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A Mechanism for Lignification in Plants

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ABSTRACT

The mechanism of lignification is explained in terms of a free radical formation after abstraction of phenolic hydrogen from coniferyl alcohol. In plants this oxidative reaction occurs in the presence of a hydrogen peroxide–peroxidase system. In xylem cell walls the lignification mechanism has three steps: initiation *(a monomer of* x-*mer radical is produced by the abstraction of the phenolic hydrogen);* propagation *(an initiated radical combines with its own monomer or* x-*mer); and* termination *(a propagated radical receives a hydroxyl radical released from the system by peroxidase). In this paper the mechanism of in-vitro synthesis of lignin from coniferyl alcohol in the presence of hydrogen peroxide and peroxidase is described.*

INTRODUCTION

Lignin occurs as one of the major components of woody tissue of plants comprising nearly 20–30% of biomass. Degradation of lignin by enzymes and microorganisms has been intensively studied and many

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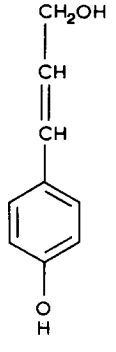
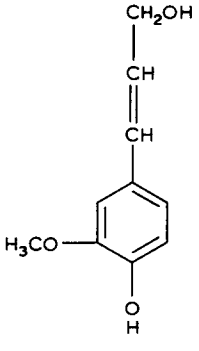
papers have been published (Crawford *et al.*, 1983; Higushi, 1983; Kirk, 1983; Srinivasan & Cary, 1987). However, not many papers on the synthesis of lignin have appeared.

The synthesis of lignin from coniferyl alcohol in plants has been described by Gross *et al.* (1977) and Halliwell (1978). Lignin precursors — e.g. *p*-coumaryl (I), coniferyl (II), and sinapyl (III) alcohols — are



(I)



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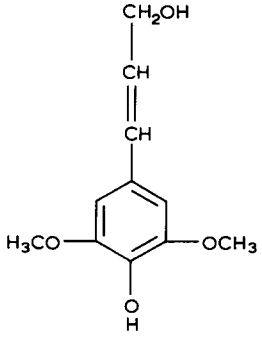

(III)

formed from D-glucose by a variety of enzymic reactions involving oxidations, reductions, aminations, deaminations, etc. The enzymic dehydrogenation reaction is initiated by an electron transfer which results in the formation of resonance-stabilized phenoxy radicals. The combination of these radicals produces a variety of dimers and oligomers, termed lignols.

In this work the lignification mechanism from coniferyl alcohol in an ethanol–water system proposed by Freudenberg and Neish, (1968) has been revised and additional in-vitro experiments have been performed in order to explain the lignin synthesis.

MATERIALS AND METHODS

Materials

Peroxidase (EC 1.11.1.7) with activity of 95 I.U./mg protein and alcohol dehydrogenase (EC 1.1.1.1.) with activity of 400 I.U./mg protein were purchased from Sigma Chemical Co. Limited. Coniferyl alcohol was obtained from Aldrich Chemical Co. Limited.

Methods

Experiment 1
Coniferyl alcohol (50 mg) was dissolved in 20 ml ethanol and mixed to a peroxidase preparation (2·5 mg/ml) in phosphate buffer (0·025 M, pH 6·8). During stirring, one drop of 1% hydrogen peroxide (H_2O_2) was added at intervals. After centrifugation, the precipitate was dissolved in tetrahydrofuran, coated on a tungsten probe and a mass spectrum was run in a Kratos spectrometer MS80RF and recorded using a field desorption technique.

Experiment 2
Coniferyl alcohol (50 mg) was dissolved in 2·1 ml of a mixture of dioxane: 0·025 M phosphate buffer pH 7·0 (1:1). Peroxidase (0·2 mg) was stirred into the mixture. After 2–5 days, the freeze-dried material was run by mass spectrometry as in experiment 1.

Experiment 3
Coniferyl alcohol (75 mg) was dissolved in 2·15 ml of a mixture of dioxane: 0·025 M phosphate buffer pH 6·8 (1·1:1) containing peroxidase (0·2 mg), alcohol dehydrogenase (7·5 units) and NAD^+ (100 μM). At the same time 5 μl of ethanol was added for polymerization. The reaction was stirred and exposed to air. After 2 days the solution was freeze-dried and run by mass spectrometry as in experiment 1.

