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Article

Transglycosilation activity of *Aspergillus oryzae*-derived α-glucosidase

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An *Aspergillus oryzae* RIB40 (NBRC100959) α-glucosidase（designed as AgdB）gene (*agdB*) was expressed at high levels in an *A. oryzae* host by self-cloning. The obtained transformant (MIBA1002) produced intracellular and extracellular α-glucosidase at levels 3- and 10-fold higher, respectively, than the parent host strain. The base sequence of *agdB* consisted of a 3036-kb structural gene containing three introns and encoding 963 amino acids, and the linear sequence thus obtained from these amino acids was identical to *A. oryzae* RIB40 unknown protein BAE64257.1. The amino acid sequence had 72% and 51% homology to α-glucosidase B from *Aspernillus nidulans* and *Acremonium implicatum*, respectively, which exhibit transglycosylation activity. The sequence has conserved residues specific to glucosyl hydrase family 31 (GH31) α-glucosidases. Tyr296 present in the β→α Loop1 in GH31 is important for transglycosylation. The enzyme produced 2.2% isomaltose, 0.4% maltotriose, and 0.3% kojibiose from 20% maltose substrate. This is the first report of the transglycosylation activity of α-glucosidase B cloned in *A. oryzae* (unknown protein BAE64257.1).

Key words: glycosyl hydrolases family 31, nitrate reductase gene-deficient strain, vitamin glycoside, maltooligosaccharide,

Introduction

Alpha-glucosidase (α-glucosidase, EC 3.2.1.20) hydrolyzes the terminal, non-reducing α-1,4-glucoside bond of oligosaccharides such as starch and maltooligosaccharides (1–4). Water is typically an excellent glucosyl receptor for carbohydrate hydrolases during catalysis. However, when the glucosyl receptor is a sugar, α-glucosidase can catalyze

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transglycosylation. Transglycosylation is the transfer of a glucosyl group at the non-reducing terminus of α-glucoside, maltooligosaccharide, or α-glucan to the hydroxyl group at the 6- or 3-position of glucose or an oligosaccharide. α-Glucosidase is widely distributed in animals, plants, and microbes (5–9).

The α-glucosidases are classified into three types according to their substrate specificity. Type 1 α-glucosidases are members of the GH13 and hydrolyze hetero-substrates containing aryl groups, such as *p*-nitrophenyl-α-D-glucopyranoside, faster than maltooligosaccharides. However, GH13 enzymes have almost no effect on α-glucan. Type 2 α-glucosidases have higher activity towards maltooligosaccharides than towards hetero-substrates and can hydrolyze α-glucan. Type 3 α-glucosidases are similar to those classified as Type 2, and can hydrolyze oligosaccharides and starch at similar rates. Type 2 and 3 enzymes belong to the GH31. In addition to hydrolysis, α -glucosidases catalyzes transglucosylation to form new glucosidic linkages, and thus it is used for the production of α -glucosides(10–13).

The fungus *A. oryzae* is essential in the traditional Japanese fermentation industry and is used in the production of sake, soybean paste, and soy sauce. The long history of *A. oryzae* in the food and pharmaceutical industries demonstrates the safety of enzymes produced by this organism. The *A. oryzae* genome project was completed in 2005 (14), Although *A. oryzae* likely produces α-glucosidases with transglycosylation activity, to our knowledge there has been no report confirming this.

Here we investigated the enzymatic characteristics of α-glucosidase (AgdB) from *A. oryzae* exhibiting transglycosylation activity and obtained high-producing strains through self-cloning of the gene in *A. oryzae*.

