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Laccase-catalyzed oxidation of 1-(3,4-dimethoxyphenyl)-1-propene using ABTS as mediator

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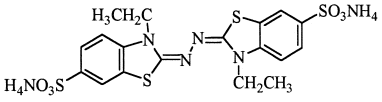
Abstract

The fungal laccases catalyzed oxidation of 1-(3,4-dimethoxyphenyl)-1-propene (**2**) with dioxygen in acetate buffer (pH 4.5) producing 1-(3,4-dimethoxyphenyl)propane-1,2-diol (**4**) and its 1-*O*-acetyl and 2-*O*-acetyl derivatives **5** and **6**, and 3,4-dimethoxybenzaldehyde (**7**). However, in phosphate buffer (pH 5.9), the same reaction produced only **4** and **7**. When **4** was treated in the same fashion in the phosphate buffer, it was converted into **7** with more than 95 mol% yield. This, together with the formation of **5** and **6** in the acetate buffer, showed that **2** is converted into **3**–**5** via 1-(3,4-dimethoxyphenyl)propane-1,2-epoxide (**3**) in the acetate buffer in the presence of ABTS. The major reaction of fungal laccase-catalyzed oxidation of **2** with dioxygen in the presence of ABTS is epoxidation of the double bond conjugated to the aromatic ring.

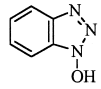
Keywords: Fungal laccases; Enzyme-catalyzed oxidation; 1-(3,4-Dimethoxyphenyl)-1-propene; Laccase-mediator system

1. Introduction

Fungal laccase (EC 1.10.3.2) is a group of enzymes that occurs in nature in white-rot fungi that decompose woody materials [1–3]. It is well established that fungal laccases readily catalyze the oxidation of phenols with dioxygen, producing the corresponding dimeric, oligomeric and high molecular mass dehydrogenative polymerization products, but not *O*-etherified phenols. Recently, Bourbonnais and Paice [4] found that laccase isolated from *Coriolus versicolor* catalyzed the oxidation of the side-chain of *O*-etherified phenols, such as 3,4-dimethylbenzyl alcohol, to the corresponding benzaldehydes in the presence of a specific compound usually called a mediator, such as 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) or 1-hydroxybenzotriazole (HOBT) [4,5].



2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS)



1-Hydroxybenzotriazole (HOBT)

A review of the literature shows that only four types of oxidation reaction are catalyzed by fungal laccases in the presence of a mediator.

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Kawai et al. [6] found that the *Coriolus* laccase catalyzed oxidation of 3,4-dimethylbenzyl alcohol with dioxygen to 3,4-dimethylbenzaldehyde in the presence of a small amount of syringaldehyde (Type 1). Bourbonnais and Paice [4] found further that the same reaction occurred when ABTS was used as the mediator instead of syringaldehyde (Type 1). The *Coriolus* laccase-ABTS system also catalyzed the oxidative decarboxylation of homoveratric acid to veratryl alcohol and 3,4-dimethoxybenzaldehyde (Type 2) [7], the oxidation of the α-hydroxyl group in 1-(3,4-dimethoxyphenyl)-2-(2-methoxyphenoxy)propane-1,3-diol to the corresponding α-carbonyl group (Type 3), and the oxidative cleavage of the C_α–C_β bond in 1-(3,4-dimethoxyphenyl)-2-phenylethanediol to yield benzaldehyde and 3,4-dimethoxybenzaldehyde (Type 4) [4]. In dimeric compounds, the oxidative cleavage of the C_α–C_β bond is dependent on the nature of the β-substituent, as demonstrated by type 2 and 3 reactions. However, none of these reactions includes the reactivity of fungal laccase-catalyzed oxidation with dioxygen towards double bonds conjugated to aromatic rings, such as 1-(4-hydroxy-3-methoxyphenyl)-1-propene (**1**; isoeugenol) and 1-(3,4-dimethoxyphenyl)-1-propene (**2**; 4-*O*-methylisoeugenol), in the presence of ABTS. Thus, the objectives of this investigation are: (1) to study fungal laccase-catalyzed oxidation of 1-(3,4-dimethoxyphenyl)-1-propene (**2**) with dioxygen in the presence of ABTS in acetate buffer (pH 4.5) and in phosphate buffer (pH 5.9), and (2) to clarify the reaction mechanism of the reaction as compared to the reaction of 1-(4-hydroxy-3-methoxyphenyl)-1-propene (**1**).

