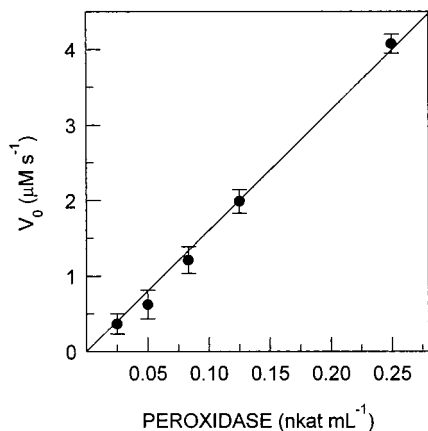
J. Agric. Food Chem., Vol. 49, No. 8, 2001 **4067**

Figure 4. Dependence of the initial oxidation rate of Bd on the purified red beet peroxidase concentration. The medium contained 0.2 mM Bd, 1 mM H_2O_2, and enzyme at variable concentrations in 100 mM phosphate buffer (pH 3.5). Bars indicate SE of three independent determinations.

Figure 5. Dependence of the initial oxidation rate of Bd on the substrate concentration in reaction media composed of variable concentrations of Bd, 1 mM H_2O_2, and 0.002 nkat of purified peroxidase in 100 mM phosphate buffer (pH 3.5). (Insert) Lineweaver–Burk plot of the results. Bars indicate SE of three independent determinations.

by the reaction product of the enzymatic oxidation of Bd (Figure 2)

The relative effectiveness of beet root peroxidase in the oxidation of Bt and Bd was also studied. For comparative purposes, the progress of both Bt and Bd oxidations was followed. The reaction media contained either 100 μM Bd or 100 μM Bt, 1 mM H_2O_2, and 0.05 nkat of enzyme in 100 mM phosphate buffer (pH 3.5). Bd was a more efficient substrate for this peroxidase preparation than Bt. Fifteen seconds after the reaction was started, the peak area of the remaining Bd in the reaction medium was 24% of the initial area, whereas that of Bt was 82% of the initial peak area. These results are in accordance with the findings described for several phenols and phenol glycosides. Thus, although flavonols and anthocyanins are efficiently oxidized by peroxidase, their corresponding glycosides are poor substrates of the enzyme (*16, 17*). These differences in the efficiency of red beet peroxidase in the catalysis of Bd and Bt points to the possible involvement of both β-glycosidase and peroxidase in betalain catabolism. This possibility is lent weight by the ability of β-glycosidase to hydrolyze Bt and by the well-established vacuolar localization of glycosidases (*18*). The involvement of glycosidases and peroxidase was also proposed for the degradation of phenol glycosides in vivo (*16, 17*).

The kinetics of Bd oxidation by beet root peroxidase was also studied. The progress of Bd oxidation approximately followed a first-order reaction (results not shown), and so the initial rate is difficult to estimate. However, the plot of the natural logarithm of the substrate concentration versus time allowed us to calculate the reaction constant (k), which, when multiplied by the initial substrate concentration, provides the initial rate. The linearity observed in the plot of initial rate against protein concentration (Figure 4) confirms the validity of the above-described method for measuring the enzymatic activity with Bd as substrate.

Because red beet peroxidase is a mixture of three basic isoenzymes (Figure 1), a reliable K_M value cannot be defined for the substrate. However, for comparative purposes, the apparent K_M value was determined. The dependence of the initial oxidation rate of Bd by red beet peroxidase on substrate concentration (Figure 5) showed a Michaelis–Menten type kinetics, which was confirmed by the linearity observed in the Lineweaver–Burk plot
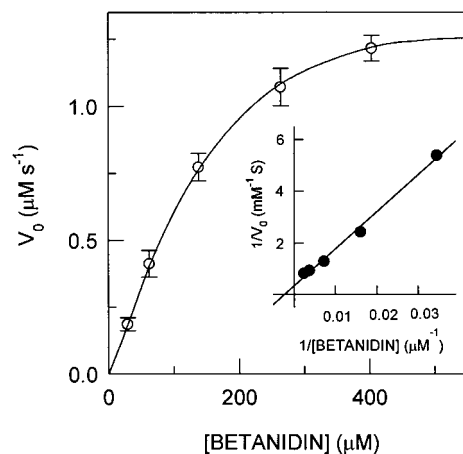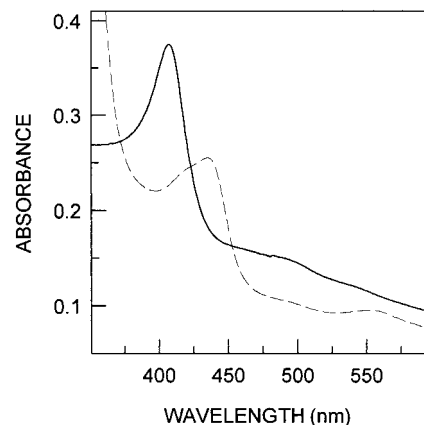

