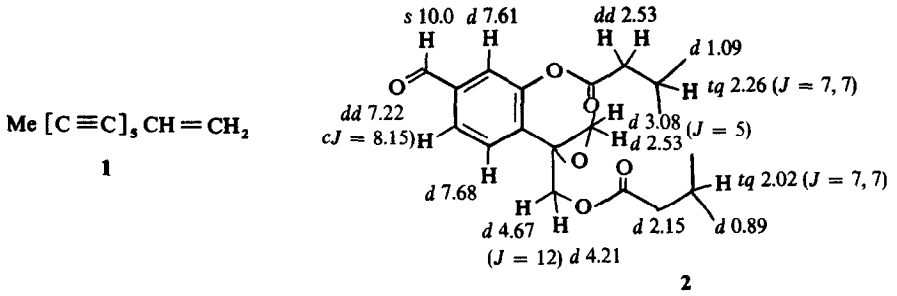

$$Me\,[C\equiv C]_5\,CH=CH_2$$

1

2

Werte in ppm, $CDCl_3$, TMS als innerer Standard. Die lufttrockenen Wurzeln (560 g, im Februar 1977 in Natal gesammelt, Herbar Nr. 77/56) wurden zerkleinert und mit Ether–Petrol (1:2) bei RT extrahiert. Den Extrakt trennte man zunächst grob durch SC (Si gel, Akt.-St. II) und anschließend durch DC (Ether–Petrol 1:3). Man erhielt 0.2 mg **1** und 1.8 g **2**.

7-Oxo-10-isovaleryloxy-8,9-dihydro-8,9-epoxy-thymolisovalerat (**2**). Farbloses Öl. IR: PhOCOR 1765; ROCOR 1740; CHO 2710, 1703; Aromat 1610, 1565 cm^{-1}. MS: M^+ *m/e* 362 (<0.1%) (mit Cl (Isobutan also Stoßgas: 363); $-Me_2CHCH_2CO_2H$ 260.105 (6) (ber. für $C_{15}H_{16}O_4$ 260.105); 260 $-Me_2CH-CH=O$ 176 (22); $C_4H_9CO^+$ 85 (100); 85 $-CO$ 57 (96).

$$[\alpha]_{24}^{\lambda} = \frac{589 \quad 578 \quad 546 \quad 436\,nm}{-37.4 \quad -39.2 \quad -45.4 \quad -87.7^\circ} (c = 7.55,\ CHCl_3)$$

Anerkennung—Frau Dr. O. Hilliard, Dept. of Botany, Univ. of Natal, danken wir für die Hilfe bei der Beschaffung und Bestimmung des Pflanzenmaterials, der Deutschen Forschungsgemeinschaft für die Bereitstellung von Mitteln für das WH 270.

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MECHANISM OF ACTION OF LIGNINS AND LIGNIN MODEL COMPOUNDS WITH HORSERADISH PEROXIDASE*

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Key Word Index—lignins; lignin model compounds; horseradish peroxidase.

Abstract— Horseradish peroxidase displayed a ping-pong kinetic reaction mechanism with lignin model compounds and lignins. Oxidation of the α carbon on acetosyringone or acetovanillone failed above pH 6.5, while conversion of α-methylsyringyl (or guaiacyl) alcohol to acetosyringone (or vanillone) occurred optimally at pH 7.8. Small MW fragments were not formed from lignins at pH 6.4 and 7.8. These observations provide evidence for the growing concept that freely soluble peroxidase is not a lignolytic enzyme.

INTRODUCTION

Lignins are heteropolymeric molecules composed mainly of singly or doubly methoxylated phenylpropyl phenolic units [1]. They are degraded by microorganisms [2–4]. The enzymological approaches are virtually unknown [5], though polyphenoloxidases such as peroxidase and laccase have long been suspected [3]. The reaction between laccase and maple milled wood lignin involves simultaneous depolymerization processes [6], and although long-lived phenoxy radicals are produced during the oxidative reaction of monomeric and dimeric syringyl derivatives with horseradish peroxidase [7], subsequent coupling reactions between mesomeric aryloxy radicals that are produced in these reactions, as well as in laccase-catalyzed reactions, virtually negate any lignolytic effect these enzymes may have [8].

In lignin model studies it has been shown that α-methylsyringyl alcohol is converted by horseradish peroxidase to acetosyringone, which is oxidized further to a mixture of syringyl hydroquinone and 3-methoxy-5-acetyl-*o*-hydroquinone [9]. Free radical intermediates are involved in these reactions, as well as in reactions between horseradish peroxidase and hardwood lignins

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[9]. A mechanism for the enzyme's reaction is given in the present paper, and evidence is provided to support the observation that stable low MW compounds are not produced as a result of the reaction [8].

