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PEROXIDATIVE PROPERTIES OF BETANIN DECOLORIZATION BY CELL WALLS OF RED BEET

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Key Word Index—*Beta vulgaris*; Chenopodiaceae; beet; horseradish peroxidase; betacyanin; betalain; cell wall; phenoloxidase.

Abstract—The effect of H_2O_2 on betanin decolorization by horseradish peroxidase and a red beet cell wall preparation was examined. Both reactions were stimulated by the addition of micromolar concentrations of H_2O_2 and were accompanied by the formation of an unstable product with an absorbance maximum of *ca* 452 nm. In the absence of added H_2O_2, the cell wall reaction was inhibited by catalase. When cell walls alone were placed in assay buffer, pH 3.4, H_2O_2 generation occurred. The addition of betanin caused a further production of H_2O_2. These results suggest that the beet cell wall-mediated decolorization reaction proceeds via a peroxidatic mechanism.

INTRODUCTION

The red beet (*Beta vulgaris* L.) contains a group of red and yellow pigments known as betalains [1], which are localized within the vacuole of the intact cell [2]. Although the thermal degradation of isolated beet pigments has been well documented [3], information concerning their enzymatic decomposition is limited. Soboleva *et al.* [4] identified and characterized a betalain-decolorizing enzyme activity in an isolated red beet cell wall fraction. Later studies confirmed these results [5, 6]. A soluble betanin decolorizing enzyme was recently partially purified from *Amaranthus tricolor* [7].

Soboleva et al. [4] suggested that decolorization might be due to a cell wall-bound peroxidase and found that the red pigment betanin could be decolorized by horseradish peroxidase in the presence of 150 mM H_2O_2 and dissolved oxygen. The isolated cell wall fraction was then assayed in the presence of this concentration of H_2O_2. Rather than having a stimulatory effect, an inhibition of the total amount of betacyanin decolorized was observed. This led to the conclusion that a phenoloxidase, rather than a peroxidase, was involved in the cell wall-mediated decolorization reaction.

In this paper, we describe a series of experiments which examine the role of H_2O_2 in betanin decolorization. Our results suggest that a peroxidase is involved in the cell wall-mediated decolorization reaction.

RESULTS

To distinguish between horseradish peroxidase (HRP) and phenoloxidase (PPO) mediated betanin decolorization, purified preparations of both enzymes were incubated with the substrate at pH 3.4, the pH optimum of the cell wall-mediated decolorization reaction. Whereas phenoloxidase had no effect, rapid decolorization occurred with HRP in the presence of H_2O_2. To eliminate the possibilty that the phenoloxidase preparation was inactive, it was incubated with catechol (Experimental). This reaction proceeded at the expected rate.

General characteristics of HRP-mediated decolorization

At 15 μM betanin and 10 μM H_2O_2, initial reaction rates were linear between 0 and 1.0 μg per ml HRP. The reaction had a pH optimum of 3.4 with 50% activity at pH values of 3.0 and 4.0. The shape of the pH profile was identical to the one obtained by Soboleva *et al.* [4] with isolated cell walls.

HRP-mediated decolorization was accompanied by the formation of a product with a λ_{max} at *ca* 453 nm (Fig. 1A), which gradually diminished, suggesting that the initial

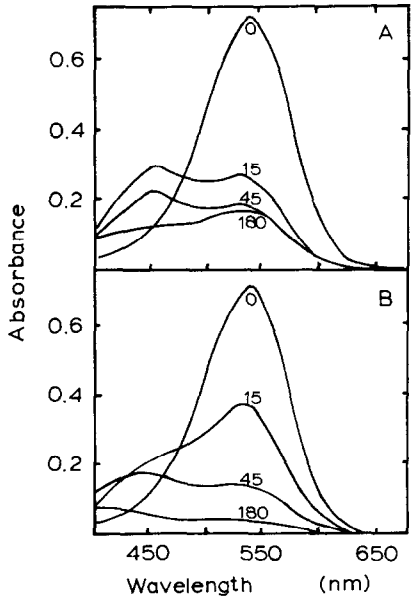

Fig. 1. Wavelength scan of the time course of decolorization. (A) HRP-mediated decolorization, conducted with 10^{-5}M H_2O_2. (B) The cell wall-catalysed reaction. HRP (0.305 μg) and cell walls (0.27 mg) were assayed as described under Experimental.

breakdown product may also be a peroxidase substrate. Decolorization by beet cell wall was accompanied by the formation of a peak with λ_{max} at *ca* 451 nm (Fig. 1B) which gradually shifted downwards. These λ_{max} are somewhat higher than those reported with the *Amaranthus* decolorizing enzyme [7].

