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The Brown-Rot Basidiomycete *Fomitopsis palustris* Has the Endo-Glucanases Capable of Degrading Microcrystalline Cellulose

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Abstract Two endoglucanases with processive cellulase activities, produced from *Fomitopsis palustris* grown on 2% microcrystalline cellulose (Avicel), were purified to homogeneity by anion-exchange and gel filtration column chromatography systems. SDS-PAGE analysis indicated that the molecular masses of the purified enzymes were 47 kDa and 35 kDa, respectively. The amino acid sequence analysis of the 47-kDa protein (EG47) showed a sequence similarity with fungal glycoside hydrolase family 5 endoglucanase from the white-rot fungus *Phanerochaete chrysosporium*. N-terminal and internal amino acid sequences of the 35-kDa protein (EG35), however, had no homology with any other glycosylhydrolases, although the enzyme had high specific activity against carboxymethyl cellulose, which is a typical substrate for endoglucanases. The initial rate of Avicel hydrolysis by EG35 was relatively fast for 48 h, and the amount of soluble reducing sugar released after 96 h was 100 μg/ml. Although EG47 also hydrolyzed Avicel, the hydrolysis rate was lower than that of EG35. Thin layer chromatography analysis of the hydrolysis products released from Avicel indicated that the main product was cellobiose, suggesting that the brown-rot fungus possesses processive EGs capable of degrading crystalline cellulose.

Keywords: Brown-rot fungus *Fomitopsis palustris*, endoglucanase, purification, microcrystalline cellulose, hydrolysis

Cellulose, which is a linear polymer of glucose units linked by β-1,4-D-glucosidic bonds, is the main constituent of woody biomass. Cellulose biodegradation by fungi has generally been considered to involve only three types of hydrolytic enzymes: endoglucanase (EG), exoglucanase (cellobiohydrolase; CBH), and β-glucosidase (BGL). EG randomly cleaves the β-1,4-linkage of the internal cellulose chain, CBH acts processively on the free ends of cellulose polymer chains, and BGL hydrolyzes cellobiose, removing a strong inhibitor of both EG and CBH [24, 19]. In general, EGs can only attack the amorphous regions of cellulose molecules, and CBH can do the crystalline regions to release soluble reducing sugar from nonreducing ends of cellulose chains [5]. However, processive EGs capable of degrading crystalline cellulose like CBHs have been reported in bacterial species such as *Bacillus circulans* [16] and *Clostridium thermocellum* [8], and the fungus *Gloeophyllum trabeum* [4].

Brown-rot basidiomycetes cause the most destructive type of wood decay and are important contributors to biomass recycling [9]. These basidiomycetes are unusual since they rapidly depolymerize the cellulose in wood without removing the surrounding lignin that normally prevents microbial attack [15]. However, there is little information available on the mechanism of hydrolysis of cellulose by brown-rot fungi. In particular, these fungi are generally thought to lack the exoglucanases that can hydrolyze crystalline cellulose [11, 13]. Recently, brown-rot fungi such as *G. trabeum* [4] and *Fomitopsis palustris* [27] have been reported to hydrolyze microcrystalline cellulose.

The *F. palustris* used for this study causes a typical brown-rot and is as a model fungus for wood-preservative efficacy tests both in Korea and Japan. Extracellular enzyme preparations from this fungus were reported to degrade carboxymethylcellulose, hemicelluloses, and several β-glycosides, but not to exhibit cellulolytic activity on crystalline cellulose [13]. However, our previous paper

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reported that this fungus is able to degrade both the crystalline and amorphous forms of cellulose from wood [27]. To establish the cellulase system in relation to degradation of crystalline cellulose, we purified and characterized two endoglucanases that hydrolyze the microcrystalline cellulose. Here, we report that *F. palustris* has endoglucanases degrading microcrystalline cellulose.

MATERIALS AND METHODS

Microorganism and Culture Conditions

The brown-rot basidiomycete *F. palustris* FFPRI 0507 (Berkeley et Curtis) Murill used for the experiment originated from the Forest Products Research Institute (FFPRI) of Japan. The mycelia of *F. palustris*, which had been incubated on a potato dextrose agar plate at 28°C for 7 days, were punched out and inoculated into 100 ml of potato dextrose broth in a 500-ml Erlenmeyer flask. After the plugs were incubated in this medium at 28°C for 7 days on a rotary shaker at 105 rpm, 5 ml of *F. palustris* precultures were aseptically inoculated into 5 l of cellulolytic medium in a 10-l bioreactor (Samsung Science, Seoul, Korea). The culture medium contained 0.8% (w/v) peptone, 0.2% (w/v) yeast extract, 0.5% (w/v) KH_2PO_4, 0.5% (w/v) K_2HPO_4, and 0.3% (w/v) $MgSO_4 \cdot 7H_2O$. The two percents (w/v) of microcrystalline cellulose (Avicel; Fluka, Switzerland) was used as a carbon source for the cultivation of this fungus. The culture in a 10-l bioreactor was incubated at room temperature for 14 days.

