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Trametes versicolor ligninase: isozyme sequence homology and substrate specificity

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The substrate specificity of three ligninase isozymes from the white-rot fungus *Trametes versicolor* has been investigated using stereochemically defined synthetic dimeric models for lignin. The isozymes have been found to attack non-phenolic β-*O*-4 as well as β-1 lignin model compounds. This finding confirms the classification of the isozymes from *T. versicolor* as ligninases. The amino-terminal residues of the three isozymes from *T. versicolor* have been determined using Edman degradation. Minor differences found between the sequences suggest the existence of several structural genes for ligninase in *T. versicolor*. Comparisons have been made with the sequences of three previously reported ligninases from *Phanerochaete chrysosporium*, another lignin-degrading fungus. One of the sequences from *P. chrysosporium* is distinctly more similar to the *T. versicolor* isozymes than to the other two sequences from *P. chrysosporium*.

Ligninase isozyme; Amino acid sequence homology; Lignin model compound; (*Trametes versicolor*)

1. INTRODUCTION

The white-rot fungus *Trametes (Coriolus) versicolor* has been shown to excrete ligninase [1], one of the enzymes believed to be involved in the degradation of lignin. In previous papers the purification of isozymes of ligninase and Mn(II)-dependent peroxidase from *T. versicolor* has been described [2,3]. The heme-containing exoenzyme ligninase was first reported in 1983 [4,5] from studies of another white-rot fungus: *Phanerochaete chrysosporium*. Since then, this peroxidase has been the subject of extensive investigations in several laboratories to elucidate the mechanism for the enzymic degradation of lignin [6]. Sequences of isolated cDNA clones have been published for some of the *P. chrysosporium* ligninase isozymes [7,8]. In this paper we present the amino-terminal sequences of three isozymes of *T. versicolor* ligninase and compare them with those previously reported from *P. chrysosporium*. We also include some observations concerning the substrate specificity of the *T. versicolor* ligninase isozymes.

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Abbreviations: SDS-PAGE, SDS-polyacrylamide gel electrophoresis; DMSO, dimethyl sulfoxide

2. MATERIALS AND METHODS

2.1. *Organism and culture conditions*

Trametes versicolor (PRL 572) was grown as described in [2]. The carbon-limited culture medium (150 ml medium in 1000 ml Erlenmeyer flasks) was harvested after 8 days.

2.2. *Purification of ligninase isozymes*

Ligninase isozymes were isolated by the procedure described previously [2], which includes anion-exchange chromatography and chromatofocusing. After a thorough dialysis against distilled water, the purified isozymes were lyophilized for further analysis by amino-terminal sequencing. Here we describe the sequencing and enzymic properties of the three isozymes isolated in largest amounts. It should be emphasized that *T. versicolor* possesses several additional forms of ligninase that occur in smaller amounts and have not yet been characterized. The isozymes examined here are referred to as A, B and C, respec-

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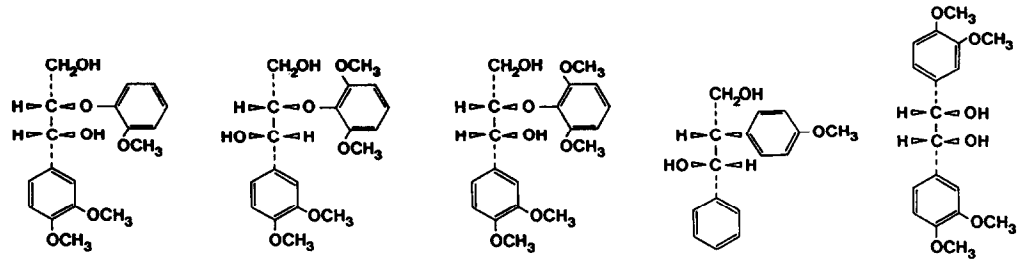

Fig.1. Dimeric lignin model compounds.

tively. They are eluted from the chromatofocusing column (in alphabetical order) between pH 3.4 and 3.2. The molecular masses, obtained by SDS-PAGE, range from 43 to 45 kDa (isozyme C having an apparently higher molecular mass).

2.3. *Amino acid sequencing*

Samples were applied to an Applied Biosystems 470 A protein sequencer. Analysis of the phenylthiohydantoin derivatives was performed with an Applied Biosystems 120 A PTH analyzer.

2.4. *Substrates*

Veratryl alcohol was used for routine assays of ligninase activity [9]. Dimeric lignin model compounds (see fig.1) were synthesized and their structure determined, some of them by X-ray diffraction, as described elsewhere [10–13].

2.5. *Enzyme assays*

Reaction mixtures contained 0.1 M sodium tartrate, pH 2.9, 0.4 mM H_2O_2 and 2 mM substrate. Compounds I, II and III (see fig.1) were dissolved in ethanol (at a concentration of 40 mM) and IV was dissolved in DMSO (also at a concentration of 40 mM). Final concentration of ethanol or DMSO in the reaction mixtures was 5%.

Reactions were monitored at room temperature (approx. 21°C) using a Shimadzu model UV-160 recording spectrometer. The oxidation of veratryl alcohol to veratraldehyde was followed at 310 nm. Susceptibility of the dimeric lignin model compounds towards ligninase was examined by spectrophotometric scanning from 600 nm to 200 nm after mixing of the reactants.

