Journal of Bioscience and Bioengineering
VOL. 128 No. 6, 669–676, 2019

ELSEVIER

www.elsevier.com/locate/jbiosc

Purification and characterization of a glycoside hydrolase family 5 endoglucanase from *Tricholoma matsutake* grown on barley based solid-state medium

Hiroki Onuma, Kento Hara, Kayo Sugita, Akiko Kano, Yasuhisa Fukuta,* and Norifumi Shirasaka

Department of Applied Biological Chemistry, Graduate School of Agriculture, Kindai University, 3327-204 Naka-machi, Nara 631-8505, Japan

Received 17 January 2019; accepted 23 May 2019
Available online 27 June 2019

An endoglucanase was isolated from solid-state culture of the ectomycorrhizal fungus *Tricholoma matsutake* (*TmEgl5A*) grown on rolled barley and vermiculite. The enzyme was purified by ammonium sulfate fractionation, ion-exchange, hydrophobic, and gel filtration. TmEgl5A showed a molecular mass of approximately 40 kDa as determined by SDS–PAGE. The single band of the protein was analyzed by peptide-mass-finger-printing using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and the trypsin-digested peptide sequences were matched to a putative endoglucanase sequence (protein ID1465229) in the JGI *T. matsutake* 945 v3.0 genome database. Based on the sequence information, the gene encoding TmEgl was cloned and expressed in *Pichia pastoris* KM71H. The deduced amino acid sequence was similar to GH5 family endoglucanases from Basidiomycetes. The enzyme acts on barley β-glucan, lichenan, and CMC-Na. The hydrolyzation products from these substrates were detected by thin-layer chromatography as oligosaccharides with minimal disaccharides. These results suggested that *T. matsutake* produces a typical endoglucanase in solid-state culture, and the fungus has the potential to degrade β-linkage polysaccharides.

[**Key words:** Endoglucanase; Gene cloning; Heterologous expression; Purification; Solid-state cultivation; *Tricholoma matsutake*]

Tricholoma matsutake is an economically important ectomycorrhizal fungus in Japan where it holds its exceptional commercial value as a highly sought-after edible mushroom (1). As part of Japanese culture, this mushroom is more than just a seasonal delicacy. However, local production of this valuable mushroom has declined severely over the last few decades, and as a result Japan now imports matsutake from other countries (2). Because the fungus grows on living plant roots (i.e., *Pinus densiflora*) and mycelial growth is slow *in vitro*, it scarcely produces fruiting bodies in artificial cultivation. For the formation of a fruiting body in purely artificial cultivation, there have only been three reports of fruiting-body formation in artificial culture (3–5). There have also been problems with reproducibility along with known extremely slow mycelial growth and limitations with sugar utilization by the fungi (glucose, fructose, maltose, trehalose, cellobiose, or starch) (6,7).

In 1996, artificial cultivation of an ectomycorrhizal fungus, *Lyophyllum shimeji* (Kawam.) Hongo, was carried out successfully in bottle cultivation using barley (*Hordeum vulgare*) grains and sawdust medium. These reports indicated that barley starch can be used as a carbon source and provide factors for the production of the fruiting bodies in the medium (8–10). Barley grains contain high concentrations of starch and mixed-linkage (1,3;1,4)-β-D-glucans in the endosperm (11). Starch is the main component of barley, constituting approximately 75% of the endosperm (12). Some recently developed barley cultivars contain starches with a broad range of amylose content, varying between 0% and 40% (13). In addition, the water absorption coefficient of barley is 73.2%–78.2% of grain weight after 2 h immersion, 3.3 to 3.5 times higher than that of rice (14). Barley has not only superior water absorption but also high starch content, which is useful in solid-state cultural substrates for the artificial cultivation of ectomycorrhizal mushrooms.

Kusuda et al. (15) described that *T. matsutake* shows β-glucosidase activity, and the enzyme was purified and characterized. However, the fungus showed very weak activity of cellulases (endo-type glucanase and cellobiohydrolase), xylanase, and glucoamylase in synthetic liquid culture (16,17). For these reasons, it seemed difficult to use this cultivation method for saprophytic mushrooms for artificial cultivation of *T. matsutake*. However, we found that a glucoamylase (*TmGlu1*) is induced by cultivation with soluble starch as a carbon source, and an enzyme from *T. matsutake* strain NBRC 30605 was purified and its enzymatic properties were characterized (18). In addition, *TmGlu1* was secreted when expressed in the methylotroph yeast *Pichia pastoris*, revealing that the translated gene showed glucoamylase activity. This work demonstrated that *TmGlu1* has a potentially valuable function for starch degradation in the vegetative mycelial growth of *T. matsutake*. Recently, a xylan-degradation enzyme was purified from *T. matsutake* and characterized (19). This enzyme belongs to glycoside hydrolase family 3 protein β-xylosidase, which shows exo-type activity according to its ability to convert xylan to xylose directly. These results indicated that *T. matsutake* has the potential to degrade polysaccharides and change simple sugars by itself. However, there have been no detailed investigations on the degradation of β-linkage

* Corresponding author. Tel./fax: +81 742 43 7362.
E-mail address: yfukuta@nara.kindai.ac.jp (Y. Fukuta).

1389-1723/$ – see front matter
https://doi.org/10.1016/j.jbiosc.2019.05.012

polysaccharides such as cellulose or β-glucan using the solid-state cultivation with this fungus.

In this paper, we describe the purification and identification of an endoglucanase produced by *T. matsutake* in barley based solid-state cultivation, its molecular cloning, and heterologous expression of the gene in *P. pastoris*.

MATERIALS AND METHODS

Strain and culture conditions *T. matsutake* strain NBRC30605 was obtained from the NITE Bioresource Research Center (Chiba, Japan). *T. matsutake* mycelia were grown in SY medium (2% soluble starch from corn, 0.3% yeast extract and 2% agar, w/v, pH 5.1) for 40 days at 24°C with constant conditions. Standard solid-state medium contained 30 g of rolled barley and 15 g of vermiculite with a 60% moisture content in 100 mL Erlenmeyer flask. Liquid contents of the media were prepared with 0.3% yeast extract, although the water uptake of rolled barley absorbed sufficiency at 4°C for 12 h. The initial pH of the media was adjusted to 5.1 before sterilization at 121°C for 90 min. To cultivate the mycelia, the strains were cut into 5-mm diameter mycelial discs from SY agar medium, and solid-state medium was inoculated with five mycelial discs. Inoculated flasks were placed in an incubation chamber (Nippon Medical & Chemical Instruments, Osaka, Japan) for 40 days at 24°C with 70% humidity. In the experiment of effect on the solid-state medium contents for the enzyme activity, weight ratio of rolled barley and vermiculite were prepared as 1:0 (only 45 g of rolled barley), 2:1 (30 g of rolled barley and 15 g of vermiculite), 1:1 (22.5 g of rolled barley and 22.5 g of vermiculite), and 0:1 (only 45 g of vermiculite). For liquid cultivation, *T. matsutake* mycelia were inoculated into glucose-yeast extract complex liquid medium (GYL) containing 2% glucose, 0.5% yeast extract, pH 5.1. After incubation of the cultures at 24°C for 30 and 60 days, the culture medium was filtered through filter paper (Toyo Roshi, Tokyo, Japan) to remove mycelia, and the culture filtrate was then dialyzed in 50 mM sodium acetate buffer (pH 5.0).