Materials and Methods

Chromosomal DNA library construction

Chromosomal DNA was isolated from *A. oryzae* strain RIB40 using a routine procedure (15). The DNA was digested with restriction enzyme *Bam*HI, separated by agarose gel electrophoresis, and then subjected to Southern hybridization using AlkPhos Direct (Amersham Biosciences, Inc. Piscataway, NJ, USA). The probes used were generated by nested polymerase chain reaction (PCR) as described below. *A. nidulans* belongs to the same family as *A. oryzae*. The amino acid sequence of α-glucosidase B (5) is conserved between *A. oryzae* and *A. nidulans*. The following primers were designed: SP1 degenerate primer (5′-TGGATHGAYATGAAYGARCC-

3′), ASP2 primer (5′-TGRTCNCKRAARAA NGTRTA-3′), and ASP3 primer (5′-CCNCC RAANCCRCANACRTC-3′), where H represents A, C, or T, Y represents C or T, R represents A or G,, and N represents A, C, G, or T. Nested PCR was performed first using the chromosomal DNA, SP1 primer, and ASP3 primer, and then with the obtained PCR product, SP1 primer, and ASP2 primer, to provide a PCR fragment approximately 900 bp long (15) which was used as a probe for Southern blot analysis.

A band approximately 5.5 kb in size was detected using Southern blot analysis. The chromosomal DNA was digested again with *Bam*HI and separated by agarose electrophoresis as follows. The 5.5 kb band was cut out of the gel using a razor, crushed in a micro-centrifuge tube using a plastic pipet tip, and incubated with constant shaking in 50 mM Tris-HCl /80 mM NaCl (pH 7.5) at 37°C for 1 h. The agarose pieces were then removed by centrifugation at 3,000 x *g* at room temperature for 1 min. The fragment recovered from the supernatant by ethanol (final concentration: 75%) precipitation was dissolved in 10 mM Tris-HCl (pH7.5). The DNA in an aliquot of this sample was ligated into the *Bam*HI site of λDASH II phage using T4 ligase. The obtained phage DNA was packaged using an *in vitro* packaging kit (Gigapack-Gold: TOYOBO Co. Ltd, Osaka, Japan). The construct was then transfected into *Escherichia coli* strain LE392 to generate a chromosomal DNA library.

Cloning into phage

The chromosomal DNA was cloned into phage and plaques were formed. Clones containing the *agdB* were selected by plaque hybridization using ECL Direct (Amersham Biosciences, Tokyo, Japan). The probes used were the same as those used to construct the chromosomal DNA library. Several positive plaques were obtained.

Sequencing of agdB gene

A DNA fragment from *A. oryzae* was amplified from the positive plaques. PCR was performed using the T7 and T3 promoter sequences adjacent to the vector-cloning site and the 3′- and 5′-terminal sequences of the probe. The nucleotide sequence of the vector insert was determined by sequencing the amplified PCR fragment using a BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Life Technologies, Tokyo, Japan).

Construction of vectors for use in A. oryzae

PCR was performed on the obtained plaques using a primer containing a start codon and *Not*I sequence and another primer containing a stop codon and *Xba*I sequence to amplify

the α-glucosidase B structural gene. The obtained PCR fragment was ligated into the pNEN8142 vector, which can replicate in *A. oryzae* (16). In brief, 10 μg of the PCR fragment was double-digested with *Not*I and *Xba*I and subjected to 0.8% agarose gel electrophoresis to separate a 3-kb fragment. This fragment was cut from the gel and recovered in TE solution (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) using a Gene Clean Kit-Bio 101 (MP Biomedicals, Japan, Tokyo, Japan). The pNEN8142 vector was double-digested with *Not*I and *Xba*I and then ligated with the 3-kb. DNA fragment, obtained as described above, to generate pNEN142-agdB(17).

Preparation of A. oryzae producing high levels of agdB

A.oryzae was transformed with the pNEN142-*agdB* (17). The cells were cultured at 30°C in dextrin Czapek-Dox (DC; 2% dextrin, 0.3% $NaNO_3$, 0.2% KCl, 0.1% KH_2PO_4, 0.05% MgSO4・$7H_2O$, 0.002% $FeSO_4$, pH 5.5) containing 0.8 M NaCl. Since pNEN142-agdB contains the nitrate reductase gene (*niaD*), transformants were selected by their ability to grow on DC medium.

