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BIOENERGY/BIOFUELS/BIOCHEMICALS

Construction of a starch-inducible homologous expression system to produce cellulolytic enzymes from *Acremonium cellulolyticus*

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Abstract A starch-inducible homologous expression system in *Acremonium cellulolyticus* was constructed to successfully produce recombinant cellulolytic enzymes. *A. cellulolyticus* Y-94 produced amylolytic enzymes and cellulolytic enzymes as major proteins in the culture supernatant when grown with soluble starch (SS) and Solka–Flock cellulose (SF), respectively. To isolate a strong starch-inducible promoter, glucoamylase (GlaA), which belongs to glycoside hydrolase family 15, was purified from the SS culture of Y-94, and its gene was identified in the genome sequence. The 1.4-kb promoter and 0.4-kb terminator regions of *glaA* were amplified by polymerase chain reaction (PCR) and used in the construction of a plasmid that drives the expression of the cellobiohydrolase I (Cel7A) gene from *A. cellulolyticus*. The resultant expression plasmid, containing *pyrF* as a selection marker, was randomly integrated into the genome of the *A. cellulolyticus* Y-94 uracil auxotroph. The prototrophic transformant, Y203, produced recombinant Cel7A as an extracellular protein under control of the *glaA* promoter in the SS culture. Recombinant and wild-type Cel7A were purified from the SS culture of Y203 and the SF culture of *A. cellulolyticus* CF-2612, respectively. Both enzymes were found to have the same apparent molecular weight (60 kDa), thermostability (T_m 67.0 °C), and optimum pH (pH 4.5), and showed similar catalytic properties for soluble and insoluble substrates. These results suggest that the *A. cellulolyticus* starch-inducible expression system will be helpful for characterization and improvement of fungal cellulolytic enzymes.

Keywords *Acremonium cellulolyticus* · Protein expression · Cellulase production · Homologous expression · Glucoamylase · Cellobiohydrolase I

Introduction

The development of lignocellulose conversion technology to produce alternative fuels and chemicals has advanced rapidly over the past few years. Cellulolytic enzymes, such as cellobiohydrolase I (CBHI; Cel7A), cellobiohydrolase II (CBHII), endo-1,4-β-glucanase, and endo-1,4-β-xylanase, are key enzymes that hydrolyze cellulose and hemicellulose components in lignocellulosic biomass to glucose and other fermentable sugars, respectively [15, 16]. Filamentous fungi are the major source of industrial cellulolytic enzymes, which are abundantly produced in mutant strains of these fungi, such as in *Trichoderma reesei* [18].

Heterologous and homologous expression of cellulolytic enzymes have been widely used to develop effective cellulase systems, including strain improvement [25], protein engineering [17], and optimization of enzyme composition [3, 26]. Heterologous expression systems such as *Schizosaccharomyces pombe*, *Pichia pastoris*, and *Aspergillus awamori* have been used to produce *Trichoderma* cellulolytic enzymes, often with limited success. These expression systems often result in hyperglycosylation, which may negatively impact protein structure, function, and stability

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[2, 3, 9, 13, 23]; on the other hand, the performance of recombinant cellulolytic enzymes would not be impaired when using the homologous expression host [17]. Successful homologous expression of cellulolytic enzymes in *T. reesei* has been observed in a medium containing glucose, using the constitutive promoters of the *tef1*, *pdc*, and *eno* genes from *T. reesei*, and the *gpdA* promoter from *Aspergillus nidulans* [4, 19, 22].

Acremonium cellulolyticus is one of several fungi that are promising alternatives to *T. reesei* for use in industrial production of cellulolytic enzymes [10]. Cellulosic carbon sources, such as Solka–Flock cellulose (SF), pretreated rice straw, and waste paper sludge, are strong inducers of cellulase production in *A. cellulolyticus* [6, 11, 24]. In a previous study, we demonstrated homologous expression of β-xylosidase in *A. cellulolyticus*, using the cellulose-inducible *cbh1* promoter, to improve xylan digestibility in the *Acremonium* cellulase system [14]. However, a cellulose-inducible expression system may not be suitable for improvement and characterization of recombinant cellulolytic enzymes, because a variety of cellulolytic enzymes that have similar enzymatic activities and molecular weights are simultaneously produced in cellulose cultures of *A. cellulolyticus* [29, 30]. These facts significantly increase the difficulties faced in detecting and purifying recombinant cellulolytic enzymes for further characterization.

