Simultaneous Determination of Manganese Peroxidase and Lignin Peroxidase by Capillary Electrophoresis Enzyme Assays

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ABSTRACT: Here, we developed an enzyme assay of manganese peroxidase (MnP) by capillary electrophoresis using an in-capillary reaction and applied it to a simultaneous assay of MnP and lignin peroxidase (LiP). The enzyme activity of MnP was determined from the peak area corresponding to Mn(III)—malonate produced by the plug—plug reaction between MnP and Mn(II) in a separation capillary. A background electrolyte containing 250 mM malonate buffer (pH 4.5) and 5 mM cetyltrimethylammonium bromide was employed for the separation of Mn(III)—malonate from MnP at −10 kV after a plug—plug reaction for 5 min. Although the assay permitted the determination of purified MnP, we found that both LiP and MnP have similar activities against their substrates, that is, LiP catalyzed the oxidation reaction of Mn(II) as well as MnP, whereas MnP catalyzed the oxidation reaction of veratryl alcohol which was the substrate used in the LiP assay developed previously. Thus, we proposed a method to discriminate MnP from LiP based on the difference in the activities of these enzymes to each substrate. Amounts of MnP and LiP in a mixture were successfully evaluated by the proposed method.

INTRODUCTION

Lignin-degrading basidiomycete, Phanerochaete chrysosporium (P. chrysosporium), produces enzymes that catalyze the oxidative depolymerization of lignin.1,2 The enzymes include lignin peroxidase (LiP), manganese peroxidase (MnP), and their isozymes which help oxidation of lignin directly and/or indirectly through Mn(III)-mediated oxidation.

These enzymes are important for degrading aromatic hydrocarbons not only in nature but also in industry because they facilitate the decomposition of lignin and its analogues under mild conditions. In fact, they are expected to be useful in the pulp and paper industry as a possible alternative to the conventional chlorine bleaching of chemical pulps.3 The energy industry uses these enzymes to achieve an optimal biological conversion of lignocellulosic biomass to biofuels,4 and the mining industry uses them to improve the recovery of gold by reducing the adsorption of gold cyanide on the carbonaceous matter found in gold ores via cleavage of the C—C bond in the carbonaceous matter.5,6

When using the enzymes produced by P. chrysosporium, the quality and activity of the enzymes must be measured, so a sensitive and accurate enzyme assay is necessary. We previously reported a method for the determination of LiP using micellar electrokinetic chromatography coupled with an in-capillary enzymatic reaction. This method was more sensitive than a conventional spectrophotometric assay of LiP and permitted the determination of LiP in a culture medium of P. chrysosporium.

In a conventional assay of MnP, a spectrophotometric method is employed to monitor the enzymatic reaction where Mn(II) is oxidized to Mn(III) in a medium containing an organic ligand such as lactate or malonate that forms the complex of Mn(III) with an absorption band in a UV region.7 The activity of MnP is determined via an increase in the absorbance of the Mn(III) complex. However, the spectrophotometric method is only applicable to the assay of MnP that has been isolated and preconcentrated by gel permeation chromatography because several interferences in the culture medium of P. chrysosporium and MnP itself exhibit absorption in the UV region. Therefore, it is difficult to measure the Mn(III) complex produced by MnP without purification.

In the present study, we developed an enzyme assay of MnP using an in-capillary enzymatic reaction followed by separation via capillary electrophoresis (CE). Solutions of MnP dissolved in water and MnSO₄ containing H₂O₂ and malonate buffer were injected consecutively into the capillary in which the enzymatic reaction was conducted for a constant amount of time, which resulted in a Mn(III)—malonate complex. The complex was separated from MnP and other components in the...
sample by applying an electric potential in a background electrolyte (BGE) containing cetyltrimethylammonium bromide (CTAB) that accelerated the migration of the anionic Mn(III)−malonate complex by reversing the electroosmotic flow. The enzyme assay permitted the determination of activity for standard MnP solutions.

