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Polysaccharide-Inducible Endoglucanases from *Lentinula edodes* Exhibit a Preferential Hydrolysis of 1,3−1,4-β-Glucan and Xyloglucan

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Ⓢ *Supporting Information*

ABSTRACT: Three genes encoding glycoside hydrolase family 12 (GH12) enzymes from *Lentinula edodes*, namely *Lecel12A*, *Lecel12B*, and *Lecel12C*, were newly cloned by PCR using highly conserved sequence primers. To investigate enzymatic properties, recombinant enzymes encoded by *L. edodes* DNAs and GH12 genes from *Postia placenta* (PpCel12A and PpCel12B) and *Schizophyllum commune* (ScCel12A) were prepared in *Brevibacillus choshinensis*. Recombinant LeCel12A, PpCel12A, and PpCel12B, which were grouped in GH12 subfamily 1, preferentially hydrolyzed 1,3−1,4-β-glucan. By contrast, LeCel12B, LeCel12C, and ScCel12A, members of the subfamily 2, exhibited specific hydrolysis of xyloglucan. These results suggest that two subfamilies of GH12 are separated based on the substrate specificity. Transcript levels of *L. edodes* genes increased 72 h after growth of *L. edodes* mycelia cells in the presence of plant cell wall polymers such as xyloglucan, 1,3−1,4-β-glucan, and cellulose. These results suggest that *L. edodes* GH12 enzymes have evolved to hydrolyze 1,3−1,4-β-glucan and xyloglucan, which might enhance hyphal extension and nutrient acquisition.

KEYWORDS: *glycoside hydrolase family 12, endoglucanase, 1,3−1,4-β-glucan, xyloglucan, Lentinula edodes*

■ INTRODUCTION

Cell wall-degrading enzymes (CWDEs) occur widely in microorganisms, plants and some animals are thought to play a significant role in the degradation of cell wall polysaccharides to enhance plant-pathogen invasion, cell growth, fruit softening and nutrient acquisition. Furthermore, CWDEs from some ascomycetes are routinely used in the manufacture of beverages and industrial products, for example, beer and wine, animal feed, paper, textiles, laundry detergents and food ingredients.[1]

Wood decay basidiomycetes, often categorized as white-rot and brown-rot fungi, are essential contributors to carbon cycling in forest soils by means of their degradation of plant cell wall components. White-rot fungi degrade cellulose, hemicellulose and lignin by using hydrolytic and oxidative enzymes. Brown-rot fungi employ a different approach, in which lignin is modified extensively but the products remain *in situ* as a polymeric residue.[2,3] The difference has also been demonstrated by comprehensive transcriptome analysis in which white- and brown-rot fungi were found to exhibit distinct expression patterns of genes encoding glycoside hydrolases and oxidoreductases.[4−6] In addition, hydroxyl radicals generated via the Fenton reaction have been implicated as diffusible oxidants capable of depolymerizing cellulose. On the other hand, the basidiomycetes produce endo- and exo-1,3-β-glucanases and chitinases to reconstruct their own cell walls.[7] Thus, enzymes related to polysaccharide degradation are thought to be involved in nutrient assimilation and in morphological changes, such as cell expansion and division, and hyphal branching.

CWDEs are classified into glycoside hydrolase (GH) families on the basis of amino acid sequence analysis,[8] which assists in the prediction of enzymatic properties such as substrate preference. However, substrate specificity is defined based on the results from enzymatic analyses. Enzymes belonging to the GH12 family have been well studied because of their ability to hydrolyze naturally occurring β-1,4-linked glucans in plant cell walls such as crystalline and amorphous cellulose, xyloglucan and 1,3−1,4-β-glucan. Some enzymes, including xyloglucan-specific endoglucanase (XEG) (EC 3.2.1.151) from *Aspergillus aculeatus* and 1,3−1,4-β-glucanases (EC 3.2.1.73) from *Cochliobolus carbonum* and *Magnaporthe oryzae*, show substrate-specific hydrolysis.[9−11] Although GH12 enzymes share high similarity in amino acid sequence, analyses of substrate specificity are to determine their actual enzymatic classification. Proteomic analysis of extracellular proteins of *Postia placenta* grown on solid aspen revealed 34 probable glycoside hydrolases, including a GH12 enzyme, that degraded slightly amorphous and crystalline cellulose, although transcriptome analysis did not show higher levels of GH12 mRNA.[4−6,12] Furthermore, GH12 enzymes with hydrolytic activity toward various β-glucans have been identified from the culture medium of *Phanerochaete chrysosporium* and *Fomitopsis palustris*.[13,14] Thus, GH12 enzymes from white- and brown-rot basidiomycetes seem to play an important role in the degradation of plant hemicellulose and cellulose.

In the present study, we describe the identification of GH12 endoglucanase genes from the white-rot fungus *Lentinula edodes*, an edible fungus called shiitake mushroom, the expression of these genes induced by carbohydrates, and the enzymatic properties of the recombinant enzymes. Furthermore, clustering analysis of GH12 enzymes with amino acid sequences from basidiomycetes and ascomycetes was carried out. This study demonstrates a new use of an edible fungus for

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producing enzymes that degrade polysaccharides included in vegetable foods.