RESULTS AND DISCUSSION

The process of a free radical polymerization involves 3 steps — namely, initiation, propagation and termination. In general, the energy of activation in the termination step is lower than in the propagation step. The propagation of coniferyl alcohol is similar to that of styrene.

In the biosynthesis of lignin a radical initiates addition polymerization by the attack on the double bond *beta* carbon of coniferyl alcohol monomer, resulting in hydrogen abstraction and peroxide reduction in the presence of peroxidase (Higuchi, 1985*a*, *b*; Hwang, 1985).

The results obtained from the experiments show that the lignification mechanism can be explained as follows.

Polymerization

A radical is formed by the removal of the phenolic hydrogen and oxidation of hydrogen peroxide catalysed by peroxidase — see eqn (1).

$$\text{Per—}H_2O_2 + H^+ + e^- \rightleftharpoons \text{Per—OH} + H_2O \qquad E = -0{\cdot}27\ \text{V}$$

$$\text{ArOH (M1)} \rightleftharpoons H^+ + \text{ArO.(M1.)} + e^- \qquad E > 0{\cdot}27\ \text{V}$$

$$\text{ArOH(M1)} + \text{Per—}H_2O_2 \rightleftharpoons \text{ArO.(M1.)} + \text{Per—OH} + H_2O \qquad (1)$$

where Per = peroxidase, and Ar = aryl.

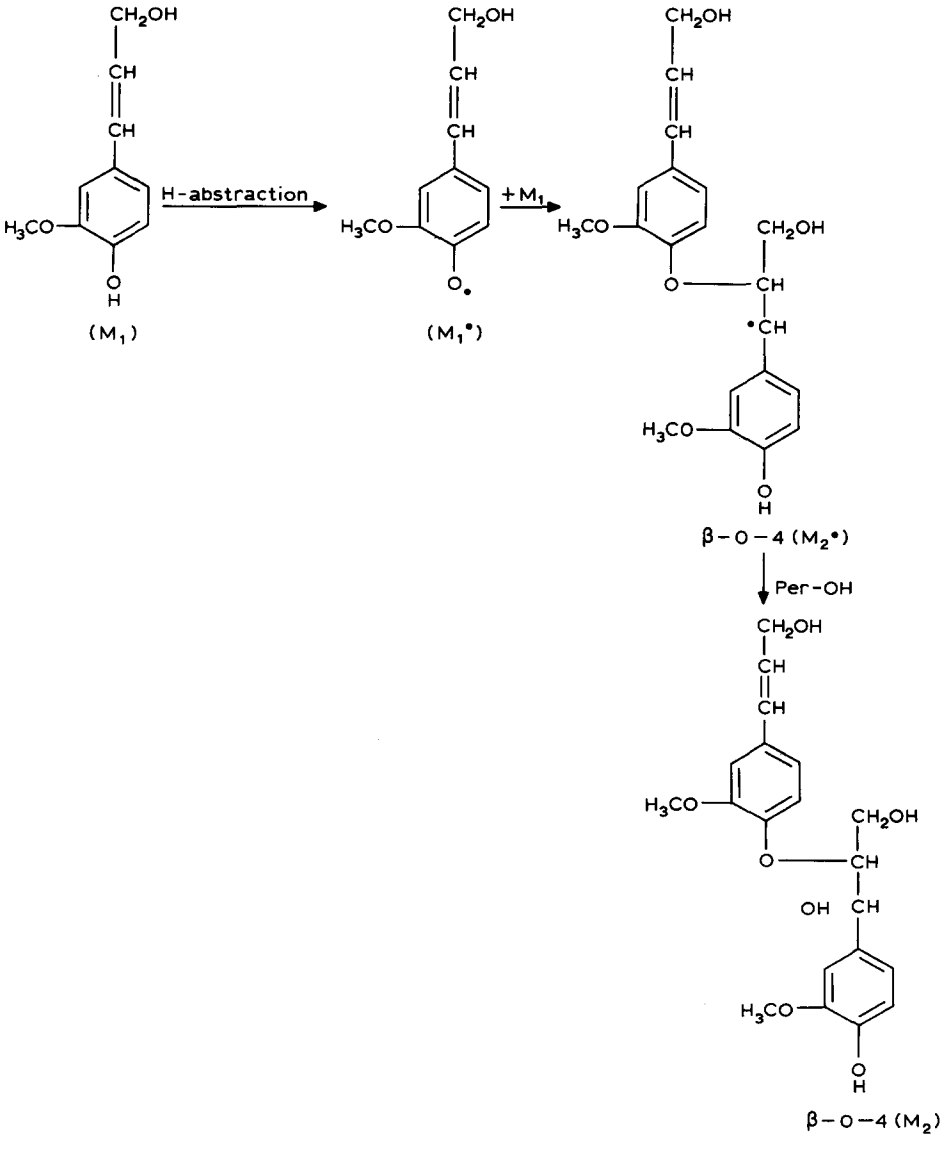

Fig. 1. The mechanism of lignification from coniferyl alcohol in plants (β–O–4 combination).

