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Difference in enzymatic dehydrogenative polymerization of dilignols using horseradish peroxidase and crude enzyme obtained from Japanese cypress (*Chamaecyparis obtusa*)

Yasuyuki Matsushita*, Daisuke Baba, Dan Aoki and Kazuhiko Fukushima

Abstract

Lignin is a biopolymer consisted of monolignols but the growth mechanism of lignin has not yet been elucidated. In this study, enzymatic dehydrogenative polymerization of dilignols, guaiacylglycerol-β-coniferyl ether (**I**), dehydrodiconiferyl alcohol (**II**), and pinoresinol (**III**), was conducted using crude enzyme obtained from Japanese cypress (*Chamaecyparis obtusa*) in order to investigate the step following the initial radical coupling between monolignols. The results suggested the occurrence of radical transfer during the reaction, similar to the reaction using horseradish peroxidase (HRP). The behavior of *erythro* and *threo* of **I** during the reaction was analyzed, and it was found that the crude enzyme has a substrate specificity toward *erythro* rather than *threo* forms, but HRP shows negligible specificity. And that *erythro* of **I** shows a slightly greater tendency to form a radical by radical transfer from **II** or **III** than the *threo* form.

Keywords: Lignin, Dehydrogenative polymerization, Radical transfer, Enzyme, Substrate specificity

Introduction

Lignin is a natural polymer comprising monolignol as the monomer unit. The polymerization mechanism for lignin formation is unique. The first step of the polymerization, radical coupling, proceeds between two monolignol radicals generated by enzymatic oxidation to yield mainly three dilignols, guaiacylglycerol-β-coniferyl ether (**I**), dehydrodiconiferyl alcohol (**II**), and pinoresinol (**III**), which have β-*O*-4, β-5, and β–β linkages, respectively. These radicals are consumed after the first step, and hence, new dilignol radicals must be generated for the growth of the polymer chain. Lignin is thought to grow by the repeated formation of monolignol, oligolignol, and polylignol (i.e., lignin) radicals and subsequent coupling between two radicals. However, the steric hindrance at the attachment between the enzyme and the oligo- or polylignol would make it difficult to oxidize them. Recently, a specific enzyme for lignin growth was proposed; the redox-active sites are located on the protein surface, resulting in the except the considering of the steric hindrance [1–3]. A radical transfer model was also proposed, according to which the monolignol radical site is transferred to the oligo- or polylignol, which led to generate the corresponding radical [4]. In this hypothesis, the monolignol plays the role of a redox shuttle. In addition, a radical transfer system between coniferyl alcohol and sinapyl alcohol [5–8], *p*-coumarate and sinapyl alcohol [9, 10], superoxide radical in the presence of calcium and coniferyl alcohol [11], and manganese oxalate and monolignols [12] were reported. Previously, we investigated the enzymatic oxidation of dilignols using horseradish peroxidase (HRP) and proposed a radical transfer system, where one dilignol radical transfers its radical site to another dilignol [13, 14].

HRP is generally used for studies on lignin, but it is not obtained from woody plants. It is unclear that the enzyme from woody plants acts the same reaction as HRP. In this research, we used the crude enzyme obtained from

*Correspondence: ysmatsu@agr.nagoya-u.ac.jp
Graduate School of Bioagricultural Sciences, Nagoya University, Furo-cho Chikusa-ku, Nagoya, Aichi 464-8601, Japan

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the differentiating xylem of Japanese cypress (*Chamaecyparis obtusa*) to investigate the chain growth from dilignols and shed light on the difference between the polymerization modes observed in the presence of HRP and this crude enzyme.

Materials and methods

Materials

Compounds **I**, **II**, and **III** were synthesized according to the method in our previous study [13, 14]. Briefly, after the oxidation of coniferyl alcohol by silver (I) oxide, these three compounds were isolated by flash column chromatography (Agilent 971-FP, Agilent Technologies) using a silica gel column (Super Flash SF, Agilent Technologies). HRP was purchased from Wako Pure Chemical Industries.

Crude enzyme extraction

The crude enzyme was isolated using the method reported by Hiraide et al. [15]. After debarking, the differentiating xylem was scraped off using a peeler and frozen by immersing in liquid nitrogen. The frozen differentiating xylem was ground into powder using a mortar cooled with liquid nitrogen.

