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Note

Recombinant α-Glucosidase from *Aspergillus niger*. Overexpression by *Emericella nidulans*, Purification and Characterization

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Abstract: An expression plasmid containing the *agl*A gene encoding *Aspergillus niger* GN-3 α-glucosidase was constructed and inserted into *Emericella nidulans* JCM10259. The transformant secreted about 61 mg/L of the recombinant α-glucosidase into its culture medium. The recombinant enzyme was purified from the culture filtrate through ammonium sulfate precipitation and three chromatographic steps. It was confirmed that, like wild-type *A. niger* GN-3 α-glucosidase, the purified recombinant enzyme consisted of two subunits. Although the molecular mass of the recombinant enzyme was slightly smaller than that of wild-type *A. niger* α-glucosidase (attributed to differences in glycosylation), the pH optima and substrate specificities of the wild-type and recombinant enzymes were comparable.

Key words: α-glucosidase, *Aspergillus niger*, recombinant enzyme, enzyme expression, enzyme characterization

Exo-glycosidases are enzymes that hydrolyze monosaccharides from synthetic glycosides and from the nonreducing ends of oligosaccharides and polysaccharides. Glycosidases are generally classified based on their specificity for the glycon structure and anomer configuration of their substrates; substrate specificities are expressed in terms of the enzyme's relative activities towards substrates having various aglycons. α-Glucosidase (EC 3.2.1.20) is one of the *exo*-glycosidases widely found in microorganisms, plants, insects and mammals. Many α-glucosidases have been purified and their substrate specificities were investigated. Using partially modified α-glucosides (*e.g.*, several monodeoxy and mono-*O*-methyl analogs of *p*-nitrophenyl α-glucopyranoside, *p*NP-α-Glc),[1–6] we previously elucidated the glycon specificities of α-glucosidases purified from various organisms. The GH family 31 α-glucosidases isolated from rice, sugar beet, flint corn and the mold *Aspergillus niger* exhibited high hydrolytic activity towards 2-deoxy analogs of *p*NP-α-Glc (*p*NP-2D-α-Glc). In contrast, GH family 13 α-glucosidases isolated from the bacterium *Geobacillus stearothermophilus*, the yeast *Saccharomyces cerevisiae*, and from the honeybee could not hydrolyze these partially-modified glucosides. *Exo*-glycosidases not only hydrolyze glycosides and oligosaccharides, but can also synthesize them *via* a transglycosylation reaction. The specificity of the *A. niger* α-glucosidase for 2-deoxy glucoside was employed to synthesize glycon 2-deoxy derivatives of isomaltose and maltose using *p*NP-2D-α-Glc as the donor sugar and ethyl β-D-thioglucopyranoside as the acceptor sugar.[5–7] The controllable condensation of various modified monosaccharides by an *exo*-glycosidase could be a useful general approach for synthesizing modified oligosaccharides.

In an attempt to obtain glycosidases exhibiting broad glycon specificities, mutants of GH family 13 α-glucosidase from *G. stearothermophilus* were generated by site-specific mutagenesis[6]; however, enzymes with the desired characteristics were not obtained. It has previously been reported that the substrate specificity of GH family 13 α-glucosidase of bacteria *Bacillus* sp. SAM1606 and GH family 31 enzyme of yeast *Schizosaccharomyces pombe* can be altered.[8–10] For our study, it is necessary to overproduce the recombinant family 31 α-glucosidase from *A. niger*. Previouly, the α-glucosidase gene from *A. niger* had been cloned and inserted into the vector pSal23, which does not contain an autonomously replicating sequence, to construct expression plasmid.[11] In the report, although the gene product was produced in *Emericella nidulans* harboring the plasmid, the recombinant enzyme was not purified and characterized. With the intent of eventually producing glycosidases with broad glycon specificities, we here report the construction of an autonomously replicating plasmid for the expression of recombinant *A. niger* α-glucosidase, the overexpression of this recombinant enzyme in *E. nidulans*, and the purification and characterization of the product.

The protocol for constructing the plasmid is shown in Fig. 1. Plasmid pGTT55 containing the *A. niger* GN-3 α-glucosidase gene (*agl*A, GenBank accession no. D45356) was donated by Amano Enzyme Inc. Cloning, DNA ma-

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Abbreviations: GH, glycoside hydrolase; BAP, bacterial alkaline phosphatase; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography.