Propagation of the polymerization

$$M1. + M1 \rightarrow M2. \qquad (2)$$

where M1 = monomer, M2 = dimer, etc.

Termination of the polymerization

$$M2. + Per{-}\cdot OH \rightarrow M2 + \text{peroxidase} \qquad (3)$$

Experiment 1 is a repetition of Freudenberg's experiment, using coniferyl alcohol in ethanol-water with drops of H_2O_2 in the presence of peroxidase. The 5–5′ combination (Hwang, 1989) has the lowest ΔL value and the highest rate of reaction. The β–O–4 and β–β combinations are the second and third immediate reactions. The lignification mechanisms in β–O–4 and in β–β combinations are illustrated in Figs 1 and 4, respectively.

This radical attacks the beta position of the vinyl bond of its own monomer to form a 1-hydroxy-2(2-methoxy)-4(3-hydroxy-propenyl) phenoxy-3(4-hydroxy-3-methoxy)-phenyl-propyl radical. The dimer radical is then terminated when a hydroxyl radical is released from peroxidase to form a dimer guaiacyl-glycerol-β-coniferyl ether (β–O–4). If the dimer β–O–4 loses a water molecule, it will have a 1–7 sigmatropic rearrangement forming the 55, β–O–4 (Hwang *et al.*, 1989) (see Fig. 2).

The mechanism of lignification produces mostly the β–β combination (M2 358) (see Figs 3 and 4, and Table 1) rather than the β–O–4 combination as shown in Fig. 1.

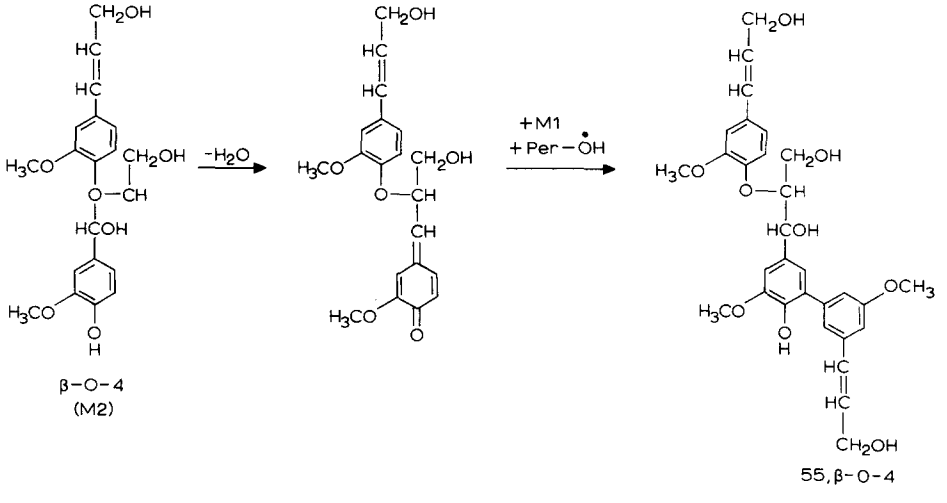

Fig. 2. Sigmatropic rearrangement forming the 55, β–O–4.

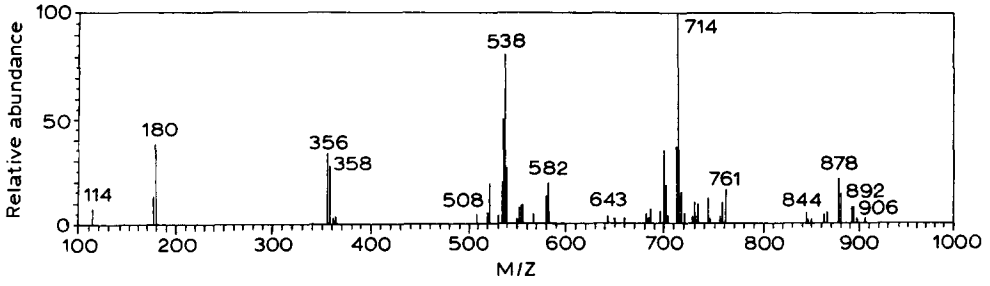

Fig. 3. Mass spectrum of experiment 1.