■ MATERIALS AND METHODS

Materials. The commercially cultivated *L. edodes* (Shiitake mushroom, H600) was obtained from Hokken Co. Ltd. (Tochigi, Japan). *Schizophyllum commune* (NBRC 4928) was purchased from National Institute of Technology and Evaluation (Chiba, Japan). *L. edodes* mycelia were grown in YPG medium (1% yeast extract, 2% peptone and 1% glucose, w/v) for 20 days at 25 °C with gyratory shaking at 130 rpm. *Escherichia coli* (Dh5α, Takara Bio, Shiga, Japan) was used for preparing plasmid DNA. *Brevibacillus choshinensis* (Takara Bio, Shiga, Japan) was grown on MT agar plates or TM medium as described previously.[11] Polysaccharides tested as substrates included: cellulose (Sigmacell 20, Sigma-Aldrich, St. Louis, MO), phosphoric acid-swollen cellulose (PSC) made from Sigmacell 20, barley 1,3−1,4-β-glucan (Megazyme, Wichlow, Ireland), xyloglucan (Megazyme, Wichlow, Ireland), laminarin (Sigma-Aldrich, St. Louis, MO), glucomannan (Megazyme, Wichlow, Ireland), carboxymethyl cellulose (CMC, Sigma-Aldrich, St. Louis, MO) and oat spelt xylan (Sigma-Aldrich, St. Louis, MO).

cDNA Cloning. *L. edodes* and *S. commune* mature fruiting bodies were ground in liquid nitrogen and the powdered sample was used for total RNA extraction using an RNeasy Plant Mini kit (Qiagen, Hilden, Germany). First-strand cDNA pools were synthesized from total RNA using SuperScript III (Invitrogen, Carlsbad, CA). PCR was performed in a 50 μL reaction mixture using GXL DNA polymerase (Takara Bio, Shiga, Japan). Degenerate primer pairs for cloning internal DNA fragments encoding *L. edodes* GH12 enzymes, termed Le-F1 and Le-R1, and Le-F2 and Le-R2, were designed on the basis of the DNA sequences registered for ascomycetous and the basidiomycetous GH12 enzymes from *Gloeophyllum trabeum* (HQ730918), *Phanerochaete chrysosporium* (AY682744), *Polyporus arcularius* (AB187524), *P. placenta* (XM_002474197), *Aspergillus nidulans* (XM_652964), *Laccaria bicolor* (XM_001879533), *Penicillium marneffei* (XM_002145431) and *Serpula lacrymans* (GL945488) (Figure S1, Supporting Information). Cloning of the 5′ and 3′ regions was performed by PCR using a GeneRacer kit (Invitrogen, Carlsbad, CA) and the following gene-specific primers: LeA-F and LeA-R for *LeScel12A*, LeB-F and LeB-R for *Lecel12B*, and LeC-F and LeC-R for *Lecel12C*. Amplified DNA fragments were cloned into pGEM-easy (Promega, Madison, WI) and verified by DNA sequencing using a 3130 Genetic Analyzer (Applied Biosystems, Carlsbad, CA).

Cloning of the *Sccel12A* gene (XM_003037119) was performed by PCR from *S. commune* cDNA pools using the specific primers ScA-F and ScA-R. *Ppcel12A* (XM_002472809) and *Ppcel12B* (XM_002474197) genes were artificially synthesized by Takara Bio (Shiga, Japan), and amplified by PCR using the following primers: PpA-F and PpA-R for *Ppcel12A*, and PpB-F and PpB-R for *Ppcel12B*. The primers used for cloning DNAs are summarized in Table S1, Supporting Information.

Sequence Analysis. The search for conserved domains was done using protein blast program via the National Center for Biotechnology Information Web site (NCBI, http://www.ncbi.nlm.nih.gov/). The N-terminal signal sequence and potential *N*-glycosylation site were predicted by the SignalP 4.0 Server (http://www.cbs.dtu.dk/services/SignalP/) and NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/), respectively. The phylogenetic tree was constructed on the basis of multiple alignments using ClustalW software (http://www.genome.jp/tools/clustalw/). Bars represent 0.1 amino acid substitutions per site.

Preparation of Recombinant Proteins. For protein purification and immunoblot analysis of recombinant proteins, DNA sequences encoding contiguous histidine residues at the 3′ end, and the restriction nuclease digestion sites of *Bam*HI at the 5′ end and *Hin*dIII at the 3′ end were fused in-frame by a PCR extension. The PCR products were digested with *Bam*HI and *Hin*dIII, ligated into a secretary expression vector (pNCMO2) driven by a MWP constitutive promoter,[15] and introduced into *B. choshinensis* (Takara-Bio, Shiga, Japan) according to the manufacturer's instructions. Transformants were screened on MT agar plates containing neomycin (50 μg/mL), and cultured in TM medium containing neomycin (50 μg/mL) for 4 days at 30 °C for protein production.

Protein Purification. Culture filtrates of *B. choshinensis* transformants collected by centrifugation at 8000× *g* for 5 min at room temperature was added ammonium sulfate (420 g/L) and kept on ice for 30 min. The pellet obtained by centrifugation at 22000× *g* for 15 min at 4 °C was dissolved in equilibration buffer (50 mM sodium phosphate buffer (pH 7.0) containing 300 mM NaCl) and applied to a polyhistidine (polyHis)-binding resin (Takara-Bio, Shiga, Japan). The resin was washed sequentially with equilibration buffer and 0.2× elution buffer (10 mM sodium phosphate (pH 7.0), 60 mM NaCl and 30 mM imidazole), and then bound protein was eluted with 1× elution buffer (50 mM sodium phosphate (pH 7.0), 300 mM NaCl and 150 mM imidazol) containing 40 mM EDTA. After desalting and equilibration with 10 mM sodium phosphate buffer (pH 7.0) by ultrafiltration (Amicon Ultra-4, Millipore, Billerica, MA), the enzyme preparation was further fractionated on an anion-exchange column (MonoQ, GE Healthcare, Buckinghamshire, UK). The active fractions were concentrated by ultrafiltration and used for analysis. To confirm the purity of the proteins, each recombinant protein (2.0 μg) was subjected to SDS-PAGE, followed by staining with Coomassie Brilliant Blue R-250 (CBB). Protein concentration was determined using a Bradford protein assay kit (Thermo-Fisher, Worcester, MA) with bovine serum albumin (Sigma-Aldrich, St. Louis, MO) as the standard.

