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Physical and Chemical Properties of an Extracellular Low-Molecular-Weight Substance from the Brown-Rot Basidiomycete *Fomitopsis palustris*

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A low-molecular-weight, extracellular substance with one-electron-oxidizing activity was isolated from wood-degrading cultures of the brown-rot basidiomycetous fungus *Fomitopsis palustris*. The substance was partially purified by acetone precipitation, gel-filtration chromatography on Sephadex G-50 and G-25, and DEAE-Sepharose ion-exchange chromatography. The partially-purified material was a glycopeptide composed of (54%, w/w) protein and (42%, w/w) neutral carbohydrate, containing (0.06%, w/w) Fe(II) by weight. The molecular weight as estimated by Tricine-SDS-PAGE was 7,200-10,000; whereas the molecular weight as determined by size-exclusion or gel-filtration chromatography was 1,000-5,000. The glycopeptide reduced O_2 to H_2O_2 and Fe(III) to Fe(II), and thus could generate hydroxyl radical via a Fenton reaction. One mg of the partially-purified glycopeptide reduced 1.7 μ mol of Fe(III) to Fe(II) and contained at least 0.6 μ mol of α -hydroxyketone or endiol groups. Most of the α -hydroxyketone groups were 1-amino-2-ketoses produced by the condensation of side-chain amino groups and carbohydrate aldehydes. The function of the glycopeptide in brown-rot decay is discussed.

Key words : *Fomitopsis palustris*/Brown-rot decay/Glycopeptide/One-electron oxidation/Hydroxyl radical.

INTRODUCTION

Although brown-rot fungi slightly degrade the lignin in wood, they preferentially degrade the polysaccharide components, leaving an amorphous, brown, crumbly residue composed largely of lignin (Filley et

al., 2002; Highley, 1987). Brown-rot fungi degrade cellulose in wood even though they lack enzymes which can attack and hydrolyze crystalline or natural cellulose. However, brown-rot fungi can transform lignin-related model compounds under conditions in which the fungi actively degrade crystalline cellulose while brown-rot fungi are unable to degrade the cellulose substrate under conditions in which the fungi are unable to transform the lignin-related compounds (Enoki et al., 1988; Hirano et al., 1997). This indicates that brown-rot fungi may possess a unique wood-degrading system that is capable of fully degrading natural cellulose and modifying lignin.

In the early stages of wood degradation by brown-rot fungi, the S_2 layer of the wood cell wall is degraded extensively. However the S_3 layer adjacent to the cell lumen is less affected when it is attacked by

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Abbreviations: KTBA: 2-keto-4-thiomethylbutyric acid; $\cdot OH$: hydroxyl radical; T-SDS-PAGE: tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DMSO: dimethyl sulfoxide; SOD: superoxide dismutase; DMNA: *N*, *N'*-dimethyl-4-nitrosoaniline; TCA: trichloroacetic acid; TBA: thiobarbituric acid; 5-HMF: 5-hydroxymethylfurfural

fungus hyphae from the lumen (Enoki et al., 1988; Kuo et al., 1988). Fungal enzymes, such as cellulase and peroxidase, are too large to penetrate wood cell walls at early stages of degradation (Flournoy et al., 1991; Srebotnik and Messner, 1991). The one-electron-oxidizing activity in cultures of brown-rot fungi is proportional to the degradation of lignin-related model compounds, natural cellulose substrates, and wood in the cultures. This one-electron-oxidizing activity appears to result from hydroxyl radicals ($\cdot\text{OH}$) is a strong oxidant that severely degrades the cellulose in wood, but does not delignify wood significantly (Chirat and Lachenal, 1997). However $\cdot\text{OH}$ attacks lignin through a variety of reactions (Filley et al., 2002; Gierer et al., 1992). Some brown-rot fungi degrade lignin-related model compounds to yield relatively large amounts of the same products that are obtained when the lignin model compounds are attacked by $\cdot\text{OH}$ (Espejo et al., 1990; Zohar et al., 1998). Furthermore, endoglucanases isolated from cultures of a brown-rot fungus hydrolyze very little of the cellulose in wood, unless the wood has been pretreated with $\cdot\text{OH}$ (Rättö et al., 1997). These results suggest that brown-rot fungi have a unique non-enzymatic degradative system that may involve the diffusion of $\cdot\text{OH}$ into secondary cell walls, with the degradation occurring at some distance from the fungal hyphae in the lumen.

