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Article

Characterization of Two α-Glucosidases Purified from *Bifidobacterium longum* subsp. *longum* JCM 7052

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Two α-glucosidases, which hydrolyzed 4-nitrophenyl (4NP)-α-D-glucopyranoside, were purified and characterized from *Bifidobacterium longum* subsp. *longum* JCM 7052 grown on gum arabic. These enzymes, named BlAglA1 and BlAglA2, had apparent molecular masses of 72.0 and 72.8 kDa estimated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and 70.1 and 92.7 kDa by PAGE without SDS, respectively. The amino acid sequences analyzed by MALDI-TOF-MS were found in the genomes of several strains of *B. longum* subsp. *longum*. BlAglA1 and BlAglA2 showed hydrolyzing activities toward isomaltose, trehalose, palatinose, isomaltotriose and panose, but did not toward sucrose, maltose, amylose, amylopectin, and oyster glycogen, indicating that each enzyme belongs to oligo-1,6-glucosidase (EC 3.2.1.10). Both enzymes showed optimal activities with 4NP-α-D-glucopyranoside at pH 5.5-6.0 and at 45-50°C. Values of K_m and V_{max} for 4NP-α-D-glucopyranoside, trehalose, palatinose, panose, isomaltose, and isomaltotriose were also determined. The two oligo-1,6-glucosidases were shown to have transglycosylation activities to synthesize oligosaccharides from trehalose, palatinose, isomaltose, panose, and isomaltotriose to their selves. Both enzymes also catalyzed transglucosylation from palatinose to ethanol, 1-propanol, 2-propanol, 1-butanol, and 1-hexanol.

Keywords: α-glucosidase, oligo-1,6-glucosidase, transglycosylation, ethyl-α-D-glucoside, *Bifidobacterium longum*

Introduction

Bifidobacteria, which are strictly anaerobic Gram-positive bacteria, have many kinds of genes to be adapted to grow on host produced glycan such as mucins and milk oligosaccharides and dietary plant-derived oligo- and polysaccharides. Many novel enzymes involved in utilization of carbohydrates, therefore, have been discovered, isolated and characterized [1-3].

Among carbohydrate-hydrolyzing enzymes α-glucosidases have been purified and characterized from only two species of *Bifidobacteria, Bifidobacterium adolescentis* [4, 5] and *Bifidobacterium breve* [6-8]. α-Glucosidase is a general term for the enzymes able to hydrolyze α-glucoside bonds as shown in Fig. 1. Maltitol-hydrolyzing enzyme of *B. adolescentis* type a, E194a is a maltase acting as α-1,4-glucosidase [4]. AglA of *B. adolescentis* DSM20083 (BaAglA) has substrate specificity toward α-1,6-linkage of glucosides, AglB of this strain (BaAglB) prefers to α-1,4-glucoside bond [5]. *B. breve* UCC2003 contains Agl1

This article is dedicated to Dr. Lars Ljungdahl on the occasion of his 90th birthday.

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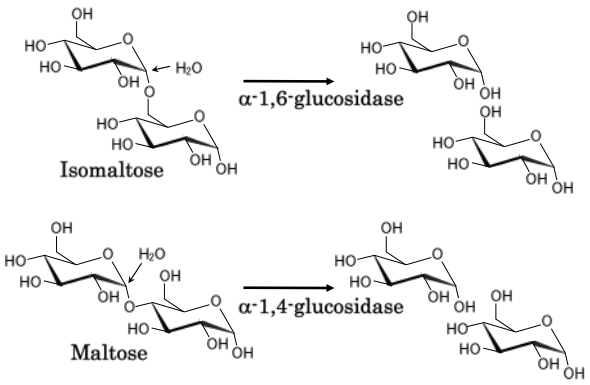

Fig. 1. Hydrolytic reactions catalyzed by α-1,6-glucosidase and α-1,4-glucosidase.

(BbAgl1) and Agl2 (BbAgl2), these enzymes showing substrate specificity toward α-1,6-glucoside linkage [6], Agl3 which can hydrolyze α-1,1-, α-1,3-, α-1,4- and α-1,5-glucosidic linkages [7], and MelD being able to hydrolyze α-1,3-glucoside bond of melezitose [8].

Bifidobacterium longum subsp. *longum* JCM 7052 has been shown that enzyme activities of several glycoside hydrolases, α- and β-galactosidases, α-L-arabinofuranosidase, α-L-arabinopyranosidase, α-L-fucosidase, and α-glucosidase, are increased during growth on gum arabic [9]. Two α-galactosidases and one β-galactosidase have been purified and characterized from this strain [9-11]. The β-galactosidase encoded in the *lacA* gene appears to have multiple functions with activities of α-L-fucosidase and α-L-arabinopyranosidase. In this study we focused on α-glucosidase, especially its transglycosylation activity to produce oligosaccharides and alkyl glucosides which are promised to exert beneficial effects on maintenance of human health.

