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PEROXIDASE CATALYSED OXIDATIVE DECARBOXYLATION OF VANILLIC ACID TO METHOXY-*p*-HYDROQUINONE

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Key Word Index—Peroxidase; vanillic acid; vanillin; vanillyl alcohol; syringic acid; methoxy-*p*-hydroquinone; methoxy-*p*-benzoquinone.

Abstract—Horseradish peroxidase catalysed the oxidative decarboxylation of vanillic acid to methoxy-*p*-hydroquinone and subsequent oxidation of the hydroquinone to methoxy-*p*-benzoquinone. Peroxidase also catalysed the oxidation of vanillyl alcohol to vanillin and vanillic acid; however, neither vanillyl alcohol nor vanillin appeared to give rise to methoxyhydroquinone directly. Correspondingly, peroxidase catalysed the oxidative decarboxylation of syringic acid to 2,6-dimethoxy-*p*-hydroquinone and subsequent oxidation of the hydroquinone to 2,6-dimethoxy-*p*-benzoquinone.

INTRODUCTION

Phenoloxidases are widely distributed among plants [1], and have been found as extracellular and intracellular enzymes [2] in white rot fungi where they have been implicated in lignin degradation [3, 4]. Evidence has accumulated that these enzymes catalyse the formation of a phenoxy radical as an initial step in the oxidation process [5, 6]. The unpaired electron is then stabilized by delocalization throughout the molecule. The radicals can then condense to form a polymer (lignification) or react with H_2O_2 or O_2 to form higher oxidation product(s). The importance of phenoloxidases in oxidative or dehydrogenative polymerization is well established [7]. Their role in the oxidative degradation of phenols, however, has been limited to syringyl compounds. 2,6-Dimethoxy-*p*-benzoquinone has been shown to be a phenoloxidase oxidation product of syringylglycerol-β-guaiacyl ether [5, 8], of syringic acid [9], of syringaldehyde and acetosyringone [5]. When similar studies were performed with guaiacylglycerol-β-guaiacyl ether [10] and other guaiacyl compounds [5], however, no methoxy-*p*-benzoquinone (MQ) was detected. Where fungal cultures were used [5], further degradation of MQ cannot be ruled out.

Since previous studies have indicated that vanillic acid is an intermediate in the fungal degradation of lignin [11], we were interested in examining the possible oxidative degradation of this compound by peroxidase. In this study we demonstrate that vanillic acid can be oxidatively decarboxylated to methoxy-*p*-hydroquinone (MHQ) by H_2O_2–peroxidase with further oxidation of MHQ to MQ. In bacteria specific monooxygenases have been found which oxidatively decarboxylate aromatic acids. Thus salicylate (2-hydroxybenzoic acid) hydroxylase [12] and anthranilate hydroxylase [13] catalyse the oxidative decarboxylation of salicylate and anthranilate to catechol.

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RESULTS

*Isolation and characterization of methoxy-*p*-hydroquinone*

After incubation of vanillic acid (1.2 mM) with peroxidase–H_2O_2 for 10 min at 38°, most of the vanillic acid remained unchanged (>95% recovery). A small amount (0.3%) of a new compound was produced, however, which after silanation co-chromatographed on the GC with authentic MHQ-TMSi. The simple mass spectrum of this compound *m/e* 284 (M^+), 269 (M − 15) and 254 (base, M − 30) and a few less intense peaks at lower mass was similar to that of authentic MHQ-TMSi. No MHQ was formed if the enzyme was boiled for 2 min prior to incubation. In addition no MHQ was formed if either H_2O_2 or the enzyme was omitted from the reaction mixture. Finally, although this experiment was routinely performed with Type II peroxidase, the more highly purified Type VI enzyme gave essentially identical results. All these results indicate that peroxidase–H_2O_2 is catalysing the reaction.

In an attempt to detect methoxy-*p*-benzoquinone in the reaction mixture, an underivatized sample was chromatographed at 100° in the isothermal mode. (MQ is unstable under the conditions used for silanation.) The retention times for MQ and MHQ were 4 and 12 min, respectively, under these conditions. GC analysis in-

dicated no MQ was formed during this short reaction; however, again a peak emerged which co-chromatographed with authentic MHQ. The mass spectrum of this compound (*m/e*, rel. int. = 140, 62%; 125, 70%; 97, 100%; and 69, 38%) was similar to that of MHQ. These results indicate that MHQ is an immediate product of the peroxidase-catalysed oxidation of vanillic acid.