2. Results and discussion

2.1. Fungal laccase-catalyzed oxidation of 1-(3,4-dimethoxyphenyl)-1-propene (O-methylisoeugenol) in acetate buffer at pH 4.5

1-(3,4-Dimethoxyphenyl)-1-propene (**2**) underwent the *Pycnoporus* laccase-catalyzed oxi-

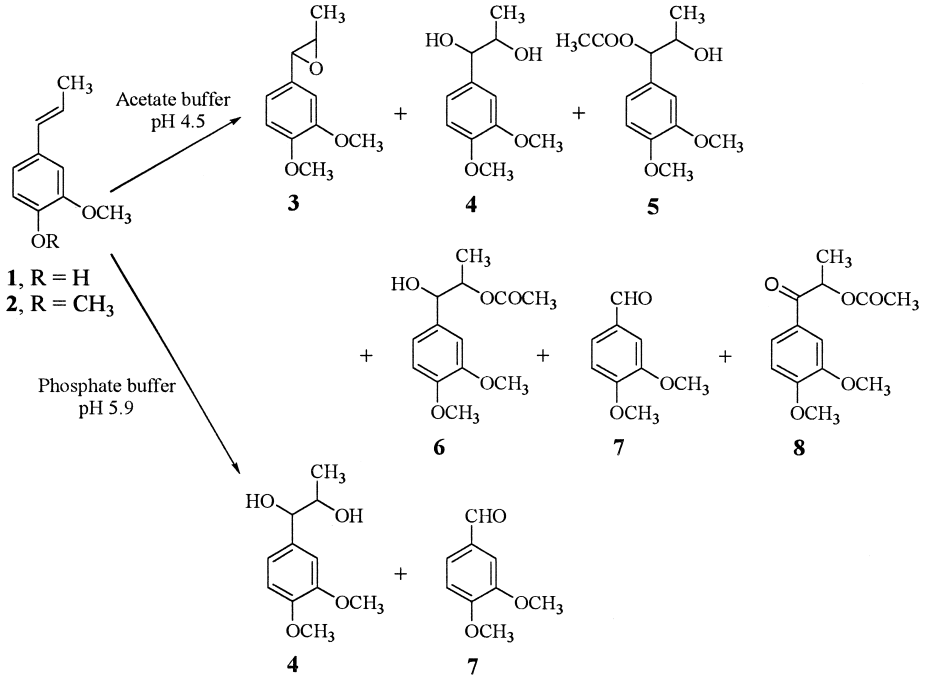

Fig. 1. Reaction products from fungal laccase-catalyzed oxidation of 1-(3,4-dimethoxyphenyl)-1-propene (**2**) with dioxygen in both acetate buffer at pH 4.5 and phosphate buffer at pH 5.9 at 40°C in the presence of ABTS.

dation in an acetate buffer solution (pH 4.5) at 40°C. Within a reaction time of 12 h all of the substrate disappeared. As shown in Fig. 1, 1-(3,4-dimethoxyphenyl)propane-1,2-diol (**4**), 1-acetoxy-1-(3,4-dimethoxyphenyl)propan-2-ol (**5**), 2-acetoxy-1-(3,4-dimethoxyphenyl)-propan-1-ol (**6**) and 3,4-dimethoxybenzaldehyde (**7**) were isolated in a molar ratio of 22:10:6:62 with a total yield of 95 mol% from the resulting reaction mixture.

The *Coriolus* laccase-catalyzed oxidation of **2** gave a small amount of 1-(3,4-dimethoxyphenyl)propane-1,2-epoxide (**3**), the products **4**, **5**, **7**, and a small amount of 2-acetoxy-1-(3,4-dimethoxyphenyl)-1-propanone (**8**). The yield of **4**, **5** and **7** is similar in magnitude with the same oxidation with *Pycnoporus* laccase as catalyst under the same reaction conditions. The *trans*-isomer (*E*-isomer) of **2** shows a higher reactivity than that of the *cis*-isomer (*Z*-isomer). After a reaction time of 10 h, the *E*-isomer had been completely consumed while the *Z*-isomer was still present in the reaction mixture.