In this study, we developed a starch-inducible homologous expression system in *A. cellulolyticus* to express cellulolytic enzymes. Cel7A, which is recognized as the most important single enzyme component in the fungal cellulase system, was used as a model protein to evaluate this expression system. A gene encoding glucoamylase (GlaA), a major protein in starch culture, was identified in the *A. cellulolyticus* genome sequence. Using the *glaA* promoter and terminator regions, an expression plasmid was constructed. Our results demonstrate that the recombinant Cel7A (rCel7A) purified from the soluble starch (SS) culture of *A. cellulolyticus* exhibits similar physicochemical and enzymatic properties to those of wild-type Cel7A (wtCel7A) purified from SF culture.

Materials and methods

Fungal strains and plasmids

The fungal strains and plasmids used in this study are summarized in Table 1. *Acremonium cellulolyticus* Y-94 wild type (FERM BP-5826) [28] and CF-2612 high-cellulase-producing mutant (FERM BP-10848) [6] were maintained on potato dextrose agar plates. The *A. cellulolyticus* YP-4 uracil autotroph mutant was isolated from Y-94 according to the method of Fujii et al. [7] in this study and maintained on potato dextrose agar plates containing uracil and uridine at final concentrations of 1 g/L each. Transformants of *A. cellulolyticus* YP-4 were maintained on MM agar plates [7].

Production of amylolytic and cellulolytic enzymes

Y-94 and YP-4 transformants were grown on a medium (pH 4.0) containing, per liter: 20 g carbon source, 24 g KH_2PO_4, 1 g Tween 80, 5 g $(NH_4)_2SO_4$, 1.2 g $MgSO_4{\cdot}7H_2O$, 0.01 g $ZnSO_4{\cdot}7H_2O$, 0.01 g $MnSO_4{\cdot}6H_2O$, 0.01 g $CuSO_4{\cdot}7H_2O$, and 0.8 g urea, at 30 °C, 200 rpm, for 96 h in an Erlenmeyer flask. SS (Wako Pure Chemical Industries, Osaka, Japan), SF (Fiber Sales & Development, Urbana, OH, USA), or glycerol was used as carbon source. CF-2612 was grown on the same medium containing 50 g of SF/L and 4.0 g of urea/L as described previously [6].

Table 1 Fungal strains and plasmids

Strain or plasmid	Description	References
Strain		
A. cellulolyticus Y-94	Wild type (FERM BP-5826)	[28]
CF-2612	High-cellulase-producing mutant (FERM BP-10848) to produce wtCel7A, source of *cel7A* and *glaA* promoter and terminator regions	[6]
YP-4	Host strain for homologous expression, uracil autotroph mutant of Y-94	This study
Y202	YP-4 prototrophic transformant harboring pANC202	This study
Y203	YP-4 prototrophic transformant harboring pANC203 to produce rCel7A	This study
Plasmid		
pbs-pyrF	Amp^r $PyrF^r$; pBluescript KS(+) derivative containing 2.7-kb fragment harboring *pyrF* from Y-94	[7]
pANC202	Amp^r $PyrF^r$; expression vector, pbs-pyrF derivative containing 1.4-kb promoter and 0.4-kb terminator regions of *glaA*	This study
pANC203	Amp^r $PyrF^r$; expression vector of *cel7A*, pANC202 derivative containing *cel7A*	This study