RESULTS AND DISCUSSION

The conversion of α-methylguaiacyl alcohol to acetovanillone occurred optimally at pH 7.8, while further oxidation to quinone proceeded optimally at pH 5.2 (Fig. 1). Quinone production failed above pH 6.5, suggesting that if lignin depolymerization is to occur by way of cleavage at phenylpropyl junctions, the reaction might proceed most favorably in a slightly acid medium. The oxidation of α-methylsyringyl alcohol and acetosyringone

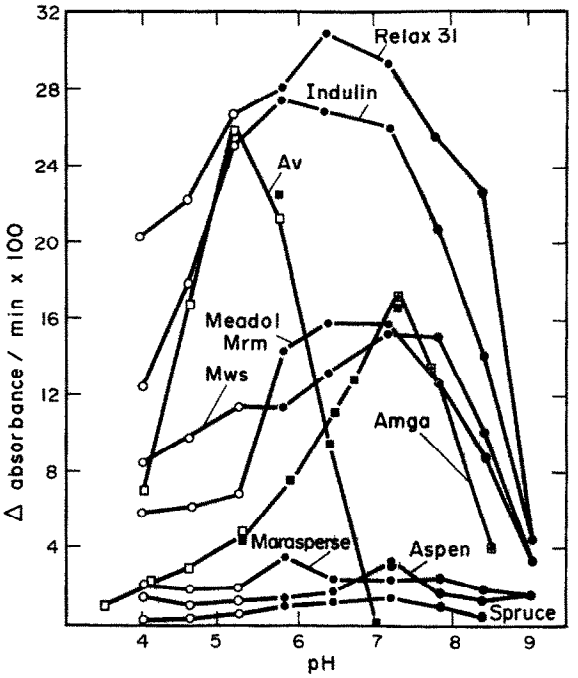

Fig. 1. pH and peroxidase activity. 20 mM acetovanillone (AV) or α-methylguaiacyl alcohol (AMGA) (squares), or 20 μl 0.5% lignin in dimethylsulfoxide was reacted with 1 mM H_2O_2 and 2.8×10^{-8} M horseradish peroxidase in a final vol. of 2 ml 0.1 M sodium acetate (hollows), potassium phosphate (solids), or Tris (hydroxymethyl) aminomethane–HCl (dots). The changes in *A* for acetovanillone were 20 fold greater than those indicated by the scale.

occurred optimally at pH 7.8 and 4.7 respectively, giving nearly identical curves to those of the guaiacyl analogs.

Relatively strong spectrophotometric signals at 300 nm, signifying a reaction between peroxidase and lignin were obtained over a pH range of 5.7 to 7.7 for two, weakly (1.4%) sulfonated lignins (reax 31 and indulin AT) and two soda lignins (meadols MRM and MWS), while weak signals were obtained for a strongly sulfonated (6%) hardwood lignin, cellulase spruce lignin and cellulase aspen lignin (Fig. 1).

To establish whether low MW products were being produced, 0.005% reax 31 and meadol MRM lignins in 0.05 M KPi were reacted within benzoylated [10] or

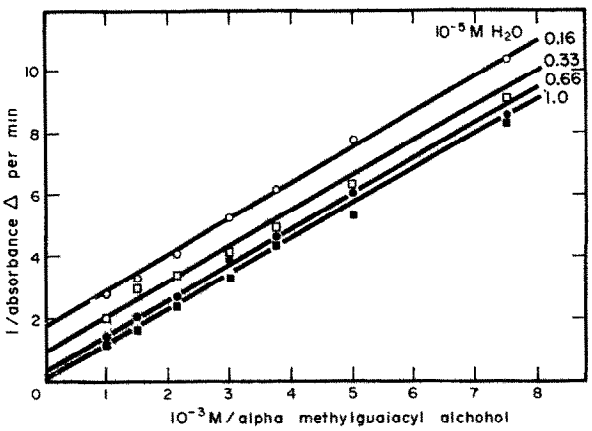

Fig. 2. Reactivity of α-methylguaiacyl alcohol at pH 4.7 with horseradish peroxidase and H_2O_2.

ordinary dialysis sacs for 4 hr at pH 6.4 and 7.8 to attain absorbancy increases in excess of 3. H_2O_2 was added hourly to maintain a concentration of about 1 mM. The sacs, containing 10 ml reaction medium, were dialyzed during the reaction period against 50 ml 0.05 M KPi dialyzing solution. The latter was examined spectrophotometrically for product formation, but in all cases the result was negative. Regardless of pH or quantity of brownish colored material produced within a sac, no UV-absorbing or colored material passed to the outside of the membrane. This observation suggests that depolymerization did not proceed to the point of yielding monomeric or small polymeric products having a MW lower than *ca* 3000 (benzoylated sacs) or 15000 (ordinary sacs) and that small MW substances which may have been formed, were quickly repolymerized.