2.2. Fungal laccase-catalyzed oxidation of 1-(3,4-dimethoxyphenyl)-1-propene (2, O-methyl-isoeugenol) in phosphate buffer at pH 5.9

When the acetate buffer was replaced by a phosphate buffer (pH 5.9), the *Pycnoporus* laccase-ABTS-catalyzed oxidation of **2** produced only **4** and **7** in a molar ratio of 5:95 with a total yield of 95 mol% at 40°C, and a reaction time of 120 h (Fig. 1). At 50°C, the reaction time was shortened to 48 h with the same results. When **4** was treated in the same manner, **7** was

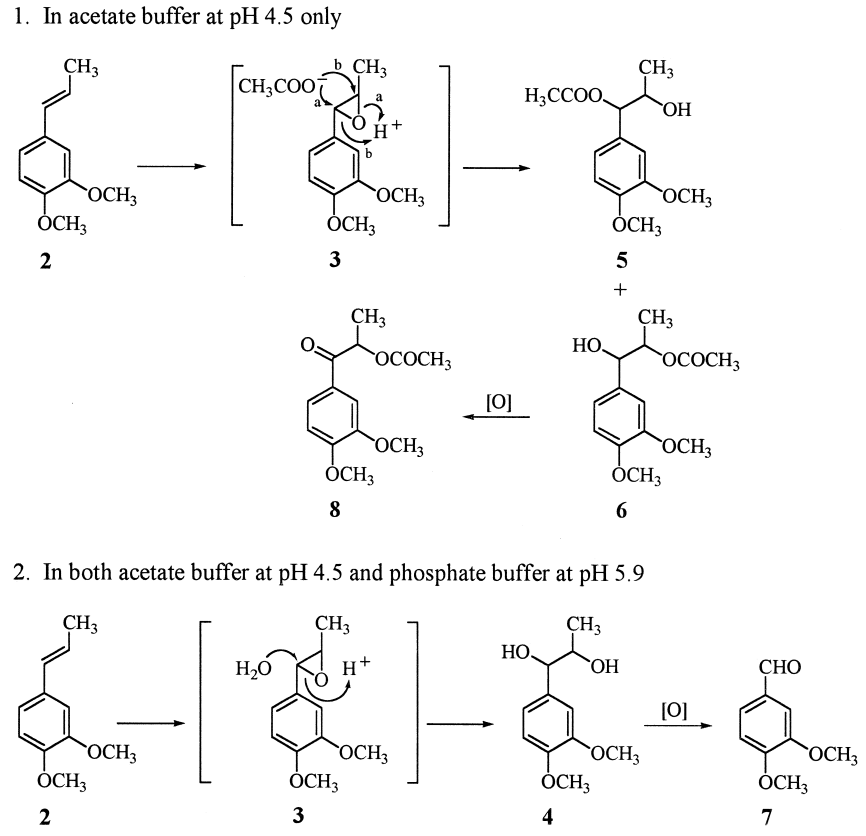

Fig. 2. Reaction mechanism for the fungal laccase-catalyzed oxidation of 1-(3,4-dimethoxyphenyl)-1-propene (**2**) with dioxygen in both acetate buffer at pH 4.5 and phosphate buffer at pH 5.9 at 40°C in the presence of ABTS.

produced in quantitative yield. This clearly shows that the latter is not produced directly from the oxidation of **2**, but is produced from **2** via oxidation of **4**.