An extracellular metabolite, 2,5-dimethoxy-1,4-benzoquinone, has been isolated from liquid cultures of the brown-rot fungus, *Gloeophyllum trabeum*. This benzoquinone can reduce Fe (III) to Fe (II) and O_2 to H_2O_2 , thereby promoting a Fenton reaction between Fe (II) and H_2O_2 (Zohar et al., 1998). A low-molecular-weight peptide (<5,000) that is a high-affinity Fe (III) chelator and that can reduce Fe (III) to Fe (II) also has been isolated from culture extracts of *G. trabeum* (Wang and Gao, 2003).

Extracellular low-molecular-weight substances that catalyze a redox reaction between O_2 and an electron donor to produce $\cdot\text{OH}$ have been isolated from wood-degrading cultures of the brown-rot fungi *G. trabeum* and *Fomitopsis palustris* (Enoki et al., 1992; Hirano et al., 1995 and 1997). The low-molecular-weight substance from *F. palustris* cultures reduces Fe (III) to Fe (II) and strongly chelates Fe (II) (Hirano et al., 1995). Most of the extracellular $\cdot\text{OH}$ produced in intact cultures of *F. palustris* is generated by a redox reaction between O_2 and an electron donor that is catalyzed by this low-molecular-weight substance (Hirano et al., 1995). During the early stages of wood degradation by *F. palustris*, the low-molecular-weight substance is localized in the fungal cytoplasm, the

fungus cell wall, the extracellular sheath surrounding the fungal cell wall, and throughout the wood cell wall, suggesting that it diffuses from the S_3 layer into the S_2 layer and the middle lamella (Hirano et al., 2000). Herein we report on the physical and chemical properties of this extracellular low-molecular-weight substance from cultures of *F. palustris*.

MATERIALS AND METHODS

Organism

The brown-rot fungus, *F. palustris* (Berk. Et Curt Murr. FRI) 0507, was used throughout this study.

Culture conditions

Basal agar medium was prepared as previously described (Enoki et al., 1988) except that the medium contained 0.2%, (w/v) glucose and ten-fold concentrated trace elements. Wood meal from Japanese beech (*Fagus crenata* Blume) was extracted twice with acetone and dried. The wood meal (2 g), wetted with 1.2 ml water, was sterilized and sprinkled evenly over the surface of the basal medium (30 ml) in 300-ml Erlenmeyer flasks. The cultures were inoculated with a small piece of fungal mat and incubated at 28 °C.

Isolation and partial purification of the extracellular substance

After 30 days of incubation, 30 ml of distilled water was added to the cultures. The mixtures were stirred with a glass rod and centrifuged at $10,000 \times g$ at 4 °C for 20 min. The supernatants were filtered. The extracellular medium from 100 flasks was acetone-precipitated (70%, v/v), as described previously (Hirano et al., 1995 and 1997). The precipitate was dissolved in 100 ml of distilled water and centrifuged at $10,000 \times g$ for 20 min. Aliquots (10 ml) of the resulting supernatant were loaded on a distilled-water-equilibrated Sephadex G-50 column ($3.8 \times 60\text{cm}$) and the column was eluted with distilled water. Fractions were collected, the absorbance at 280 nm was measured, and the ethylene generation from 2-keto-4-thiomethylbutyric acid (KTBA) of the fractions was assayed as described below. Fractions comprising the major peak of ethylene-generating activity were pooled and lyophilized. The lyophilized material was combined and dissolved in 50 ml of distilled water. Aliquots (10 ml) were loaded on a distilled-water-equilibrated Sephadex G-25 column and eluted with distilled water. Fractions were collected and assayed for ethylene generation from KTBA. Fractions comprising the major peak of ethylene-generating activity were pooled and lyophilized. The lyophilized material