Assay for Hydrolytic Activity. Hydrolytic activity of the enzymes toward polysaccharides was determined by measuring the increase in reducing power using *p*-hydroxybenzoic acid hydrazide.[16] A reaction mixture (50 μL) containing purified enzyme (0.2 μg), 0.1% (w/v) polysaccharide and 100 mM sodium acetate (pH 5.0) (or 100 mM sodium phosphate for the *S. commune* GH12 enzyme, ScCel12A) was incubated at 30 °C for 30 min. To the reaction mixture, 175 μL of 1% (w/v) *p*-hydroxybenzoic hydrazide-HCl was added and then the mixture was boiled for 5 min. The absorbance of the solutions was measured at 410 nm. The increase in reducing power was calculated on the basis of a glucose standard curve. A unit of enzyme activity was defined as the amount of enzyme required to produce 1 μmol of reducing sugar equivalent to glucose in 1 min. Determining the effects of pH on hydrolytic activity was performed by equilibrating reaction mixtures with 100 mM sodium acetate (pH 3.5−5.5) or 100 mM sodium phosphate (pH 5.5−8.0) at 40 °C. The temperature dependency for hydrolytic activity was determined by incubating reaction mixtures at 4−70 °C.

Production of Oligosaccharides from Xyloglucan and 1,3−1,4-β-Glucan using *L. edodes* GH12 Enzymes. 1,3−1,4-β-Glucan and xyloglucan were individually treated with LeCel12A and LeCel12C (2.0 μg each), respectively, in 100 μL of 100 mM sodium acetate buffer (pH 5.0) at 40 °C for 24 and 48 h. The solutions were kept in a boiling water for 5 min to inactivate enzymes and then applied to ion-exchange resins, Dowex 50WX8 (Sigma-Aldrich, St. Louis, MO) and Dowex 1 × 8−100 (Sigma-Aldrich, St. Louis, MO). Hydrolysates were subjected to thin layer chromatography (TLC) on silica gel plates (60 F_{254}, Merck, Darmstadt, Germany) in butan-1-ol/acetic acid/water (2:1:1, v/v) and stained with 0.5% (w/v) thymol in $EtOH/H_2SO_4$ (19:1, v/v).

Quantitative Real-Time PCR. *L. edodes* mycelia grown in YPG medium for 20 days were transferred to modified Czapek-Dox medium containing 1% (w/v) glucose, xyloglucan, 1,3−1,4-β-glucan, cellulose, xyloglucan hydrolysates or 1,3−1,4-β-glucan hydrolysates, and further cultured for 24−72 h in the same conditions.[17] Hydrolysates obtained by the treatment with LeCel12A and LeCel12C for 24 h shown in Figure 5A were used as 1,3−1,4-β-glucan and xyloglucan oligosaccharides. The mycelia were collected by filtration through cheesecloth and ground in liquid nitrogen. Total RNA extraction and first-strand cDNA synthesis were carried out as described in the section of cDNA Cloning. Real-time PCR was carried out using a Quantitect SYBR Green PCR Kit (Qiagen, Hilden, Germany) and specific DNA primers (Table S2, Supporting Information) in a StepOnePlus Real-Time PCR system (Applied