Enzyme assay and protein determination Endoglucanase activity was detected by the release of reducing sugars from barley β-glucan (Megazyme, Wicklow, Ireland) and carboxymethyl cellulose sodium salt (CMC-Na; Nacalai Tesque, Kyoto, Japan) using the 3,5-dinitrosalicylic acid (DNS) method (20). Each reaction mixture containing 1.0% (w/v) substrate in 180 μL sodium acetate buffer (50 mM, pH 4.0) and 20 μL of diluted enzyme solution was incubated in block incubator at 37°C for 15 min. Enzyme reactions were terminated with 600 μL of DNS reagent, and colorization by boiling for 5 min. After the coloring reaction, the samples were cooled to room temperature and absorbance measured at 500 nm using an absorption spectrometer. One unit of endoglucanase activity was defined as the amount of enzyme that released 1 μmol of reducing sugar per minute. Protein concentration was determined using a micro BCA protein assay kit (Thermo Fisher Scientific, Carlsbad, CA, USA) with BSA as a standard (21).

Purification of endoglucanase from *T. matsutake* NBRC 30605 Unless otherwise stated, all purification steps were performed below 4°C. Forty-five flasks of *T. matsutake* mycelia cultivated in standard solid-state medium (30 g of rolled barley and 15 g of vermiculite) at 24°C for 40 days (1.04 kg) were extracted in 3.0 L of 50 mM sodium acetate buffer (pH 4.0) by immersion for 24 h and filtering through cheesecloth. The extract was centrifuged at 10,000 × *g* for 30 min at 4°C to remove the fine vermiculite and barley debris, which yielded a crude extract. Ammonium sulfate was added to the crude extract, with gentle stirring, to 50% saturation. After equilibration, for 4 h at 4°C, the precipitate was removed, and ammonium sulfate was added again to 75% saturation. The solution was allowed to equilibrate for 4 h, and the resulting precipitate was collected after centrifugation (12,000 × *g* for 30 min at 4°C). The precipitate was dissolved in 50 mM potassium acetate buffer (pH 4.0) and dialyzed for 24 h against the same buffer. Then, the dialyzed solution was centrifuged (8000 × *g* for 10 min at 4°C) and the supernatant was loaded onto Toyopearl Sulfate-650F (φ2.5 × 25 cm) pre-equilibrated with 50 mM sodium acetate buffer, pH 4.0. The active flow-through fraction was then brought to 30% ammonium sulphate saturation and loaded onto a Toyopearl Butyl-650M column (φ2.5 × 10 cm) that had been equilibrated with 30% saturated ammonium sulfate. The bound proteins were eluted with a 300 mL gradient of ammonium sulfate at 30%–0% saturation in ddH_2O. The active fractions were combined and dialyzed for 24 h with 20 mM Tris–HCl buffer (pH 7.0). The dialyzed protein solution was then concentrated using an Amicon Ultra-15 Centrifugal Filter Units (10 kDa cut off; Merck Millipore, CA, USA). The concentrated solution was loaded onto a MonoQ 5/50 GL (GE Healthcare UK, Buckinghamshire, UK) column that has been equilibrated with 20 mM Tris–HCl buffer (pH 7.0). The flow-through fraction was concentrated using an Amicon Ultra-15 Centrifugal Unit. The protein samples obtained in the previous step were applied to a Superdex 200 increase 10/300 GL column (GE Healthcare UK) equilibrated with 20 mM Tris–HCl buffer (pH 7.0) containing 150 mM NaCl. Then, the column was eluted by high-performance liquid chromatography (HPLC) at 0.5 mL min^{-1}, and the active fractions were collected.

SDS polyacrylamide gel electrophoresis analysis SDS polyacrylamide gel electrophoresis (SDS–PAGE) was performed using a Tris–HCl/glycine buffer system and 12.5% polyacrylamide gel described by Laemmli (22). Protein samples were boiled for 10 min in the presence of 10% SDS, 2-mercaptoethanol, and bromophenol blue and applied to sample wells and electrophoresed. The separated proteins were stained with Coomassie Brilliant Blue R-250. The following molecular mass standards was used as Precision Plus Protein Standards (Bio-Rad Laboratories, Hercules, CA, USA).

Peptide-mass-finger-printing of purified endoglucanase protein Purified endoglucanase (50 μg) was separated by SDS–PAGE and was treated by in-gel digestion with trypsin (Fujifilm Wako Pure Chemicals, Tokyo, Japan). The digested samples were analyzed using an Autoflex Speed KN2 (Bruker, MD, USA). The peptide-mass-finger-prints from MS/MS data were acquired and processed using Flexanalysis software (Bruker) and converted to LIFT files using Proteinscape 3.0.3 software (Bruker). The LIFT files were analyzed using the MASCOT search engine (Matrix Science, London, UK). Then local BLAST analysis was performed using NCBI BLASTP search and Joint Genome Institutes (JGI) *T. matsutake* 945 v3.0 protein sequences (https://genome.jgi.doe.gov/Trima3/Trima3.home.html).

Genomic and cDNA cloning of the endoglucanase gene (*TmEgl5A*) For genomic DNA and cDNA cloning, mycelia of the fungus grown in solid-state culture were ground in liquid nitrogen, and the powdered sample was used for total RNA and genomic DNA extraction. Genomic DNA was extracted using ISOPLANT II (Nippon Gene, Tokyo, Japan) in accordance with the manufacturer's instructions. Total RNA was extracted using an FastGene RNA Basic Kit (Nippon Genetics, Tokyo, Japan). First strand cDNA was synthesized from total RNA using ReverTra Ace (Toyobo, Osaka, Japan). Primer pairs were designed by prediction gene using the JGI *T. matsutake* 945 v3.0 genome database and *T. matsutake* NBRC 30605 genome database (DDBJ/EMBL/GenBank accession no. BDDP01000001-BDDP01088884) as a reference. The cDNA and genomic DNA of the *TmEgl5A* gene was amplified using general PCR methods with a forward primer (5′-ATGAAGTCTTCCTTAGCATT-3′) and reverse primer (5′-TTACAAGAACGGCTTCAGGG-3′). PCR was performed in a 50 μL reaction mixture using KOD plus DNA polymerase (Toyobo). Amplified cDNA and genomic DNA fragments were cloned into T-vector pMD20 (Takara Bio, Shiga, Japan) and sequences ascertained by DNA sequencing using an ABI PRIZM 310 Genetic Analyzer (Applied Biosystems Japan, Tokyo, Japan).

Heterologous expression of *TmEgl5A* in *P. pastoris* The cDNA fragment encoding *TmEgl5A* without a signal peptide sequence was flanked by *Xho*I and *Xba*I restriction sites at the 5′ and 3′ ends, respectively. Then cDNA fragment was amplified by PCR using the following primer pair: 5′-TTTTCTCGAGAAAAGA-GAGGCTGAAGCTGTCGTTCCTGTCTGG-3′ and 5′-TTTTTCTAGATTACAAGAACGG CTTCAGGG-3'. After digestion, the fragment was ligated into the pPICZαA vector to yield the construct pPICZαA-Tmegl5A, which was digested using *Sac*I and linearized. Transformation of *P. pastoris* KM71H with the linearized pPICZαA-Tmegl5A was performed using Electro Gene Transfer Equipment GTE-10 (Shimadzu, Kyoto, Japan). Heterologous expression of *TmEgl5A* were done in accordance with the methods described by Onuma et al. (18).

Sequence analysis Searches for conserved domains were done using the protein BLAST program via the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/) database. The signal peptide was predicted using the SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/). The amino acid sequences were aligned using the NCBI BLASTP (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and GENETYX ver. 13 programs. The putative *N*-glycosylation and *O*-glycosylation sites were predicted using online servers (NetOGlyc 4.0 server; http://www.cbs.dtu.dk/services/NetOGlyc/, and NetNGlyc 1.0 server; http://www.cbs.dtu.dk/services/NetNGlyc/).