Construction of expression plasmid

A plasmid pbs-pylF [7], containing the *A. cellulolyticus* orotate phosphoribosyltransferase gene (*pyrF*) as a selection marker inserted into pBluescript KS(+), was used to construct the expression plasmid pANC202 (Fig. 1). *A. cellulolyticus* CF-2612 chromosomal DNA was used as the template to amplify genes that were then introduced into the expression vector. The chromosomal DNA was prepared using the QIAGEN Genomic-tip 100/G together with Genomic DNA Buffer Set (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. The 1.4-kb promoter region of the glucoamylase gene (*glaA*) was amplified by PCR using the forward primer 5′-TAAGAATTCTGAA GATCGAAAGTATTTACATG with an *Eco*RI site (underlined) and the reverse primer 5′-TATGATATCAATA GACAGACTTCAAGGTTCGG with an *Eco*RV site (underlined). The 0.4-kb terminator region of *glaA* was amplified using the forward primer 5′-TTTGATATCCTG CAGGAGAATTACCGGGGTAGGATATG with *Eco*RV and *Sbf*I sites (underlined) and the reverse primer 5′-TAAGTCGACTCGACTGATTACTAATCGTTTGAT AG with a *Sal*I site (underlined). The primers for the promoter and terminator regions were designed based on information obtained from an *A. cellulolyticus* genome database (unpublished data). The Cel7A gene (*cbh1*) [21] was amplified using the forward primer 5′-ATTGTTAA CACAATGTCTGCCTTGAACTCTTTCA with an *Hpa*I site (underlined) and the reverse primer 5′-AATCCTG CAGGTTACAAACATTGAGAGTAGTAAGG with an *Sbf*I site (underlined).

The 1.4-kb PCR fragment of the *glaA* promoter region was digested with *Eco*RI/*Eco*RV, and ligated into the *Eco*RI/*Eco*RV site of pbs-pylF. The 0.4-kb terminator fragment digested with *Eco*RV/*Sal*I was ligated into *Eco*RV/*Sal*I of the resulting plasmid to obtain pANC202 (Fig. 1). The *cbh1* expression plasmid pANC203 was constructed by introducing the *cbh1* fragment digested with *Hpa*I/*Sbf*I into the *Eco*RV/*Sbf*I of pANC202. All ligated gene fragments and their ligation sites were verified by sequencing.

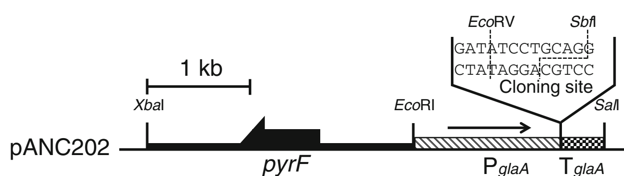

Fig. 1 Restriction map of the expression vector pANC202. The 1.4-kb promoter region (P_{glaA}) and 0.4-kb terminator region (T_{glaA}) from *glaA* and the *pyrF* gene containing its original promoter and terminator region were inserted into the *Xba*I–*Sal*I site of the plasmid vector pBluescript KS(+)

Transformation of the *A. cellulolyticus* uracil auxotroph

Protoplasts of *A. cellulolyticus* YP-4 were transformed with pANC202 or pANC203 by nonhomologous integration into the host chromosomal DNA [7]. The gene integrated into the prototrophic transformants was verified by genomic PCR, using the forward primer for the *glaA* promoter region and the reverse primer 5′-GAAACAGC TATGACCATGATTACGC within the downstream of the 3′-end of the *glaA* terminator on pANC202. The chromosomal DNA of the transformants was purified using the Gentra Puregene Yeast/Bact. Kit (Qiagen).

Purification and identification of GlaA

The Y-94 culture grown in medium containing 20 g SS/L was centrifuged, and the resulting supernatant was filtered through a 0.22-µm polyether sulfone membrane. The culture filtrate was loaded onto a HiPrep 26/10 desalting column (GE Healthcare, Buckinghamshire, UK) equilibrated with 20 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer, pH 6.5 (buffer A). The desalted sample was subjected to anion-exchange chromatography on a Source 15Q column (GE Healthcare) equilibrated with the same buffer, and the protein was eluted with a linear gradient of 0–0.25 M NaCl. Fractions with GlaA activity were pooled, brought to 1.4 M $(NH_4)SO_4$, and subjected to hydrophobic interaction chromatography on a Source 15ISO column (GE Healthcare) with a 1.4–0.8 M $(NH_4)SO_4$ gradient in 20 mM sodium acetate buffer, pH 5.5 (buffer B). The eluted GlaA fractions were pooled, desalted, and stored in buffer B containing 0.01 % NaN_3 at 4 °C. The purity and size of the protein were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE clean-up kit (GE Healthcare) was used prior to SDS-PAGE analysis of the culture supernatant. The GlaA in the gel was digested by lysyl endopeptidase. The resulting peptide fragments were separated by reversed-phase high-performance liquid chromatography (HPLC) and collected, and the amino acid sequence was determined by using a Procise 494-HT protein sequencer (Applied Biosystems, Foster City, CA, USA). The *glaA* coding region in the genome sequence of *A. cellulolyticus* was searched using the gene analysis software in silico Molecular Cloning (in silico Biology, Yokohama, Japan) based on the internal amino acid sequence. The nucleotide sequence of *glaA*, including the promoter and terminator regions, was submitted to the DDBJ/EMBL/GenBank database under accession number AB781686.