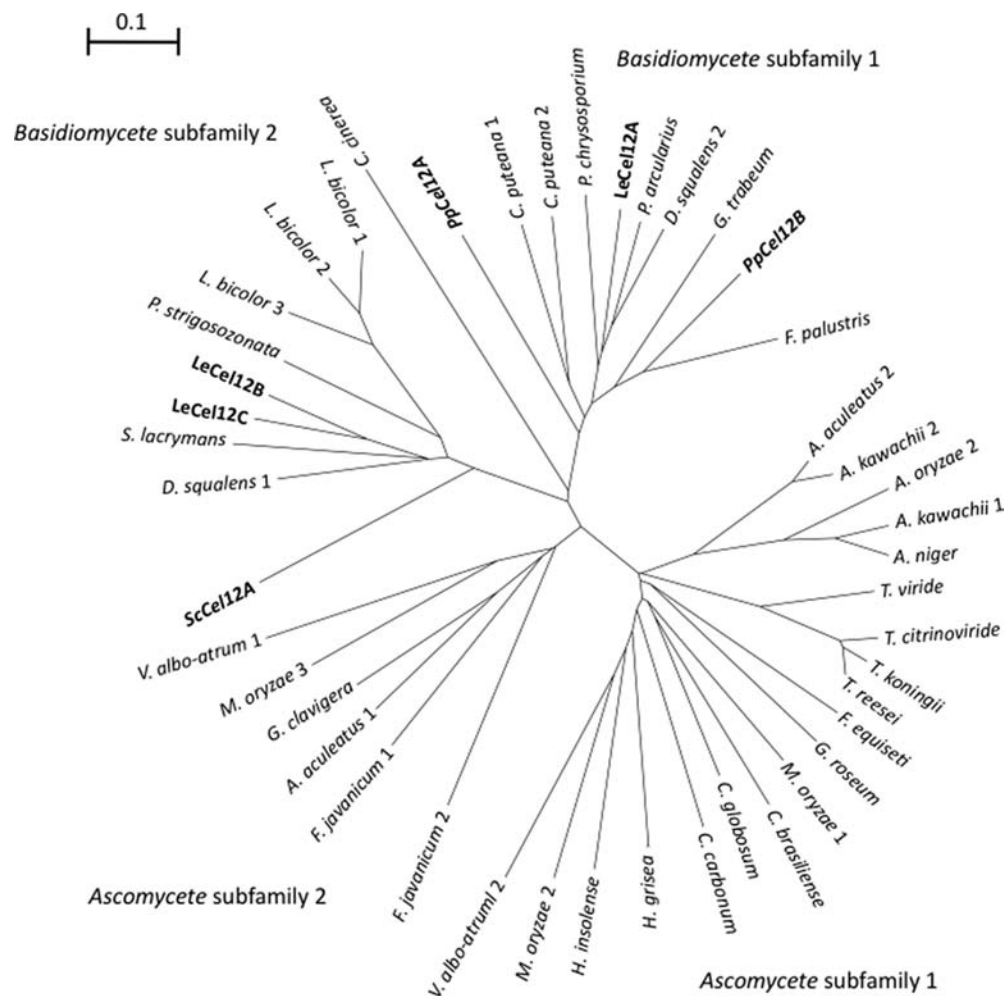

Figure 1. Phylogenetic tree of the basidiomycete and the ascomycetous GH12 enzymes. Proteins enzymatically assayed in this study are indicated in bold.

Biosystems, Carlsbad, CA) using SYBR GreenER qPCR Super Mix (Invitrogen, Carlsbad, CA). Gene expression levels were calibrated by the expression of glyceraldehyde-3-phosphate dehydrogenase gene (*Legpd*, AB013136).[18]

Nucleotide Sequence Accession Number. The accession numbers of the *L. edodes* genes were AB771719 (*Lecel12A*), AB771720 (*Lecel12B*), AB771721 (*Lecel12C*) and AB013136 (*Legpd*). The DDBJ/GenBank/EBI accession numbers of the fungal GH12 genes used in the phylogenetic tree were as follows: *Aspergillus aculeatus* 1 (AF043595), *A. aculeatus* 2 (D00546), *A. kawachii* 1 (AF435072), *A. kawachii* 2 (D12901), *A. niger* (AJ224451), *A. oryzae* (AP007159), *Caprinopsis cinerea* (XM_002910519), *C. brasiliense* (AF434180), *Chaetomium globosum* (XM_001222999), *C. carbonum* (AF229447), *Coniophora puteana* 1 (JH711576), *C. puteana* 2 (JH711573), *Dichomitus squalens* 1 (JH719402), *D. squalens* 2 (JH719443), *Fusarium javanicum* 1 (AF434183), *F. javanicum* 2 (AF434184), *Fomitopsis palustris* (AB299016), *Fusarium equiseti* (AF434182), *Gliocladium roseum* (AF435063), *Gloeophyllum trabeum* (HQ730918), *Grosmannia clavigera* (GL629729), *Humicola grisea* (AF435071), *H. insolens* (A22907), *Laccaria bicolor* 1 (XM_001879533), *L. bicolor* 2 (XM_001886624), *L. bicolor* 3 (XM_001890579), *M. oryzae* 1 (XM_368567), *Phanerochaete chrysosporium* (AY682744), *Polyporus arcularius* (AB187524), PpCel12A (*P. placenta* 1 XM_002472809), PpCel12B (*P. placenta* 2 XM_002474197), *Punctularia strigosozonata* (JH687542), ScCel12A (*S. commune* XM_003037119), *Serpula lacrymans* (GL945488), *Trichoderma citrinoviride* (AF435068), *T. koningii* (AF435069), *T. reesei* (AB003694), *T. viride* (AF435070), *Verticillium albo-atrum* 1 (DS5985216), *V. albo-atrum* 2 (DS5985227).

■ RESULTS

Cloning of *L. edodes* GH12 Genes. Three genes, *Lecel12A, Lecel12B and Lecel12C*, encoding putative GH12 enzymes from *L. edodes* were amplified by PCR using degenerated DNA primers, and their DNA sequences were determined by sequencing. The *Lecel12A, Lecel12B and Lecel12C* genes encode proteins of 247, 259, and 258 amino acids, respectively, that contain a GH12 catalytic domain and secretion signal peptide sequences but no carbohydrate-binding module. LeCel12B was found to have the highest amino acid similarity to LeCel12C at 76.5%, whereas LeCel12A showed 35.8% similarity to LeCel12B and LeCel12C, suggesting that LeCel12B and LeCel12C may possess similar enzymatic properties.

Evolutional Diversity of the Basidiomycetous GH12 Enzymes. A phylogenetic tree of fungal GH12 family enzymes showed that the basidiomycetous GH12 enzymes were separated from the ascomycetous ones and divided into two clusters, termed subfamily 1 and subfamily 2 (Figure 1). LeCel12A was grouped into subfamily 1, and LeCel12B and LeCel12C were grouped into subfamily 2.

Hydrolytic Activity of the Basidiomycetous GH12 Enzymes. To investigate the enzymatic properties of the

