Direct Determination of Lignin Peroxidase Released from *Phanerochaete chrysosporium* by In-Capillary Enzyme Assay Using Micellar Electrokinetic Chromatography

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Highlights

- A sensitive in-capillary enzyme assay for lignin peroxidase was developed.
- The analysis time is less than 15 min including the incubation time.
- The method achieves low consumption of the reagents and samples.
- A crude culture medium sample can be measured without any preconcentration.
- The limit of detection is much lower than a conventional spectrophotometry.

ABSTRACT

Here we describe the application of an in-capillary enzyme assay using micellar electrokinetic chromatography (MEKC) in the determination of enzyme activity in a crude culture medium containing lignin peroxidase released from *Phanerochaete chrysosporium* (*P. chrysosporium*). The method consists of a plug-plug reaction between lignin peroxidase and its substrate, veratryl alcohol, the separation of the product, veratraldehyde, from the other components including the enzyme and the culture medium, and the determination of the enzyme activity from the peak area of veratraldehyde produced by the plug-plug reaction. This method is more sensitive than conventional spectrophotometry since the background originates from the enzyme and the culture medium can be removed via MEKC separation. Veratraldehyde was separated at -10 kV in a background electrolyte containing 50 mM tartrate buffer (pH 2.5) and 50 mM sodium dodecyl sulfate (SDS) after a plug-plug reaction in the capillary for 5 min. The calibration curve of veratraldehyde was linear up to 4 pmol (500 μM) with a limit to quantification of 0.026 pmol (3.2 μM) (SN=10). The activity of lignin peroxidase was directly measured from the peak area of veratraldehyde. The activity of lignin peroxidase released from *P.*

chrysosporium into the medium for 7 days was successfully determined to be 3.40 U L^{-1}.

Keywords:
Lignin Peroxidase
Phanerochaete chrysosporium
Micellar electrokinetic chromatography
Capillary electrophoresis
Enzyme assay

1. Introduction

Lignin peroxidase is an enzyme that catalyzes the oxidative depolymerization of lignin [1,2]. A well-known source of lignin peroxidase is lignin-degrading basidiomycete, *Phanerochaete chrysosporium* (*P. chrysosporium*), which produces a group of isozymes that catalyzes the cleavage of the C-C bond in lignin. Therefore, *P. chrysosporium* helps to decompose the lignin contained in wood, resulting in a naturally efficient carbon cycle.

Recent research results have led to speculation that lignin peroxidase released from *P. chrysosporium* could be utilized in industry since it facilitates the decomposition of lignin and its analogues under mild conditions. In the pulp and paper industry, lignin peroxidase is a possible alternative to the conventional chlorine bleaching of chemical pulps since enzymatic digestion is mild and environmentally friendly [3]. The degradation of lignin is also important for the achievement of an optimal biological conversion of lignocellulosic biomass to biofuels [4]. In addition, lignin peroxidase has

the potential to reduce the adsorption of gold cyanide on the carbonaceous matter found in gold ores via cleavage of the C-C bond in the carbonaceous matter, which leads to improvement in the recovery of gold [5].

In the industrial application of lignin peroxidase produced by *P. chrysosporium*, a sensitive and accurate enzyme assay is essential when assessing the quality and activity of the enzyme. Spectrophotometry is employed in a conventional enzyme assay of lignin peroxidase where the substrate, veratryl alcohol, is converted to veratraldehyde in the presence of hydrogen peroxide [6]. However, a sample with a low concentration of lignin peroxidase cannot be measured by conventional spectrophotometry due to the interference from the culture medium of *P. chrysosporium*.

To determine the trace levels of enzymes, electrophoretically mediated microanalysis (EMMA) was developed as a sensitive enzyme assay based on capillary electrophoresis (CE) by Regnier et al. in early investigations using CE [7]. In EMMA, an enzyme and its substrate were reacted in a capillary either under static conditions or during an electrophoretic run. There were several modes for the assay [8,9]: continuous modes that included zonal sample introduction and moving boundary sample introduction; plug–plug modes with the consecutive introduction of the plugs of an enzyme and a substrate; and, a partial-filling technique.