Benzylsulfonyl fluoride (PMSF; 17.4 mg) was dissolved in 1 mL of dehydrated ethanol to prepare a 100 mM PMSF EtOH solution. Polyvinyl pyrrolidone (PVP; 200 mg) was dissolved in 100 mL of 50 mM acetic acid buffer (pH 5.0). To the PVP solution, 35 μL of 2-mercaptoethanol, 500 μL of PMSF ethanol solution, and 15 g of calcium chloride dihydrate were added to obtain an extraction buffer for soluble proteins. The prepared buffer was stored at 4 °C.

The powdered frozen differentiating xylem was extracted using the solution buffer at 4 °C for 24 h to obtain the crude enzyme. After extraction, the buffer was dialyzed to remove calcium chloride using 50 mM acetate buffer (pH 5.0). To concentrate the protein, the dialyzed solution was centrifugated using an ultrafiltration tube (Amicon Ultra-15 mL, Merck Millipore, Darmstadt, Germany) at 3500 rpm and 4 °C.

Enzyme activity

The procedure for estimating the enzyme activity was based on our previous report [13], and the activity was determined from the oxidation rate of guaiacol. The activities of HRP and the crude enzyme obtained from cypress were 387.2 and 5.24 U/mg, respectively.

Enzymatic dehydrogenative polymerization

Enzymatic dehydrogenative polymerization was carried out by a modification of our previously reported method [13]. In this study, we employed two systems: a single-component system and mixed-component system.

Single-component system

A solution of the dilignol substrate **I**, **II**, or **III** (1.5 μmol in 10 mL of distilled water) was subjected to enzymatic dehydrogenative polymerization catalyzed by the crude enzyme (0.2 U) or HRP (0.4 U) using H_2O_2 (0.125 μg). At certain times, a 0.3 mL aliquot was withdrawn from the reaction mixture and poured into catalase aqueous solution (100 μL of 0.01 mg/mL) to stop the enzymatic reaction. The solution mixture was dried under reduced pressure, and the residue was dissolved in methanol (150 μL). The samples were then prepared by membrane filtration (PTFE 0.50 μm, DISMIC-13JP, ADVANTEC), and liquid chromatography (LC) analysis was conducted. All experiments were performed in triplicate.

Mixed-component system

A mixture of two different dilignols (**I**+**II**, **I**+**III**, and **II**+**III**; 0.75 μmol+0.75 μmol) was subjected to enzymatic dehydrogenative polymerization similar to the case of the single-component system. All experiments were performed in triplicate.

Liquid chromatography (LC) analysis

LC analysis was carried out according to our previous study [14]. The LC system was composed of an Agilent 1100 series binary pump, an Agilent 1100 series variable wavelength detector, a column compartment, an Agilent 1260 Infinity high-performance degasser, and an Agilent 1200 series high-performance autosampler (Agilent Technologies Inc, USA). The equipment was fitted with a Luna phenyl-hexyl column (liquid layer thickness: 5 μm, 150 mm × 2.0 mm, Phenomenex, USA). Ultrapure water (solvent A) and a methanol/acetonitrile (6:1, v/v; solvent B) mixture served as the mobile phase (0.2 mL/min). The column temperature was 40 °C. The elution gradient program was as follows: 0–4 min, 20% of B; 4–10 min, linear gradient from 20 to 30% of B; 10–15 min, 30% of B; 15–40 min, linear gradient from 30 to 50% of B; 40–80 min, linear gradient from 50 to 60% of B; 80–90 min, linear gradient from 60 to 20% of B. Substrate determination was performed at 280 nm.

The *erythro* and *threo* peaks in the chromatogram of **I** were identified based on the LC analysis of *erythro*-rich **I**.

I (*erythro*): retention time (RT): 24.9 min, **I** (*threo*): RT: 23.1 min, **II**: RT: 36.1 min, **III**: RT: 43.0 min.

Results and discussion

Enzyme activity against dilignols

The enzyme activity against dilignols for HRP and the crude enzyme was investigated. **II** was selected as the

dilignol. Although the same activity dosage (0.4 U) against guaiacol was added for both enzymes, the consumption rate of **II** was higher when the crude enzyme was used (Additional file 1: Figure S1). This suggested that the crude enzyme contained many types of phenol oxidase. Accordingly, the experiments in this study were performed by reducing the crude enzyme dosage. The dosages of both enzymes were determined based on their activity against guaiacol: crude enzyme, 0.2 U; HRP, 0.4 U.