The HRP-mediated reaction was accompanied by the rapid uptake of O_2. The extent of O_2 uptake, estimated polarographically, was variable and ranged between 1.0 and 2.0 nmol per nmol betanin decolorized. O_2 uptake occurred in one rapid burst and ended long before decolorization was complete. Decolorization by the cell wall fraction was also O_2 dependent [4].

The K_4 of the reaction in the presence of H_2O_2 was found to be 1.95×10^{-4} sec^{-1}. M^{-1}. This fits within the expected range of K_4 values for HRP substrates [8]. The energy of activation was 8.4 kcal per mol, which is similar to that obtained with the cell wall enzyme [4]. Ascorbate ($K_4 = 1.2 \times 10^{-4}$. sec^{-1} M^{-1}) was a strong inhibitor of the HRP reaction at levels above 1 μM. A concentration of 3 μM gave 50% inhibition and the reaction was completely inhibited at ascorbate levels of 10 μM. The addition of Mn^{2+} had no effect on the reaction rate.

Effect of H_2O_2 *on the initial rate of HRP and cell wall-mediated decolorization*

During the initial investigation of enzyme-catalysed betacyanin decolorization [4] the possibility that a peroxidase was the cell wall component responsible for this reaction was examined. This was done by incubating the cell wall fraction and substrate in the presence of 150 mM H_2O_2. The expected acceleration of the reaction was not obtained, leading to the conclusion that peroxidase was not involved. Since a number of peroxidase reactions have now been shown to be inhibited at this level of H_2O_2 [9, 10], its effect on beet cell wall catalysed decolorization was reinvestigated and compared with the HRP-mediated reaction.

The data presented in Fig. 2 compares the effects of H_2O_2 on the initial rate of decolorization by HRP and on the isolated beet cell wall preparation. The results show that both enzymes were sensitive to H_2O_2, with an accelerated reaction rate occurring upon the addition of low concentrations of H_2O_2, and a marked inhibition at higher levels. The maximal initial rate of decolorization was obtained at an H_2O_2 concentration of 10 μM with HRP and 100 μM with the cell wall fraction.

Peroxide concentrations of greater than 10 and 100 μM in the HRP and cell wall-catalysed reactions, respectively, caused a marked inhibition of both enzymes (Fig. 2). Of particular significance was that 10 mM H_2O_2 caused reduction of cell wall activity to a level below that obtained in the absence of added H_2O_2. These results demonstrate that the inability of Soboleva *et al.* [4] to observe an acceleration of decolorizing activity was due to the use of concentrations of H_2O_2 which were inhibitory to the enzyme.

Cell wall-mediated H_2O_2 *generation*

In the absence of added H_2O_2, the cell wall fraction exhibited 58% of its maximal activity whereas HRP was only 10% as active. The relatively high initial rate of the cell wall enzyme in the absence of added H_2O_2 suggests that the cell wall possesses an H_2O_2 generating system. If H_2O_2 is being produced, the addition of catalase should decrease the rate of decolorization. Figure 3 shows that saturating levels of catalase caused a significant reduction of the rate of cell wall-mediated decolorization. The fact that only partial inhibition was observed may be due to the inability of the catalase, MW 380 000, to penetrate the cell wall matrix [11].

Direct measurements of H_2O_2 generation were then performed. Cell wall fragments were transferred into Assay Buffer, pH 3.4, which did not contain betanin, and were incubated for various lengths of time. The cell wall fragments were then removed by centrifugation and H_2O_2 present in the supernatant was measured spectrophotometrically (Experimental). Figure 4 shows that upon transfer to pH 3.4 buffer, isolated beet cell walls begin to produce H_2O_2. After 10 min the concentration was 80 μM. To test the pH dependence of H_2O_2 production, an experiment was performed where cell walls were transferred into 100 mM citrate phosphate buffers ranging in pH between 3.0 and 7.0, and incubated for 5 min. Figure 5 demonstrates that H_2O_2 production is pH dependent, with maximal levels formed at low pH values.

The time course of H_2O_2 production in the presence of betanin is shown in Fig. 6. After 10 min of decolorization, the concentration of H_2O_2 was 150 μM, approximately double the concentration formed in the absence of betanin.

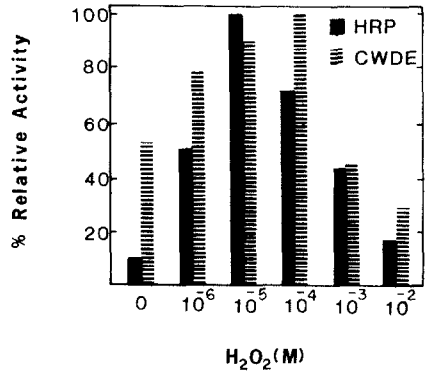

Fig. 2. Effect of H_2O_2 on the initial rate of decolorization. HRP (≡); Cell walls (■). Maximal activities of HRP (0.305 μg) and the cell wall suspension (0.27 mg) were 4.2 and 1.6 pkat, respectively.