Purification and identification of Cel7A

wtCel7A was purified from a CF-2612 culture grown in the medium containing 50 g of SF/L. The culture filtrate was

desalted with buffer A. The desalted sample was applied to a Source 15Q column equilibrated with the same buffer, and the protein was eluted with a linear gradient of 0–0.25 M NaCl. The presence of Cel7A in the eluted fractions was determined by assaying for activity on *p*-nitrophenyl β-D-lactoside (PNP-Lac) and Avicel PH101. The Cel7A fractions were pooled, brought to 1.2 M $(NH_4)SO_4$, and applied to a Source 15ISO column with a 1.2–0 M $(NH_4)SO_4$ gradient in buffer B. The eluted major Cel7A fractions were pooled and subjected to affinity chromatography on a 4-aminophenyl β-D-cellobioside-conjugated Affi-Gel 10 column (Bio-Rad, Hercules, CA, USA) [1] equilibrated with buffer B containing 20 mM gluconolactone. Cel7A was eluted with buffer B containing 20 mM gluconolactone and 40 mM cellobiose. Cel7A was once again bound to the Source 15Q column equilibrated with buffer A, washed with the same buffer, and eluted with a linear gradient of 0–0.5 M NaCl. The eluted enzyme was stored in buffer B containing 0.01 % NaN_3 at 4 °C. The purity and size of Cel7A was analyzed by SDS-PAGE. The identification of Cel7A was verified by analyzing the peptide fragment masses of the purified enzyme (Stanford Mass Spectrometry Services, Stanford University, Stanford, CA, USA).

rCel7A was purified from a Y203 (YP-4 transformed with pANC203) culture grown in medium containing 20 g of SS/L. rCel7A was purified and stored by the same procedure used for wtCel7A.

Enzyme assay

Glucoamylase and α-glucosidase activities were measured using a glucoamylase/α-glucosidase assay kit (Kikkoman, Chiba, Japan), and α-amylase activity was determined using an α-amylase assay kit (Kikkoman) according to the manufacturer's instructions. One unit of glucoamylase and α-glucosidase activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol PNP per minute from PNP-β-D-maltoside (with β-glucosidase in the kit) and PNP-α-D-glucoside at 37 °C, respectively. One unit of α-amylase activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol 2-chloro-4-nitrophenol per minute from 2-chloro-4-nitrophenyl 6^5-azido-6^5-deoxy-β-maltopentaose (with β-glucosidase in the kit) at 37 °C. Glucoamylase activity was also assessed by determining the amount of glucose released from 1 % SS, 10 mM maltoheptaose, or 10 mM maltose in 50 mM sodium acetate (pH 5.0) at 37 °C. The concentration of glucose was measured by using a BF-5 biosensor with a glucose oxidase electrode (Oji Scientific Instruments, Amagasaki, Japan).