Enzymatic characterization of a *Tm*Egl5A The optimum pH of the purified recombinant *Tm*Egl5A was determined by measuring barley β-glucan hydrolytic activity at 37°C containing 50 mM of pH buffer: McIlvain buffer (pH 2.2–6.0), sodium acetate buffer (pH 4.0–6.0), sodium phosphate buffer (pH 6.0–8.0), or Tris–HCl (pH 7.0–10.0). pH stability was estimated by measuring residual activity in standard conditions (pH 4.0, 37°C for 10 min) after preincubation at 37°C in the same buffer for 30 min. The optimum temperature for *Tm*Egl5A activity was determined in a temperature range of 4–80°C in sodium acetate buffer (pH 4.0) for 30 min. For the determination of thermal stability, *Tm*Egl5A was preincubated in temperature ranges of 4–80°C in sodium acetate buffer (pH 4.0). After cooling on ice, residual activity was measured in standard conditions.

To determine of the effects of chemicals on *Tm*Egl5A activity, metal ions (Li^+, Na^+, K^+, Ag^+, Ca^{2+}, Mn^{2+}, Hg^{2+}, Fe^{2+}, Pb^{2+}, Ba^{2+}, Zn^{2+}, Cu^{2+}, Co^{2+}, Ni^{2+}, Mg^{2+}, Fe^{3+}, or Al^{3+}), chemical reagents (ethylenediamine tetra acetic acid [EDTA], or dithiothreitol [DTT], or β-mercaptoethanol) were added to the standard reaction conditions at a final concentration of 1 mM. The activity of reaction controls was determined without metal ions or chemical reagents.

To evaluate the substrate specificity, polysaccharides substrates tested as follows: Avicel PH-101 (Sigma–Aldrich, St. Louis, MO, USA), CMC-Na (Nacalai Tesque), barley β-glucan (Megazyme), lichenan (Megazyme), pachyman (Megazyme), curdlan (Fujifilm Wako Pure Chemical), laminaran (Tokyo Chemical Industry, Tokyo, Japan), pustulan (Invitrogen, San Diego, CA, USA), beech wood xylan (Serva, Heidelberg, Germany) at a final concentration of 1.0% (w/v). *p*-Nitrophenyl-β-glucopyranoside (pNPG; Nacalai Tesque) and *p*-nitrophenyl-β-cellobioside (pNPC; Tokyo

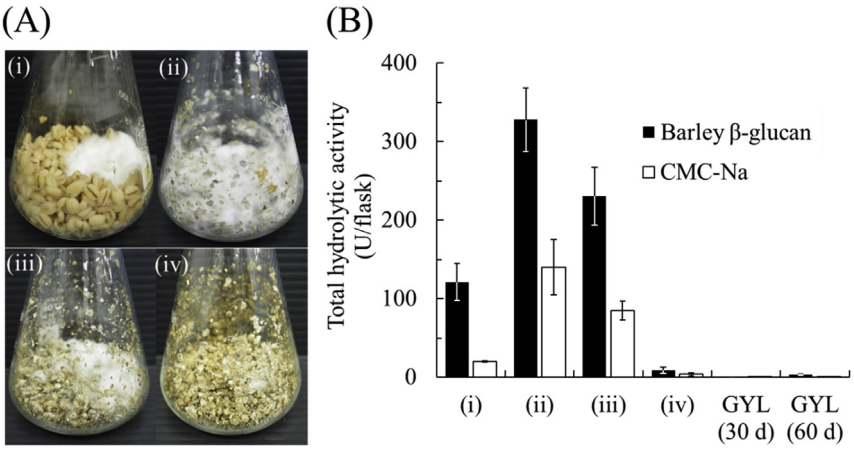


FIG. 1. Cultivated *Tricholoma matsutake* strain NBRC 30605 grown on solid-state media and hydrolysis of barley β-glucan and CMC-Na. (A) (i) Rolled barley medium, (ii) 2:1 weight ratio of rolled barley and vermiculite medium, (iii) 1:1 weight ratio of rolled barley and vermiculite medium, (iv) vermiculite medium. (B) Enzyme assays were performed using 1% barley β-glucan or CMC-Na in 50 mM sodium acetate buffer (pH 4.0) as substrates. Error bars indicate standard deviations (±SD) of experiments performed in replicates of five.

Chemical Industry) were used at a final concentration of 1.0 mM to determine the substrate specificity of the enzyme. The reaction was terminated by adding 0.5 M sodium carbonate and color developed. The activity was measured by reading the absorbance at 405 nm as the amount of *p*-nitrophenol.

Thin-layer chromatography analysis of β-glucan and CMC hydrolysis by *Tm*Egl5A Barley β-glucan, lichenan, or CMC-Na (1.0%, w/w) were individually treated with *Tm*Egl5A (20 μg/mL) in 100 μL of 20 mM sodium acetate buffer (pH 4.0) at 37°C for 12 h. The reaction solutions were kept in a boiling water for 5 min to inactivate enzymes and then applied to ion-exchange resin, Dowex 50WX8 (Fujifilm Wako Pure Chemical). Hydrolysates were subjected to thin layer chromatography (TLC) on silica gel 60 F254 plates (Merck Millipore) in acetic acid/methanol/water (8:4:3, v/v) and stained with 50% H_2SO_4 (v/v).

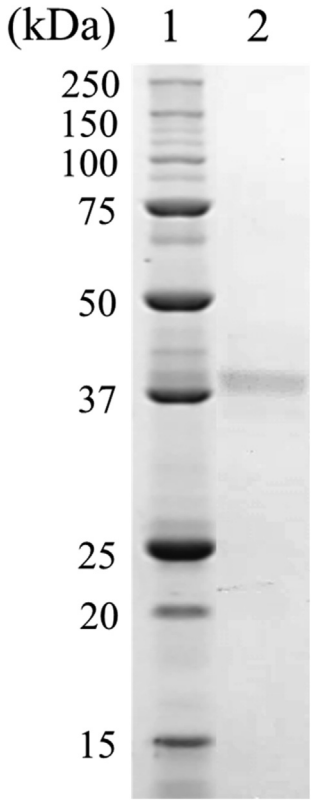


FIG. 2. SDS–PAGE analysis of purified endoglucanase. Lane 1, molecular marker; lane 2, purified endoglucanase.

Nucleotide sequence accession number The nucleotide for the *T. matsutake* NBRC30605 endoglucanase gene (*TmEgl5A*) has been submitted to the DDBJ/EMBL/GenBank databases under accession number LC424191.