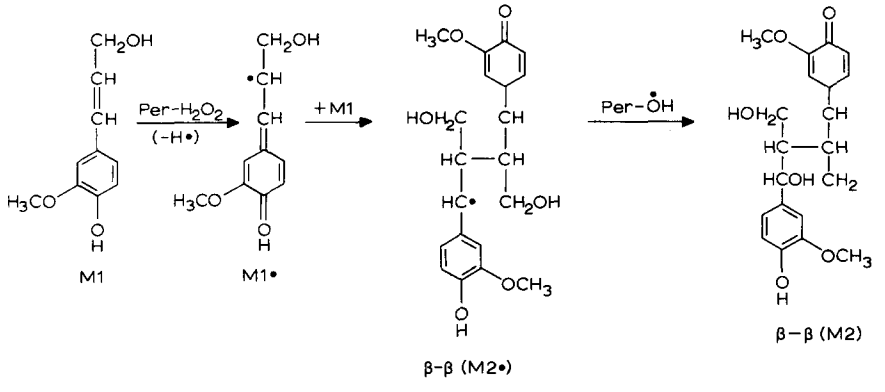

Fig. 4. The mechanism of lignification from coniferyl alcohol in plants (β-β combination).

Formation of peroxidase–H_2O_2

Peroxidase is a heme-protein. The heme is a porphyrin containing an iron atom at the centre. The electron configuration in iron has 18 electrons of argon core. The 6 electrons are in *d*-orbitals. In chemical reaction, the iron atom can accept electrons into the extranuclear core until 36 electrons are present. The dx^2–y^2, *s*, *px* and *py* orbitals can be on the *xy* plane. The dz^2 and *pz* orbitals can be either above or below the *xy* plane to form an octahedral configuration. A hydroxyl radical when joined to another radical forms a pair. Hydrogen peroxide or diradical oxygen also in pairs could occupy the vacant 4 *pz* orbital. The NADH-depending dehydrogenase contains a nicotinamide and a sulphydryl group of glutatione which could be at the other apex. This explains how an enzyme model for ligand-binding receptor can form a peroxidase–O_2 or a peroxidase–H_2O_2.

TABLE 1
Mass Tabulation Obtained From Experiment 1

M/Z	*Composition (%)*	*Combinations*
178	12·6	M
180	36·2	M
356	33·1	M2 $\beta\beta$
358	28·7	M2 $\beta\beta$
522	19·9	M3 $\beta\beta$, 55 554–CH_3OH
534	10·1	
536	55·4	
537	34·5	
538	80·2	
539	29·4	$\beta\beta$, 55 554–OH
540	25·3	
556	10·8	
580	14·2	M4 732–153 $C_6H_5(OCH_3)(OH)CHOH$
582	20·5	
698	12·8	732–CH_3OH
699	11·1	732–CH_2OH
700	34·5	
702	19·4	
703	10·7	
712	37·1	732–H_2O
713	17·2	
714	100·0	
715	41·2	
716	39·4	
717	12·4	750–CH_3OH
718	12·7	
730	10·1	
734	10·5	M4
744	12·4	M5 928–153–CH_3OH
758	10·4	928–153–H_2O
761	10·7	
762	17·4	946–153–CH_3OH
878	22·5	928–H_2O–CH_3OH
880	15·9	

The formation of a green-coloured primary complex (compound I) when H_2O_2 is added to peroxidase has been described by Chance (1949). After H-abstraction of phenol, the compound I is rapidly converted into pale red-coloured compound II as follows:

$$\text{per—}H_2O + H_2O_2 \rightleftharpoons \text{per—}H_2O_2\,(\text{compound I}) + H_2O$$

$$\text{compound I} + AH_2 \rightarrow \text{compound II} + AH\cdot + H_2O$$

$$\text{compound II} + AH\cdot + AH_2 \rightarrow \text{per—}H_2O + A + AH_2$$

where AH_2 = electron donor; $AH\cdot$ = half-oxidized electron donor.