Carboxymethyl cellulase (CMCase) activity was measured by assaying the reducing sugars released after 30 min of enzyme reaction with 1 % carboxymethyl cellulose (CMC, low viscosity) at pH 5.0 and 45 °C. β-Xylanase activity was measured using 1 % (w/v) birchwood xylan using the same method as for CMCase activity. Avicelase activity was determined by analyzing the reducing sugars released after 60 min in the reaction mixture with 20 mg of Avicel PH 101/mL at pH 5.0 and 45 °C. The reducing sugars were detected by reaction with 3,5-dinitrosalicylic acid. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of reducing sugar per minute. β-Glucosidase and Cel7A activities were determined using PNP-β-D-glucoside and PNP-Lac, respectively, at pH 5.0 and 45 °C in reaction mixtures including 1 mM PNP substrate. One unit of these enzyme activities was defined as the amount of enzyme that catalyzed the formation of 1 μmol PNP per minute. Protein concentration was determined by using the BCA protein assay reagent (Pierce, Rockford, IL, USA), using bovine serum albumin as the protein standard. All measurements were performed in triplicate.

Protein thermal shift assay

The fluorescence-based thermal shift assay [20] was performed in a 96-well plate format, with a volume of 20 μL per well, using a real-time PCR detection system (CFX Connect, Bio-Rad). The protein sample was diluted to 0.1 mg/mL in 50 mM sodium acetate buffer, pH 5.0, and SYPRO orange dye [5,000-fold stock solution in dimethyl sulfoxide (DMSO); Invitrogen, Carlsbad, CA, USA] was added at fivefold final concentration. The sample was heated at 0.5 °C per 5 s from 25 to 85 °C. The fluorescence intensity (Ex/Em: 450–490 nm/560–580 nm) was measured every 0.5 °C. Protein melting temperatures (T_m) were determined by the CFX Manager Program (Bio-Rad) based on the calculation of the negative first derivative.

Results and discussion

Production of amylolytic enzymes in *A. cellulolyticus*

In a previous study, Yamanobe et al. [28] reported that *A. cellulolyticus* Y-94 displayed not only cellulolytic activity but also amylolytic activity for starch in culture grown with cellulose. We recently found that the *Acremonium* commercial cellulase preparation was highly efficient in saccharification of potato pulp starch [8]. Based on these observations, we examined the production of amylolytic enzymes (α-amylase, glucoamylase, α-glucosidase) using SS or SF as carbon source.

A. cellulolyticus Y-94 was cultured in medium containing 20 g SS or 20 g SF by adding a 5 % culture

Table 2 Enzyme production of *A. cellulolyticus* Y-94 in starch and cellulose cultures

Enzyme	Specific activity (U/mg) in		Ratio (A/B)
	SS culture (A)[a]	SF culture (B)[b]	
α-Amylase	1.143	0.036	31.8
Glucoamylase	0.865 ± 0.007	0.086 ± 0.001	10.1
α-Glucosidase	0.136	0.032	4.25
Avicelase	0.09 ± 0.015	0.77 ± 0.09	0.12
CMCase	0.32 ± 0.018	4.79 ± 0.08	0.07
Xylanase	0.79 ± 0.03	4.79 ± 0.08	0.16
β-Glucosidase	2.59	2.11 ± 0.03	1.23

[a] The enzymes were produced in a culture containing 20 g soluble starch/L as carbon source

[b] The enzymes were produced in a culture containing 20 g Solka–Flock/L as carbon source

solution grown in medium containing 20 g glycerol, which is neither an inducer nor a repressor of the production of cellulolytic enzymes in *A. cellulolyticus* [12]. Y-94 produced 1.1 ± 0.03 and 3.1 ± 0.08 g/L of extracellular protein in the SS and SF culture conditions, respectively. A small quantity of amylolytic enzymes was observed in the SF culture (Table 2), supporting previous evidence for starch degradation. Furthermore, the activities of α-amylase and glucoamylase were significant in the SS culture (Table 2). These enzymes in the SS culture exhibited 10- to 30-fold higher specific activities than those in the SF culture, indicating that they are starch-inducible enzymes. On the other hand, cellulase (Avicelase and CMCase) and xylanase were hardly detectable in the SS culture (Table 2). These results suggest that *A. cellulolyticus* has different inducible systems for the production of cellulolytic and amylolytic enzymes. Therefore, a starch-inducible homologous expression system is expected to be effective in producing a recombinant cellulolytic enzyme, reducing the contamination from other cellulolytic enzymes induced in the SF culture. Interestingly, the same levels of β-glucosidase activity were observed in both cultures. β-Glucosidase plays an important role in cellulose degradation to produce glucose from cellobiose; however, its role in starch metabolism remains unknown.