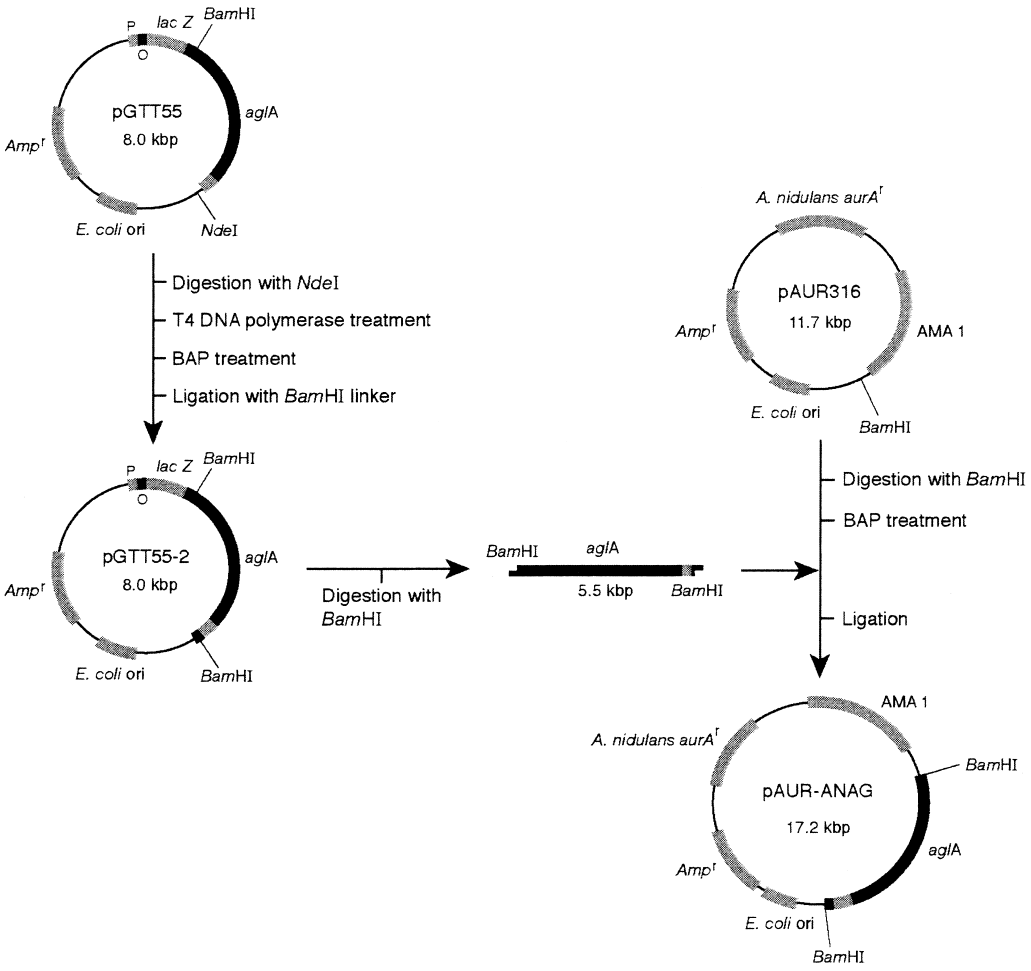

Fig. 1. Construction of the pAUR-ANAG plasmid for α-glucosidase overexpression.

