N-linked carbohydrate chains protect laccase III from proteolysis in Coriolus versicolor

AKIFUMI YOSHITAKE,† YOSHIHIRO KATAYAMA,‡ MASAYA NAKAMURA,§ YOUSUKE IIMURA, SHINYA KAWAI and NORIYUKI MOROHOSHI

Laboratory of Wood Chemistry, Faculty of Agriculture, Tokyo University of Agriculture and Technology (Tokyo Noko University), Fuchu, Tokyo 183, Japan

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The structure and function of the N-linked carbohydrate chains in laccase III, one of the ligninolytic glycoenzymes from the white-rot fungus Coriolus versicolor, have been partially characterized using endoglycosidases (Endo F and Endo H) and the N-asparagine-specific inhibitor, tunicamycin. In the presence of 10 μg tunicamycin ml⁻¹ laccase and proteinase activities in culture filtrates of C. versicolor were measured over 3 weeks. Laccase activity was slightly decreased by the addition of tunicamycin, whereas proteinase activity was increased. The N-linked carbohydrate chains were not necessary for laccase secretion and activity. Endo-glycosidase digestion showed that laccase III contained at least four N-linked carbohydrate chains, of which two were high-mannose type or hybrid type and two were complex type. Judging from the differences in the resistance of the native and the carbohydrate-depleted laccase to proteolytic digestion and high temperature, the four N-linked carbohydrate chains have important protective functions against proteolytic attack and elevated temperature.

Introduction

Lignin is a complex random phenylpropanoid polymer that comprises 20–30% of woody plants, to impart structural rigidity. Lignin is extremely resistant to microbial attack in nature, but white-rot basidiomycetes are able to degrade lignin by the action of extracellular enzymes such as laccase and lignin peroxidase (Evans & Frazer, 1987; Reid et al., 1982).

Laccase (EC 1.10.3.2) is synthesized in higher plants (Zouari et al., 1987; Takahashi & Hotta, 1986) and fungi (Froehner & Eriksson, 1975; De Vries et al., 1986; Wood, 1980). Coriolus versicolor produces several laccases, including laccase III, which plays an important role in lignin biodegradation by this fungus. These laccases are secreted into the extracellular medium as isozymes. Laccase III is the most abundant isoenzyme synthesized by this micro-organism during wood decay (Morohoshi et al., 1989). Laccase III is an anionic glycoprotein, containing 10% carbohydrate, that is able to degrade and solubilize ¹³C- and ¹⁴C-labelled high-molecular-mass synthetic lignin (Limura et al., 1991, 1992), and some lignin model compounds (Kawai et al., 1988). Recently, we determined the genomic DNA sequences encoding laccase III and found four possible N-glycosylation sequences (Limura et al., 1992).

However, it has not yet been shown how laccase III is synthesized and secreted by this fungus, nor have roles for the carbohydrate chains in this glycoenzyme been elucidated. In particular, it is unclear how the secreted enzyme is protected from proteolytic enzymes produced by the fungus (Venables & Watkinson, 1989). In the nitrogen nutrition of the wood rot fungus, proteinases have an important role in mycelium growth by hydrolysis and utilization of fungal protein in senescent mycelium.

The aim of this work is to investigate the structure of the N-linked carbohydrate chains in laccase III, and their function in the secretion and stability of the enzyme. Tunicamycin and endoglycosidase were used to produce...
laccase III deficient in N-linked carbohydrate chains. The resulting enzyme displayed increased proteinase and temperature sensitivities. The implications of these data for wood degradation are discussed.

Methods

Micro-organism cultivation. Coriolus versicolor IFO8753 was cultivated in a liquid medium consisting of 30 g glucose, 10 g peptone, 1.5 g KH₂PO₄, 0.5 g MgSO₄·7H₂O, 2 mg thiamine hydrochloride and 16 mg CuSO₄·5H₂O per litre of distilled water, at 28 °C with orbital shaking, as described previously (Morohoshi et al., 1987a). Tunicamycin (TM) was added to the culture medium as a 1 mg ml⁻¹ stock solution. If the TM concentration in the culture medium increased to 1 mg laccase III in complete Freud’s adjuvant. Blood samples were taken after four weeks. The specificity and activity of the antisera was characterized using enzyme-linked immunosorbent assay (Nakane & Pierce, 1967) and double radial immunodiffusion (Crowle, 1961). It was shown previously that the antisera cross-reacts with laccase III strongly, and the other isozymes weakly. The antisera did not cross-react with other proteins produced by Coriolus versicolor.