*2.3. Reaction mechanism for the fungal laccase-catalyzed oxidation of 1-(3,4-dimethoxyphenyl)-1-propene (**2**, O-methylisoeugenol) with dioxygen*

Compounds **5** and **6**, 1-*O*-acetyl and 2-*O*-acetyl derivatives of **4**, were formed in the reaction mixture of *Pycnoporus* laccase-catalyzed oxidation of **2** in an acetate buffer solution at pH 4.5, but not in a phosphate buffer solution at pH 5.9. In addition, 1-(3,4-dimethoxyphenyl)propane-1,2-epoxide (**3**) was identified in the reaction mixture of *Coriolus* laccase-catalyzed oxidation of **2** in the acetate buffer solution at pH 4.5. Thus, as shown in Fig. 2, these results clearly indicate that **5** and **6** are produced via nucleophilic attack of an acetate anion ($CH3COO^-$) on C-1 (pathway a) or C-2 (pathway b) of 1-(3,4-dimethoxyphenyl)-propane-1,2-epoxide intermediate **3**, respectively. A nucleophilic attack of water on C-1 of **3** produces 1-(3,4-dimethoxyphenyl) propane-1,2-diol (**4**). Since the phosphate buffer solution does not contain acetate anions, **5** and **6** cannot be produced by the laccase-catalyzed oxidation of **2** with dioxygen in this solution. Thus, epoxidation of the double bond conjugated to the aromatic ring is the initial step in the laccase-catalyzed oxidation of **2** with dioxygen in the presence of ABTS. The epoxidation leads to the formation of 1-(3,4-dimethoxyphenyl)propane-1,2-epoxide (**3**), which in turn undergoes nucleophilic attack by nucleophiles present in the reaction system to give the corresponding products. The role of ABTS in the epoxidation is not known yet.

The *Coriolus* laccase-catalyzed oxidation of **2** also produced 2-acetoxy-1-(3,4-dimethoxyphenyl)-1-propanone (**8**) in addition to the epoxide intermediate **3**. The compound **8** must be produced by oxidation of α-hydroxyl groups in **6** under the reaction conditions (Fig. 2). This is further evidence for the ability of the laccase-mediator system to catalyze oxidations of non-phenolic compounds containing an α-substituted benzyl alcohol structure, a reaction that was referred to as type 3 above. However, this reaction is a minor pathway compared with the epoxidation of the conjugated double bond, the nucleophilic attack of nucleophiles on the epoxide, and the oxidative cleavage of the C_α–C_β bond in phenylglycol derivatives.

*2.4. Fungal laccase-catalyzed oxidation of 1-(4-hydroxy-3-methoxyphenyl)-1-propene (**1**; isoeugenol) in the presence of ABTS*

When 1-(4-hydroxy-3-methoxyphenyl)-1-propene (**1**; isoeugenol) was oxidized with dioxygen catalyzed by the *Pycnoporus* laccase in the presence of ABTS, the rate of dehydrogenative polymerization became considerably slower than that without ABTS or HOBT (Table 1). Compared with the results of the reaction without a mediator, the yield of acetone-insolu-

Table 1
Laccase-mediator catalyzed oxidation of isoeugenol[a]

Isoeugenol (mg)	*Pycnoporus* laccase[b] (ml)	Mediator (mg)	DHP[c] (mg)	Oligomer[d] (mg)	M_w of oligomer
329	1.0	–	209	126	3200
333	1.0	ABTS (5.5)	61	276	2568
319	1.0	HOBT (1.4)	97	222	2598

[a] In 40 ml acetone–water (1:4 v/v), reaction temperature 40°C, reaction time 24 h.
[b] Laccase activity: 2.6×10^4 units/ml measured by the syringaldazine assay method [8].
[c] DHP, acetone-insoluble.
[d] Oligomer, acetone-soluble.

ble dehydrogenative polymer (DHP) decreased appreciably, while the yield of the acetone-soluble oligomer fraction increased accordingly. In addition, the weight-average molecular weight (M_w) of the oligomer fraction decreased from 3200 to the 2568–2598 range, i.e., from an oligomer consisting of approximately 20 C_9-units to that consisting of approximately 16 C_9-units in average, assuming that the C_9-units in the oligomer fractions are unaltered. Investigations on the dehydrogenative polymerization of phenols by the laccase-mediator system [9,10] have shown that this decrease in the M_w is caused by oxidative degradation of the initially produced oligomeric and polymeric substances, rather than by a slower reaction rate because of an inhibition by the mediator. This results in build-up of the oligomer fractions and decrease in the DHP fraction in the reaction mixture from the laccase-catalyzed oxidation of **1** in the presence of either ABTS or HOBT.