nipulations and *Escherichia coli* transformation were conducted using standard techniques.[12] DNA was recovered from agarose gel slices using the GENECLEAN II kit (Q · Biogene), plasmid recovery from *E. coli* cells was achieved using the QIAprep Spin Miniprep Kit (QIAGEN), and ligation was accomplished using a Ligation Pack (Nippon Gene). After isolation from cell-free extracts of *E. coli* DH5α, pGTT55 was digested with *Nde*I, the linear DNA was treated first with T4 DNA polymerase to form blunt ends and then with BAP, and was ligated finally with *Bam*HI linker (Nippon gene) to afford pGTT55-2. Following isolation from cell-free extracts of *E. coli* DH5α, pGTT55-2 was digested with *Bam*HI to afford a DNA fragment containing the *agl*A gene. In order to construct the expression vector pAUR-ANAG, this DNA fragment was ligated into vector pAUR316 (Takarabio), which was treated with *Bam*HI and then with BAP. pAUR-ANAG was cut with *Bam*HI, and the DNA fragment containing the *agl*A gene was subcloned into the *Bam*HI sites of plasmid pBluescript II SK (+) (Stratagene) for nucleotide sequencing. DNA sequence analysis was performed using the dideoxynucleotide method.[13] The nucleotide sequence of the *agl*A gene was determined in both orientations using a ThermoSequenase fluorescence-labeled primer cycle sequencing kit (Amersham) and an automated DNA sequencer (DSQ-2000L; Shimadzu). *E. nidulans* JCM10259 was transformed with the pAUR-ANAG expression vector according to the method of Ballance *et al*.[14] The transformants were selected on an agar plate containing 1% polypeptone-S (Wako), 2% glucose, 0.8 M NaCl, 2% agar, and 2 μg/mL aureobasidin A (Takarabio).

E. nidulans harboring either pAUR-ANAG or pAUR 316 (control transformant) were grown at 25°C by shaking 100 mL cultures at 150 rpm. The medium consisted of 0.3% $NaNO_3$, 0.2% KCl, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.002% $FeSO_4 \cdot 7H_2O$, 3% soluble starch, 0.5% polypeptone, 0.25% yeast extract and 1.0 μg/mL aureobasidin A (pH 5.0). Secreted α-glucosidase was monitored using *p*NP-α-Glc as substrate; α-glucosidase activities in both cultures peaked on the 13th day. The *E. nidulans* culture containing the *agl*A gene exhibited 10.6-fold higher activity than the control transformant (Fig. 2). *E. nidulans* harboring pAUR-ANAG (500 mL cultures) were grown as above for 13 days, the cells were filtered (Toyo filter paper no. 2), and the recombinant *A. niger* α-glucosidase was precipitated with $(NH_4)_2SO_4$ (30–90% saturation) and collected by centrifugation (10,000$\times g$, 30 min). The precipitate was dissolved in 50 mM sodium acetate buffer (pH 5.0) and the enzyme solution was dialyzed against 15 mM sodium phosphate buffer (pH 6.8). Then the crude enzyme was passed over a DEAE-Toyopearl 650M resin column (Toso; column size, ϕ2.5$\times$30 cm; buffer, 15 mM sodium phosphate, pH 6.8; elution, 0–0.32 M linear gradient of NaCl). The partially-purified recombinant α-glucosidase was dialyzed against 15 mM so-

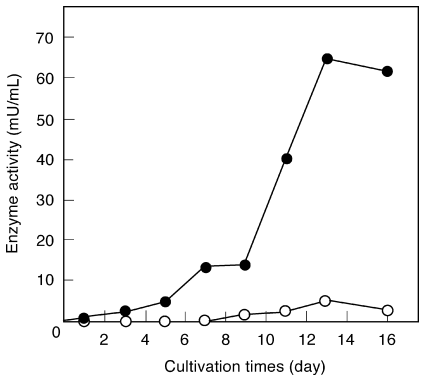

Fig. 2. Time course of α-glucosidase production.

Culture aliquots were withdrawn at the time points indicated and filtered. Culture filtrate (20 μL) was added to 80 μL of 1.25 mM *p*NP-α-Glc in 50 mM sodium acetate buffer (pH 4.5) and incubated for 10 min at 37°C. The reaction was stopped by adding 0.5 M aqueous Na_2CO_3 (0.1 mL). The concentration of liberated *p*-nitrophenol was measured spectrophotometrically at 405 nm. One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 μmol of *p*-nitrophenol/min under these assay conditions. ●, α-glucosidase activity in the filtrate from cultures of *E. nidulans* harboring pAUR-ANAG; ○, α-glucosidase activity in the culture filtrate from *E. nidulans* harboring pAUR316 (control transformant).