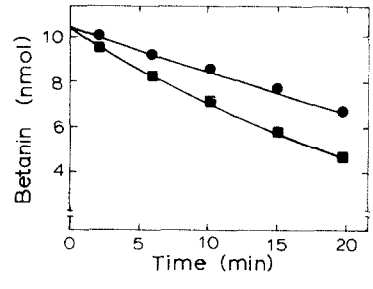

Fig. 3. Effect of catalase on cell wall-catalysed decolorization. Control (■); Catalase present (●). The assay contained 0.27 mg of cell wall material and 88 units of *A. niger* catalase (Calbiochem-Behring, La Jolla, CA). *A. niger* catalase maintains full activity over the pH range of 2–11 [21].

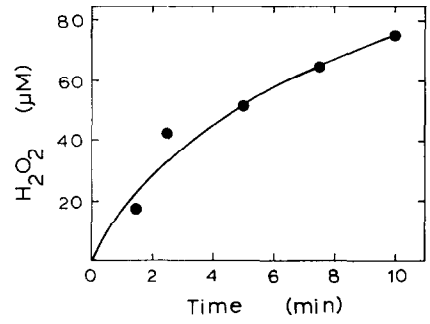

Fig. 4. Time course of H_2O_2 production by beet cell walls placed in pH 3.4 Assay Buffer. H_2O_2 was measured as described in Experimental. 0.51 mg of cell wall material were present in each assay.

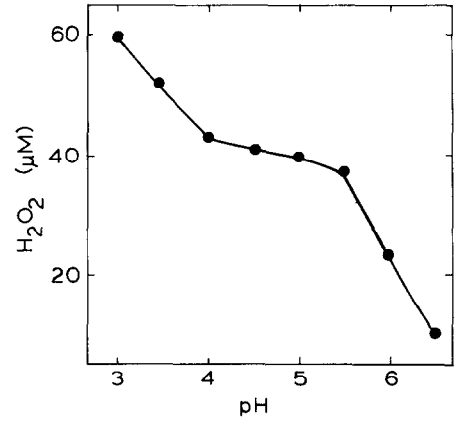

Fig. 5. Effect of pH on H_2O_2 production by beet cell walls. Cell walls (0.51 mg) were transferred into Assay Buffer at the indicated pH values, held 5 min at 25° and assayed for H_2O_2 as indicated in Experimental.

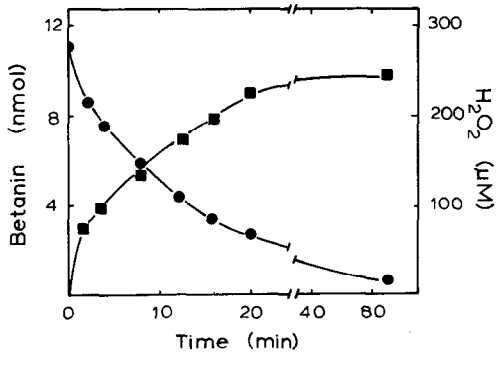

Fig. 6. Time course of H_2O_2 production during decolorization. Nmol betanin remaining (●); H_2O_2 concentration (■). Each reaction mixture contained 0.51 mg of cell wall and was incubated under standard conditions.

DISCUSSION

The results presented here demonstrate that the characteristics of betanin breakdown by HRP and red beet cell walls are very similar. Both reactions proceed optimally at pH 3.4 and both are stimulated by the presence of 10^{-5} to 10^{-4}M H_2O_2 with inhibition at higher levels (Fig. 2). In addition, both reactions resulted in the formation of an intermediate having a λ_{max} of 453 nm, which then underwent a slow decomposition (Fig. 1). These results suggest that a peroxidase-type enzyme may be responsible for betanin decolorization.

Bound cell wall peroxidase activities have now been observed in a number of plants [9, 10, 12–14], including the red beet [15]. In at least one, horseradish [12–14], H_2O_2 production has been demonstrated. In horseradish, H_2O_2 is produced as the result of NADH oxidation and utilized for the polymerization of phenolic compounds to lignin [13, 16]. This study demonstrated that H_2O_2 is a by-product of cell wall-catalysed betanin decolorization. Thus, the cell wall decolorizing enzyme in beet may function in the lignification process.