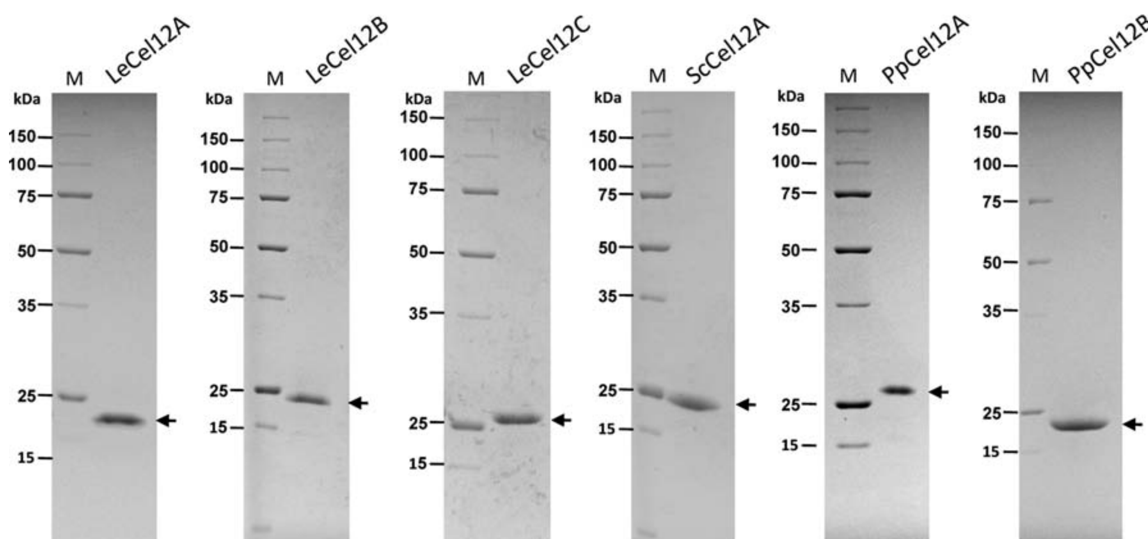

Figure 2. SDS-PAGE of six recombinant GH12 family enzymes. Recombinant enzymes of the basidiomycetous GH12 family were prepared in *B. choshinensis* and purified by polyHis-affinity chromatography. Purified proteins (2.0 μg) were subjected to SDS-PAGE and stained with CBB.

Table 1. Hydrolytic Activity of Recombinant GH12 Enzymes on Polysaccharides

	relative activity[a], %					
substrate	LeCel12A	LeCel12B	LeCel12C	PpCel12A	PpCel12B	ScCel12A
xyloglucan	1.0 ± 0.4	**100.0 ± 11.7 (0.34 ± 11.7)**	**100.0 ± 7.2 (2.26 ± 0.18)**	11.3 ± 0.8	3.2 ± 2.4	**100.0 ± 11.7 (0.73 ± 0.14)**
1,3−1,4-β-glucan	**100.0 ± 4.0 (1.17 ± 0.06)**	1.5 ± 2.6	0.7 ± 0.6	**100.0 ± 6.9 (0.56 ± 0.07)**	**100.0 ± 11.7 (0.51 ± 0.08)**	8.5 ± 2.0
CMC	16.3 ± 0.2	ND	0.4 ± 0.3	23.9 ± 1.8	23.7 ± 2.9	1.8 ± 0.8
HEC	6.5 ± 1.0	ND	ND	25.0 ± 4.4	31.1 ± 3.3	4.4 ± 3.0
laminarin	6.1 ± 0.8	ND	1.2 ± 0.3	2.8 ± 0.2	2.6 ± 0.7	ND
xylan	2.6 ± 0.8	3.1 ± 2.9	0.8 ± 0.3	0.9 ± 0.3	1.3 ± 0.8	1.1 ± 0.6
glucomannan	14.0 ± 5.2	3.1 ± 2.9	7.6 ± 0.7	4.2 ± 1.6	20.9 ± 6.7	ND
PSC[b]	ND	ND	0.8 ± 0.9	4.0 ± 1.3	5.5 ± 1.9	ND
cellulose	1.1 ± 1.2	ND	0.3 ± 0.1	1.6 ± 0.9	5.7 ± 1.8	ND

[a]ND, values were not determined due to low activity. [b]Parentheses represents the value of the specific activity (unit/mg protein).

basidiomycetous GH12 enzymes, polyHis-tagged recombinant proteins of not only LeCel12A, LeCel12B and LeCel12C but also GH12 enzymes from *S. commune* (ScCel12A) and *P. placenta* (PpCel12A and PpCel12B) were produced in *B. choshinensis*, and purified by polyHis-affinity chromatography (Figure 2). Prepared enzymes showed single band on SDS-PAGE followed by staining with CBB, hence these enzymes were used for assaying hydrolytic activity (Table 1). LeCel12A showed preferential hydrolysis of 1,3−1,4-β-glucan and slight hydrolysis of CMC, and glucomannan, but significant activity was not detected on cellulose. By contrast, LeCel12B and LeCel12C hydrolyzed xyloglucan preferentially. Similar to LeCel12A, both PpCel12A and PpCel12B showed highest activity toward 1,3−1,4-β-glucan. PpCel12A and PpCel12B also showed slight hydrolysis of XG, glucomannan and cellulose. ScCel12A showed higher activity toward xyloglucan, identical to the properties of LeCel12B and LeCel12C. These results indicate that LeCel12A, PpCel12A and PpCel12B are endo-1,4-β-glucanases exhibiting preferential hydrolysis of 1,3−1,4-β-glucan concomitant with cleavage of various β-glucans, whereas LeCel12B, LeCel12C and ScCel12A are XEGs. Furthermore, given the clustering of GH12 family enzymes in the phylogenetic tree, we propose that enzymes belonging to basidiomycete subfamily 1 are endo-1,4-β-glucanases with hydrolytic activity toward various β-1,4-glucans, and enzymes in basidiomycete subfamily 2 are XEGs.

Temperature and pH Dependencies. Effects of temperature and pH on hydrolytic activities of the recombinant enzymes were examined (Figure 3 and Figure 4). LeCel12B and PpCel12B exhibited high activities ranging from 20 to 40 °C, and the activities decreased dramatically at 50 °C. LeCel12A, LeCel12C and PpCel12A showed increased activity as reaction temperature increased up to 40 or 50 °C. ScCel12A showed maximum activity at 20−30 °C and 60% of maximal activity at 4 °C, but the activity decreased dramatically at 40 °C. LeCel12A, LeCel12B and LeCel12C showed high activities at pH 4.0−6.0, 4.5−7.0 and 4.0−6.0, respectively. ScCel12A had high activities when pH was adjusted to 5.5 and 6.0 with sodium phosphate buffer. To the contrary, PpCel12A and PpCel12B showed high activities at pH 4.5−5.5 and 5.0−5.5 in sodium acetate buffer.