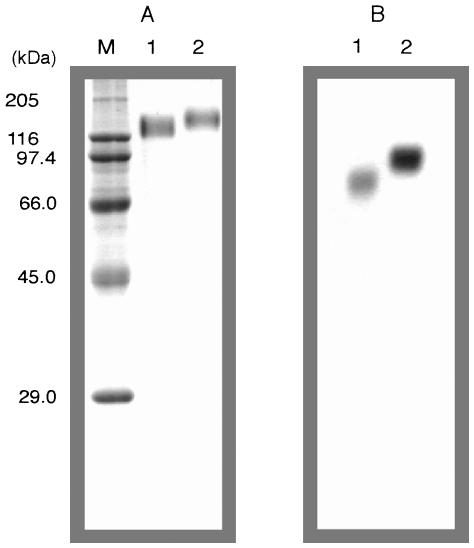

Fig. 3. Polyacrylamide gel electrophoresis of purified α-glucosidases.

A, SDS-PAGE. B, native-PAGE. Lane M of A shows molecular mass standards (Sigma). Lane 1 contains the purified recombinant α-glucosidase, and Lane 2 contains the purified wild-type α-glucosidase. Proteins in each gel were stained with Coomassie Brilliant Blue R250 (Tokyo Kasei Kogyo).

dium phosphate buffer (pH 6.8), and then the enzyme was passed over a DEAE-Sepharose Fast Flow resin column (AmershamBioscience; column size, ϕ2.5×10 cm; buffer, 15 mM sodium phosphate, pH 6.8; elution, 0–0.25 M linear gradient of NaCl). Passage over these two anion-exchange columns separated the recombinant enzyme from wild-type *E. nidulans* α-glucosidase. The recombinant α-glucosidase was further purified by gel filtration chromatography using Toyopearl HW-55F resin (Toso; column size, ϕ1.8×90 cm; buffer, 50 mM sodium acetate, pH 5.0). A total of 8.17 mg of recombinant *A. niger* GN-3 α-glucosidase was purified from 500 mL of *E. nidulans* culture broth. By HPLC using POROS HQ/20 anion-exchange column (Applied Biosystems; column size, ϕ4.6×100 mm; buffer, 15 mM sodium phosphate (pH 6.8); elution, 0–100 mM linear gradient of NaCl; flow rate, 10 mL/min; BioCAD 700E perfusion chromatography workstation), no contamination of *E. nidulans* α-glucosidase (retention time; 3.60 min) was observed in the purified preparation of recombinant *A. niger* enzyme (retention time; 5.56 min) (data not shown). Wild-type *A. niger* GN-3 α-glucosidase from Transglucosidase L (a crude preparation of *A. niger* GN-3 α-glucosidase, donated by Amano Enzyme Inc.) was purified as previously reported.[15] Both the recombinant and wild-type *A. niger* GN-3 α-glucosidase were confirmed to be homogeneous by native- and SDS-PAGE (Fig. 3).

The molecular mass of the recombinant enzyme was slightly smaller than that of wild-type *A. niger* α-glucosidase (120 kDa *vs*. 125 kDa, respectively; Fig. 3). The wild-type enzyme was confirmed to contain >25% sugars by weight.[15] We postulate that the difference in molecular mass between the recombinant and wild-type enzymes is due to differences in glycosylation rather than to differences in the amino acid sequences. The mature enzyme from wild-type *A. niger* GN-3 has been reported to be composed of two subunits (33 and 98 kDa),[15] which are derived from the same open reading frame and proteolytically generated.[11,16] These two subunits were not dissociated during SDS-PAGE[15] probably because their association was extremely tight, and they were separated by HPLC using reverse phase column.[15] Both the recombi-

Table 1. Substrate specificities of recombinant and wild-type α-glucosidases.

