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Article

α-Galactosidase purified from *Bifidobacterium longum* JCM 7052 grown on gum arabic

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Among some strains, *Bifidobacterium longum* JCM 7052 grew well anaerobically in a medium with gum arabic, and showed high activities of α- and β-galactosidases. α-Galactosidase was purified 66-fold from *B. longum* JCM 7052 grown on gum arabic by ammonium sulfate fractionation, and chromatographies on Sepharose 4B, Q-Sepharose, Butyl-S Sepharose, and hydroxyapatite. The enzyme had an apparent molecular mass of 79 kDa by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and 150 kDa by PAGE without SDS, suggesting the dimeric nature of the enzyme. This α-galactosidase showed optimal activity at pH 8.0 and at 40–45°C. Hydrolysis of α-galactosides showed normal saturation kinetics: K_m values for 4-nitrophenyl-α-D-galactopyranoside, raffinose, and stachyose were 0.15, 88.3, and 126 mM, respectively. The activity of α-galactosidase was inhibited competitively by tris (hydroxymethyl) aminomethane: its K_i was 32 mM. Transgalactosylation activity was also observed from 4-nitrophenyl-α-D-galactopyranoside to galactose and melibiose.

Keywords: α-galactosidase, purification, *Bifidobacterium longum*, gum arabic

Introduction

Bifidobacteria are Gram-positive saccharolytic anaerobes that are natural commensals of the gastrointestinal tract of humans and mammals and are thought to have health-promoting effects, like the inhibition of pathogenic microorganisms, antimutagenic and anticarcinogenic activities, prevention of diarrhea, immune modulation, and reduction of serum cholesterol levels [1-3]. *Bifidobacterium* species can ferment many kinds of complex carbohydrates including gum arabic and probably can use them as important sources of carbon and energy [4-7].

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Gum arabic is a highly heterogeneous complex polysaccharide which consists of three main fractions, arabinogalactan protein complex (AGP, 10%), arabinogalactan (AG, 90%), and glycoprotein (1%) [8]. Compositions of AG and carbohydrate block of AGP are similar, galactose (40%), arabinose (30%), rhamnose (12%), and glucuronic acid (15%) [8-10]. It has a branched structure with a β-1,3 linked galactan backbone [9]. Gum arabic is highly water soluble and is used as a food additive. It has been known that among *Bifidobacterium* species some strains of *Bifidobacterium longum* ferment gum arabic [4-6]. The genome sequence of *B. longum* NCC 2705 revealed that this bacterium is able to produce many kinds of glycoside hydrolase including arabinosidases, α- and β-galactosidases, α- and β-glucosidases, α-mannosidase and so on [11]. Recently, novel enzymes relating to carbohydrate utilization were found in *B. longum*

[12-14].

α-Galactosidases (EC 3.2.1.22) hydrolyze the α-1,6 linkages which are present in galactosaccharides such as melibiose, raffinose and stachyose. The enzymes are known to be widely distributed in mammals, plants and microorganisms [15-19]. Degradation of oligosaccharides in plants is known to involve two distinct types of α-galactosidase, acid α-galactosidase active in acidic environments and alkaline α-galactosidase active under neutral or alkaline conditions [19-21]. Some species of *Bifidobacterium* including *B. longum* also have α-galactosidases whose activities are optimal at pH 5.5-6.0 [22-25]. Glycolytic enzymes such an α-galactosidase are often described as glycoside hydrolases, but some of them also show transglycosylation activity, which is expected to produce new oligosaccharides as dietary fibers.

In this study, we examined glycoside hydrolase activities in *B. longum* JCM 7052, which was identified to be able to grow on gum arabic, and α-galactosidase activity among glycoside hydrolase activities tested was observed to be highest in gum arabic-grown cells. We described purification and characterization of α-galactosidase from *B. longum* JCM 7052 grown on gum arabic.

Materials and Methods

Chemicals Gum arabic (from *Acacia senegal*) and raffinose were obtained from Wako Pure Chemical Industries (Osaka, Japan). 4-Nitrophenyl (4-NP) glycosides, melibiose, stachyose, and 3,5-dinitrosalycylic acid were purchased from Sigma-Aldrich Co. (St. Louis, USA). Other chemicals were of reagent grade and were products of Wako Pure Chemical Industries.