Enzyme Purification

The culture solution (5.0 l) was filtered through filter paper (Toyo Roshi Kaisha, Ltd., Japan), and concentrated with a stirred ultrafiltration cell (model 8400; Millipore Corp., Bedford, MA, U.S.A.) equipped with a 10-kDa cutoff polyethersulfone membrane (PM 10 membrane, Millipore Corp.) under a nitrogen pressure of 4.0 kgf/cm^2 and dialyzed against 20 mM potassium phosphate buffer (pH 7.0). The concentrated solution (30 ml) was loaded onto a Toyopearl DEAE650S (Tosoh, Tokyo, Japan) column (2.0×20 cm), which had been equilibrated with the same buffer. Proteins were eluted with a step gradient of 0 mM, 100 mM, 200 mM, 300 mM, 400 mM, and 500 mM KCl in a volume of 900 ml at a flow rate of 3 ml/min. The fractions including EG activity were pooled at a KCl concentration of 200 mM. The pooled fractions including EG activity were concentrated by a stirred ultrafiltration cell (model 8050; Millipore Corp.). The dialyzed enzyme solution (2 ml) was applied to a Sephacryl 300-S HR (Amersham Biosciences) column (1.6×60 cm), which had been equilibrated with the same buffer, and then eluted with the buffer at a flow rate of 0.5 ml/min. The gel filtration fractions containing EG activity were pooled and concentrated. The resulting enzyme solution (1.0 ml) was then loaded onto a MonoQ HR5/5 (Amersham Biosciences) column (0.5×5.0 cm) equilibrated with the same buffer. The enzyme solution was eluted with a linear gradient from 0 to 0.5 M of KCl in the buffer at a flow rate of 1.0 ml/min. Proteins with EG activities were eluted at about 0.2 M and 0.35 M of KCl, respectively. Fractions of proteins that were eluted around 0.2 M KCl were pooled and the purity was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the procedure of Laemmli [18]. Fractions that were eluted around 0.35 M KCl were pooled and concentrated by ultrafiltration. The concentrated enzyme solution (1.0 ml) was then loaded onto a MonoQ HR5/5 (Amersham Biosciences) column (0.5×5.0 cm) equilibrated with the same buffer. The enzyme solution was eluted with a linear gradient from 0 to 0.5 M of NaCl in the buffer at a flow rate of 0.5 ml/min. Fractions with high EG activity were pooled, concentrated, and then subjected to gel filtration on a TSK-3000SW$_{XL}$ (7.8×1.0 cm) column (Tosoh, Tokyo, Japan) column (1.6×60 cm) equilibrated with the same buffer, and then eluted with the buffer at a flow rate of 0.5 ml/min. Gel filtration fractions highly enriched in EG were pooled and the purity was checked by SDS-PAGE as described above. The molecular masses of the purified enzymes were calculated with the following molecular mass standards: Myocin (220 kDa), β-galactosidase (115 kDa), bovine serum albumin (96 kDa), ovalbumin (51 kDa), carbonic anhydrase (37 kDa), soybean trypsin inhibitor (30 kDa), and lysosome (20 kDa).

Enzyme Assays and Protein Concentration

Carboxymethyl cellulase (CMCase) activity was assayed in 50 μl reaction mixtures using 1.0% carboxymethyl cellulose (CMC; Sigma/Aldrich) in 100 mM sodium acetate buffer (pH 5.0) at 50°C. Reducing sugars were determined by the Somogyi-Nelson method [13]. One unit (U) of CMCase activity is defined as the amount of enzyme catalyzing the release of 1 μmol of glucose equivalent per min. Avicelase was determined in 50 ml reaction mixtures that contained 1.0% (w/v) Avicel (Fluka) in 100 mM sodium acetate (pH 5.0) buffer. The reaction mixtures were incubated by the rotary shaker at 50°C for 21 h. Soluble reducing sugars released from Avicel were determined as described above. One unit (U) of Avicelase activity is defined as the amount of enzyme catalyzing the release of 1 μmol of glucose equivalent per hour.