Production of Oligosaccharides using *L. edodes* Enzymes. 1,3−1,4-β-Glucan and xyloglucan were treated with LeCel12A and LeCel12C and reaction products were analyzed by TLC. By the treatment with LeCel12A for 24 h, various oligosaccharides in 1,3−1,4-β-glucan hydrolysate were observed on TLC (Figure 5A). By this separation solvent, cellohexaose and laminariheptaose mobilize from the origin of

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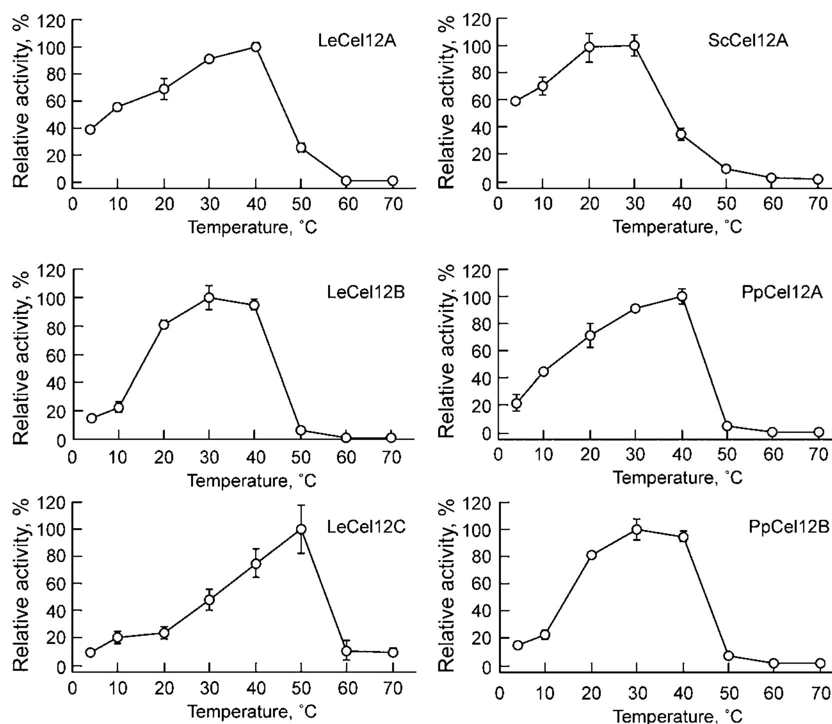

Figure 3. Effects of temperature on hydrolytic activities. Hydrolysis of 1,3−1,4-β-glucan by LeCel12A, PpCel12A and PpCel12B, or xyloglucan by LeCel12B, LeCel12C and ScCel12A was conducted in the presence of 100 mM sodium acetate (pH 5.0) (or 100 mM sodium phosphate, pH 5.5 for the ScCel12A) at 4−70 °C. The activities were determined by measuring the increase in reducing power. The highest values were taken as 100%. Data are the means of three determinations ± SE. Some measurements had smaller SE than the size of the symbols.

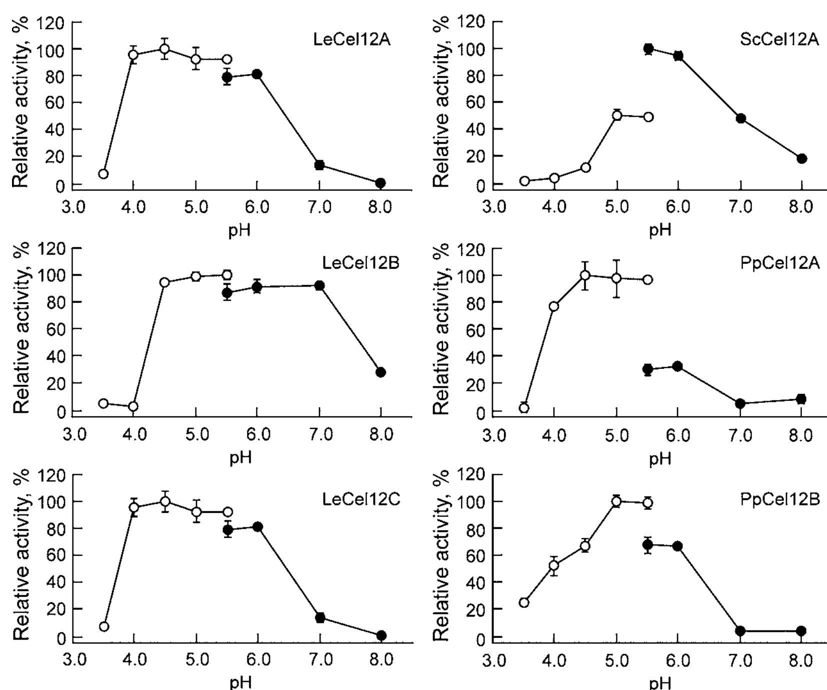

Figure 4. Effects of pH on hydrolytic activities. Hydrolysis of 1,3−1,4-β-glucan by LeCel12A, PpCel12A and PpCel12B, or xyloglucan by LeCel12B, LeCel12C and ScCel12A was conducted at 30 °C in the presence of 100 mM sodium acetate (pH 3.5−5.5, ○) or 100 mM sodium phosphate (pH 5.5−8.0, ●). The activities were determined by measuring the increase in reducing power. The highest values were taken as 100%. Data are the means of three determinations ± standard errors (SE). Some measurements had smaller SE than the size of the symbols.

