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Purification, Molecular Cloning, and Enzymatic Properties of a Family 12 Endoglucanase (EG-II) from *Fomitopsis palustris*: Role of EG-II in Larch Holocellulose Hydrolysis[▽]

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A family 12 endoglucanase with a molecular mass of 23,926 Da (EG-II) from the brown-rot basidiomycete *Fomitopsis palustris* was purified and characterized. One of the roles of EG-II in wood degradation is thought to be to loosen the polysaccharide network in cell walls by disentangling hemicelluloses that are associated with cellulose.

The cellulolytic enzyme system of brown-rot fungi is thought to differ from that of *Trichoderma reesei*, one of the most powerful cellulose-degrading fungi (5, 18). Although brown-rot fungi have not been thought to possess exoglucanases (12), they cause the most destructive type of decay in wooden structures by a complex decay process. *Fomitopsis palustris* is a well-known brown-rot basidiomycete (4, 14–17). In our previous research, we showed that two extracellular endoglucanases from *F. palustris* FFPRI 0507 degraded acid-swollen cellulose but were unable to degrade microcrystalline cellulose (Avicel) and filter paper (15, 27). On the other hand, Yoon et al. (31, 32) reported that endoglucanases from *F. palustris* are processive endoglucanases, based on the analysis of initial degradation products. Cohen et al. demonstrated processive cellulase from the brown-rot basidiomycete *Gloeophyllum trabeum* (2). They suggested that the conventional wisdom which holds that brown-rot fungi lack cellulases that degrade crystalline cellulose must be reconsidered. Clearly, more-detailed information is required to determine how endoglucanases from brown-rot fungi contribute to the wood decay process, particularly those from *F. palustris*. Toward this end, we purified an endoglucanase from culture filtrate of *F. palustris*, characterized its enzymatic properties, and sequenced its genomic DNA and cDNA.

Holocellulose, glucomannan, and xylan from larch (*Larix leptolepsis*) were prepared from wood meal as described previously (14). Soluble xylan was prepared from birchwood xylan (Fluka, Buchs, Switzerland). Pachyman from *Poria cocos* and pustulan from *Umbilicaria papulosa* were obtained from MP Biomedicals (Solon, OH) and Calbiochem (La Jolla, CA), respectively. Lichenan from *Cetraria islandica* and laminarin from *Laminaria digitata* were obtained from Sigma (St. Louis, MO). Xyloglucan from tamarind was obtained from Megazyme (Wicklow, Ireland). Cellooligosaccharides were purchased from Seikagaku Kogyo (Tokyo, Japan). Glucohexaose (Seikagaku Kogyo) was purified before use. Other chemicals, all reagent grade, were obtained from Sigma, Wako (Tokyo, Japan), or GE Healthcare (Buckinghamshire, United Kingdom).

Fomitopsis palustris FFPRI 0507 (NBRC 30339) is maintained in our laboratory. The enzyme production medium consisted of 0.5% α-cellulose, 1% glucose, 1% polypeptone, 0.1% yeast extract, 0.1% KH_2PO_4, and 0.01% $MgSO_4 \cdot 7H_2O$ (pH 6.0). Fungal mycelia were grown on a polydextrose agar plate for 1 to 3 weeks, and then a 1-cm^2 piece of the mycelium mat was inoculated with 100 ml of the medium in a 300-ml Erlenmeyer flask. The culture flask was placed on a reciprocal shaker at 26°C. After 21 days of cultivation, the culture broth was filtered through filter paper (Advantec no. 2), and the filtrate was used as a crude enzyme solution. The crude enzyme solution (346 ml) was brought to 90% saturation with $(NH_4)_2SO_4$ under continuous stirring. The $(NH_4)_2SO_4$-precipitated protein pellets were collected by centrifugation, redissolved in deionized water, and then lyophilized. The lyophilized enzyme powder was dissolved in 50 mM sodium acetate buffer (pH 4.0) containing 150 mM NaCl. The enzyme solution was applied to a Sephacryl S-200 HR column (32 by 700 mm; GE Healthcare) that was equilibrated with the same buffer at a flow rate of 0.5 ml/min. The active fractions of low-molecular-weight endoglucanase (EG-II) were combined and dialyzed against 50 mM sodium acetate buffer (pH 5.0) with a Biomax 5000 ultrafiltration membrane (Millipore, Bedford, MA). The dialyzed enzyme solution was applied to a Q Sepharose FF column (16 by 200 mm; GE Healthcare) that was equilibrated with the same buffer. The enzyme was eluted with a linear gradient of 0 to 0.2 M NaCl in the same buffer at a flow rate of 0.5 ml/min. The active fractions were collected, and purity was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (19) alongside a molecular weight standard (BenchMark protein ladder; Invitrogen, Carlsbad, CA). The N-terminal amino acid sequence of the enzyme was analyzed by Edman degradation with an automated protein sequencer (model 477A; Applied Biosystems, Foster City, CA) after the protein had been blotted onto a polyvinylidene difluoride membrane (Millipore).