Many applications of CE-based enzyme assays have been reported [10-13]. The

in-capillary enzyme assays are easy to use and are suitable for routine analyses since a commercially available CE system permits automated operations, which significantly enhances throughput.

In the present study, we developed a sensitive enzyme assay of lignin peroxidase produced by *P. chrysosporium* using an in-capillary enzymatic reaction followed by separation via micellar electrokinetic chromatography (MEKC). Veratryl alcohol and lignin peroxidase were reacted for a constant period of time in a capillary via consecutive injections of the enzyme and the substrate plugs into the capillary. After the in-capillary reaction, veratraldehyde was separated from veratryl alcohol, lignin peroxidase, and other components in the sample by applying an electric potential in a buffer solution containing sodium dodecyl sulfate (SDS). The enzyme assay showed sufficient sensitivity to permit the determination of activity for lignin peroxidase released from *P. chrysosporium* into the culture medium, which was difficult to determine using a conventional spectrophotometric method since the absorption spectra of both the culture medium and the lignin peroxidase overlapped that of veratraldehyde.

2. Materials and methods

2.1. 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). Lignin peroxidase was purchased from Santa Cruz Biotechnology (Dallas, TX). Hydrogen peroxide was purchased from Kanto Chemical (Tokyo, Japan). Veratryl alcohol was obtained from Tokyo Kasei Kogyo (Tokyo, Japan). The other reagents were purchased from Wako Pure Chemicals (Osaka, Japan).

A tartrate buffer solution (50 mM, pH 2.5) was prepared by dissolving an appropriate amount of tartaric acid and adjusting the pH using a 1 M sodium hydroxide solution. A tartrate buffer solution that included 50 mM SDS was employed as a background electrolyte. The background electrolyte was prepared by disolving 2.5 mmol of SDS in 50 mL of the buffer solution. The change in pH value after the addition of SDS was less than ± 0.1 pH. The standard solutions of lignin peroxidase, veratryl alcohol, and veratraldehyde were prepared by being dissolved with water. A mixture containing 2 mM veratryl alcohol and 0.4 mM H_2O_2 was prepared with a tartrate buffer without SDS (pH 2.5) for use as the substrate solution in the in-capillary reaction.

2.2. Apparatus

A capillary electrophoresis system, model 3^DCE (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, 34.6 cm; effective length, 26.1 cm) were obtained from GL

Sciences (Tokyo, Japan). The capillaries were flushed with 0.1 M NaOH, 0.1 M HCl, and water successively for 5 min before each run, followed by filling with the running buffer. Absorbances at 228 and 308 nm represented the absorption maxima of the substrate and the product, respectively, and were recorded in order to obtain the chromatograms. To prevent deactivation of the lignin peroxidase, the temperature in the autosampler of the CE system was maintained at 7 °C (the controllable lowest temperature) while the temperature of the capillary was controlled at 30 °C for the enzyme reaction. The solutions of the enzyme and substrate were injected for 5 s at 3.5 kPa with an injection volume that corresponded to 8.2 nL, which was calculated using the Poiseuille equation and the viscosity of the background electrolyte measured at 30 °C by Ostwald viscometer (η_r = 1.186). The amounts and concentrations of the product and enzyme were calculated using an injection volume of 8.2 nL.Chromatograms were recorded via Hewlett-Packard ChemStation software, which permitted an automatic measurement of peak height, peak area, and migration time.

2.3. Enzyme assay

The method consisted of a plug-plug reaction between lignin peroxidase and its substrate, varatryl alcohol, the separation of the product, veratraldehyde, from the other components including the enzyme and the culture medium, and the determination of the enzyme activity from the peak area of veratraldehyde produced by the plug-plug reaction.

