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Lignin transformation by a versatile peroxidase from a novel *Bjerkandera* sp. strain

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Abstract

A versatile peroxidase, purified from a novel strain of *Bjerkandera* sp. (B33/3), was tested for its reactivity on a lignin fraction obtained from straw pulping. The effects of such processing parameters as reaction time, pH, and lignin:enzyme ratio were evaluated. Gel filtration chromatography was employed to characterise the molecular mass distribution of the lignin fragments produced by the enzyme-mediated reaction. Our results have shown that such a versatile peroxidase can directly bring about transformations of lignin, even in the absence of external mediators.

Keywords: Enzyme; Wood material; Gel filtration chromatography

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1. Introduction

Attention has recently been paid to a novel class of ligninolytic peroxidases, versatile peroxidases, which combine typical properties of both manganese and lignin peroxidases, coupled with a broad substrate range (Ruiz-Duenas et al., 1999; Camarero et al., 2000; Martinez, 2002). Such a new class of enzymes has high affinity for manganese and dyes, but is also able to oxidise 2,6-dimethoxyphenol (DMP) and veratryl alcohol (VA) in a manganese-independent reaction (Martinez, 2002). Unlike manganese peroxidases that are only able to oxidise phenolic substrates in the presence of manganese, versatile peroxidases are able to oxidise such compounds both in manganese dependent and independent reactions.

Versatile peroxidases have also raised a great deal of interest in the biotechnology field, because they do not seem to require any mediators for substrate oxidation. Most studies to date pertaining to versatile enzymes have focused on characterisation of their oxidation ability toward several model compounds; however, little effort has been devoted to demonstrate the activity of the members of this enzyme group on what is thought to be one of its natural substrates, i.e., lignin. There is little data available relating to oxidation of lignin (or lignin-derivatives) by versatile peroxidases. A recent communication (Moreira et al., 2001a) has reported results of the first in vitro testing of a versatile peroxidase from *Bjerkandera* sp. strain BOS55, in biobleaching of a eucalyptus kraft pulp; those authors acknowledged a 6% reduction in the pulp kappa number, but no further assessment was carried out. This study calls attention to the potential use of versatile peroxidases for applications in the pulp and paper industry, namely modification of lignins.

The presence of lignin, or lignin products resulting from various pulping and bleaching operations in pulp and paper industries, is responsible for the brownish colour of their effluents, which can cause environmental problems; although colour is considered a nonconventional discharge parameter, several countries have already limited colour levels in discharge effluents via strict environmental legislation.

Versatile peroxidases have to date been isolated only from *Pleurotus ostreatus, Pleurotus eryngii, Pleurotus*

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pulmonarius, Bjerkandera adusta, and *Bjerkandera* sp. strain BOS55 (Camarero et al., 1996, 1999, 2000; Heinfling et al., 1998a, b, c; Mester and Field, 1998; Ruiz-Duenas et al., 1999, 2001; Giardina et al., 2000). Their pH optima for oxidation of Mn(II) (pH 5.0) and aromatic compounds and dyes (pH 3.0) are quite different, but are similar to the optima of MnP and LiP activities. *Pleurotus eryngii* produces two versatile peroxidase isoenzymes (PS1 and MnPL1), which can be isolated from distinct culture media (Camarero et al., 1999; Ruiz-Duenas et al., 1999). PS1 presents similar activities on Mn(II), DMP, and methoxy-*p*-hydroquinone, but lower than MnPL1 on VA, *p*-methoxybenzene, and lignin model dimers, hence suggesting a lower redox potential (Ruiz-Duenas et al., 2001). The catalytic properties of *Pleurotus* and *Bjerkandera* sp. versatile peroxidases are similar to each other (Martinez, 2002).

During a selection program aimed at isolating new potential lignin degraders, one of the most promising strains, tentatively named B33/3, was further tested and consequently identified as a member of the *Bjerkandera* genus. Further investigation provided a detailed characterization of B33/3 ligninolytic enzymes, leading to description and molecular characterisation of a novel versatile peroxidase in this genus (Moreira et al., 2001b, 2005, 2006). The direct interaction of the enzyme with natural lignin molecules remains to be demonstrated.