Conditions for cell culture and enzyme extraction

Dextrin peptone medium (100 mL; 2% dextrin, 1% polypeptone, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, pH 5.5) was inoculated with 2.5×10^6 spores, then cultured at 30°C for 1–9 d with shaking. The cultures were passed through a 3GI glass filter and the filtrate was used as the extracellular crude enzyme preparation. Cells were washed with ultrapure water and homogenized in buffer (50 mM NaH_2PO_4 (pH 7.0), 10 mM EDTA, 0.1% Triton X-100, 0.1% *N*-lauroylsarcosine sodium salt, 10 mM β-mercaptoethanol) and centrifuged. The supernatant was used as the intracellular crude enzyme preparation.

α-Glucosidase activity (hydrolytic assay)

α-Glucosidase activity was measured with an α-glucosidase assay kit (Kikkoman Co., Tokyo, Japan) (18). using *p*-nitrophenyl-α-D-glucopyranoside as the substrate and measuring the amount of *p*-nitrophenol released after incubating at 37°C for 10 min. One unit of α-glucosidase was defined as the amount of enzyme releasing 1 μmol of product per minute. McIlvaine broad buffer (0.1 M citric acid /0.2 M di-sodium phosphate buffer: pH 3.0–8.0) was used to determine the optimal pH for enzyme activity and its pH stability. The pH of the solution was adjusted to 5.0 with 10 vol. each of 0.1 M citric acid or 0.2 M Na_2HPO_4, then glucose was assayed using a Glucose C

test kit (Wako Pure Chemical Co., Osaka, Japan).

Transglycosylation activity and conditions for HPLC analysis

To measure the transglycosylation activity of α-glucosidase, 20% maltose, 10mM acetate buffer (pH 5.5), and 0.023 U of enzyme (50 μL total volume) were incubated at 37°C, the enzyme was heat denatured at 100°C for 5 min to terminate the reaction, then the mixture was analyzed by high performance liquid chromatography (HPLC). HPLC column (6 x 150 mm) of Shodex SUGAR Series® 6 μm SZ5532 100 Å was used and eluted at 1 mL/min with an acetonitrile to water ratio ranging from 80:20 (0–10 min) to 70:30 (10–30 min). A RID-10A differential refractometer (Shimadzu, Kyoto, Japan) was used as a detector.

Results and Discussion

Base sequence of the agdB gene

Initially, the present study was conducted throughout under lacking the references about the corresponding genome structure since total genome sequences of *A. oryzae* were kept unknown at that time. We obtained those sequences by applying the information of *A. nidulans agdB* genes to those that reportedly had transglycosylation activity. At present it seems to be more suitable for us to transform the respective strain by using BLAST search *A. oryzae* (geneBank BAE64257.1: 82% identity).

Sequencing the insert of the recombinant phage showed that the *agdB* gene was a 3036-bp structural gene containing three introns and encoding 963 amino acid residues. Homology searches of this base sequence using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (19) returned an unnamed protein product from *A. oryzae* (GenBank: BAE64257.1; 100% sequence identity), a putative lysosomal α-glucosidase from *Aspergillus flavus NRRL3357* (XP 002380576.1; 99% identity), α/β-glucosidase AgdC from *A. oryzae* (XP 001825390.2; 95% identity), and Pc21g13670 from *Penicillium chrysogenum* Wisconsin 54-1255 (XP 002568384.1; 88% identity). These proteins with high sequence identity had been speculated based on the base sequence of the corresponding isolated DNA (GH31) and their functions likewise were speculative.

The results for α-glucosidases that reportedly have transglycosylation activity were α-glucosidase B from *A. nidulans* FGSC A4 (XP 682222.1, 82% homology) (5) and α-glucosidase from *Acremonium implicatum* (BAD08418.1, 51% homology) (6). These results indicate that the isolated enzyme may be involved in the synthesis of novel

glycosides.

