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Characterization of two acidic β-glucosidases and ethanol fermentation in the brown rot fungus *Fomitopsis palustris*

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ABSTRACT

Two acidic β-glucosidases (βGI and βGII) from the brown rot fungus *Fomitopsis palustris* were purified to homogeneity by several chromatographic steps. βGI and βGII had molecular weights of 130 and 213 kDa, respectively, and exhibited optimum activity at pH 2.5 and 55 °C. The K_m values of βGI and βGII for *p*-nitrophenyl-β-D-glucopyranoside were 0.706 and 0.971 mM, respectively. Although the effect of metal ions and inhibitors differed between the two enzymes, both β-glucosidases exhibited preferential glucose release during hydrolysis of cello-oligosaccharides, indicating that βGI and βGII possess effective exo-type activities. Notably, *F. palustris* was able to produce ethanol when cultured on medium containing 20 g/l of glucose, mannose, cellobiose, and maltose, in which the maximum ethanol concentrations measured were 9.2, 8.7, 9.0, and 8.9 g/l, corresponding to 90.2%, 85.3%, 88.2%, and 87.3% of the theoretical yield, respectively. These findings suggest that *F. palustris* has the ability not only to secrete β-glucosidase enzymes effective at low pH, but also to function as a biocatalyst, which may be suitable for the conversion of lignocellulosic materials into ethanol.

1. Introduction

The most abundant type of biomass on Earth is cellulose, which is a key substance in the cell walls of all plants. Cellulose is a straight-chain polymer composed of glucose units connected by β-1,4-glycosidic bonds, and can be converted into various chemicals following complete hydrolysation to glucose. Presently, the most common approaches employed for the breakdown of cellulose to glucose are treatment with acid and enzymatic hydrolyses. The production of ethanol from renewable plant resources requires the utilization of lignocellulosic materials; however, commercial ethanol production from biomass remains economically unfeasible as a suitable saccharification process for hydrolysing lignocellolosic materials has yet to be developed.

Endoglucanases (EC 3.2.1.4), cellobiohydrolases (EC 3.2.1.91), and β-glucosidases (EC 3.2.1.21) represent three types of cellulolytic enzymes that act synergistically to hydrolyse cellulose. Endoglucanases hydrolyse cellulose chains internally providing new chain ends for cellobiohydrolases that subsequently hydrolyse cellulose processively from the ends to form predominantly cellobiose, while β-glucosidases hydrolyse cellobiose to glucose [1]. Therefore, the decomposition of cellulose is not readily achieved by the actions of endoglucanases and cellobiohydrolases alone, but also requires the activity of β-glucosidases for efficient hydrolysis. The presence of β-glucosidase has been shown to improve the efficiency of cellulose hydrolysis through reduction of the inhibitory effect caused by the end product cellobiose [2]. Thus, β-glucosidase is considered a crucial enzyme for the efficient conversion of cellulose to glucose.

Numerous microorganisms in nature, which are mostly fungi, have complex cellulolytic enzyme systems that are associated with wood decomposition and are responsible for hydrolysing cellulose into simple sugars [3]. Among these species, the cellulose-degrading fungi *Trichoderma reesei* possesses strong cellulolytic activity and secretes three types of extracellular cellulolytic enzymes, including five endoglucanases, two cellobiohydrolases, and two β-glucosidases [4–7]. Several cellulolytic enzymes have also been isolated from the well-known brown rot fungus *Fomitopsis palustris* [8–11], which grows on wood and causes a rapid and extensive depolymerization of cellulose [12]. *F. palustris* is of particular interest as it causes a dramatic pH decrease in culture medium during growth that is likely due to the secretion of oxalic acid [13]. To date, no β-glucosidase active at strongly acidic pH values (<3) have been reported, which are considered necessary for the saccharification of cello-oligosaccharides in the acid hydrolysates of lignocellulose.

In this study, we report the purification and characterization of two acidic β-glucosidases from the culture filtrate of *F. palustris*, and describe the efficient production of ethanol by this fungus.