Initially, the capillary was filled with the background electrolyte containing 50 mM tartrate buffer (pH 2.5) and 50 mM SDS. The plug-plug reaction was accomplished by introducing plugs in the following order: water—substrate—enzyme—substrate—water. The substrate plug consisted of 2 mM veratryl alcohol and 0.4 mM H_2O_2, and 50 mM tartrate buffer (pH 2.5) without SDS. Veratryl alcohol was reacted with lignin peroxidase for 5 min by diffusion-based mixing in the capillary for its conversion to veratraldehyde. The background electrolyte containing 50 mM tartarate buffer (pH 2.5) and 50 mM sodium dodecyl sulfate (SDS) permitted the separation of varatryl alcohol from veratraldehyde whereas the same solution without SDS was employed for the reaction buffer to prevent the deactivation of lignin peroxidase. The enzyme activity was directly calculated from the peak area of veratraldehyde produced by the in-capillary reaction.

2.4. Preparation of the crude enzyme samples

Crude enzyme released from *P. chrysosporium* into the culture medium (cell-free extract, CFE) was prepared as follows. A trace element solution (the solution is referred to in ref. 6) and a stock culture solution were prepared according to a process reported in the literature [6]. The trace element solution contained 3 g of $MgSO_4$, 0.5 g of $MnSO_4$, 1 g of NaCl, 0.1 g of $FeSO_4$, 0.1 g of $CuSO_4$, and 10 mg of boric acid in 1 L of water. A 500 ml portion of the stock culture solution (pH 4.0 ±0.1) was prepared by adding 5 g of

glucose, 0.59 g of succinic acid, 0.1 g of ammonium tartrate, 1 g of KH_2PO_4, 0.25 g of $MgSO_4$, 0.05 g of $CaCl_2$, 0.5 mg of thiamine hydrochloride, and 35 ml of the trace element solution to water. A white rot fungus, *P. chrysosporium*, was cultured in the culture medium at 37 °C for 7 days under stationary conditions [6]. The CFE was harvested after the culturing period by vacuum filtration using a 0.22 μm sterilized filter cap, and was stored at 4 °C.

3. Results and discussion

3.1. Absorption spectra

In the conventional spectrophotometric method for lignin peroxidase, veratryl alcohol is generally used as a substrate that can be converted to the product, veratraldehyde, in the presence of H_2O_2. The absorbance of veratraldehyde at 310 nm was monitored after incubating the mixture of veratryl alcohol, H_2O_2, and a sample solution containing lignin peroxidase [6]. Therefore, the sample solution was expected to exert no absorbance at the detection wavelength of veratraldehyde, which allowed an accurate and sensitive enzyme assay.

Veratraldehyde showed its absorption maxima at 228, 277 and 308 nm, whereas the absorption maxima of veratryl alcohol were located at 228 and 277 nm. Since no absorption of veratryl alcohol was observed at 308 nm, the veratraldehyde could spectrally

be separated from veratryl alcohol via detection at 308 nm. However, the culture medium showed broad absorption bands that were gradually decreased in the ultraviolet region. Therefore, the culture medium had to be removed from the sample solutions since its absorption band overlapped the maximum absorption band of veratraldehyde, resulting in a high background signal.

3.2. Measurement of enzyme activity

According to the absorption spectra, veratraldehyde should be separated from the culture medium. In the present study, a tartrate buffer solution (pH 2.5) was employed to separate them, since it is generally employed in the enzyme assay of lignin peroxidase via spectrophotometry [6]. Due to the lack of an electric charge of veratryl alcohol and veratraldehyde at pH 2.5, SDS was added to the separation buffer. A background electrolyte with pH 2.5 helped prevent the degradation of the enzyme, which occurs above pH 3 [6], and suppressed the generation of the electroosmotic flow that deaccelerates the migration of SDS micelles, and results in a longer analysis time. The chromatogram of a mixture containing veratryl alcohol, veratraldehyde, lignin peroxidase, and the culture medium of *P. chrysosporium* is shown in Fig. 1a and 1b. When using 50 mM tartrate buffer with 50 mM SDS, veratraldehyde was separated completely with no overlapping of the peaks (Fig. 1a). No peak of veratryl alcohol was detected at the detection wavelength

of 308 nm (Fig. 1b), as expected from the absorption spectrum. Conversely, the peaks of lignin peroxidase and the culture medium were detectable at both wavelengths of 228 and 308 nm and were completely separated from veratraldehyde. The chromatogram of the culture medium is shown in Fig. 1c, and indicates two negative peaks that can be attributed to the culture medium. Therefore, these separation conditions were employed for the in-capillary enzyme assay of lignin peroxidase.

