Biodegradation **13:** 383–394, 2002.

A peptide-mediated and hydroxyl radical HO·-involved oxidative degradation of cellulose by brown-rot fungi

Wei Wang[1,2] & Peiji Gao[1,*]
[1]*The State Key Laboratory of Microbial Technology, Shandong University, Jinan, 250100;* [2]*School of Chemical and Environmental Engineering, Jinan University, Jinan, 250002, The People's Republic of China (*author for correspondence: e-mail: gaopj@sdu.edu.cn)*

Accepted 29 November 2002

Key words: atomic force microscopy, brown-rot fungi, cellulose degradation, electron paramagnetic resonance, hydrogen bonds, hydroxyl radical HO·

Abstract

A special low-molecular-weight peptide named Gt factor, was isolated and purified from the extracellular culture of brown-rot fungi *Gloeophyllum trabeum* via gel filtration chromatography and HPLC. It has been shown to reduce Fe^{3+} to Fe^{2+}. Electron paramagnetic resonance (EPR) spectroscopy revealed Gt factor was able to drive H_2O_2 generation via a superoxide anion $O_2^{\cdot-}$ intermediate and mediate the formation of hydroxyl radical HO· in the presence of O_2. All the results indicated that Gt factor could oxidize the cellulose, disrupt the inter- and intrahydrogen bonds in cellulose chains by a HO·-involved mechanism. This resulted in depolymerization of the cellulose, which made it accessible for further enzymatic hydrolysis.

Abbreviations: AFM, atomic force microscopy; CMC - carboxymethylcellulose; DMPO, 5,5-dimethylpyrroline-1-oxide; EPR, electron paramagnetic resonance; SEM, scanning electron microscopy; TBA, thiobarbituric acid

Introduction

Brown-rot fungi is a physical group of fungi that cause rapid and extensive degradation of cellulose. The biodegradation of lignocellulose by brown-rot fungi has drawn increasing attention recently not only because of the potential utilization of wood sources, but also because of the requirement for decay protection, especially for commercial antifungal products. However, the mechanism of the cellulose degradation by brown-rotters has not been fully understood. Although brown-rot fungi are able to degrade cellulose, they were thought to have a deficient cellulase system and no cellobiohydrolase (CBH) was found in these fungi (Highley 1988). Enzyme is thought not to be involved in the initial step of wood decay because its size is larger than intact wood cell wall micropores (Carpita et al. 1979; Flournoy et al. 1991). Therefore, some low-molecular-weight biochemical agent which is capable of penetrating cell wall pores is proposed to be involved in depolymerizing the cellulose, thus making the cellulose accessible to further degradation. (Koenigs 1974; Fekete et al. 1989; Jellison et al. 1991; Enoki et al. 1992, 1997; Goodell et al. 1997; Zohar et al. 1999). In 1965, Halliwell found that the hydroxyl radical HO· generated by Fenton's reagent is destructive to cellulose (Halliwell 1965),

$$H_2O_2 + Fe^{2+} \rightarrow HO^{\cdot} + OH^{-} + Fe^{3+}$$

the effects of H_2O_2 and Fe^{2+} on cellulose is similar to that of the brown-rot fungi on cellulose. Therefore hydroxyl radical HO· has been hypothesized to be involved in the cellulose decomposition (Koenigs 1974; Enoki et al. 1989, 1992, 1997; Goodell et al. 1997; Zohar et al. 1999; Murmanis et al. 1988; Veness & Evans 1989; Xu & Goodell 2001) and searching for some low-molecular-weight agent which can mediate the formation of HO· has been the focus of the studies on brown-rotters. One hypothesis concerning the mechanism related to fungal wood degradation

was proposed in 1992, Enoki et al. isolated and partially purified extracellular substances from wood-containing cultures of *Gloeophyllum trabeum*. These substances were characterized to be a glycopeptide with a molecular weight of about 1 k $\sim$ 5 kDa (Enoki et al. 1992). Since then, there almost has been no report about further purification and the role of this glycopeptide in decomposition of cellulose. Other hypotheses concerning low-molecular-weight agents include high affinity iron-binding compounds (Fekete et al. 1989; Jellison et al. 1991; Goodell et al. 1997; Xu & Goodell 2001). These compounds were phenolate chelators, named "Gt chelator", which referred to the mixture of ultrafiltered (<1 kDa), ethyl acetate soluble, iron-binding compounds produced by *G. trabeum* (Goodell et al. 1997). Gt chelator had not only high affinity for ferric iron ion but could reduce it to ferrous form. It was also reported to be able to mediate the generation of hydroxyl radical HO$^{\cdot}$ and the degradation of lignocellulose (Goodell et al. 1997). Almost all the hypotheses proposed HO$^{\cdot}$ be involved, but no evidence has been provided showing the direct relationship between cellulose degradation and HO$^{\cdot}$ production; besides this, the formation of HO$^{\cdot}$ and its redox mechanism in cellulose decay has not been well studied; How HO$^{\cdot}$ is generated and whether or not HO$^{\cdot}$ is generated in a Fenton-type reaction are still not quite clear; Furthermore, the role of the low-molecular-weight agent in HO$^{\cdot}$ formation, is it just a electron carrier or a catalyst of cellulose oxidation, also needs further investigation. More detailed work are necessary to elucidate the low molecular weight agent-mediated, HO$^{\cdot}$-involved oxidative mechanism by brown-rot fungi.

Therefore, in this paper, based on the study of properties of a novel purified peptide (named Gt factor) isolated from the extracellular cultures of *G. trabeum*, we have used electron paramagnetic resonance (EPR) spin trapping method to measure the HO$^{\cdot}$ generation mediated by Gt factor, employed electrochemical method to detect the redox reaction between cellulose and Gt factor, and studied the effects of Gt factor on the cellulose degradation by brown-rot fungi. We found that brown-rot fungi produced HO$^{\cdot}$ in a H_2O_2/Fe^{2+} reaction, Gt factor mediated the formation of HO$^{\cdot}$ and played an important role in the initial cellulose degradation by destroying the hydrogen bonds in cellulose chains and causing cellulose depolymerization via HO$^{\cdot}$.

Materials and methods

Chemicals

Ferrozine (Fe^{2+} indicator), 2-D-deoxyribose, cellobiose, thiobarbituric acid (TBA), catalase, carboxymethylcellulose sodium (CMC–Na), Avicel (microcrystalline cellulose powder, Type 50), were purchased from Sigma Co., 5,5-dimethylpyrroline-1-oxide (DMPO) was purchased from Aldrich Chemical Co., Sephadex G-10 from Pharmacia Co.

Microorganism and culture conditions

Gloeophyllum trabeum CTCC 7047 was obtained from the Forestry Institute of China. *Gloeophyllum trabeum* was grown in a 200-ml flask on 60 ml solid basal salts medium on which sterilized Chinese willow wood chips (1.5×1.5 cm) had been layered. The composition of basal salts medium was as following (g/l): KH_2PO_4 1.5, $MgSO_4$ 0.5, $(NH_4)_2SO_4$ 0.5, $CaCL_2$ 0.1, $MnSO_4$ 0.01, $FeSO_4$, 0.01. The medium was inoculated with a plug (0.5×0.5 cm) from potato agar culture and then incubated for 28 days at 28 °C with 80% relative humidity.