was combined and dissolved in distilled water (20 ml). Aliquots (1 ml) of the solution were applied to a column of Hiprep 16/10 DEAE FF (Amersham Biosciences, Sweden), previously equilibrated with 0.02 M Tris-HCl buffer (pH 8.0) and eluted with a linear gradient of 0 to 2 M NaCl by fast-protein liquid chromatography (Amersham Biosciences, Sweden). Fractions containing the major peak of one-electron oxidizing activity, as measured by ethylene generation from KTBA, were pooled and desalted on a column of HiTrap™ Desalting (Amersham Biosciences, Sweden). The desalted solution was lyophilized. A final yield of 50 mg of the extracellular one-electron-oxidizing substance was obtained from 100 culture flasks.

Ethylene generation from KTBA

Ethylene generation from KTBA was measured as described previously (Enoki et al., 1989). Reaction mixtures contained 300 μ l of eluate, 300 μ l of 0.01 M H₂O₂, 300 μ l of 0.16 M Na-acetate buffer (pH 4.5), and 50 μ l of 0.1 M KTBA. After 1 h at 30 °C, 1 ml of gas was removed from the headspace and the ethylene concentration was measured directly by gas chromatography as described previously Enoki et al., 1989).

Electrophoresis

Sample preparation and Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoreses (Tricine-SDS-PAGE) were performed as described by Schägger and Jagow (1987). The slab gels (0.75 mm thick) consisted of three sections: a 15-cm separation gel (16.5% T, 3% C), a 3-cm spacer gel (10% T, 3% C), and a 2-cm stacking gel (4% T, 3% C). T denotes the total percentage concentration of both (acrylamide and bisacrylamide). C denotes the percentage concentration of the crosslinker relative to the total concentration T. The mounted gels were allowed to polymerize overnight at room temperature. Electrophoresis of the samples was performed at room temperature. The initial running conditions were 30 V for 1-2 h. After the samples had entered the stacking gel, electrophoresis was at 80 V. Proteins were visualized by staining with silver nitrate.

Hydroxyl radical production

Hydroxyl radicals were measured by the method of Fukui et al. (1993). The basic reaction mixture contained 1 mg of the partially-purified preparation, 3×10^{-4} mol of dimethyl sulfoxide (DMSO), and Na-acetate buffer (40 mM, pH 4.5) in a total volume of 2 ml in a 33-ml test tube. Hydrogen peroxide (3×10^{-6} mol), NADH (3×10^{-6} mol), NADH plus su-

peroxide dismutase (SOD) (0.3 mg, 2,670 units mg^{-1}), NADH plus catalase (0.3 mg, 42,000 units mg^{-1}), NADH plus *N,N'*-dimethyl-4-nitrosoaniline (DMNA) (6×10^{-6} mol), or ascorbic acid (3×10^{-6} mol) were added to the basic reaction mixture. After 1.5 h of incubation at 30 °C under 100% O₂ or N₂, 1 ml of 2% (w/v) Fast Yellow GC Salt reagent (Sigma Chemical Co., St. Louis, MO, USA) was added to the reaction mixture. The mixture was shaken and allowed to stand for 30 min. Ethyl acetate (10 ml) was added and the mixture was shaken well. After the ethyl-acetate layer was washed with distilled water (10 ml, 3 times), 8 ml of the ethyl-acetate layer was removed and evaporated off under reduced pressure. The residue was dissolved in ethyl acetate (500 μ l), filtered through a Millipore filter (pore size 0.2 μ m), and analyzed by high-performance liquid chromatography as previously described (Lowry et al., 1951).