The calibration curve for veratraldehyde was constructed in a concentration range of 10 μM (0.082 pmol) to 500 μM (4.11 pmol) at a detection wavelength of 308 nm (y = y = 0.1821x). The calibration curve exhibited good linearity with 0.9971 of the correlation coefficient, and the limits of detection (LOD) and of quantification (LOQ) were estimated to be 0.96 μM (0.0079 pmol) at S/N=3 and 3.2 μM (0.026 pmol) at S/N=10, respectively.

The concentrations of veratryl alcohol and H_2O_2 in the substrate solution were adjusted to 2 and 0.4 mM, according to the literature of the enzyme assay [6], in which the Michaelis constants (K_m) corresponded to the substrate concentrations wherein the reaction rate was half of the maximum reaction rate, V_{max}, and reported to be 60 μM for veratryl alcohol and 80 μM for H_2O_2. Thus, 2 mM of veratryl alcohol and 0.4 mM of H_2O_2 would be sufficient to obtain V_{max} in the enzymatic reaction. Consequently, we employed 50 mM tartrate buffer containing 2 mM veratryl alcohol and 0.4 mM H_2O_2 as the substrate solution.

To measure the enzyme activity of lignin peroxidase, the veratraldehyde that was produced was determined at various concentrations of lignin peroxidase. As described in the experimental section, the plugs of water, substrate, and enzyme were injected in the order of water—substrate—enzyme—substrate—water. When the plugs of water (or the buffer without SDS) were injected, the peak area of the product was increased 3-fold. Therefore, water was injected as the first and the last plugs although the buffer solution without SDS could be employed instead of water.

With the pressurized sample injections, the enzyme and the substrate plugs are expected to be mixed efficiently by transverse diffusion of the laminar flow profiles (TDLFP), as described by Krylov's group [14]. The conditions employed in the literature were 0.5 psi for 3 s, whereas we injected the plugs at 3.5 kPa (= 0.508 psi) for 5 s. In addition, they assumed that the length of every solution plug would be at least 30 times greater than the diameter of capillary (50 μm). According to our conditions, the plug length calculated from the injection volume was 4.2 mm, which was 84-fold greater than the diameter of the capillary. Therefore, TDLFP seemed to take place under the conditions employed in this study.

Under these conditions, we simulated the plug profiles using the program available at the website of the Krylov group [15], as shown in Fig. 2. When the injection time of the enzyme was fixed at 5 s, the injection times of substrate plugs and the water plugs were

varied, as shown in Fig. 2, which shows that all plugs were injected for 5 s, and two substrate plugs overlapped in the enzyme plug, although the maximum concentration of the enzyme in the plug was roughly half. Increases in the injection times of either the substrate plugs (Fig. 2b) or the water plugs (Fig. 2c) elongated the enzyme plug, resulting in the reduced concentration. Fig. 2a shows that the concentrations of the substrates were decreased to 14% at the center of the enzyme plug, i.e., the concentrations decreased to 280 μM for veratryl alcohol and 56 μM for H_2O_2. The concentration of veratryl alcohol remained higher than the K_m value (60 μM) while H_2O_2 seemed insufficient to obtain V_{max}. However, when the concentration of H_2O_2 was increased in the range of 0.4 to 2.8 mM, no change in the product peak was observed. Therefore, a constant injection time of 5 s (Fig. 2a) was employed in this study.

The relationship wherein the amount of the product was proportional to the amount of lignin peroxidase in a range of from 0 to 117 U $L^{-1}$is shown in Fig. 3 ($y = 0.0327x$, $R^2 = 0.9903$). In general, 1 U of the enzyme was the amount that would convert 1 μmol of the substrate to the product in 1 min. According to the relationship between the enzyme activity and the amounts of veratraldehyde, the LOD and LOQ for lignin peroxidase was estimated to be 0.0020 and 0.0065 μU, which correspond to 0.24 U L^{-1} and 0.79 U L^{-1}, respectively.