Figure 6. Absorption spectra of the purified red beet peroxidase (15 nkat mL^{-1}) (—) and of the reduced enzyme after addition of 300 μg of sodium dithionite (- - -).

of the results (Figure 5 insert). From this plot, an apparent K_M for the substrate of 0.46 mM was calculated.

The ability of cell wall fragments and beet extracts to decolorize Bt has been described (*3–6*). Although the decolorizing activity of beet preparations was stimulated by H_2O_2 (*6, 8*), which suggests that peroxidase is the enzyme responsible for Bt catabolism in vivo, the true nature of the enzyme involved in the process has not been demonstrated. To ascertain the nature of the protein eluted in the CM-cellulose, the absorption spectrum of this protein fraction was recorded. The purified protein showed absorption maxima at 407 nm (Figure 6), which shifted to 550 nm when the ferric enzyme was reduced by the addition of sodium dithionite (Figure 6). These spectral characteristics are typical and unequivocal for heme-containing high-spin secretory class III peroxidases and differentiate them from low-spin peroxidases as the latter show, in their oxidized form, a Soret peak at 420 nm (*19*). For this reason, the involvement of peroxidase in both Bd and Bt catabolism seems to be very probable. The decolorizing activity shown by both solubilized protein and cell wall fragments from beet roots in the absence of H_2O_2 (*6, 8*) can be explained by the ability of peroxidase to generate H_2O_2 at the expense of molecular oxygen and a suitable reductant, as has been demonstrated in the oxidation of different substrates (*20–22*).

ABBREVIATIONS USED

Bd, betanidin; Bt, betanin; HRP, horseradish peroxidase; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Tris, tris[hydroxymethyl]aminomethane; SE standard error of the mean.

LITERATURE CITED

(1) Piattelli, M. The betalains: Structure, biosynthesis, and chemical taxonomy. In *The Biochemistry of Plants*; Stumpf, P. K., Conn, E. E., Eds.; Academic Press: New York, 1981; Vol. 7, pp 557−575.

(2) von Elbe, J. H. Stability of betalains as food colorants. *Food Technol.* **1975**, *29*, 42−46.

(3) Soboleva, G. A.; Ul'yanova, M. S.; Zakharova, N. S.; Bokuchava, M. A. Betacyanine-decolorizing enzyme. *Biochemistry (USSR)* **1976**, *41*, 794−799.

(4) Lashley, D.; Wiley, R. C. A betacyanine decolorizing enzyme found in root beet tissue. *J. Food Sci.* **1979**, *44*, 1568−1569.

(5) Shih, C. C.; Wiley, R. C. Betacyanine and betaxanthine decolorizing enzymes in the beet (*Beta vulgaris* L.) roots. *J. Food Sci.* **1981**, *47*, 164−199.

(6) Wasserman, B. P.; Guilfoy, P. Solubilization of the red beet cell wall betanine decolorizing enzyme. *J. Food Sci.* **1984**, *49*, 1075−1077.

(7) Elliot, D. C.; Schultz, C. G.; Cassar, R. A. Betacyanin decolorizing enzyme in *Amaranthus tricolor* seedlings. *Phytochemistry* **1983**, *22*, 383−387.

(8) Wasserman, B. P.; Guilfoy, P. Peroxidative properties of betanine decolorization by cell walls of red beets. *Phytochemistry* **1983**, *22*, 2653−2656.

(9) Martínez-Parra, J.; Muñoz, R. An approach to the characterization of betanine oxidation catalyzed by horseradish peroxidase. *J. Agric. Food Chem.* **1997**, *45*, 2984−2988.