3. Experimental

3.1. Materials

Laccase from *Pycnoporus coccineus* in 10 mM phosphate buffer was purchased from Koken Tokyo, Japan. Laccase activity: 2.6×104 units/ml. The laccase activity was measured by the syringaldazine assay method as modified by Grassin and Dubourdien [8]. One unit of laccase is defined as the amount of laccase oxidizing 1 nmole of syringaldazine per min in 0.1 M acetate buffer solution (pH 5) at 20°C.

Laccase from *C. versicolor* was obtained from Mercian, Fujizawa, Japan, as a suspension in 0.1 M sodium phosphate buffer solution (pH 6). Laccase activity: 51 units/ml. The laccase activity was measured by the *p*-hydroxymandelic acid assay method [11]. One unit of laccase is defined as the amount of laccase producing 1 μmole of 4-hydroxybenzaldehyde per min in 50 mM sodium acetate buffer solution (pH 5.4) at 30°C with 4-hydroxymandelic acid as substrate.

ABTS, HOBT, 1-(4-hydroxy-3-dimethoxyphenyl)-1-propene (**1**) and 1-(3,4-dimethoxyphenyl)-1-propene (**2**) were obtained from Aldrich, Milwaukee, WI. All other chemicals were purchased from commercial sources or synthesized according to common procedures.

3.2. Enzymatic oxidations

3.2.1. Oxidation of 1-(4-hydroxy-3-methoxyphenyl)-1-propene (1)

A mixture of **1** (0.3 mmol) with or without 5.5 mg ABTS or 1.5 mg HOBT was dissolved in 8 ml of Me_2CO. The resulting solution was added to 32 ml of acetate buffer (pH 4.5) in a 100 ml flask. To this solution was added 1 ml of *Pycnoporus* laccase. The reaction vessel was flushed with O_2 for 1 min, closed, and mechanically stirred at 40°C for 24 h. In the case of ABTS, the colorless solution turned deeply blue-green immediately upon addition of the enzyme. The precipitate in the reaction mixture was centrifuged, washed thoroughly with deionized H_2O, then air-dried. The air-dried remainder was then treated with Me_2CO to obtain the Me_2CO-soluble (oligomeric) and Me_2CO-insoluble (DHP) fractions.

3.2.2. Oxidation of 1-(3,4-dimethoxyphenyl)-1-propene (2) and 1-(3,4-dimethoxyphenyl)propane-1,2-diol (4)

A mixture of **2** or **4** (0.3 mmol) with 5.5 mg ABTS was dissolved in 8 ml of Me2CO. The resulting solution was added to either 32 ml of acetate buffer (pH 4.5) or 32 ml of phosphate buffer (pH 5.9) in a 100 ml flask. To this solution was added either 1 ml of *Pycnoporus* laccase or 1 ml of *Coriolus* laccase. The reaction vessel was flushed with O_2 for 1 min, closed, and then mechanically stirred continuously at 40°C. Upon addition of the enzyme, the colorless solution turned deeply blue-green immediately. At 3 h intervals, 0.5 ml samples of the reaction mixture were taken, extracted with CH_2Cl_2, and the resulting organic solution was analyzed by GC and GCMS. If the substrate

was still present, the reaction mixture was further flushed with O_2 for 1 min and incubated again while shaking. This procedure was repeated until the substrate disappeared completely. The reaction mixture was extracted three times with CH_2Cl_2. The organic phase was chromatographed on silica gel (column 50 cm $\times$ 1.5 cm, i.d.) using $CHCl_3$–CH_3OH (20:1, v/v) to isolate the individual oxidation products.

3.3. Identification of the oxidation products

The organic extracts obtained according to the procedure described above were analyzed by GC and GCMS. The final reaction mixtures were analyzed either by comparing GC-retention times or by co-injection of an authentic sample.