The conditions were optimized using a standard solution of lignin peroxidase dissolved

in water, whereas the practical samples contained a culture medium with pH 4.0. In the in-capillary reaction, an enzyme sample was sandwiched between the plugs of the substrate solution containing a buffer solution with pH 2.5, which was required to have sufficient buffering capacity. Therefore, the effect of pH was investigated using standard solutions of lignin peroxidase dissolved in water and in the culture medium. When comparing the electropherograms of these enzyme solutions, no difference in the peak area of veratraldehyde was found. This fact indicated that the sample solutions were sufficiently buffered at pH 2.5 by the buffer components in the substrate solution.

3.3. Determination of lignin peroxidase in CFE

The present enzyme assay was applied to the determination of lignin peroxidase in CFEs. Instead of a standard solution of lignin peroxidase, a CFE was directly injected to determine the activity of lignin peroxidase released from *P. chrysosporium*. The MEKC chromatogram is shown in Figure 4. The chromatogram clearly shows a peak for veratraldehyde with a S/N ratio of more than 10. The activity of lignin peroxidase was directly determined based on the amount of veratraldehyde that was obtained using the calibration curve of veratraldehyde standard solutions. The activity of the lignin peroxidase contained in the CFE was 3.40 ± 0.30 U L^{-1}.

The concentration was too low to determine the activity of lignin peroxidase in the

CFE via conventional spectrophotometry because of the large background signal that originated from the culture medium at 308 nm. For example, in the standard protocol of conventional spectrophotometry [6], the concentration of lignin peroxidase should be high enough to initiate an absorbance change in 0.2 min^{-1}, so that the veratraldehyde would be produced at a rate of 21.5 μM min^{-1}. Assuming that the volume of the reaction mixture is 5 mL, veratraldehyde is produced at a rate of 108 nmol min^{-1} when determined via conventional spectrophotometry. This means that the reaction mixture must contain 0.1 U of lignin peroxidase in 5 mL, which corresponds to 20 U L^{-1}. Obviously, the concentration of lignin peroxidase in the CFE (3.40 U L^{-1}) was too low to be determined via conventional spectrophotometry. This fact was also supported by our experiments; we attempted to measure increases in the absorbance of veratraldehyde at 308 nm via the reaction of the culture medium with veratryl alcohol under conditions similar to those in the literature [6], but no significant increase in absorbance was observed.

According to the results of the activity determined by the in-capillary enzyme assay, the sample employed here must be concentrated at least 5.5-fold to obtain an activity of 20 U L^{-1} when using the chromatographic preconcentration stipulated in the literature [16]. Therefore, the present method is useful when evaluating the enzyme activity of lignin peroxidase released from *P. chrysosporium* into a culture medium without the conditions of purification and preconcentration that are always required in conventional

spectrophotometry [6, 16].

4. Conclusions

An enzyme assay of lignin peroxidase was developed using an in-capillary enzymatic reaction followed by MEKC separation of the product from the substrate and the components in the sample. The sensitivity of the method was much higher than that of a conventional spectrophotometric method since the separation permitted quantification for an amount of the product that was as low as 0.026 pmol (3.2 μM)with no interference from the coexisting components in the sample. Lignin peroxidase in a CFE was directly determined to be 3.40 U L^{-1} without preconcentration. The developed method is superior to the conventional spectrophotometric method in terms of sensitivity, rapidity, and low consumption of the reagents. Therefore, this in-capillary enzyme assay shows promise for use in routine analyses to evaluate the enzyme activity of lignin peroxidase released from *P. chrysosporium*, which is expected to be a useful source of lignin peroxidase in industrial applications.

Acknowledgements

This research was supported by Grants-in-Aid for Scientific Research, Scientific Research (B) (No. 26288067) from the Japan Society for the Promotion of Science (JSPS).