Conversely, we found that LiP had enzymatic activity similar to that of MnP; that is, LiP catalyzed the oxidation of Mn(II), whereas MnP catalyzed the oxidation of veratryl alcohol, which is a substrate of LiP. Therefore, the activity of MnP would be overestimated in the presence of LiP and vice versa. Thus, we proposed a method to discriminate MnP from LiP in the mixture of enzymes by using two enzymatic assays for MnP and LiP. We demonstrated how the method can be used to successfully measure the amounts of both MnP and LiP in a mixture without purification of the enzymes.

RESULTS AND DISCUSSION

Determination of Mn(III)−Malonate. In a conventional enzyme assay, Mn(III) is complexed with organic ligands because organic ligands form stable Mn(III) complexes with UV absorption. In this study, malonate ion was selected because it has an absorption maximum that is larger than that of other ligands such as oxalate, lactate, tartrate, and malate. However, when using a malonate buffer with pH 4.5, which is the optimum for an enzymatic reaction, the electroosmotic flow is too slow to detect anionic species under conventional CE conditions where the BGE flows from anode to cathode by electroosmosis. In fact, our preliminary results indicated that Mn(III)−malonate was anionic and its migration time was too long to be detected at pH 4.5. Therefore, we added a cationic surfactant, CTAB, to reverse the direction of the electroosmotic flow and applied a negative potential at the inlet of the capillary. No significant difference in the migration time of Mn(III)−malonate was observed in a concentration of CTAB that ranged from 0.5 to 10 mM, so 5 mM CTAB was employed in the present study. A sharp peak of Mn(III)−malonate was observed when using a BGE that consisted of 250 mM malonate buffer and 5 mM CTAB, as shown in Figure 1. However, the peak area gradually decreased with an increase in the time following the preparation of the sample solution (Figure 1a−c), probably because of the disproportional reaction of Mn(III).

Therefore, we evaluated the disproportional reaction of Mn(III) by measuring the time dependence of the decreased peak area. Standard solutions of Mn(III) with different concentrations were prepared with a 250 mM malonate buffer at pH 4.5. After completely dissolving Mn(III) acetate in the 250 mM malonate buffer, the solution was injected into the capillary in a time interval of 6 min. The first injection was carried out at 15 min because it takes more than 10 min to dissolve Mn(III) acetate in the 250 mM malonate buffer. The disproportional reaction of Mn(III) was approximated to the first-order reaction by plotting the logarithm of the peak area to time. At concentrations of 0.187, 0.373, 0.533, and 0.761 mM Mn(III)−malonate, the logarithm of the decreased peak areas was plotted against time, resulting in good linear relationships. Therefore, the intercepts of the lines should indicate the peak areas at t = 0.

The relationship between the concentrations of Mn(III)−malonate and the peak areas obtained from the intercepts showed good linearity with a correlation coefficient of 0.9923. We employed the calibration curve for determining the amount of Mn(III) produced by the enzymatic reaction of MnP to correct the influence of the disproportional reaction of Mn(III) during the preparation of the standard solutions. The limits of detection and quantification for Mn(III)−malonate were estimated to be 11.9 µM at S/N = 3 and 40.9 µM at S/N = 10, respectively.

Enzyme Assay of MnP. In the enzyme assay of MnP, a standard solution of MnP was sandwiched by a substrate solution containing MnSO₄ and H₂O₂. The injection times were optimized using a program for the simulation of transverse diffusion of laminar flow profiles that was developed by Krylov. We selected injection times for the front substrate plug, the enzyme plug, and the back substrate plug of 5, 5, and 8 s, respectively.

When a standard solution of MnP was reacted with Mn(II), the electrophoretic run was begun immediately. The electropherogram obtained by the reaction between MnP and Mn(II) is shown in Figure 2, where a peak of Mn(III)−malonate produced by the in-capillary enzymatic reaction can be found clearly. In Figure 2, the peak of MnP (peak 2) is broadened because proteins adsorb on the surface of the capillary as shown in an early study on CE separations. The migration time corresponded to the Mn(III)−malonate complex prepared by

![Figure 1. Electropherograms of the Mn(III)−malonate complex. (a) 62, (b) 144, and (c) 226 min after the preparation of the sample solution. BGE, 250 mM malonate buffer with 5 mM CTAB (pH 4.5); sample, 187 µM Mn(CH₃COO)₃ dissolved in 250 mM malonate buffer (pH 4.5); and voltage, −10 kV.](image1)