Oxidation of syringic acid by peroxidase–H_2O_2

Incubation of syringic acid (1.2 mM) with peroxidase for 10 min at 38° correspondingly yielded 2,6-dimethoxyhydroquinone rather than 2,6-dimethoxy-*p*-benzoquinone. The hydroquinone, however, was apparently easily air-oxidized with appreciable amounts of the quinone being formed within 24 hr. The mass spectra of the hydroquinone product (*m/e*, rel. int. = 170, 100% 155, 67%; 140, 25%; 139, 30%; 127, 78%; 112, 44%; 67, 82%) was consistent with 2,6-dimethoxyhydroquinone.

Oxidation of methoxyhydroquinone by peroxidase–H_2O_2

Incubation of MHQ (1.2 mM) with peroxidase at 38° yielded an immediate yellow colour which gradually turned dark brown. If the reaction was stopped after 1 min, subsequent GC analysis of the underivatized product showed a single peak which co-chromatographed with MQ. The mass spectrum (*m/e*, rel. int. = 140, 3%; 139, 3%; 138, 31%; 123, 13%; 111, 4%; 110, 46%; 108, 48%; 95, 25%; 82, 34%; 69, 100%) was similar to that of authentic MQ.

Quantitative assay of the formation of MHQ/MQ

MHQ/MQ formed in the oxidation of vanillic acid was quantitated by GLC as the trimethylsilyl derivative of the MHQ. A known amount of *p*-hydroquinone was added to the reaction mixture just prior to extraction as an internal standard. The combined organic fractions were shaken with a few crystals of sodium dithionite to reduce any quinone formed during the reaction or extraction procedure. If 1.2 mM vanillic acid was present in the reaction mixture, methoxyhydroquinone was formed rapidly (Fig. 1), reaching a level of 3.5 μM or 0.3% of the original substrate after approximately 2 hr. After this time there was still an appreciable amount of vanillic acid in the reaction mixture. If 12 μM vanillic acid was present in the reaction mixture (Fig. 1), the amount of MHQ formed reached a level of 0.86 μM or 7% of the original substrate after 40 min; 85% of the vanillic acid was recovered. Further incubation led to the disappearance of MHQ.

Oxidation of vanillyl alcohol and vanillin by peroxidase–H_2O_2

With 1.2 mM vanillyl alcohol in the reaction mixture the solution turned yellow-brown and cloudy. If the reaction was terminated after 10 min, the major monomers present were vanillyl alcohol, vanillin (1% of the original substrate) and trace amounts of vanillic acid. No MHQ or MQ were detected. After further treatment, however, MHQ (0.01% of the initial substrate) could be detected. Similarly, when vanillin (1.2 mM) was the substrate only vanillic acid and the original substrate could be detected after 10 min; after 30 min, however, a small amount of MHQ was also detectable. Cumulatively these results suggest that the pathway for the oxidation of vanillyl alcohol to MHQ went through vanillin and vanillic acid; they also suggest that only the acid can give rise directly to MHQ.

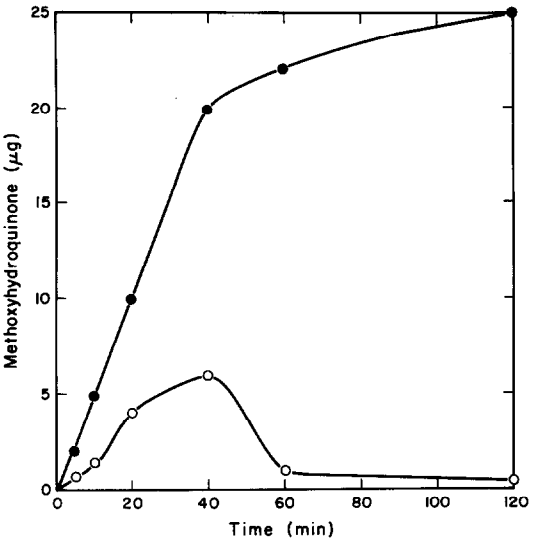

Fig. 1. Production of MHQ from the peroxidase-catalysed oxidation of vanillic acid. 1.2 mM(●) or 12 μM(○) vanillic acid was incubated with peroxidase–H_2O_2. Aliquots of the reaction were removed periodically and the amount of MHQ was measured by GLC as described in the text.

DISCUSSION

These results indicate that vanillic acid, vanillin and vanillyl alcohol are oxidized to MHQ by peroxidase–H_2O_2. They suggest, in addition, that vanillyl alcohol and vanillin are first oxidized to the acid and that only the acid is oxidatively decarboxylated to MHQ with subsequent oxidation of MHQ to MQ. Although Ishihara and Ishihara [9] reported that laccase from *C. versicolor* oxidized syringic acid to 2,6-dimethoxy-*p*-benzoquinone, our results indicate 2,6-dimethoxyhydroquinone was the immediate product of the peroxidase-catalysed oxidation of syringic acid. The discrepancy may be due to the different phenoloxidases used. Under the conditions used by the previous workers [9], however, any 2,6-dimethoxyhydroquinone produced may have been subsequently oxidized to 2,6-dimethoxy-*p*-benzoquinone prior to analysis.