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Figure legends

Figure 1. Chromatograms of the culture medium of *P. chrysosporium* with and without veratryl alcohol, veratraldehyde, and lignin peroxidase. (a) and (b), the culture medium with veratryl alcohol, veratraldehyde, and lignin peroxidase; (c), the culture medium. 1, the culture medium of *P. chrysosporium*; 2, Lignine peroxidase (5 mg mL^{-1}); 3, veratraldehyde (100 μM): 4, veratryl alcohol (300 μM). Running buffer; 50 mM tartrate buffer (pH 2.5) containing 50 mM SDS. Other experimental conditions are provided in the main text.

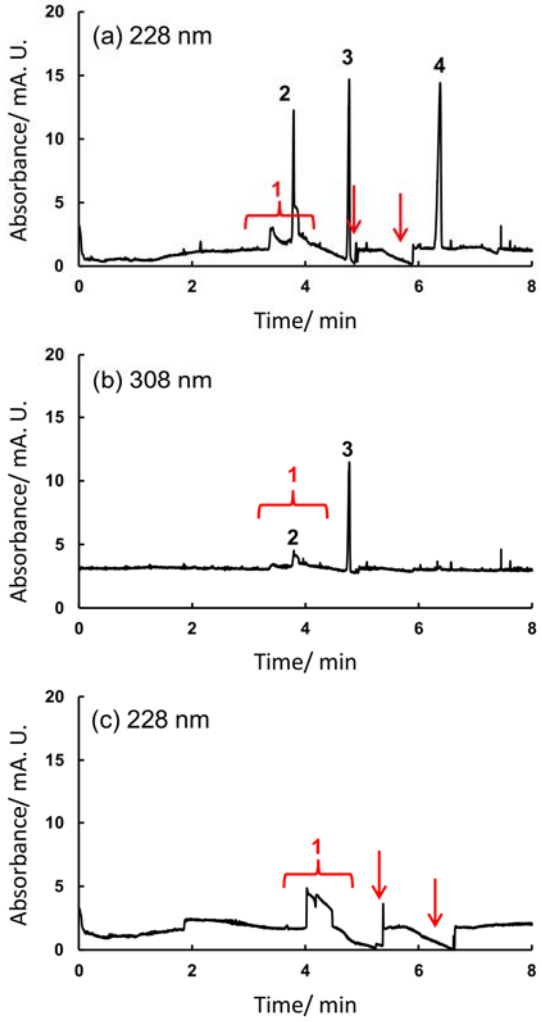

Figure 1

Figure 2. Simulation of the profile for the injection plugs in the capillary. Red, enzyme; blue, substrate; green, water. Conditions: injection pressure, 3.5 kPa (0.5075 psi); viscosity, 0.945 mPa s. Diffusion coefficients of the enzyme and substrate were assumed to be 5.0×10^{-7} and 5.0×10^{-6} cm^2 s^{-1}, respectively. Injection times: (a) water, 5 s; substrate, 5 s; enzyme, 5 s, (b) water, 5s; substrate, 10 s; enzyme, 5s, (c) water, 10 s; substrate, 5 s; enzyme, 5 s. The dimensionless concentration indicates the concentration relative to the initial concentration except for water. The dimensionless concentration for water indicates the mixing ratio of water and the adjacent solution.

