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Cloning, Heterologous Expression, and Characterization of *Thielavia terrestris* Glucoamylase

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Abstract

Thielavia terrestris is a soil-borne thermophilic fungus whose molecular/cellular biology is poorly understood. Only a few genes have been cloned from the *Thielavia* genus. We detected an extracellular glucoamylase in culture filtrates of *T. terrestris* and cloned the corresponding *glaA* gene. The coding region contains five introns. Based on the amino acid sequence, the glucoamylase was 65% identical to *Neurospora crassa* glucoamylase. Sequence comparisons suggested that the enzyme belongs to the glycosyl hydrolase family 15. The *T. terrestris glaA* gene was expressed in *Aspergillus oryzae* under the control of an *A. oryzae* α-amylase promoter and an *Aspergillus niger* glucoamylase terminator. The 75-kDa recombinant glucoamylase showed a specific activity of 2.8 μmol/(min·mg) with maltose as substrate. With maltotriose as a substrate, the enzyme had an optimum pH of 4.0 and an optimum temperature of 60°C. The enzyme was stable at 60°C for 30 min. The K_m and k_{cat} of the enzyme for maltotriose were determined at various pHs and temperatures. At 20°C and pH 4.0, the enzyme had a K_m of 0.33 ± 0.07 m*M* and a k_{cat} of (5.5 ± 0.5) × 10^3 min^{-1} for maltotriose. The temperature dependence of k_{cat}/K_m indicated an activation free energy of 2.8 kJ/mol across the range of 20–70°C. Overall, the enzyme derived from the thermophilic fungus exhibited properties comparable with that of its homolog derived from mesophilic fungi.

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Index Entries: Glucoamylase; sequence; enzymology; expression; *Thielavia terrestris*.

Introduction

Thielavia terrestris (synonym, *Allescheria terrestris*; anamorph, *Acremonium alabamense*) is a soil-borne thermophilic ascomycete that can grow at relatively low pH (e.g., pH 4.5) and elevated temperature (40–45°C) *(1)*. This fungus has a wide geographic distribution and a homothallic mating behavior *(2)*. Thermophilic fungi such as *T. terrestris* constitute a rich source of thermostable enzymes, and thermal tolerance is a beneficial attribute for many industrial enzyme applications *(3)*.

T. terrestris has been listed as a safe microorganism for food enzyme production by the Association of Manufacturers of Fermentation Enzyme Products, and it is a noted source of commercial cellulase. Additionally, thermostable xylanase, β-glucanase, β-glucosidase, mannanase, mannosidase, arabinase, and galactanase activities have been detected in culture filtrates of *T. terrestris (4–17)*. There are no published reports of starch- or maltooligosaccharide-hydrolyzing enzymes from *T. terrestris*. Given the previously described thermostable enzymes from *T. terrestris*, we hypothesized that it also might be a suitable source of thermally tolerant catalysts for starch hydrolysis.

The molecular/cellular biology of *Thielavia* is poorly understood, with only a few genes being cloned from the entire *Thielavia* genus. There is significant interest in, for the field of applied enzymology and biotechnology, exploring the potential of *T. terrestris* as a safe source for industrial enzymes, as well as in evaluating *T. terrestris* enzymes as thermally tolerant industrial biocatalysts. We report here the first systematic characterization of a *T. terrestris* gene that encodes an extracellular glucoamylase (GA). We cloned the gene, determined its structure, analyzed its regulatory sequence, expressed it in a heterologous *Aspergillus oryzae* host, and studied the biochemical and catalytic properties of the recombinant enzyme.

Materials and Methods

Chemicals and Equipment

Chemicals used as buffers and substrates were commercial products of at least reagent grade. Britton and Robinson (B&R) buffer (pH 2.7–11.0, made by mixing 0.1 *M* boric acid–0.1 *M* acetic acid–0.1 *M* phosphoric acid with 0.5 *M* NaOH to the desired pH) was used unless indicated otherwise. The protocols for molecular biology experiments were adapted from either the manufacturer's instructions or standard procedures *(18)*. DNA sequences were determined using Prizm dye terminator chemistry on an Applied Biosystems 377 DNA sequencer (Foster City, CA). A primer walking strategy was used to generate the nucleotide sequence of the entire *glaA* gene.