Purification and identification of *A. cellulolyticus* glucoamylase

To obtain a strong promoter suitable for use in a starch-inducible protein expression system, we identified the major protein induced in SS culture. SDS-PAGE analysis of the SS culture supernatant from Y-94 revealed the presence of a major protein with apparent molecular mass of 68 kDa (Fig. 2, lane 1). This protein was considered a glucoamylase based on preliminary analyses (SDS-PAGE and enzyme assay) of the fractions separated by anion-exchange chromatography (data not shown), and was purified to homogeneity by further chromatography (Fig. 2, lane 2). The specific activity of the purified enzyme was 2.59 and 0.21 U/mg for PNP-β-D-maltoside and PNP-α-D-glucoside, respectively. Amylase activity using 2-chloro-4-nitrophenyl 6^5-azido-6^5-deoxy-β-maltopentaose was not detected for the purified enzyme. Glucoamylase (α-1,4-glucan glucohydrolase, EC 3.2.1.3) catalyzes hydrolysis of terminal α-1,4-linked D-glucose residues successively from the nonreducing ends of amylose chains with release of D-glucose. The production rates of glucose from SS (45.8 ± 0.39 μmol glucose min^{-1} mg^{-1} protein) and maltoheptaose (46.0 ± 0.33 μmol glucose min^{-1} mg^{-1} protein) were nearly identical, whereas the production rate from maltose (8.56 ± 0.10 μmol glucose min^{-1} mg^{-1} protein) was significantly lower, indicating that cleavage of the α-1,4 glycoside bond in maltose was 10-fold lower than in SS and maltoheptaose. This demonstrates that the purified enzyme is a glucoamylase, preferring the increased degree of polymerization of malto-oligosaccharides as substrates, rather than α-glucosidase [31].

A gene encoding the glucoamylase was discovered by searching the *A. cellulolyticus* genomic sequence for the internal amino acid (aa) residues TYADGYMSIAQT of the purified glucoamylase. The glucoamylase coding region (*glaA*) in the chromosome included three introns

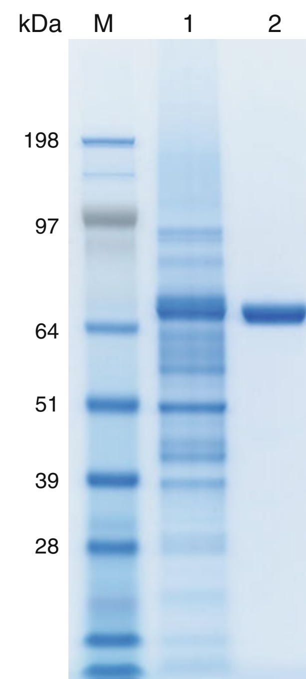

Fig. 2 SDS-PAGE analysis of glucoamylase from *A. cellulolyticus* Y-94. *Lanes*: *M* protein marker, *1* culture supernatant of Y-94 grown in SS culture, *2* purified glucoamylase

(AB781686). The deduced open reading frame was 1,924 bp and encoded 638 aa. A BLAST search (blastp) and Conserved Domain Database search in the National Center for Biotechnology Information (NCBI) revealed that GlaA consists of an N-terminal domain of glycoside hydrolase family 15 (GH15) and a C-terminal carbohydrate-binding module 20 (CBM20) connected by a Ser/Thr-rich linker region. GlaA exhibited high similarities with ascomycete GH15 glucoamylases, sharing the same domain architecture, with 66 % identity to *Aspergillus awamori* (640 aa, 1508161A) and 62 % identity to *Rasamsonia emersonii* (618 aa, CAC28076).