The protein concentration in the enzyme solution was measured with a Protein Assay kit II (Bio-Rad Laboratories, U.S.A.) based on Bradford's method [2].

Amino Acid Sequencing

The purified enzymes were submitted for internal amino acid sequencing of selected peptides that were digested by

trypsin and eluted by HPLC (HP1100, Agillent Technologies, CA, U.S.A.) and N-terminal amino acid sequencing at the Korea Basic Science Institute of Seoul Center (Seoul, Korea). Protein sequencing was performed using the Procise 492 cLC protein sequencer (Applied Biosystems, U.S.A.).

Thin Layer Chromatography (TLC) Analysis of Soluble Reducing Sugars Released from Avicel

Each enzyme solution (10 μg/ml of purified enzyme) was incubated in the reaction mixture (0.8 ml) that contained 50 mM sodium acetate (pH 5.0) and 1.0% (w/v) Avicel at 50°C, and the changes of reducing sugars released from Avicel at various incubation times were analyzed by the Somogyi-Nelson method [18]. Hydrolysis products from Avicel were separated on 0.2 ml silica gel 60F254 plate (Merck KGaA, Darmstadt, Germany) with ethyl acetate/water/methanol (40:15:20 vol/vol) and the spots were detected by the method described by Gilad *et al.* [8].

Results and Discussion

Purification of Glycosylhydrolases Produced from *Fomitopsis palustris*

The brown-rot basidiomycete *F. palustris* produced the major cellulolytic enzymes such as endoglucanases and exoglucanases when it was grown on Avicel as a carbon source [27]. In this study, the two endoglucanases (EGs) containing high activities toward the β-1,4-glucanase substrates such as CMC and Avicel were purified to electrophoretic homogeneity from the culture filtrates grown on Avicel. Peak fractions containing EG activities at each purification step were checked by SDS-PAGE. The molecular masses of two purified enzymes were about 47 kDa and 35 kDa, respectively. A 47-kDa protein (EG47) was purified 68-fold from peak I fractions and a 35-kDa protein (EG35) was purified 107-fold by gel filtration on a TSK-3000SW$_{XL}$ after two step purification steps (Fig. 1A). The purified EG47 and EG35 had specific activities of 57.5 and 89.9 U/mg of protein, respectively. The results of the purification are summarized in Table 1.

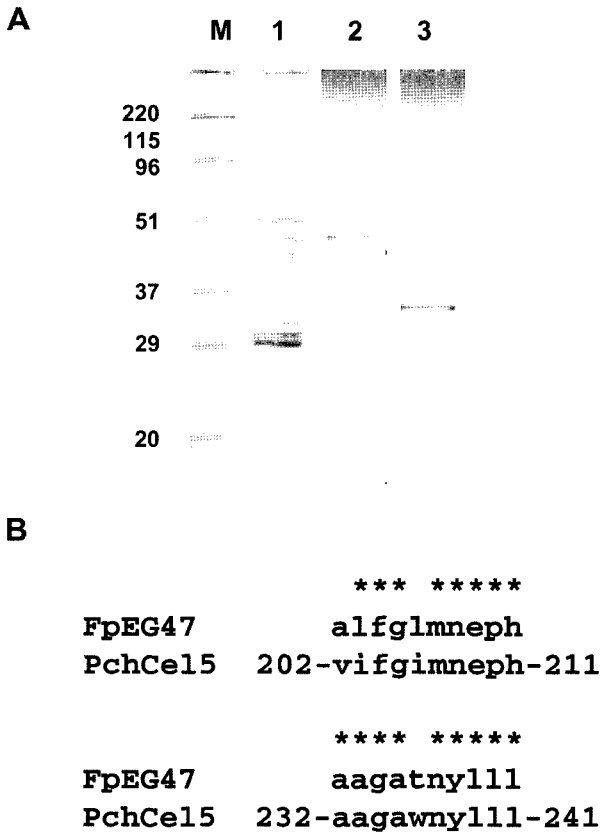

Fig. 1. A. SDS-PAGE analysis of extracellular proteins and purified endoglucanases from *F. palustris*.
M, molecular marker (kDa); lane 1, crude enzyme; lane 2, purified EG47; lane 3, purified EG35. **B.** Comparison of partial amino acid sequences between EG47 purified from *F. palustris* (FpEG47) and a GH family 5 EG from *Phanerochaete chrysosporium* (PchCel5, Accession No. AAU12275).