Protein, amino acid, carbohydrate, and Fe analysis

The protein concentration of the partially-purified preparation was determined by a modification of the method of Lowry et al. (1951). The amino acid composition was determined with an amino acid autoanalyzer (HITACHI, L-8500, Japan), following hydrolysis of the sample with 6 N HCl. The neutral carbohydrate content was determined by the phenol-sulfuric acid assay (Dubois et al., 1956). Monosaccharide composition was measured with a saccharide composition analysis kit (Honen Corporation, Tokyo, Japan) according to the manufacturer's instructions. Following the hydrolysis of the sample, the released monosaccharides were coupled with *p*-aminobenzoic ethyl ester at the reducing end by reductive amination, according to the method of Yasuno et al. (1997). The *p*-aminobenzoic ethyl ester-derivatized monosaccharides were injected into a Honenpak C18 column (75 $\times$ 4.6 mm i.d., Honen Corp., Tokyo, Japan) and detected by fluorescence monitoring according to the method of Yasuno et al., 1997. Ferrous iron content was measured with ferrozine by the procedure of Sørensen (1982).

Fe (III) - and Cu (II) -reducing activity

The reduction of Fe (III) to Fe (II) by the preparation was measured using ferrozine, according to the procedure of Sørensen (1982). Distilled water (500 μ l), with or without 10 ppm Fe (III), was added to an aqueous solution of the partially-purified preparation (35 μ g in 500 μ l). For the control, an aqueous solution (500 μ l) containing 10 ppm Fe (II) or Fe (III) was added to distilled water (500 μ l). After 24 h of incubation at 30 °C, 500 μ l of 1%

ferrozine in distilled water was added. After the solutions were left standing for 24 h at 30 °C, their absorption spectra were measured at 400 nm to 750 nm with a spectrophotometer. The Cu (II) reducing ability of the preparation was measured by the method of Somogyi-Nelson (Somogyi, 1951).

Determination of carbonyl groups

The carbonyl groups, other than those in the peptide bonds, were assayed by the method described in Uchida et al. (1998). Distilled water (500 μ l), with or without 3.5 mg of sample, was added to 0.1% (w/v) 2,4-dinitrophenylhydrazine in 2 N HCl. After 1 h at room temperature, 500 μ l of 20% (w/v) trichloroacetic acid (TCA) was added to each assay. Following centrifugation at 9,500 $\times g$ for 10 min, the supernatants were scanned at 800 nm to 300 nm. The number of carbonyl groups was calculated based on the rate of decrease in absorbance at 365 nm.

Determination of aldehyde groups

Aldehyde groups in the preparation were assayed using methone (dimethyldihydroresorcin). Distilled water (1 ml), with or without 10 mg of the sample, was mixed with 100 μ l of 0.1 M methone in methanol and shaken vigorously for 30 min. Controls contained 100 μ l of 0.1 M methone and 1 ml aqueous solution of 0.012 M formaldehyde or 0.012 M 2,4-dihydroxybenzaldehyde. The mixtures were extracted with 1 ml ethyl acetate. Methone in the ethyl acetate fraction was measured directly by gas chromatography at 160 °C in the isothermal mode using a glass column packed with 3% OV-101 on chromsorb Q 100/120 (Applied Science, Japan). The number of aldehyde groups was calculated based on the loss of methone.

Determination of protein glycosylation

Glycosylation of the N-terminal and side-chain amino acids in the glycopeptide was measured by the thiobarbituric acid (TBA) colorimetric technique of Fluckiger and Gallop (1984). Following the addition of 500 μ l of 0.5 M oxalic acid to 1-ml aqueous solutions containing 0, 1 mg, or 2 mg of the sample, or 25 μ mol of cellobiose (8.6mg), glucose (4.5mg), galactose (4.5mg), or mannotriose (12.6mg), the tubes were mixed and capped with rubber stoppers vented with 26-gauge needles. The tubes were placed in a heating block at 100 °C and the needles were removed after a 10-min equilibration period. After 5 h, the tubes were cooled in an ice bath. The volumes of the solutions were adjusted with water to 1.5 ml. Cold 40% (w/v) TCA (500 μ l) was added to each tube and the tubes were mixed and centrifuged