Isolation of N-linked-carbohydrate-chain-depleted laccase III from Coriolus versicolor treated with tunicamycin. Preliminary experiments examined the effect of tunicamycin and DMSO on mycelial growth, the laccase activity and the total extracellular proteins. DMSO at 1% (v/v) had no influence on any of the experiments carried out. TM was added to the culture medium as 100-fold concentrated solution in DMSO (100 x stock solution). If the TM concentration in the culture medium was increased from 10 or 13 μg ml⁻¹ to 15 μg ml⁻¹, laccase activity in the medium slowly decreased. Accordingly, in the present study, TM was added to the culture medium to a final concentration of 10 μg ml⁻¹ three days after the beginning of cultivation. Eleven days after TM addition, N-linked-carbohydrate-chain-depleted laccase III (TM-laccase III) was isolated from culture media by column chromatography on DEAE-Toyopearl 650M (Tosoh, Japan), as described previously (Morohoshi et al., 1987b). In the isolated enzyme preparation, three or four different sizes of protein were detected by SDS-PAGE, but all showed the same P-I value (3.5) as that of native laccase III by isoelectric focusing analysis.

Deglycosylation of laccase III. Laccase III was purified according to the methods described above. The enzyme was then digested with endo-β-N-acetylglucosaminidase F or H (Endo F/H; Boehringer Mannheim). Laccase III (1 mg) was dissolved in 1 ml 100 mm-sodium acetate buffer (pH 6.0) and incubated for 6–24 h at 37 °C with 1 unit of Endo F or Endo H.

Enzyme assay. Laccase III activity was determined using a colorimetric assay at 25 °C. The assay mixture consisted of 100 μl enzyme solution (5–10 μg), 500 μl 1 mm-guaiacol solution dissolved in 200 mm-sodium acetate buffer (pH 4.0) and 3 ml 200 mm-sodium acetate buffer (pH 4.0). The reaction was initiated by the addition of guaiacol as the substrate and the absorbance at 420 nm was monitored. One unit of laccase III activity was defined as the amount of enzyme that oxidized 1 μmol guaiacol min⁻¹. The proteinase activity was determined using an azocasein hydrolysis method (Venables & Watkinson, 1989). Units of proteolytic activity in culture filtrate were expressed as (μg azocasein hydrolysed) h⁻¹ (ml culture filtrate)⁻¹.

SDS-PAGE. Western blotting and immunostaining of laccase III. SDS-PAGE was done with 10% (v/w) acrylamide gel and 0.1% SDS according to Laemmli (1970). Protein was stained with Coomassie Brilliant Blue R.

Following electrophoresis, the protein on the gel was electrophoretically transferred to nitrocellulose membrane using a Semidry blotter (Sartorius). The immunoblotting assay was carried out using the Bio-Rad Immun-Blot Assay Kit. Anti-laccase-III rabbit antiserum was used as a first antibody. Alkaline phosphatase anti-rabbit IgG conjugate was employed as a second antibody. The conjugate was detected by its capacity to cleave 5-bromo-4-chloro-3-indolyl phosphate (provided with the Immun-Blot Kit).

Protease susceptibility and thermal stability of carbohydrate-depleted-laccase III. Native and carbohydrate-depleted laccase III (100 μg) were incubated with 400 μg proteinase F (Sigma protease Type XIV) in 1 ml 100 mm-sodium phosphate buffer (pH 7.5) at 37 °C. Laccase III activity was assayed periodically, and the reaction mixtures were subjected to SDS-PAGE. The thermal stability of laccase III was determined by preincubation at various temperatures for 10 min in 200 mm-sodium acetate buffer (pH 4.0). The pH stability was measured over the pH range 3–8, using 200 mm-sodium acetate buffer (pH 3.0–5.5) and 200 mm-sodium phosphate buffer (pH 6.0–8.0). After 2 h at various pHs in 100 μl buffer, the enzyme solutions were added to 3 ml 200 mm-sodium phosphate buffer (pH 6.0) and remaining enzyme activity was measured.

Analytical methods and chemicals. Protein was determined by the Bio-Rad protein assay kit with bovine serum albumin as the standard. TM, prepared by a previously described method (Takatsuki et al., 1970), was a gift from Professor M. Yamasaki of the University of Tokyo, Japan.

Results

Effects of tunicamycin on carbohydrate chains of laccase

The effect of tunicamycin (TM) on mycelial growth, on the total activity of laccase III and production of extracellular proteins in culture media was determined. TM concentrations below 10 μg ml⁻¹ had little effect on mycelial growth, the laccase activity and total extracellular proteins (data not shown). Laccase and proteinase activities in culture media were measured over a 3 week period (Fig. 1). The high level of proteinase activity detected in the culture filtrate was elevated in cultures containing 10 μg TM ml⁻¹. Laccase activity in the culture filtrate was slightly reduced by the addition of TM.

