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Synthesis of polycardanol from a renewable resource using a fungal peroxidase from *Coprinus cinereus*

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Abstract

A fungal peroxidase from *Coprinus cinereus* (CiP) was successfully used for oxidative polymerization of cardanol in water–organic solvent mixtures. Cardanol is a phenol derivative from a renewable resource having the meta-substituent of a C15 unsaturated hydrocarbon chain mainly with one to three double bonds. So far, only uneconomic plant peroxidases, such as soybean peroxidase (SBP), have been used to polymerize cardanol. The fungal peroxidase used was easily produced by cultivating *C. cinereus*, and was purified by ultrafiltration and size exclusion chromatography. The purified peroxidase had a specific activity of 4960 U/mg. The CiP-catalyzed polymerization of cardanol was carried out in aqueous/organic solvents. Microbial CiP catalyzed the cardanol polymerization as efficiently as SBP. The structure and molecular weight of the polycardanol produced by CiP were comparable to those produced by SBP. A low reaction temperature of 10 and 15 °C produced polycardanol in high yield and the hydrogen peroxide feed rate was found to affect the initial reaction rate and the final conversion. From a practical point of view, it is believed that microbial CiP will be found more useful for the synthesis of a range of polyphenols from renewable resources than plant peroxidases.
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Keywords: Enzymatic polymerization; *Coprinus cinereus*; Peroxidase; Cardanol; Renewable resource

1. Introduction

Strong demand exists for polymers that can be synthesized from renewable resources rather than petroleum-based feedstocks. This reflects a global requirement for sustainability without resource depletion. Cashew nut shell liquid (CNSL) is a side-product from mechanical processing (hot-bath process) for the edible use of the cashew nut of *Anacardium occidentale*, and cardanol, the main component of CNSL, is a phenol derivative having the meta-substituent of a C15 unsaturated hydrocarbon chain containing mainly one to three double bonds [1]. Cardanol has potential use in resins, friction lining materials, and surface coatings [2]. Since conventional phenolic resins produced from cardanol need formaldehyde, there is a strong need for a new formaldehyde-free synthesis method.

Major advantages of the enzymatic polymerization of phenol derivatives are as follows: (i) enzyme-catalyzed polymerization of phenols proceeds under mild conditions without the use of toxic reagents such as formaldehyde; (ii) phenol monomers having various substituents are polymerized to give a new class of functional polyaromatics; (iii) the structure and solubility of the polymer can be controlled by changing the reaction condition; (iv) the polymerization procedures and polymer isolation are straightforward [3].

Only soybean peroxidase (SBP) has been successfully used in aqueous organic solvents for the oxidative polymerization of cardanol. Horseradish peroxidase (HRP) has been shown to be incapable of catalyzing cardanol polymerization [4,5]. Recently we reported that cardanol can be polymerized by HRP in the presence of mediators [6]. However,

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because these peroxidases are derived from plants, their properties depend on cultivation conditions such as weather and soil. In addition, their production in the large amounts required is limited. These problems represent major limitations regarding their industrial applications. Therefore, the application of microbial peroxidases, which are far more economical than plant peroxidases, is attractive. Of many microbial peroxidases, a fungal peroxidase from *Coprinus cinereus* (identical to *A. ramosus* peroxidase) appears useful for industrial applications because it is easily produced and it has high specific activity [7,8]. Shinmen et al. were the first to isolate and characterize this novel peroxidase from the medium of the fungal mycelia [9]. *C. cinereus* peroxidase (CiP) has been proposed as an alternative to HRP for various commercial assays because of its stability and its accessible active site [10]. CiP has also been successfully used to remove phenols from wastewaters by polymerization and precipitation [11].

In the present study, we examined the potential use of CiP for the industrial oxidative polymerization of cardanol. The effects of reaction conditions on the CiP-catalyzed polymerization of cardanol were also investigated. To the best of our knowledge, no previous report has been issued on the oxidative polymerization of cardanol using microbial peroxidases.