TLC. Hence, 1,3−1,4-β-glucan could be hydrolyzed by LeCel12A into oligosaccharides with less than 6 glucose units linked β-1,3- and/or β-1,4 linkage(s). These oligosaccharides were further hydrolyzed by LeCel12A to generate three major spots after 48 h treatment (Figure 5B). By contrast, three spots, XXXG, XXLG and XLLG, from xyloglucan hydrolysate treated with LeCel12C for 24 and 48 h were observed (Figure 5A and B). This result indicates that LeCel12C enable to hydrolyze

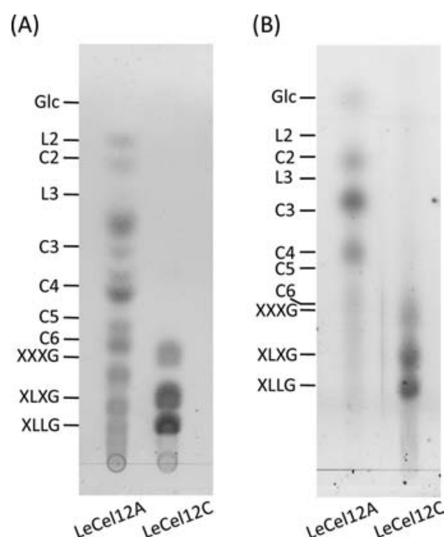

Figure 5. Production of oligosaccharides using the basidiomycetous GH12 family enzymes. 1,3−1,4-β-Glucan and LeCel12A, and xyloglucan and LeCel12C, were incubated in 100 mM sodium phosphate buffer (pH 5.0) at 30 °C for (A) 24 h or (B) 48 h. Hydrolysates were subjected to TLC in butan-1-ol/acetic acid/water (2:1:1, v/v) and stained with 0.5% (w/v) thymol in $EtOH/H_2SO_4$ (19:1, v/v). Bars indicate the positions of glucose (Glc) and oligosaccharides (C2, cellobiose; C3, cellotriose; L2, laminaribiose; L3, laminaritriose; XXXG, xyloglucan heptasaccharide; XXLG, xyloglucan octasaccharide; XLLG, xyloglucan nonasaccharide) on TLC plate.

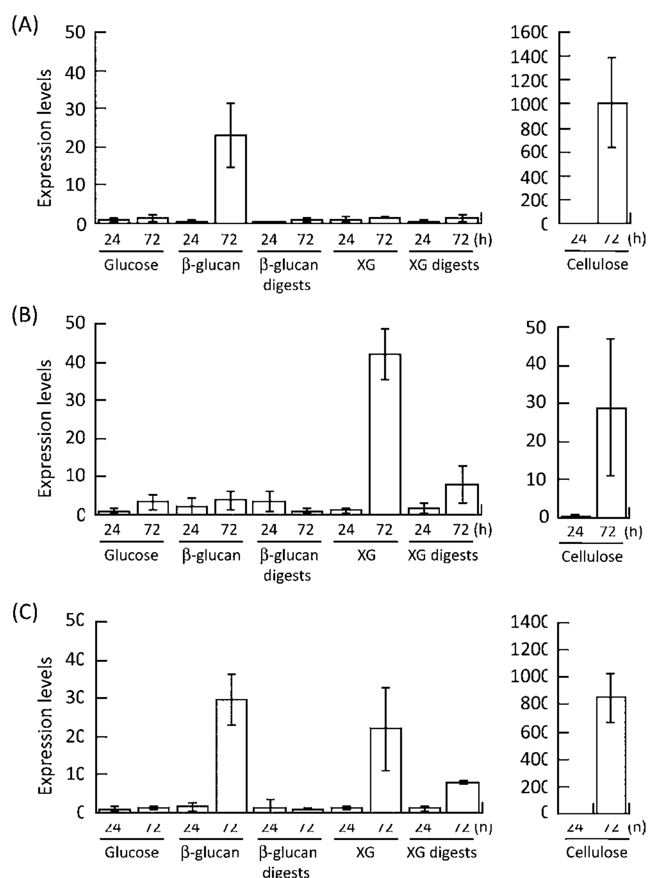

Figure 6. Transcript levels of the *L. edodes* GH12 enzymes. Shown are relative transcript levels of (A) *Lecel12A*, (B) *Lecel12B* and (C) *Lecel12C* after growth of *L. edodes* mycelia in culture medium with glucose, 1,3−1,4-β-glucan (β-glucan), 1,3−1,4-β-glucan hydrolysates (β-glucan digests), xyloglucan (XG), xyloglucan hydrolysates (XG digests) and cellulose. 1,3−1,4-β-Glucan hydrolysates and xyloglucan hydrolysates as shown in Figure 5A were used for this experiment. Gene expression levels in mycelia grown in culture medium with glucose for 24 h were taken as 1. Data are the means of individual three determinations ± SE.

xyloglucan into minimum xyloglucan unit after a long incubation.

Transcript Levels of *L. edodes* GH12 Genes. Transcript levels of *Lecel12A, Lecel12B and Lecel12C* were assayed after growth of *L. edodes* mycelia in liquid media containing various polysaccharides and oligosaccharides shown in Figure 5A as carbon source (Figure 6). The transcript level of *Lecel12A* increased at 72 h after treatment with 1,3−1,4-β-glucan or cellulose, but not after treatment with 1,3−1,4-β-glucan hydrolysates. Similarly, *Lecel12B* transcripts increased 72 h after treatment with xyloglucan or cellulose. Xyloglucan hydrolysates slightly enhanced the transcript level of *Lecel12B*. The transcript level of *Lecel12C* increased after treatment with xyloglucan, xyloglucan hydrolysates, 1,3−1,4-β-glucan or cellulose. Notably, expression of both *Lecel12A* and *Lecel12C* greatly increased during culture in the presence of cellulose, whereas that of *Lecel12B* only slightly increased. Thus, expression of the three genes was induced by treatment with various β-1,4-glucans.

