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Inactivation of *Coprinus cinereus* peroxidase during the oxidation of various phenolic compounds originated from lignin

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ABSTRACT

In this study, the inactivation of *Coprinus cinereus* peroxidase (CiP) during the oxidation of various phenolic compounds originating from lignin was investigated. The CiP was significantly inactivated during the oxidation of phenolic compounds, such as vaniline, *p*-coumaric acid, 2,6-dimethoxy phenol, 4-hydroxybenzoic acid, 4-hydroxybenzaldehyde, *p*-cresol, *m*-cresol and phenol. Conversely, the CiP nearly maintained its initial activity for the oxidation of syringic acid, vanillic acid and ferulic acid. Hydrogen peroxide affected the CiP inactivation, while the polymerized reaction product hardly affected the CiP inactivation. The thermodynamic parameter ($\Delta\Delta G^0_{f298K}$) and turnover capacity ($\Delta S/\Delta E$) were adapted to explain the CiP inactivation due to covalent bonding between the enzyme and phenolic compounds. In the cases of syringic acid, vanillic acid and ferulic acid, which maintained high residual CiP activities after reaction, the $\Delta\Delta G^0_{f298K}$ were more negative and the turnover capacities were higher than the other values. This means that these compounds prefer to form a dimer rather than an enzyme–phenolics complex. Among the inactivation factors, the formation of covalent bonding between the enzyme and phenolic radicals was concluded to be the main mechanism for the inactivation of CiP. The new thermodynamic parameter ($\Delta\Delta G^0_{f298K}$) used in this study could help to quantitatively show the reaction tendency of phenolic compounds to form a dimer or covalent bonding with the enzyme, which could be used to predict the degree of CiP inactivation.

1. Introduction

The enzyme peroxidase catalyzes the oxidation of phenolic compounds to generate phenoxy radicals, which react with each other to form dimeric, oligomeric and polymeric compounds. This enzymatic polymerization method has previously been exploited for the treatment of wastewater polluted with phenolic compounds and for the production of industrially useful polymers [1–5]. Recently, the production of biofuel from lignocelluloses, such as ethanol and butanol, results in the discharge of a huge amount of lignin by-products. The phenolic compounds originating from lignin could be used as a source for the synthesis of new phenolic resin with this enzyme [6]. Enzymatic polymerization of phenolics using peroxidase has several advantages over conventional polymerization, as follows: (i) phenolic monomers having various substituents are polymerized to give a new class of functional polyaromatics and (ii) the structure and solubility of polymer can be controlled by changing the reaction conditions [7].

The peroxidases originating from plants, such as soybean peroxidase (SBP) and horseradish peroxidase (HRP), are economically inefficient for industrial application. On the other hand, fungal peroxidase can be readily produced in a large quantity in a bioreactor, which would make it economical to oxidize some phenolic compounds [8,9]. It has recently been reported that polycardanol and poly (bisphenol A) can be successfully synthesized by enzymatic polymerization using the fungal peroxidase, CiP (*Coprinus cinereus* peroxidase) [10,11].

However, CiP and other peroxidases significantly lose their activity during the oxidation of phenolic compounds, which results in a limitation to their wide application to industrial processes. There are theories according to the mechanism of peroxidase inactivation during phenolic compound oxidation: firstly, inactivation due to the addition of excessive amounts of hydrogen peroxide in the reaction solution [12–14]; secondly, the inactivation by adsorption of polymeric reaction products onto the enzyme [15,16]; thirdly, the inactivation by covalent bonding between phenolic compounds and the amino acid residues of peroxidase as a result of the reaction of free phenoxyl radicals with peroxidase [17,18]. The first two are well known mechanisms, which can be proved easily using experimental procedures, but the third remains hypothetical. Experimental proof for HRP inactivation by radical attack has

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recently been obtained through various spectroscopic analyses [19].