Materials and Methods

Chemicals Trehalose, palatinose, isomaltotriose, melibiose and gum arabic (from *Acacia senegal*) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Panose and isomaltose were obtained from Hayashibara Co. Ltd (Okayama, Japan). Isomaltotriose was obtained from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan). 4-Nitrophenyl (4NP) glycosides were purchased from Sigma-Aldrich Co. (St. Louis, USA). Other chemicals were of reagent grade and were products of Wako Pure Chemical Industries, Ltd.

Bacterial strain and growth conditions *Bifidobacterium longum* subsp. *longum* JCM 7052 was used in this study. Bacterial cells were grown in a medium of slightly modified Bifidobacterium medium, which contained (per liter) Bacto-peptone (Difco Laboratories, USA), 10 g; yeast extract (Oriental Yeast Co., Ltd., Japan), 5 g; meat extract (Wako Pure Chemical Industries, Ltd., Osaka, Japan), 5 g; K_2HPO_4, 3 g; sodium ascorbate, 10 g; cysteine hydrochloride, 0.5 g; and Tween 80, 1 ml. Gum arabic (10 g/l) was added as carbon source instead of glucose. pH was adjusted to 6.8. Cells were anaerobically grown at 37°C for 20 h. Anaerobic conditions were obtained by filling the neck of screw-capped bottles with the medium.

Assay of enzyme activities Standard assay of α-glucosidase activity was done with 4NP-α-D-glucopyranoside as substrate. The reaction mixture (1.0 ml) contained an enzyme preparation, 80 mM K-phosphate (pH 6.0) and 1.0 mM 4NP-α-D-glucopyranoside. The reactions were carried out at 40°C for 10 min and were terminated by the addition of 0.5 ml of 0.2 M Na_2CO_3. The amount of 4-nitrophenol released was determined at 400 nm with an extinction coefficient of 18.3 mM^{-1} cm^{-1}. One unit of enzyme activity was defined as the amount of enzyme that released 1 μmol of 4-nitrophenol per min from 4NP-α-D-glucopyranoside.

Glucose released by glycoside hydrolyzing activity was assayed by using a glucose assay kit (LabAssay™ Glucose, Wako Pure Chemical Industries, Ltd., Osaka, Japan). The reaction mixture (50 μl) for glycoside hydrolyzation consisted of 20 mM saccharide, 80 mM K-phosphate (pH 6.0), and a purified enzyme. The enzyme reaction was carried out at 40°C and stopped by heating in boiling water for 5 min. When trehalose, isomaltose, palatinose, isomaltotriose, or panose were used as substrate, glucose production was observed proportionally during 5 min incubation, while in the incubation for 10 min products of transglycosylation were detected by thin layer chromatography (TLC) described below. Therefore, the incubation for 5 min was chosen to

determine K_m and V_{max} values. The reaction mixtures with the other saccharides (shown in Table 2) as substrate were incubated for 30 min.

Determination of transglycosylation activity was performed by TLC on HPTLC Silica gel 60 plates (Merck Ltd., Germany). Glucoside, 0.4 M concentration of isomaltose, trehalose, palatinose, isomaltotriose, or panose in 80 mM K-phosphate (pH 6.0), was incubated with 0.14-0.22 U ml^{-1} of the purified enzyme at 35°C for 0.5, 1, 3, 6, 12, 24, 48 and 72 h. Transglycosylation activities from 0.5 M palatinose to 1.0 M alcohols, ethanol, 1-propanol, 2-propanol, 1-butanol, 1-hexanol, or 1-octanol, were also determined in 80 mM K-phosphate (pH 6.0), with 0.2 U ml^{-1} of each enzyme at 35°C at intervals indicated above. When 1-butanol, 1-hexanol, and 1-octanol were used as acceptor molecules, 0.1% Tween 80 was added in the reaction mixtures. The reactions were stopped by heating in boiling water for 5 min. After centrifugation supernatants (0.5 or 1.0 μl) were spotted on a silica gel plate and saccharides were developed twice in a 2-propanol:1-butanol:water (2:2:1) solvent system, and detected with spray of 10% sulfuric acid in ethanol and heating for 15 min at 130°C.

Thermal stability of enzyme was assayed by incubating a purified enzyme (about 0.02 mg of protein/ml) at 20-60°C and drawing aliquots at intervals. The enzyme activity with 4NP-α-D-glucopyranoside was then determined according to the standard assay procedure.

Activity staining of α-glucosidase in polyacrylamide gel after electrophoresis without SDS was performed by putting the gel on a plastic sheet with a few drops of 2 mM 4NP-α-D-glucopyranoside as substrate. Incubation for color development was done at room temperature.

The protein concentrations were determined by using Coomassie brilliant blue (Bio-Rad Laboratories, USA). Bovine serum albumin was used as the standard. All reactions were carried out in triplicate.

