Structure and Characteristics of an Endo-β-1,4-glucanase, Isolated from *Trametes hirsuta*, with High Degradation to Crystalline Cellulose

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*Trametes hirsuta* produced cellulose-degrading enzymes when it was grown in a cellulosic medium such as Avicel or wheat bran. An endo-β-1,4-glucanase (ThEG) was purified from the culture filtrate, and the gene and the cDNA were isolated. The gene consisted of an open reading frame encoding 384 amino acids, interrupted by 11 introns. The whole sequence showed high homology with that of family 5 glycoside hydrolase. The properties of the recombinant enzyme (rEG) in *Aspergillus oryzae* were compared with those of the En-1 from *Irpex lacteus*, which showed the highest homology among all the endoglucanases reported. The rEG activity against Avicel was about 8 times higher than that of En-1 when based on CMC degradation. A remarkable structural difference between the two enzymes was the length of the linker connecting the cellulose-binding domain to the catalytic domain.

Key words: *Trametes hirsuta*; endoglucanase; cellulose degradation

*Trametes hirsuta* is a strong ligninolytic, cellulosytic fungus expected to be utilized in biomass conversion technologies. There are two primary applications of this fungus. The first is its utilization in the removal of environmental pollutants and in the bleaching of pulp in the paper-making industry taking advantage of its strong lignin-degrading activity. To this end several ligninolytic enzymes have been identified and the possibilities of utilization investigated.† Another is their utilization in the production of sugar available in alcohol fermentation using strong cellulosic activity. The problem in the former is the suppression of cellulase activity, because biological treatment changes the paper strength and product yield due to cellulase action.† In the latter, it is important to increase cellulosytic activity as much as possible. To solve these conflicting problems, it might be necessary to obtain information about the cellulosomes produced by this fungus, but there has been no report about cellulosytic enzymes from this fungus, except for cellobiose dehydrogenase.†

When studying cellulase, it is interesting to elucidate the mechanism of strong cellulose degradation in this fungus. The major problem in the biodegradation of cellulose might be the low reactivity of cellulase against high crystalline cellulose. When it was based on the release of reducing sugar, the activity of endo-type cellulase (En-1 from *I. lacteus*) to high crystalline cellulose such as cotton was below 1/20,000 as compared with that of a soluble substrate such as CMC.‡ Even an exo-type cellulase such as cellobiohydrolase (CBH), which releases cellobiose in the initial reaction stage, showed remarkably low reactivity (20.9 mU/mg for Avicel, Ex-1 from *I. lacteus*).‡ It is desirable to increase the reactivity by improving the enzyme by gene manipulation, and to cause synergistic degradation by combining with enzymes that show different reactivities.‡ For this reason, we expect to find a new enzyme that shows high synergistic effect on cellulose degradation together with the known cellulases.

The aim of this study was to investigate the cellulase components of the strong cellulosytic fungus *T. hirsuta*, and to obtain a new enzyme that shows different reactivity against crystalline cellulose.

**Materials and Methods**

*Fungus and chemicals.* The white-rot fungus *Trametes hirsute* and a cDNA library constructed in a lambda ZAP II vector were obtained from Dr. A. Tsukamoto (Oji Paper Co., Ltd., Tokyo). *Aspergillus oryzae* niaD, a deficient mutant of a nitrate reductase gene, and a high-level expression vector pNAN8142 were kindly provided by Dr. K. Ozeki (Ozeki Corp., Hyogo, Japan). Phosphoric acid-swollen cellulose, Avicel, and cotton were prepared, and crystallinity was determined according to the method of Kanda et al.⁸ Pulp (LBKP, hardwood bleached kraft pulp) was