After 28 days of incubation, the medium was extracted with 0.05 M acetate buffer (pH 4.5) for 4 h and the extract was centrifuged at 10,000 g for 10 min and the supernatant was collected for purification.

Isolation and purification of low-molecular-weight active agent

The crude extracellular extract was ultrafiltrated (MW cut-off 5 kDa) and the filtrat was concentrated, applied to Sephadex G-10 ($\Phi 1.6 \times 100$ cm) column equilibrated with 0.05 M acetate buffer (pH 4.5) at a flowrate of 9 ml/h. All fractions were assayed for cellulase activity, effects on CMC viscosity, formation of hydroxyl radical HO$^{\cdot}$ and others.

Then the active fractions were pooled, concentrated and purified by HPLC (Waters, USA, Waters Model 510 HPLC Pump, Waters 486 Detector) and a Hypersil ODS2 RP C_{18} column (4.4×50 mm, particle 5 μm) was used under the conditions as below: buffer A, triethyamine-phosphate (20 μM pH 5.05) and buffer B, methanol, gradient condition 100%, elute with buffer A for 10 min, then use buffer A (95%) + buffer B (5%) to elute, gradient curve was No. 6. The flowrate was 1 ml/min, injection 10 μl, temperature 27 °C, wavelength 280 nm.

The active peak obtained on first HPLC was further purified on column Hypersil ODS2 RP C_{18} (8.0×250 mm, particle 10 μm. Buffer A, water, buffer B, methanol. Flowrate 2 ml/min, injection 100 μl, temperature 27 °C, wavelength 280 nm.

Reduction of Fe^{3+} to Fe^{2+}

Ferrozine was used in the detection of reduced iron (Gibbs 1976). 2 ml active agent (0.5 μM) was mixed with 0.5 ml ferrozine (15 mM) and 0.5 ml freshly prepared $FeCL_3$ (1.2 mM) solution. The reduction of ferric ion to ferrous ion was indicated by the increase of the absorbance at 562 nm after the sample was added.

TBA assay for $HO^{\cdot}$ generation

$HO^{\cdot}$ was detected with the thiobarbituric acid (TBA) method as described by Halliwell & Getteridge (1985): 1 ml active agent (0.5 μM) was mixed with 1 ml citrate buffer (0.05 mM, pH 4.5) containing 2-D-dexyribose (4 mM), cellobiose (0.8 mM) and $FeCL_3$ (0.1 mM), incubated shaking under 30 °C for 1 h, then 1 ml TBA (1%, in 0.05mM NaOH solution) and 1 ml trichloroacetic acid (2.8%) were added. The mixture was heated in boiling water for 15 min, then the absorbance at 532 nm was measured. The formation of $HO^{\cdot}$ with different substrates as electron donors was measured according to the steps described above except substituting cellobiose for different cellulosic substrates.

Redox reaction between cellulosic substrate and active agent

The cyclic voltammetry method was used to conduct this experiment using electrochemical analyzer (CHI 800, Shanghai Co., China). Three-electrode system consists of a saturated calomel (SCE) reference electrode, a platinum counter electrode and a carbon fiber working electrode. The potential was scanned from –1.0 V $\sim$ 1.2 V at rate 50 mV/s. 200 μl soluble cellulosic substrate cellobiose or carboxylmethyl cellulose sodium (CMC-Na, 0.2 mM) in HAC-NaAC buffer (0.05 M, pH 4.5) was scanned as reference and the change of redox potential was recorded after 800 μl active agent (0.5 μM) was added.

Electron paramagnetic resonance (EPR) spin trapping study

5,5-dimethylpyrroline-1-oxide (DMPO) at a final concentration of 1mM was used as spin trapper for $HO^{\cdot}$ in EPR study. EPR spectra were recorded at room temperature using Bruker Esp 300E spectrometer. The spectrometer settings were: modulation frequency, 100 kHz; modulation amplitude: 2.024 G; microwave power: 10 Mw; microwave frequency: 9.8 GHz; center field: 3483 G; sweeping time: 1 min. The samples were vortex-mixed and then aspirated to a quartz aqueous cell, EPR data collection started 30 s after the addition of the final reagent.

Estimation of reducing ends in cellulosic substrate (Halliwell 1961)

Twenty mg dewaxed cotton powder was suspended in 3 ml distilled water. One ml cyanide-carbonate solution (0.064% potassium + 0.52% sodium carbonate anhydrous) followed by 1ml potassium ferricyanide (0.05%) was added. The mixture was heated in boiling water bath for 15 min, then cooled and treated with 2 ml ferric alum-Duponol-phosphoric acid reagent. The absorbance at 600 nm was measured (Halliwell 1961).

FT-IR spectroscopy of cotton powder

Samples were prepared by mixing dewaxed cotton powder with KBr and detected with a 710 FT-IR spectrometer. Spectra were recorded in the transmission mode with a resolution of 4 cm^{-1} in the range of 4000–400 cm^{-1}.

Cellulose crystallinity

The present crystallinity of dewaxed cotton powder (100 mg) was determined using D/max-rB X-ray diffractometer. The wavelength of the Cu/Ka α-radiation source was 0.154 nm, and the spectra were obtained at 30 mA with a voltage of 40 kV. The crystallinity index (CrI) of the cellulose sample was estimated by the following equation: $CrI = I_{002} - I_{am}/I_{002}\%$, where I_{002} is the intensity of the 002 peak (at $2\theta = 22°$), and I_{am} is the intensity at $2\theta = 18°$; The I_{002} peak corresponds to the crystalline fraction and I_{am} amorphous intensity corresponds to the amorphous fraction.

Scanning Electron Microscopy (SEM) observation of dewaxed cotton fiber

The cotton fibers for SEM observation were coated with 4–6 gold particles and observed with KYKY 2000 Scanning Electron Microscope.

Atomic forcing microscopy (AFM) observation of dewaxed cotton fiber

A Digital Instruments NanoScope III controller with a MultiMode AFM head was used to image the dewaxed cotton fiber. Several dried cotton fibers were deposited onto freshly cleaved mica and images were acquired in tapping mode using commercial, unmodified silicon nitride cantilevers. Scan rates were 0.69 Hz, with the scan angle being varied to obtain optimum contrast.

Results

Isolation and purification of low molecular weight active peptide

The ultrafiltered components (<5 kDa of the extracellular extract of *G. trabeum* applied on Sephadex G-10 column ($\Phi 1.6 \times 100$ cm) was shown in Figure 1. The elution peak III could form hydroxyl radical $HO^{\cdot}$. When 1 ml of this fraction was mixed with 1 ml CMC–Na (4%) and incubated under 50 °C for 30 min, the viscosity of the CMC-Na solution was found to be significantly decreased by 50%, which indicated the depolymerization of CMC; the III fraction was pooled, concentrated and further purified on HPLC reverse C_{18} column. The active fraction obtained on first HPLC was concentrated, applied to HPLC again and a symmetric sharp peak was obtained (figure not shown). Different purification conditions such as different polarity of the eluent or different detection wavelength etc., which were usually used in HPLC chromatography to separate different materials, always resulted in the same sharp peak, which indicated the peak contained the pure component. Because this material was obtained from *G. trabeum*, we named it as Gt factor. Gt factor had no cellulase activity, and it was identified as a peptide by absorbance at 280 nm, by biuret reaction and amino acid analysis. Its amino acid composition was as following: Asp Thr Ser Glu Gly Ala Cys Val Met Ile Leu Tyr Phe Lys Arg Trp. According to the abundance of its amino acid and estimation by gel filtration, the MW of Gt factor was about 4 kDa. In view of its peptide characteristic, Gt factor