N-terminal amino acid sequencing was done on an Applied Biosystems 476A Protein Sequencer (Foster City) following sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotting onto polyvinyl difluoride membranes. Spectral data were recorded on either a spectrophotometer (Shimadzu UV160U; Columbia, MD) with a 1-cm quartz cuvet or a microplate reader (Molecular Devices Thermomax; Sunnyvale, CA) with 96-well microplates. Differential scanning calorimetry (DSC) was performed on a MicroCal VP-DSC instrument (Northhampton, MA). Chromatography was made in a Pharmacia FPLC (Piscataway, NJ). Carbohydrate analysis was performed on a Dionex CarboPac PA1 column (Sunnyvale, CA) connected to a Beckman Gold high-performance liquid chromatograph (Fullerton, CA), equipped with a pulsed amperometric detector, eluted by 0–0.6 *M* Na acetate in 0.1 *M* NaOH.

Enzymic Assays

After considering the sensitivity and background reaction, we chose maltotriose as the substrate for GA and a Sigma GO kit (St. Louis, MO) to assay the glucose-releasing activity of GA. The parameters K_m and k_{cat} were obtained by nonlinear regression using the GraphPad Prism software (San Diego, CA). A molar absorptivity of 124 mM^{-1}cm^{-1} at 280 nm was used to calculate *T. terrestris* GA (tGA) concentration.

The pH-activity profile was measured as follows: ten microliters of 0.11 g/L or 0.18 *M* tGA, 10 µL of 0.63 to 100 m*M* maltotriose (to cover from the initial to the saturating phase at different pHs), and 5 µL of B&R buffer were preincubated for 3 min. Then 50 µL of GO assay reagent and 25 µL of H_2O were added, and the absorbance change was monitored at 490 nm (the available wavelength on the microplate reader closest to the 540 nm suggested by Sigma for its GO kit).

The thermal profile was measured at 4, 22, 30, 37, 50, 60, 70, and 80°C. First, 100 µL of 10 m*M* maltotriose, 50 µL of B&R buffer (pH 4.0), and 95 µL of H_2O were preincubated at selected temperatures. After 30 min, 5 µL of 0.11 g/L tGA was added. After a 20-min reaction, the solutions were chilled in ice water and briefly centrifuged. A 25-µL aliquot was subjected to GA assay by mixing with 50 µL of GO reagent and 25 µL of H_2O.

To measure thermal stability, 10 µL of 0.11 g/L tGA and 5 µL of B&R buffer, pH 4.0, were incubated at 4, 22, 30, 37, 50, 60, 70, and 80°C, respectively, for 30 min. The solutions were then chilled in ice water and briefly centrifuged before being transferred to a 96-well microplate for GA activity assay. One assay was made by adding simultaneously 10 µL of 10 m*M* maltotriose, 50 µL of GO assay reagent, and 25 µL of H_2O with the tGA solution to start the reactions, whereas another assay was made by preincubating the tGA solution with the maltotriose stock for 3 min before adding the GO/H_2O and monitoring. To measure the thermal transition by DSC, 2.2 mg of tGA in 0.5 mL of 50 m*M* Na acetate, pH 4.5, was scanned from 20 to 110°C at a scan rate of 90°C/h.

Screening of tGA-producing transformants was done by culturing the strains in 24-well plates followed by measuring GA activity with a *p*-nitrophenyl-glucoside substrate. For this assay, 100 µL of 50 m*M* Na acetate, pH 4.3, containing 1 g/L of substrate was added to 50 µL of supernatant from each transformant and incubated at 37°C for 30 min. The reaction was terminated by adding 150 µL of 1 *M* Tris, pH 8.0, and the absorbance at 405 nm was measured.

Saccharification

Saccharification substrate was made by dissolving 30% maltodextrin in boiling water and then adjusting the pH to 4.5 at 60°C. Aliquots of substrate corresponding to 15 g of dry solids were transferred to 50-mL glass flasks; mixed with 0.06–0.12 mg/g of GA; and incubated at pH 4.5 and 60, 65, or 70°C. Sugar was analyzed by first inactivating GA in boiling water for 15 min, the mixing with Bio-Rad AG 501/X8(D) (Hercules, CA) resin for 30 min, filtering on a 0.2-µm filter, and analyzing on a high-performance liquid chromatograph.

Fermentation

Spores of *T. terrestris* strain E373 (Novozymes) were germinated in a 125-mL shake flask at pH 4.2 and 45°C in 25 mL of MY50 medium consisting of 5% maltodextrin with 2 g/L of $MgSO_4 \cdot 7H_2O$, 10 g/L of KH_2PO_4, 2 g/L of K_2SO_4, 0.5 g/L of $CaCl_2 \cdot 2H_2O$, 2 g/L of citric acid, and 10 g/L of yeast extract. The *A. oryzae* transformants harboring *T. terrestris glaA* gene were grown in a 2-L fermentor in a fed-batch mode with maltodextrin, urea, and yeast extract at pH 7.0 and 34°C for 188 h.