Endoglucanase activity and β-D-glucosidase activity were as-

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sayed as described previously (26). The protein concentration was measured using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL), with bovine serum albumin used as a standard (28). The concentration of protein in column eluates was routinely monitored by measuring absorbance at 280 nm.

To analyze the changes in the molecular size distribution of carboxymethyl cellulose (CMC) by EG-II treatment, the reaction was carried out in a mixture consisting of 50 μl enzyme solution, 0.25 ml 1% CMC, and 0.2 ml buffer (0.1 M sodium acetate buffer, pH 4.0). The mixture was incubated at 50°C for 6 h, and the reaction was stopped by heating the mixture at 100°C for 5 min. The reaction products were analyzed by size exclusion chromatography (SEC) using a TSK gel G3000 PWXL column (300 by 7.8 mm; Tosoh, Tokyo, Japan) that was equilibrated with a 50 mM sodium acetate buffer (pH 5.0) at 45°C with a flow rate of 0.5 ml/min. The elution patterns were monitored using a differential refractometer.

Cellooligosaccharides (from cellobiose to cellohexaose) were subjected to enzymatic degradation to analyze the degradation pattern of EG-II. The reaction mixture consisted of equal portions of cellooligosaccharides (4 mM) and enzyme solution in the buffer. The reaction was performed at 50°C for 30 min and was terminated by heating the mixture. The products were analyzed by high-performance anion-exchange chromatography with a pulsed amperometric detector (HPAEC-PAD; Dionex, Sunnyvale, CA) equipped with a CarboPac PA1 column (Dionex). The column was equilibrated with 20 mM NaOH at 30°C with a flow rate of 1 ml/min. Cellooligosaccharides and glucose were eluted in a linear-gradient elution from 0 to 0.3 M sodium acetate in 100 mM NaOH within 15 min.

The substrate specificity of the enzyme toward various soluble polysaccharides was tested at a final concentration of 0.2%. The mixture was incubated at 50°C for 30 min, and then the reaction was stopped by heating it at 100°C for 5 min. The reaction products were detected as reducing sugar groups using corresponding monosaccharides as standards.

The depolymerization of larch holocellulose and Avicel by EG-II was performed at 40°C. The 1-ml reaction mixtures were stirred continuously and contained 0.01% bovine serum albumin, 0.02% NaN_3, a 20-mg sample, and enzyme (0.14 units) in 50 mM sodium acetate buffer (pH 4.0). After 96 h or 168 h of incubation, the reactions were stopped by heating the mixtures for 5 min. The supernatants were recovered for analyses of the soluble products. The insoluble pellets were washed with cold water and lyophilized. The percent solubilization of the substrate was calculated from the amount of released reducing sugars detected by the dinitrosalicylic acid method, using glucose as the standard (21). To determine the relative neutral sugar composition, water-insoluble samples were hydrolyzed in 4% H_2SO_4 (3), and corresponding neutral monosaccharides were analyzed by HPAEC-PAD with a CarboPac PA1 column. The monosaccharides were eluted with 1 mM NaOH containing 0.3 mM acetic acid at 30°C at a flow rate of 1 ml/min. To determine the relative neutral sugar composition of water-soluble substrates, aliquots of samples were lyophilized and hydrolyzed with 2 M trifluoroacetic acid to form free glycoses and then analyzed by HPAEC-PAD. Molecular size distributions of the water-insoluble substrates, such as holocellulose, were analyzed by SEC of tricarbanilate derivatives using two TSK gel GMH_6 columns (300 by 7.5 mm; Tosoh) connected in a series as described previously (26).