Purification of α-glucosidase Wet cells (40 g) were suspended in four volumes of 50 mM Tris-HCl buffer (pH 7.2). The cells were sonicated (TOMY Ultrasonic Disruptor UD-200, Japan) in an ice bath at 20 kHz, 140 W for 10 min and centrifuged at 20,000 x *g* for 20 min at 4°C. The supernatant was used as crude extract (Step 1. Crude extract, 165 ml). The precipitate obtained between 30 and 60% saturation of $(NH_4)_2SO_4$ was dissolved in 50 mM Tris-HCl, pH 7.2 (Step 2. Ammonium sulfate fraction, 13 ml). This solution was loaded on a column (2.5 x 90 cm) of Sepharose 4B (GE Healthcare, Sweden) equilibrated in 50 mM Tris-HCl (pH 7.2) containing 0.1 M NaCl and eluted at a flow rate of 0.3 ml/min. Fractions containing α-glucosidase activity were pooled (Step 3. Sepharose 4B, 76 ml). The pooled fractions were diluted with 10 volumes of cold distilled water and loaded on a column (1.5 x 10 cm) of Q-Sepharose (GE Healthcare, Sweden) equilibrated in 50 mM Tris-HCl (pH 7.2). Proteins were eluted at a flow rate of 0.3 ml/min with a linear concentration gradient of NaCl (0.1-0.32 M). In this step two peaks of α-glucosidase activity were found. One of the enzymes was named BlAglA1 and another BlAglA2. Fractions containing each enzyme were pooled separately (Step 4A. 1st Q-Sepharose, 40 ml, and Step 4B, 30 ml). This process was repeated twice for each enzyme (Step 5A. 2nd Q-Sepharose, 44 ml, and Step 5B, 25 ml). The solution pooled was mixed with $(NH_4)_2SO_4$ at a concentration of 1.84 M, then was loaded on a column (1.5 x 4 cm) of Butyl-S Sepharose 6 Fast Flow (GE, Sweden) equilibrated with 50 mM Tris-HCl (pH 7.2) containing 1.84 M $(NH_4)_2SO_4$. Proteins were eluted using a decreasing stepwise gradient of $(NH_4)_2SO_4$ (1.1-0.7 M for both enzymes) at a flow rate of 0.5 ml/min. Fractions containing α-glucosidase activity were pooled (Step 6A. Butyl-S Sepharose, 50 ml; and Step 6B, 42 ml). Each enzyme pooled was desalted and concentrated by centrifugation with a filtration tube Vivaspin 4 (Sartorius, Germany). Concentrated preparations were subjected to 12.5% polyacrylamide gel electrophoresis (PAGE) without sodium dodecyl sulfate (SDS). An area stained with α-glucosidase activity was cut out of the polyacrylamide gel and was soaked in 50 mM Tris-HCl (pH 7.2). The enzyme was filtrated using a gel extraction spin column (Bio-Rad, USA) and was concentrated by centrifugation with a filtration tube Vivaspin 4 (Step 7A. Native PAGE, 0.61 ml; and Step 7B, 0.46 ml).

Table 1. Purification of α-glucosidases BlAglA1 and BlAglA2 from *B. longum* subsp. *longum* JCM 7052 grown on 1% gum arabic. 4NP-α-D-glucopyranoside was used as substrate.

	Purification step	Protein (mg)	Total activity (units)	Specific activity (units/mg of protein)	Yield (%)
BlAglA1, BlAglA2	1. Crude extract	679	614	0.90	100
	2. Ammonium sulfate fraction	243	283	1.16	46
	3. Sepharose 4B	207	264	1.27	43
BlAglA1	4A. 1st Q-Sepharose	54.0	155	2.87	25
	5A. 2nd Q-Sepharose	41.3	151	3.66	25
	6A. Butyl-S Sepharose	18.5	136	7.34	22
	7A. Native PAGE	0.29	130	451	21
BlAglA2	4B. 1st Q-Sepharose	32.4	100	3.09	16
	5B. 2nd Q-Sepharose	21.5	87	4.03	14
	6B. Butyl-S Sepharose	9.9	83	8.45	14
	7B. Native PAGE	0.14	39	284	6

Molecular mass determination SDS-PAGE was carried out with a 5-20% (w/v) linear gradient polyacrylamide slab gel (Wako Pure Chemical Industries, Ltd.) according to Laemmli [12]. Molecular weight marker proteins for SDS-PAGE (WAKO PLUS-VIEW™ Protein Size Marker) were used for calibration. Native PAGE was done using the 5-20% (w/v) polyacrylamide gel and molecular mass was estimated with a molecular weight marker kit (NativeMark™, Invitrogen, Carlsbad, CA, USA).

Protein identification by mass spectrometry The Coomassie-stained protein bands were excised from the SDS-PAGE gel, and subjected to tryptic digestion as previously described [13]. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) and tandem mass spectrometry (MS/MS) were performed on an Ultraflex MS system (Bruker Daltonik GmbH, Bremen, Germany). Peptide mass fingerprinting (PMF) searches were performed by using the Mascot Search engine against the NCBI protein database. BLAST [14, 15] was also used to analyze distribution of the obtained peptides in proteins on the DDBJ database.