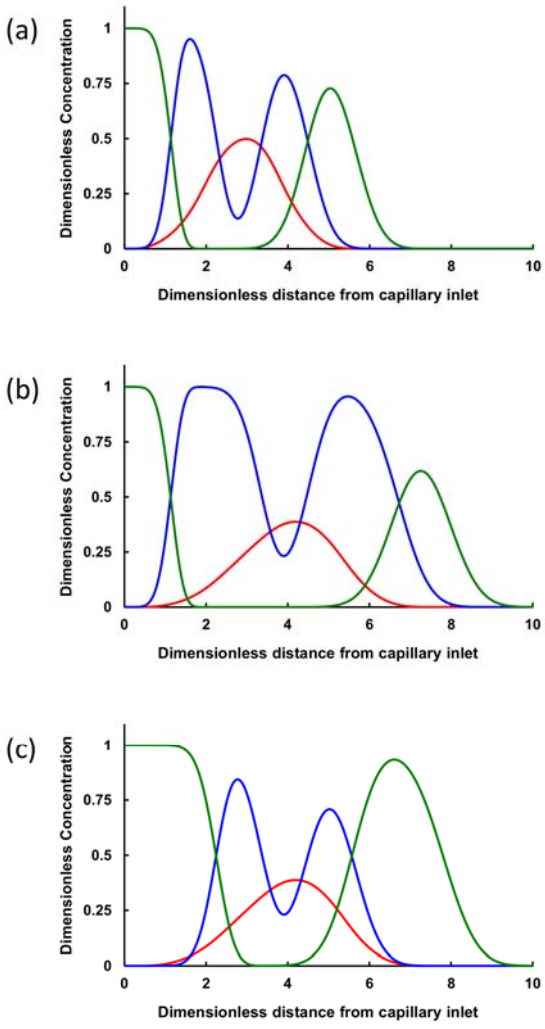

Figure 2

Figure 3. The relationship between enzyme activity and the amounts of veratraldehyde. Enzyme solutions were sandwiched by a substrate solution containing 2 mM veratryl alcohol and 0.4 mM H_2O_2. Incubation time, 5 min; running buffer, 50 mM tartrate buffer (pH 2.5) containing 50 mM SDS: injection volume, 8.2nL for each solution. Other experimental conditions are provided in the main text.

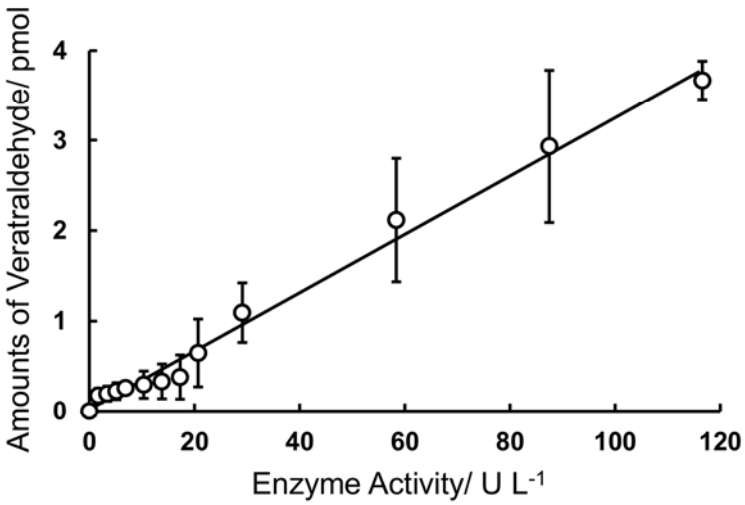

Figure 3

Figure 4. Chromatogram of a CFE sample. The peaks were identified by the number used in Fig. 1. Detection wavelength, 228 nm. The experimental conditions are the same as those described in Fig. 3.

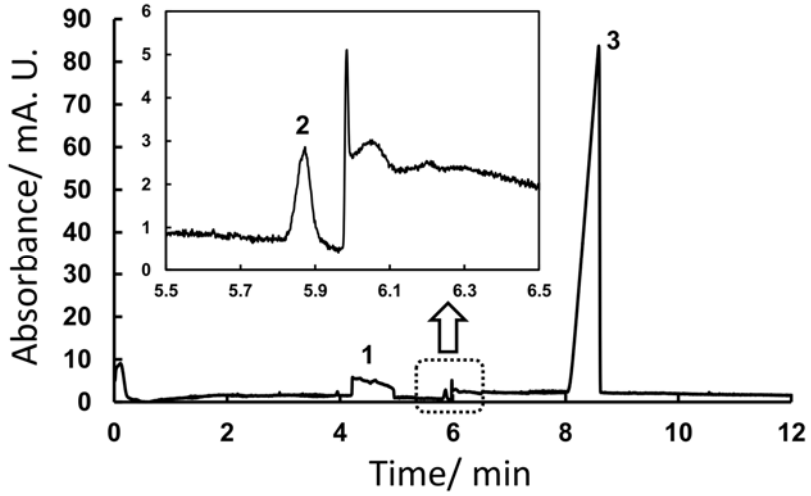

Figure 4