Homologous expression of recombinant Cel7A in *A. cellulolyticus*

The promoter and terminator regions of starch-inducible *glaA* were isolated and used to construct a new expression plasmid. The resultant plasmid pANC202 was designed to enable the expression of various cellulolytic enzymes under the control of the *glaA* promoter together with *pyrF* as a selection marker (Fig. 1). The various gene cassettes can be inserted into two specific cloning sites, namely *Eco*RV (blunt-end restriction site) and *Sbf*I (8-base restriction site), in pANC202. In addition, the new host strain YP-4, which is a uracil autotroph mutant, was isolated from ultraviolet (UV)-irradiated protoplasts of wild-type *A. cellulolyticus* Y-94. The transformation efficiency of YP-4 was increased more than 20-fold (18.7 ± 3.8 transformants μg^{-1} pANC202), when compared with that of Y-94 using *hph* as the selective marker described previously [14]. This increased transformation efficiency is beneficial; because of the variability that occurs when using nonhomologous integration of the target gene, it is necessary to screen a number of transformants to identify clones with high expression levels.

Most cellulolytic enzymes secreted by fungi are glycoproteins. Cel7A, which is the most abundant and important enzyme in industrially relevant fungal cellulolytic systems, has both *O*- and *N*-linked glycosylation sites, and its successful heterologous expression has been limited due to stringent co- and posttranslational requirements, including the formation of multiple disulfide bridges and native-like glycosylation [2]. Therefore, we selected *A. cellulolyticus* Cel7A as a model protein to verify successful homologous expression using the pANC202 expression system.

A Y203 transformant harboring pANC203 has enabled the homologous expression of extracellular rCel7A under the 20 g SS/L culture condition, during which endogenous Cel7A is hardly produced. A 60-kDa protein was detected by SDS-PAGE analysis of the culture supernatant from Y203 (Fig. 3, lane 2). The protein band of similar size was also detected in the culture supernatant from Y202, a

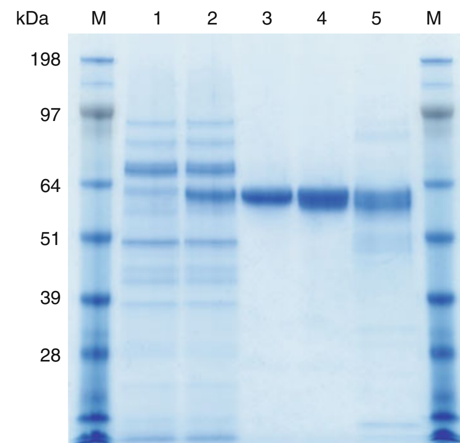

Fig. 3 SDS-PAGE analysis of Cel7A. *Lanes*: *M* protein marker, *1* culture supernatant of *A. cellulolyticus* Y202 grown in SS culture (control), *2* culture supernatant of *A. cellulolyticus* Y203 grown in SS culture, *3* rCel7A purified from Y203, *4* wtCel7A purified from *A. cellulolyticus* CF-2612 grown in SF culture, *5* culture supernatant of *A. cellulolyticus* CF-2612 grown in SF culture

transformant harboring the pANC202 control plasmid lacking the *cbh1* gene sequence (Fig. 3, lane 1). The Cel7A activities in the supernatant of Y203 and Y202 were 35.9 and 2.1 mU/mg, respectively, using PNP-Lac as substrate, strongly suggesting that the protein expressed in Y203 but not Y202 was rCel7A. The specific activities of amylolytic enzymes were similar between Y203 and Y202 regardless of the overexpression of rCel7A (data not shown). This observation implies that the production of amylolytic enzymes is not negatively influenced by the introduction of additional glucoamylase promoters.

The culture supernatants of *A. cellulolyticus* in the SF and SS culture conditions showed different expression patterns on SDS-PAGE (Fig. 3, lanes 1, 2, 5). The protein corresponding to glucoamylase (68 kDa) was not observed in the SF culture supernatant. The 60-kDa protein, a major band in the SF culture supernatant, was expected to be wtCel7A; however, it should be noted that other cellulolytic enzymes having similar activity and molecular weights are also produced in cellulose culture of *A. cellulolyticus* [29, 30].