Identification of the Purified Proteins by Analysis of Amino Acid Sequences

To identify the two purified enzymes, N-terminal and internal amino acid sequences of the proteins were analyzed. Although we failed to determine the N-terminal sequence of EG47, its proteolytic fragments were determined as ALFGLMNEPH and AAGAWNYLLL. BLAST searches of the amino acid sequences indicated that the protein has a

Table 1. Purification of two endoglucanases from *F. palustris*.

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)
Crude extracts	386	326	0.84	1
Toyopearl DEAE650S	110	250	2.28	3
Sephacryl S-300HR	30.6	209	6.82	8
1st Mono Q HR5/5				
Peak I	0.24	13.8	57.5	68
Peak II	8.54	132	15.5	18
Peak II purification 2nd Mono Q HR5/5	0.27	17.2	63.7	76
TSK-3000SW$_{XL}$	0.11	9.80	89.9	107

Table 2. Substrate specificities of glycosylhydrolases from *F. palustris*

Substrate[a]	Specific activity (U/mg of protein)[b]	
	EG47	EG35
CMC	89.5±1.1	57.0±7.4
Xylan	0	11.8±5.1
pNPC	0.54±0.01	0.49±0.05
pNPG	0	0
pNPL	ND[c]	ND[c]
pNPX	0	0
Avicel	0.25±0.02	0.79±0.04

[a]pNPC, p-nitrophenyl-β-cellobioside; pNPG, p-nitrophenyl-β-glucopyranose; pNPL, p-nitrophenyl-β-lactopyranoside; pNPX, p-nitrophenyl-β-xylopyranoside. The enzyme activities for pNPC, pNPG, pNPL, and pNPX were determined as the amount of enzyme necessary to release 1 μmol of p-nitrophenol per minute. Values reported are averages from duplicate experiments for each substrate.
[b]The presented values are averages (±SD) of triplicate experiments.
[c]ND, not determined (<0.005 μmol of p-nitrophenol/min/mg of protein).

sequence similarity with endo-β-1,4-glucanse (GH family 5) of the white-rot fugus *Phanerochaete chrysosporium* (Fig. 1B). Although the N-terminal and internal amino acid sequences of EG35 were determined as N-ATTLTGQYSXATTGN, it has no homology with any glycosylhydrolase sequences available through the CAZy Web site (http://afmb.cnrs-mrs.fr/CAZY) [10].

Substrate Specificity of Purified EGs

The specificities of the purified EG47 and EG35 for a variety of standard glycosylhydrolase substrates are presented in Table 2. The results showed that the EG47 has a high specific activity with CMC, which is a typical substrate for EGs, suggesting that EG47 is an endo-β-1,4-glucanae that belongs to GH family 5. Hydrolytic activity of the purified EG47 against crystalline cellulose was relatively weak, because specific activity of EG47 with Avicel was considerably lower than that of the EG35. On the other hand, EG35 had a relatively high specific activity for EG substrate, although its N-terminal amino acid sequence had no homology with any known glycosylhydrolase sequences.

The purified EG35 from *F. palustris* also degraded xylan and the specific activity was 11.8±5.1 U/mg (Table 2). There have been some reports that microbial cellulases act not only on cellulose but also on xylan [20, 25]. Kim [16] suggested that the activity of EG towards xylan displayed a flexible specificity for the C-6 position of the glucopyranosyl unit of cellulose. The xylanases from some microorganisms such as alkaliphilic *Aspergillus niger*, *Bacillus* sp. strain BK, and alkaliphilic *Bacillus halodurans* C-1 were able to hydrolyze xylans in woody biomasses [3, 17, 22]. *F. palustris* was also found to produce xylanase, which is able to hydrolyze xylans in the lignocellulosic materials (data not shown).