Results

Purification of α-glucosidases from *B. longum* subsp. *longum* JCM 7052

For purification of α-glucosidase *B. longum* subsp. *longum* JCM 7052 grown on 1% gum arabic was used to obtain a much amount of the enzyme. The purification of α-glucosidase is summarized in Table 1. Two peaks of α-glucosidase activity measured with 4NP-α-D-glucopyranoside were separated by the 1st Q-Sepharose ion exchange chromatography (Fig. 2). The enzymes released at 0.3 and 0.2 M concentrations of NaCl were named BlAglA1 and BlAglA2, respectively. In hydrophobic chromatography with Butyl-S Sepharose 6 Fast Flow, BlAglA1 and BlAglA2 were released at 0.9 and 1.0 M concentrations of $(NH_4)_2SO_4$ in Tris-HCl (pH 7.2), respectively. Finally, each α-glucosidase was purified as a single protein band, which was evidently separated by native PAGE. In consequence, α-glucosidases BlAglA1 and BlAglA2 were purified 450- and 285-folds, respectively.

Molecular masses of α-glucosidases

SDS-PAGE showed that BlAglA1 and BlAglA2 were purified homogeneously and had

apparent molecular masses of 72.0 and 72.8 kDa, respectively (Fig. 3A). Molecular masses of BlAglA1 and BlAglA2 were also estimated to be 70.1 and 92.7 kDa by PAGE without SDS, respectively (Fig. 3B).

Identification of two α-glucosidases by mass spectrometry

The purified BlAglA1 was analyzed by MALDI-TOF-MS, MS/MS, and PMF search to identify its putative gene. The PMF result (Accession number, gi|494117734; sequence coverage, 42.2%; score, 147) and two MS/MS results (1631.760 (285-301), EGFLTVGEAPGVTAQR; score, 85) and (2301.925 (398-417), GTPYIYQGEELGMTD-AHFTR; score, 75) were obtained. For the purified BlAglA2, the PMF result (Accession number, gi|658455158; sequence coverage, 62.2%; score, 230) and two MS/MS results (2823.350 (515-540), HGNATVATGEWNLVAADSEQVYSFTR; score, 106) and (1919.841 (353-368), NAGWASLFFCN-HDQPR; score, 131) were obtained. The two couples of peptide sequences were also searched against the genomic sequence of *B. longum* subsp. *longum* JCM7052, which is still unpublished. The couple of peptides obtained from BlAglA1 were found in an α-amylase, and the couple of peptides from BlAglA2 were also found in another α-amylase.

BLAST search showed that the amino acid sequences of BlAglA1 and BlAglA2 were completely identical with those of the products of genes (locus tags of BLNIAS 02948 and BLNIAS 02851, respectively) in the genome of *B. longum* subsp. *longum* KACC 91563 [16]. BlAglA1 consisted of 607 amino acids and had the molecular mass of 68256.70 Da. This molecular mass is approximately consistent with 72.0 kDa determined by SDS-PAGE (Fig. 3A). BlAglA2 was composed of 606 amino acids and its molecular mass was 68547.12 Da. This value of molecular mass was approximately consistent with 72.8 kDa estimated by SDS-PAGE (Fig. 3A).

In addition, similar amino acid sequences of the α-glucosidases were found in the characterized enzymes of two *Bifidobacterium* species. The amino acid sequence of BlAglA1 is highly identical to that of BaAglA (85%) of *B. adolescentis*

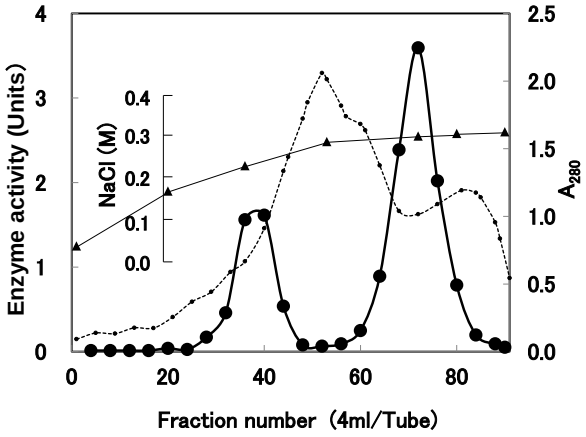

Fig. 2. Ion exchange chromatography on Q-Sepharose of α-glucosidases from *B. longum* subsp. *longum* JCM 7052. Enzyme activities were determined with 4NP-α-D-glucopyranoside as substrate. Symbols: ●, enzyme activity; ▲, NaCl (0.10-0.32 M); and •, A_{280}.

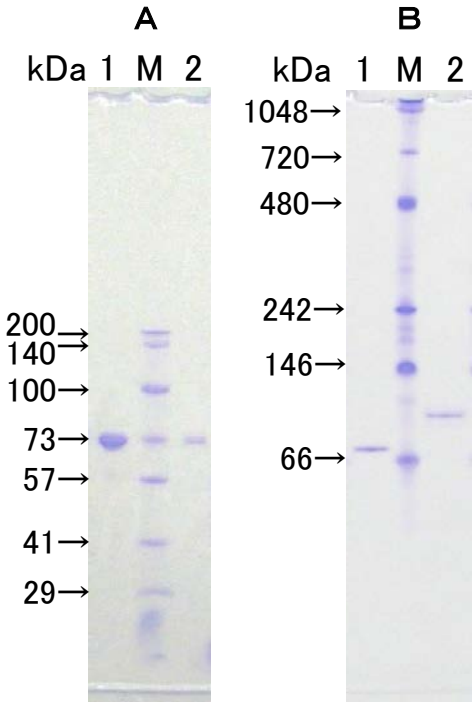

Fig. 3. Molecular mass determination of α-glucosidases by SDS-PAGE [A] and native PAGE [B]. Lane M, marker proteins; lane 1, purified BlAglA1; and lane 2, purified BlAglA2. Proteins were stained by Coomassie blue R-250.