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2. Materials and methods

2.1. Microorganism and culture conditions

F. palustris BC315 collected from pine stumps on the Tottori University campus, Japan were maintained on 1.5% (w/v) agar slants consisting of 10 g/l malt extract, 4 g/l yeast extract, and 4 g/l glucose, pH 6.3 (MYG medium). Fungal mycelia were grown on an agar plate for two weeks, and three 0.5 cm^2 pieces of the mycelium mat were then inoculated into MYC liquid medium (10 g/l malt extract, 4 g/l yeast extract, and 4 g/l cellobiose, pH 6.3). For enzyme induction and purification, *F. palustris* was cultivated in 500-ml Erlenmeyer flasks containing 50 ml of MYC medium. For the fermentation experiments, cells were cultivated for 9 days in MYG medium and subsequently filtered and transferred aseptically to a 500-ml Erlenmeyer flask with 50 ml of T medium (20 g/l glucose, 10 g/l yeast extract, 10 g/l KH_2PO_4, 2 g/l $(NH_4)_2SO_4$, and 0.5 g/l $MgSO_4{\cdot}7H_2O$, pH 6.0). The cultures were incubated statically at 28 °C under anaerobic conditions. Glucose in the T medium was replaced by other sugars at similar concentrations when investigating the fermentation characteristics of the fungus towards various sugars.

2.2. Verification of strain identity

Identification of the strain BC315 based on standard morphological and biochemical analysis was carried out in TechnoSuruga Laboratory Co., Ltd. (Shizuoka, Japan). The identity of this strain was determined by sequencing of the 28S rDNA D1/D2 region and the internal transcribed spacer (ITS) region with 5.8S rDNA. DNA was extracted from mycelia using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, and PCR was performed using puRe*Taq* Ready-To-Go PCR Beads (Amersham Biosciences, South Plainfield, NJ). Partial sequences were amplified with two primers for the D1/D2 region (NL1 and NL4) [14] and two primers for the ITS1-5.8S-ITS2 region (ITS4 and ITS5) [15]. DNA sequencing was performed using an ABI Prism 3130 × 1 Genetic Analyzer System (Applied Biosystems, Foster City, CA, USA). DNA sequences were compared with those of the GenBank database using BLAST [16], while multiple alignments were conducted using CLUSTAL W [17]. The sequences of 28S rDNA (EU024964) and ITS1-5.8S-ITS2 rDNA (EU024965) from the *F. palustris* strain available at GenBank/DDBJ/EMBL both displayed 100% identity to those of strain BC315, respectively. According to the morphological, biochemical and phylogenetic analyses, strain BC315 was verified as *F. palustris*. The sequence data on 28S rDNA and ITS1-5.8S-ITS2 rDNA from *F. palustris* BC315 determined in this study have been deposited in the GenBank/DDBJ/EMBL databases under accession numbers AB604155 and AB604156, respectively.

2.3. Analytical methods

Ethanol and residual sugar concentrations in the culture filtrates were determined by high-performance liquid chromatography (Shimadzu Co., Ltd., Kyoto, Japan) with a refractive index detector and a Shodex KS-801 column (8.0 mm × 300 mm; Showa Denko Co., Ltd., Tokyo, Japan) operated at 80 °C with a distilled water mobile phase at a flow rate of 0.6 ml/min. The theoretical yield of ethanol was defined as 0.51 g of ethanol per g of glucose (2 mol of ethanol/1 mol of glucose).

2.4. Enzyme activity assay

β-Glucosidase activity was determined by monitoring the release of *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG). The enzyme solution (100 μl) was incubated with 1 mM *p*NPG in 1 ml of 100 mM sodium acetate buffer, pH 4.0 at 37 °C for 5 min. The enzyme reaction was stopped by the addition of 200 μl of 1 M sodium carbonate, and the absorbance was read at 400 nm. The molar coefficient for *p*-nitrophenol is $\varepsilon_{400} = 18.3\ mM^{-1}\ cm^{-1}$. One unit of β-glucosidase activity was defined as the amount of enzyme liberating 1 μmole of *p*-nitrophenol per minute under the assay conditions.