![Figure 2. Electropherogram obtained by the in-capillary enzymatic reaction between MnP and Mn(II). 1 = Mn(III)−malonate complex; 2 = MnP; injection times: 5 s for the first substrate solution, 5 s for 1 mg mL⁻¹ MnP, and 8 s for the second substrate solution; and reaction time: 20 min. Other conditions are the same as in Figure 1.](image2)
dissolving Mn(III) acetate in a 250 mM malonate buffer, and the peak was increased by increasing the reaction time from 5 to 50 min. These facts indicate that the peak detected at around 2 min was produced by the enzymatic reaction.

**Simultaneous Determination of MnP and LiP.** As shown in Figure 2, the Mn(III)—malonate complex produced by the enzymatic reaction can be detected using an in-capillary reaction. However, it is known that MnP and LiP have poor specificity to substrates, that is, these enzymes can catalyze the oxidation reactions of several compounds.\(^{15–17}\) Therefore, if LiP reacts with Mn(II) to produce Mn(III) or MnP catalyzes the oxidation reaction of veratryl alcohol, which is the substrate employed in the LiP assay, it would be impossible to determine the individual enzymes by the proposed method.

Thus, we evaluated the activities of LiP to Mn(II) and MnP to veratryl alcohol. Figure 3 shows the electropherograms for MnP and LiP using veratryl alcohol and Mn(II) as the substrates. (a) substrate, veratryl alcohol; enzyme, 5 mg mL\(^{-1}\) MnP; BGE, 250 mM malonate buffer with 5 mM CTAB (pH 4.5); and reaction time, 5 min. (b) Substrate, Mn(II); enzyme, 5 mg mL\(^{-1}\) LiP; BGE, 50 mM tartrate with 50 mM SDS (pH 2.5); and reaction time, 5 min.

Figure 3. Electropherograms obtained by the enzyme assays using Mn(II) and veratryl alcohol as the substrates. (a) substrate, veratryl alcohol; enzyme, 5 mg mL\(^{-1}\) MnP; BGE, 250 mM malonate buffer with 5 mM CTAB (pH 4.5); and reaction time, 5 min. (b) Substrate, Mn(II); enzyme, 5 mg mL\(^{-1}\) LiP; BGE, 50 mM tartrate with 50 mM SDS (pH 2.5); and reaction time, 5 min.

MnP and LiP, and Mn(II) and veratryl alcohol, are catalyzed by MnP and LiP, respectively. The results indicate that both MnP and LiP catalyze the oxidation reactions of veratryl alcohol and Mn(II). Therefore, when a sample contains both MnP and LiP, MnP cannot be discriminated from LiP by the MnP assay and vice versa.

The kinetic parameters of the enzymes, including \(K_m\) and \(V_{max}\), were calculated using two substrates, veratryl alcohol and Mn(II), according to the Lineweaver–Burk plot, as shown in Table 1. The \(K_m\) and \(V_{max}\) values for MnP (substrate, Mn(II)) and for LiP (substrate, veratryl alcohol) are in good agreement with the reported values,\(^{17}\) whereas the activities (\(V_{max}\)) are significantly small. The small \(V_{max}\) values might be attributed to the lack of purity of the standard enzymes or to the degradation of the enzymes during the storage period.

To determine the individual enzymes in a mixture, we proposed a method for the simultaneous determination of MnP and LiP using two enzyme assays, that is, the CE enzyme assays for MnP and LiP. Here, we describe how 1 mg of MnP and LiP produces \(X_{MnP}\) mol and \(X_{LiP}\) mol of Mn(III) and \(Y_{MnP}\) mol and \(Y_{LiP}\) mol of veratraldehyde. When a mixture contains \(A\) mg of MnP and \(B\) mg of LiP, the amounts of the products, Mn(III) and veratraldehyde, are given by

\[
N_{Mn(III)} = AX_{MnP} + BX_{LiP}
\]

\[
N_{VAld} = AX_{MnP} + BX_{LiP}
\]

where \(N_{Mn(III)}\) and \(N_{VAld}\) are the moles of Mn(III) and veratraldehyde produced by the enzymatic reactions. Therefore, if we know \(X_{MnP}\), \(X_{LiP}\), \(Y_{MnP}\) and \(Y_{LiP}\), and \(A\) and \(B\) can be calculated by solving eqs 1 and 2, that is, MnP and LiP are determined simultaneously.