In this study, the factors responsible for the inactivation of CiP were investigated. Phenolic compounds originating from lignin were selected as the substrates for the reaction. The effects of hydrogen peroxide and polymerized reaction product on the inactivation of CiP were examined. A thermodynamic parameter was introduced to explain the inactivation of CiP due to covalent bonding between CiP and the phenolic compounds. The difference in the inactivation of CiP in the presence of various phenolic compounds was also explained via a thermodynamic approach and turnover capacity.

2. Materials and methods

2.1. Chemicals

The phenolic compounds, syringic acid, vanillic acid, ferulic acid, vanillin, *p*-coumaric acid, 2,6-dimethoxy phenol, 4-hydroxybenzoic acid, 4-hydroxybenzaldehyde, *p*-cresol, *m*-cresol, and phenol were purchased from Sigma chemicals (St. Louis, MO, USA). Water, methanol, and acetic acid of high performance liquid chromatography (HPLC) grade were supplied by Mallinkrodt Backer Inc. (Philipsburg, NJ, USA). Microbiological culture media such as yeast extract, peptone, tryptone were purchased from Becton Dickinson Co. (Sparks, MD, USA). The other chemicals and laboratory media components were obtained from either Sigma chemicals (St. Louis, Mo, USA) or Fluka chemicals (Buchs, Switzeland), and they were of analytical grade.

2.2. Preparation of Coprinus cinereus peroxidase (CiP)

C. cinereus IFO 8371 was used as the peroxidase-producing strain. The medium used for the production of the peroxidase contained 30.0 g/L glucose, 5.0 g/L peptone, and 3.0 g/L yeast extract. The details of the production and purification of fungal peroxidase have previously been reported [10]. Finally, the purified CiP was concentrated to a final concentration of 25,000 U/mL.

2.3. CiP-catalyzed reaction

The batch reactors consisted of a vial (100 mL) containing 30 mL of 100 mM phosphate buffer and 10 mM phenolic compound. Before the addition of hydrogen peroxide, the CiP was added to the batch reactor to a final concentration of 25 U/mL. 10 mM of hydrogen peroxide was added to the reactor to initiate the phenolic compound oxidation, with the reactor stirred strongly using a Teflon-coated magnetic bar at room temperature for 10 min. After initiating the phenolic oxidation, the mixture was centrifuged for 15 min at 4000 rpm to remove polymerized precipitates. The supernatant was analyzed for the final concentration of phenolic compound.

To investigate the effect of polymerized reaction products on the inactivation of CiP, the phenol polymer was obtained as follows: to precipitate the phenol polymer from the reaction solution, 10 mM phenol solution was mixed with 25 U/mL of CiP and hydrogen peroxide. After the reaction, the mixture was centrifuged, with the supernatant removed. The resultant precipitates were washed with deionised distilled water until no enzyme activity remained, and then added to the solution with 25 U/mL of fresh CiP.

2.4. Analysis

The CiP activity (U/mL) was measured as follows: 10 μL of reaction solution was added to 3 mL of a 0.18 mM 2,2′-azino-bis(3-ethylbenzthia-zoline-6-sulfonic acid) (ABTS) solution in 50 mM phosphate-citrate buffer (pH 5.0) at room temperature. One microliter of 15% hydrogen peroxide solution was added to initiate the color generation reaction. One unit of peroxidase was defined as the amount of enzyme required to catalyze the conversion of 1 μmol of ABTS ($\epsilon = 34{,}700\ \mathrm{cm^{-1}\ M^{-1}}$).

Phenolic compound concentrations were determined using an Agilent model 1200 liquid chromatograph with a diode-array detector working at 280 nm. Separation was carried out using a Zorbax XDB-C_{18} column (150 mm × 3.0 mm, 3.5 μm) at 25 °C, with a mobile phase of 0.3% acetic acid (70%) and methanol (30%) at a flow rate of 1.0 mL/min. The concentrations of the phenolic compounds were quantified using calibration curves prepared from external standards.