2.5. Purification of β-glucosidases

The culture medium of *F. palustris* was filtered through glass wool to remove mycelial fragments. Polyethylene glycol (PEG) 6000 was slowly added to the culture filtrate with stirring to a final concentration of 30% (w/v). After 12 h of mixing at 4 °C, the suspension was centrifuged at 12,000 × *g* for 30 min at 4 °C. The precipitate was dissolved in 50 mM sodium acetate buffer (pH 5.0) and the dialyzed overnight at 4 °C against the identical buffer. The dialysate was applied to an anion exchange HiTrap Sepharose Q column (1.6 cm × 2.5 cm; GE Healthcare UK Ltd., Buckinghamshire, UK) equilibrated with the dialyzing buffer. The absorbed proteins were eluted with a linear gradient from 0 to 0.5 M NaCl at a flow rate 1.0 ml/min, and 2.5 ml fractions were collected. The fractions were analysed for activity and absorbance at 280 nm. The fractions containing two distinct active β-glucosidases, named βGI and βGII, were pooled separately, concentrated on an Amicon YM10 membrane (Millipore Corp., Billerica, MA, USA), and applied to a column of hydroxyapatite (1 cm × 5 cm; Bio-Rad Laboratories, Hercules, CA, USA) equilibrated with 50 mM potassium phosphate buffer (pH 6.0). The proteins in each fraction were eluted with a stepwise gradient of 50–500 mM potassium phosphate buffer at a flow rate 0.2 ml/min, and fractions containing the active proteins were pooled and concentrated. The βGI fraction was then applied to a Superdex 200 column (1 cm × 30 cm; GE Healthcare Bio-Sciences, Piscataway, NJ, USA) equilibrated with potassium phosphate buffer (pH 6.0) containing 0.1 M NaCl. The proteins were eluted with the identical buffer at a flow rate 0.5 ml/min, and the fraction containing active protein was pooled and concentrated as before. The activities of the two β-glucosidases, βGI and βGII, purified to homogeneity by the above steps, were then characterized.

2.6. Protein determination

Protein concentrations were determined by the method of Bradford [18] using bovine serum albumin as the standard. Protein content was monitored by the absorbance at 280 nm during chromatographic separation.

2.7. Electrophoresis

Polyacrylamide gel electrophoresis (PAGE) under non-denaturing conditions was performed using 7.5% polyacrylamide gels in Tris–HCl (pH 8.8) by the method of Davis [19]. Sodium dodecyl sulfate-PAGE (SDS-PAGE) was carried out using 10% gels in the presence of 0.1% SDS under denaturing conditions according to Laemmli [20]. Protein bands were stained with Coomassie Brilliant Blue R-250, and the carbohydrate-containing bands were stained with periodate-Schiff reagent.

2.8. Molecular weight determination

The molecular weights of βGI and βGII were determined by SDS-PAGE with Precision Protein Standards (Bio-Rad). The glycosylation state was determined by mobility profiles in SDS-PAGE before and after treatment with endoglycosidase H and O-glycosidase.

2.9. Effect of pH and temperature

The optimal pH values and temperatures for the activities of βGI and βGII were determined over a pH range of 1.5–7.0 (100 mM KCl–HCl [pH 1.5–2.0], glycine–HCl [pH 2.0–3.5], sodium acetate [pH 3.5–5.5], potassium phosphate [pH 5.5–7.0]), and a temperature range of 20–70 °C.

2.10. Other assays

The effects of various metallic ions and reagents (4 mM) on the activity of the purified enzymes were tested. Residual activity was measured as described above in Section 2.4. K_m values were determined from Lineweaver–Burk plots at *p*NPG concentrations varying from 0.1 to 5 mM.