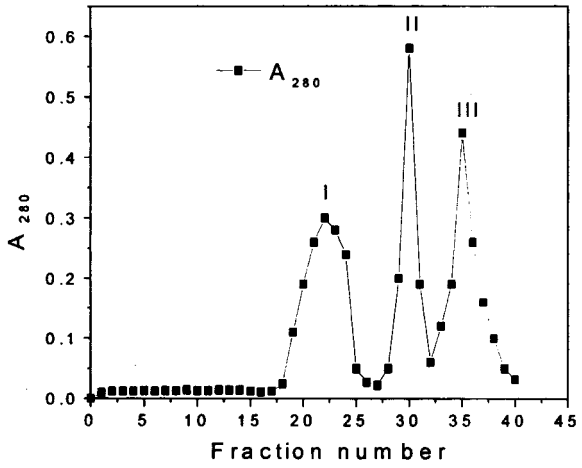

Figure 1. Chromatography of extracellular compounds (MW 5 kDa from *G. trabeum* on Sephadex G-10 column. The concentrated compounds were applied to the column ($\Phi 1.6 \times 100$ cm) equilibrated with 0.05 M acetate buffer, pH 4.5 and eluted with the same buffer at a flowrate of 9 ml/h. Peak III fraction were pooled for next purification.

was quite different from the phenolate compound – "Gt chelator" which was described by Goodell et al. (1997) Gt factor processed high thermo-stability, only 20% activity was lost after 24 h at 60 °C, which was quite different from cellulase. The iso-electric point of Gt factor was 6.6.

Reduction of Fe^{3+} to $Fe^{2+}$by Gt factor

Ferrozine chelates Fe^{2+}, forming stable red compound which has characteristic absorbance at 562 nm (Gibbs 1976). When ferrozine (15 mM) was added to a solution containing Gt factor (0.5 μM) and Fe^{3+} (1.2 mM), the absorbance of the solution at 562 nm continually increased at a fast rate over a 60-min period especially the initial 10-min period (Figure 2). The difference between the absorbance of the solution of ferrozine and Gt factor with Fe^{3+} and the absorbance of ferrozine and Gt factor indicated the amount of reduction of exogenous Fe^{3+} by Gt factor.

TBA experiment for assay for hydroxyl radical $HO^{\cdot}$

The TBA reaction is a common method for the detection of $HO^{\cdot}$. In this reaction, 2-D-deoxyribose is oxidized by $HO^{\cdot}$to malonaldehyde. The product of TBA and malonaldehyde has a characteristic absorbance at 532 nm and the absorbance is proportional to the amount of $HO^{\cdot}$ (Halliwell & Getteridge 1985). As shown in Figure 3, Gt factor (0.5 μM) could produce

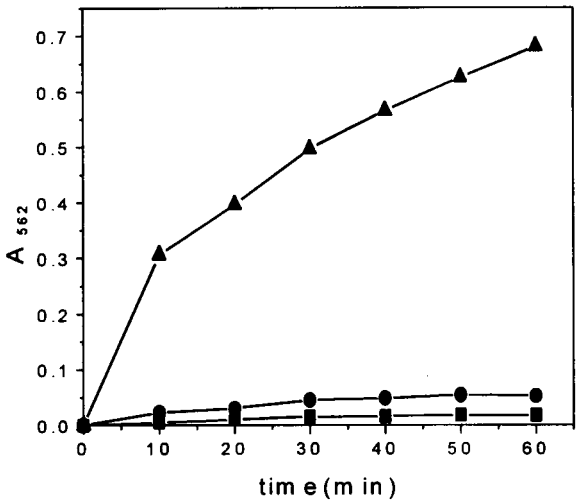

Figure 2. Reduction of Fe^{3+} by Gt factor: (■) 5 mM ferrozine; (●) 5 mM ferrozine and Gt-factor; (▲) 5 mM ferrozine, 250 μM Fe^{3+} and Gt-factor.

HO·. The absorbance of the product under O_2 condition was higher (A_{532} = 0.275) than that in normal air (A_{532} = 0.204), which indicated that O_2 is necessary in the formation of HO·. Appropriate amount of Fe^{3+} (10^{-5} M) could increase the production of HO·, indicating iron ion is related to the formation of HO·. The addition of catalase decreased the amount of HO·, suggesting the formation of H_2O_2 in reaction system. When the HO·-specific scavengers (10 mM) such as mannitol, benzoate sodium, thiourea, or dimethyl sulfoxide were added, HO· production was decreased, the inhibition by thiourea could amount to 40%, which proved the radical produced by Gt factor was exactly the hydroxyl radical HO·.

TBA experiment with different substrates as electron donors

When cellobiose is replaced by different cellulosic substrates (0.2 mM) in the TBA assay, HO· is still formed whatever electron donor is amorphous CMC, or microcrystalline cellulose, even native cellulose like cotton fiber, which suggested that the oxidative reaction of HO· with cellulose is not as specific as enzymes like cellobiohydrolase or endoglucanase. It is well-known that the large amounts of hydrogen bonds in cellulose chains make cellulose difficult to be degraded. Therefore the significant decrease in the DP of cellulose in the initial stage of decay by brown-rot fungi might be related to non-specific action of hydrogen bonds by HO· produced by Gt factor.

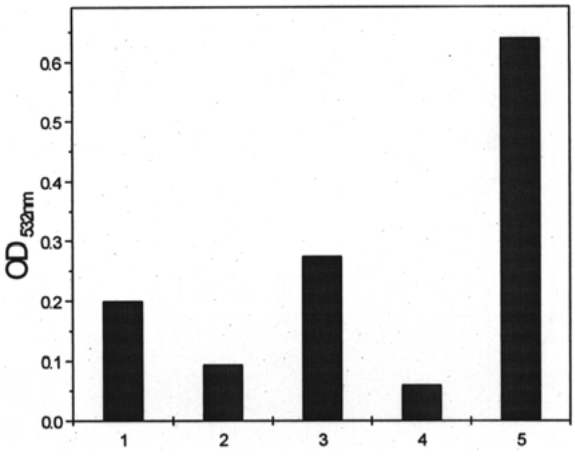

Figure 3. TBA assay for HO· production by Gt factor: (1) Gt factor; (2) half amount of Gt factor; (3) under pure O_2; (4) catalase added; (5) addition of 10^{-4}M Fe^{3+}.

Electron transfer between electrode and cellobiose/Gt factor system

In order to analyze the effects of Gt factor on cellulosic substrates, a sensitive electrochemical method was used. Electrochemical analysis of cellobiose/Gt factor at carbon fiber electrode by Cyclic voltammetry was shown in Figure 4. Cycle 1 is the background substrate cyclic voltammogram obtained from 0.2 mM cellobiose in 0.05 M HAC–NaAC buffer (pH 4.5). This cyclic voltammogram demonstrated the cellobiose, HAC-NaAC system is insulated in the absence of mediator. After Gt factor (0.5 μM) is added, a cyclic voltammetric oxidative wave, cycle 2, is immediately developed. This change of redox potential could result in oxidation of cellobiose in which Gt factor acted as an electron carrier, i.e., it could catalyze the electron transfer between carbon fiber electrode and cellobiose. The electron transfer between electrode and CMC/Gt factor was also detected as described above and similar results were obtained.

