Vol. 105, No. 2, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 30, 1982 Pages 509-514

An Approach to Lignification in Plants

Robert Hwang, Chemist,

Transit Authority Lab.,960 Carrol St. Brooklyn, N.Y.

Received October 24, 1981

Summary: A lignin-like substance was synthesized in vitro from coniferyl alcohol in the presence of peroxidase with coenzymes NADH and flavin. Eugenol was also polymerized in the same way.

Introduction:

Lignin is a widely occurring polymer in higher plants. Its monomer units are coniferyl alcohol, sinapyl alcohol and p-coumaryl alcohol. Lignin's detailed structure has not been elucidated. It is, however, suspected that the polymerization in vivo is a free radical process. Polymerization of coniferyl alcohol and its homologous propanoid compounds can be accomplished in vitro on the addition of hydrogen peroxide[1]. Analogous reactions may be taking within the plant cell; peroxide flavoprotein, pyridine nucleotide (NADPH) and peroxidase have been proposed to be involved in the biosynthesis of lignin. Flavin, for example, can form a peroxide[2]. In the presence of peroxidase, the hydrogen peroxide or flavin peroxide undergoes H-abstraction of the phenolic hydrogen to form an aroxyl radical[3]. In the experiments described herein, the author wishes to demonstrate that in vitro polymerization of lignin monomers can be accomplished with these suspected biosynthetic intermediates. Specifically, these experiments were undertaken to determine whether the aroxyl radical formed with flavin peroxide in the presence of peroxidase can initiate the in vitro polymerization of coniferyl alcohol and eugenol to polymers.

0006-291X/82/060509-06$01.00/0

Materials and Methods:

Half a ml of riboflavin 5' phosphate monosodium (FMN) ca 112 mg/ml, half a ml of NAD^+, 141 µmols/ml, half a ml of alcohol dehydrogenase concentrate[4], 50 µL peroxidase solution and 20 µL coniferyl alcohol, 100mg/ml, were mixed in a test tube and 20 µL ethyl alcohol were added. Turbidity was observed immediately. Repeated addition of ethanol and coniferyl alcohol brought out more brown precipitate. This polymer was washed with water, centrifuged and decanted several times. After drying under vacuum, it was insoluble in both chloroform and tetrahydrofuran. When eugenol was substituted for coniferyl alcohol, a white precipitate appeared immediately. The eugenol polymer was soluble in both chloroform and tetrahydrofuran.

If alcohol dehydrogenase is left out of the incubation mixture in which NADH is directly substituted for NAD^+, polymer formation does not occur. This is because NADH is not bound to dehydrogenase[5] and therefore it can be oxidized by flavin peroxide to NAD^+ [6]. Under this condition, this peroxide is no longer available to form an aroxyl radical.

Results:

The dried eugenol polymer was dissolved in tetrahydrofuran and filtered for analysis by liquid chromatography with a U.V. detector at 254 nm. The column was packed with U 8, 39 mm I.D., 30 cm long. The elution was performed with mixtures of water and methanol ranging from 20% methanol to 80% methanol. The HPLC chromatogram showed a monomer peak at the extreme right. At a separation of each additional 17 cm, a second and a third peak occurred, rich in dimer and tetramer. It appeared that there was a trimer and various kinds of tetramer and pentamer with their own peaks.

A eugenol polymer which was dried at -80°C under high vacuum, was soluble in chloroform and filtered. It was subjected to permeation chromatography (column F-1000, F-500 and E-125) packed with

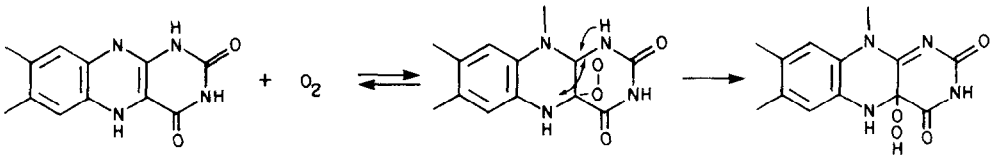

Figure 1

bongel and eluted with chloroform. A high large peak due to the monomer occurred around 75 counts. A small peak occurred between 32-45 counts. In comparison with standard polystyrene using calibration ratio Q=42, M_n, the number-average molecular weight, is 31,909. M_w, the weight-average molecular weight, is 890,991. Referring to the HPLC data, a tetramer, if in monodisperity, gives M_n = 650. The real M_n depends on the molecular weights of various molecular species and their number of molecules.