3. Results

3.1. Purification of βGI and βGII

Extracellular β-glucosidase activity was detected in the culture medium of the brown rot fungus *F. palustris* BC315. The pH of the medium decreased to below 2 as mycelial growth proceeded, and accumulation of oxalic acid was detected. Growth of the fungus on cellobiose or Avicel increased β-glucosidase production compared to that when cultured on glucose (data not shown). From the culture filtrate, the enzymes responsible for the β-glucosidase activity were purified by PEG precipitation, anion-exchange, hydroxyapatite, and gel filtration chromatography. The result of the purification is summarized in Table 1. During the HiTrap Sepharose Q column chromatography, two active enzymes were clearly eluted as separate peaks (Fig. 1). The highest level of enzyme activity appeared in βGI (fractions 23–30), which corresponded to 57.5% of the total activity loaded onto the column, whereas βGII (fractions 8–12) was characterized by lower levels of activity, representing 13.8% of the total activity. The main βGI fractions were concentrated, subjected to hydroxyapatite chromatography, and subsequently separated on a Superdex 200 gel filtration column, which resulted in the isolation of a pure enzyme that eluted as a single peak. The isozyme βGII fractions pooled from the HiTrap Sepharose Q column chromatography step were only subjected to hydroxyapatite chromatography. Finally, overall yields of 8.8% and 1.5% and specific activities of 836 and 157 U/mg protein were obtained for βGI and βGII, respectively. Purified

Table 1
Purification of βGI and βGII from the brown-rot fungus *F. palustris*.

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification fold
Culture filtrate	38.3	845	22.1	100	1
PEG ppt	21.6	633	29.3	74.9	1.3
HiTrap Q					
βGI	3.3	364	110	43.1	5.0
βGII	3.2	87.1	27.2	10.3	1.2
Hydroxyapatite					
βGI	0.3	195	650	23.1	29.4
βGII	0.08	12.6	157	1.5	7.1
Superdex 200					
βGI	0.09	75.2	836	8.8	37.8

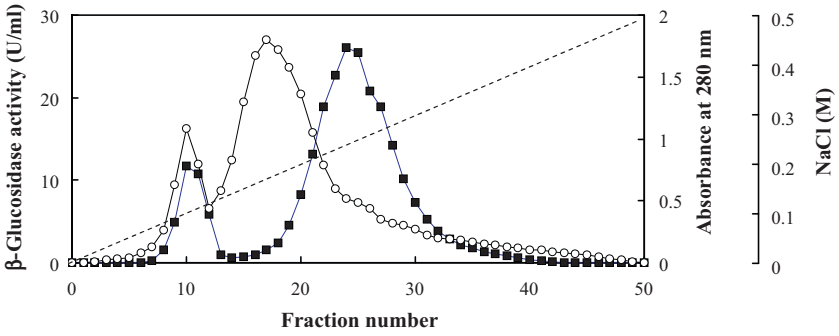

Fig. 1. Elution profile of β-glucosidase from *F. palustris* using HiTrap Sepharose Q column chromatography. Absorbance at 280 nm (○); enzyme activity in U/ml (■); NaCl concentration (– – –).

βGI and βGII appeared as single bands on SDS-PAGE with estimated molecular masses of 130 and 213 kDa, respectively (Fig. 2). Additionally, these enzymes exhibited typical characteristics of glycoproteins, as each protein band on a native PAGE gel was stained by Schiff's reagent (data not shown). Incubation of βGI and βGII with endoglycosidase H resulted in decreases of the molecular masses by 10 and 40 kDa, respectively. In contrast, no significant changes in the molecular masses were observed for βGI and βGII when incubated with O-glycosidase. Taken together, these results indicated that the majority of carbohydrate structures of βGI and βGII consisted of N-linked sugar chains.

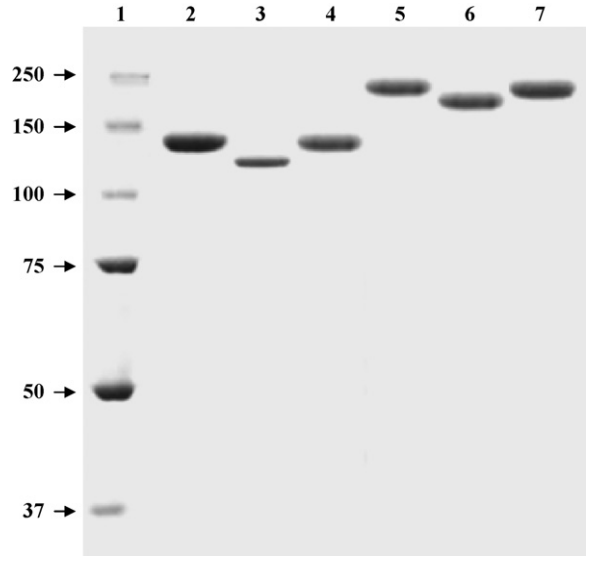

Fig. 2. SDS-PAGE of purified βGI and βGII from *F. palustris*. Lane 1, molecular weight markers with indicated molecular masses in kDa on the left; lane 2, βGI; lane 3, βGI treated with endoglycosidase H; lane 4, βGI treated with O-glycosidase; lane 5, βGII; lane 6, βGII treated with endoglycosidase H; and lane 7, βGII treated with O-glycosidase.