In this research, sulphur-free lignin obtained from a soda straw pulping process was used as substrate for biotransformation studies. Lignin in solution was made to react with the versatile peroxidase, previously isolated from *Bjerkandera* sp. (B33/3), in the absence of any external mediators. Gel filtration was used to follow the evolution in molecular mass distribution of lignin (Zimmerman et al., 1988). The effects of the prevailing reaction conditions upon the decolourisation processes were studied.

2. Materials and methods

2.1. Feedstock source

Sulphur-free lignin was obtained from Granit SA (Lausanne, Switzerland). A versatile peroxidase (named RBP) from a *Bjerkandera* sp. strain was purified, as described elsewhere (Moreira et al., 2006).

2.2. Experimental design and statistical analysis

Standard 2^3 full factorial design methodology was used to evaluate the effect of some important parameters on RBP reactions, as detailed in Table 1. The parameters tested were reaction time, initial reaction pH, and lignin:enzyme ratio. Duplicates (i.e., replicates run under the same experimental conditions) were obtained for half of the experiments, and used to estimate the standard error of the overall mean and associated with each major and interaction effects. Significance was set at the 0.05 confidence level, using Fisher's distribution. Statistical analysis was performed using the experimental design module of the STATISTICA v. 5.5 software.

2.3. Reaction conditions

In a typical experiment, the pH of a lignin preparation (0.005 g solubilized in 100 mM NaOH) was adjusted to the intended value (4.0 or 5.3) with 100 mM sodium tartrate buffer; the appropriate amount of RBP was then added to reach the desired lignin:enzyme ratio (800:1 or 1500:1). The final volume (5 mL) of the assay aliquot was made up with the same buffer. The reaction was started with addition of 0.05 mL of 20 mM hydrogen peroxide. The reaction was conducted at 30 °C, under stirring at 90 rpm, for the appropriate period (2.0 or 4.0 h), during which 0.05 mL of 20 mM hydrogen peroxide was added every 60 min. At the end of the reaction, the medium was filtered through Whatman no. 1 (Middlesex, UK) filter paper and the supernatant taken for analysis. Controls were done at the two pH values tested (4.0 and 5.3), in the presence and absence of the denatured enzyme. The enzyme was denatured by heating to 106 °C for 10 min. No difference in the decolourisation reaction was observed in the absence or presence of the denatured enzyme. Following the experimental design methodology, 12 runs (8 different conditions and 4 replicas) were carried out (see Table 2) and analysed against the controls.

Table 1
Processing variables tested (in actual and coded form) pertaining to decolourisation reactions initiated with peroxidase/H_2O_2 systems

Variable		Peroxidase reaction	
Actual	Coded	Level −1	Level +1
Reaction time (h)	1	2.0	4.0
pH	2	4.0	5.3
Lignin:enzyme ratio (w/w)	3	800	1500

Table 2
Decolourisation yields obtained in the various experiments pertaining to decolourisation reactions initiated with peroxidase/H_2O_2 systems[a]

Run	Variable			Decolourisation yield (%)	
	1	2	3	1st replicate	2nd replicate
1	−1	−1	−1	57.5	—
2	+1	−1	−1	55.7	61.2
3	−1	+1	−1	0.0	—
4	+1	+1	−1	0.0	11.8
5	−1	−1	+1	66.7	68.5
6	+1	−1	+1	74.9	—
7	−1	+1	+1	10.1	—
8	+1	+1	+1	44.7	50.6

[a] Replicate values result from two independent experiments.

2.4. Reaction features

The reaction of lignin, as catalysed by our enzyme, was quantified via decrease in the true colour of samples (20-fold diluted in 100 mM NaOH), determined at 455 nm using a DR/1020 colorimeter (Hach, Loveland, OH, USA). This method, also employed routinely in the pulp and paper industries to measure the true colour of effluents containing lignin in APHA Pt–Co colour units, was performed according to manufacturer's instructions. The apparent reaction yield was calculated based on decolourisation of reaction samples, with reference to control samples.

The molecular mass distribution of the soluble fragments of lignin (which is correlated with the true colour of the sample) was determined by gel filtration chromatography in a 40 × 2.5-cm Sephadex G-100 column (Sigma-Aldrich, St. Louis, MO, USA); elution was at 0.5 mL min^{-1}, using 100 mM NaOH (pH 13) as eluant; the effluent was monitored spectrophotometrically at 280 nm. The gel filtration system was calibrated with