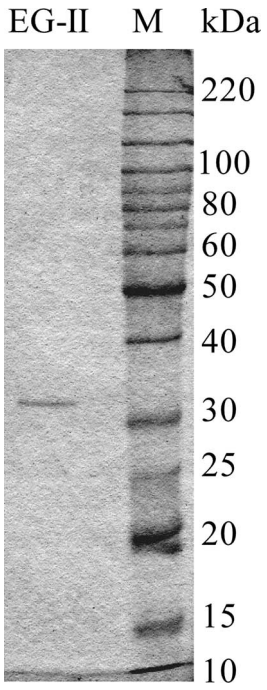

FIG. 1. SDS-PAGE analysis of purified EG-II. Lane M, molecular mass standards.

To identify the gene encoding EG-II, two degenerate primers were prepared according to the N-terminal region sequence (G-N-Y-Q-L-C-N) and the conserved region of fungal GH12 endoglucanases (Y-E-I-M-I-W-L) (7). Total RNA was extracted using the RNAgents total RNA isolation kit (Promega, Madison, WI) as described in the manufacturer's instructions. A cDNA fragment encoding a portion of *F. palustris* EG-II was amplified using the reverse transcription (RT)-PCR method (Access RT-PCR system; Promega). Chromosomal DNA was extracted as described by Raeder and Broda (25), and the 5′- and 3′-end-flanking regions of the *F. palustris* EG-II gene were obtained using the DNA walking PCR technique (DNA Walking SpeedUp premix kit; Seegene, Seoul, Korea). The full-length cDNA was determined by RT-PCR with primers that were designed based on the obtained genomic DNA sequence.

Purification and physical properties of EG-II. When grown with α-cellulose as the carbon source, *F. palustris* FFPRI 0507 produced two endoglucanases and one β-glucosidase. The molecular mass of EG-II was estimated to be 32 kDa by SDS-PAGE (Fig. 1). The sequence of the 21 amino acids at the N-terminal site of purified EG-II was A-T-T-L-T-G-Q-Y-S-C-A-T-S-G-N-Y-Q-L-C-N-D (27). In the CAZy sequence-based classification of carbohydrate-active enzymes (11), this enzyme showed high similarity to the glycoside hydrolase family member GH12. The molecular mass of EG-II is very close to that of the 35-kDa endoglucanase (EG35) reported by Yoon et al. (31). In addition, the reported sequence of 15 amino acids at the N-terminal site of EG35 showed high similarity with that of EG-II. Thus, EG-II and EG35 are considered to be almost

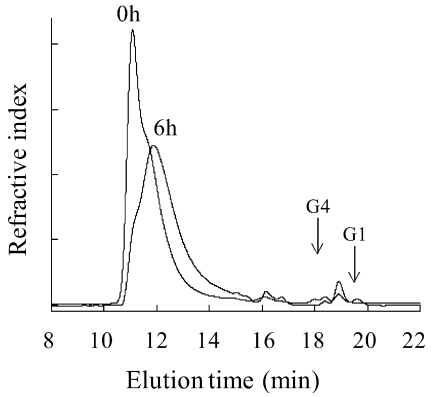

FIG. 2. Changes in the molecular size distribution of CMC before and after EG-II treatment for 6 h. Arrows indicate elution times of standard glucose (G1) and cellotetraose (G4).

TABLE 1. Substrate specificity of purified EG-II from *F. palustris*

Substrate (source)	Main linkage type(s)	Activity (U/mg)[a]
Lichenan	β-1,4; β-1,3	29.9
Glucan (barley)	β-1,4; β-1,3	20.5
Cellohexaose	β-1,4	15.8
Pachyman	β-1,3	12.3
Laminarin	β-1,3	8.3
Carboxymethyl cellulose	β-1,4	7.3
Pustulan	β-1,6	<0.1
Xyloglucan (tamarind)		5.4
Glucomannan (konjac)		1.1
Glucomannan (larch)		1.0
Xylan (birch)		ND
Xylan (larch)		ND
Galactomannan (locust bean)		ND

[a] ND, activity not detected.

identical endoglucanases. The cloning of the EG-II gene showed that the coding sequence encoded a signal sequence of 18 amino acids and 226 amino acids of mature EG-II, comprising a protein with a molecular mass of 23,926 Da. The deduced amino acid was 59% and 56% identical to GH12 enzymes from *Polyporus arcularius* (DDBJ/EMBL/GenBank accession no. BAD98315) and *Phanerochaete chrysosporium* (DDBJ/EMBL/GenBank accession no. AAU12276), respectively.