An unexpected result of this investigation was the observation that decreasing the pH of the cell wall suspension resulted in the release of significant amounts of H_2O_2 (Figs. 4 and 5). The nature of the electron donor is unknown at this time. One can speculate that since the plant cell wall contains many phenolic compounds, some are being released as the pH is lowered and utilized for H_2O_2 generation. The shape of the pH profile suggests that one class of compounds is released as the pH is decreased from 7 to 5 and that a second class is released below pH 4. Gross *et al.* [13] had also observed H_2O_2 generation by cell wall suspensions in the absence of added cofactors.

EXPERIMENTAL

Substrate preparation. Slices of red beet (*Beta vulgaris* L., cv. Detroit Dark Red), grown locally, were suspended in a homogenization medium (1 g tissue fr. wt per ml) consisting of 10 ppm ascorbic acid in 10 mM sodium phosphate buffer, pH 6.9 and homogenized for 10 min at 4°. The homogenate was filtered through two layers of cheesecloth and centrifuged at 100 000 *g* for 45 min.

The supernatant was collected, lyophilized and stored at −20°. To purify betanin, 1.0 g of the freeze dried crude pigment was then resuspended in 10 ml deionized distilled water and applied to a chromatography column (5 × 30 cm) containing Bio-Gel P-6 (Bio-Rad, Richmond, CA). The column was eluted with distilled deionized H_2O at a flow rate of 1 ml per min. The major betacyanin fraction was collected, freeze dried, dissolved in 10 ml deionized, distilled H_2O and passed over the column a second time. Analysis of the betacyanin fraction by HPLC [17] showed a betacyanin distribution of *ca* 90% betanin and 10% betanidin.

Cell wall isolation. Beet cell walls were purified by a series of low speed centrifugations according to the procedure of Shih and Wiley [5]. The final ppt. was resuspended in 10 mM phosphate buffer, pH 7.0, divided into 1.5 ml microcentrifuge tubes and stored at −80° until use. Although total activity varied among the various preparations, enzymological properties were consistent. The final concn of cell wall material in each preparation was measured by evaporation of dialysed aliquots of cell wall suspensions.

HRP-mediated decolorizing activity. Purified HRP was obtained from Sigma Chemical Co. Betanin decolorization was

measured spectrophotometrically at 25° by determining the rate of betanin loss at 538 nm. The standard assay mixture of 1.0 ml contained 15 μM betanin ($\varepsilon = 65\,000\ L\ cm^{-1} \cdot M^{-1}$), 50 nM HRP and Assay Buffer, 100 mM citrate-phosphate buffer, pH 3.4. As controls, rates of non-enzymatic betanin decolorization were measured, and subtracted from values obtained in the presence of enzyme.

Cell wall bound decolorizing activity. Cell wall bound decolorizing activity was measured in 1.5 ml polyethylene centrifuge tubes. Unless otherwise indicated, each assay mixture (1.0 ml final vol.) contained 100 mM citrate-phosphate buffer, pH 3.4 and 15 μM betanin. The decolorization reaction was initiated by the addition of 50 μl of the cell wall suspension and incubated in the dark for various times. Each assay was stopped by a 1.5 min centrifugation at 15 000 g to remove cell walls. The absorbance of the supernatant was measured immediately at 538 nm to determine betanin loss.

H_2O_2 *measurement.* H_2O_2 formation was measured spectrophotometrically by the enzymatic method of Mader *et al.* [18]. To measure H_2O_2 production by the cell wall fraction, reactions were terminated by centrifugation as described above, and 0.8 ml of the supernatant transferred to a cuvette. A background absorbance reading was then taken and supernatant brought to 6.25 mM guaiacol and 4.5 μg per ml peroxidase. The increase in absorbance at 470 nm was then measured. Standards contained known concns of H_2O_2. The system was shown to function over the pH range of 3–7.

Betanin K_4 *determination.* The K_4 or dissociation constant value for betanin was determined by the method of Chance [19, 20]. The reaction mixture contained 1.1 μM HRP, 6.5 μM betanin and 5 μM H_2O_2. The reaction was conducted in both 100 mM sodium acetate buffer, pH 4.5 [18] and 100 mM citrate phosphate buffer, pH 3.4.

Polyphenoloxidase assay. PPO was assayed according to technical literature provided by Sigma Chemical Company. Its activity was tested on catechol and betanin at pH 3.4 and 6.5. Catechol assays contained 100 mM citrate phosphate buffer, 150 μg enzyme, 10 μM EDTA and 42 μM ascorbic acid in a final vol. of 1.0 ml. Catechol activity was measured by monitoring the increase in absorbance at 280 nm at 25°. The betanin decolorizing activity of PPO was tested similarly, except absorbance was monitored at 538 nm and tandem experiments were performed in which the EDTA and ascorbate were omitted.

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