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Cultivation methods. *T. hirsuta* was maintained on potato dextrose agar medium (PDA, Nissan, Tokyo, Japan). For liquid culture, the PDA (four pieces of circular medium, each 1 cm in diameter) was inoculated into 100 ml of a medium (0.1% Avicel or glucose, 0.2% polypropen, and 0.05% yeast extract) in a 300-ml Erlenmeyer flask. The medium was then incubated at 25°C on a rotary shaker at 130 rpm. The supernatant was collected with a sterile pipette and used in the subsequent experiment. In the case of solid culture, the mycelium (four pieces of PDA) was inoculated onto 100 g of wheat bran with 64% moisture in a 1-liter cubic culture bottle (As One, Osaka, Japan). After being cultured at 25°C, 100 ml of distilled water was added to the medium, and it was stirred until the mycelium dispersed completely. The supernatant obtained by centrifugation was subjected to the subsequent experiment.

Assay of cellulase activity and measurement of protein. Degradation activity for various celluloses was determined in a reaction mixture consisting of 0.25% substrate, 50 mM sodium acetate buffer (pH 5.5), and a suitable amount of enzyme in a total volume of 0.4 ml. After being incubated at 30°C, the reducing sugar produced was determined by the method of Somogyi–Nelson. Against insoluble cellulose, the reaction was done by shaking at 50 strokes/min, and the supernatant was subjected to analysis. The activity for *p*-nitrophenol (pNP)-derivatives was determined in a mixture consisting of 1.25 mM substrate, 50 mM sodium acetate buffer (pH 5.5), and a suitable amount of enzyme in a total volume of 0.4 ml. After being incubated at 30°C, the reducing sugar produced was determined by the method of Somogyi–Nelson. Against insoluble cellulose, the reaction was done by shaking at 50 strokes/min, and the supernatant was subjected to analysis. The activity for *p*-nitrophenol (pNP)-derivatives was determined in a mixture consisting of 1.25 mM substrate, 50 mM sodium acetate buffer (pH 5.5), and a suitable amount of enzyme in a total volume of 0.4 ml. After being incubated at 30°C, the reducing sugar produced was determined by the method of Somogyi–Nelson. Against insoluble cellulose, the reaction was done by shaking at 50 strokes/min, and the supernatant was subjected to analysis. The activity for *p*-nitrophenol (pNP)-derivatives was determined in a mixture consisting of 1.25 mM substrate, 50 mM sodium acetate buffer (pH 5.5), and a suitable amount of enzyme in a total volume of 0.4 ml. After being incubated at 30°C, the reducing sugar produced was determined by the method of Somogyi–Nelson.

Purification of cellulase components. Fifteen ml of the enzyme extract (cultured for 18 d on a solid medium) was mixed with 0.25 g of Avicel and 3 ml of 0.15 M sodium acetate buffer (pH 5.0), and then incubated for 1 h on ice. It was centrifuged again, and the precipitation was washed twice with 50 ml of 20 mM sodium acetate buffer (pH 5.0). The adsorbed proteins were eluted with 1% SDS solution by boiling for 5 min. The supernatant was subjected to the subsequent experiments.

Amino acid sequencing. The supernatant was subjected to SDS–PAGE using a 12% polyacrylamide gel. The N-terminal sequence was determined for the protein transferred to a PVDF-membrane using an automated protein sequencer (PPSQ-21, Shimadzu, Kyoto, Japan). The internal sequence was also determined for the protein digested by protease as follows: The SDS–PAGE gel was stained with the reverse stain kit (Atto, Tokyo, Japan), and the protein was recovered by Attoprep MF (Atto). The protein obtained (2 µg) was digested with lysyl endopeptidase (0.05 µg, Wako Pure Chemical Industries, Osaka, Japan) in 50 mM Tris–HCl buffer (pH 9.0), V8 protease (0.05 µg, Daiichi Pure Chemicals, Tokyo, Japan) in 50 mM Na2HPO4-citric acid buffer (pH 7.8), and trypsin (0.05 µg, Wako) in 50 mM Tris–HCl buffer (pH 8.0). It was incubated for 45 min at 30°C, and then subjected to electrophoresis and sequencing as described above.