Gibbs free energy of formation (ΔG^0_{f298K}) was estimated by Joback method using ChemOffice 2004 software. Joback method is based on the assumption that each fragment of a molecule contributes to the value of its physical property [21]. According to this assumption, Gibbs free energy of formation was calculated at standard state (298.15 K, 1 atm, 1 mol) as follows:

$$\Delta G^0_{f298K} = 53.88 + \sum n_i G_i$$

where n_i and G_i represent the number of occurrences and a group contribution, respectively.

The new thermodynamic parameter, $\Delta\Delta G^0_{f298K}$, was defined as follows:

$$\Delta\Delta G^0_{f298K} = \Delta G^0_{f298K} - \Delta G'^0_{f298K}$$

where $\Delta\Delta G^0_{f298K}$ and $\Delta G'^0_{f298K}$ represent the Gibbs free energy of the formation of dimer and Gibbs free energy of the formation of phenylalanine–phenolic complex (PPC) or tyrosine–phenolic complex (TPC), respectively.

The turnover capacity is expressed as the ratio of exhausted substrate (ΔS) per enzyme used (ΔE) during 10 min of reaction

3. Results and discussion

3.1. Inactivation of CiP during the oxidation of various phenolic compounds

In this study, several phenolic compounds were selected according to their structure. The tested phenolic compounds are categorized into three main structures, these being cinnamic, benzoic, and phenolic derivatives, which have a different numbers of methoxy groups. Fig. 1 shows the structures of the phenolic compounds used in this experiment.

Fig. 2 shows the residual CiP activity after 10 min of reaction. The residual activity of CiP (RAC) in the presence of phenolic compounds depended on the structure of the tested materials. The results examined can be represented as follows: (1) The RAC was higher in the presence of benzoic and cinnamic derivatives than the phenolics. (2) The greater the number of methoxy side groups on the phenolic compounds, the greater the amount of RAC remained. (3) The RAC was much higher in the compounds in the acid than aldehydic form. With most of the benzoic and cinnamic derivatives the RAC remained above 17.0%, while the RAC was below 1.5% with phenol, *p*-cresol, and *m*-cresol. The difference in the RAC showed a harsh contrast in the presence and absence of compounds with methoxy groups. The RAC with vanillic acid (4-hydroxy-3-methoxybenzoic acid), vanillin (4-hydroxy-3-methoxy-benzaldehyde), and 2,6-dimethoxyphenol were 95.3%, 34.3%, and 19.9%, respectively. However, the RAC with 4-hydroxybenzoic acid, 4-hydroxybenzaldehyde, and phenol were 17.0%, 0.3%, and 1.2%, respectively. Regardless of the presence of methoxy groups, the RAC with vanillic acid and 4-hydroxybenzoic acid were much higher than with vanillin and 4-hydroxybenzaldehyde.

3.2. Inactivation of CiP by hydrogen peroxide

The CiP inactivation by hydrogen peroxide experiment was also performed in a reaction solution without phenolic compounds. It is well known that peroxidase is inactivated by hydrogen peroxide [12–14]. As shown in Fig. 2, the activity of CiP decreased about 50% due to hydrogen peroxide inactivation. Interestingly, the RAC with vanillic acid, syringic acid, and ferulic acid were higher than those of the reaction solutions containing only hydrogen peroxide. This result shows that these compounds significantly prevent the inactivation of CiP caused by hydrogen peroxide. Except for these compounds, most of the tested compounds caused much higher inactivation of CiP than the inactivation by hydrogen peroxide. These results indicate that there are other factors involved in the inactivation of CiP during the oxidation of phenolic compounds.

3.3. Inactivation of CiP by polymerized product

In addition to the inactivation of peroxidase by hydrogen peroxide, two inactivation mechanisms have been proposed. First, the peroxidase is inactivated by polymerized reaction products, which are adsorbed onto the enzyme [15,16]. Second, free radicals produced from the oxidation reaction cause covalent bonding between the enzyme and substrate, which inactivates the per-