The optimum pH and temperature for the activities were measured by standard assays using CMCs as substrates. The optimum pH was 3.5 at 40°C with sodium acetate buffer (pH 3.5 to 4.0) and McIlvaine buffer (pH 2.5 to 6.0). The optimum temperature was 55°C in 50 mM sodium acetate buffer (pH 4.0). The enzyme was stable up to 55°C. After incubation at 55°C for 1 h, 91% of the enzyme activity remained.

Substrate specificity and action pattern of EG-II. Changes in the molecular size distribution of CMC before and after EG-II treatment were analyzed by SEC to investigate the depolymerization pattern (Fig. 2). The peaks of CMC became broader and shifted toward a lower molecular size after the enzyme reaction. This result suggests that EG-II is an endoglucanase. The hydrolysis products of soluble cellooligosaccharides after enzymatic treatment by HPAEC-PAD are shown in Fig. 3. Since cellobiose was not degraded by EG-II, the final reaction products from cellooligosaccharides (degree of polymerization, 3 to 6) were cellobiose and glucose. The enzyme did not degrade xylotetraose or mannotetraose (data not shown).

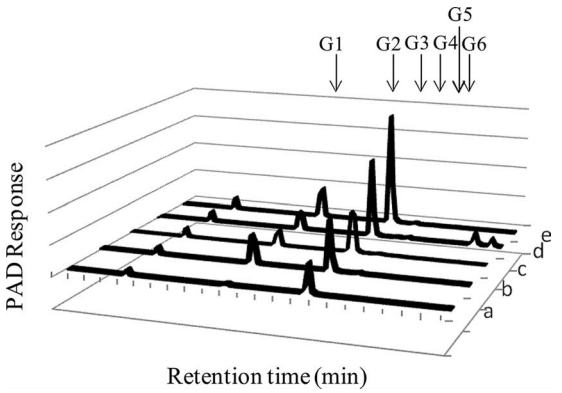

FIG. 3. HPAEC-PAD analysis of soluble cellooligosaccharides after treatment with purified EG-II. Traces a to e indicate each reaction product, from cellobiose (G2) to cellohexaose (G6). The elution times of standard glucose and cellooligosaccharides (G2 to G6) are also indicated.

The incubation of Avicel with EG-II for 96 h resulted in only 0.8% solubilization. The soluble products consisted largely of cellobiose (data not shown). Low levels of cellotriose and cellotetraose were also detected. Yoon et al. described the endoglucanase from *F. palustris* (EG35) as a processive endoglucanase that could degrade Avicel. We do not consider the possibility that EG-II preferentially degraded crystalline regions of Avicel as a processive endoglucanase, because only trace activity of EG-II was detected against Avicel, and there are considerable amorphous regions contained in this substrate (crystallinity index of 63% determined by X-ray diffraction analysis [9]). Like other GH12 enzymes (10, 22, 23), the predicted molecular mass of EG-II (24 kDa) is low relative to those of most cellulases. These GH12 endoglucanases do not have a cellulose binding domain (29) and, thus, preferentially degrade amorphous cellulose rather than crystalline cellulose. The cellulose binding module was critical for the processive activity of endoglucanase Cel9A-90 from *Thermobifida fusca* on crystalline-cellulose hydrolysis (13, 20).

