Lignin Synthesis: The Generation of Hydrogen Peroxide and Superoxide by Horseradish Peroxidase and its Stimulation by Manganese (II) and Phenols

Barry Halliwell
Department of Biochemistry, King’s College, University of London, Strand, London WC2R 2LS, U.K.

Abstract. The enzyme horseradish peroxidase (EC 1.11.1.7) catalyses oxidation of NADH. NADH oxidation is prevented by addition of the enzyme superoxide dismutase (EC 1.15.1.1) to the reaction mixture before adding peroxidase but addition of dismutase after peroxidase has little inhibitory effect. Catalase (EC 1.11.1.6) inhibits peroxidase-catalysed NADH oxidation when added at any time during the reaction. Apparently the peroxidase uses hydrogen peroxide (H₂O₂) generated by non-enzymic breakdown of NADH to catalyse oxidation of NADH to a free-radical, NAD⁺, which reduces oxygen to the superoxide free-radical ion, O₂⁻. Some of the O₂⁻ reacts with peroxidase to give peroxidase compound III, which is catalytically inactive in NADH oxidation. The remaining O₂⁻ undergoes dismutation to O₂ and H₂O₂. O₂⁻ does not react with NADH at significant rates. Mn²⁺ or lactate dehydrogenase stimulate NADH oxidation by peroxidase because they mediate a reaction between O₂⁻ and NADH. 2,4-Dichlorophenol, p-cresol and 4-hydroxycinnamic acid stimulate NADH oxidation by peroxidase, probably by breaking down compound III and so increasing the amount of active peroxidase in the reaction mixture. Oxidation in the presence of these phenols is greatly increased by adding H₂O₂. The rate of NADH oxidation by peroxidase is greatest in the presence of both Mn²⁺ and those phenols which interact with compound III. Both O₂⁻ and H₂O₂ are involved in this oxidation, which plays an important role in lignin synthesis.

Key words: Cell wall — Hydrogen peroxide — Lignification — Peroxidase — Superoxide.

Introduction

The lignification of plant cell walls involves the oxidative condensation of various phenols derived from cinnamic acid. Polymerisation of these phenols is initiated by the peroxidase-dependent formation of phenoxy radicals, a reaction which requires H₂O₂ (Harkin and Obst, 1973; Stafford, 1974). The H₂O₂ required can be generated in situ by the peroxidase-catalysed oxidation of NADH produced by a cell-wall-bound malate dehydrogenase (Elstner and Heupel, 1976; Gross, 1977; Gross et al., 1977). H₂O₂ generation by isolated horseradish cell walls was stimulated by addition of monophenols and Mn²⁺ (Gross et al., 1977), cofactors which increase the rate of NADH oxidation by isolated horseradish peroxidase (Aka-zawa and Conn, 1958). In the present paper, I report studies designed to examine the mechanisms of oxidation of NADH by purified horseradish peroxidase and of the stimulatory effects of Mn²⁺ and phenols.

It is generally accepted that oxidations catalysed by horseradish peroxidase may be represented by the series of reactions below, where SH₂ is the substrate being oxidised.

peroxidase(Fe³⁺) + H₂O₂ → compound I

compound I + SH₂ → compound II + SH.

compound II + SH₂ → peroxidase(Fe³⁺) + SH.

The “resting” (Fe³⁺) enzyme loses two electrons to form compound I; the electrons are replaced in 2 one-electron steps in which a substrate SH₂ is converted into a radical SH. Compounds I and II are intermediates that can be distinguished spectrally.

However, peroxidase also oxidises certain substrates without it being necessary to add H₂O₂; such substrates include dihydroxyfumaric acid and NADH (Yamazaki and Yokota, 1973). During oxidation of dihydroxyfumaric acid by peroxidase oxygen is taken up and both hydrogen peroxide and the superoxide free radical ion (O₂⁻) are formed in the reaction mixture (Halliwell, 1977a). Most of the peroxidase is also converted into a different species, known as Compound III. Compound III is an oxygenated fer-
roperoxidase (enzyme – Fe$^{2+}$ – O$_2$) which may be formed by a reaction between O$_2$" and the Fe$^{3+}$-enzyme (4); (Sawada and Yamazaki, 1973).

Peroxidase(Fe$^{3+}$)+O$_2$" → Peroxidase(Fe$^{2+}$ – O$_2$). (4)

Compound III is incapable of oxidising dihydroxyfumarate (Halliwell, 1977a) or NADH (Yokota and Yamazaki, 1965) and merely breaks down slowly to yield Fe$^{3+}$-enzyme, O$_2$" and H$_2$O$_2$ (Rotilio et al., 1975).

NADH oxidation by peroxidase occurs rapidly at acidic and neutral pH values in the presence of Mn$^{2+}$ and certain phenols (Akazawa and Conn, 1958). In the absence of these cofactors, NADH oxidation is much slower and occurs only at acidic pH values (Yokota and Yamazaki, 1965). In both cases, O$_2$ is taken up and compound III can be detected in the reaction mixtures (Yokota and Yamazaki, 1965).

