Oxidative Decarboxylations of 4-Hydroxymandelic Acid and 2-(4-Hydroxyphenyl)glycine by Laccase from *Coriolus versicolor* and Bilirubin Oxidases from *Trachyderma tsunoda* and *Myrothecium verrucaria*

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Laccase from *Coriolus versicolor* and bilirubin oxidases from *Trachyderma tsunoda* and *Myrothecium verrucaria* converted 4-hydroxymandelic acid (HMA) and 2-(4-hydroxyphenyl)glycine into 4-hydroxybenzaldehyde (HBA), which was a single product and was not converted further. The reactions were oxidative decarboxylations that were considered to be caused by the enzyme-catalyzed abstractions of hydrogen from the phenolic hydroxyl groups. The decarboxylation of HMA was used for a new colorimetric measurement of the activities of these enzymes. One unit of the enzymes was defined as the amount that catalyzed the formation of 1 μmol of HBA per minute. When HMA was used for a substrate, the optimum pHs of laccase, bilirubin oxidase from *T. tsunoda*, and bilirubin oxidase from *M. verrucaria* were 4.5, 5.0, and 8.5, respectively, and their Kₘ values were 31.3 mM, 34.5 mM, and 22.3 mM, respectively.

Laccase (benzenediol: oxygen oxidoreductase; EC 1.10.3.2) is a multicopper oxidase¹¹ and has been found in fungal strains belonging to various classes.² The enzyme catalyzes only abstractions of hydrogen from hydroxyl groups of phenolic compounds to give phenoxy radicals, which nonenzymatically undergo free-radical additions and the related reactions to produce large amounts of final products.³–⁷ These free-radical reactions are generally not single reactions but are rather composed of several steps. Consequently, the unit of laccase activity has not been definable as the formation rate of a product.

During our investigations on enzymatic transformations of antibiotics, we found a laccase-catalyzed decarboxylation⁸ of the 7β-(4-hydroxyphenylmalonylamin) side chain of latamoxef, a 1-oxacephalosporin antibiotic.⁹ The reaction indicated that α bonds of carboxyl groups of carboxylic acid salts tend to be cleaved by unpaired electrons. Therefore, we noted the decarboxylation of phenolic compounds by laccase to find single reactions by the enzyme. Various phenolic compounds para-substituted by an alkyl group containing a carboxyl group were incubated with the laccase from *Coriolus versicolor*. We found that 4-hydroxymandelic acid (HMA) and 2-(4-hydroxyphenyl)glycine (HPG) were converted to 4-hydroxybenzaldehyde (HBA), which is a single product from each substrate and not converted further. From stoichiometric studies described in our previous paper,¹⁰ we concluded that these reactions are represented by the following equations: HMA + 1/2O₂ → HBA + CO₂ + H₂O, and HPG + 1/2O₂ → HBA + CO₂ + NH₃.

In this paper, we proposed reaction pathways for the oxidative decarboxylations of HMA and HPG by laccase. As an application of the reaction, we present a new method for measurement of laccase activity. In addition, we show that bilirubin oxidases (bilirubin: oxygen oxidoreductase; EC 1.3.3.5) from *Trachyderma tsunoda*¹¹ and *Myrothecium verrucaria*¹²–¹⁶ also have decarboxylation activity like laccase, and compare these enzymes with the laccase from *C. versicolor*.

**Materials and Methods**

**Chemicals.** 4-Hydroxymandelic acid (HMA) and 2-(4-hydroxyphenyl)glycine (HPG) were purchased from Sigma Chemical Co. and Tokyo Chemical Industry Co., Ltd., respectively. All other chemicals were of reagent grade from commercial sources.

**Enzymes.** Laccase from *C. versicolor* was purified from culture solutions of *C. versicolor* IFO 9791 in this laboratory by the method of Fähraeus and Reinhammer.¹⁷ One unit of laccase was defined as the amount of enzyme producing 1 μmol of HBA per minute in 50 mM sodium acetate buffer (pH 4.5) at 30°C with HMA as a substrate. The protein concentration of laccase solutions was measured by the method of Bradford¹⁸ using the Bio-Rad protein assay kit. Bovine serum albumin was used as the standard.

**Tyrosinase** (EC 1.14.18.1) from mushroom, peroxidase (EC 1.11.1.7) from horseradish, ascorbate oxidase (EC 1.10.3.3) from cucurbita species, and ceruloplasmin (EC 1.16.3.1) from human were purchased from Sigma Chemical Co. Bilirubin oxidase from *M. verrucaria* was purchased from Amano Pharmaceutical Co., Ltd. Bilirubin oxidase from *T. tsunoda* was kindly supplied by Takara Shuzo Co., Ltd.