EPR spin trapping for HO· with DMPO

HO· is short-lived and highly reactive. So HO· generation by Gt factor in the presence of cellobiose was monitored using the spin trap DMPO. By the characteristic spectrum of DMPO spin adduct, we can easily distinguish HO· radical from $O_2^{\cdot-}$ radical et al. The Gt factor (2.5 μM) was added to HAC–NaAC buffer (50 mM pH 4.5) containing 2 mM cellobiose. Thirty seconds after DMPO was added, EPR spectrum was obtained as shown in Figure 5, it was a 1 : 2 : 2 : 1

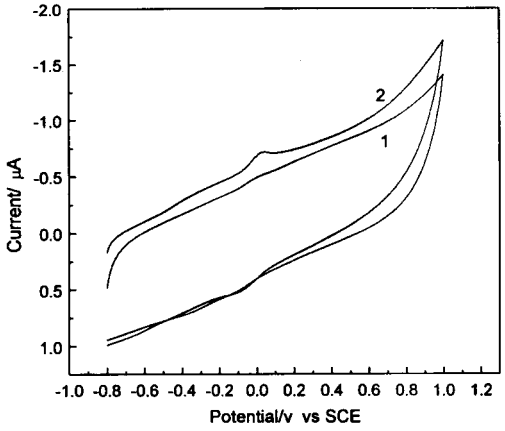

Figure 4. Cyclic voltammograms of cellobiose/Gt factor: (1) Reference: HAC–NaAC buffer containing cellobiosel; (2) Gt factor added.

quartet originating from the hydroxyl radical adduct of DMPO- OH $\alpha_N = \alpha_M = 14.9$ G). This confirmed the generation of $HO^{\cdot}$ in the presence of cellulosic substrate, Gt factor, Fe^{3+} and O_2.

$HO^{\cdot}$ formation by Gt factor is Fe^{3+}-dependent and involved H_2O_2

To prove that the formation of $HO^{\cdot}$by Gt factor is Fe^{3+}-dependent, $HO^{\cdot}$ production in the presence of metal chelators was analyzed. The addition of EDTA (100 mM) diminished TBA activity in the presence of Fe^{3+}, supporting the obligatory presence of redox-active Fe^{3+} in $HO^{\cdot}$ production. In the TBA assay above, when catalase was added, TBA activity is decreased, indicating $HO^{\cdot}$ was generated from H_2O_2. If $HO^{\cdot}$ is generated via the reduction of H_2O_2, enhanced production of $HO^{\cdot}$ upon addition of exogenous H_2O_2 would be expected. In Figure 6, after 1 mM H_2O_2 was added, EPR signal of DMPO-OH was significantly intensified compared to that in absence of H_2O_2.

Generation of H_2O_2 via $O_2^{\cdot-}$ and reduction of Fe^{3+} to Fe^{2+} by Gt factor drove $O_2^{\cdot-}$ formation

EPR spin trapping was also employed to investigate the chemistry of H_2O_2 generation. In Figure7, EPR signal of DMPO-OH originated in a Fenton reaction (1 mM H_2O_2 and 2 mM $FeSO_4$). After Gt factor (2.5 μM) was added, DMPO-OH signal intensified, this is because Gt factor reduced Fe^{3+} to Fe^{2+}, which subsequently made $HO^{\cdot}$ generation cycling possible. Besides, a characteristic DMPO-$O_2^{\cdot-}$ adduct signal was detected after addition of Gt factor, suggesting the existence of superoxide anion $O_2^{\cdot-}$ in reaction system and $O_2^{\cdot-}$ formation may be driven by reduction of Fe^{3+} by Gt factor.

Oxidative degradation of cellulose by Gt factor via $HO^{\cdot}$

Two ml Gt factor (20 μg/ml) with addition of Fe^{3+} (10^{-4} M) was incubated with 30 mg dewaxed cotton fiber at 45 °C. Three days later, no weight loss of cotton fiber was observed and no reducing sugar was detected in the reaction system; Modified procedures using potassium ferricyanide was employed to measure reducing ends in cellulose material (Halliwell 1961). As shown in Figure 8, the reducing ends in cotton fiber were gradually increased with incubation went on; When $HO^{\cdot}$-specific scavenger such as thiourea was added, 5 days later, $OD_{600} = 0.458$, much less than the absorbance of the system without thiourea (0.694), which proved it was exactly the hydroxyl radical $HO^{\cdot}$ that increased the reducing ends in cotton fiber.

FT-IR spectrum of dewaxed cotton fiber before and after treatment with Gt factor was obtained using a FT-IR 710 spectrmeter (Nicolet). Nelson–O'Conner methods was used for estimating the crystallinity index of cellulose (Tripp 1971), which was represented by the ratio of absorbance at 1372 cm^{-1} (C–H bending) and 2900 cm^{-1} (C–H stretching), corresponding to –OH stretching and flexural vibration frequencies of the intra- and itermolecular hydrogen bonds of cellulose. After incubation with Gt factor, with addition of 10^{-4} M Fe^{3+}, at 45 °C for 3 days, the relative intensity of this band was reduced by 5.3%, which indicated some hydrogen bonds between –OH groups in cellulose were disrupted after treated with Gt factor.

X-ray diffraction analysis was used to detect the effect of Gt factor on cellulose crystallinity. It was measured that the average crystallinity of native cotton fiber was 57%. After incubation with Gt factor (6 ml, 2 μg/ml) and 10^{-4} M Fe^{3+} at 45 °C for 3 days, cellulose crystallinity level was decreased to 49.8% and the crystallinity continuously decreased with longer time of incubation, which showed the discruption by Gt factor on crystalline region of cellulose.

Figure 9(a) shows the SEM image of native cotton fiber. It was observed that the native cotton fiber had a smooth and uniform surface; However after treated with 2 ml Gt factor (2 μg/ml) at 45 °C for 3 days, obvious roughening and swelling on outer surface of the cotton fiber was observed, as shown in

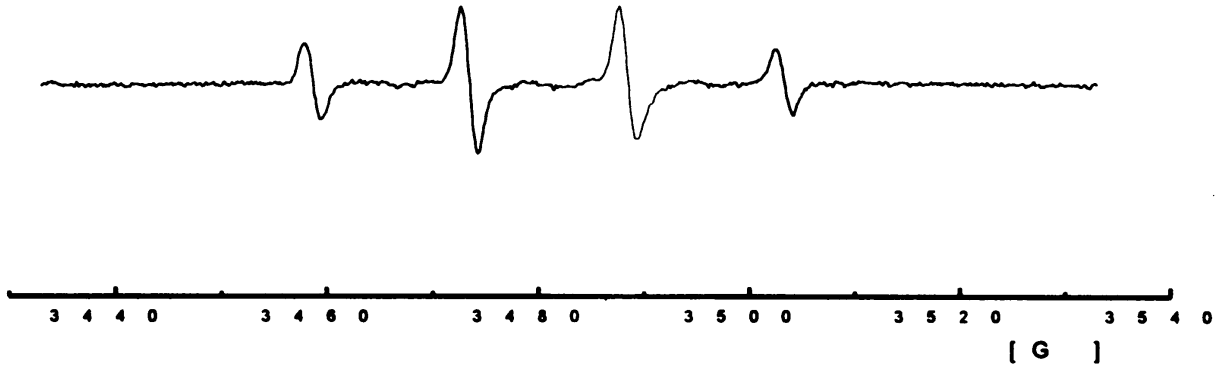

Figure 5. EPR spectrum of DMPO adduct formed in presence of Gt factor,cellobiose, Fe^{3+} and O_2. The data acquisition parameters were: modulation frequency, 100 kHz; modulation amplitude: 2.024 G; microwave power: 10 mW; microwave frequency: 9.8 GHz; center field: 3483 G; sweeping time: 1 min.