RESULTS

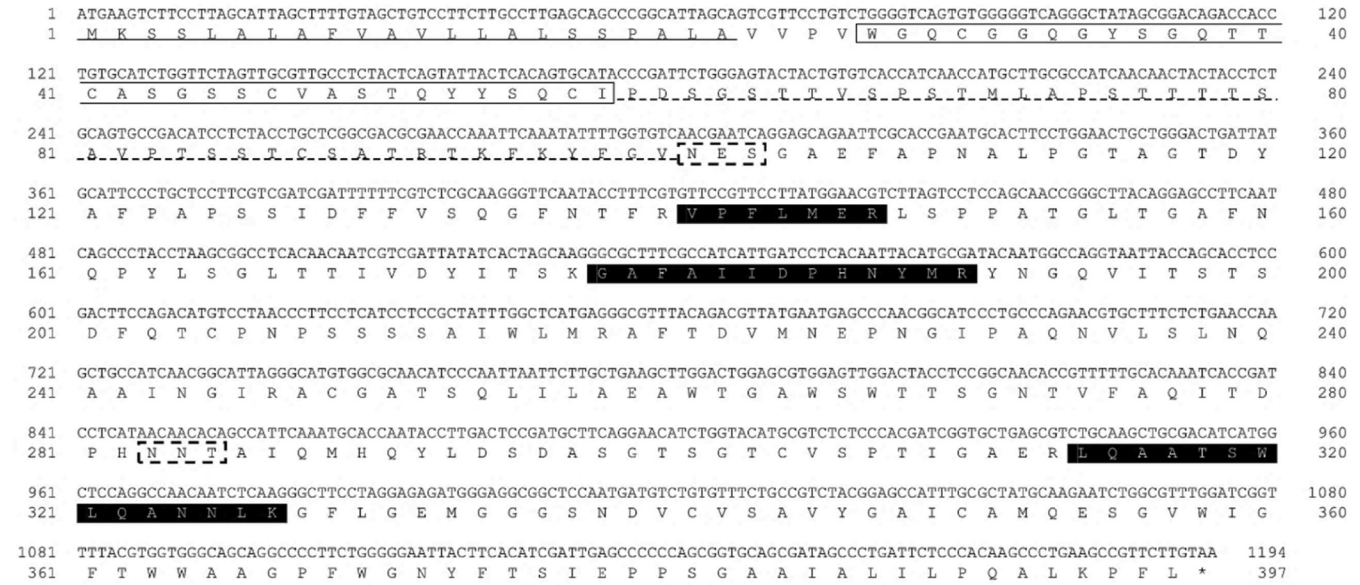
Purification and identification of the endoglucanase from *T. matsutake* grown in solid-state culture To determine the productivity of the endoglucanase activity in *T. matsutake*, we examined cultures in the presence of several compositions of barley based solid-state medium (40 days incubation) and GYL medium (30 and 60 days incubation), as shown in Fig. 1. Of the media tested, rolled barley gave the fastest vegetative mycelial growth, followed by a mixed weight ratio of 2:1 of rolled barley and vermiculite (Fig. 1A). Moreover, this condition had the highest activities with barley β-glucan and CMC-Na hydrolysis of 327.9 ± 40.4 U/flask and 140.4 ± 35.2 U/flask (Fig. 1B). In contrast, the hydrolytic activities with barley β-glucan and CMC-Na were extremely weak on GYL medium after both cultivation periods.

For purification of the endoglucanase protein, active protein bound to only Butyl-650M in the initial step was separated as a single peak by Superdex 200 column chromatography. However, this protein did not bind to ion-exchange chromatography columns. The endoglucanase protein purified from the active fraction by gel filtration was shown to be homogeneous by SDS–PAGE analysis (Fig. 2). After these purification steps, the specific activity of endoglucanase was 414.6 U/mg protein and increased 93.1-fold with a 0.5% recovery yield (Table 1). The molecular mass of purified endoglucanase was estimated by SDS–PAGE to be 40 kDa.

TABLE 1. Purification of endoglucanase from *Tricholoma matsutake* NBRC 30605.

Purification steps	Protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (-fold)
Crude extract	9002	40100	4.45	100	1.0
Ammonium sulfate fractionation (50–75%)	508.7	5700	11.2	14.2	2.5
Sulfate-650F	74.9	1022	13.7	2.5	3.1
Butyl-650M	11.4	516.4	45.4	1.3	4.0
Mono Q 5/50 GL	0.63	232.1	368.7	0.6	82.8
Superdex 200 Increase 10/300 GL	0.52	216.3	414.6	0.5	93.1

(A)



(B)

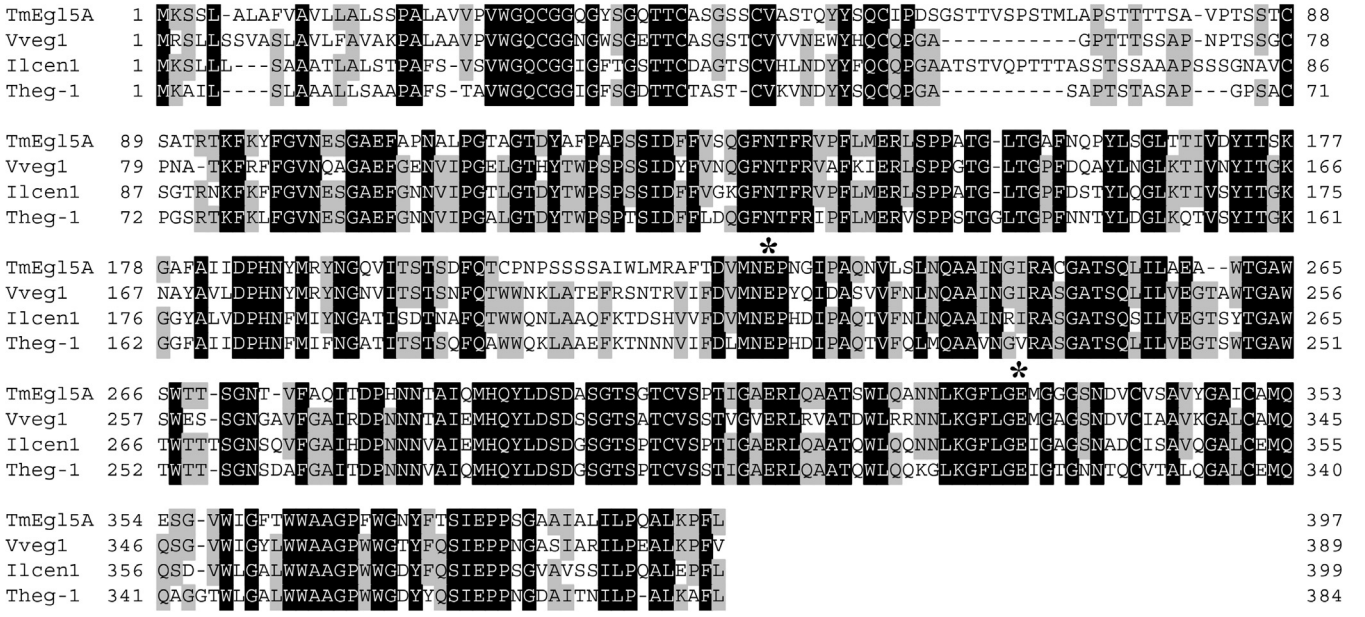


FIG. 3. Nucleotide sequence and deduced amino acid sequences of *TmEgl*5A, and sequence alignment of *TmEgl*5A and its homologous basidiomycete GH5 enzymes. (A) Underlined, potential signal peptide sequence; boxed, cellulose-binding domain; dotted line, internal sequence of Ser/Thr-rich linker amino acids; dotted box, putative *N*-glycosylation sites (Asn−Xaa−Ser/Thr, in which Xaa is not proline); outline, identified tryptic fragments from the purified enzyme. (B), The sequence alignment analysis was performed using Genetyx Ver. 13. Black and gray boxes indicate amino acids identical and similar to *TmEgl*5A, respectively. *Vveg1*, from *Volvariella volvacea* (accession no. AAG59832); *Ilcen1*, from *Irpex lacteus* (accession no. BAD67544); *Theg-1*, from *Trametes hirsuta* (accession no. BAD01163). Two deduced catalytic glutamate residues are indicated by asterisk.

The purified endoglucanase was analyzed using a matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) system. We identified three trypsin-digested peptides as VPFLMER, GAFAIIDPHNYMR, and LQAATSWLQANNLK corresponding to the deduced amino acid sequence in the JGI *T. matsutake* 945 v3.0 genome database. These peptide sequences were matched to the sequence of protein ID 1465224 annotated as a putative endoglucanase in the genome database. In addition, these peptide sequences were identified as *Irpex lacteus* MC-2 endoglucanase (AB194135) (23) analyzed by NCBI BLAST search (Fig. 2A). The result indicated that the purified protein is an endoglucanase protein.

Cloning and sequence analysis of *TmEgl5A* A 2107-bp DNA fragment starting at ATG and terminating at a TAA codon was found in the nucleotide sequences encoding *TmEgl*5A in the genomic DNA sequence of *T. matsutake* NBRC 30605. Based on the nucleotide sequence of the full-length cDNA, the endoglucanase protein was cloned by reverse-transcription PCR. Alignment with the *TmEgl*5A cDNA indicated that this genomic sequence contained the

TABLE 2. Substrate specificities of purified *TmEgl*5A.