2. Experimental

2.1. Production and purification of the fungal peroxidase

C. cinereus IFO 8371 was used as the peroxidase-producing strain. The medium used for peroxidase production contained 30 g/L glucose, 5 g/L peptone (Difco Lab., USA), and 3 g/L yeast extract (Difco Lab., USA). The culture was started by inoculating a spore suspension, which was prepared by adding 5 mL of the above medium to a solid culture and vibrating for 30 s, into 70 mL of the culture medium in a 500 mL Erlenmeyer flask. It was incubated on a shaking incubator at 120 rpm and 30 °C. The culture was carried out in 10 flasks and the culture broth in each flask was used up for sampling. The culture medium was filtered through a membrane filter (pore size: 0.45 μm, Whatman) and the filtrate was then assayed for peroxidase activity, glucose concentration, and pH. Cells retained by the filter were dried in an oven for 2 days and then weighed.

The supernatant from the culture broth was concentrated by ultrafiltration (Amicon Ultra-4 centrifugal filter, 10 kDa MWCO) and desalted with 0.1 M phosphate buffer (pH 5.0). The desalted CiP solution was purified by size exclusion chromatography (SEC) using a silica-based column (Bio-Sil SEC 125, Bio-Rad) and 0.1 M phosphate buffer (pH 5.0) as an eluent. The CiP fraction collected was concentrated by ultrafiltration (Amicon Ultra-4 centrifugal filter, 10 kDa MWCO) again.

2.2. Enzymatic polymerization of cardanol

The reaction scheme for the polymerization of cardanol is shown in Fig. 1. Cardanol (technical grade; product no. 1500-1, Palmer International, USA) is a straw colored liquid comprised of 90–95% cardanol and 5–10% cardol. This material was used without further purification. The enzymatic polymerization of cardanol was carried out as follows: 1.8 mmol of cardanol was dissolved in a mixture of 12.5 g 2-propanol and 12.5 g phosphate buffer (100 mM, pH 7.0). Six thousand units of CiP was then added to the reaction mixture, and stirred for 5 min. The reaction was started by adding H_2O_2 solution diluted with distilled water at 0.3 mmol/h continuously at room temperature (20 °C) with gentle stirring for 5 h. The reaction mixture was then concentrated under reduced pressure. Ethyl acetate (20 mL) was added to the residue, and the organic top layer was separated, followed by removal of the solvent under reduced pressure. Methanol was added to the oily residue to remove unreacted cardanol. The methanol-insoluble material was separated by centrifuge and dried in a vacuum to give polycardanol yield.

2.3. Analytical methods

Glucose concentrations in culture media were determined using a glucose assay kit (GAGO-20, Sigma, USA). Peroxidase activity (U/mL) was measured as follows. Several microliters of filtrate was added to 2 mL of 0.18 mM of ABTS (2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)) solution in 50 mM phosphate-citrate buffer (pH 5.0). One microliter of a 15% H_2O_2 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 ($\varepsilon = 34{,}700\ \mathrm{cm}^{-1}\ \mathrm{M}^{-1}$) per min at 25 °C.

Cardanol concentrations during the enzymatic polymerization were analyzed by HPLC using a μBondapak C18 column (Waters, USA). The mobile phase was composed of acetonitrile:distilled water:acetic acid (8:2:1, v/v/v) at a flow rate of 1.0 ml/min. Absorbance was measured at 280 nm. The molecular weights of polycardanol were determined by gel permeation chromatography (GPC). GPC analysis was carried out using a refractive index detector under the following conditions: PL4 mixed BB columns (TOSOH, Japan) and tetrahydrofuran as solvent at 1.0 mL/min. Calibration curves for GPC analysis were obtained using polystyrene standards. FT-IR spectra were recorded on a Perkin-Elmer FT-IR 2000 to confirm the polymer structure.

3. Results and discussion

3.1. Production and purification of the fungal peroxidase

Fig. 2 shows the time course of peroxidase production from *C. cinereus* in liquid culture. Glucose was consumed continuously and the pH gradually increased from 6.0 to