DSM20083, BbAgl1 (91%) and BbAgl2 (88%) of *B. breve* UCC2003. The amino acid sequence of BlAglA2, which was homologous with that of

Table 2. Hydrolytic activities of BlAglA1 and BlAglA2 toward some saccharides.

Substrate	Hydrolytic activity	
	BlAglA1	BlAglA2
Disaccharides		
Trehalose	+	+
Sucrose	−	−
Turanose	−	−
Maltose	−	−
Cellobiose	−	−
Lactose	−	−
Palatinose	+	+
Isomaltose	++	++
Melibiose	−	−
Trisaccharides		
Melezitose	−	−
Maltotriose	−	−
Panose	+++	++
Isomaltotriose	+++	+++
Other substrates		
Glycogen	−	−
Amylose	−	−
Amylopectin	−	−

Glucose released after 30 min incubation was determined as described in Materials and Methods. The enzyme used was 63 mU in each reaction. Scales were: −, 0-0.01 μmol glucose; +, 0.01-0.05 μmol glucose; ++, 0.05-0.10 μmol glucose; and +++, 0.10-1.0 μmol glucose.

BlAglA1 (74% identical), was identical to those of BaAglA (86%), BbAgl1 (74%), and BbAgl2 (72%).

Substrate specificities of α-glucosidases

The enzymes BlAglA1 and BlAglA2 showed hydrolytic activities against 4NP-α-D-glucopyranoside but not against other 4NP-glycosides, 4NP-α-D-galactopyranoside, 4NP-α-D-mannopyranoside, 4NP-α-L-rhamnopyranoside, 4NP-α-L-fucopyranoside, 4NP-α-L-arabinopyranoside, 4NP-α-L-arabinofuranoside, 4NP-β-D-glucopyranoside, 4NP-β-D-galactopyranoside, 2-nitrophenyl-β-D-galactopyranoside, 4NP-β-D-fucopyranoside and 4NP-β-L-fucopyranoside.

Hydrolytic activities toward some saccharides were tested for BlAglA1 and BlAglA2 by measuring glucose released after 30 min incubation at pH 6.0 and 40°C in the K-phosphate buffer (Table 2). Both BlAglA1 and BlAglA2 were capable of releasing glucose from some saccharides having α-1,6-linkage, isomaltose, palatinose, isomaltotriose, and panose. Trehalose, which has α-1,1-linkage between two glucoses, was also hydrolyzed by both enzymes at lesser extent. However, no hydrolysis products were found from the other saccharides listed in Table 2, indicating that both enzymes BlAglA1 and BlAglA2 cannot catalyze hydrolysis of α-1,2-β-linkage in sucrose, α-1,3-linkage in turanose and melezitose, and α-1,4-likage in maltose, maltotriose, and amylose. These findings including the amino acid sequence

Table 3. Kinetic parameters of BlAglA1 and BlAglA2 purified from *B. longum* subsp. *longum* JCM 7052.

Substrate	BlAglA1		BlAglA2	
	K_m	V_{max}	K_m	V_{max}
4NP-α-D-glucopyranoside	1.5 ± 0.2	122 ± 8.5	2.0 ± 0.1	64.2 ± 0.6
Trehalose	14.3 ± 1.9	1.5 ± 0.4	7.4 ± 0.5	1.3 ± 0.6
Palatinose	32.2 ± 1.5	15.3 ± 2.5	33.5 ± 3.0	16.4 ± 1.8
Isomaltose	35.0 ± 2.2	17.0 ± 1.0	38.6 ± 3.6	22.6 ± 3.7
Panose	38.3 ± 0.7	47.7 ± 2.9	24.1 ± 2.3	31.6 ± 0.6
Isomaltotriose	41.7 ± 0.5	25.3 ± 2.2	43.3 ± 1.0	17.2 ± 1.1
Ethyl-α-D-glucoside	68.6 ± 2.7	24.2 ± 2.8	37.3 ± 3.8	12.0 ± 2.3

The standard assay of enzyme activity was used for 4NP-α-D-glucopyranoside as substrate. Glucose released from the other substrate was determined as described in Materials and Methods.
K_m values are expressed as mM and V_{max} values are expressed as μmol min^{-1} mg^{-1} of protein. All values are means of three experiments ± standard errors.

similarities of the two enzymes indicated BlAglA1 and BlAglA2 to be oligo-1,6-glucosidase (EC 3.2.1.10) belonging to glycoside hydrolase family 13 [17, 18].

Values of Michaelis constant K_m and maximal velocity (V_{max}) for 4NP-α-D-glucopyranoside, trehalose, palatinose, isomaltose, panose, isomaltotriose, and ethyl-α-D-glucoside were shown in Table 3.