Consumption rate of dilignols

In our previous studies [13, 14], when HRP was used for the enzymatic dehydrogenative polymerization of dilignol, the consumption rate of **I** was the lowest among three dilignols. However, the consumption rate of **I** increased when another type of dilignol was mixed, i.e., when using a mixed-component system of **I** + **II** and **I** + **III**, while the consumption rates of **II** and **III** decreased. In the case of the **II** + **III** system, the consumption rate of **II** decreased but that of **III** increased. These results indicated a radical transfer system, where the radical form of **II** transfers its radical site to **I** or **III**, and the radical form of **III** transfers its radical site to **I**.

In this study, we used the crude enzyme obtained from Japanese cypress to investigate the difference in polymerization mechanism against HRP. Figure 1 shows the residual ratio of dilignol in the single- and mixed-component system when using the crude enzyme. A similar trend in the consumption rate was seen when HRP was used, which implied that the same radical transfer

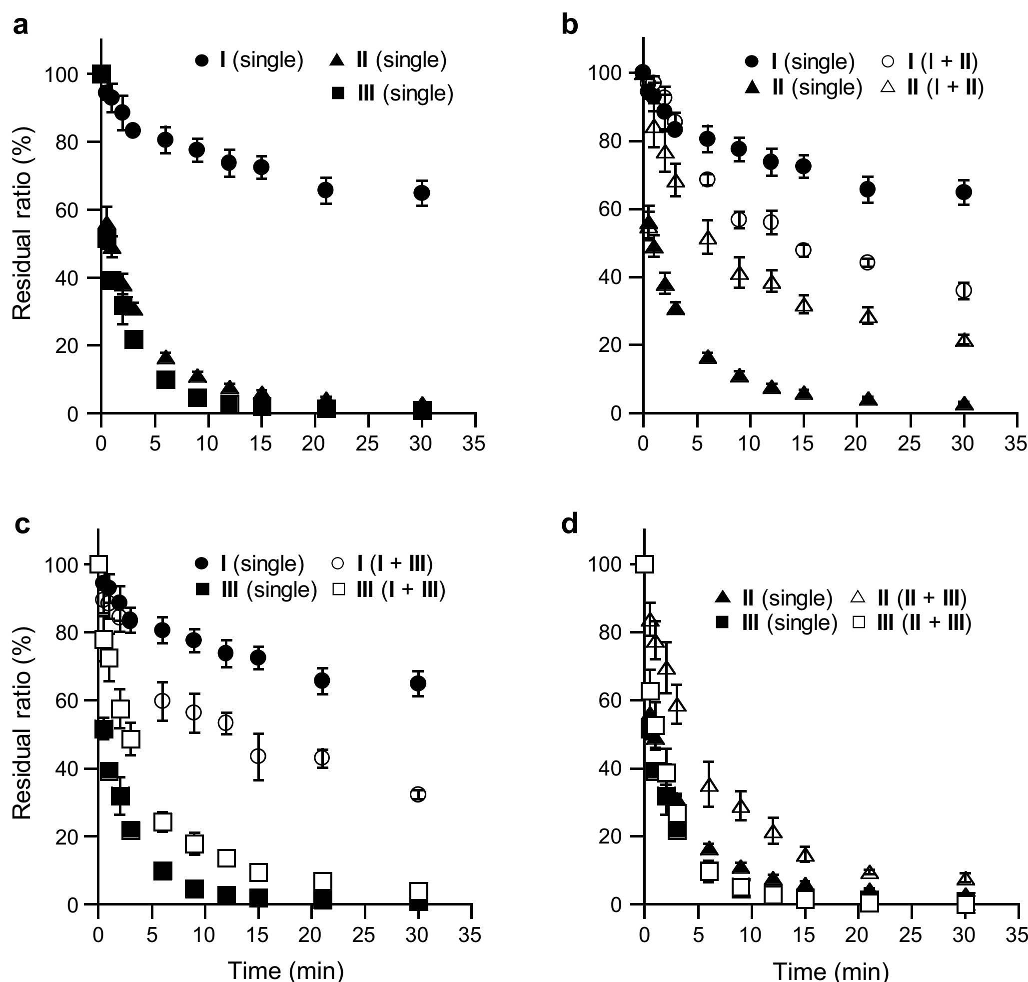

Fig. 1 Time course of residual ratio of the dilignols in the single-component and mixed-component systems. **a** Single-component system, **b** mixed-component system (**I** + **II**), **c** mixed-component system (**I** + **III**), **d** mixed-component system (**II** + **III**) **b**–**d**. Those in the single-component reactions were each described on a same figure for reference

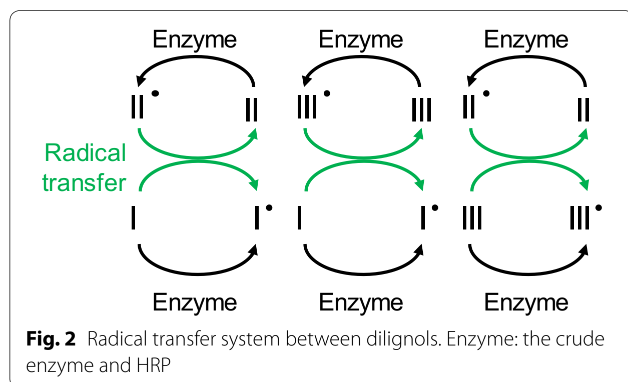

Fig. 2 Radical transfer system between dilignols. Enzyme: the crude enzyme and HRP

process occurred during the dehydrogenative polymerization using the crude enzyme (Fig. 2).

Difference in consumption rates between *erythro* and *threo* isomers of I