3.2. Effect of pH and temperature on enzyme activity

The effect of pH on the activity of βGI and βGII was next examined at pH ranging from 1.5 to 7.0 (Fig. 3A). The optimum pH for enzyme activity of both βGI and βGII was 2.5, and the activities of

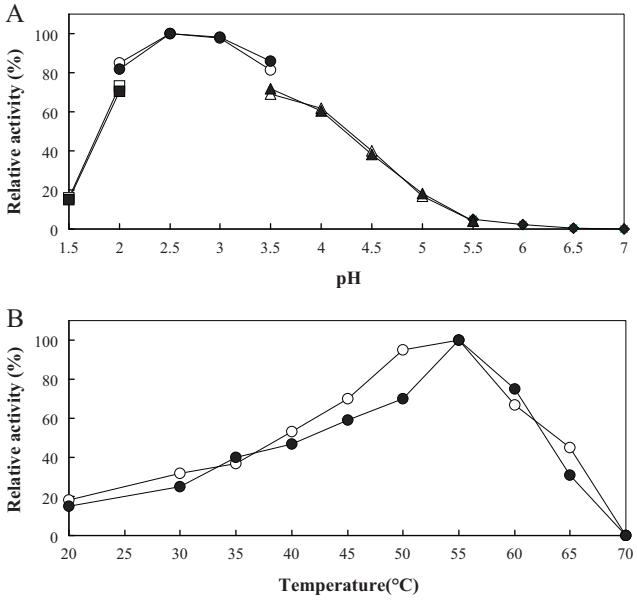

Fig. 3. The effects of pH (A) and temperature (B) on the activities of βGI and βGII of *F. palustris*. (A) Four different buffer systems: square, 100 mM KCl–HCl (pH 1.5–2.0); circle, glycine–HCl (pH 2.0–3.5); triangle, sodium acetate (pH 3.5–5.5); diamond, potassium phosphate (pH 5.5–7.0), were used to determine the effect of pH between pH 1.5 and 7.0. The activities were measured at various pH values at 37 °C under standard assay conditions. The data for βGI and βGII are presented as open and closed symbols, respectively. (B) The activities were measured at various temperatures using 100 mM glycine–HCl as the buffer (pH 2.5) under standard assay conditions. The data for βGI and βGII are presented as open and closed symbols, respectively.

Table 2
Effect of different metal ions and inhibitors on βGI and βGII.

Additive (4 mM)	Relative activity (%)[e]	
	βGI	βGII
None	100	100
$AgNO_3$	8.1	46.2
$MnCl_2$	83.1	84.7
$CaCl_2$	83.8	83.8
$FeCl_3$	104.5	108.4
$MgCl_2$	77.7	86.3
$ZnCl_2$	57.6	62.9
KCl	70.5	80.3
$NiSO_4$	91.6	96.5
$CuSO_4$	88.2	96.3
$CoCl_2$	57.7	89.8
$HgCl_2$	36.5	97.5
DTT[a]	61.9	99.6
EDTA[b]	70.5	82.4
SDS[c]	78.2	57.8
PCMB[d]	71.0	60.6

[a] Dithiothreitol.
[b] Disodium ethylenediamine tetraacetate.
[c] Sodium dodecyl sulfate.
[d] *p*-Chloromercuribenzoate.
[e] Values are averages of independent experiments performed in triplicate.

the purified enzymes between pH 2 and 4 were greater than 60% of the maximal level. The effect of temperature on the activities of βGI and βGII was determined at temperatures ranging from 20 to 70 °C (Fig. 3B). Both enzymes exhibited maximum activity at a temperature of 55 °C.