The phenoxy radical formed by the action of peroxidase on vanillic acid would be expected to distribute over the ring. Subsequent addition of a hydroxyl group either from water or H_2O_2 followed by decarboxylation would be expected to give rise to MHQ. Studies with ^{18}O would probably help to determine the origin of the new phenolic OH group on the MHQ molecule. Finally, studies recently completed in our laboratory (manuscript in preparation) indicate that the white rot fungus *Phanerochaete chrysosporium* produces an enzyme, vanillate hydroxylase, which specifically decarboxylates vanillate to MHQ in the presence of NADPH and molecular oxygen.

EXPERIMENTAL

Horseradish peroxidase Type II and Type VI were obtained from Sigma. The phenolic compounds were obtained from Sigma or Aldrich and are of the highest purity available. MQ was prepared by oxidation of MHQ with two equivalents of $FeSO_4$–H_2O_2 in water for 10 min at room temp. The resulting

product was extracted twice with an equal vol. of C_6H_6 and dried over anhydrous Na_2SO_4. GLC analysis (OV-101, 100°, isothermal program) indicated 95% purity. The MS *m/e* 138 (M^+) and UV spectrum λ_{max} 368 nm [14] were consistent with MQ.

Enzymatic reaction. Reaction mixtures (50 ml) contained 10mM sodium phosphate, pH 6.5, 10 mM H_2O_2 and various concs of phenolic substrates as indicated. Reactions were started by the addition of 50 µl peroxidase (50 µg) and were carried out at 38°. At the end of the incubation, 2 ml 2 N H_2SO_4 were added and the mixture was immediately extracted with EtOAc (2 × 30 ml). The combined organic fractions were dried and evapd to dryness in N_2. Trimethylsilanation of enzymatic products and standards was carried out by adding *bis*-(*N,O*-trimethylsilyl)trifluoroacetamide–pyridine (1:1) to the dry residue and heating at 80° for 5 min.

Gas chromatography. GLC was carried out with a Varian Model 1700 instrument fitted with a glass column (180 × 0.2 cm i.d.) packed with 3% OV-101 on chromosorb Q 100/120 (Applied Science). The oven temp. was programmed from 100 to 270° at 10°/min unless indicated otherwise. MS was carried out with a Dupont Model 21-491B equipped with the same instrument and column for GLC. The spectra were obtained at 70 eV.

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OVALIN, A NEW PIPECOLIC ACID FROM *MILLETIA OVALIFOLIA* SEEDS

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Key Word Index—*Milletia ovalifolia*; Leguminosae; ovalin.

In continuation of our earlier work [1], further examination of *Milletia ovalifolia* seeds led to the isolation of a new pipecolic acid, ovalin, from the methanol extract. Ovalin was obtained chromatographically pure as colourless needles from EtOH–Me_2CO; mp 280–281° (decomp.); R_f 0.36 (PC *n*-BuOH–HCO_2H–H_2O, 15:3:2). It analysed for $C_7H_{13}NO_3$; M^+ 159; $[\alpha]_D^{20}$ −41.2° (H_2O) was chemically neutral and insoluble in all organic solvents except alcohol in which it was partially soluble, but was readily soluble in H_2O. There was no UV absorption in the range 200–300 nm. The IR spectrum (KBr) indicated an —OH group (ν 3350 cm^{-1}), $\rangle$=O of carboxylate (1630 cm^{-1}) and a quaternary ammonium group (=N^+=) (1400 cm^{-1}). It did not respond to tests for amino acids, peptides and alkaloids. The inertness towards most of the reagents and the presence of nitrogen suggested it to be a pipecolic acid derivative, some of which are known to occur in the seeds of leguminous plants [2].

The ^1H NMR spectrum (60 MHz, solvent D_2O, values) indicated the presence of $\rangle$N—Me group, methine and methylene protons. The spectrum closely resembled that of 4-hydroxypipecolic acid recorded in D_2O [3–5]. A quintet centred at 4.3 ($J_{ae} = J_{ee} = 3.5$ Hz, 1H) was assigned to an equatorial C-4 proton. A pair of doublets centred at 3.85 ($J_{ae} = 5$; $J_{aa} = 11$ Hz, 1H) was assigned to an axial C-2 proton because of the large coupling constant, characteristic of axial–axial neigh-