CLUSTLW (20) analysis results obtained using these amino acid sequences are shown in Fig. 1. Tyrosine (Tyr)296 was present in the β→α Loop 1, which contains both conserved and catalytic residues specific to GH31 α-glucosidases (21–23), and is considered important for their transglycosylation activity (23).

A search for this gene in DOGAN, a website that annotates all *A. oryzae* proteins (http://www.bio.nite.go.jp/dogan/Top), yielded maltase-glucoamylase and related hydrolases belonging to GH31 (AO090038000471). Although several other enzymes belonging to the GH31 family are annotated for *A. oryzae*, they showed low sequence homology (Table 1).

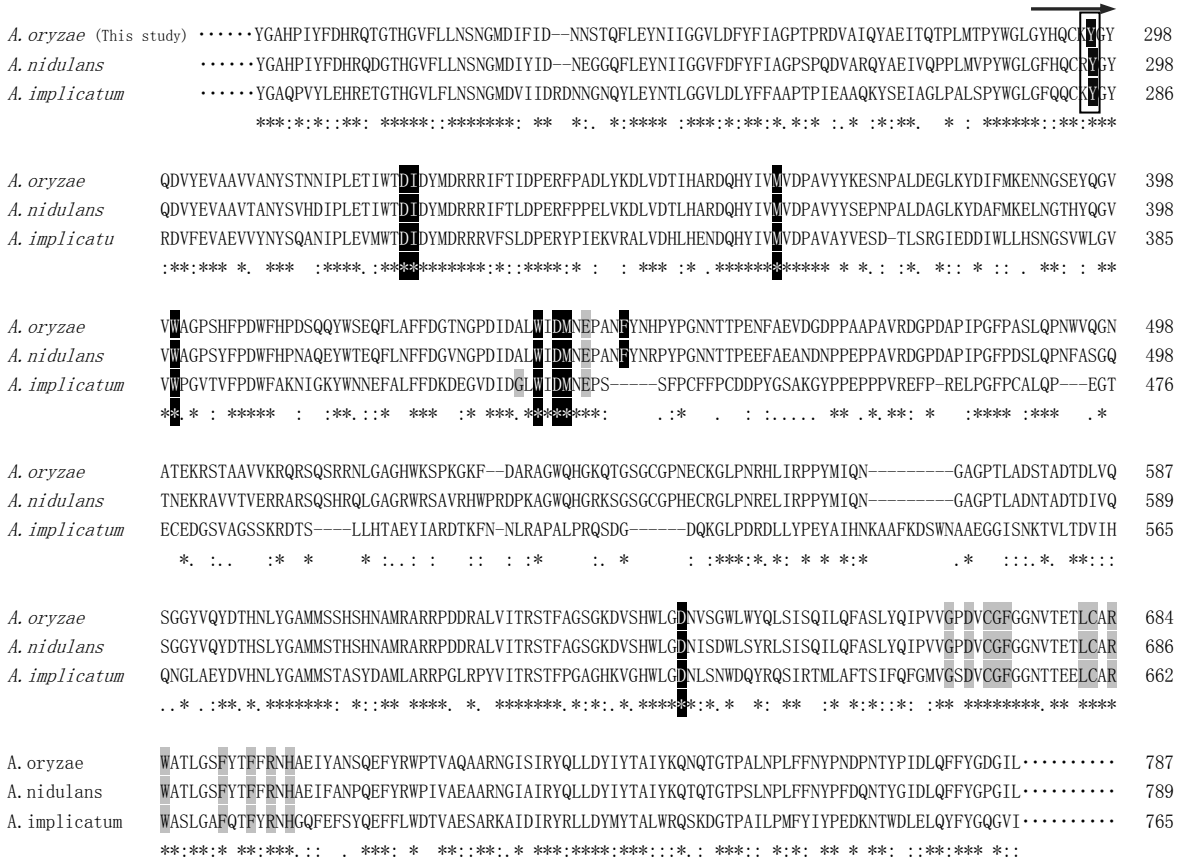

Fig.1. CLASTALW alignment of glycoside GH31 members that have reported transglycosylation activity.