I has two types of diastereomers, *erythro* and *threo*. The *erythro* ratio (*erythro*/(*erythro* + *threo*)) in residual **I** is shown in Fig. 3. Additional file 1: Figure S2 shows the consumption rates of the *erythro* and *threo* forms of **I**. In the single-component system, when HRP was used, the *erythro* ratio was initially 53.2% and remained at the same level during the reaction (53.6% at 30 min). In the case of the mixed-system, the *erythro* ratio slightly decreased during the course of the reaction. This suggested that the *erythro* form shows a slightly greater tendency to form a radical by radical transfer from **II** or **III** than the *threo* form. One of the reasons why the **II** and **III** radicals preferentially reacted with *erythro* of **I** was thought to be the less steric hindrance around phenolic hydroxide of *erythro* form than that of *threo* form (Additional file 1: Figure S3). However, it is difficult to state the reason clearly. Further studies are necessary to prove the reasons.

On the other hand, when the crude enzyme was used, a greater amount of the *erythro* form was consumed as compared to the *threo* form in the single-component system (*erythro* ratio decreased from the initial value of 53.6 to 49.7% at 30 min). This implied that the crude enzyme has a substrate specificity toward *erythro* rather than *threo* forms. The consumption trend became notable in the mixed-component system: in the mixture with **II**, the *erythro* ratio decreased from the initial value of 61.1 to 51.1% at 30 min; in the mixture with **III**, the *erythro* ratio decreased from the initial value of 60.7 to 53.2% at 30 min. This result was due to the combination of the substrate specificity of the crude enzyme and the ease of radical transfer from **II** or **III** to the *erythro* diastereomer.

In almost native softwood lignin, the proportions of *erythro* and *threo* were very similar [16]. However, this study suggested that the reactivity of *erythro* form was slightly higher than that of *threo* form. On the other hand, in the water-addition reaction to quinone methide of **I**, completely opposite result was shown recently, where *threo* was preferentially formed more than *erythro* [17]. The recent study and our experiment were conducted in vitro, and thus may not perfectly be a mimic of lignin biosynthesis. Further study should be needed to give comprehensive consideration to lignin biosynthesis.