Bacterial strains and growth conditions *Bifidobacterium longum* strains used in this study were JCM 7052, JCM 1217, JCM 7053, JCM 7055, JCM 7056, and 105-A. These strains were anaerobically grown at 37°C on following media. A medium modified from Bifidobacterium medium described in the catalogue of JCM was used as a basal medium. The basal medium contained (g/l) polypepton (Nihon Seiyaku, Japan), 10; extract from Bonito (Wako Pure Chemical Industries), 5; yeast extract (Oriental Yeast, Japan), 5; K_2HPO_4, 3; sodium ascorbate, 10; L-cysteine HCl, 0.5; Tween 80, 1 ml/l. pH was adjusted to 6.8 with 2 N NaOH. Where indicated, glucose, gum arabic, lactose, or sucrose (10 g/l) were added as carbon source. Anaerobic growth conditions were attained by flashing CO_2 for 3-5 min to butyl rubber-stoppered tubes or by filling the neck of screw-capped bottles with medium. The basal medium with and without each carbon source was inoculated with the overnight culture in the corresponding medium and incubated at intervals. The inoculum size was 1% volume of media for each culture.

Cell growth was followed by measuring the absorbance at 660 nm in a 10 mm-cuvette with a spectrophotometer (Hitachi UV-1900). When the absorbance was over 0.5, cultures were diluted with 0.85% NaCl to give an absorbance below 0.5. Cells were harvested by a centrifuge (18,000 x *g* for 15 min at 4°C) from cultures, washed three times with 0.85% NaCl, and stored at -20°C until use.

Assay of enzyme activities Glycoside hydrolase activities were determined with 4-nitrophenyl (4-NP) glycosides as substrate. Reaction mixture (1 ml) contained an enzyme preparation, 80 mM K-phosphate (pH 7.0) and 1 mM 4-NP-glycoside. The reactions were carried out at 30°C for 5 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 is defined as the amount of enzyme that released 1 μmol of 4-nitrophenol per min from 4-NP-glycoside.

Enzyme activities which hydrolyze oligosaccharides were determined by measuring the concentration of reducing sugar formed in reaction mixtures at 540 nm using 3,5-dinitrosalitylic acid. Reaction mixtures containing 80 mM K-phosphate (pH 7.0), enzyme preparation, and substrate 100 mM raffinose, stachyose, or 0.5% gum arabic were incubated at 30°C for 60 min. One unit of enzyme activity was defined as the amount of enzyme that produced 1 μmol of reducing sugar per min from the oligosaccharide.

Transglycosylation activity was assayed by detection of product in HPTLC (Silica gel 60; Merk Ltd., Germany). Reaction mixtures (1 ml) contained 80 mM NaOH-glycine buffer (pH 8.0), 15 mM 4-NP-α-D-galactopyranoside, 80 mM saccharides, and 0.2 units of purified α-galactosidase, and were incubated for 5 h. Saccharides were separated in *n*-butanol:acetic acid:water (5:4:3), and detected with spray of 5% sulfuric acid in ethanol.

Thermal stability assay was performed by incubating a purified enzyme at 40, 45, and 50°C and drawing aliquots at intervals. The enzyme activity was then determined with 4-NP-α-D-galactopyranoside as substrate described above.

The protein concentrations were determined according to the Lowry method for cells and crude extracts, and by using coomassie brilliant blue (Bio-Rad Laboratories, USA) in enzyme purification. Bovine serum albumin was used as the standard. All reactions were carried out in triplicate.