3.3. Effect of metal ions and inhibitors on enzyme activity

The purified enzymes were incubated in 100 mM glycine–HCl buffer (pH 2.5) containing various metal ions and inhibitors, and the residual activities were then determined under standard enzyme assay conditions. The effects of metal ions and inhibitors on the β-glucosidase activity are shown in Table 2. Both βGI and βGII were strongly inhibited by Ag^{2+}, whereas Hg^{2+}, Co^{2+}, and dithiothreitol (DTT) affected βGI activity, but had little effect on the activity of βGII. In addition SDS and *p*-chloromercuribenzoate (PCMB) showed inhibitory effects on βGII activity, but did not significantly affect the activity of βGI.

3.4. Kinetic property

The K_m values of βGI and βGII determined from Lineweaver–Burk plots were 0.706 and 0.971 mM, respectively, using *p*NPG as the substrate.

3.5. Hydrolysis of cello-oligosaccharides

Table 3 shows the reactivity of βGI and βGII against cello-oligosaccharides (G2–G5). It was determined that both enzymes efficiently hydrolysed all cello-oligosaccharides, regardless of DP values. The cleavage activities of βGI and βGII were found to range from 37% to 39% and 9% to 10%, respectively, within 15 min of incubation.

Table 3
Hydrolysis of cello-oligosaccharides by βGI and βGII.

Cello-oligosaccharides[a]	Liberated glucose (%)[b]	
	βGI	βGII
Cellobiose	36.6	8.7
Cellotriose	36.9	9.8
Cellotetraose	38.8	8.5
Cellopentaose	36.6	8.9

[a] Initial concentration was 10 mM.
[b] Values were calculated from the concentration of liberated glucose per that of the initial substrate. Values are averages of independent experiments performed in triplicate.

3.6. Ethanol fermentation

Finally, to examine the potential of *F. palustris* for ethanol production, the bioconversion characteristics of *F. palustris* were investigated. As shown in Fig. 4, ethanol was detected after 24 h of cultivation using various sugars as growth substrates, which reached a peak concentration after 120–144 h. Ethanol was produced with a corresponding decrease in the amount of sugar in each analysed medium. When the fungus was cultured using 20 g/l of glucose and mannose, maximum ethanol concentrations of 9.2 and 8.7 g/l, corresponding to 90.2% and 85.3% of the theoretical yields, respectively, were observed. The fungus was also cultured using 20 g/l cellobiose and maltose, which resulted in maximum ethanol concentrations of 9.0 and 8.9 g/l, corresponding to 88.2% and 87.3% of the theoretical yields, respectively. Moreover, the fungus was cultured using 20 g/l of starch and wheat bran, showing a maximum ethanol concentration of 7.9 and 2.8 g/l, corresponding to 77.5% and 76.9% of the theoretical yield, respectively. When the fungus was cultured in 20 g/l of arabinose and xylose, significantly lower maximum ethanol concentrations of 0.5 and 1.6 g/l, respectively, were observed.

4. Discussion

In this study, two extracellular β-glucosidases (βGI and βGII) produced by *F. palustris* BC315 were purified to homogeneity using a series of chromatographic techniques and subsequently characterized. Notably, although the activities of both βGI and βGII were optimal at 55 °C and an extremely low pH of 2.5, βGII exhibited a significantly higher specific activity. A number of fungi, including *T. reesei*, *Schizophyllum commune*, *Phanerochaete chrysosporium*, *Volvariella volvacea*, *Ceriporiopsis subvermispora*, and *Pichia etchellsii* also produce two or three extracellular β-glucosidases that differ slightly in properties [6,21–25]. Our results clearly indicate that two forms of β-glucosidase from *F. palustris* are excreted into the extracellular space and participate in the hydrolysation of cellulose to glucose.