■ is catalytic residues, ▒ is conserved residues, □ is important aromatic residues in GH31 α-glucosidase. The ß→α Loop1 is involved in the transglycosylation specificity of the GH31 α-glucosidase. → is the ß→α Loop1 of GH31 α-glucosidase.

Table 1. BLAST search results for *A.oryzae* GH31 members (performed using DOGAN).

	Product	ident	DOGAN ID
	maltase glucoamylase and related hydrolases, glycosyl hydrolase family 31	**53.75**	AO090005001084
	maltase glucoamylase and related hydrolases, glycosyl hydrolase family 31	**34.92**	AO090003001209
	glucosidase II catalytic (alpha) subunit and related enzymes, glycosyl hydrolase family 31	**31.48**	AO090102000559
	alpha-glucosidases, family 31 of glycosyl hydrolases	**24.75**	AO090001000649
	alpha-glucosidases, family 31 of glycosyl hydrolases	**27.83**	AO090023000288
	alpha-glucosidases, family 31 of glycosyl hydrolases	**26.67**	AO090005000767
	alpha-glucosidases, family 31 of glycosyl hydrolases	**28.47**	AO090005000768
	alpha-glucosidases, family 31 of glycosyl hydrolases	**22.78**	AO090026000111
	alpha-glucosidases, family 31 of glycosyl hydrolases	**26.34**	AO090701000639
	alpha-glucosidases, family 31 of glycosyl hydrolases	**24.39**	AO090701000558
	serine/threonine protein kinase of the CDC7 subfamily involved in DNA synthesis, repair and recombination	**23.57**	AO090020000026
	predicted protein	**35.38**	AO090003001426
	Na+/K+ ATPase, alpha subunit	**31.82**	AO090005000141

Glucosidase production by transformed MIBA1002

Self-cloning of *agdB* in *A. oryzae* yielded 65 transformant strains. The MIBA1002 strain showed the highest α-glucosidase production and was cultured with shaking in CD medium for 1–9 d to assess its enzyme production ability. MIBA1002 had approximately 10-fold higher intracellular α-glucosidase activity on day 3 of culture (0.12 U/mL) and an approximately 3-fold higher extracellular enzyme activity on day 8 (0.021 U/mL) (Fig. 2) compared with the wild-type strain.

α-Glucosidase B from *A. nidulans*, a close relative of *A. oryzae*, is reportedly localized in the cell wall. α-Glucosidase synthesized by MIBA1002 appears to have a signal sequence, as demonstrated in *A. nidulans* (5). The sequence information obtained here indicates that MIBA1002 strain releases α-glucosidase into the extracellular space following recombination.

SDS-PAGE electrophoresis

The extracellular crude enzyme preparation was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis. Electrophoresis was performed using an

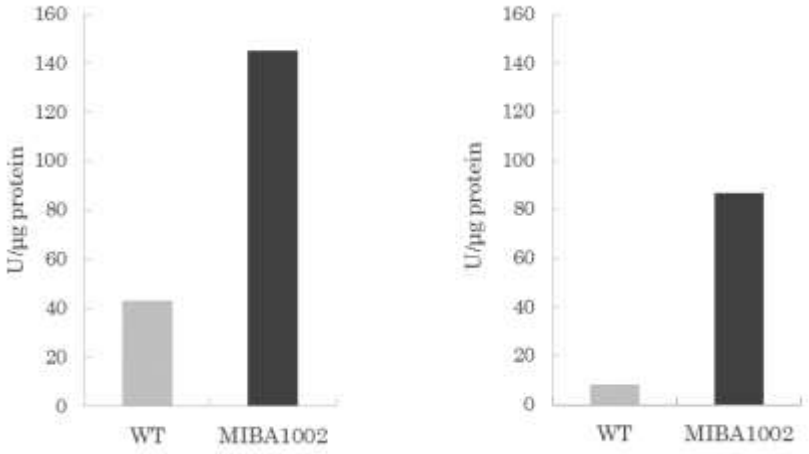

Fig. 2. Comparison of agdB production by MIBA1002 and wild-type strain.
Left panel, intracellular enzyme production by MIBA1002 and wild-type strain cells. Right panel, extracellular enzyme production by MIBA1002 and wild-type strain.