To demonstrate a possible effect of TM on N-glycoside formation in laccase III, extracellular proteins derived from cultures containing TM were isolated after various incubation periods, subjected to SDS-PAGE and immunoblotted with anti-laccase-III antiserum (Fig. 2).
N-linked carbohydrate chains of laccase III

Incubation time (d)

Fig. 1. The time course changes of laccase (a) and proteinase (b) activity in culture filtrate with (○) and without (●) tunicamycin added at time indicated by arrow. Laccase activity was expressed as light absorbance of guaiacol oxidation products ($A_{420}$, 5 min incubation). Proteinase activity was expressed as light absorbance of azocasein hydrolysates ($A_{450}$, 1 h incubation).

The native laccase III migrated with an apparent molecular mass of 66 000 Da. Besides the native laccase band, one weak and two strong protein bands were evident in Western blots of proteins isolated from TM-containing cultures (Fig. 2, lanes 5–8). These results indicated that TM inhibited N-glycosylation at three glycosylation sites under our conditions, and that N-glycosylation at these sites was not necessary for secretion of this enzyme.

To examine the enzymic properties of TM-laccase III, carbohydrate-chain-depleted laccase III was isolated from the culture filtrate after 14 d incubation (Fig. 3, lane T). These proteins had the same pI value (3.5) (data not shown). Laccase activity in this enzyme preparation was similar to native laccase III (Table 1). These results show that N-linked carbohydrate chains in laccase III are not necessary for enzyme activity.

In culture filtrate containing TM, the carbohydrate-depleted laccase III was weakly detected in 4-d-old cultures, and was absent in 5-, 7- and 9-d-old cultures. Over these incubation periods, high levels of proteolytic activity were detected (Fig. 1). After 12 d incubation, the carbohydrate-chain-depleted laccase III reappeared. These results indicate that the N-linked carbohydrate depleted laccase III was probably degraded by the high proteolytic activity evident in cultures 5–9 d old.

Structure of the N-linked carbohydrate chains of laccase III

Endo-β-N-acetylgalactosaminidase F and H hydrolyse the glycosidic bonds between the core di- N-acetychitobiose moieties of asparagine-linked oligosaccharides of various glycoproteins, resulting in the release of the carbohydrate moieties. These enzymes have been used to elucidate the structure and function of carbohydrate chains associated with glycoproteins.

Native laccase III was digested with Endo F and H to generate carbohydrate-depleted laccase III (EGF-laccase III and EGH-laccase III). The reaction mixtures were subjected to SDS-PAGE. The two carbohydrate-depleted protein bands were generated by endoglycosidase H (Fig. 3), which were designated EGH-laccase III. Endo F digestion of laccase III also generated four carbohydrate-depleted proteins which were named EGF-laccase III. These results show that laccase III contained at least four N-linked carbohydrate chains, of which two were probably high-mannose-type or hybrid-type carbohydrate chains and two chains were complex-type (Kornfeld & Kornfeld, 1985). These four protein bands differed in molecular mass by about 2000–3000 Da.

Effects of proteinase on the native and carbohydrate-depleted laccase III

To examine the proteinase susceptibilities of the native, TM- and EGF-laccase III, these enzymes were incubated with proteinase E and the remaining catalytic activity assayed. As shown in Fig. 4, there was a remarkable difference in enzyme activity on exposure to proteinase E. After incubation for 6 h, the native enzyme still showed about 75% of the original activity. On the other hand, the remaining activities of TM- and EGF-laccase III were about 40% and 25%, respectively.

We confirmed the proteolytic degradation of laccase III with SDS-PAGE (Fig. 5). The native laccase III was
largely resistant to proteolysis. In contrast, the carbohydrate-depleted proteins in TM- and EGF-laccase III were degraded and disappeared. These results indicate that four N-linked carbohydrate chains of laccase III made important contributions to protecting against proteolytic degradation.
Table 1. Kinetic properties of native and carbohydrate-depleted laccase III

<table>
<thead>
<tr>
<th></th>
<th>Activity [mU (mg protein)⁻¹]</th>
<th>$K_m$ (mM)</th>
<th>$1/K_n$ (mM⁻¹)</th>
<th>$V_{max}$ (mm min⁻¹)</th>
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<tbody>
<tr>
<td>Native laccase III</td>
<td>0.210</td>
<td>1.15</td>
<td>0.86</td>
<td>$7.7 \times 10^{-2}$</td>
</tr>
<tr>
<td>TM-laccase III</td>
<td>0.214</td>
<td>0.70</td>
<td>1.43</td>
<td>$3.2 \times 10^{-2}$</td>
</tr>
<tr>
<td>EGF-laccase III</td>
<td>0.197</td>
<td>0.69</td>
<td>1.45</td>
<td>$3.4 \times 10^{-2}$</td>
</tr>
</tbody>
</table>