βG1 and βGII were estimated to have molecular weights of 130 and 213 kDa, respectively, and be composed of 8% and 20% sugar chains, respectively, which was determined by measuring the decrease in molecular weight following endoglycosidase H treatment. A number of studies have described the purification and characterization of β-glucosidases from basidiomycetes with molecular weights ranging from 53 to 320 kDa [11,21–24,26]. Among these enzymes, a thermostable β-glucosidase with a molecular weight of 138 kDa was identified from a *F. palustris* strain [11], which is similar to the molecular weight of βGI purified here. However, the optimum pH of βGI (pH 2.5) was significantly lower than that of the β-glucosidase (pH 4.5) from the other *F. palustris* strain. In addition, a comparison of the optimum temperatures between the β-glucosidases from the two *F. palustris* strains revealed no similarity. Most β-glucosidases exhibit optimum activity in the pH range of 4–6, and a β-glucosidase with the low pH optimum observed here has not been reported. Therefore, the two identified *F. palustris* acidic β-glucosidases may be effective enzymes for the direct conversion of cello-oligosaccharides in the acid hydrolysates of lignocellulose into glucose.

Among β-glucosidases from filamentous fungi reported previously, those of *Aspergillus niger* and *A. oryzae* exhibit high specific activities of 979 and 1066 U/mg, respectively [27,28]. In contrast, the specific activities of β-glucosidases from basidiomycetes are