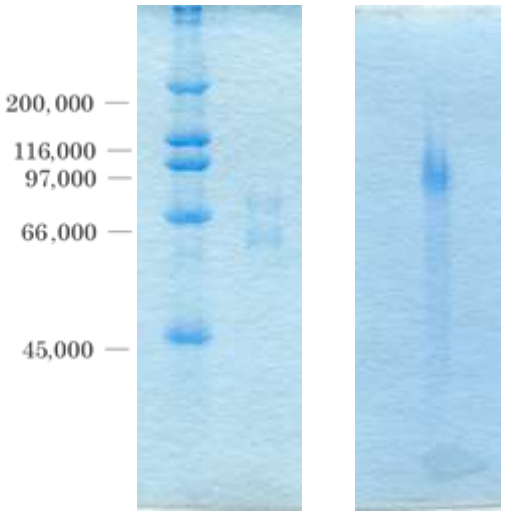

Fig. 3. Electrophoresis profiles of agdB.
Electrophoresis was performed at 25°C for 1.5h at 25 mA/gel, using 3 μg and 5 μg protein in the presence (A) or absence (B) of SDS. The numerals in the left panel denote molecular weight standards (from top to bottom): myosin (200,000), β-galactosidase (116,000), phosphorylase-b (97,000), bovine serum albumin (66,000), and ovalbumin (45,000).

polyacrylamide gel and proteins were stained using Coomassie Brilliant Blue R250 (24) (Fig. 3). Myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase b (97 kDa), serum albumin (66 kDa), and ovalbumin (45 kDa) were used as molecular weight markers.

Protein bands were observed at approximately 78 kDa and 63 kDa and had high purity. The N-terminal amino acid sequences of the 73- and 63-kDa subunits were identified at residues 21 to 28 and 515 to 522 of the derived amino acid sequence,

respectively. The first 20 amino acids at the N terminus of AgdB shows a typical feature of signal peptides.

Native PAGE of the crude enzyme on a 10% polyacrylamide gel yielded a single band, indicating that the protein was composed of two subunits. Although *Aspergillus* species generally produce enzymes in solid-state culture, *Aspergillus* was here induced to produce enzymes in liquid culture, allowing us to obtain high-purity crude enzymes.

Enzymatic characteristics

The optimal temperature, heat resistance, thermal stability, optimal pH, and pH stability of this enzyme were determined. The optimal temperature for activity was determined to be 60°C after incubating for 10 min (Fig. 4A). The enzyme was stable up to 45°C (Fig. 4B). When incubated at 4°C, 20°C (room temperature), and 30°C for 24 h, the enzyme maintained ≥ 90% activity. Even after repeated freezing and thawing, enzymeactivity remained relatively stable (data not shown). The optimal pH was 5.0 when incubated for 10 min at pH values from 3.0 to 8.0 (Fig. 4C). The enzyme maintained ≥ 60% a incubated at each pH value for 24 h and was most stable at pH 5.0 (Fig. 4D). Procedures for these assays are provided in the figure legends.

Transglycosylation activity

The hydrolyzing ability together with transglycosylation activity of MIBA1002 enzyme was analyzed with an HPLC. Maltose at an initial concentration of 20% (w/v) was decreased to 1.1% by incubationg with the enzyme for 120 min and completely degraded after reacting for 180 min. The reaction products generated after 240 min were identified as follows: 2.2 % isomaltose, 0.4% maltotriose, and 0.3% kojibiose (Fig. 5, 6).