	Recombinant enzyme	Wild-type enzyme
Specific activity (U/mg protein)		
Maltose	46.8	57.8
*p*NP-α-Glc	0.962	1.06
*p*NP-2D-α-Glc	3.69	4.25
*p*NP-3D-α-Glc	0.127	0.140
*p*NP-4D-α-Glc	—	—
*p*NP-6D-α-Glc	—	—
K_m (mM)		
Maltose	1.04	1.25
*p*NP-α-Glc	0.589	0.620
*p*NP-2D-α-Glc	6.61	6.54
*p*NP-3D-α-Glc	11.6	10.5

Hydrolytic activities of both α-glucosidases against *p*NP-α-Glc and its monodeoxy analogs were measured according to the procedure described in the literature under Fig. 2. In the case using maltose as a substrate, a solution of each α-glucosidase (30 μL) was added into 120 μL of 1.25 mM maltose in 50 mM sodium acetate buffer (pH 4.5), and the mixture was incubated at 37°C at each reaction time. After stopping the reaction by heating the mixture in a boiling water bath for 5 min, the glucose released in the mixture was determined by the glucose oxidase method using a commercially available assay kit, Glucose CII-Test (Wako). One unit of enzyme activity was defined as the amount of enzyme required to hydrolyze 1 μmol of maltose/min under the assay conditions. Protein concentrations of enzyme solutions were determined by Lowry's method using bovine serum albumin as a standard. The kinetic studies of the enzymes were carried out at the following substrate concentrations: *p*NP-α-Glc, 0.05–2.5 mM; *p*NP-α-2D-Glc, 0.5–3.0 mM; *p*NP-α-3D-Glc, 0.2–5.0 mM; Maltose, 0.4–2.0 mM. Michaelis constant (K_m) was calculated from double reciprocal plots of each reaction curve. —, Activity was too low to be measured.

nant enzyme and the wild-type one described above produced two peaks (indicative of two subunits) when chromatographed on a POROS R2/10 HPLC reverse phase column (Applied Biosystems; column size, ϕ4.6×50 mm; elution, 40–95% linear gradient of acetonitrile; flow rate, 5.0 mL/min; BioCAD 700E perfusion chromatography workstation) (data not shown). Both enzymes exhibited optimal hydrolysis of *p*NP-α-Glc at pH 4.5 in 1/2 McIlvine buffer at 37°C (data not shown). In addition, the specific activities and K_m values of the two α-glucosidases towards hydrolysis of maltose, *p*NP-α-Glc, and monodeoxy analogs of *p*NP-α-Glc (*p*NP-2D-α-Glc,[17] *p*NP-3D-α-Glc,[1] *p*NP-4D-α-Glc[18] and *p*NP-6D-α-Glc[1]) were comparable (Table 1), suggesting that the active sites of the recombinant and wild-type enzymes are the same.

From the data for enzyme production by *E. nidulans* harboring pAUR-ANAG (Fig. 2), it is estimated that 90% of whole α-glucosidase activity in the culture broth of the 13th day depends on *A. niger* recombinant enzyme. From this data and the specific activity (0.962 U/mg protein) of the recombinant α-glucosidase, it is estimated that about 61 mg/L of the recombinant α-glucosidase was secreted into 1 L of culture medium by the *E. nidulans.*

In conclusion, recombinant *A. niger* GN-3 α-glucosidase has been overproduced, purified, and shown to have properties and substrate specificities comparable to the wild-type enzyme. The approach described above is presently being investigated for the production of mutant α-glucosidases exhibiting broad glycon specificities.

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Aspergillus niger 由来組換え α-グルコシダーゼの *Emericella nidulans* での過剰発現，精製および諸性質

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Aspergillus niger GN-3 由来のα-グルコシダーゼの遺伝子（*agl*A）を含む発現用プラスミドを構築し（Fig. 1），*Emericella nidulans* JCM10259 に挿入して組換え酵素を発現させた（Fig. 2）．組換えα-グルコシダーゼは，培養液 1 リットル中に約 61mg 分泌されていると見積もられた。組換え酵素は，培養液より硫安塩析，2 回のイオン交換カラムクロマトグラフィー，およびゲル濾過カラムクロマトグラフィーにより精製された．精製組換え酵素は，*Aspergillus niger* GN-3 由来のもの（野生型酵素）と同様に，二つのサブユニットから形成されていることが確認された．組換え酵素は，野生型酵素よりも分子質量が若干小さいことが SDS-PAGE により確認された（Fig. 3）．これは，酵素タンパク質に結合している糖鎖が，両者間で異なることに起因するものと推測される．酵素活性の最適 pH や基質特異性については，組換え酵素と野生型酵素では同じであった（Table 1）．