PCR-based cloning of cDNA and gene of endoglucanase. To identify the *T. hirsuta* endoglucanase gene, nested PCR was performed with a cDNA library as template. The template DNA (0.1 ml) was prepared by ethanol precipitation following phenol/chloroform extraction of 0.5 ml of the cDNA library (6.3 × 10⁸ pfu). One µl of template DNA (about 10 ng) was used for PCR using Platinum Pfx DNA polymerase (Invitrogen, Carlsbad, CA) with high fidelity. The four degenerate primers (1, 5′-GGAAGCTTCCGIGCYWSSGGYGCICAGSWSSCA-3′; 2, 5′-GGAAGCTTTGATGCACTSCSTACACCGGSGCTGG-3′; 3, 5′-GGGATCCGAYTAGYTGGAAR-TARTCICCCCACCA-3′; 4, 5′-GGGATCCCCACCA-KGGICCCGSGCCACCACA-3′, the additional restriction sites are underlined) were synthesized on the basis of the conserved sequences of the reported fungal endoglucanase classified into the glycoside hydrolase (GH) family 5 (see http://afmb.cnrs-mrs.fr/CAZY/). The ends of the cDNA were also amplified by 5′- and 3′-RACE using two specific primers (5′, 5′-GGGATCCACCTCTTGGAACACCGGAGCGGTTC-3′; 6, 5′-GG-AAGCTTTGGTGTGCGGCTGGGTATCCGC-3′) and vector primers. The gene sequence was also amplified by PCR using two specific primers (7, 5′-GGGATCCGACGCACACGATGAAAGGCAGATCCCTC-3′; 8, 5′-GGGATCCGACGAGGAGATGAAAGGCAGATCCCTC-3′) using genomic DNA as a template. The positions of the all primers are shown in Fig. 3. The amplified fragments were connected with pCR2.1 vector (Invitrogen) by TA cloning, and sequenced from both ends with an automated DNA sequencer.

Construction of the expression system. The EG from *T. hirsuta* was expressed in *A. oryzae* niaD using pNAN8142 vector. The transformation was manipulated according to the method of Gomi *et al.* The full-length of cDNA, which was amplified by PCR using primers 7 and 8, was cloned into pCR2.1 by TA cloning. The fragment obtained by EcoRI digestion was then ligated into the same site of pBlueScript II SK+ (Stratagene, La Jolla, CA) in the desired direction (5′-end on the XhoI donor by Oji Paper Co, (Tokyo) and carboxymethyl cellulose (CMC) were purchased from Tokyo Chemical Industry (Tokyo).
side and 3′-end on the XbaI side). To delete the BamHI site in the multicloning site of this plasmid, it was digested with BamHI and treated with T4 DNA polymerase. The flush ends produced were self-ligated. After the sequence was confirmed, the fragment digested with XhoI-XbaI was ligated into the same sites of pNAN8142. The linearlized plasmid by BamHI digestion was subjected to transformation. To obtain transformants in a homokaryotic state, conidia were selected several times on a Czapek-Dox plate.

Expression and purification of the recombinant enzyme. The A. oryzae transformants were grown in 200 ml of DPY medium (2% dextrin, 1% polypeptone, 0.5% yeast extract, 0.5% KH$_2$PO$_4$, and 0.05% MgSO$_4$·7H$_2$O) in a 500-ml baffled flask with rotary shaking at 120 rpm. After incubation for 3 d at 30°C, the mycelia were removed by filtration with double layered gauze. The supernatant (167 ml) was subjected to the following purification: The precipitation, obtained in 20–80% saturation of ammonium sulfate, was dissolved in 5 ml of 20 mM sodium phosphate buffer (pH 6.0). After dialysis against the same buffer, it was applied to a DEAE Sepharose CL-6B (GE Healthcare Bio-Sciences, Tokyo, Japan) column (φ20 × 150 mm) equilibrated with the same buffer. After it was washed with the same buffer, elution was performed with a linear gradient of 0–1.0 M NaCl. The active fraction was concentrated by BIOMAX-5K (Millipore, Billerica, MA) to 2 ml. It was applied onto a Toyopearl HW50S (Tosoh, Tokyo, Japan) column (φ16 × 470 mm) equilibrated with 20 mM sodium acetate buffer (pH 5.5). The active fraction was collected and used in the experiments.