Cloning and Expression

Genomic DNA was partially digested with *Tsp*509I (New England Biolabs, Beverly, MA) and electrophorized on a 1% agarose gel. Fragments migrating between 3 and 7 kb were excised from the gel, purified using β-agarase (New England Biolabs), and ligated with λZiplox *Eco*RI arms (Gibco-BRL, Carlsbad, CA). The ligation products were packaged in bacteriophage λ particles using the Gigapack Gold kit from Stratagene (La Jolla, CA).

The polymerase chain reaction (PCR) primers 5'atgATGCGCCGT CTTCAGCTCTTG 3' and 5'gggttaattaaTTACTGCCAGGTATC3' (capital letters correspond to *glaA* coding sequences) were employed to amplify the full-length *glaA* gene from a genomic clone and incorporate a 5' ATG start codon plus a 3' *PacI* site following the stop codon. The amplified 2.2-kb *glaA* fragment was digested with *PacI* and subcloned into plasmid pBANe15 (from Beth A. Nelson of Novozymes Biotech). The resulting plasmid, pEJG20, contained an *A. oryzae* α-amylase promoter, the *T. terrestris glaA* coding region, an *A. niger* GA terminator, and the *Aspergillus nidulans amdS* selectable marker (Fig. 1). It was used to transform an *A. oryzae* recipient strain, and 29 transformants were selected on COVE medium using aceta-

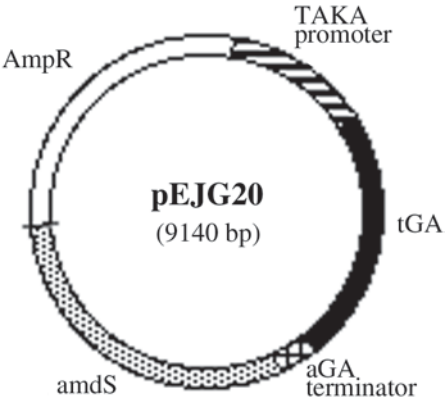

Fig. 1. Expression plasmid pEJG20 for tGA. Details regarding construction of the vector are outlined in the text. DNA segments include an *A. oryzae* α-amylase (TAKA) promoter, a *T. terrestris glaA* coding region, an *A. niger glaA* terminator, and an *A. nidulans amdS* gene selectable marker.

mide as the sole nitrogen source. Each transformant was subsequently transferred to a fresh COVE plate, and spores were obtained to inoculate 1 mL of *Aspergillus* minimal medium *(19)* in 24-well plates to grow at 34°C. Negative controls included the untransformed host and a GA-deficient *A. oryzae* strain. Aliquots of the culture medium were removed after 4, 6, and 8 d and assayed for GA activity. Genomic DNA was prepared from the GA-positive transformants, and the presence of the *glaA* gene was verified by a PCR. The highest-producing transformant was then grown in a 2-L fermentor.

Purification

Recombinant tGA was purified, with a final recovery yield of 44% and an 89-fold purification, by sequential ultrafiltration, anion-exchange, cation-exchange, and hydrophobic interaction chromatography. The cell-free broth was washed and concentrated using an Amicon Spiral-Concentrator (S1Y10 membrane cartridge; Bedford, MA). The majority of tGA did not bind to Q- and SP-Sepharose preequilibrated with 10 m*M* Tris-HCl, pH 7.5, but was separated from other components that were adsorbed. Pure tGA, with apparent homogeneity on SDS-PAGE, was obtained by the Phenyl Sepharose step (column preequilibrated with 50 m*M* Na phosphate, pH 7.0, plus 1.7 *M* $[NH_4]_2SO_4$; elution with a 1.7–0 *M* $[NH_4]_2SO_4$ gradient in 50 m*M* Na phosphate, pH 7.0). The purified tGA was dialyzed against 10 m*M* Na phosphate, pH 7.0.

Results

Detection of tGA and Cloning of Its Gene

When *T. terrestris* was grown in MY50 medium with 5% maltodextrin as the carbon source at pH 4.2, a very low level of proteins was secreted into

the culture medium. After concentrating the broth, two proteins were detected by SDS-PAGE: one had a molecular mass of 20,000 and an N-terminus of TTVSYDTGYDDPSRPLTSVA, which had a low homology to a *Coccidioides immitis* serine protease *(20)*; and the other had a molecular mass of 70,000 and an N-terminus of SVDSFIATESPIALSNLLXNIGST, homologous to *Neurospora crassa* GA *(21)*.