Oxidation of NADH by horseradish peroxidase at pH 5 was found to be inhibited on addition of superoxide dismutase (Yamazaki and Yokota, 1973), an enzyme which catalyses the breakdown of O$_2$" to O$_2$ and H$_2$O$_2$ (5) (Halliwell, 1977b; Fridovich, 1974).

O$_2$" + O$_2$" + 2H$^+$ → H$_2$O$_2$ + O$_2$. (5)

This suggests that the superoxide radical is involved in NADH oxidation. Yamazaki and Yokota (1973) proposed that the slow H$^+$-dependent non-enzymatic breakdown of NADH at pH 5 generated H$_2$O$_2$, which was used by peroxidase to catalyse oxidation of NADH to the radical NAD. whose existence has been clearly demonstrated (Willson, 1970) (eqns. 1–3; replace SH$_2$ by NADH and SH by NAD). NAD. was then proposed to reduce O$_2$ to O$_2$" (6) which could react with more NADH (7) to continue the oxidation and regenerate H$_2$O$_2$.

NAD. + O$_2$ → NAD$^+$ + O$_2$" (6)

NADH + H$^+$ + O$_2$" → NAD. + H$_2$O$_2$. (7)

Although reaction (6) has a rate constant of 1.9 × 10$^9$ M$^{-1}$ s$^{-1}$ (Willson, 1970) the rate of reaction (7) is very small (Chan and Bielski, 1974) and so it seems unlikely that it can play a role in NADH oxidation by peroxidase. Also, this scheme does not explain the great stimulation of NADH oxidation observed when Mn$^{2+}$ and phenols are added.

In view of the importance of NADH oxidation by peroxidase to lignification and also to other physiological processes such as O$_2$ metabolism by leucocytes (Segal and Peters, 1976), I have studied this reaction in more detail. First, experiments were carried out to examine the role of O$_2$" and H$_2$O$_2$ in NADH oxidation by peroxidase at pH 5, in order to test the proposals of Yamazaki and Yokota (1973). Second, the mechanisms by which Mn$^{2+}$ and certain phenols increase the rate of oxidation have been examined. The latter work has also been carried out at pH 5, so that the effects produced by the cofactors could be compared with the properties of NADH oxidation in their absence. Although pH 5 might seem to be "unphysiological", the pH of the plant cell wall during lignification is not known. In any case, I have also carried out the reactions in the presence of cofactors at pH 7 and the results are qualitatively similar to those at pH 5. NADH oxidation has been followed in 2 ways: by the fall in absorbance at 340 nm and by O$_2$ uptake using an O$_2$ electrode. The latter assay is very useful where concentrations of NADH are too high to allow accurate spectrophotometric measurements.

Materials and Methods

Materials

NADH and catalase (H$_2$O$_2$ – H$_2$O$_2$ oxidoreductase, EC 1.11.1.6) were obtained from the Boehringer Corp., London W5, UK. Contamination of the catalase with superoxide dismutase (Halliwell, 1973) was found to be negligible. Pig heart lactate dehydrogenase (L-lactate – NAD$^+$ oxidoreductase EC 1.1.1.27), 4-hydroxyccinnamic acid and horseradish peroxidase (type VI) (donor – H$_2$O$_2$ oxidoreductase, EC 1.11.1.7) were obtained from Sigma London Chemical Corp., Kingston-upon-Thames, Surrey, U.K. The peroxidase was dissolved in 50 mM acetic acid-sodium acetate buffer, pH 5, and its concentration calculated from absorbance at 403 nm (A$_{403}$ = 10$^4$ M$^{-1}$ cm$^{-1}$) (Sawada and Yamazaki, 1973). The ratio A$_{403}$/A$_{278}$ was 2.90. A superoxide dismutase (superoxide-superoxide oxidoreductase, EC 1.15.1.1) containing copper and zinc was prepared as described by McCord and Fridovich (1969). Where indicated it was denatured by heating at 100°C for 10 min and cooling before use. The manganese-containing superoxide dismutase from Bacillus stearothermophilus (Harris, 1977) was obtained from the Microbiological Research Establishment, Porton, Salisbury, Wilts, UK.

Enzyme Assays

Superoxide dismutase was assayed by the cytochrome c method: 1 unit inhibits the reduction of cytochrome c by 50% under the assay conditions of McCord and Fridovich (1969). Catalase was assayed by the fall in absorbance at 240 nm as H$_2$O$_2$ was destroyed: reaction conditions were as described by Luck (1963) except that 50 mM acetic acid-sodium acetate, pH 5, was the buffer used in the reaction mixture. One unit of catalase is that amount which catalyses the breakdown of 1 μmol of H$_2$O$_2$ in min under these conditions. Oxidation of NADH by peroxidase was followed by the fall in absorbance at 340 nm at pH 5 and 20°C: details of NADH and enzyme concentrations are given in the text and figure legends. O$_2$ uptake by these reaction mixtures was measured in a final volume of 3 ml at 25°C by using a Hansatech O$_2$ electrode (Hansatech Ltd., King's Lynn, Norfolk, UK) calibrated according to the manufacturer’s instructions.