Substrate	Major linkages[a] (monomer)	Activity (Unit/mg protein)	Relative activity[b] (%)
Barley β-glucan	1,3-1,4-β-Glu*p*	400.3 ± 6.4	100
CMC-Na	1,4-β-Glu*p*	170.9 ± 4.3	42.7
Lichenan	1,3-1,4-β-Glu*p*	81.8 ± 6.1	20.2
Avicel	1,4-β-Glu*p*	ND	–
Curdlan	1,3-β-Glu*p*	ND	–
Pachyman	1,3-β-Glu*p*	ND	–
Laminaran	1,3-1,6-β-Glu*p*	ND	–
Pustulan	1,6-β-Glu*p*	ND	–
Xylan	1,4-β-Xyl*p*	ND	–
pNPG	1,4-β-Glu*p*	ND	–
pNPC	1,4-β-Glu*p*	ND	–

[a] Glu*p*, glucopyranose; Xyl*p*, xylopyranose.
[b] ND, not determined.

complete protein-coding region. The coding region of the *TmEgl*5A gene was interrupted by 14 introns. As shown in Fig. 3A, the 1194-bp cDNA contained an open reading frame encoding 397 amino acid residues. The first 22 amino acid residues in the N-terminal region were assigned as a signal peptide, and the mature protein apparently consisted of 375 amino acids. The molecular mass of mature *TmEgl*5A protein was predicted as approximately 39.4 kDa. Two potential *N*-glycosylation sites (Asn–Xaa–Ser/Thr, in which Xaa is not proline) and several *O*-glycosylation sites (Ser/Thr-rich linker) were predicted (24,25). Homologous protein sequences were obtained through BLAST searches of the NCBI GenBank database. We estimated the location of the carbohydrate binding module family 1 (CBM1) domain in the N-terminus from the Glycoside hydrolase family 5 cellulase signature via a Ser/Thr-rich linker region. According to the amino acid sequence, *TmEgl*5A belongs to GH family 5 subfamily 5 (GH5_5). It was shown that *TmEgl*5A has identity levels of 65%, 64%, and 62% with *Volvariella volvacea eg1* (GenBank accession no. AAG59832) (26), *I. lacteus cen1* (accession no. BAD67544) (23), and *Trametes hirsuta eg-1* (accession no. BAD01163) (27) (Fig. 3B). These gene-coding amino acids exhibited cleavage ability of the β-1,4 linkage of

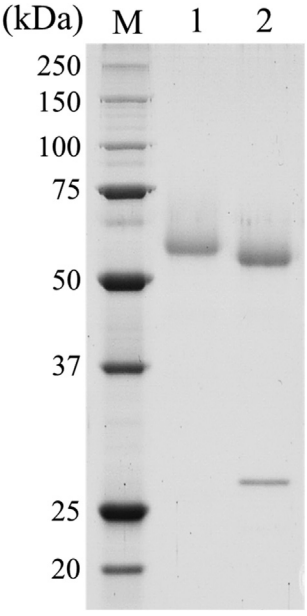


FIG. 4. SDS–PAGE of purified recombinant *TmEgl*5A with endoglycosidase H treatment. Samples were purified enzyme from culture supernatants of *Pichia pastoris* transformants after 120 h. M, molecular marker; lane 1, purified *TmEgl*5A from *P. pastoris*; lane 2, purified recombinant enzyme treated with endoglycosidase H.

CMC-Na, whereas crystalline cellulose showed little hydrolysis. The alignment of the amino acid sequence of *TmEgl*5A with a series of homologous and enzymatically characterized GH5_5 enzymes is shown in Fig. 3B. According to the sequence alignment of these enzymes, the regions containing the catalytic residues (E226 and E332) are conserved, which indicated that these share the same catalytic mechanism.

Heterologous expression, purification, and identification of recombinant *TmEgl*5A Recombinant *TmEgl*5A was secreted into the medium, with high levels on barley β-glucan hydrolytic activity (3963 ± 159 U; ~40 mg protein, per liter). No hydrolytic activity was detected in a control vector with *P. pastoris* KM71H transformant. Recombinant *TmEgl*5A was purified with a specific activity of 398.5 U/mg protein compared with a value of 414.6 U/mg protein for the native enzyme. As shown in Fig. 4, a band of glycosylated *TmEgl*5A appeared at about 60.5 kDa, as determined by comparison with the molecular mass marker. After endoglycosidase H treatment, the band appeared at about 57.5 kDa in a putative non-glycosylated state. When purified *TmEgl*5A was analyzed using a MALDI-TOF/MS system, the amino acid sequence of the tryptic fragmented protein was confirmed to be consistent with the amino acid sequence of the native endoglucanase from *T. matsutake* NBRC30605, it was shown that *TmEgl*5A had been successfully expressed in *P. pastoris* KM71H.

Enzymatic properties of *TmEgl*5A The enzymatic properties of *TmEgl*5A were determined using barley β-glucan as the substrate. Effects of temperature and pH on hydrolytic activities of the recombinant *TmEgl*5A were examined (Fig. 5). *TmEgl*5A exhibited high activity from 60°C and was stable at 4–40°C, which decreased dramatically at 50°C. *TmEgl*5A showed high activity at pH 4.0 with McIlvain buffer and sodium acetate buffer. Stability of pH was associated with high activities when the pH was adjusted to pH 3.0–5.0 and pH 4.0–6.0 with McIlvain buffer and sodium acetate buffer, respectively (~70%; residual activity).

The influence of various chemical additives as amino acid modifiers on the enzymatic activity of *TmEgl*5A was measured (Table S1). Various compounds at a final concentration 1 mM or 5 mM were added to the reaction mixture, and enzyme activity was detected using the DNS method. The hydrolytic activity was increased by the addition of 1 mM Co^{2+}, Ba^{2+}, Fe^{2+}, Fe^{3+}, Al^{3+}, Ni^{2+}, and Mn^{2+}. The enzyme was strongly inhibited by Ag^{+}. The activity of the enzyme was also inhibited by the presence of 1 mM Hg^{2+}, EDTA, DTT, and β-mercaptoethanol. In contrast, Na^{+}, K^{+}, Li^{+}, Ca^{2+}, Mg^{2+}, Zn^{2+}, Pb^{2+}, Cu^{2+}, and EDTA had no effect on the enzyme activity.

To determine the substrate specificity of *TmEgl*5A, several β-glycosidic linkages of glucans were subjected to enzymatic reactions. As shown in Table 2, *TmEgl*5A showed preferential hydrolysis with barley β-glucan and slight hydrolysis of CMC-Na and lichenan with 45.3% and 13.5% relative activities. However, significant activity of *TmEgl*5A was not detected with Avicel PH-101, xylan β-1,3-glucan, β-1,6-glucan, pNPG, and pNPC. These results indicated that *TmEgl*5A exhibited preferential hydrolysis of 1,3-1,4-β-glucan, concomitant with cleavage of 1,4-linkages in 1,3-1,4-β-glucan.

Hydrolytic products of *TmEgl*5A Barley β-glucan, lichenan, and CMC-Na were treated with recombinant *TmEgl*5A, and the reaction products were analyzed by TLC. By treatment with *TmEgl*5A for 12 h, cellobiose, β-1,3-1,4-glucotriose, β-1,3-1,4-glucotetraose, and higher DP oligosaccharides in barley β-glucan hydrolysate were subjected to TLC analysis (Fig. 6). By contrast, four spots, cellobiose, cellotriose, cellotetraose, and cellopentaose, from CMC-Na hydrate with *TmEgl*5A for 12 h were observed. In the case of lichenan, almost no spots were found.