Based on the N-terminal sequence of the 70,000 protein and the conserved sequence segments found among various fungal GAs, we designed PCR primers and successfully amplified a 0.2-kb segment of genomic DNA from *T. terrestris*. Subsequent nucleotide sequence analysis of this segment suggested that it encoded a portion of the *T. terrestris* glucoamylase gene. A Northern blot using total RNA from maltodextrin-grown cells was probed with this PCR fragment, and a hybridization signal was detected corresponding to a 2.37-kb RNA. Similarly, when *T. terrestris* genomic DNA was digested with *Apa*I and analyzed in Southern-blotting experiments, a 3.0-kb DNA fragment was observed. Similar blots employing additional restriction enzymes suggested that a single copy of the *glaA* gene was present in the *T. terrestris* genome. The complete *glaA* gene was cloned from a *T. terrestris* genomic library. Several positive clones were picked and purified through single plaques. Preliminary DNA sequencing results indicated that one clone contained the entire *glaA* coding region, and it was subsequently excised from the λZipLox vector as a pZL1 derivative *(22)*. Figure 2 shows the nucleotide and deduced amino acid sequences derived from this isolate.

Structure of T. terrestris *glaA Gene*

As shown in Fig. 2, five introns were identified within the coding region of *glaA* based on the consensus rules for intron features in filamentous fungi *(23)*. In each of these introns, the consensus for the 5' splice donor site is GTANGY, whereas the consensus for the 3' splice acceptor site is CAG. The exons encoding tGA are significantly more GC rich than the introns (66% G+C vs 57%). The pattern of codon usage within the *glaA* gene suggested a marked bias for codons ending in C. Among the four codon families, codons ending in G are preferred over those ending in T when C is not used. For the two codon families not using C, codons ending in G are preferred. Codons ending in A were seldom used.

The 5'-untranslated region of the *T. terrestris glaA* gene contains a putative TATA-box (TATAA) 96 bp upstream of the start codon. In addition, a 12-bp palindrome, 5'GCGGGGCCCCGC3', was found 201 bp upstream of the ATG. Within the palindrome lies a consensus binding site (SYGGGG) for the glucose-responsive repressor protein *CreA (24)*. A few base pairs upstream of the palindrome, the sequence 5'TCGGTCCTTTATCCC3' was observed. This sequence is very similar (12 of 15 bp) to a regulatory motif known as region IIIa found in the promoters of several starch-induced genes in *Aspergillus (25)*. Further analysis will be required to demonstrate the function of these motifs in the *T. terrestris glaA* gene.