**HPLC analysis.** Products of the enzyme reaction were analyzed by HPLC on a YMC-Pack ODS A-312 column (4.6 × 150 mm; YMC Co., Ltd., Japan) using a methanol concentration gradient in 20 mM sodium phosphate buffer (pH 6.8) from 10% to 60% for 30 min. The flow rate was 0.5 ml/min. The products were detected by UV monitoring at 190 nm to 400 nm with a photodiode array detector (model Waters 991J). The conditions for the measurement of HMA, HPG, and HBA in the reaction mixture were the same as above except that mobile phase was methanol/20 mM sodium phosphate buffer (pH 6.8) (6:4) and the detection was at UV 280 nm.

**Measurement of the activities of laccase and bilirubin oxidase.** The standard reaction mixture contained 50 μmol of HMA, 50 μmol of buffer, and enzyme in a final volume of 1.01 ml. For laccase, acetate buffer (pH 4.5) was used. For bilirubin oxidase from *T. tsunoda*, acetate buffer (pH 5.0) was used. For bilirubin oxidase from *M. verrucaria*, borate buffer (pH

**Abbreviations:** HMA, 4-hydroxymandelic acid; HPG, 2-(4-hydroxyphenyl)glycine; HBA, 4-hydroxybenzaldehyde.
8.5) was used. The reaction mixture was incubated at 30°C. After various incubation times, 100 μl of the reaction mixture was collected and was added to 2.8 ml of 2,4-dinitrophenyldrazine solution. After the reaction mixture had stood for 10 min at room temperature, 100 μl of 5 N NaOH was added to it. Then its absorbance at 475 nm was measured on a spectrophotometer. The amount of HBA was calculated using a standard calibration curve. The activities of the enzymes were expressed in terms of μmol of HBA produced/minute/ml (i.e., mm/min). The 2,4-dinitrophenyldrazine solution described above was prepared by 20-fold dilution with water of 1 N HCl saturated with 2,4-dinitrophenyldrazine.

Results

Oxidation of phenolic compounds by laccase

Various phenolic compounds listed in Table I were dissolved in 50 mM sodium phosphate buffer (pH 6.0) and incubated with laccase. The resulting reaction mixture was analyzed by HPLC with a linear gradient system as described above. As shown in Table I, HMA and HPG were converted into HBA as a single product. No product except HBA was produced in these reactions. 4-Hydroxybenzoic acid was scarcely oxidized. On the other hand, other compounds were converted into many products, which were presumed to be coupling compounds or polymers of each substrate.

Oxidation of HMA by other oxidases

We examined whether other oxidases that oxidize phenolic compounds, such as ascorbate oxidase, bilirubin oxidase, ceruloplasmin, tyrosinase, and peroxidase, catalyze the decarboxylation of HMA to produce HBA like laccase. Table II summarizes the results. No reaction occurred with ascorbate oxidase and tyrosinase, while bilirubin oxidase, ceruloplasmin, and peroxidase converted HMA into HBA. No product except HBA was detected by HPLC analysis in the reaction mixture of bilirubin oxidase and ceruloplasmin. HMA and HBA in the reaction mixture were measured by HPLC at each incubation time (Fig. 1). Figure 1A and 1B show that the number of moles of consumed HMA was equal to that of produced HBA. The results indicated that HBA was a single product in the reaction by bilirubin oxidase. In the reaction with ceruloplasmin, the reaction rate was too slow to study the stoichiometry of it in the same way. On the other hand, in the reaction by peroxidase, the sum of the number of moles of HMA and that of HBA did not remain constant (Fig. 1C), which indicated that other products than HBA were produced. HBA was also oxidized by peroxidase (data not shown).

Measurement of laccase activity using the decarboxylation of HMA

The decarboxylation of HMA was used for the measurement of laccase activity. The formation rate of HBA, laccase activity, was measured by the colorimetric method as described in Materials and Methods. The principle of the method was as follows: the produced HBA undergoes a coupling reaction with 2,4-dinitrophenyldrazine, which is a color-producing reagent for the determination of aldehydes and ketones, to form hydrazone under acidic conditions, and then the compound is converted to a quinone imine compound under alkaline conditions by addition of sodium hydroxide. The absorption maximum of the quinone compound was 475 nm. The molar absorptivity against the amount of HBA were calculated to be 33,400 at 475 nm. Figure 2 shows the linear relationship of HBA formation by laccase in the standard reaction mixture. However, the amount of produced HBA didn't increase more than about 0.38 mM. The oxygen concentration in the reaction mixture containing 7.2 μg of