In experiment 2 the spontaneous redox reaction (5) occurs as follows.

$$2\,\text{per—}O_2 + 2H^+ + 2e^- \rightleftharpoons \text{per-}H_2O_2 \qquad Eo^1 = 1{\cdot}1$$

$$2Ml \rightleftharpoons 2Ml\cdot + 2H^+ + 2e^- \qquad Eo^1 > 0{\cdot}27$$

$$2Ml + 2\,\text{per—}O_2 \rightleftharpoons 2Ml\cdot + \text{per—}H_2O_2 \qquad Eo^1 > 1{\cdot}37 \qquad (4)$$

It seems to be that the two hydroxyl radicals in 4 *pz* orbital of peroxidase would donate a hydroxyl radical which can abstract the phenolic hydrogen or can terminate the radical propagation. The dimerization of the radicals is possible; however, the propagation does not go very long. The expected combination products and their m/z are shown in Table 2.

$$2Ml\cdot + 2Ml \rightleftharpoons 2M2\cdot$$

$$2M2\cdot + \text{per—}H_2O_2 \rightarrow 2M2 + \text{peroxidase}$$

$$M1\cdot + M1 \rightleftharpoons M2\cdot \qquad (5)$$

$$M2\cdot + M1\cdot \rightarrow M3$$

TABLE 2
Mass Tabulation Obtained From Experiment 2

M/Z	*Composition (%)*	*Combinations*
163	33·2	M M–H_2O
305	74·6	M2 $\beta\beta$, 55 358–C_2H_2–CH_2OH
333	100·00	358–C_2H_2
341	20·2	358–OH
357	10·3	
358	18·0	
359	14·6	M3 β–O–4, $\beta\beta$, 55 = 538
532	94·2	
533	33·5	

Malate and oxaloacetate are intermediate compounds of the Krebs cycle. In the Krebs cycle, in plants, malate is oxidized to oxaloacetate by malate dehydrogenase as follows:

$$\text{Malate} + \text{NAD}^+ \rightarrow \text{oxaloacetate} + \text{NADH} + \text{H}^+ \qquad (6)$$

TABLE 3
Mass Tabulation Obtained From Experiment 3

M/Z	*Composition (%)*	*Combinations*
163	40·7	M 180–OH
342	10·3	M2 $\beta\beta$, 55 358–OH
343	25·5	
358	12·5	M2 358
359	11·8	
360	23·0	M2 $\beta\beta$, 55 with an open furan ring
376	20·9	
522	44·1	M3 554–CH_3OH
523	17·8	
524	33·2	
532	100·0	M3 536
533	33·1	
538	7·1	
540	5·3	
686	7·4	M4 β–O–4, $\beta\beta$, 55 732–CH_2OH–OH
1061	22·8	M6 β–O–4, β–O–4, $\beta\beta$, 55 1124–2 × CH_2OH
1062	15·5	
1063	12·7	

In experiment 3 (see Table 3) the enzyme alcohol dehydrogenase has been used to promote ethanol oxidation and nicotinamide adenine dinucleotide (NAD^+) reduction. The $NADH + H^+$ is produced in the same manner as in malate and lactate oxidative reaction in which the coenzyme NAD^+ is reduced. The reduction takes place when the hydrogen radical is transferred from the malate or lactate molecule to NAD^+. The reaction is catalysed by the dehydrogenase. The peroxidase–O_2 therefore can be converted to peroxidase–H_2O_2 by the reoxidation of the $NADH + H^+$. $NADH + H^+$ can oxidize the ferric form of the iron present in the peroxidase molecule into its ferrous form, but not the

formation of peroxidase–H_2O_2 (Halliwell, 1978), as follows:

$$NADH + H^+ \rightleftharpoons NAD^+ + H^+ + 2e^- \qquad Eo^1 = 0{\cdot}32$$

$$2\ per\text{—}O_2(Fe^{3+}) + 2e^- \rightleftharpoons 2\ per\text{—}O_2(Fe^{2+}) \qquad Eo^1 = 1{\cdot}19$$

$$NADH + H^+ + 2\ per\text{—}O_2(Fe^{3+}) \rightleftharpoons H^+ + NAD^+ + 2\ per\text{—}O_2(Fe^{2+}) \qquad Eo^1 = 1{\cdot}51 \quad (7)$$

However, when the enzyme superoxide dismutase is added before the peroxidase, the oxidation of $NADH + H^+$ is prevented and the NADH bound to the superoxide dismutase can also be bound on to peroxidase. The produced amount of peroxidase–H_2O_2 is sufficient to promote lignification in plants.