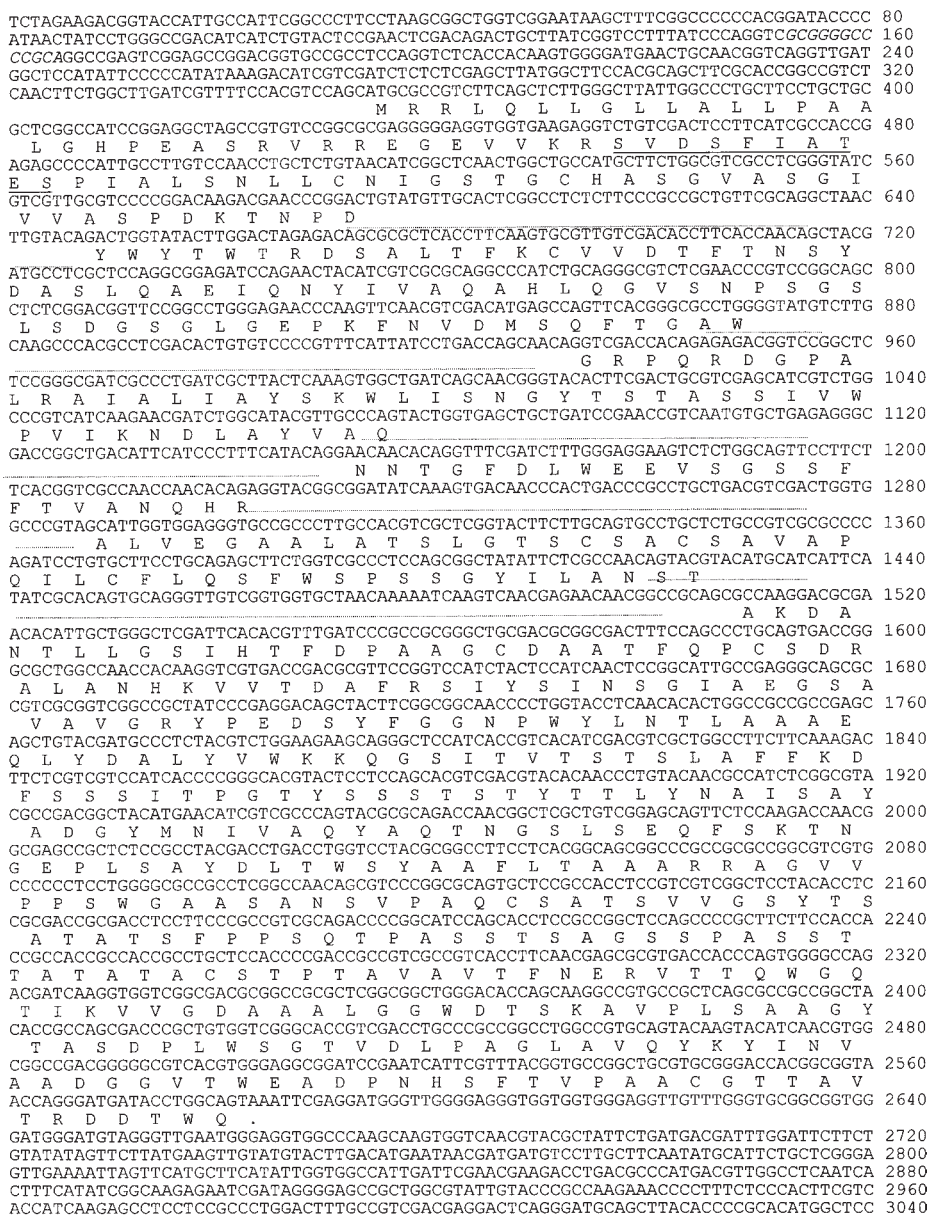

Fig. 2. Nucleotide sequence of the cloned tGA gene (Genbank accession no. AAE85601) and its deduced amino acid sequence. The result from the protein sequencing is underlined. The palindrome 5'GCGGGGCCCCGC and the flanking 5'CCGCA3' are in italics.

The open reading frame of the *glaA* gene encoded a primary translation product of 630 amino acids, which had a 64.5% identity and an 86.5% similarity to *N. crassa* GA. Signal peptide prediction software *(26)* sug-

gested that the first 18 amino acids of the gene product comprise a secretory signal peptide. The next 18 residues likely encompass a propeptide whose proteolytic removal (after a KEX2-like KR- sequence) would yield a mature tGA of 596 amino acids. By analogy with other fungal GAs *(27,28)*, a putative starch-binding domain (Q540 to Q630), a catalytic domain (S37 to A491), and an S/T-rich linker (T492 to T539) were identified (Fig. 2). The catalytic domain belonged to the glycosyl hydrolase family 15, which includes most fungal GAs *(29)*. Corresponding to the catalytically active D176/E179/E180 of *A. niger* GA (aGA) *(30,31)*, the residues D208/E211/E212 most likely constituted the catalytic triad of tGA.

Heterologous Expression of tGA in A. oryzae

Because the *A. oryzae* host produced an endogenous GA, the presence of *T. terrestris glaA* gene in the genome of the transformants was verified by PCR. The highest GA activity among the *A. oryzae* transformants secreting tGA was about five times that of the untransformed control strain, corresponding to an expression of approx 50 mg/L.

Characterization of Recombinant tGA

On SDS-PAGE, the purified recombinant tGA showed a mass of approx 75,000, higher than the 62,000 predicted by the DNA sequence. Since there are five potential sites for N-linked carbohydrate addition in tGA, this observed difference was likely owing to glycosylation. The purified tGA had an N-terminus of SVDSFIATESPIALSNLLXNIGST, which matched 100% to the sequence predicted from the *glaA* gene, suggesting a correct posttranslational processing by the host.

At pH 3.0–7.0, tGA showed initial rates dependent on concentration [maltotriose]. The rate exhibited a Michaelis pattern when [maltotriose] ≤ 2 m*M*. Above 2 m*M*, a reduction in rate was observed, indicating a substrate inhibition on tGA (Fig. 3). Figure 4A shows the pH-activity profiles corresponding to 0.25 and 0.50 m*M* maltotriose, a concentration range where the rate dependence on [maltotriose] was linear. Both profiles showed an optimal pH (pH_{opt}) of 4.0. The K_m and k_{cat} were calculated over the [maltotriose] ranges where no substrate inhibition took place. The pH dependence of K_m, k_{cat}, and k_{cat}/K_m is shown in Fig. 4B,C. Although k_{cat}/K_m became optimal at pH 4.0, k_{cat} reached the highest value at pH 5.0.