3. RESULTS

The amino acid sequences obtained for the *T. versicolor* isozymes are shown in fig.2. For comparison, the corresponding portions of ligninase isozymes from *P. chrysosporium* have also been included.

With the synthetic dimeric lignin model compounds, substantial reactions could be observed for all three *T. versicolor* isozymes with compounds I, II and IV. With compound III, no reaction could be observed for any of the isozymes.

4. DISCUSSION

The peroxidases from *T. versicolor* studied here were originally isolated on the basis of their capaci-

Ligninase isozyme	Amino-terminal end												
	1	2	3	4	5	6	7	8	9	10	11	12	13
T. versicolor A	Val	Thr	?	Pro	Asp	Gly	Lys	Asn	Thr	Ala	Thr	Asn	Ala
T. versicolor B	Val	Thr	?	Pro	Asp	Gly	Val	Asn	Thr	Ala	Thr	Asn	Ala
T. versicolor C	Val	Ala	?	Pro	Asp	Gly	Val	Asn	Thr	Ala	Thr	Asn	Ala
P. chrysosporium LG4	Val	Ala	Cys	Pro	Asp	Gly	Val	His	Thr	Ala	Ser	Asn	Ala
P. chrysosporium LG5	Ala	Thr	Cys	Ser	Asn	Gly	Lys	Val	Val	Pro	Ala	Ala	Ser
P. chrysosporium ML-1	Ala	Thr	Cys	Ser	Asn	Gly	Lys	Thr	Val	Gly	Asp	Ala	Ser

Fig.2. Amino-terminal sequences of ligninase isozymes from *T. versicolor* (this paper) and *P. chrysosporium* [7,8].

ty to oxidize veratryl alcohol [2]. The finding presented here, that they can also attack dimeric model compounds for lignin strengthens their similarity with lignin-degrading peroxidases previously described from *P. chrysosporium* and supports a classification of the isozymes from *T. versicolor* studied here as ligninases.

Of the model compounds tested, the arylglycerol-β-aryl ethers (I and II) mimic the most frequent type of intermonomeric linkage (β-O-4) in lignins [14]. Compound I is a well-known substrate for the ligninase from *P. chrysosporium* and veratraldehyde has been identified as one of the reaction products [15]. Compounds, related to I, have been shown to become degraded in cultures of *T. versicolor* [16]. As expected, compound I is attacked by the *T. versicolor* ligninase isozymes. Compound II was also known to be a substrate and the *erythro* and *threo* forms were both found to be attacked.

No reaction was detected with the 1,2-diaryl-1,3-propanediol (compound III), which also represents an intermonomeric linkage (β-1) in lignin. A compound with a similar linkage, 1,2-bis(3,4-dimethoxyphenyl)-1,3-propanediol, has previously been reported as a substrate for ligninase from *T. versicolor* [1]. A discrepancy in susceptibility between the two diols could be anticipated from the difference in methoxy substituents which are known to affect the susceptibility towards oxidation [15].

Compound IV, *meso*-1,2-bis(3,4-dimethoxyphenyl)-1,2-ethanediol, was found to be readily attacked by the *T. versicolor* isozymes. A related compound, 1-(3,4-dimethoxyphenyl)-2-phenyl-1,2-ethanediol, has been investigated by Hammel et al. [17] and shown to exhibit a simple pattern of degradation, being quantitatively cleaved to give two molecules of aldehyde: veratraldehyde and benzaldehyde. The compound examined here (IV) has a symmetrical structure and would be anticipated to give two molecules of veratraldehyde upon cleavage. Compared with veratryl alcohol, it would be expected to provide twice the amount of veratraldehyde per oxidizing equivalent. The initial increase in absorbance at 310 nm was found to be considerably larger than for veratryl alcohol. However, the low solubility in the reaction medium makes compound IV more difficult to handle than veratryl alcohol. This drawback could be largely eliminated by replacing IV with the diastereomeric (±)-1,2-bis(3,4-dimethoxyphenyl)-1,2-ethanediol which is more soluble in the reaction medium. The synthesis of water-soluble substrates chemically related to compound IV is in progress.

As indicated in fig.2, the identity of the residue at position 3 in the *T. versicolor* sequences has not yet been finally settled. The amino-terminal regions of the three isozymes from *T. versicolor* differ slightly in sequence. This finding would imply the presence of at least three structural genes for ligninase in *T. versicolor*. It is interesting to note that the isozymes from *T. versicolor* are quite similar to one of the forms of ligninase from *P. chrysosporium*, namely LG4. In fact, the amino-terminal end of LG4 is more similar to the *T. versicolor* isozymes than to the other isozymes from *P. chrysosporium*, LG5 and ML-1, which are closely related to each other as known from the sequences of their structural genes [7,8]. The finding that the sequence homology of amino-terminal regions can be greater between species than within species indicates that this portion of the structure is important for the function of the isozymes and might reflect the occurrence of groups of ligninase isozymes differing in physiological role. A structure corresponding to the amino-terminal ends of LG5 and ML-1 has not yet been found in *T. versicolor*, but several additional *T. versicolor* ligninase isozymes remain to be characterized.

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