The relationship between the amounts of the enzymes and the products produced by the enzymatic reactions is shown in Figure 4. Surprisingly, LiP exhibited higher activity in the oxidation of Mn(II) than MnP and vice versa in the oxidation of veratryl alcohol. The slopes in Figure 4 represent \(X_{MnP}\), \(X_{LiP}\), \(Y_{MnP}\) and \(Y_{LiP}\) with the values of 0.0065, 0.040, 0.036, and 0.011 pmol ng\(^{-1}\), respectively (the reaction times were consistent at 5 min).

When a mixture of 5 mg mL\(^{-1}\) MnP and 5 mg mL\(^{-1}\) LiP was reacted with Mn(II) and veratryl alcohol, the amounts of Mn(III) and veratraldehyde were 2.74 and 1.75 pmol, respectively. According to eqs 1 and 2, the concentrations of the enzymes using the experimental values were calculated at 3.8 mg mL\(^{-1}\) for MnP and 7.8 mg mL\(^{-1}\) for LiP. Therefore, the proposed method could be employed to estimate the amounts of MnP and LiP in a mixture sample without purification of each enzyme, although each concentration would deviate slightly from the expected value (5 mg mL\(^{-1}\)).

**CONCLUSIONS**

Here, we developed a CE enzyme assay of MnP using Mn(II) as a substrate. The method is applicable to the determination of MnP when the sample does not contain LiP. However, we found that LiP had an activity that was similar to that of MnP, resulting in Mn(III) by catalyzing the oxidation reaction of Mn(II), that is, the developed method cannot be employed for a sample containing both MnP and LiP. In addition, MnP also oxidized veratryl alcohol to veratraldehyde which is the product of the enzyme assay for LiP. Surprisingly, MnP showed higher activity in the oxidation of veratryl alcohol compared with LiP, but LiP was more efficient in the oxidation of Mn(II) than MnP. To discriminate MnP from LiP in the mixture, the enzyme assays for MnP and LiP can be combined because their

| enzyme | substrate | \(K_m/mM\) | \(V_{max}/pmol\, s^{-1}\) | \(k_{cat}/s^{-1}\) |
|--------|-----------|------------|-----------------|--------------|
| MnP    | Mn(II)    | 0.22 (0.03) | 5.0 (25.50 U mg\(^{-1}\)) | 2.9 (7.97) |
| LiP    | Mn(II)    | 0.63 | 15 | 7.3 |
| MnP    | veratryl alcohol | 0.22 | 3.4 | 1.9 |
| LiP    | veratryl alcohol | 2.0 (0.10) | 43 (15.20 U mg\(^{-1}\)) | 1.9 (8.44) |

*The values in parentheses are reported in ref 17. Molecular weights of MnP and LiP were assumed to be 45 and 36 kDa, respectively.*\(^{17}\)
activities are different in the oxidation reactions of Mn(II) and veratryl alcohol. Two enzyme assays successfully permitted the simultaneous determination of MnP and LiP in a mixture sample. Therefore, the present enzyme assays are a promising method for the determination of enzymes in practical samples containing both MnP and LiP without separation and purification. Finally, it is noteworthy that this simultaneous determination method is, in principle, applicable to a sample containing more than two enzymes with different substrate selectivities, but the number of different substrates must equal the number of enzymes.

**EXPERIMENTAL SECTION**

**Materials.** All chemicals were of analytical grade and were used without further purification. Deionized water was prepared by means of an Elix water purification system (Millipore Co. Ltd., Molsheim, France). Standards of MnP and LiP were purchased from Santa Cruz Biotechnology (Dallas, TX). Hydrogen peroxide was purchased from Kanto Chemical (Tokyo, Japan). Malonic acid was obtained from Nacalai Tesque, Inc. (Kyoto, Japan). Ethanol was obtained from Sigma-Aldrich Japan (Tokyo, Japan). Manganese(II) sulfate pentahydrate was purchased from Kishida Chemical Co. (Osaka, Japan). Veratryl alcohol was obtained from Tokyo Kasei Kogyo (Tokyo, Japan). The other reagents were purchased from Wako Pure Chemicals (Osaka, Japan).