The thermal stability of tGA is illustrated in Fig. 5. The two experiments (*see* Materials and Methods) produced very similar profiles. Under the assay conditions employed, tGA appeared stable at temperatures as high as 60°C. At 70°C, tGA retained 60–70% of its original activity after 30 min of incubation. In its DSC thermogram, a phase transition around 71°C was observed for tGA. Because of the precipitation of tGA (exothermic transition following the endothermic transition derived from the thermal unfolding of tGA), the exact phase-transition (protein unfolding) temperature could not accurately be determined but was estimated to lie

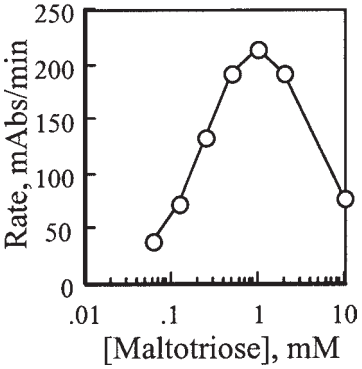

Fig. 3. Activity dependence on substrate concentration at pH 4.0. The rate unit was 0.001 absorbance/min.

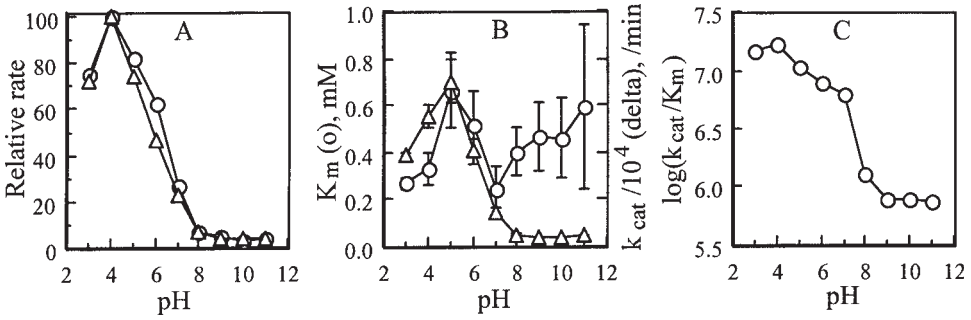

Fig. 4. Dependence on pH. **(A)** Initial rate. Initial [maltotriose]: (○) 0.25 m*M*; (△) 0.5 m*M*. **(B)** K_m (○) and k_{cat} (△). **(C)** log(k_{cat}/K_m). k_{cat}/K_m unit: $M^{-1}min^{-1}$.

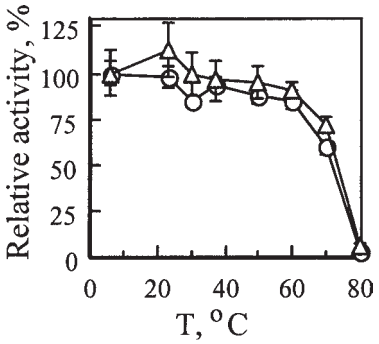

Fig. 5. Thermal stability. Residual activity was assayed after heat-treated tGA was mixed with maltotriose for 0 (△) or 3 min (○).

between 69 and 72°C. Figure 6 shows the slightly superior thermal stability of tGA at 70°C in comparison with aGA.

At high temperatures, tGA exhibited a Michaelis-type rate dependence on [substrate] (Fig. 7A). Figure 7B shows the profile of the initial rate

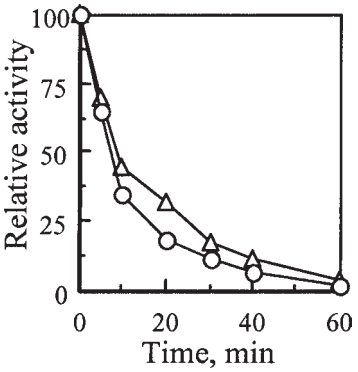

Fig. 6. Thermal stability of tGA (△) and aGA (○) at pH 4.3 and 70°C.

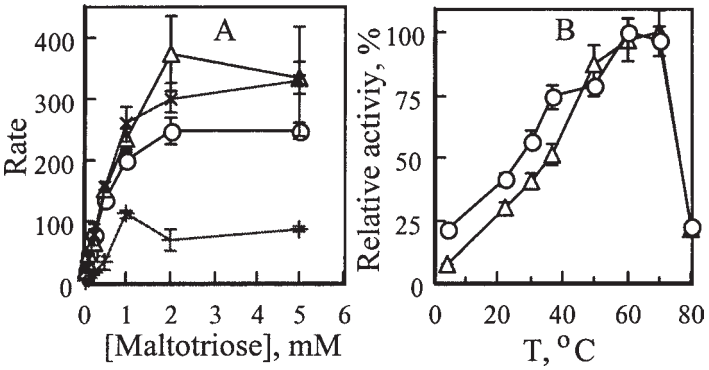

Fig. 7. Thermal activity. **(A)** Rate profiles at 50 (○), 60 (△), 70 (×), and 80°C (+). **(B)** Relative activity for 0.25 (○) and 0.5 m*M* (△) maltotriose. [tGA] = 1.1 mg/L.