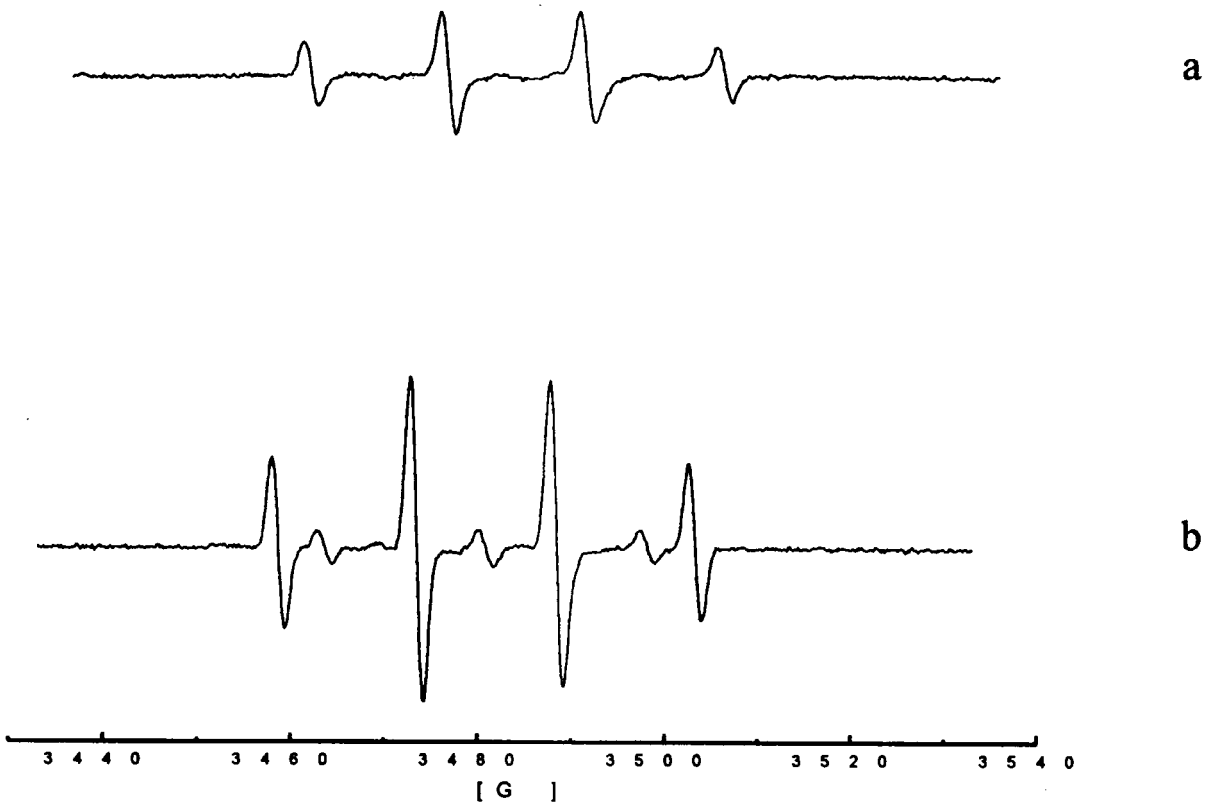

Figure 6. EPR spectra of DMPO-OH adduct obtained from Gt factor, cellobiose, Fe^{3+} and O_2: (a) without H_2O_2; (b) 1 mM H_2O_2 added. The instrumental conditions are given in the legend to Figure 6.

Figure 9(b). The structure of cotton fiber seemed to be "looser" than native state, which made cellulose accessible to further degradation. AFM image of cotton fiber showed the change clearly. Figure 10 is the AFM image of native cotton fiber, the lateral diameter of the cotton fibril was about 30–50 nm, and had a relatively smooth and uniform surface shown in Phase Image. After 6 days incubation with Gt factor, from Height Image and Phase Image in Figure 11, we can both observe "roughening" on cotton fibril; more clearly in Phase Image, numerous splits appeared along and across the long axes of fibril, which may subsequently

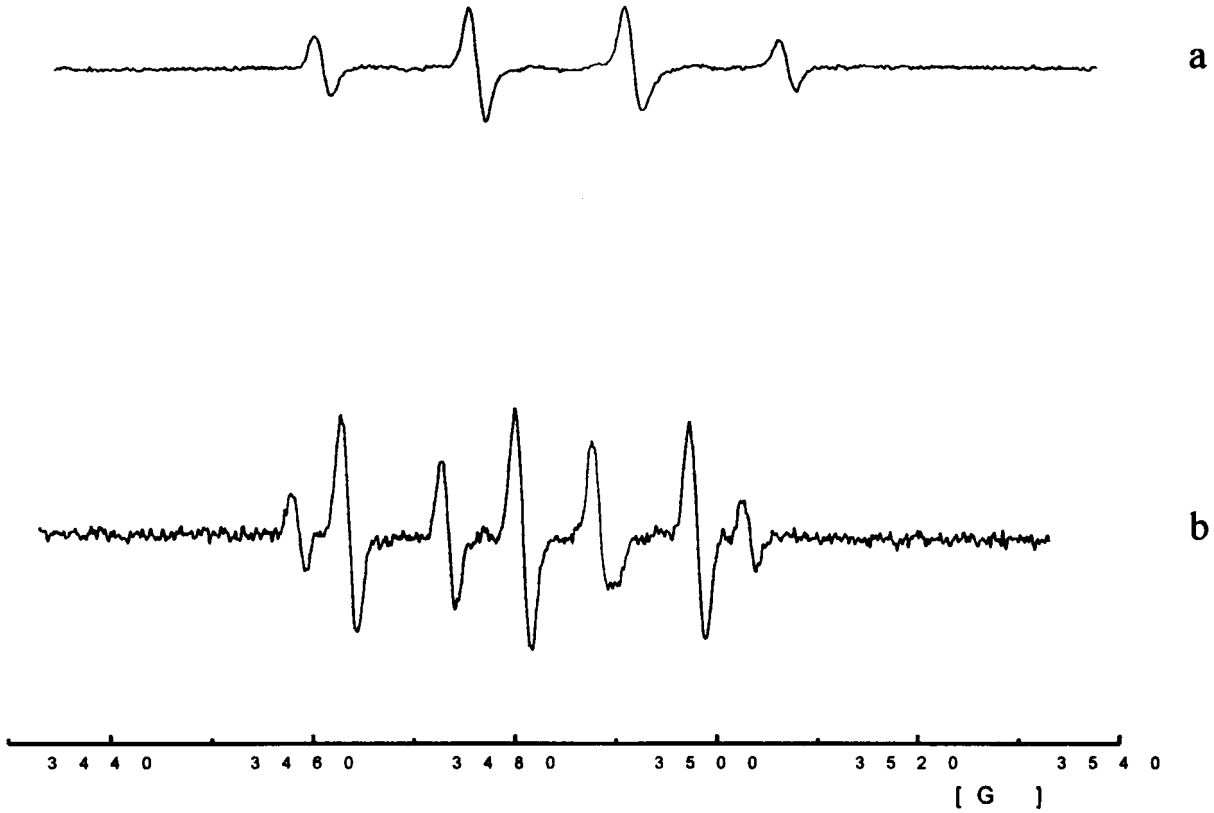

Figure 7. EPR spectra of DMPO adduct obtained from H_2O_2 and Fe^{2+}, and Gt factor: (a) spectrum obtained from H_2O_2 (1 mM) and Fe^{2+} (2 mM); (b) as in (a), but with addition of Gt factor. The instrumental conditions are given in the legend to Figure 6.