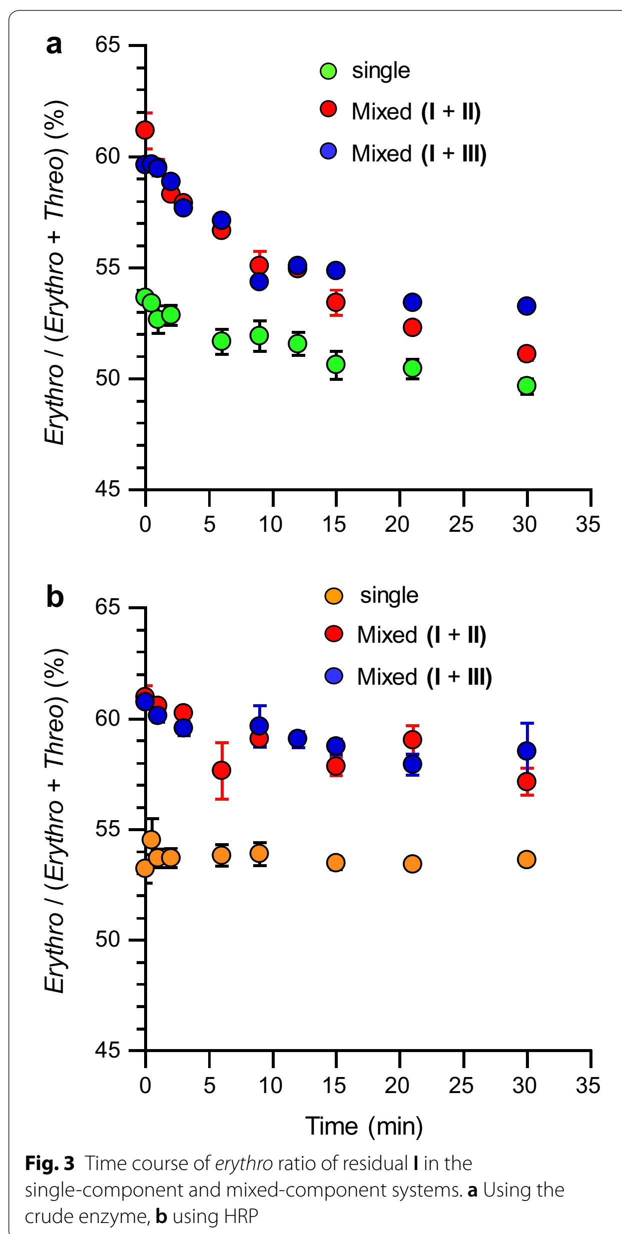

Fig. 3 Time course of *erythro* ratio of residual **I** in the single-component and mixed-component systems. **a** Using the crude enzyme, **b** using HRP

Conclusion

- The polymerization behavior of dilignols in the presence of the crude enzyme obtained from Japanese cypress was similar to that observed with HRP, suggesting that radical transfer also occurred when using the crude enzyme.
- When using the crude enzyme, the reactivity of the *erythro* form of **I** was higher than that of the *threo* form, which implied that the crude enzyme showed substrate specificity toward *erythro* rather than *threo* forms. On the other hand, HRP does not have substrate specificity toward the *erythro* nor *threo* forms.
- During the radical transfer system, **II** and **III** radicals somewhat preferentially reacted with *erythro* form of **I** rather than the *threo* form.

Additional file

Additional file 1: Figure S1. Enzymatic activity of HRP and the crude enzyme against **II.** The unit shows the activity against guaiacol. **Figure S2.** Time course of residual ratio of the erythro and threo diastereomers of **I** in the single-component and mixed-component systems. (a, c) Using the crude enzyme, (b, d) using HRP dilignols, (a, b) mixed-component system (**I** + **II**) within single-component system (b, d) mixed-component system (**I** + **III**) within the single-component reaction Ie: *erythro*, It: *threo*. **Figure S3.** Display of conformation of *erythro* and *threo* isomers of **I**. A-ring and O-B-ring is located at *Anti*-position. The steric hindrance around the A-ring in *erythro* is less than that of in *threo*.

Abbreviations
HRP: horseradish peroxidase; PMSF: benzylsulfonyl fluoride; PVP: polyvinyl pyrrolidone; LC: liquid chromatography; RT: retention time.

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Authors' contributions
YM mainly planned and analyzed data obtained in this research. YM was a major contributor in writing the manuscript. DB performed almost the experiments and also analyzed data obtained in this research. YM is a corresponding author. DA and KF also analyzed the data obtained in this research. All authors read and approved the final manuscript.

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Competing interests
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