Discussion:

These experiments suggest the following mechanism shown in scheme 1.

Scheme 1

$$NAD^+ + CH_3CH_2OH \underset{\text{dehydrogenase}}{\overset{\text{alcohol}}{\rightleftharpoons}} CH_3CHO + NADH + H^+$$

$$NADH + H^+ + \underset{\text{flavin}}{F} \rightleftharpoons \underset{\text{reduced flavin}}{FH_2} + NAD^+$$

$$FH_2 + O_2 \longrightarrow \underset{\text{flavin peroxide}}{FHOOH}$$

Figure 1 shows the structure of flavin and its peroxide

The proposed mechanism consists of four consecutive steps. The first reaction is the formation of an enzyme complex substrate between peroxidase and flavin peroxide. The second reaction between the enzyme complex and a molecule of monomer produces a diradical enzyme complex and water. Reaction 3 shows the addition of a second monomer unit to the diradical complex, and the last reaction shows the decomposition of the latter product, regenerating the enzyme and flavin, producing a dimer of the original monomer (AH). The

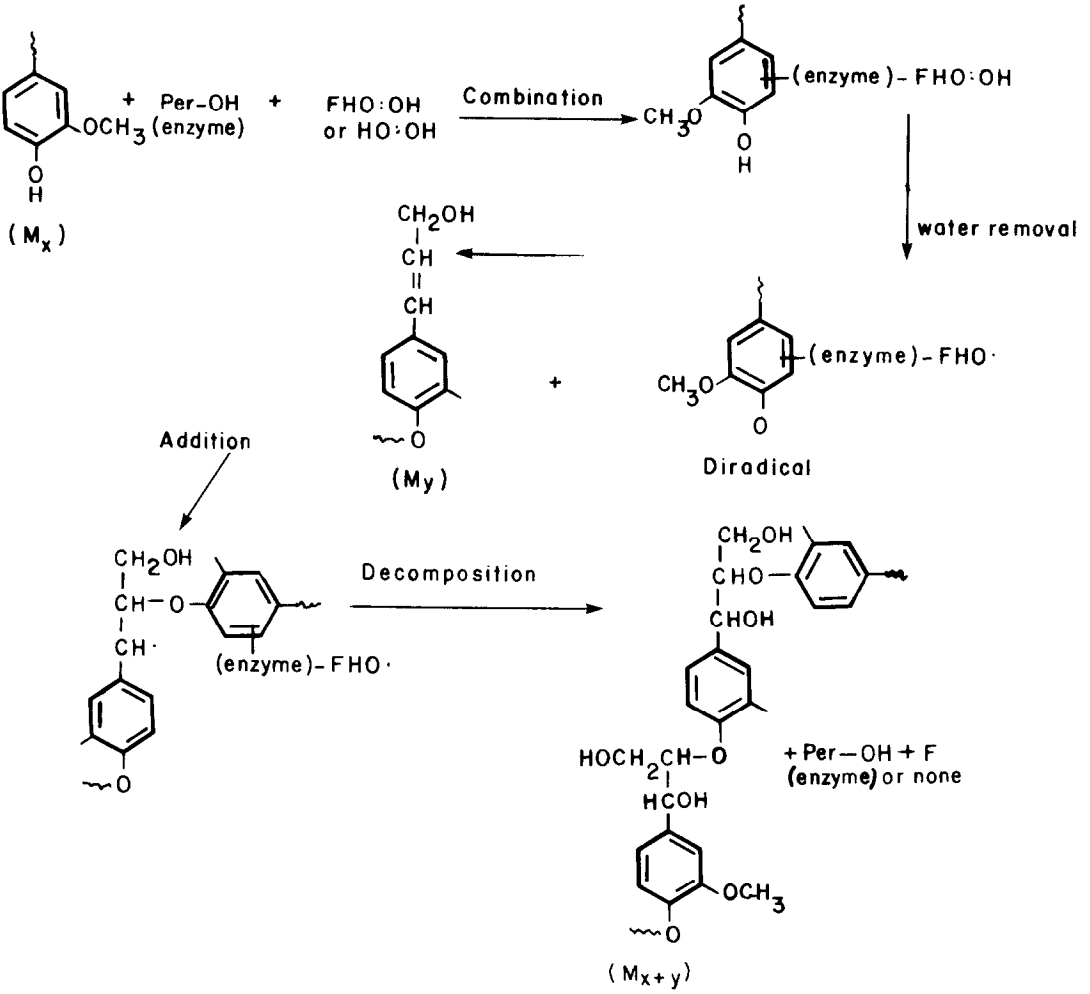

Figure 2

process can continue: peroxidase and newly formed flavin peroxide can again form their complex and this can react with the already formed dimer to yield a diradical which can attack another monomeric molecule, forming a trimer; or another dimer molecule, forming a tetramer. Polymerization can continue by these paths and others yield a n-mer.

The four consecutive steps are shown below in scheme 2[7]:

Scheme 2

(1) Per-OH (peroxidase) + HO:OHF (flavin peroxide) $\rightleftharpoons$ (Per-OH-HO:OHF) enzyme complex substrate

(2) (Per-OH-HO:OHF) + AH monomer (M_1) $\rightleftharpoons$ ($\dot{A}$-Per-OH-$\dot{O}$HF) + H_2O diradical complex

(3) ($\dot{A}$-Per-OH-$\dot{O}$HF) + AH monomer $\rightleftharpoons$ (H$\dot{A}$-A-Per-OH-$\dot{O}$HF)

(4) (H$\dot{A}$-A-Per-OH-$\dot{O}$HF) $\longrightarrow$ Per-OH + F + HA-AOH (=A_2H, M_2)

The structural mechanism can be expressed[8] more clearly as follows in Fig. 2 .

In conclusion, lignification in plants is visualized as in scheme 3:

Scheme 3

$$NADP^+ + H_2O \underset{\text{photosynthesis in Chloroplast}}{\overset{\text{Hill Reaction}}{\rightleftharpoons}} NADPH + H^+ + \tfrac{1}{2} O_2$$

These experiments support the idea that the coenzymes NADPH and flavin in vivo, produce flavin peroxide, when exposed to air. Phenyl propanoid compounds convert to their alcohols and lignin is polymerized through a peroxidase complex substrate[9].

ACKNOWLEDGMENTS:

The author wishes to express his thanks to the Union Industrial Research Institute of Taiwan for the use of their instrumental facilities and is indebeted to Professor J. Steigman and Dr. F. Aronson of the Downstate Medical Center for their assistance.

References:

1. L. Kalb and T. Lieser, Chem. Ber. 61, 1007, 1928 in K. V. Sarkanen and C.H. Ludwig, "Lignin Occurance, Formation, Structure and Reaction" Interscience Inc. 1971, p116-155
2. W. Bereude, J. Posthus, J.S. Sussenbach and H.I.X. Mayer "On Mechanism of some Flavin-photosensitized Reaction" in E.S. Slatter "Flavin and Flavoprotein" Elsevier Pub. 1966 p29; V. Massey, S. Palmer and D. Ballow,"On the Reaction of Reduced Flavin and Flavoprotein with molecular Oxygen" in H. Kamin, "Flavin and Flavoprotein" University Press, Baltmore, 1971, p349
3. K. Freudenberg, G.L. Chen, J.M. Harkin, M. Nimz and H. Renner, Chem. Commun. 224, 1963
4. S.P. Clowick and N.D. Kaplan,"Methods in Enzymology", Acad Press, 1955, p500

5. M. Florin and G.H. Stotz, "Comprehensive Biochemistry" Elsevier Pub. Vol 14, p63-72

6. C.H. Suetter and D. Motzler, Bio. Biophy. ACTA, 44, 23, 1940; T.P. Binger and E.B. Kearney, J. Biochemistry, 183, 409, 1950

7. E. Chance, Advances in Enzymatology, 12, 153, 1951; Helen H. Stofford, An. Rev. Plant Physiology, 25, 459, 1974

8. K. Freudenberg and A.C. Neish,"Constitution and Biosynthesis of Lignin" Springer-Verlag, New York, 1968

9. K. Hahlbrook and H. Grisebach, "Enzymatic Controls in the synthesis of Lignin and Flavoids", An. Rev. Plant Physiology, 30, 107, 1979