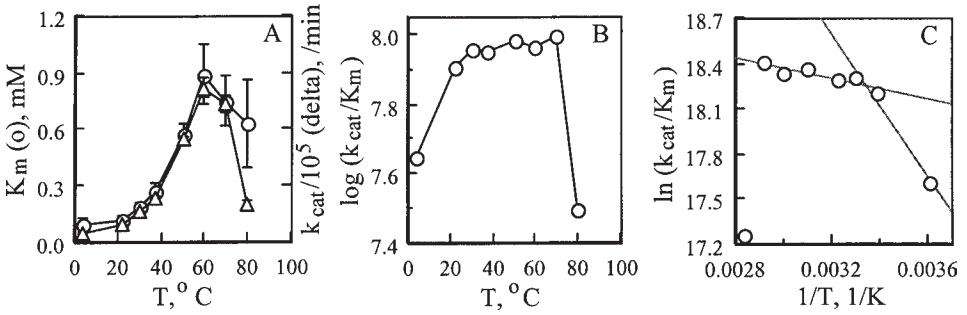

Fig. 8. Thermal profiles. **(A)** K_m (○), k_{cat} (△); **(B)** $\log(k_{cat}/K_m)$ (unit: $M^{-1}\text{min}^{-1}$); **(C)** $\ln(k_{cat}/K_m)$ (T unit: K). Correlation lines: $\ln(k_{cat}/K_m) = 26 - 2360/T$ (for 4–30°C); $\ln(k_{cat}/K_m) = 19 - 342/T$ (for 22–70°C).

when [maltotriose] = 0.25 and 0.50 m*M*. A temperature optimum between 60 and 70°C was observed. Figure 8 shows the temperature dependence of K_m and k_{cat} extracted from the data obtained at [maltotriose] ≤ 5 m*M*. Both

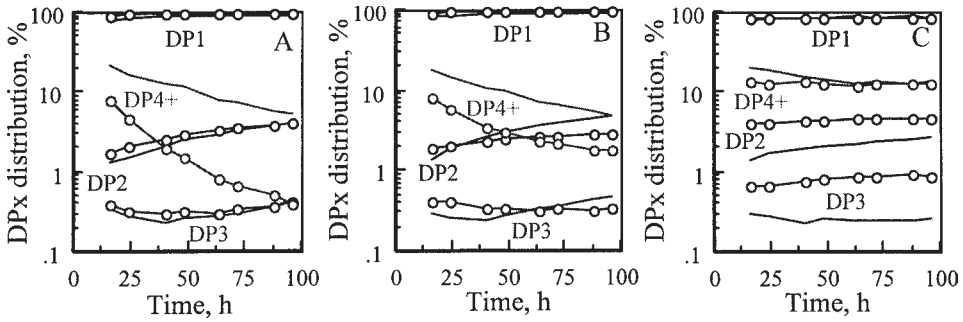

Fig. 9. Maltodextrin saccharification. Temperature tested: **(A)** 60°C, **(B)** 65°C, and **(C)** 70°C. Enzyme tested: (——) tGA; (—○—) aGA. Hydrolyzed product: DP1, glucose; DP2, maltose; DP3, maltotriose; DP4+, maltotetraose and larger maltooligomers.

parameters had a maximum at 60°C. The dependence of $\log(k_{cat}/K_m)$ on the inverse of temperature over the range of 30–70°C suggested an apparent activation free energy ($\Delta G^{\neq}$) of 2.8 kJ/mol.

Saccharification by tGA

Figure 9 shows the hydrolysis of maltodextrin by tGA in comparison with aGA. Recombinant tGA yielded 90% glucose (DP1) after 48 h, similar to that found with aGA. The performance of aGA generally declined with temperature, showing increased levels of maltotetraose or larger maltooligomers ($DP4^+$) and decreased glucose levels. Using tGA, more $DP4^+$ was hydrolyzed at 65°C compared with 60°C, but the corresponding increased product was mainly maltose (DP2), not glucose. The level of isomaltose formed by tGA hydrolysis (62% of total DP2 at 60°C) was higher than that found with aGA (55%). This difference increased when the temperature reached 70°C (50 vs 6% of total DP2).