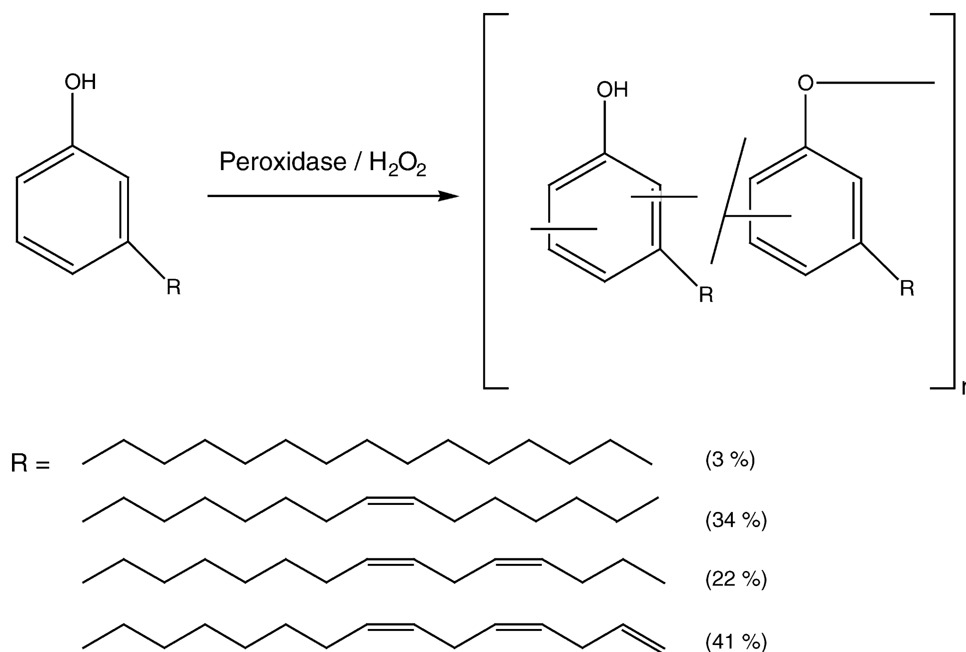

Fig. 1. Reaction scheme of cardanol polymerization catalyzed by peroxidase.

7.3. The cell concentration increased until the 10th day and slightly decreased thereafter, even though glucose remained in the medium. This may have been due to the exhaustion of nitrogen sources. Peroxidase activity was observed in the culture broth from the 6th day and peaked on the 10th day. It was considered that the peroxidase was produced inside the cells in connection with the growth and was then secreted to the medium [12]. The reduction of CiP activity observed after the 11th day may have resulted from peroxidase degradation by extracellular proteases.

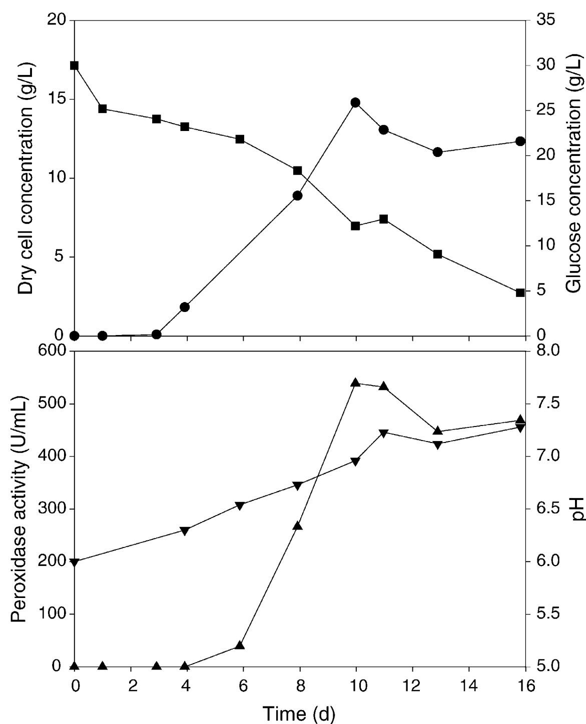

Fig. 2. Time-course profile of CiP production. Culture conditions: initial pH 6.0; temperature, 30 °C; medium volume, 70 mL; shaking speed, 120 rpm. *Symbols:* (●) dry cell concentration; (■) glucose concentration; (▲) peroxidase activity; (▼) pH.

The fungal peroxidase from *C. cinereus* was purified as described in Section 2. Table 1 shows the results of the CiP purification. Specific activity was significantly enhanced 3.2 times after ultrafiltration (UF) and then to 4.5 times by SEC and UF. The purity of peroxidases is often represented by the *Rz* (Reinheitszahl) number, which is the ratio of absorbance at 405 nm to that at 280 nm. As shown in Table 1, the purity of CiP was also elevated to *Rz* 1.26, which is higher than the reported value ($Rz = 0.96$) [13].