On the other hand, when, instead of the extracellular recombinant protein, we used similar amounts of enzyme obtained from the wild type strain, 7.9% of the maltose substrate was left unreacted, and no significant amount of oligosaccharides was detected after 300 min incubation.

These results confirmed that *agdB* encoded a protein exhibiting transglycosylation activity and indicated that 20% maltose as a substrate likely favored transglycosylation over hydrolysis. Liquid culture offers the advantage of easy control of culture conditions compared to solid-state culture. Liquid culture of MIBA1002 enabled the release of highly pure AgdB product into the extracellular space. MIBA1002 can facilitate amylolysis, producing glucose in the presence of amylase, and has many applications in the fermentation, food, and pharmaceutical in

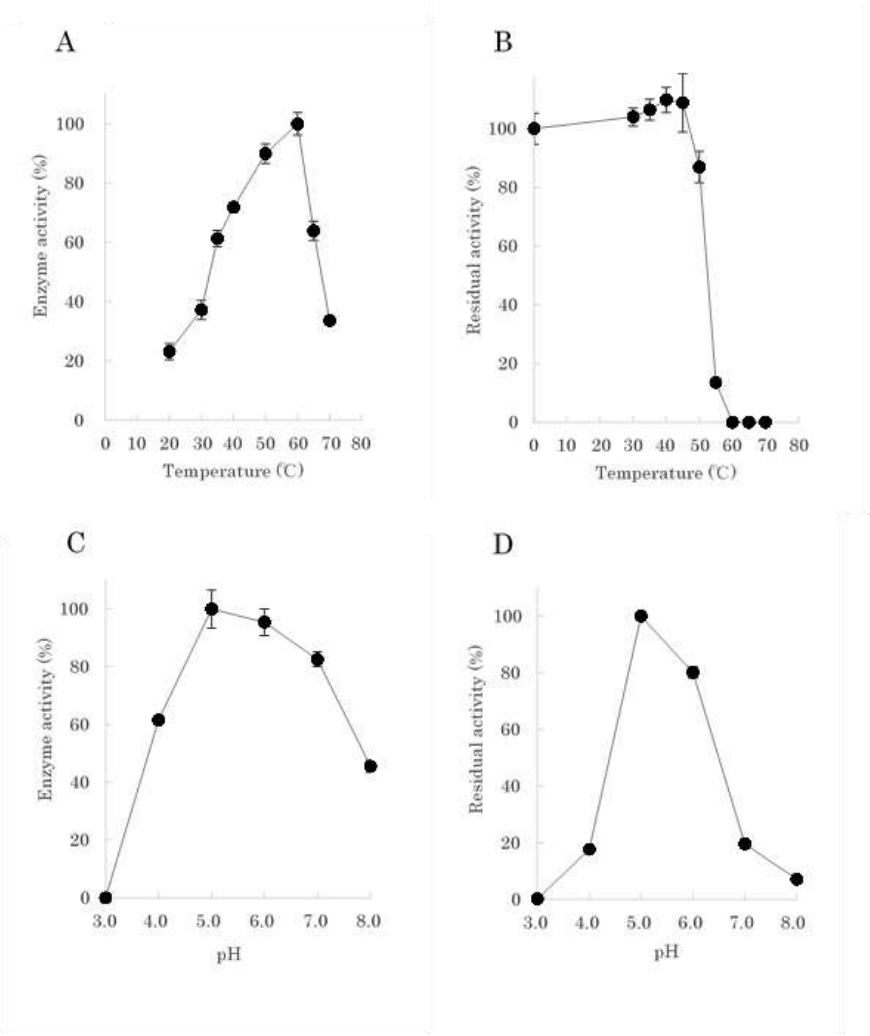

Fig. 4. Effects of temperature and pH on the activity and stability of agdB.