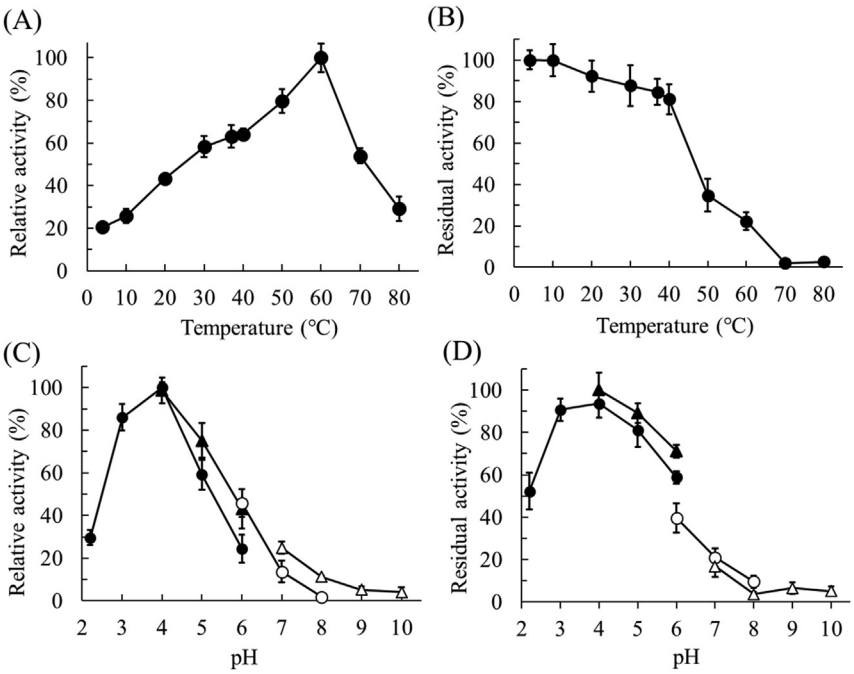


FIG. 5. Enzymatic characterization of *TmEgl5*A activity. (A) The optimum temperature; (B) the thermostability; (C) the optimum pH; the pH stability. (C, D) The buffers used were 50 mM of McIlvain buffer (closed circles), sodium acetate (closed triangles), sodium phosphate (open circles), and Tris–HCl (open triangles). The maximal and residual activity of each enzyme was defined as 100%. Data are presented as the mean of 3 trials (±SD).

DISCUSSION

Complete hydrolysis of cellulose requires the cooperative actions of three types of cellulases: endoglucanase (EC 3.2.1.4) that randomly cleaves the internal β-1,4-glycosidic bonds; two types of cellobiohydrolases, CBHI and CBHII (EC 3.2.1.176 and EC 3.2.1.91) that processivity act on the chain termini to release cellobiose; and β-glucosidase (EC 3.2.1.21) that hydrolyzes cellobiose to glucose

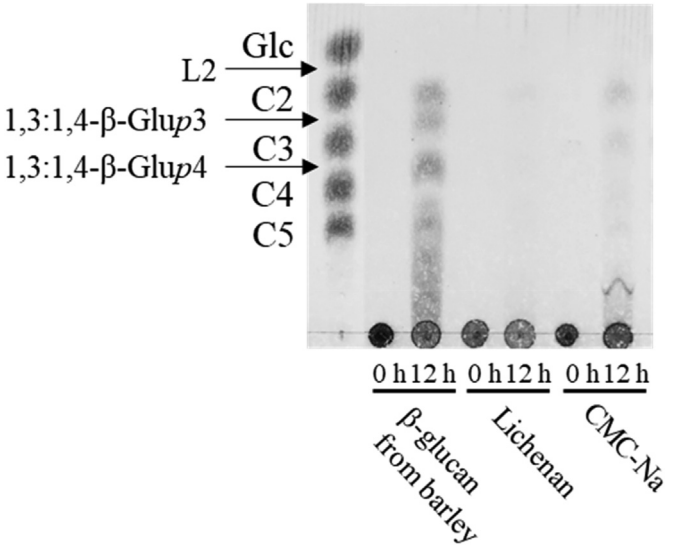


FIG. 6. Production of oligosaccharides using the *TmEgl5*A. Barley β-glucan, lichenan, and CMC-Na were incubated in 50 mM sodium acetate buffer (pH 5.0) at 30°C for 12 h. Hydrolysates were subjected to TLC in ethyl acetate/methanol/water (8:4:3, v/v) and stained with 50% (v/v) H_2SO_4. Markers indicate the positions of glucose (Glc) and oligosaccharides (C2, cellobiose; C3, cellotriose; C4, cellotetraose; C5, cellopentaose) on TLC silica gel plate. L2, laminaribiose; 1,3:1,4-β-Glup3, 3-β-D-glucosyl-cellobiose; 1,3:1,4-β-Glup4, 3-β-D-cellotriosyl-glucose.

(28). Endoglucanase also hydrolyzes the β-1,3-1,4-branched β-glucans converting into oligosaccharides. So far, ectomycorrhizal fungus, *T. matsutake* is known to produce hydrolytic activity of hemicellulose, cellulose and carboxymethyl cellulase (CMCase), although it has been described there are slightly weak (16,17). Previous enzymatic studies revealed that *T. matsutake* produces only β-glucosidase when it grown in synthetic liquid culture (15). However, the test results in these reports were based on synthetic liquid culture, and there is no biochemical knowledge about solid-state culturing system. This study is the first report regarding the efficient production of endoglucanase by solid-state cultivation of *T. matsutake*.

Results from the experiment confirmed that *T. matsutake* is able to grow on solid-state medium containing rolled barley and vermiculite. Here, we found that when *T. matsutake* was grown on barley-based solid-state culture, it specifically produced main extracellular protein that showed barley β-glucan (β-1,3-1,4-glucan) and CMC-Na hydrolyzing activity (Fig. 1). The trypsin-digested internal amino acid sequences of the purified enzyme were analyzed by peptide-mass-finger-printing, and these digested peptides were matched to that of the putative protein-coding gene (contig 21512) in the *T. matsutake* NBRC30605 strain translated genome database (Fig. 3A). Then, this gene was applied to the NCBI BLAST search system, which classified it in GH family 5 subfamily 5 based on amino acid sequence similarities (Fig. 3B). Based on the sequence and structure similarity of CAZymes (http://www.cazy.org), endoglucanases are grouped into 13 GH families, including GH5, 9, 12, 44, 45, 48, 51, 74, 124, and 131 (29). Among them, GH5 is the largest and the most functionally diverse group, and those from fungi are mainly confined to subfamily GH5_5 with endo-β-1,4-glucanase activity (30). So far, seven eukaryotic GH5 endoglucanases from *Piromyces rhizinflata* (PrEglA) (31), *Thermoascus aurantiacus* (TaCel5A) (32), *Trichoderma reesei* (TrCel5A) (33), *Ganoderma lucidum* (GlCel5A) (34), *Aspergillus niger* (AnCel5A) (35), *Penicillium verruculosum* (PDB No. 5I6S), and *Talaromyces emersonii* (TgEgl5A) (36) have been resolved. In addition, the endoglucanases activity of GH5_5 endoglucanases from basidiomycetous fungus,

I. lacteus (*cen*1) (23), *V. volvacea* (*eg*1) (26), *T. hirsuta* (*Th*EG) (27), and *G. lucidum* (GlCel5A) (34) have been reported.