The $NADH + H^+$ can be utilized to convert peroxidase–O_2 into peroxidase–H_2O_2 which is the main primer for the initiation of a radical.

$$NADH + H^+ \xrightarrow{*} NAD^+ + 2H^+ + 2e^- \qquad E = 0{\cdot}32\ V$$

$$2H^+ + per\text{—}O_2(Fe^{3+}) + 2e^- \rightarrow per\text{—}H_2O_2(Fe^{3+}) \qquad E = 1{\cdot}1\ V$$

$$NADH + H^+ + per\text{—}O_2 \rightarrow NAD^+ + per\text{—}H_2O_2 \qquad E = 1{\cdot}43\ V \quad (8)$$

*–NADH-depending dehydrogenase

The eqns (1) and (8) occur fast and the eqns (2) and (3) occur almost instantaneously. However, the velocity of the enzymatic eqn (6) depends on the utilization of oxaloacetate when the steady state is reached, otherwise NADH cannot be produced. For this reason, the lignification products cannot be a high-molecular-weight lignol and the low-molecular-weight polymer also needs a suitable medium to be able to initiate a radical. Nevertheless, the radical and *X*-mer could be increased to form a higher molecular weight polymer (*n*-mer).

The peroxidase seems to have 5 hybrid orbitals with a *pz* orbital to form an octahedral configuration. The electron in *py* orbital is not easily transferred to *d*-orbital which is filled by *s*-covalent bond from NADH-dehydrogenase. Our proposal for the ligand–receptor binding is shown in Fig. 5.

The mechanism of lignification (see Fig. 6) is coupled to an enzyme-catalysed reaction. Coniferyl alcohol is almost insoluble in water, and its polymerization leads to a total insolubility. Coniferyl alcohol when exposed to air can be polymerized in the presence of peroxidase or H_2O_2/peroxidase or dehydrogenase bound NADH/peroxidase in a

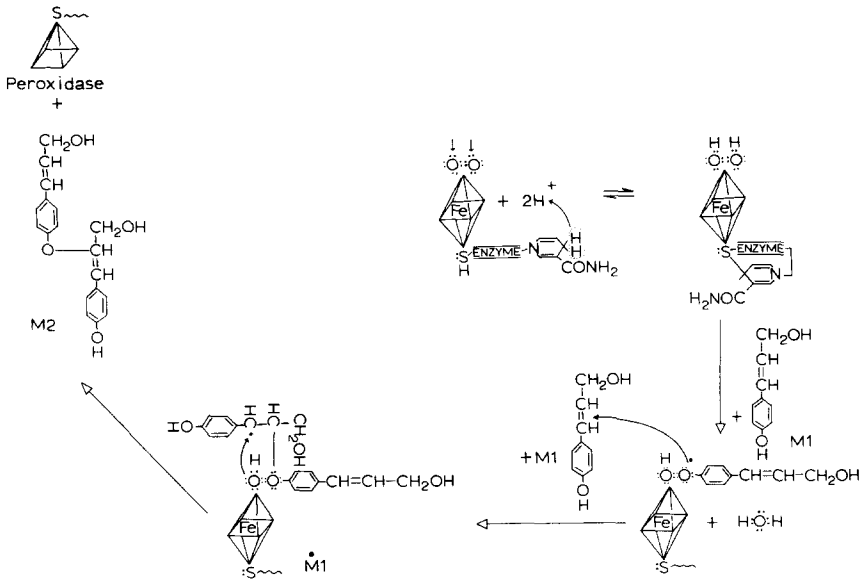

Fig. 5. Model for ligand–receptor binding.

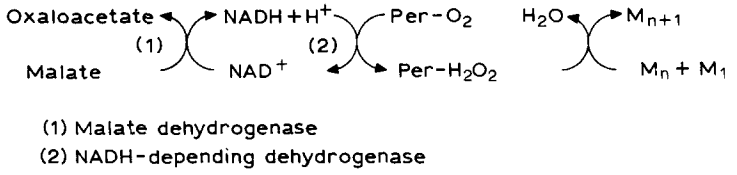

Fig. 6. Mechanism of lignification coupled to the enzyme-catalysed reaction.

dioxan–water system. However, the high-molecular-weight polymers cannot be produced in this dioxan–water system. In an ethanol–water system 4- to 5-mers are distinctly produced.

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