Table 1 shows the relative activities of EG-II against different soluble substrates. Among the polysaccharides tested, lichenan was the most suitable substrate for EG-II. The enzyme also showed high activity against barley glucans. These glucans are polymers with mixed β-(1→3)- and β-(1→4)-glycoside linkages. The enzyme showed activity toward pachyman and laminarin [β-(1→3)-glucan] but showed little activity toward pustulan [β-(1→6)-glucan]. Glucomannans from konjac and larch were somewhat less degraded by EG-II than xyloglucan. EG-II had no activity against xylans from birch and larch or against galactomannan from locust bean. Many GH12 endoglucanases are known for their broad substrate specificities (6, 8, 10, 24, 30). EG-II from *F. palustris* also showed high catalytic activity in degrading lichenan, laminarin, and xyloglucan; thus, it is not limited to cellulose-based substrates.

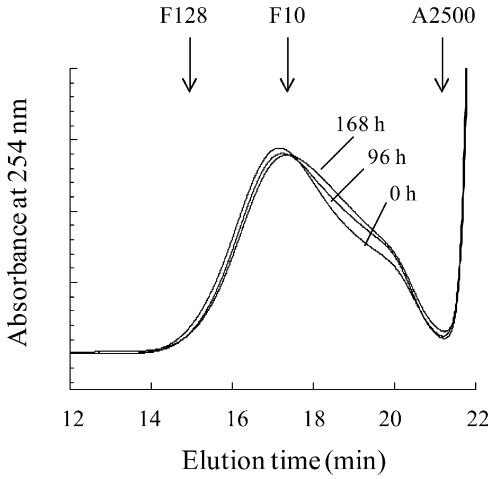

FIG. 4. Changes in the molecular size distribution of larch holocellulose during EG-II hydrolysis. The incubation time (h) corresponding to each curve is indicated. Arrows indicate the elution times of polystylene standards: F128 (molecular weight, 1.26×10^6), F10 (1.07×10^5), and A2500 (2.8×10^3).

Larch holocellulose hydrolysis. The incubation of larch holocellulose with EG-II resulted in 41% solubilization after 96 h and 10.3% after 168 h. The rate of solubilization of the holocellulose was calculated from the amount of released reducing sugars detected by the dinitrosalicylic acid method, using glucose as a standard; therefore, the value is only a guide for enzymatic hydrolysis. Changes in molecular size distribution curves for holocellulose during hydrolysis are shown in Fig. 4. The peak of holocellulose shifted toward a lower molecular size after enzymatic degradation, though the shift in the position of the major peak was not as clear. As the incubation continued, the minor shoulder peak located in the lower molecular size began to disappear.

Before enzyme treatment, relative neutral sugar composition analysis of larch holocellulose showed 77% glucose, 15% mannose, 6.4% xylose, 1.4% galactose, and 0.8% arabinose (mol%). Since the ratio of glucose to mannose of the larch glucomannan was about 1:3 (mol%), it was deduced that the larch holocellulose consisted of 72% cellulose, 19.5% glucomannan, and 6.4% xylan. After 168 h of EG-II treatment, sugar composition analysis of the solubilized products of larch holocellulose showed a high percentage of hemicellulose monosaccharides: 54% glucose, 24% mannose, and 15% xylose. This result suggests that EG-II solubilized cellulose as well as glucomannan and xylan. As shown in Table 1, EG-II was less active against larch glucomannan than CMC or tamarind xyloglucan and showed very low activity against larch xylan. In addition, EG-II did not degrade xylotetraose or mannotetraose. This finding suggests that one of the roles of EG-II in larch holocellulose hydrolysis may be to disentangle hemicelluloses from the polysaccharide network in the cell walls but that the enzyme does not always show powerful activity against larch holocellulose itself. The action of this enzyme might result in increased enzymatic accessibility for other *F. palustris* hemicellulases and cellulases.

Brown-rot fungi cause a diffuse removal of polysaccharide from decayed wood (1). The EG-II enzyme is a small protein without a cellulose binding domain. Considering its roles in degradation, these structural features would be advantageous in allowing the enzyme to diffuse into the cell wall polysaccharide network. The broad substrate specificity of this enzyme might be another advantage for breaking down a comprehensive cell wall network. The hypothesized function of EG-II, to disentangle hemicelluloses from the polysaccharide network in cell walls, may be shared by other GH12 endoglucanases, a function that is in addition to their roles in swelling and dispersing cellulose.

Nucleotide sequence accession number. The DNA sequence of *F. palustris* EG-II was deposited in DDBJ/EMBL/GenBank (accession number AB299016).

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