3.4. General conditions of GCMS measurements

GC analysis was carried out on a Hewlett-Packard Model 5890 Series II apparatus, employing a DB-5 bonded phase, fused silica capillary column (30 m $\times$ 0.32 mm i.d.; film thickness 0.25 mm) with flame ionization detection (FID) and following parameters: carrier gas He; flow rate 2 ml/min; temperature profile: initial temp. 45°C (1 min), ramp 5°C/min (45–140°C), 10°C/min (140–260°C), final temp. 260°C (10 min); injection port temp. 200°C; FID temp. 280°C. GCMS analysis was performed on a Hewlett-Packard Model 5985B quadrupole GCMS, electron impact (70 eV) apparatus.

*3.4.1. Analytical data of compounds identified in the reaction mixture of Pycnoporus laccase-catalyzed oxidation of **2** in acetate buffer solution at pH 4.5*

3,4-Dimethoxybenzaldehyde (**7**): GCMS-retention time 7.59 min; EIMS, *m/z* (rel. int.), 166 (M^+, 100), 165 (65) 151 (15), 137 (5), 95 (35), 77 (30).

1-(3,4-Dimethoxyphenyl)-1-propene (**2**): GCMS-retention time 9.03 min; EIMS, *m/z* (rel. int.), 178 (M^+, 100), 163 (35), 147 (10), 115 (15), 107 (35), 91 (30), 77 (15).

1-(3,4-Dimethoxyphenyl)propane-1,2-diol (**4**): GC-retention time 8.7 min; EIMS, *m/z* (rel. int.) 212 (M^+, 10), 178 (5), 167 (100), 151 (5), 139 (60), 124 (20), 108 (15).

2-Acetoxy-1-(3,4-dimethoxyphenyl)propan-1-ol (**6**): GC-retention time 14.85 min; EIMS, *m/z* (rel. int.) 254 (M^+, 10), 194 (5), 167 (100), 151 (10) 139 (50), 124 (10), 108 (5).

1-Acetoxy-1-(3,4-dimethoxyphenyl)propan-2-ol (**5**): GC-retention time 14.98 min; EIMS, *m/z* (rel. int.) 254 (M^+, 10), 209 (25) 194 (5), 167 (100), 151 (5) 139 (45), 124 (10), 108 (5).

*3.4.2. Analytical data of compounds identified in the reaction mixture of Coriolus laccase-catalyzed oxidation of **2** in acetate buffer solution at pH 4.5*

In addition to **4**, **6** and **7**, the following compounds were identified:

1-(3,4-Dimethoxyphenyl)propane-1,2-epoxide (**3**): GC-retention time 9.43 min; EIMS, *m/z* (rel. int.), 194 (M^+, 15), 151 (100), 135 (5), 107 (15).

2-Acetoxy-1-(3,4-dimethoxyphenyl)-1-propanone (**8**): GC-retention time 15.20 min; EIMS, *m/z* (rel. int.) 252 (M^+, 10), 194 (5), 165 (100), 137 (5).

*3.4.3. Analytical data of compounds identified in the reaction mixture of Pycnoporus laccase-catalyzed oxidation of **2** in phosphate buffer solution at pH 5.9*

3,4-Dimethoxybenzaldehyde (**7**): GCMS-retention time 7.59 min; EIMS, *m/z* (rel. int.), 166 (M^+, 100), 165 (60) 151 (10), 137 (5), 95 (30), 77 (30).

1-(3,4-Dimethoxyphenyl)propane-1,2-diol (**4**): GC-retention time 8.7 min; EIMS, *m/z* (rel. int.) 212 (M^+, 5), 178 (5), 167 (100), 151 (5), 139 (65), 124 (20), 108 (15).

1-(3,4-Dimethoxyphenyl)-1-propene (**2**): GCMS-retention time 9.03 min; EIMS, *m/z* (rel. int.), 178 (M^+, 100), 163 (35), 147 (10), 115 (15), 107 (35), 91 (30), 77 (15).

*3.4.4. Analytical data of compounds identified in the reaction mixture of Pycnoporus laccase-catalyzed oxidation of **4** in phosphate buffer solution at pH 5.9*

From the reaction mixture, the yield of 3,4-dimethoxybenzaldehyde (**7**) was determined to be quantitative. The compound was then isolated on a silica gel column according to the procedure described in Section 3.2.2.

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