3.2. Peroxidase-catalyzed polymerization of cardanol

The peroxidase-catalyzed polymerization was carried out in a mixture of phosphate buffer (pH 7.0) and organic solvents. Even though the peroxidase activity in buffer was much higher than that in aqueous organic solvent, the yield of phenolic polymers obtained by peroxidases in buffer has been reported to be far lower than that obtained in aqueous organic solvent. On the whole a high yield was achieved in about 50% water–organic solvent mixtures [14]. Experimentation with 2-propanol concentrations of 10, 30, and 50 wt% also showed that highest polycardanol yield was obtained in 50 wt% 2-propanol (Table 2). When the CiP-catalyzed polymerization of cardanol was performed in various water-miscible organic solvents/phosphate buffer (50:50, w/w) mixes, 2-propanol produced the highest yield of 74%, and polycardanol was not obtained in aqueous *t*-butanol or 1,4-dioxane. The molecular weight of the polycardanol formed was also affected by

Table 1
Purification of the fungal peroxidase from *Coprinus cinereus*

Step	Total activity (U)	Specific activity (U/mg protein)	Yield (%)	Rz (A_{405}/A_{280})
Culture broth	32810	1100	100	<0.001
UF	25920	3520	79	0.49
SEC and UF	17720	4960	54	1.26

the nature of the solvent. The M_w increased from 5221 to 10,808 when 2-propanol was replaced by methanol. A similar tendency was found for SBP in the polymerization of cardanol [5]. Future study of the correlation between polycardanol yield and organic solvent type would be informative. Torres et al. introduced a new hydrophobicity parameter (H) to describe the catalytic behavior of peroxidases in water–organic solvent mixtures [15]. H was defined as the organic solvent activity (a_s) divided by the transition energy as Dimroth–Reichardt parameter [E_T(30)]. As shown in Table 2, polycardanol yields did not correlate with H values well.

The enzymatic polymerization of cardanol was significantly dependent on the natures of the peroxidase as well as the organic solvents used. Only SBP, but not HRP, is known to be able to catalyze the oxidative polymerization of cardanol in appropriate solvents [4,5]. Although there is little structural homology between CiP and HRP, CiP has many similarities to HRP including active site, pH optimum, observed oxidation potential, and susceptibility to inhibition by H_2O_2 [13]. However, unlike HRP, the CiP-catalyzed polymerization of cardanol proceeded satisfactorily. This may be because CiP has broader substrate specificity than HRP [13]. Since HRP has a high catalytic activity toward phenols with a small meta-substituent [14], cardanol, a phenol with a C15 hydrocarbon at the meta-position, may be too large for HRP. Only in the presence of proper redox mediators does the HRP-catalyzed polymerization of cardanol proceed [6]. For comparison purposes, cardanol polymerization catalyzed by SBP with the same peroxidase activity as CiP was carried out under identical conditions. As shown in Table 2, SBP produced a slightly lower yield and a higher molecular weight polycardanol than CiP.

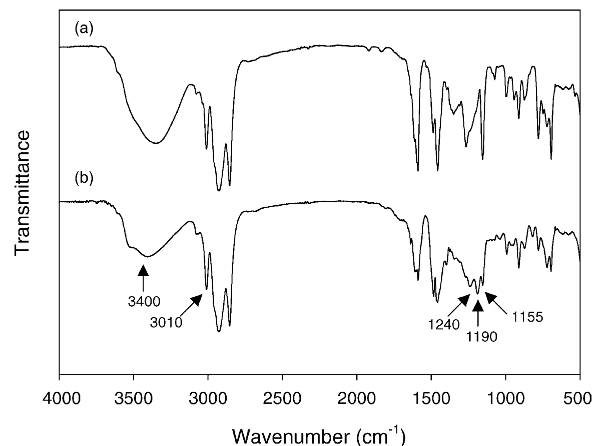

Fig. 3. FT-IR spectra of (a) cardanol and of (b) polycardanol synthesized using CiP.

FT-IR spectra, which were recorded on a Perkin-Elmer FT-IR 2000, confirmed a structure of the polymer synthesized by the fungal peroxidase from *C. cinereus*. In Fig. 3, are shown the FT-IR spectra of (a) cardanol and of (b) polycardanol formed by CiP. The broad peak at 3400 cm^{-1} is due to the vibration of the O-H linkage of phenolic group. Fig. 3(b) shows three characteristic peaks at 1240, 1190, and 1155 cm^{-1} ascribed to the vibrations of the C(Ar)–O–C(Ar) and/or C(Ar)–OH linkages. These results reveal that polycardanol formed by CiP consisted of a mixture of phenylene and