(10) Welinder, K. G. Superfamily of plant, fungal and bacterial peroxidases. *Curr. Opin. Struct. Biol.* **1992**, *2*, 388−393.

(11) Pedreño, M. A.; Bernal, M. A.; Calderón, A. A.; Ferrer, M. A.; López-Serrano, M.; Merino de Cáceres, F.; Muñoz, R.; Ros Barceló, A. A general pattern for peroxidase isoenzyme localization and function in *Vitaceae, Solanaceae* and *Leguminoseae*. In *Plant Peroxidases: Biochemistry and Physiology*; Welinder, K. G., Rasmusen, S. K., Penel, C., Grepin, H., Eds.; University of Geneve: Geneve, Switzerland, 1993; pp 307−314.

(12) Ferrer, M. A.; Calderón, A. A.; Muñoz, R.; Ros Barceló, A. 4-Methoxy-α-naphthol as a specific substrate for kinetic, zymographic and cytochemical studies on plant peroxidase activities. *Phytochem. Anal.* **1990**, *1*, 63−69.

(13) Pourrat, A.; Lejeune, B.; Grand, A.; Pourrat, H. Betalains assay of fermented red beet root extract by high performance liquid chromatography. *J. Food Sci.* **1988**, *53*, 294−295.

(14) Bernal, M. A.; Calderón, A. A.; Pedreño, M. A.; Muñoz, R.; Ros Barceló, A.; Merino de Cáceres, F. Capsaicin oxidation by peroxidasefrom *Capsicum annuum* (var. annuum) fruits. *J. Agric. Food Chem.* **1993**, *41*, 1041−1044.

(15) Bernal, M. A.; Calderón, A. A.; Pedreño, M. A.; Muñoz, R.; Ros Barceló, A.; Merino de Cáceres, F. Dihydrocapsaicin oxidation by *Capsicum annuum* (var. annuum) peroxidase. *J. Food Sci.* **1993**, *58*, 611−613.

(16) Morales, M.; Pedreño, M. A.; Muñoz, R.; Ros Barceló, A. Oxidation of flavonols and flavonol glycosides by a hypodermal peroxidase isoenzyme from Gamay rouge grape (*Vitis vinifera*) berries. *J. Sci. Food Agric.* **1993**, *62*, 385−391.

(17) Calderón, A. A.; García-Florenciano, E.; Muñoz, R.; Ros Barceló, A. Gamay grapevine peroxidase: Its role in vacuolar anthocyani(di)n degradation. *Vitis* **1992**, *31*, 139−147.

(18) Zapata, J. M.; Calderón, A. A.; Muñoz, R.; Ros Barceló, A. Oxidation of *p*-hydroquinone by both cellular and extracellular grapevine peroxidase fractions. *Biochimie* **1992**, *4*, 143−148.

(19) Marty, F.; Branton, D.; Leigh, L. A. Plant vacuoles. In *The Biochemistry of Plants*; Tolbert, N. E., Ed.; Academic Press: New York, 1980; Vol. 1, pp 625−658.

(20) Zapata, J. M.; Calderón, A. A.; Muñoz, R.; Ros Barceló, A. Oxidation of natural hydroxybenzoic acids by grapevine peroxidases: Kinetic characteristics and substrate specifity. *Am. J. Enol. Vitic.* **1992**, *43*, 134−138.

(21) Yamazaki, I.; Yokota, K. N. Oxidation states of peroxidase. *Mol. Cell. Biochemistry* **1973**, *2*, 39−52.

(22) Pedreño, M. A.; Ros Barceló, A.; García-Carmona, F.; Muñoz, R. Oxidation of dihydroxyfumaric acid in the absence of H_2O_2 by cell-wall bound peroxidases from lupin: A possible general model. *Plant Physiol. Biochem.* **1990**, *28*, 37−42.

(23) Ferrer, M. A.; Pedreño, M. A.; Muñoz, R.; Ros Barceló, A. Oxidation of coniferyl alcohol by cell wall peroxidases at the expense of indole-3-acetic acid and O_2. A model for the lignification in the absence of H_2O_2. *FEBS Lett.* **1990**, *276*, 127−130.

(24) Ros Barceló, A.; Aznar-Asensio, G. J. Coniferyl alcohol oxidase of a cell-wall-located class III peroxidase. *Aust. J. Plant Physiol.* **1999**, *26*, 411−419.