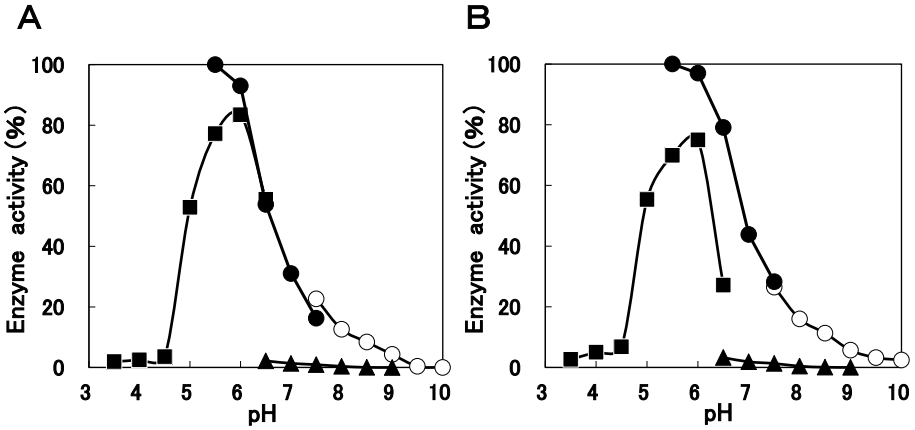

Fig. 4. Effects of pH on the activity of the purified α-glucosidases BlAglA1 [A] and BlAglA2 [B]. Buffers used were ■, 80 mM acetate (pH 3.5-6.5); ●, 80 mM K-phosphate (pH 5.5-8.0); ○, 80 mM glycine-NaOH (pH 7.5-10.0); and ▲, 80 mM Tris-HCl (pH 6.5-9.0).

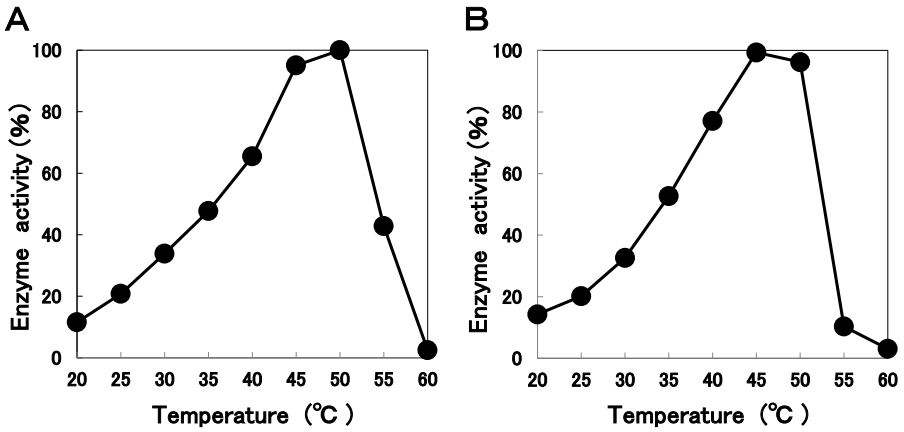

Fig. 5. Effects of temperature on the activity of the purified α-glucosidases BlAglA1 [A] and BlAglA2 [B].

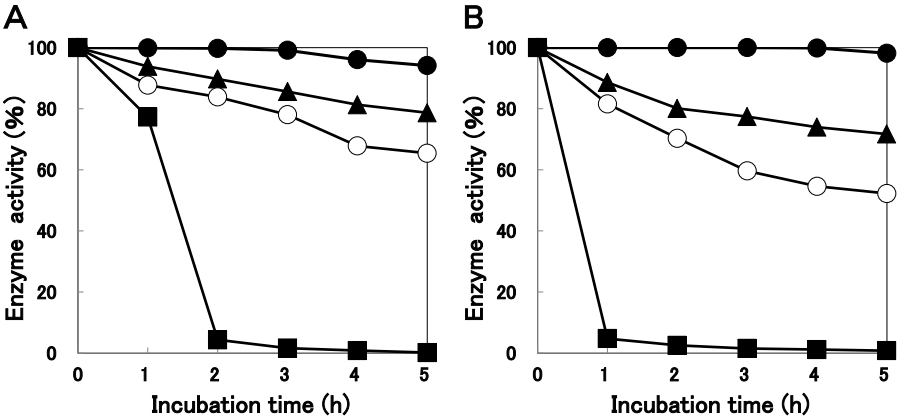

Fig. 6. Effects of temperature on the stability of the purified BlAgl1 [A] and BlAgl2 [B]. Enzyme activities were assayed at intervals after incubation of enzyme solutions at 40°C (●), 45°C (▲), 50°C (○), and 55°C (■).

Effects of pH, temperature, and chemicals on the enzyme activity.

The optimal pH was assayed by incubating the purified enzyme with 4NP-α-glucopyranoside in 80 mM buffers ranging from pH 3.5 to pH 10.0. For both enzymes BlAglA1 and BlAglA2, the highest activity of α-glucosidases were obtained at pH 5.5-6.0 in 80 mM K-phosphate buffer (Fig. 4A and B).