Table 2
Effects of organic solvents on the enzymatic polymerization of cardanol

Solvent[a]	Enzyme[b]	Yield (%)	a_s[c]	H[d] (μmol/cal)	M_w[e]	M_n[f]
Methanol (50 wt%)	CiP	55	0.44	7.9	10808	3540
Ethanol (50 wt%)	CiP	62	0.51	9.7	8974	4096
t-Butanol (50 wt%)	CiP	0	0.58	13.3	–	–
1,4-Dioxane (50 wt%)	CiP	0	0.56	15.7	–	–
2-Propanol (10 wt%)	CiP	0	0.37	7.6	–	–
2-Propanol (30 wt%)	CiP	19	0.56	11.6	3460	2950
2-Propanol (50 wt%)	CiP	74	0.55	11.4	5221	3411
2-Propanol (50 wt%)	HRP	0	0.55	11.4	–	–
2-Propanol (50 wt%)	SBP	70	0.55	11.4	10600	5320

[a] The mixture of organic solvents and phosphate buffer amounted to 25 g.
[b] 6000 units of peroxidases were used.
[c] The thermodynamic activity of organic solvent (a_s) in the mixture was determined using isothermal data for vapor–liquid equilibrium at 25 °C and calculated using the NRTL equation [16].
[d] Solvent hydrophobicity (H) was calculated from a_s and E_T(30) [15].
[e] Weight average molecular weight.
[f] Number average molecular weight.

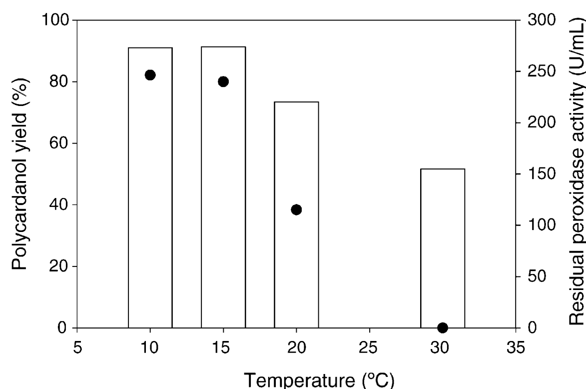

Fig. 4. Effects of reaction temperature on the enzymatic polymerization of cardanol (1.8 mmol) at 0.3 mmol H_2O_2/h in 2-propanol/phosphate buffer (50:50, w/w) for 5 h. *Symbols:* (bar) polycardanol yield; (●) residual peroxidase activity.

oxyphenylene units. This structure is highly similar to that of the polycardanol formed by SBP [4]. Although cardanol has two groups subject to polymerization (the phenolic moiety and the unsaturated hydrocarbon group), only the phenolic moiety was polymerized. In Fig. 3(a), the spectrum of cardanol shows a characteristic peak at 3010 cm^{-1} due to C–H vibration of the unsaturated hydrocarbon moiety. As shown in the FT-IR spectra of Fig. 3(b), the peak at 3010 cm^{-1} remained almost unchanged, indicating no reaction of the unsaturated groups during the CiP-catalyzed polymerization.

3.3. Effects of reaction temperature and hydrogen peroxide feed rate

Fig. 4 shows the effect of reaction temperature on the enzymatic polymerization of cardanol. It was surprising that the polycardanol yield after 5 h at a low temperature of 10 and 15 °C was much higher than that at 30 °C. We expected that the polycardanol yield would increase with increasing the reaction temperature from 10 to 30 °C, because maximum CiP activity occurs between 40 and 50 °C [17]. This result was probably due to CiP inactivation during the enzymatic polymerization, which was supported by the finding that the residual CiP activity after a 5 h reaction at low temperatures (10 and 15 °C) was higher than that at 20 and 30 °C as the polycardanol yield was. At 25 °C, CiP showed no significant loss of activity over 30 days with an extrapolated half-life of 120 days, but at 85 °C the half-life of CiP was 42 min [13]. Therefore, it is believed that a reaction temperature of 30 °C is insufficient to inactivate CiP. Masuda et al. reported that peroxidases could be inactivated by several mechanisms during phenol polymerization in water [18]: (i) reactions between hydrogen peroxide and intermediates of the enzyme's catalytic cycle [19,20]; (ii) irreversible reactions between the enzyme and phenyl or phenoxy radicals [21]; (iii) adsorption of the enzyme on polymerized phenols, which consequently hinders substrate access to the enzyme's active site [22]. They also demonstrated that the differences in CiP inactivation due to temperature are mainly caused by (ii) reactions between the enzyme and free radicals formed by the one-electron oxidation of phenolic substrates. Increases in reaction rate caused by raising the reaction temperature enhance the rate of free radical generation. As a result, the probability that peroxidases contact with free radicals would increase, that is, the rate of enzyme inactivation might be accelerated [17]. In addition, in our case, water–miscible organic solvents can cause CiP deactivation during cardanol polymerization, and enzyme inactivation by organic solvents can become more severe at higher temperatures; for example, when the temperature was increased from 25 to 37 °C, the apparent α-chymotrypsin deactivation constant increased 20-fold in water and 100-fold in 16% ethanol or 16% acetone [23]. Weight average molecular weights (M_w) of the polycardanols formed using CiP were almost the same irrespective of the reaction temperature and ranged from 5500 to 6500 (data not shown).