The activity of agdB was determined as described in the Materials and Methods, except that the temperature was changed. The maximum activity is taken as 100%. **A**, agdB (0.12 U/mL) was incubated for 10 min at different temperatures in acetate buffer (pH 5.0). **B**, agdB was divided into aliquots (0.18 U/mL each) and one aliquot was heated for 15 min at the indicated temperature (30 – 70°C). Each mixture was then assayed for residual activity. The activity observed after incubation at 4°C was considered to be 100%. **C**, The activity of agdB at different pH values was assessed as described in the Materials and Methods. The maximum activity was considered to be 100%. **D**, agdB was divided into aliquots (0.18 U/mL each) and one aliquot was incubated at 20°C for 24 h at the indicated (pH3.0 – 8.0). The pH of each mixture was then adjusted to 5.0 and the activity was assayed. The activity measured after incubation for 24 h at pH 6.8 and 4°C was considered to be 100%.

Maltose : α-D-Glucosyl-(1→4)-D-glucose
Isomaltose : α-D-Glucosyl-(1→6)-D-glucose
Maltotriseo : α-D-Glucosyl-(1→4)-D-Glucosyl-(1→4)-D-glucose
Kojibiose : α-D-Glucosyl-(1→2)-D-glucose

Fig.5. Structures of the glucooligosaccharides used in this study.

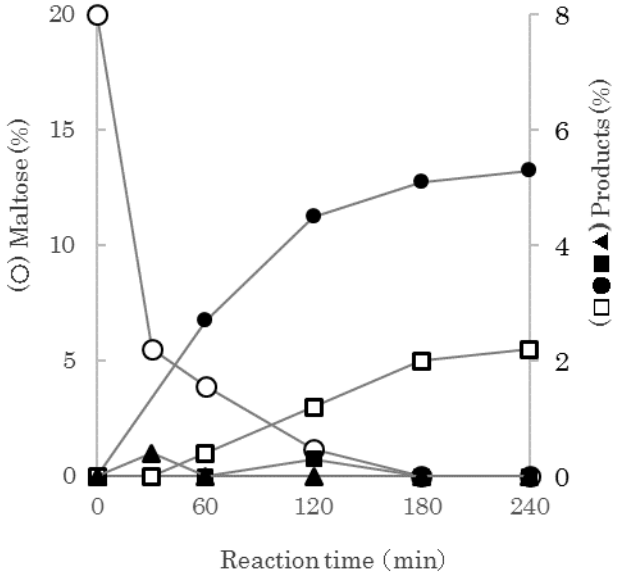

Fig.6. HPLC chromatography analysis of transglycosylation by MIBA1002 enzyme.

Using 20% maltose as a substrate, transglycosylation reactions were performed with 0.023 U enzyme in 10mM acetate buffer (pH 5.5) in a total volume of 50 µL at 37°C for 30, 60, 120, 180, 240, and 300 min. Maltose (○), isomaltose (□), and glucose (●), maltotriose (■), and kojibiose (▲) were used as standard markers.

dustries. Although isomaltose and kojibiose can be produced from sucrose using sucrose phosphorylase from *Leuconostoc mesentroides*, the process is complicated and the yields are extremely low (10). MIBA1002 shows great promise for the development of foods and cosmetics based on non-conventional carbohydrate processing. MIBA1002 enzyme may be useful as a transglycosylation catalyst for these applications since it formed 2.2% isomaltose, 0.4% maltotriose, and 0.3% kojibiose from 20% maltose substrate. Currently, α-glucosaccharides are too expensive for the mass production of these oligosaccharides.

Very recently, *A.niger* α-glucosidase was expressed in yeast and optimized (25) and the similar enzyme was purified from thermophilic fungus to achieve more effective production (26). Our aim to synthesize useful oligosaccharides from maltose will be accomplished in future by using AgdB transglycosylation activity. The present study is the first report of the enzymatic character-

istics and transglycosylation ability of an unnamed protein product from *A. oryzae*.

MIBA1002 was deposited with the Fermentation Research Institution (deposit number: NITE P-214) (Tokyo, Japan).

Disclosure statement

A patent relating to this work has been published (Patent Publication No. 2008-72956) (Tokyo, Japan).

Acknowledgements

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