at 9,500 $\times g$ for 4 min. A 500 μ l aliquot of the supernatant was removed and 500 μ l of a 0.05 M aqueous 2-thiobarbituric acid solution was added. The solutions were incubated at 37 °C for 15 min and at room temperature for 20 min. The absorption spectra were measured at 400 nm to 700 nm. The standard curves were prepared using the μ mol range of 5-hydroxymethylfurfural (5-HMF) and 500 μ l of a 0.05 M aqueous TBA.

RESULTS AND DISCUSSION

Partial purification of the extracellular substance

The Sephadex G-50 gel-filtration chromatography of the acetone precipitate of the extracellular fluid from 30-day-old *F. palustris* cultures was carried out. Only one major peak of one-electron-oxidation activity, as measured by ethylene generation from KTBA, was observed in the low-molecular-weight fractions (1000-5000). This peak corresponded to the maximum absorbance peak at 280 nm. The major peak was pooled and lyophilized. Then the Sephadex G-25 gel-filtration chromatography of this pooled material was carried out. Low molecular-weight fractions (1000-5000), constituting the major peak of the one-electron-oxidation activity, were pooled, lyophilized, and subjected to anion exchange chromatography on HiPrep 16/10 DEAE FF. Fractions, constituting the major peak of the one-electron-oxidation activity were pooled, desalted, and lyophilized. The yield of this partially-purified preparation was 50 mg from 100 30-ml, 30-day-old cultures of *F. palustris*.

Composition and molecular weight

The molecular weight of the partially-purified preparation, as determined by gel-filtration on Sephadex G-25 or Sephadex G-50, was in the range of 1,000-5,000 (data not shown). Tricine-SDS-PAGE of the partially-purified preparation yielded two bands of 7.2 kDa and 10.0 kDa. The partially purified preparation was 42% neutral carbohydrate by weight and 54% protein by weight. The true molecular weight of the preparation could be lower than 7.2 kDa and 10.0 kDa because molecular weights of glycopeptides determined by SDS-PAGE are higher than their true molecular weights (Leach et al., 1980).

Hydroxyl radical generation

The transformation of DMSO to methane sulfonic acid is a specific assay for $\cdot\text{OH}$ (Hirano et al., 1997). The generation of $\cdot\text{OH}$ by the partially-purified glycopeptide in the presence of the electron donor NADH (3×10^{-6} mol) under a 100% O_2 atmosphere (9.2×10^{-8} mol ml^{-1} 1.5h^{-1}) was given a relative

value of 100% (Table 1). In the absence of H_2O_2 or an electron donor such as NADH or ascorbic acid, only a very small amount of $\cdot OH$ was generated under 100% O_2 and almost no $\cdot OH$ was generated under an N_2 atmosphere. In the presence of NADH or ascorbic acid under 100% O_2 , $\cdot OH$ generation was 15-fold and 47-fold higher, respectively, than in the absence of an electron donor. In the presence of NADH, under 100% O_2 , catalase almost completely blocked $\cdot OH$ generation, and the $\cdot OH$ -scavenging agent DMNA suppressed $\cdot OH$ generation. Likewise, SOD inhibited $\cdot OH$ generation in the presence of NADH by about 40% (Table 1). These results indicate that the extracellular glycopeptide from *F. palustris* catalyzes redox reactions between electron donors such as NADH or ascorbic acid and O_2 to produce H_2O_2 via superoxide and further reduces H_2O_2 to $\cdot OH$. We previously demonstrated that the production

of $\cdot OH$ in *F. palustris* cultures is related to the degradation rates of wood, crystalline cellulose, and lignin-model compounds in the cultures (Hirano et al., 1997).

Amino acid, monosaccharide, and Fe content

Amino acid analysis of the partially-purified preparation indicates that it contains 10.4% aspartic acid, 9.1% glutamic acid, 1.3% lysine, and 25.1% NH_3 (Table 2). Thus the substance contains an abundance of amino acids with side-chain amino groups.