Because hydrogen peroxide acts as an enzyme denaturant as well as an oxidant for peroxidase-catalyzed polymerizations, the H_2O_2 concentration must be maintained at a proper level. In this respect, the continuous feeding of hydrogen peroxide produced a better result than intermittent feeding [5]. The effects of the amounts of hydrogen peroxide added were investigated by increasing the feed rate from 0.3 to 3 mmol/h for 5 h in aqueous 2-propanol. In the case of 0.3 mmol H_2O_2/h, the reaction was allowed to proceed for 7 h, and the cardanol monomer after the 7 h reaction was not detected by HPLC (data not shown). The initial reaction rate increased with increasing the flow rate to 1.5 mmol/h, and then seemed to decrease slightly at 3 mmol/h (Fig. 5). This is probably because a higher concentration of H_2O_2 can form more com-

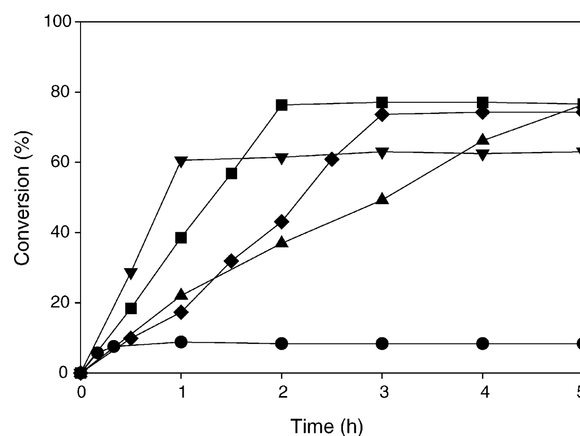

Fig. 5. Effects of the H_2O_2 feed rate on the enzymatic polymerization of cardanol (1.8 mmol) in 2-propanol/phosphate buffer (50:50, w/w) at 20 °C for 5 h. *Symbols:* (▲) 0.3 mmol/h; (♦) 0.5 mmol/h; (■) 0.75 mmol/h; (▼) 1.5 mmol/h; (●) 3 mmol/h.

pound I (a highly oxidized state of peroxidase), which is able to catalyze cardanol polymerization. However, hydrogen peroxide can also react with intermediates of peroxidase's catalytic cycle to inactivate the enzyme [19,20]. Thus, a higher concentration of H_2O_2 can accelerate the suicide inactivation of peroxidases and reduce the final conversion (Fig. 5). Therefore, the H_2O_2 feeding strategy is essential for successful oxidative polymerizations catalyzed by peroxidases.

4. Conclusions

In this study, a fungal peroxidase from *C. cinereus* was successfully applied to the oxidative polymerization of cardanol from a renewable resource. The peroxidase was produced by cultivating *C. cinereus* and was purified by ultrafiltration and size exclusion chromatography. The purified peroxidase had a specific activity of 4960 U/mg. The structure and molecular weight of the polycardanol synthesized using CiP were comparable with those produced using SBP. The effects of reaction temperature and H_2O_2 concentration on the CiP-catalyzed polymerization of cardanol were investigated in aqueous 2-propanol. Low temperature (10 and 15 °C) increased polycardanol yield (to 91%) and the feeding rate of hydrogen peroxide also affected the initial reaction rate and final conversion. From a practical point of view, it is expected that CiP will be found to be more useful for the synthesis of polyphenols from renewable resources than plant peroxidases.

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