Stabilities and enzymic properties of carbohydrate-depleted laccase III

The catalytic properties of carbohydrate-depleted laccase III were compared with those of the native enzyme (Table 1). $K_m$ and $V_{max}$ increased reproducibly as the enzyme become more heavily glycosylated. These results indicate that $N$-linked carbohydrate chains might have an effect on the accessibility to the substrate ($K_m$) and the rate of reaction ($V_{max}$), but the apparent nature of the enzymic action was not influenced.

To examine the thermal stability of these enzymes, the remaining activity at various temperatures was determined. As shown in Fig. 6, the native laccase III retained catalytic activity at 50 °C. In contrast, TM- and EGF-laccase III were less stable than the native enzyme above 40 °C. Differences in the pH stability and optimum pH range for the three enzyme types could not be detected.
Discussion

Laccase III, a ligninolytic phenoloxidase produced by the typical wood-rot fungus *Coriolus versicolor*, is an anionic glycoprotein (pI 3.5). In wood-rotting by the fungus, laccase III was secreted onto the wood surface in substantial quantity. Recently, we determined the enzymic functions of laccase III on polymeric lignin by the use of $^{13}$C- and $^{14}$C-labelled synthetic high-molecular-mass lignin and $^{13}$C-NMR spectroscopy (Iimura et al., 1991, 1992). In these experiments, we observed that laccase III could attack $^{13}$C-labelled polymeric lignin, degrade the framework structure loosely and introduce additional hydrophilic groups. Water-soluble material was also produced from $^{14}$C-labelled polymeric lignin by laccase III.

In this study, we elucidated the function of the $N$-linked carbohydrate chains on laccase III in lignin degradation by this fungus, based on experiments using TM and endoglycosidases H and F. In order to assess the physiological significance of the $N$-linked carbohydrate chains, we used TM, which is able to inhibit $N$-glycosylation of glycoproteins (Tamura, 1982). If $N$-linked carbohydrate chains of laccase III contributed to the proper folding of the enzyme for catalytic activity, enzyme activity of TM-laccase III would be expected to be lower than that of native laccase. In this study, we observed that the activity of TM-laccase III was similar to that of the native enzyme (Fig. 3 and Table 1), and deduced that the $N$-glycosylation process was not essential for proper folding of the peptide chain. Furthermore, the $N$-linked carbohydrate chains did not constitute a signal for protein secretion.

Structural analysis of the carbohydrate chains by endoglycosidase H and F showed that laccase III contained at least four $N$-linked carbohydrate chains, in which two chains were high-mannose-type or hybrid-type and two were complex-type structures. The carbohydrate-depleted laccase III derivatives (EGF-laccase III and TM-laccase III) were sensitive to proteolysis. The carbohydrate-depleted laccase III was not detected in 5-, 7- and 9-d-old culture filtrates, in which proteinase levels were high, implying that the carbohydrate chains of laccase III have a protective function against proteolytic attack.

Recently, Venables & Watkinson (1989) investigated the occurrence of proteolytic activity in many wood-rotting basidiomycetes and detected strong proteolytic activity in the growing mycelia. Wood can act as sole substrate, but alone does not supply sufficient nutrients to maintain growth of wood rot fungi. In the nitrogen nutrition of these wood rot fungi, proteinases have essential functions for growth in acting on senescent mycelium to utilize fungal protein as a nutrient. However, it was generally observed that ligninolyis by wood rot fungi such as *Phanerochaete chrysosporium* or *C. versicolor* was suppressed by nitrogen compounds in the media (Fenn & Kirk, 1981; Dodson et al., 1987). If laccase is secreted on the wood surface in large quantities and is rapidly degraded to produce free amino acids under proteolytic conditions, production of other ligninolytic enzymes such as lignin peroxidase could be suppressed and consequently wood degradation inhibited.

In this study, the $N$-linked carbohydrate chains of laccase III were shown to have important protective functions. For effective wood rotting and mycelial growth in wood, lignin degradation and cellulolytic reactions must proceed efficiently in the presence of proteinase. Therefore, the protective function of the $N$-linked carbohydrate chains on laccase III may be important in achieving effective wood degradation in vivo.

References