Results and Discussion

Cultivation of T. hirsuta

The ability of T. hirsuta to produce cellulolytic enzymes was investigated using a liquid medium containing Avicel and glucose as a carbon source, and a natural solid medium of wheat bran. The activities of CMC and Avicel degradation were measured as an index of cellulose degradation ability. The activity of β-glucosidase was detected using pNP β-D-glucoside as substrate. pNP β-D-lactoside is known as a selective substrate digested by some cellulases. All these activities were induced by a cellulosic substance, and their activities in glucose medium were undetectable (data not shown). As shown in Fig. 1, the specific activities of all the enzymes on solid medium were about 10 times higher than in liquid medium. Especially, CMC degradation and β-glucosidase activities were high in both media. It might be convenient that β-glucosidase prevents the product inhibition of endo- and exo-type cellulases.

![Fig. 1. Time Course of Cellulase Activity during Cultivation. T. hirsuta was cultured in a liquid medium containing Avicel as the carbon source (A), and a wheat bran solid medium (B).](image1)

![Fig. 2. SDS-PAGE of the Adsorbed Proteins on Avicel and Its Digestion by Proteases. A. Each fraction from Avicel purification was analyzed. Lane 1, total proteins; lane 2, non-adsorbed protein; lane 3, absorbed protein. Arrow indicates the 44 kDa protein. B. The purified 44 kDa protein (lane 1) and the hydrolysates by lysyl endopeptidase (lane 2), V8 protease (lane 3), and trypsin (lane 4) were analyzed. L1, L2, V1, and T1 fragments were subjected to protein sequencing. UD, undigested 44 kDa protein. All markers were the same as that used in A.](image2)
On the other hand, only slight activity against Avicel was found. These findings indicate that most enzymes responsible for Avicel degradation were not in the supernatant of the extract. Rather, they might have bound with the cellulosic substance in the medium. We tried to identify cellulase components with higher activity in the extract of the solid medium.

**Purification of cellulase components**

Conventional purification was performed by Avicel-affinity. The adsorbed activity and protein content were evident in the difference between the non-adsorbed fraction and the crude extract. The adsorbed CMC degradation activity was estimated to be 16.8%, and the protein adsorbed was less than 1%. As shown in Fig. 2A, the three adsorbed proteins were revealed by SDS-PAGE, which showed two major proteins (58 and 54 k) and a minor protein (44 k). The N-terminal sequences of the 58- and 54-k proteins corresponded almost exactly with those of En-1 from *I. lacteus* and endo-β-1,4-glucanases belonging to GH family 5.21 These cellulases.21 On the other hand, only slight activity against Avicel was found. These findings indicate that most enzymes responsible for Avicel degradation were not in the supernatant of the extract. Rather, they might have bound with the cellulosic substance in the medium. We tried to identify cellulase components with higher activity in the extract of the solid medium.

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fungal enzymes contain CBM in the N-terminal. On this basis, the 44-k protein was postulated to be an endo-β-1,4-glucanase belonging to GH family 5. It was named ThEG.

**Sequencing of the cDNA and the gene**

To determine the structure of ThEG, the gene, named eg1, and the cDNA were sequenced. The cDNA encoded 384 amino acids with a calculated Mr of 40.6 k. As shown in Fig. 3, a comparison of amino acid sequences in the BLAST search (only of those whose gene was registered) revealed high homology with the sequences of *Irpex lacteus* En-1 (73% identity, AB194135), *Humicola grisea* EG (46%, D84470), and *A. niger* EglB (49%, AJ224452). These are all endo-β-1,4-glucanases belonging to GH family 5 containing family 1 CBM, except for *A. niger* EglB. In comparing the sequences of the catalytic domain, ThEG and En-1 from *I. lacteus* were 89% similar. The deduced structure of ThEG consisted of a fungal CBM, a connecting linker region, and a catalytic domain. But the length of the linker was much shorter than that of the other linkers. The N-terminal sequence determined by the protein sequencer presented downstream of the signal sequence containing 21 hydrophobic amino acids. Consequently, the N-terminal of the mature protein was 7 amino acids shorter than that of En-1, and the cleavage site was located in the CBM. But the adsorption capacity might have been maintained, because the essential aromatic amino acids for adsorption were completely conserved.