Characterization of recombinant and wild-type Cel7A

wtCel7A was purified to an electrophoretically homogeneous form from the cellulose culture of *A. cellulolyticus* CF-2612. Cellobiose-conjugated affinity chromatography was found to be highly effective in removing β-glucosidase activity in the Cel7A fraction. Peptide fragment masses from purified wtCel7A were assigned to the primary structure of *A. cellulolyticus* Cel7A (Table 3), corresponding to 18 % amino acid sequence coverage for Cel7A. Although *A. cellulolyticus* Cel7A has been

identified as the major protein in cellulose culture [21], to our knowledge, this is the first example of Cel7A purified from *A. cellulolyticus*. The purified wtCel7A displayed maximum activity at pH 4.5 and fourfold higher performance on PNP-Lac than on PNP-β-D-cellobioside (PNP-Cel) (Table 4). No activity was detected toward CMC and PNP-β-D-glucoside (data not shown).

The purification of rCel7A was achieved using the same chromatography techniques. The yield of purified rCel7A was approximately 20 mg/L in the Y203 culture supernatant; this will be sufficient for detailed structural and kinetic studies in the future. The apparent molecular masses of rCel7A and wtCel7A on SDS-PAGE gel were nearly identical at about 60 kDa (Fig. 3, lanes 3, 4), and their SDS-PAGE bands seemed to be broader. The molecular weight of the purified proteins was higher than the calculated molecular mass (54,962 kDa) of Cel7A. These observations suggest that the molecular weights of the purified proteins were increased due to glycosylation. The band of wtCel7A appears more diffuse than that of rCel7A, probably due to increased microheterogeneity of glycosylation resulting from inherent differences in culture conditions, i.e., carbon source and urea concentration [5].

Table 3 Identification of peptides in purified *A. cellulolyticus* Cel7A

Residues	MS/MS peptide sequence
46–64	K.SGGSCTTNSGAITLDANWR.W
117–129	R.LNFVTGSNVGSR.T
185–200	K.AGAQYGVGYCDSQCPR.D
274–289	R.YAGTCDPDGCDFNPYR.L
290–300	R.LGVTDFYGSGK.T
331–345	R.YYVQNGVVIPQPSSK.I

Table 4 Physicochemical and enzymatic properties of Cel7A from *A. cellulolyticus*

Property	rCel7A	wtCel7A
Molecular weight (kDa)[a]	60	60
Thermostability (°C)[b]	67.0	67.0
Optimum pH[c]	4.5	4.5
Substrate specificity		
Avicel (U/mg)	0.222 ± 0.013	0.204 ± 0.008
PNP-Cel (U/mg)	0.045 ± 0.001	0.047
PNP-Lac (U/mg)	0.188 ± 0.001	0.186 ± 0.003

[a] The apparent molecular weight of the protein was determined by SDS-PAGE

[b] Thermostability was determined by thermal shift assay as described in "Materials and methods"

[c] Optimum pH of enzyme activity was determined using PNP-Lac as substrate in McIlvaine's buffer (pH 3.0–6.0)

A. cellulolyticus Y-94 produced a large amount of extracellular proteins in the SF culture rather than in the SS culture. The differences in the protein expression level between the SF and SS cultures may also contribute to the microheterogeneity of glycosylation. These conditions should not be confused with the far more dramatic hyperglycosylation which occurs in heterologous expression.

rCel7A and wtCel7A had identical protein melting temperature and optimum pH, and highly similar substrate specificity toward soluble and insoluble substrates (Table 4). These results demonstrate that both proteins undergo similar posttranslational events and protein folding during the secretion process in the SS and SF culture conditions, and are for all practical purposes, identical.

The starch-inducible homologous expression system designed in this study will enable the construction and evaluation of various *A. cellulolyticus* cellulolytic enzymes: not only Cel7A but also CBHII, endo-1,4-β-glucanase, and endo-1,4-β-xylanase, including mutant forms of these proteins and other putative enzymes of interest encoded in the genome. Cellulolytic enzymes produced in SS culture allow for much easier detection and purification, due to the clean cellulase background resulting from SS culture. Refinements to the designs of homologous expression systems for these enzymes are ongoing. Recently, analysis of transcripts from *A. cellulolyticus* has shown high similarity with putative genes from the genomic sequences of *Talaromyces marneffei* and *Talaromyces stipitatus* [12]. Some species of *Talaromyces* are known to produce highly effective cellulases [10, 27], thus the *A. cellulolyticus* protein expression system could also be applied for heterologous expression of cellulolytic enzymes from *Talaromyces* or other species of interest.

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