For the assay of MnP, a malonate buffer solution (250 mM, pH 4.5) was prepared by dissolving an appropriate amount of malonic acid and adjusting the pH using 1 M sodium hydroxide solution. A malonate buffer solution that included 5 mM CTAB was employed as a BGE. The BGE was prepared by dissolving 0.25 mmol CTAB in 50 mL of the buffer solution. The change in pH after the addition of CTAB was less than ±0.1 pH. The standards of MnP and LiP were dissolved with water. Standard solutions of Mn(III)—malonate were prepared by dissolving appropriate amounts of Mn(III) acetate in a 250 mM malonate buffer. The standard solutions were prepared freshly in all experiments. A mixture containing 2 mM Mn(II) sulfate and 0.2 mM H2O2 was prepared with the malonate buffer without CTAB (pH 4.5) for use as the substrate solution in the in-capillary reaction.

The solutions used for the assay of LiP were similar to those reported previously. Briefly, a tartrate buffer solution that included 50 mM sodium dodecyl sulfate (SDS) was employed as a BGE, and a mixture containing 2 mM veratryl alcohol and 0.4 mM H2O2 was prepared with a tartrate buffer without SDS (pH 2.5) for use as the substrate solution in the in-capillary reaction.

**Apparatus.** A CE system, model 3DCE (Agilent Technologies, CA, USA), was used throughout the study. Fused-silica capillaries with an i.d. of 50 μm and an o.d. of 375 μm (total length, 33.5 cm; effective length, 25 cm) were obtained from GL Sciences (Tokyo, Japan). The capillaries were flushed with 0.1 M NaOH and water successively for 5 min before each run, followed by filling with the BGE.

In the MnP assay, absorbance at 270 nm represented the absorption maxima of Mn(III)—malonate and was recorded to obtain the electropherograms. To prevent deactivation of MnP, the temperature in the autosampler of the CE system was maintained at 7 °C (the controllable lowest temperature), whereas the temperature of the capillary was controlled at 25 °C for the enzyme reaction, that is, the Mn(III)—malonate complexes are formed at 25 °C. The solutions of the enzyme and the substrate were injected in the order of the substrate for 5 s, the enzyme for 5 s, and the substrate for 5 or 8 s at 3.5 kPa. The injection volume of the enzyme corresponded to 7.62 nL, which was calculated using the Poiseuille equation, and the viscosity of the BGE was measured at 25 °C by an Ostwald viscometer (ηv = 1.052). The amounts and concentrations of the product and enzyme were calculated using an injection volume of 7.62 nL. Electropherograms were recorded via Hewlett-Packard ChemStation software, which permitted an automatic measurement of peak height, peak area, and migration time. The LiP assay was conducted according to the procedure in our previous paper.

**Enzyme Assay for MnP.** The method consisted of a plug—plug reaction between MnP and its substrate, Mn(II), the separation of the product, Mn(III)—malonate, from the other components including the enzyme and the culture medium, and the determination of the enzyme activity from the peak area of Mn(III)—malonate produced by the plug—plug reaction. Initially, the capillary was filled with the BGE containing 250 mM malonate buffer and 5 mM CTAB (pH 4.5 ± 0.1). The plug—plug reaction was accomplished by introducing plugs in the following order: substrate—enzyme—substrate. The substrate plug consisted of 2 mM MnSO4, 0.2 mM H2O2, and 250 mM malonate buffer (pH 4.5) without CTAB. The substrate, Mn(II), was reacted with MnP for 5–50 min by diffusion-based mixing in the capillary for its conversion to Mn(III)—malonate. The enzyme activity was directly calculated from the peak area of Mn(III)—malonate produced by the in-capillary reaction.
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Notes
The authors declare no competing financial interest.

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