The optimal temperature of BlAglA1 was observed in hydrolyzation of 4NP-α-D-glucopyranoside at 40-45°C (Fig. 5A). The enzyme activity gradually decreased during 5 h incubation at 45 and 50°C (Fig. 6A), and a remarkable decline in the activity occurred during 2 h incubation at 55°C (Fig. 6A). The optimal temperature for BlAglA2 was observed at 45-50°C (Fig. 5B). BlAglA2 was unstable rather than BlAglA1, so that the activity of BlAgl2 was lost after 1 h incubation at 55°C (Fig. 6B)

To determine the effects of chemicals, hydrolytic activities of BlAglA1 and BlAglA2 were measured using 4NP-α-D-glucopyranoside in the presence of 1 mM metal chloride salts or EDTA (Table 4). For both enzymes Cu^{2+} inhibited about 60% of their enzyme activities and Ca^{2+}, Co^{2+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, and Zn^{2+} did about 10–20%. EDTA was less active as a chelating reagent for both enzymes.

Table 4. Effects of metal ions and EDTA on the hydrolytic activities of purified BlAglA1 and BlAglA2. 4NP-α-D-glucoside was used as substrate.

Reagents	Relative activity (%)	
	BlAglA1	BlAglA2
None	100	100
$BaCl_2$	95	97
$CaCl_2$	90	89
$CoCl_2$	86	89
$CuCl_2$	43	39
$FeCl_2$	94	96
$MgCl_2$	90	87
$MnCl_2$	82	82
$NiCl_2$	87	89
$ZnCl_2$	89	92
NaCl (10 mM)	97	99
KCl (10 mM)	103	101
EDTA	99	99

Divalent metal ions and EDTA were added at 1.0 mM to the reaction mixture.

Transglycosylation activity of the enzymes

α-Glucosidases are known to have transglycosylation activity [5, 6, 19, 20]. The purified enzymes BlAglA1 and BlAglA2 were briefly tested to have glucosyl transferring activity. TLC showed that oligosaccharides were synthesized in the reaction mixtures containing isomaltose, palatinose, isomaltotriose, or panose. Especially, tetra- and pentasaccharides were produced from panose and isomaltotriose. When isomaltose was used as a glucosyl donor, isomaltotriose was obviously produced (Fig. 7, lanes 10 and 11), suggesting the formation of α-1,6-linkage in the products of transglucosylation by BlAglA1 and BlAglA2.

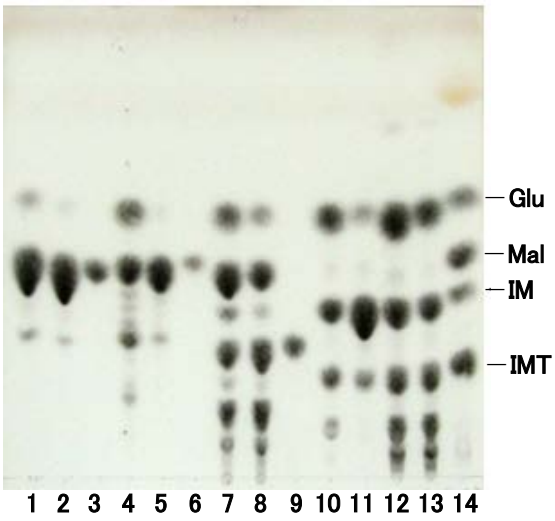

Fig. 7. TLC analysis of the transglycosylation activities of BlAglA1 and BlAglA2. Standard carbohydrates used were trehalose (lane 3), palatinose (lane 6), panose (lane 9), and a mixture of glucose (Glu), maltose (Mal), isomaltose (IM), and isomaltotriose (IMT) (lane 14). The reaction mixtures contained trehalose (lanes 1 and 2), palatinose (lanes 4 and 5), panose (lanes 7 and 8), isomaltose (lanes 10 and 11), and isomaltotriose (lanes 12 and 13). In the reaction mixture BlAglA1 was added in lanes 1, 4, 7, 10 and 12, and BlAglA2 was in lanes 2, 5, 8, 11, and 13.

Transglucosylation to alcohols was also examined with purified BlAglA1 and BlAglA2 by TLC (Fig. 8). Under the used conditions the standard ethyl-α-D-glucoside moved faster than glucose, suggesting alkyl-glucosides formed in the reactions would be detected at upper region of the gel plate. In the reactions with palatinose as a donor and ethanol, 1-propanol, 2-propanol, 1-butanol, or 1-hexanol as acceptors, products of the respective reactions were found at upper place than the position of glucose or fructose developed in the silica gel plate, and ethyl-α-D-glucoside was identified as the product of transglycosylation to ethanol. This result suggests that both oligo-1,6-glucosidases BlAglA1 and BlAglA2 transfers glucose to alcohol to produce alkyl-α-D-glucoside. In the reaction with 1-octanol, only a faint spot was found at an upper place of TLC gel. When glycerol was used as an acceptor of glucose, no products were detected by TLC (data not shown).