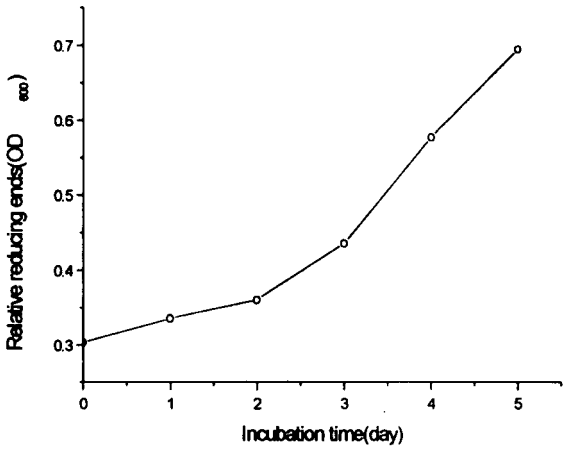

Figure 8. Change of reducing ends in cotton fiber after treated with Gt factor.

lead to formation of short fibril and decrease of depolymerization degree. Oxidative effects of $HO^{\cdot}$ is very strong, for brown-rot fungi void of cellobiohydrolase, the effects of $HO^{\cdot}$ may be very important in early stage of cellulose depolymerization. To prove that, we treated cotton fiber at 45 °C with the member-retained MW > 5000 Da enzyme components (ultrafiltration, cut-off 5000 Da) from *G. trabeum* containing endoglucanase and β-glucosidase et al., 6 days later, no reducing sugar was produced and as shown in Figure 12 (little dots showing enzyme protein absorbed), almost no change was observed; whereas when cotton fiber was pretreated with Gt factor, then treated with MW > 5000 Da components, much more reducing sugar was produced.

Disscussion

Fungi including basidiomycetes, can secrete many types of low-molecular-weight metal-chelators, such as siderophore, melanin and phenolate derivatives which were named by Goodell as "Gt chelators". Gt factor, which we purified via HPLC, is a peptide quite different from any cellulase components; Nor is it the "Gt chelator", as proposed by Goodell et al. (1997).

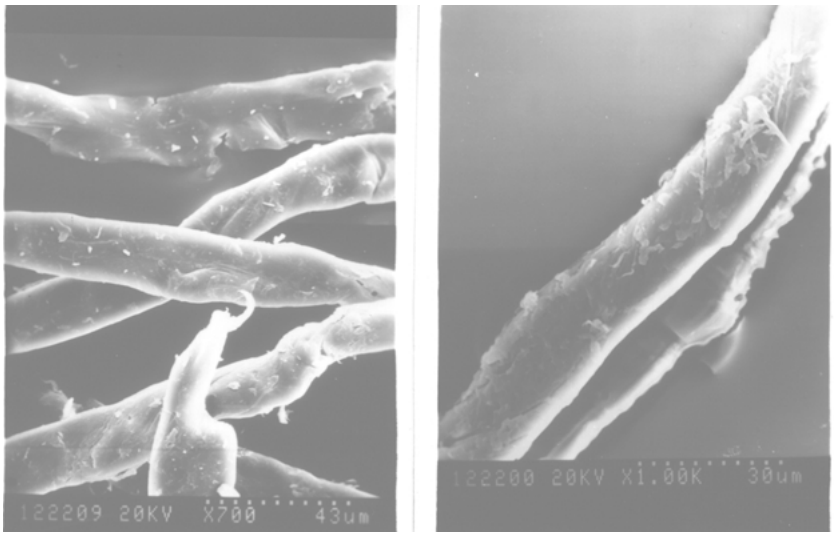

Figure 9. SEM photo of cotton fiber before (a) and after (b) treated with Gt factor.

Gt factor strongly reduced Fe^{3+} to Fe^{2+}. As we know, Fe^{2+} is a reactant in Fenton reaction and 'Redox cycling' is important in $HO^{\cdot}$production. Though it is not clear which mechanism drove this reduction to happen, the fact of reduction of Fe^{3+} to Fe^{2+} might somewhat make the redox cycle possible. All results of TBA assay and electrochemical experiments indicated Gt factor could act as a catalyst to oxidize cellulosic substrates in presence of O_2. Gt factor, cellulose, O_2 and Fe^{3+} make up a redox system to produce $HO^{\cdot}$. EPR studies further verified the $HO^{\cdot}$ production mediated by Gt factor. EPR study also indicated H_2O_2 involvement in $HO^{\cdot}$formation, combined with the experiment result of Fe^{3+}-dependent, we hypothesized that $HO^{\cdot}$formation occurred in a Fenton-type reaction. But where does H_2O_2 come from? The appearance of DMPO- $O_2^{\cdot-}$ adduct signal upon addition of Gt factor in Figure 8 indicated the $O_2^{\cdot-}$ formation is caused by Gt factor. Reduction of Fe^{3+} drove the $O_2^{\cdot-}$ formation as $Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^{\cdot-}$. This might somewhat enlighten on the mechanism of H_2O_2 generation. H_2O_2 can potentially form by a two-electron transfer to O_2 to generate the peroxide anion O_2^{2-} or by a one-electron transfer generating superoxide anion $O_2^{\cdot-}$. Figure 8 showed the $O_2^{\cdot-}$ generation, we conclude that H_2O_2 is generated through $O_2^{\cdot-}$. As for biological system, $O_2^{\cdot-}$ easily undergoes disproportionation to H_2O_2, $2\ O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2$ or by a two one-electron transfer from each of two iron atoms to bridging dioxygen molecule resulting in H_2O_2, $2Fe^{2+} + O_2^{\cdot-} + 2H^+ \rightarrow 2Fe^{3+} + H_2O_2$. In our research work,

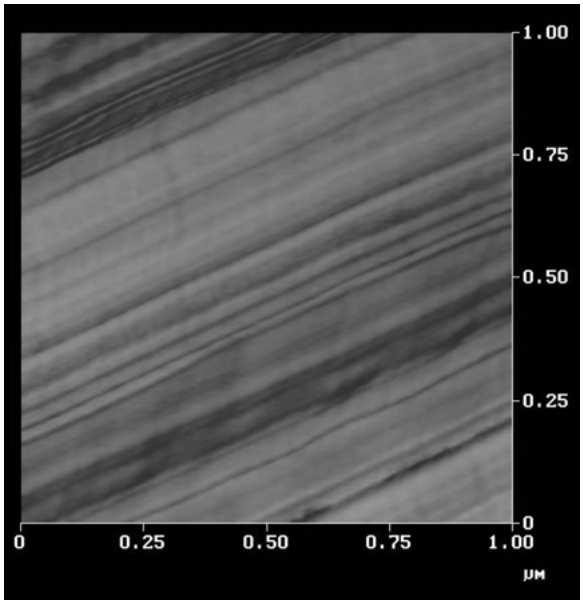

Figure 10. AFM image of the surface of native cotton fiber.

we don't know by which way above H_2O_2 is generated, but we conclude that generation of H_2O_2 by Gt factor was via a superoxide anion intermediate-$O_2^{\cdot-}$. Gt factor acted as a reductant of Fe^{3+}, Gt factor and Fe^{2+} facilitated the reduction of O_2 to H_2O_2.