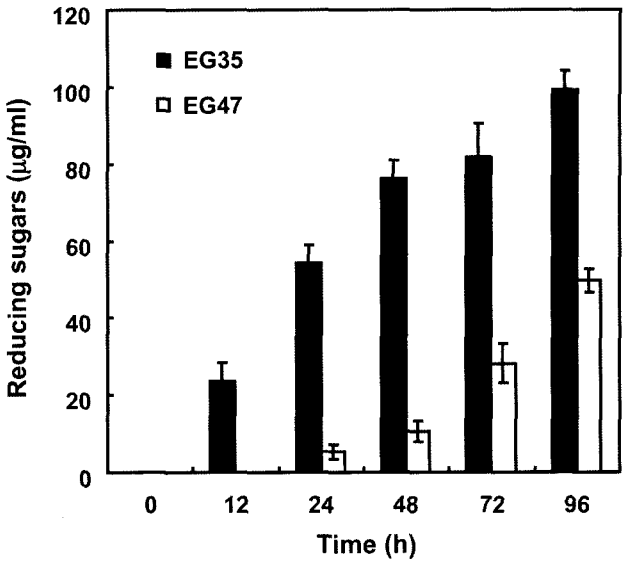

Fig. 2. The time course of production of reducing sugars released from hydrolysis of Avicel by the purified EG47 and EG35, respectively.
Values represent the mean of three replicate determinations; error bars indicate standard deviation.

Hydrolysis of Avicel by Purified Enzymes

Avicel hydrolysis by the purified EG47 and EG35 from *F. palustris* was studied at 50°C and pH 5.0. The time course of production of soluble reducing sugar released by hydrolysis of Avicel is shown in Fig. 2. The initial hydrolysis rate of EG35 was relatively fast for 48 h and the amount of soluble reducing sugar released after 96 h was 100 μg/ml. However, the hydrolysis of Avicel by EG47 increased slowly after 24 h and the hydrolysis rate was lower than that of EG35. Furthermore, TLC analysis of the hydrolysis products released from Avicel indicated that the main product was cellobiose (G2) together with a small amount of glucose (G1) (Fig. 3) and the enzymes did not degrade cellobiose (data not shown). These results indicated that the property of EG35 from *F. palustris* is similar to that of CBH from *Trichoderma viride* [1]. The action of EG35 from *F. palustris* appears to be similar to that of EGs from *B. circulans* [16], *C. thermocellum* [8], and *G. trabeum* [4].Yamanobe *et al.* [26] has reported that a cellulase from the fungal Y-94 strain hydrolyzed Avicel and was classified as a specific endocellulase rather than an exocellulase.

Brown-rot basidiomycetes are the principal cause of biodegradation of wooden structures. Cellulose degradation by brown-rot fungi is critical for loss of wood strength, as this type of fungi rapidly depolymerize cellulose [12]. The pore size in wood is too small for the known brown-rot enzymes to diffuse freely through intact wood cell walls [7]. Therefore, the extracellular cellulases from these fungi act on cellulose released after the action by extracellular agents, such as reactive oxygen species (ROS), which are

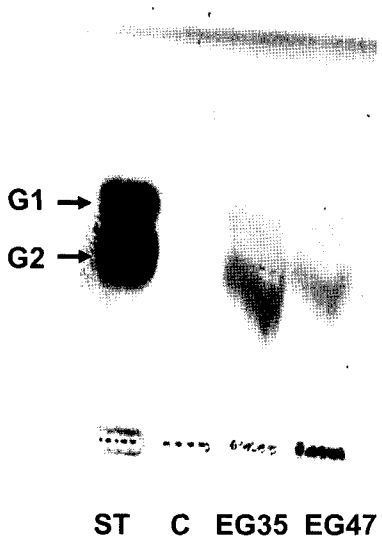

Fig. 3. Thin layer chromatography analysis of soluble sugars following degradation of Avicel by purified EG47 and EG35, respectively.
ST, standard of glucose (G1) and cellobiose (G2); C, control (without enzymes).

capable of diffusing through the wood cell wall as reported previously in other brown-rot fungi [4, 9, 14]. Although brown-rot fungi extensively degrade cellulose in wood, enzyme preparations from fungal cultures appeared to lack the exoglucanase component; they ineffectively degrade crystalline cellulose but do modified celluloses such as CMC [11, 13]. In contrast to other studies, our previous results indicated that the brown-rot fungus *F. palustris* produced both EG and CBH and was able to degrade the cellulose in wood, in both crystalline and amorphous forms [27]. Moreover, the fungus grown on Avicel produces β-glucosidase that hydrolyzes cellobiose to glucose [27]. Cohen and his coworkers have also reported that the brown-rot fungus *G. trabeum* produces a processive EG that belongs to GH family 5, capable of yielding assimilable glucose from crystalline cellulose [4]. Therefore, our results clearly indicate that the brown-rot basidiomycete possesses processive EGs capable of degrading crystalline cellulose.

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