Table II. Oxidation of HMA by Copper-containing Enzymes and Peroxidase

<table>
<thead>
<tr>
<th>Enzyme (Origin)</th>
<th>Reaction conditions</th>
<th>Specific activity (U/mg)</th>
<th>Product</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Protein (μg/ml)</td>
<td>Buffer, Temp.</td>
<td></td>
</tr>
<tr>
<td>Laccase (C. versicolor)</td>
<td>13</td>
<td>50 mM acetate (pH 4.9), 30°C</td>
<td>42°</td>
</tr>
<tr>
<td>Ascorbate oxidase (cucumber)</td>
<td>11.4</td>
<td>25 mM acetate (pH 5.5), 25°C</td>
<td>1750°</td>
</tr>
<tr>
<td>Bilirubin oxidase (M. verrucaria)</td>
<td>20</td>
<td>50 mM borate (pH 8.5), 30°C</td>
<td>3.9°</td>
</tr>
<tr>
<td>Bilirubin oxidase (T. trunca)</td>
<td>22.3</td>
<td>50 mM acetate (pH 5.0), 30°C</td>
<td>60°</td>
</tr>
<tr>
<td>Ceruloplasmin (human)</td>
<td>784</td>
<td>25 mM acetate (pH 5.5), 37°C</td>
<td>16.1°</td>
</tr>
<tr>
<td>Tyrosinase (mushroom)</td>
<td>—</td>
<td>50 mM phosphate (pH 6.5), 25°C</td>
<td>100°</td>
</tr>
<tr>
<td>Peroxidase (horse radish)</td>
<td>—</td>
<td>50 mM acetate (pH 4.0, 30°C)</td>
<td>(430 U/ml)°</td>
</tr>
</tbody>
</table>

Table I. Oxidations of Various Phenolic Compounds by Laccase

<table>
<thead>
<tr>
<th>Compound</th>
<th>Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-Hydroxybenzaldehyde (HBA)</td>
<td>—</td>
</tr>
<tr>
<td>Vanillin</td>
<td>+</td>
</tr>
<tr>
<td>4-Hydroxybenzoic acid</td>
<td>±</td>
</tr>
<tr>
<td>Vanillic acid</td>
<td>+</td>
</tr>
<tr>
<td>Syringic acid</td>
<td>+</td>
</tr>
<tr>
<td>4-Hydroxyphenylacetic acid</td>
<td>+</td>
</tr>
<tr>
<td>4-Hydroxy-3-methoxyphenylacetic acid</td>
<td>+</td>
</tr>
<tr>
<td>2-(4-Hydroxyphenyl)isovaleric acid</td>
<td>+</td>
</tr>
<tr>
<td>2-(4-Hydroxyphenyl)glycine</td>
<td>HBA</td>
</tr>
<tr>
<td>4-Hydroxymandelic acid</td>
<td>HBA</td>
</tr>
<tr>
<td>4-Hydroxy-3-methoxymandelic acid</td>
<td>+</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>+</td>
</tr>
<tr>
<td>p-Coumaric acid</td>
<td>+</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>+</td>
</tr>
</tbody>
</table>

° The described buffers containing 50 mM HMA and each enzyme were incubated. The buffer for peroxidase contained 94 mM hydrogen peroxide.

—, no reaction; ±, slight reaction; +, many products were produced.

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Oxidative Decarboxylations by Laccase and Bilirubin Oxidase

Fig. 1. Measurement of HMA and HBA during the Reactions by Two Bilirubin Oxidases from \textit{T. tsunoda} (1A) and \textit{M. verrucaria} (1B), and Peroxidase (1C).

The reaction mixture contained 4 μl of enzyme solution and 400 μl of 10 mM HMA in the following buffers: 50 mM acetate buffer (pH 5.0) (1A), 50 mM borate buffer (pH 8.5) (1B), and 50 mM acetate buffer (pH 4.0) containing hydrogen peroxide (100 μmol) (1C). Incubation was at 25°C. At regular times, HMA and HBA in the reaction mixture were measured by HPLC analysis as described in Materials and Methods. [ ], HMA; [ ], HBA.

Laccase was measured by an oxygen electrode. The oxygen concentration at the incubation times of 0 and 5 min were 0.40 and 0 mM oxygen, respectively. The result indicated that the phenomenon was caused by complete consumption of dissolved oxygen in the reaction mixture and the number of moles of consumed oxygen was equal to that of produced HBA. Figure 3 shows laccase activity measured by this method increased linearly with laccase concentration.