The glycopeptide contains unusually high levels of galactose (47%), glucose (22%), and xylose (18%); whereas there is less than 1% *N*-acetyl-*D*-glucosamine and a negligible amount of *N*-acetyl-*D*-galactosamine (Table 3). In glycopeptides, *N*-acetyl-*D*-glucosamine usually combines with the side-chain amino group of asparagine and *N*-acetyl-*D*-

TABLE 1. Generation of the hydroxyl radical by the partially-purified glycopeptide isolated from wood-degrading cultures of *Fomitopsis palustris*.^a

Addition to reaction mixture	Atmosphere ^b	$\times 10^{-8}$ mol ml ⁻¹ 1.5h ^{-1c}	Relative value (%)
None	100% O_2	0.6	6
None	100% N_2	0.3	3
3×10^{-6} mol H_2O_2	100% N_2	4.0	44
3×10^{-6} mol NADH	100% O_2	9.2	100
3×10^{-6} mol NADH, 0.3mg SOD ^d	100% O_2	3.6	39
3×10^{-6} mol NADH, 0.3mg catalase	100% O_2	0.9	10
3×10^{-6} mol Ascorbic acid	100% O_2	28.2	307
3×10^{-6} mol NADH, 6×10^{-6} mol DMNA	100% O_2	3.3	36

^aReaction mixtures contained 1.0mg of the preparation, 3×10^{-4} mol DMSO, and acetate buffer (40mM, pH4.5) in a total volume of 2 ml. Reactions and detection of methane sulfuric acid were as described in the text.

^bReaction mixtures were incubated under 100% O_2 or N_2 , as described in the text.

^cEach value represents the average of triplicate assays.

^dSuperoxide dismutase, 2,670unit/mg.

TABLE 2. Amino acid composition of the partially-purified glycopeptide isolated from wood-degrading cultures of *F. palustris*.^a

Amino acids	%	Amino acids	%
Asparagine	ND ^b	Tryptophan	ND
Aspartic acid	10.4	Threonine	8.9
Alanine	7.8	Valine	3.6
Arginine	1.1	Histidine	0.4
Isoleucine	3.2	Phenylalanine	3
Glycine	7.1	Proline	ND
Glutamine	ND	Methionine	ND
Glutamic acid	9.1	Lysine	1.3
Cysteine	ND	Leucine	5.4
Serine	9.6	NH_3	25.1
Tyrosine	3.7		

^aDetermined with an amino acid autoanalyzer, following hydrolysis of the sample with 6 N HCl.

^bND: Not detectable.

TABLE 3. Monosaccharide composition of the partially-purified glycopeptide isolated from wood-degrading cultures of *F. palustris*.^a

Monosaccharide	% ^b
Galactose	47
Mannose	8
Glucose	22
Arabinose	4
Xylose	18
<i>N</i> -acetyl- <i>D</i> -glucosamine	1
<i>N</i> -acetyl- <i>D</i> -galactosamine	0

^aMonosaccharide composition was determined using a saccharide composition analysis kit, as described in the text.

^bPercentage of total monosaccharides.

FIG. 1. Fe (III) reduction by the partially-purified extracellular glycopeptide from *Fomitopsis palustris* cultures. Absorption spectra were determined at 400 to 750 nm. Fe (III) reduction was measured using 1% ferrozine, according to the procedure of Sørensen (1982), as described in the text. A: 10/3 ppm Fe (II); B: 10/3 ppm Fe(III), 35 μ g glycopeptide sample; C: 10/3 ppm Fe(III); D: 35 μ g glycopeptide sample. Numbers in parentheses indicate the absorbance at 560nm

galactosamine combines with the OH side chains of serine or threonine. These results suggest that carbohydrates other than *N*-acetyl-*D*-glucosamine and *N*-acetyl-*D*-galactosamine are attached to the side-chain amino groups in this glycopeptide.