Discussion

To our knowledge, this is the first report describing the characterization of a *Thielavia* enzyme gene combined with its heterologous expression and biochemical analysis of the gene product. Although *T. terrestris* has been tested as a host for heterologous enzyme production *(1)*, there is a paucity of information regarding gene structure, expression, and regulation in this species. A recent literature and database search yielded gene sequence information on just six DNA sequences from this organism: a partial xylanase gene *(32)*, two partial ribosomal RNA genes (*33*; Cano, J. F., EMBL accession no. AJ271589, 2000), a cellobiose dehydrogenase gene (Subramaniam, S. S., Nagalla, S. R., and Renganathan, V., EMBL accession no. AF074951, 1998), a decarboxylase gene, and an endoglucanase gene *(35)*. The present article discloses the molecular characterization of a gene encoding a secreted GA from *T. terrestris*. The deduced amino acid sequence of tGA indicated its overall architecture belonging to family 15 of

Table 1
Comparison Between tGA and Other Fungal GA[a]

Source	pH_{opt}	K_m (m*M*)	k_{cat} (min^{-1})	T_{opt} (°C)	Reference
Aspergillus hennebergi	5.0			70	*39*
Aspergillus awamori	4.0	0.73	3700		*46*
Aspergillus niger	3.5–5.0			60–65	*40*
Aspergillus spp. K-27	4.5	0.45	3900		*41*
Chalara paradoxa	5.0–6.0			50	*45*
Humicola resinae				55–60	*43*
Hormoconis resinae	4.0				*43*
Humicola grisea	6.0			60	*37*
Thielavia terrestris	4.0	0.33	5500	60–70	This work
Trichoderma reesei	5.5	0.5	1200	70	*44*

[a]Optimal pH (pH_{opt}) was measured at ambient temperature; K_m and k_{cat} were measured at pH_{opt} and ambient temperature, with maltotriose as substrate; and optimal temperature (T_{opt}) was measured at pH_{opt}, with maltotriose as substrate.

glycosyl hydrolases *(29,36)*. A cursory analysis of the 5'-untranslated region of the *T. terrestris glaA* gene suggests that it may be subject to regulation by carbon source in a manner similar to some starch/maltose-induced genes from other filamentous fungi.

Native tGA was secreted only at a very low level by *T. terrestris*, making its purification difficult. However, the *T. terrestris glaA* gene could be effectively expressed in *A. oryzae*. The recombinant tGA possessed properties comparable with other fungal GAs. Although few reports are available regarding GAs from thermophilic fungi *(37)*, our data indicate that cloning and expression of a GA gene from a thermophilic fungus does not necessarily yield an enzyme thermally much more stable/active than its counterparts from mesophilic organisms. Nevertheless, several noticeable differences were found between tGA and other GAs in terms of catalytic parameters and pH/thermal dependence (Table 1). At pH 4.0, tGA had a thermal stability (60% activity retention after incubation at 70°C for 30 min) that appeared comparable with that of *A. terreus* GA *(38)* and greater than the themostabilities reported for GAs from *A. hennebergi (39)*, *A. niger (40)*, *A.* spp. K-27 *(41)*, *A. awamori (42)*, *H. resinae (43)*, *T. reesei (44)*, *H. grisea (37)*, and *C. paradoxa (45)*. Thus, it appears that the thermal properties of tGA are slightly enhanced when compared with GA originated from several mesophilic fungi.

Although the pH/thermal activity profiles of various GAs are available, few reports have studied the pH/temperature dependence of K_m and k_{cat}. With maltotriose, tGA showed a k_{cat} and K_m that became maximal at pH 5.0 (Fig. 4B). Thus, the observed pH_{opt} in the pH-activity profile (Fig. 4A) was mainly caused by k_{cat}. The decline of k_{cat} at elevated pH might reflect the deprotonation of key amino acid residues involved in the hydrolysis *(46)*. Our study showed that at pH 4.0 and with maltotriose as the substrate, both the K_m and k_{cat} of tGA reached a maximum at 60°C (Fig. 5A). Assuming that

k_{cat}/K_m represented the apparent second-order rate constant and that $-\Delta G^{\neq} = RT \times \ln(k_{cat}/K_m)$, then the catalysis of tGA in hydrolyzing maltotriose seemed to involve two phases: a phase with a $\Delta G^{\neq}$ of 19 kJ/mol from 4 to 30°C, and another with a $\Delta G^{\neq}$ of 2.8 kJ/mol from 20 to 70°C. However, further experiments need to be done to confirm whether high temperature could reduce $\Delta G^{\neq}$ and accelerate catalysis by enhancing k_{cat}.

We have reported on the characterization of a *Thielavia* glucoamylase gene, its heterologous expression, and the biochemical analysis of the encoded protein. This work should enable us to better understand the gene regulation and protein secretion in *Thielavia*, as well as to explore further various applications of glucoamylase *(47)*.

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