Figure 4 shows the domain structures of these enzymes and the positions of the introns. The sequence of *eglI*, 1,932 bp, was interrupted by 11 introns, which confirmed the GT-AG rule with regard to RNA splicing. Especially, the positions of the introns were almost the same with that of *I. lacteus En-1* which has three introns more in the catalytic domain than does *eglI*. In comparing the three genes of fungal endo-β-1,4-glucanase, it was found that only two introns, located at both ends of the catalytic domain, were completely conserved. The cDNA and gene sequences have been deposited in the GenBank databases under accession nos. AB125596 and AB125597 respectively.

**Expression of ThEG and enzyme purification**

The *A. oryzae* transformant was cultured in DPY
medium for 3 d, and the supernatant was subjected to activity assay and SDS–PAGE. The transformant produced 5.6 U/ml of CMC degradation activity with 0.58 U/mg of specific activity. Analysis by SDS–PAGE revealed the appearance of a recombinant protein with an Mr around 50 k (Fig. 5). This was different from the native enzyme (44 k) and the calculated Mr from cDNA (38.6 k). The recombinant enzyme (rEG) was purified easily in three steps, as shown in Table 1. Finally, the specific activity for CMC degradation reached 45 U/mg, and the recovery was estimated to be 57%. These values indicate that 126 mg of the recombinant enzyme was produced in 1 liter of culture.

**Enzymatic properties of rEG**

The substrate specificity of rEG was determined using various celluloses with different crystallinities as substrate (Table 2). The activity decreased remarkably as the crystallinity index increased. Especially, the activities against high crystalline celluloses such as cotton, Avicel, and LBKP were less than 1/1,000 as compared with that of CMC. The activity ratio (CMC/Avicel = 2,703) was much lower than that of En-1 (21392) from *I. lacteus*. The specific activity of the enzymes did not differ very much (rEG, 45 U/mg; En-1, 25.9 U/mg against CMC). This indicates that the rEG activity against Avicel was higher than that of En-1. Kanda *et al.* reported that the activity ratio of endo-2 from *Trichoderma reesei* was 7.027, and that it showed an intermediate type of depolymerization between exo- and endo-type cellulase. It was also reported that the length of the linker affected adsorption and catalytic efficiency. The linker length of ThEG was 13 amino acids shorter than that of En-1. It is possible that the short linker resulted in higher accessibility of the catalytic domain with crystalline cellulose. As shown in Fig. 6, rEG showed optimum activity at pH 5.0, and was stable between pH 3–10 (> 80%). At pH 3.5, which was the optimum pH of En-1 from *I. lacteus*, rEG activity was less than 10%. The optimum temperature was 50 °C, and it was stable (> 80%) below 50 °C.

Among the cellulase components, we identified ThEG, which showed high homology with En-1 from *I. lacteus*. But the reactivity on crystalline cellulose and the pH profile between the enzymes were remarkably different. In the domain structure, the main difference was the length of the linker. In addition, it is possible that partial alteration of amino acids in the catalytic domain.
domain and modification of sugar chains influenced it. To confirm this, it is necessary to construct the chimeric enzyme of both. The synergistic effect of cellulose degradation by ThEG together with other cellulases is also interesting. A different enhancement mechanism of cellulose degradation is to be expected if it were to be combined with an exo-type cellulase as well as an endo-type cellulase such as En-1. This information might be effective in attempts to increase the degradation of crystalline cellulose by cellulases.

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