The partially-purified glycopeptide contained 0.06% ferrous iron by weight, as determined with ferrozine.

Fe (III)- and Cu (II) -reducing activity

The ferric-iron-reducing activity of the partially-purified glycopeptide was measured using ferrozine (Fig. 1, Table 4). Fig. 1 shows that 1 mg of the glycopeptide reduced about 1.7 μ mol of Fe (III) to Fe (II); that is, a single mole of the glycopeptide reduced about 17 moles of Fe (III) to Fe (II), assuming that the molecular weight is 10,000.

The Cu (II)-reducing activity of the glycopeptide, measured by the method of Somogyi-Nelson, was 1.3 μ mol/mg (Table 4).

Carbonyls

The carbonyl groups in the glycopeptide, excepting those in the peptide bonds, were determined with 2,4-dinitrophenylhydrazine, which combines with carbonyl groups, with the loss of water, to form 2,4-dinitrophenylhydrazone, which is insoluble in water. The number of carbonyl groups is calculated based on the decrease in absorbance at 365 nm. Carbonyl groups were present 0.6 μ mol mg^{-1} in the glycopeptide (Table 4).

The aldehyde groups in the glycopeptide were determined using methone, which readily condenses stoichiometrically with aldehyde groups but not with ketones. Aldehyde groups were present in the glycopeptide at less than 3×10^{-9} μ mol mg^{-1} or 0.04 mol mol^{-1} (Table 4). This suggests that most of the

TABLE 4. Fe (III)- and Cu (II)-reducing activities and carbonyl and aldehyde content of the partially-purified glycopeptide isolated from wood-degrading cultures of *F. palustris*.

	μ mol mg^{-1}	mol 10^{-4}g^{-1}
Fe (III) reduction ^a	1.7	17
Cu (II)-reduction ^b	1.3	13
Carbonyl groups ^c	0.6	6
Aldehyde groups ^d	negligible	negligible
1-amino-2-ketoses ^e	0.5	5

^aDetermined using 1% ferrozine, according to the procedure of Sørensen, as described in the text.

^bDetermined by the method of Somogyi-Nelson.

^cDetermined using 2,4-dinitrophenylhydrazine method of Uchida et al. as described in the text.

^eAssayed using methone, as described in the text.

carbonyl groups in the glycopeptide are ketone or enediol groups rather than aldehydes. However 0.6 μ mol of α -hydroxyketone per mg of glycopeptide could reduce, at most, only 1.2 μ mol of Fe (III), less than the measured Fe (III) reduction of 1.7 μ mol per mg of glycoprotein (Table 4).

Glycosylation

Glycosylation of the N-terminal and the side-chain amino groups in the glycopeptide was measured using the TBA method. In the TBA colorimetric method, 1-amino-2-deoxyketoses are dehydrated in boiling oxalic acid and released as 5-HMF. The sugar-free protein is removed by TCA precipitation and the 5-HMF concentration is determined colorimetrically after condensation with TBA (Furth, 1988). One mg of the glycopeptide produced about 0.5 μ mol of 5-HMF, whereas 25 μ mol of cellobiose (8.6mg) produced 0.11 μ mol of 5-HMF (Fig. 2). Glucose and galactose (25 μ mole, 4.5mg) each formed 0.06 μ mol of 5-HMF. Mannotriose (25 μ mol, 12.6 mg) produced 0.15 μ mol of 5-HMF (data not shown). These results suggest that under the acidic conditions polysaccharides were dehydrated in very low yields and were hydrolyzed in high yields (or were hydrolyzed in high yields and were hydrated in very low yields) to 5-HMF in less than 0.3% yields. Thus, most of the 5-HMF produced from the partially-purified preparation came from the carbohydrates condensing with the N-terminal and side-chain amino groups in the glycopeptide. The mole number of side-chain amino groups condensing with carbohydrates to form the glycopeptide could be larger than 0.5 μ mol mg^{-1} , since 5-HMF formation from N- and O-glycosides also is small under acidic conditions (Fluckiger and Gallop, 1984).