The initial rates of reaction between peroxidase, H_2O_2, and α-methylguaiacyl alcohol at pH 4.7 as a function of initial substrate concentration, are shown in Fig. 2. Similar parallel slope relationships were obtained for the same substrate at pH 7.8. Also, parallel slope relationships were obtained when α-methylsyringyl alcohol, acetosyringone, acetovanillone, reax 31, and indulin AT were tested as substrates. In all cases a ping-pong mechanism of action [11] was exhibited. This suggests that the oxidation of the monomeric lignin model compounds and the lignins themselves involves an initial peroxidation of the enzyme, after which two electrons can be removed consecutively from a single reactant molecule or one electron from each of two reactant molecules. The removal of two electrons from single phenylpropyl moieties with a secondary alcohol on the α carbon, and also from the resulting carbonyl products, would thus account for peroxidase catalyzed oxidations reported for α-methylsyringyl alcohol [9, 12], acetosyringone [9], α-ethylvanillyl alcohol [13], and guaiacyglycerol β-guaiacyl ether [13]. The reaction mechanism also accounts for the removal of one electron from each of two reactant molecules, resulting in free radicals capable of combining with one another, and thus polymerizing, as was shown by one of us for guaiacol [14], and as is known to occur for the types of lignin model compounds described in this paper [13], for guaiacylglycerol β-guaiacyl ether [13], and for phenols in general [15].

Taken together, the data of the present paper support

the growing concept that the reactivity of peroxidase with lignins, at least in cell-free systems, results at best in an internal rearrangement of the polymeric substrate (*cf.* refs [5, 8, 12, 13]), and not in depolymerization.

EXPERIMENTAL

Horseradish peroxidase (type VI) was purchased from Sigma Chemical Co., and acetovanillone and acetosyringone from Aldrich Chemical Co. Alphamethylguaiacyl alcohol and α-methylsyringyl alcohol were gifts from Dr. Carlton Dence, meadol MRM and MWS hardwood soda lignins from Dr. Conrad Schuerch, sitka spruce cellulase and aspen cellulase lignins from Dr. T. Kent Kirk, marasperse hardwood lignosulfonate lignin from American Can Co., and reax 31 and indulin AT Kraft pine lignins from Westvaco Co. The benzoylated dialysis membranes were prepared according to Pearse [10]. The kinetic measurements as a function of pH were made with a spectrophotometer in cells of 1 cm light path at 25°. The double reciprocal plots were based on measurements made in cells of 10 cm light path at room temp. All *A* changes were recorded on a 25 cm scale representing 0.1 *A* units.

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AN ACYLATED DELPHINIDIN 3-RUTINOSIDE-5,3′,5′-TRIGLUCOSIDE FROM *LOBELIA ERINUS*

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Key Word Index—*Lobelia erinus*; Campanulaceae; acylated anthocyanin; caffeylferulyl-*p*-coumaryl delphinidin 3-rutinoside-5,3′,5′-triglucoside.

A blue pigment was isolated from blue petals of garden *Lobelia*. The absorption peaks of this pigment in H_2O were at 316, 530, 568 and 612 nm, and the R_fs were 0.80, 0.90 and 0.92 in 50% EtOH, 2PW and H_2O, respectively. The absorption peaks in MeOH–HCl (0.01%) were at 302, 320 and 544 nm; the values of $E_{440}/E_{vis\ max}$ and $E_{acid\ max}/E_{vis\ max}$ being 19% and 142%, respectively. No bathochromic shift of the visible max. occurred upon addition of $AlCl_3$ (5% in EtOH), indicating the absence of an *o*-dihydroxyl grouping. The R_fs of the chloride were 0.24, 0.15, 0.60 and 0.30 in BAW, BuN, AAH and 1% HCl, respectively. Upon acid hydrolysis, the pigment gave delphinidin, glucose, rhamnose, and *p*-coumaric, ferulic and caffeic acids, the molar ratio of delphinidin, glucose and rhamnose being 1:4:1 respectively.

The deacylated anthocyanin (**1**) had an orange red colour and fluoresced light orange in UV light on the chromatogram. The R_fs were 0.05, 0.00, 0.77 and 0.71 in BAW, BuN, AAH and 1% HCl, respectively. That **1** is pentaglycoside follows from its higher R_f values in aqueous solvents and lower in alcoholic solvents than those of related di- and tri-glycosides of delphinidin. **1** showed λ_{max} at 522, 275nm in 0.01% MeOH–HCl, and the values of $E_{440}/E_{vis\ max}$ and $E_{UV\ max}/E_{vis\ max}$ were 17% and 60%, respectively. The 3′- and/or 5′-hydroxyls must be glycosylated, because the visible max. was at a lower wavelength than that of delphinidin glycosides having sugars at the 3- and/or 5-positions [1]. **1** furnished rutinose upon H_2O_2 degradation. Upon partial hydrolysis, **1** gave delphinidin and the 3-rutinoside-5,3′-glucoside, 3-rutinoside-5-glucoside, 3,5,3′-triglucoside, 3,5-diglucoside and 3-monoglucoside of delphinidin.

The absence of substitution at the 7- and 4′-positions