Discussion

In *B. longum* subsp. *longum* JCM 7052 the increase in α-glucosidase activity by growing on gum arabic seemed to be attributed to one or both of two oligo-1,6-glucosidases. Both enzymes BlAglA1 and BlAglA2 had similar properties relative to the amino acid sequence (74% identical), pH- and temperature-dependency of enzyme activities, and substrate specificity. These two oligo-1,6-glucosidases did not produce glucose from amylopectin and glycogen, indicating these enzymes to have no hydrolytic activities against α-1,6-glucosidic linkages present inside the polysaccharides. *B. longum* subsp. *longum* including the strain JCM 7052 cannot grow on starch, amylopectin, and pullulan [21], while maltodextrins were shown to be the most favorable carbon source for bifidobacteria [22]. When *B. longum* subsp. *longum* BBMN68 was grown on maltodextrins as sole carbon source, oligo-1,6-glucosidase beside α-1,4-glucosidases was observed to be induced and 2 components of ABC-type sugar transporters to be more abundant [23]. Utilization of carbohydrates have been understood to be controlled by transcriptional regulation with DNA- and/or sugar-binding regulatory proteins in bifidobacteria [24, 25]. Such sugar-binding regulators might respond to hydrolysates of gum arabic, and subsequently activate the expression of genes involved in hydrolysis of other oligosaccharides.

Oligosaccharides have attracted attention to their beneficial functions as food ingredients (low

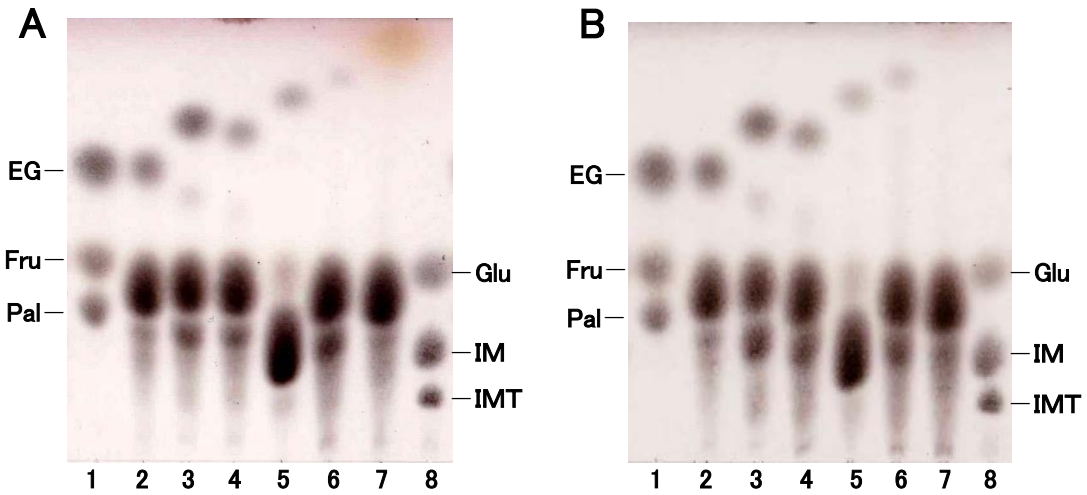

Fig. 8. TLC analysis of the transglucosylation activities of the purified oligo-1,6-glucosidases BlAglA1 [A] and BlAglA2 [B]. Standard carbohydrates used were a mixture of ethyl-α-D-glucosidse (EG), fructose (Fru) and palatinose (Pal) in lane 1, and that of glucose (Glu), isomaltose (IM), and isomaltotriose (IMT) in lane 8. The reaction mixtures containing each purified enzyme, 500 mM palatinose as a glucosyl donor and the following 1.0 M alcohol: ethanol (lane 2), 1-propanol (lane 3), 2-propanol (lane 4), 1-butanol (lane 5), 1-hexanol (lane 6), and 1-octanol (lane 7).

calorie, mildly sweet, and viscous compounds), prebiotics, dental caries preventives, drug coatings, and so on. Both oligo-1,6-glucosidases BlAglA1 and BlAglA2 showed transglycosylation activity to produce higher oligosaccharides (tetra- and pentasaccharides). Since both enzymes showed to use several α-glucosides as substrate in transglucosylation, a variety of oligosaccharide would be synthesized by using different sugars as acceptor molecules.

Alcohols also served as glucosyl acceptor molecules in transglucosylation of BlAglA1 and BlAglA2. Alkyl glucosides are also attractive compounds because of their health beneficial properties. Especially, ethyl-α-D-glucoside has been known as one of the components in traditional Japanese sake wine [26]. This compound has been known to have a moisture-retaining effect on skin through oral ingestion or skin application [27], and hepatoprotective effects against galactosamine-induced liver injury in mice [28]. Production of ethyl-α-D-glucoside has been performed through alcohol fermentation with rice [29], and transglucosylation of enzymes. α-Glucosidase of *Mortierella alliacea* transfers glucose from starch to ethanol [30], while *Leuconostoc mesenteroides* dextransucrase uses sucrose to form ethyl-α-D-glucoside [31]. In this study we tested only palatinose as glucosyl donor in the synthesis of alkyl glucoside by the oligo-1,6-glucosidases BlAglA1 and BlAglA2. To confirm the method for more convenient and effective production of alkyl glucosides, modification of enzymes, selection of glucosyl donors, and improvement of reaction conditions are required in further investigations.

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