**Effects of pH on the activities of laccase and bilirubin oxidase**

The activities of laccase and bilirubin oxidases from \textit{T. tsunoda} and \textit{M. verrucaria} were measured with HMA or HPG as a substrate at various pHs. The following 25 mM buffers were used: acetate buffer (pH 4.0–5.5), phosphate buffer (pH 6.0–7.5), and borate buffer (pH 7.5–10.0). As shown in Fig. 4, when HPG was used for a substrate, the optimum pHs of these enzymes were about 5.5, 7.5, and 8.5–9.0, respectively. In each enzyme the optimum pH for the decarboxylation of HPG was slightly higher than that of HMA. In the case of laccase and bilirubin oxidase from \textit{T. tsunoda}, the optimum pHs were on the acidic side, and the reaction rate of HMA was much faster than that of HPG. On the other hand, in the case of bilirubin oxidase from \textit{M. verrucaria}, the optimum pHs were on the alkaline side, and the difference in the reaction rate between HMA and HPG was much smaller than those in the former two enzymes.

**Measurement of \( K_m \) values of laccase and bilirubin oxidase**

The enzyme activities were measured with various concentrations of HMA and HPG each at the optimum pH. Incubation was at 30°C. \( K_m \) values were calculated based on Lineweaver–Burk plots (1/y – 1/s plots) as shown in Table III.

**Discussion**

From the screening for single reactions by laccase, we...
found two single reactions in which the substrates were HMA and HPG and the product only HBA, a compound inert to laccase. Neither mandelic acid nor 2-phenylglycine were converted by laccase (data not shown). From the results obtained thus far, we propose the reaction pathways for the decarboxylation of HMA and HPG by laccase as shown in Fig. 5. Each reaction is considered to be caused by laccase-catalyzed abstractions of hydrogen from phenolic hydroxyl groups, and an unpaired electron produced and located at para position cleaves the $\alpha$ bond of carboxyl group to produce carbon dioxide. The decarboxylation of HPG is presumed to involve the formation of an imine as an intermediate. Therefore, the differences in the reaction rate between HMA and HPG being large under acidic conditions (Figs. 4A and 4B) and small under alkaline conditions (Fig. 4C) can be explained because an imine is produced more easily under alkaline conditions than under acidic conditions. The reason for the inactivation of HBA against laccase seems to be that its reactant is stabilized more than the transition state by delocalization of a produced unpaired electron between an $\alpha$ carbon and a carbonyl oxygen.

For the assay of laccase activity, some chromogenic reagents have been developed. The method is based on the measurement of an increase in absorbance due to the formation of colored products resulting from the reaction of laccase with these reagents. However, there are some problems. For example, $N,N$-dimethyl-1,4-phenylenediamine, that has been used routinely, is highly toxic, irritating, and autoxidizable. Syringaldazine, which has been adopted as a sensitive and reliable substrate for the estimation of laccase activity, is less soluble and chemically unstable in water. The produced syringaldazine quinone is also unstable. The unit of laccase activity obtained from these assays cannot be defined as the formation rate of a product, because their reactions are not based on a single reaction. Our method for the measurement of laccase activity will satisfy the following criteria: (a) The substrate, HMA, is easily soluble in water and stable. (b)
The product, HBA, is stable without being further oxidized and is estimated accurately by the HPLC method and simply by the colorimetric method using 2,4-dinitrophenylhydrazine. (c) This reaction is suitable for measuring reaction rates, because one product is produced and there are no other reactions to occur. (d) It is possible to measure lower enzyme levels by increasing the reaction time. (e) This method isn't interfered with by quinone reducing enzymes, because this method is not dependent on a quinone color. So this method is more reliable when crude samples such as crude mycelial extracts or culture solutions of fungi, where quinone-reducing enzymes are present, are used. 21)

However, this method is interfered with by peroxidase with hydrogen peroxide similarly to the methods already developed.

At present, two kinds of bilirubin oxidase have been isolated and characterized. One of them is obtained from M. verrucaria and has two atoms of type 1 copper per mole. 22) The other is obtained from T. tsunoda and is a multi-copper enzyme like the laccase from C. versicolor. 23)

The laccase activity at pH 8.0 with HMA as a substrate was 0.5% of that at the optimum pH, while the activity of bilirubin oxidase from T. tsunoda at pH 8.0 was 2.2% of that at the optimum pH. The difference in the activity between the two enzymes at pH 8.0 is presumed to contribute to the difference of the reactivity to bilirubin, because bilirubin dissolves only on the alkaline side and the activity for it is usually measured at pH 8.0. Bilirubin oxidase from M. verrucaria was a specific enzyme whose optimum pH was about 8.5. We expect that the enzyme will be useful for enzymatic conversions of phenolic compounds under alkaline conditions.

References

1) D. M. Dooley, J. Rawlings, J. H. Dawson, P. J. Stephens, L-E.