The results described here showed Gt factor could act as electron catalyst between cellulose and O_2, mediate the generation of $HO^{\cdot}$ with cellulose as electron donor; besides, in an aqueous system and in the presence of Fe^{3+} and O_2, Gt factor also generated $HO^{\cdot}$

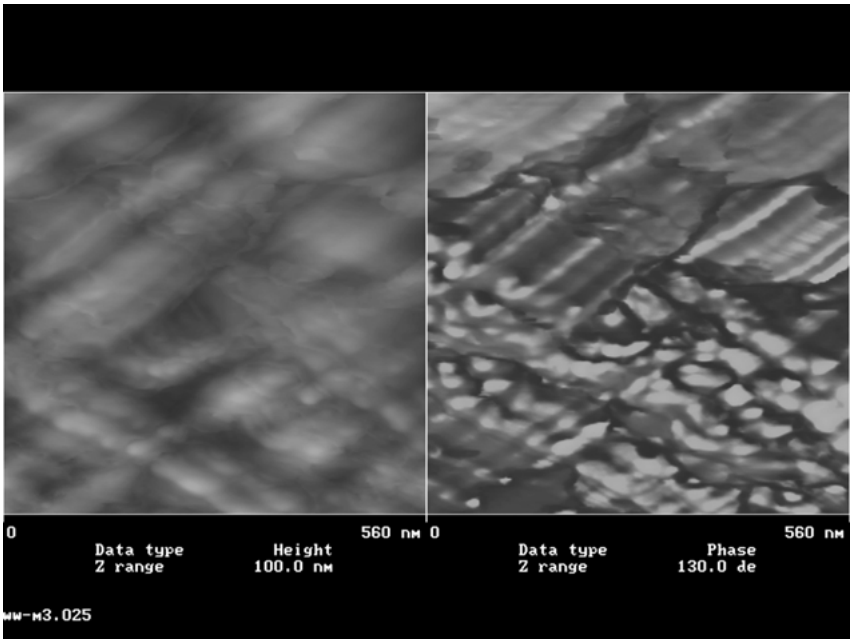

Figure 11. AFM image of the surface of cotton fiber treated with Gt factor. Amplitude set point: 1.705 V; drive frequency: 289.08 kHz; drive amplitude: 40.5 mV; scan rate: 0.598; scan angle: 0 degree.

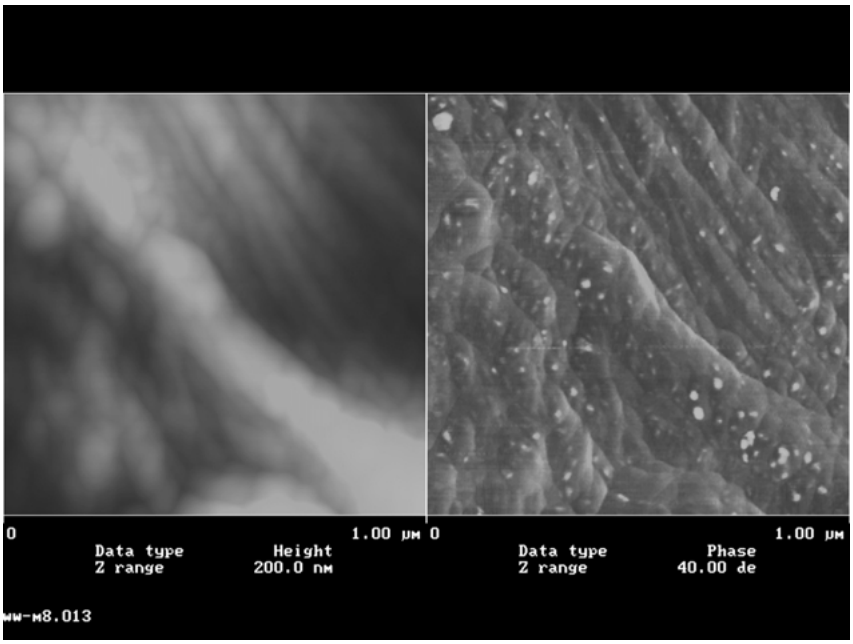

Figure 12. AFM image of cotton fiber treated with MW > 5000 Da enzyme components from *G. trabeum*. Amplitude set point: 1.695 V; drive frequency: 289.01 kHz; drive amplitude: 40.5 mV; scan rate: 0.598; scan angle: 0 degree.

void of cellulose. According to the experiment results above, we infer HO· generation may proceed as follows:

1. Gt factor reduces Fe^{3+} to Fe^{2+} ; and Gt factor could be reduced to its reduced state with cellulose substrate as electron donor;

$$Fe^{3+} + \text{Gt factor} \rightarrow Fe^{2+} + \text{Gt factor}(+) \quad (1)$$

$$\text{Gt factor}(+) + \text{cellulose substrate} \rightarrow \text{Gt factor} + \text{cellulose substrate (oxidized)}$$

2. In the presence of O_2, Fe^{2+} oxidizes molecular oxygen by one-electron oxidation to $O_2^{\cdot -}$, then forms H_2O_2 in either way below:

$$Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^{\cdot -}$$

$$2O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2 + O_2 \text{ or } 2Fe^{2+} + O_2^{\cdot -} + 2H^+ \rightarrow 2Fe^{3+} + H_2O_2 \quad (2)$$

3. HO· produces by Fenton reaction between H_2O_2 and Fe^2:

$$H_2O_2 + Fe^{2+} \rightarrow HO^{\cdot} + HO^- + Fe^{3+} \quad (3)$$

As we know, HO·is strongly oxidative, it could abstract electron from hydroxyl group on glucose monomer of cellulose, leave a carbonyl in place of hydroxyl group, thus causing disturbance to interchain and intrachain hydrogen bonds and then cleavaging of a polysaccharide chain (Uchida & Kawakishi 1988).