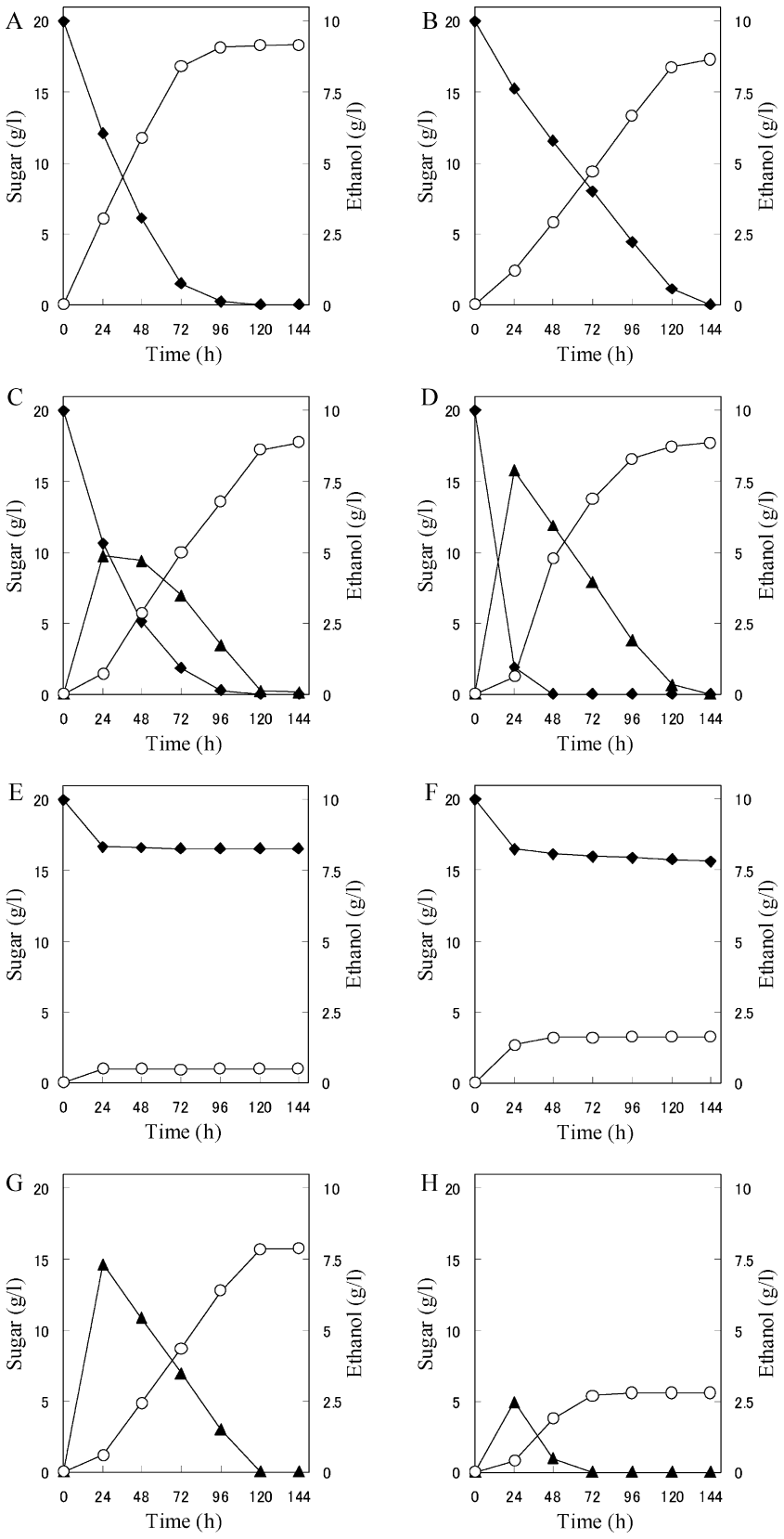

Fig. 4. Time course of ethanol production by *F. palustris* using 20 g/l carbohydrate as the sole carbon source: glucose (A), mannose (B), cellobiose (C), maltose (D), arabinose (E), xylose (F), starch (G), and wheat bran (H). Total sugar (□); liberated glucose (▲); ethanol (○). Values are averages of independent experiments performed in triplicate.

typically in the range of 8.5–191 U/mg [11,22,23,26,29]. A comparison of specific activities shows that βGI exhibits a significantly higher value than those of β-glucosidases from basidiomycetes. Thus, due to their unique characteristics, the β-glucosidases from *F. palustris* described in the present study appear to represent novel enzymes, with βGI in particular exhibiting high activity.

Although βGI and βGII were found to have similar properties, the effect of metal ions and inhibitors on activity differed between the two enzymes. Notably, βGI was inhibited by the sulfhydryl oxidant metals (Ag^{2+} and Hg^{2+}), but was not influenced strongly by the thiol-specific inhibitor PCMB. In contrast, βGII was not significantly affected by these compounds. The differences in susceptibility may be due to differing catalytic residues; for example, the inhibitory actions of Ag^{2+} and Hg^{2+} suggest that a sulfhydryl group may be involved in the active catalytic site of βGI, but has no function in the active site of βGII. In addition, βGI and βGII were not strongly inhibited by Cu^{2+}, as is generally reported for fungal β-glucosidases [23–25,28]. The lack of effects of Cu^{2+} and Ni^{2+} on the activities of βGI and βGII was considered to arise from the copper and nickel metal resistance exhibited by *F. palustris* [30]. Taken together, these results suggest that the structure of the active catalytic sites of βGI and βGII from *F. palustris* are unique from those of fungal β-glucosidases previously reported.

Although hydrolysis of cello-oligosaccharides (G2–G5) by purified βGI and βGII exhibited efficient release of glucose at the beginning of the reaction, the activity of βGI was four times higher than that of βGII. This difference in performance may be due to the specific activity of the two enzymes, which was significantly higher for βGII. The results indicate that the two β-glucosidases possess effective exo-type activities, as no differences in the rates of release of glucose among the cello-oligosaccharides used as substrates were detected.

Finally, we found that *F. palustris* was able to efficiently ferment hexose sugars into ethanol, which is a similar property exhibited by the white rot fungi *Peniophora cinerea* and *Trametes suaveolens* [31]. There appears to be no distinct difference in ethanol fermentation between the white rot fungi which are mainly able to degrade lignin and the brown rot fungi that predominantly degrade cellulose. Interestingly, *F. palustris* directly produced ethanol from starch and wheat bran without prior acid or enzymatic hydrolysis. The wheat bran used in this study contained 35.7% (w/w) glucose (mainly in the form of starch) as a fermentable sugar of the fungus. Therefore, it is possible that the ethanol conversion rate between starch and wheat bran correlated with each other. In previous studies, it was suggested that cellulolytic enzymes from brown rot fungi, such as *F. palustris*, are useful for the bioconversion of woody biomasses into fermentable sugars or bioethanol by the enzymatic hydrolysis of cellulose. The present study has further illustrated that not only isolated β-glucosidase enzymes from a brown rot fungus, but also the fungus itself, can potentially act as a biocatalyst for ethanol production.

Acknowledgments

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