Side-chain amino groups in proteins condense with carbohydrate aldehyde groups to yield glycosylamines (Schiff bases), which undergo nucleophile-catalyzed rearrangements to form 1-amino-1-deoxy-D-fructose derivatives (Amadori compounds). These compounds reduce O_2 to H_2O_2 and Fe (III) to Fe (II) (Oak et al., 2000). Thus, iron-containing glycosylated peptides could reduce O_2 to $\bullet\text{OH}$ via H_2O_2 as shown in Fig. 3.

Proposed mechanism for wood decay by brown-rot fungi

On the basis of earlier findings and the results presented here, we propose the following mechanism for wood decay by brown-rot fungi: Fungal hyphae in the wood cell lumen secrete a glycosylated peptide of lower than 10,000 Da. This effector is able to diffuse through the S_3 layer into the S_2 layer and middle

FIG. 2. Absorption spectra of 5-HMF produced from TBA and the carbohydrate released from the partially-purified extracellular glycopeptide from *F. palustris* cultures. Experimental procedures were as described in the text. A: 0.5 mg of the glycopeptide per ml; B: 0.25 mg of the glycopeptide per ml; C: 0.125 μ mol of 5-HMF per ml; D: 0.125/2 μ mol of 5-hydroxymethylfurfural per ml; E: 8.6 mg (25 μ mol) cellobiose per ml; F: 4.5 mg (25 μ mol) of glucose per ml; G: 4.5 mg (25 μ mol) of galactose per ml.

lamella of the cell wall, where it reduces Fe (III) to Fe (II) and chelates Fe (II). Alternatively it may reduce Fe (III) and chelate Fe (II) prior to diffusion into the S_3 layer. The glycopeptide with Fe (II) catalyzes redox reactions between O_2 and an electron donor to produce $\bullet\text{OH}$ via H_2O_2 as shown in Fig. 3. The $\bullet\text{OH}$ attacks cell wall constituents, causing the depolymerization of both crystalline and noncrystalline cellulose. The $\bullet\text{OH}$ also attacks the lignin in the cell wall, causing a variety of reactions. These processes transform the cell wall by cutting channels through the S_3 layer for enzyme diffusion. Enzymes, including endoglucanases, then can penetrate the cell wall and act on hemicellulose and noncrystalline regions of the cellulose that have been attacked by $\bullet\text{OH}$.

Reactions caused by the attack of $\bullet\text{OH}$ on lignin, including the hydroxylation of aromatic rings, demethylation of methoxyl groups on aromatic rings, and

FIG. 3. Proposed mechanism for the generation of $\cdot\text{OH}$ and the reduction of Fe (III) to Fe (II) by the extracellular glycosylated peptide from *F. palustris*.

β -O-4 alkyl aryl ether-cleavage, produce a significant number of dihydroxy aromatic structures (Filley et al., 2002; Henriksson et al., 2000). These dihydroxy aromatic structures could reduce O_2 to H_2O_2 , Fe (III) to Fe (II), and the oxidized form of the glycopeptide to the reduced form (Jensen Jr. et al., 2001; Paszczyński et al., 1999).

The reaction of $\cdot\text{OH}$ with saccharides is initiated by hydrogen subtraction, followed, under aerated conditions, by oxidative C-C cleavage, which can cause depolymerization of saccharides and the formation of new aldehyde compounds (Uchida and Kawakishi, 1988). This series of reactions produces superoxide, which reduces Fe (III) to Fe (II) or generates H_2O_2 by either non-enzymatic or SOD-catalyzed dismutation. Some of the newly-produced aldehydes also may reduce Fe (III).

The ongoing production of $\cdot\text{OH}$ by the glycopeptide in wood cell walls requires a constant supply of O_2 and an electron donor for the reduction of the oxidized form of the glycopeptide. The identification of the electron donor will be the subject of further investigation.

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