$$>CH(OH) + HO^{\cdot} \rightarrow > C{\cdot}(OH) + H_2O$$

$$>C{\cdot}(OH) + Fe^{3+} \rightarrow > C = O + H^+ + Fe^{2+}$$

Since Gt factor can mediate the generation of HO·, it must have some effects on cellulose. It has been shown that Gt factor could increase the number of reducing ends in cotton fiber. Oxidation of hydroxyl group to a carbonyl on glucose monomer of cellulose and hence the following break of hydrogen bonds account for increase of reducing ends in cotton fiber. Although HO· produced by Gt factor couldn't firstly break the glucosidic bonds in cellulose, it might destroy the inter- and intrachain hydrogen bonds of cellulose and make more reducing ends exposed, thus making it easier for cellulose to be further degraded. FT-IR spectroscopy and X-ray diffraction provided evidence that Gt factor could disrupt hydrogen bonds and decrease the cellulose crystallinity. Cellulose crystallinity and large amount of hydrogen bonds are two essential factors affecting the biodegradation of native cellulose. Studies on super-structure of cellulose proved that there are large amounts of intra- and iterhydrogen bonds in cellulose. Although disruption of hydrogen bonds only involves change of tri-dimension structure, not cleavage of glucosidic bonds, it might be initial step of cellulose biodegradation (Sinnott 1988). Investigations established that the attack of HO· on cellulose is initiated by hydrogen abstraction from hydroxyl group on glucose monomer, and followed by oxidative –C–C– cleavage of sugar under aerobic condition, which can cause depolymerization of cellulose and an increase of new carbonyl compounds (Uchida & Kawakishi 1988). According to the recent research related to low-molecular-weight metal chelators (Enoki et al. 1997; Goodell et al. 1997; Zohar et al. 1999; Xu & Goodell 2001) and combined with our results, we believe that a non-enzymatic agent with HO·-producing activity generally exists in the early degradation of cellulose by brown-rot fungi, and hydroxyl radical HO· plays a critical role in the mechanism. It is possible that the following complete degradation is a combined action of several cellulase components, but the fact that Gt factor could mediate the formation of HO· suggested Gt factor might play an important role in early cellulose degradation by brown-rot fungi by destroying hydrogen bonds with HO·. In cellulolytic system void of cellobiohydrolase, the function of Gt factor may have its necessity of existence, even vital.

In our research work, we also isolated the same peptide which could mediate HO·production from the extracellular culture of other 10 brown-rotters, such as *Lentinus lepideus*, *Poria placeura*, *Laetiporus sulplureus*, *Poria cocos* and others, which indicated Gt factor and HO·-involved oxidative mechanism generally existed in brown-rot fungi. Concerning the HO·-producing ability and the oxidative effects on cellulose, Gt factor is similar to cellobiose dehydrogenase (CDH) whose function in cellulose degradation has been widely discussed recently. However, in HO· production, CDH is specific for electron donor – cellobiose, whereas Gt factor is non-specific whatever electron donor is cellobiose, amorphous CMC or other cellulosic substrate. Furthermore, compared with CDH, Gt factor is much smaller, which enables it penetrate into the wood cell wall.

The hydroxyl radical HO·is the most potent oxidizing agent in biological systems, however its half-life

is very short, its effective oxidation must occur in a short distance between the generation site and the action site; Furthermore, the oxidative effects of $HO^{\cdot}$ is non-specific. As we know, all reactions in cell are highly regulated. Thus, we postulate that a mechanism which makes $HO^{\cdot}$ in close proximity to cellulose and regulates the oxidative effects of $HO^{\cdot}$ on cellulose decomposition must exist. But until now, it is not clear and still needs a lot of investigations. Further study of Gt factor, including its molecular structure and its oxidative mechanism was being conducted, all of which will help to explain the mechanism of cellulose degradation by brown-rot fungi, even contribute to wood decay preservation. Furthermore, the free radical generating system described above has a great potential in variety of industrial processes and pollution control applications.

Acknowledgement

This work was supported by Grant No. 39970004 from the National Natural Science Foundation of China. We thank Dr. Wang L.S. for AFM observation and Dr. Li M. for critical reading and helpful discussion.

References

Carpita N, Sabularse D, Montezinos D & Delmer DP (1979) Determination of the pore size of cell walls of living plant cells. Science 205: 1144–1147.

Enoki A, Tanaka H & Fuse G (1989) Relationship between degradation of wood and production of H_2O_2-producing or one-electron oxidases by brown-rot fungi. Wood Sci. Technol. 23: 1–12.

Enoki A, Hirano T, Tanaka H (1992) Extracellular substance from the brown-rot basidiomycete *Gloeophyllum trabeum* that produces and reduces hydrogen peroxide. Mater Org. 27: 247–261.

Enoki A, Itakura S & Tanaka H (1997) The involvement of extracellular substances for reducing molecular oxygen to hydroxyl radical and ferric iron to ferrous iron in wood degradation by wood decay fungi. J. Biotechnol. 53: 265–272.

Fekete FA, Chandhoke V & Jellison J (1989) Iron-binding compounds produced by wood-decaying Basidiomycetes. Appl. Environ. Microbiol. 55: 2720–2722.

Flournoy DS, Kirk TK & Highley TL (1991) Wood decay by brown-rot fungi: Changes in pore structure and cell wall volume. Holzforschung 45: 383–388.

Gibbs CR (1976) Characterization and application of ferrozine iron reagent as a ferrous iron indicator. Anal. Chem. 48: 1197–1201.

Goodell B, Jellison J, Liu J, Daniel G, Paszczynski A, Fekete F, Krishnamurthy S, Jun L & XU G (1997) Low molecular weight chelators and phenolic compounds isolated from wood decay fungi and their role in the fungal biodegrdation of wood. J. Biotechnol. 53: 133–162.

Halliwell G (1961) The action of cellulolytic enzymes from *Myrothecium verrucaria*. Biochem. J. 79: 185–192.

Halliwell G (1965) Catalytic decomposition of cellulose under biological conditions. Biochem. J. 95: 35–40.

Halliwell B & Getteridge JMC (1985) Hydroxyl radicals assayed by aromatic hydroxylation and dexyribose degradation. In: GreenWald RA (Ed) Handbook of Methods for Oxygen Radical Research (pp 177–180). CRC Press, Boca Raton, FL.

Highley TL (1988) Cellulolytic activity of brown-rot and white-rot fungi on solid media. Holzforchung 42: 211–216.

Jellison J, Chandhoke V, Goodell B & Fekete F (1991) The isolation and immunolocalization of iron-binding compounds produced by *Gloeophyllum trabeum*. Appl. Microbiol. Biotechnol. 35: 805–809.

Koenigs JW (1974) Hydrogen peroxide and iron: A proposed system for decomposition of wood by brown-rot Basidiomycetes. Wood fiber 6: 66–80.

Murmanis L, Highley TL & Palmer JG (1988) The action isolated brown-rot cell-free culture filtrate, H_2O_2-Fe(II), and the combination of both on wood. Wood Sci. Technol. 22: 59–66.

Sinnott ML (1988) The cellobiohydrolases of Trichoderma reesei: A review of indirect and direct evidence that their function is not just glycoside bond hydrolysis. Biochem. Soc. Trans. 26: 160–164.

Tripp VW (1971) Measurement of crystallinity. In: Bikales NM & Segel L (Eds) Cellulose and Cellulose Derivatives (pp 319–320). Wiley Iterscience, New York.

Uchida K & Kawakishi S (1988) Interaction of (1 → 4)- and (1 → 6)-linked disaccharides with the fenton reagent under physiological conditions. Carbohydrate Res. 173: 89–99.

Veness RG & Evans CS (1989) The role of hydrogen peroxide in the degradation of crytalline cellulose by basidiomycete fungi. J. Gen. Microbiol. 135: 2799–2806.

Xu G & Goodell B (2001) Mechanism of wood degradation by brown-rot fungi: chelator-mediated cellulose degradation and binding of iron by cellulose. J. Biotechnol. 27: 43–57.

Zohar K, Kenneth AJ & Kenneth EH (1999) Low molecular weight chelators and phenolic compounds isolated from wood decay fungi and their role in the fungal biodegrdation of wood. FEBS Lett. 446: 49–54.