We have focused on the enzymatic characterization of *TmEgll*5A for its production. Besides the distinct enzymatic properties, recombinant *TmEgl*5A expressed in methylotroph yeast *P. pastoris* was obtained. The recombinant enzyme had the same enzymatic characteristics as most fungal endoglucanases, which have acidic or neutral optimum pH values (pH 2.5–7.0) (20), *TmEgl*5A activity was maximal at pH 4.0 and stable at 3.0–6.0. This was compatible with the growth characteristics of the mycelia, which grow well over a pH range of 5–6. In terms of substrate specificity, *TmEgl*5A belongs to the endo β-1,4-glucanases; it acts more on the β-1,3-1,4-mixed linkages of barley β-glucan. However, *TmEgl*5A cannot breakdown β-1,3-glucan, β-1,6-glucan, xylan, and crystalline cellulose. *Th*EG (*rEG*) activities against high crystalline celluloses such as Avicel, were much lower than 1/2703 compared with that of CMC hydrolysis (45 U/mg; against CMC) (27). A much lower ratio of activity toward Avicel and CMC hydrolytic activity of En-1 was found from *I. lacteus*. Its coding gene, *cen1* expressed in *Saccharomyces cerevisiae* had strong synergistic action for a hydrolysis of Avicel and PASC with cellobiohydrolase (23). An endoglucanase, EG1 from *V. volvacea* (*eg1*) that catalyzed the hydrolysis of CMC has maximal activity at pH 7.5 and 55°C (26). EG1 also hydrolyzed phosphoric acid swollen-cellulose and filter paper (at rates of 29% and 6%, respectively, compared with CMC), but did not hydrolyze crystalline cellulose, cotton, oat spelt xylan, or birchwood xylan. Moreover, Li and Walton (37) described that its gene coding product (VvCel5A) showed a very high specific activity toward oat β-glucan against CMC hydrolysis. Additionally, the GH5_5 enzyme group that they tested in the same way had the same effect. This substrate selectivity can also be supported from the structural characterization of GH5_5, GlCel5A had about 4.9-fold higher hydrolytic activity against β-glucan than CMC (514 ± 135 U/mg and 104 ± 3 U/mg, respectively). Therefore, common structural features and substrate specificities from the GH5_5 endoglucanases suggest that *TmEgl*5A shows high efficiency hydrolyzing activity toward barley β-glucan and releases oligosaccharides.

TLC analysis described in Fig. 6 revealed that *TmEgl*5A can cleave the only β-1,4 linkages, whereas links to a glucose residue through a CMC-Na and β-1,3 branched β-1,3-1,4-glucan. We found that *TmEgl*5A had a glycosidic bond cleavage pattern for β-1,3-1,4-glucan and CMC-Na by TLC analysis. *TmEgl*5A is able to hydrolyze β-1,4 linkages in β-1,3-1,4 bond-linked polysaccharides, but with a low catalytic efficiency compared with the same linked polysaccharide as lichanan and linear β-1,4-linkages of CMC-Na. The molecular structures of β-1,3-1,4-glucans from barley endosperm are usually constructed from the analysis of oligomers obtained by digestion of the polymers with a specific β-1,3-1,4-D-glucan hydrolase that releases 3-*O*-β-D-cellobiosyl-D-glucose and 3-*O*-β-D-cellotriosyl-D-glucose, accounting for 90–95% of the total oligosaccharides, and longer oligosaccharides (DP $\geq$ 5) accounting to 5–10% of the total contents. Despite the non-random arrangement of individual 1,3- and 1,4-β-linkages, cellotriosyl, cellotetraosyl, and longer cellooligomers are arranged in an essentially independent and random β-glucan chain (38,39). In contrast, lichenan (β-1,3-1,4-glucan) is particularly abundant in the endosperm cell walls of cereals and lichens. Its linear polysaccharides of up to 1200 β-D-glucosyl residues usually composed of 25–30% β-1,3 and 70–75% β-1,4 linkages with a branching pattern. Comparison of the molar ratios of tri-to tetrasaccharides (DP3/DP4) indicated that they followed the order of lichenan (24.5) > barley (2.8–3.0) (40). Therefore, we concluded that *TmEgl*5A preferentially acts on structures with a long chain length of cellooligosaccharide units in β-1,3-1,4-glucan, but lichenan has a higher β-1,3-branching frequency, causing lower hydrolytic ability than barley β-glucan. Additionally, this is able to be supported because it does not act on β-1,3-glucan, such as straight-chain curdlan, or β-1,3-1,6-mixed linkage of β-glucan such as laminaran.

Although, putative endoglucanase genes exist in the genome of ectomycorrhizal fungi, to the best of our knowledge, there are no reports regarding enzymatic characterization of purified endoglucanases from *T. matsutake*. *T. matsutake* had been thought to lack plant cell wall degrading enzymes, because it had been characterized that it forms mycorrhiza in the roots of softwoods such as red pine (hardwoods also exist) (41,42). However, in this study we found that the fungus produces GH5 endoglucanase, which degrades barley glucan by converting into its oligosaccharides using the formation of hyphal growth on barley-based solid-state cultivation without forming mycorrhiza.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jbiosc.2019.05.012.

ACKNOWLEDGMENTS

This study was supported in part by a grant by the Strategic Research Foundation Grant-aided Project for Private Universities from the Ministry of Education, Culture, Sports, Science, and Technology (MEXT), 2015-2017 (S1512004).

References

1. **Hall, I. R., Yun, W., and Amicucci, A.:** Cultivation of edible ectomycorrhizal mushrooms, Trends Biotechnol., **21**, 433–438 (2003).
2. **Yun, W., Hall, I. R., and Evans, L. A.:** Ectomycorrhizal fungi with edible fruiting bodies 1. *Tricholoma matsutake* and related fungi, Econ. Bot., **51**, 311–327 (1997).
3. **Ogawa, M. and Hamada, M.:** Primordia formation of *Tricholoma matsutake* (Ito et Imai) Sing. in pure culture, Trans. Mycol. Soc. Jpn., **16**, 406–415 (1975).
4. **Kawai, M. and Ogawa, M.:** Studies on the artificial reproduction of *Tricholoma matsutake* (S. Ito et Imai) Sing. IV. Studies on a seed culture and a trial for the cultivation on solid media, Trans. Mycol. Soc. Jpn., **17**, 499–505 (1976) (in Japanese).
5. **Inaba, K., Yoshida, T., Takano, Y., Mayuzumi, Y., Mitsunaga, T., and Koshijima, T.:** An instance of the fruiting–body formation of *Tricholoma matsutake*, Environ. Control Biol., **33**, 59–64 (1995).
6. **Kawai, M. and Abe, S.:** Studies on the artificial reproduction of *Tricholoma matsutake* (S. Ito et Imai) Sing. I. Effects of carbon and nitrogen sources in media on the vegetative growth of *T. matsutake*, Trans. Mycol. Soc. Jpn., **17**, 159–167 (1976) (in Japanese).
7. **Norkrans, B.:** Studies in growth and cellulolytic enzymes of *Tricholoma*, Symbolae Botanicae Upsalienses, **11**, 1–126 (1950).
8. **Ohta, A.:** Production of fruit–bodies of a mycorrhizal fungus, *Lyophyllum shimeji*, in pure culture, Mycoscience, **35**, 147–151 (1994).
9. **Watanabe, K., Kawai, M., and Obatake, Y.:** Fruiting body formation of *Lyophyllum shimeji* in pure culture, Mokuzai Gakkaishi, **40**, 879–882 (1994).
10. **Yoshida, H. and Fujimoto, S.:** A trial cultivation of *Lyophyllum shimeji* on solid media, Trans. Mycol. Soc. Jpn., **35**, 192–195 (1994) (in Japanese).
11. **Wood, P.:** Cereal β-glucans in diet and health, J. Cereal Sci., **46**, 230–238 (2007).
12. **Evers, T. and Millar, S.:** Cereal grain structure and development: some implications for quality, J. Cereal Sci., **36**, 261–284 (2002).
13. **Izydorczyc, M. S., Storsley, J., Labossiere, D., MacGregor, A. W., and Rossnagel, B. G.:** Variation in total and soluble β-glucan content in hulless barley: effects of thermal, physical, and enzymatic treatments, J. Agric. Food Chem., **48**, 982–989 (2000).
14. **Takasaki, F. and Ohya, C.:** Studies on physical and chemical properties and taste characteristics of barley, Tokyo Bunka Junior College Memoirs, **21**, 1–9 (2004) (in Japanese).
15. **Kusuda, M., Ueda, M., Miyatake, K., Araki, Y., Yamanaka, K., Nakazawa, M., Miyatake, K., and Terashita, T.:** Detection of β-glucosidase as saprotrophic ability from an ectomycorrhizal mushroom, *Tricholoma matsutake*, Mycoscience, **47**, 184–189 (2006).
16. **Terashita, T., Kono, M., Yoshikawa, K., and Shishiyama, J.:** Productivity of hydrolytic enzymes by mycorrhizal mushrooms, Mycoscience, **36**, 221–225 (1995).
17. **Kusuda, M., Ueda, M., Miyatake, K., and Terashita, T.:** Characterization of the carbohydrase productions of an ectomycorrhizal fungus, *Tricholoma matsutake*, Mycoscience, **49**, 291–297 (2008).
18. **Onuma, H., Uchiyama, H., Hara, K., Fukuta, Y., and Shirasaka, N.:** Enzymatic characterization of an extracellular glucoamylase from *Tricholoma matsutake*