■ DISCUSSION

GH12 enzymes, which occur widely in microorganisms but have not been so far observed in plants, cleave various β-1,4-linked glucans such as cellulose and hemicellulosic polymers. Some enzymes, including XEG from *Aspergillus aculeatus* and 1,3−1,4-β-glucanases from *M. oryzae* and *C. carbonum*, show substrate specific hydrolysis.[9−11] Furthermore, a *Trichoderma reesei* GH12 enzyme catalyzes a transglycosylation reaction, which can be used for the chemoenzymatic synthesis of sugars.[19−22] Basidiomycetous GH12 family enzymes have also been reported to show hydrolytic activity; however, their relative transcript and product levels are not high, as judged by transcriptome and secretome analyses.[4−6,8,12,14] Compared to the extensive information published for ascomycetous GH12 enzymes, there has been less description of the enzymes from the basidiomycetes.

L. edodes is white-rot fungus that degrades the cell wall polysaccharides of woody plants in nature, which may enhance efficient hyphal extension and nutrient acquisition. Hydrolases, endo-1,3-β-glucanases and exo-1,3-β-glucanases, which contribute to reconstruction of the *L. edodes* cell wall during morphogenesis, as well as a laccase involved in lignocellulose degradation, have been demonstrated in *L. edodes*.[23−25] In this study, gene identification, gene expression analysis and enzyme characterization of *L. edodes* GH12 enzymes were carried out in order to understand the mechanism underlying polysaccharide biodegradation.

Three genes encoding the *L. edodes* GH12 enzymes were cloned for the first time and then enzymatically active recombinant proteins were produced by expression in *B. choshinensis*. LeCel12A is an endo-1,4-β-glucanase that cleaves various β-1,4-linked glucans, among which 1,3−1,4-β-glucan, an important hemicellulose in Poacea plants,[26] is the best substrate, but it does not cleave crystalline or amorphous cellulose (Table 1). Notably, its activity toward 1,3−1,4-β-

glucan is about 7-fold higher than that toward CMC or glucomannan. On the other hand, LeCel12B and LeCel12C showed substrate preference for xyloglucan with regard to hydrolytic activity. To our knowledge, this is the first evidence that basidiomycetous fungi possess XEGs. Both 1,3−1,4-*β*-glucan and xyloglucan are hemicellulosic polymers of the primary cell wall in monocotyledonous and dicotyledonous plants, respectively, and are thought to play a significant role in determining cell wall strength.[27−29] By contrast, cell walls of the basidiomycetes *S. commune*, *Coprinopsis cinerea*, *Agaricus bisporus* and *L. edodes* are mainly composed of chitin and *β*-1,3-glucan.[30−33] These facts suggest that *L. edodes* GH12 enzymes function to cleave plant hemicellulosic polymers to enhance hyphal extension and nutrient acquisition. Furthermore, the actions of the enzymes cause the removal of hemicellulosic polymers that cover cellulose microfibrils, leading to an increased opportunity that cellulose-digestable enzymes will attack cellulose more efficiently.

Higher transcript levels of *Lecel12A, Lecel12B* and *Lecel12C* in *L. edodes* mycelia were observed 72 h after growth in culture medium containing the corresponding substrate carbohydrates; in addition, the levels of *Lecel12A* and *Lecel12C* markedly increased in the presence of cellulose, although these enzymes do not have the ability to hydrolyze cellulose. By contrast, xyloglucan hydrolysates increased transcript levels of *Lecel12B* and *Lecel12C* to some extent, whereas 1,3−1,4-*β*-glucan hydrolysates did not. Some CWDEs of *T. reesei* are strongly induced by the addition of cellulose, cellooligosaccharides, laminarioligosaccharides and gentiobiose in culture medium.[34−36] Presumably, there are proteins that are able to recognize these polysaccharides and/or oligosaccharides, resulting in the induction of CWDE genes.

Identifying proteins with amino acid similarity helps the prediction of enzymatic properties and biological functions. However, detailed functions must be determined by means of enzyme characterization using native or recombinant proteins. GH12 enzymes are thought to be a group of proteins that catalyze the cleavage of *β*-1,4-linkages; within this group, the basidiomycetous GH12 enzymes are separated from the clusters of ascomycetous enzymes and further divided into two subfamilies. Highly active enzymes toward 1,3−1,4-*β*-glucan, namely LeCel12A, PpCel12A and PpCel12B, are included in subfamily 1, whereas highly active enzymes toward xyloglucan, namely LeCel12B, LeCel12C and ScCel12A, are members of the subfamily 2. This suggests that enzymes in subfamily 1 are endo-1,4-*β*-glucanases with hydrolytic activity toward various *β*-1,4-glucans including cellulose, and enzymes in subfamily 2 are XEGs, respectively. Xyloglucan is a structural polysaccharide that gives rigidity to the walls of not only the primary cell walls but also the secondary cell walls of woody plants.[27,37] Thus, the basidiomycetous GH12 enzymes are proposed to function in the hydrolysis of plant cell wall polysaccharides, which might lead to enhanced hyphal extension and nutrient acquisition. In addition, these findings could contribute to the prediction of enzymatic actions for newly identified GH12 enzymes from the basidiomycetous fungi and to development of food products containing plant cell wall polysaccharides and oligosaccharide production.

■ ASSOCIATED CONTENT

Supporting Information

Supplemental tables and figure. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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