and its cloning and secretory expression in *Pichia pastoris*, Biosci. Biothchnol. Biochem., **82**, 2180–2190 (2018).
19. **Kamei, K., Fukuta, Y., Onuma, H., and Shirasaka, N.:** Purification and characterization of xylan-degrading enzyme from *Tricholoma matsutake*, Mushroom Sci. Biotechnol., **26**, 73–80 (2018).
20. **Miller, G. L.:** Use of dinitrosalicylic acid reagent for determination of reducing sugar, Anal. Chem., **31**, 426–428 (1959).
21. **Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., and Klenk, D. C.:** Measurement of protein using bicinchoninic acid, Anal. Biochem., **150**, 76–85 (1985).
22. **Laemmli, U. K.:** Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature, **227**, 680–685 (1970).
23. **Toda, H., Takada, S., Oda, M., Amano, Y., Kanda, T., and Shimosaka, M.:** Gene cloning of an endoglucanase from the basidiomycete *Irpex lacteus* and its cDNA expression in *Saccharomyces cerevisiae*, Biosci. Biotechnol. Biochem., **69**, 1262–1269 (2005).
24. **Kasturi, L., Chen, H., and Shakin–Esleman, S. H.:** Regulation of N-linked core glycosylation: use of a site direction mutagenesis approach to identify Asn–Xaa–Ser/Thr sequons the oligosaccharide acceptors, Biochem. J., **323**, 415–419 (1997).
25. **Steentoft, C., Vakhrushev, S. Y., Joshi, H. J., Kong, Y., Vester–Christensen, M. B., Schjoldager, K. T., Lavrsen, K., Dabelsteen, S., Pedersen, N. B., Marcos–Silva, L., and other 3 authors:** Precision mapping of the human O–GalNAc glycoproteome through SimpleCell technology, EMBO J., **32**, 1478–1488 (2013).
26. **Ding, S. J., Ge, W., and Buswell, A. J.:** Endoglucanase I from the edible straw mushroom, *Volvariella volvacea*. Purification, characterization, cloning and expression, Eur. J. Biochem., **268**, 5687–5695 (2001).
27. **Nozaki, K., Seki, T., Matsui, K., Mizuno, M., Kanda, T., and Amano, Y.:** Structure and characteristics of an endo-beta-1,4-glucanase, isolated from *Trametes hirsuta*, with high degradation to crystalline cellulose, Biosci. Biotechnol. Biochem., **71**, 2375–2382 (2007).
28. **Hoshino, E., Shiroishi, M., Amano, Y., Nomura, M., and Kanda, T.:** Synergistic actions of exo-type cellulases in the hydrolysis of cellulose with different crystallinities, J. Ferment. Bioeng., **84**, 300–306 (1997).
29. **Lombard, V., Golaconda, Ramulu, H., Drula, E., Coutinho, P. M., and Henrissat, B.:** The carbohydrate-active enzymes database (CAZy) in 2013, Nucleic Acids Res., **42**, D490–D495 (2014).
30. **Aspeborg, H., Coutinho, P. M., Wang, Y., Brumer, H., and Henrissat, B.:** Evolution, substrate specificity and subfamily classification of glycoside hydrolase family 5 (GH5), BMC Evol. Biol., **12**, 186 (2013).
31. **Lo, Leggio, L. and Larsen, S.:** The 1.62 Å structure of *Thermoascus aurantiacus* endoglucanase: completing the structural picture of subfamilies in glycoside hydrolase family 5, FEBS Lett., **523**, 103–108 (2002).
32. **Lee, T. M., Farrow, M. F., Arnold, F. H., and Mayo, S. L.:** A structural study of *Hypocrea jecorina* Cel5A, Protein Sci., **20**, 1935–1940 (2011).
33. **Liu, G., Li, Q., Shang, N., Huang, J. W., Ko, T. P., Liu, W., Zheng, Y., Han, X., Chen, Y., Chen, C. C., Jin, J., and Guo, R. T.:** Functional and structural analyses of a 1,4-β-endoglucanase from *Ganoderma lucidum*, Enzyme Microb. Technol., **86**, 67–74 (2016).
34. **Yan, J., Liu, W., Li, Y., Lai, H. L., Zheng, Y., Huang, J. W., Chen, C. C., Chen, Y., Jin, J., Li, H., Zhong, L. H., and Guo, R. T.:** Functional and structural analysis of *Pichia pastoris*–expressed *Aspergillus niger* 1,4-β-endoglucanase, Biochem. Biophys. Res. Commun., **475**, 8–12 (2016).
35. **Wang, K., Luo, H., Bai, Y., Shi, P., Huang, H., Xue, X., and Yao, B.:** A thermophilic endo-1,4-β-glucanase from *Talaromyces emersonii* CBS394.64 with broad substrate specificity and great application potentials, Appl. Microbiol. Biotechnol., **98**, 7051–7060 (2014).
36. **Béguin, P. and Aubert, J. P.:** The biological degradation of cellulose, FEMS Microbiol. Rev., **13**, 25–58 (1994).
37. **Li, B. and Walton, D. J.:** Functional diversity for biomass deconstruction in family 5 subfamily 5 (GH5_5) of fungal endo-β1,4-glucanases, Appl. Microbiol. Biotechnol., **101**, 4093–4101 (2017).
38. **Buliga, G. S., Brant, D. A., and Fincher, G. B.:** The sequence statistics and solution conformation of a barley (1→3, 1→4)-β-D-glucan, Carbohydr. Res., **157**, 139–156 (1986).
39. **Staudte, R. G., Woodward, J. R., Fincher, G. B., and Stone, B. A.:** Water–soluble (1→3), (1→4)-β-D-glucans from barley (*Hordeum vulgare*) endosperm. III. Distribution of cellotriosyl and cellotetraosyl residues, Carbohydr. Polym., **3**, 299–312 (1983).
40. **Lazaridou, A., Biliaderis, C. G., Micha–Screttas, M., and Steele, B. R.:** A comparative study on structure–function relations of mixed–linkage (1→3), (1→4) linear β-D-glucans, Food Hydrocolloids, **18**, 837–855 (2004).
41. **Yamanaka, K., Aimi, T., Wan, J., and Cao, H.:** Species of host trees associated with *Tricholoma matsutake* and close allies in Asia, Mushroom Sci. Biotechnol., **19**, 79–87 (2011).
42. **Wan, J., Koike, A., Yamanaka, K., Saotome, K., Morinaga, T., Tanaka, C., Terashima, Y., and Aimi, T.:** Genetic diversity of *Tricholoma matsutake* and close allies associated with broad–leaved trees in Asia, Mushroom Sci. Biotechnol., **19**, 167–174 (2012).