Purification of α-galactosidase Wet cells (7 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 5 min and centrifuged at 20,000 x *g* for 30 min at 4°C. The supernatant was used as crude extract (Step 1. Crude extract, 37.5 ml). The precipitate obtained between 30 and 70% saturation of ammoniun sulfate was dissolved in 50 mM Tris-HCl, pH 7.2 (Step 2. Ammonium sulfate fraction, 9.1 ml). Proteins dissolved were 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 0.4 ml/min. Fractions containing α-galactosidase activity were pooled (Step 3. Sepharose 4B, 54 ml). The pooled fractions were loaded on a column (1.5 x 5 cm) of Q-Sepharose (GE Healthcare, Sweden) equilibrated in 50 mM Tris-HCl (pH 7.2) containing 0.1 M NaCl. Proteins were eluted at a flow rate of 0.5 ml/min with a linear concentration gradient of NaCl (0.1-0.7 M). Fractions containing α-galactosidase activity were pooled (Step 4. Q-Sepharose, 14 ml). The solution pooled was mixed with ammonium sulfate at a concentration of 1.84 M, then was loaded on a column (1.5 x 5 cm) of Butyl-S Sepharose 6 Fast Flow (Amersham Biosciences, Sweden) equilibrated with 50 mM Tris-HCl (pH 7.2) containing 1.84 M ammonium sulfate. Proteins were eluted using a decreasing stepwise gradient of ammonium sulfate (1.84–0 M) at a flow rate of 1 ml/min. Fractions active with α-galactosidase were pooled (Step 5. Butyl-S Sepharose, 19.5 ml). The enzyme factions pooled were desalted and concentrated by centrifugation with a filtration tube Vivaspin 4 (Sartorius, Germany). Concentrated proteins were loaded on a column (5.2 ml) of CHT Ceramic Hydroxyapatite Type I 40 μm (Bio-Rad Laboratories, USA) equilibrated in 0.01 M K-phosphate (pH 7.2). Proteins were eluted by a stepwise gradient of the phosphate buffer (0.01–0.5 M) at a flow rate of 1 ml/min (Step 6. Hydroxyapatite, 9.9 ml).

Molecular mass determination The molecular mass of the native enzyme was determined by polyacrylamide gel electrophoresis (PAGE) without sodium dodecyl sulfate (SDS). The concentration gradient of polyacrylamide gel was 5-15% (w/v). Molecular weight marker proteins for native PAGE (HMW calibration kit, GE Healthcare, UK) were used for calibration. SDS-PAGE was carried out with 12.5% (w/v) polyacrylamide gel according to Laemmli [26]. Molecular weight marker proteins for SDS-PAGE (GE Healthcare, UK) were used for calibration. Protein bands in gels were detected by staining with Coomassie blue R-250 (Bio-Rad Laboratories, USA).

Results and Discussion

Cell growth of *B. longum* strains on gum arabic

Cell growth of some strains of *B. longum* was examined on gum arabic under anaerobic conditions. The basal medium without carbon source gave no growth of any strains of *B. longum*. All the tested strains grew well with glucose as carbon source and sedimentation of cells occurred after 8 h incubation. Gum arabic

Table 1. Enzyme activities with 4-NP-glycoside in extracts from *B. longum* JCM 7052 grown on 1% carbohydrate

Substrate	Gum arabic	Lactose	Glucose
	Enzyme activity (μmol/min/mg of protein) ± SE (3)		
4-NP α-L-Arabinofuranoside	0.444 ± 0.095	0.420 ± 0.041	0.091 ± 0.012
4-NP α-L-Arabinopyranoside	0.489 ± 0.083	0.195 ± 0.047	0.117 ± 0.003
4-NP α-D-Galactopyranoside	1.96 ± 0.059	0.404 ± 0.055	0.176 ± 0.019
4-NP β-D-Galactopyranoside	1.85 ± 0.075	0.797 ± 0.197	0.491 ± 0.046
4-NP α-D-Glucopyranoside	1.12 ± 0.116	0.864 ± 0.125	0.500 ± 0.052
4-NP β-D-fucopyranoside	0.319 ± 0.062	0.084 ± 0.008	0.148 ± 0.009

caused cell growth of strains JCM 7052 and JCM 7053, whereas there were no growth on gum arabic of the other strains JCM 1217, JCM 7055, JCM 7056, and 105-A. High turbidities of the JCM 7052 and JCM 7053 cultures were observed after 12 h and 24 h of incubation, respectively. All the tested strains failed to grow on guar gum.

Glycoside hydrolase activities in *B. longum* JCM 7052

Some glycoside hydrolases presumed to be involved in the degradation of gum arabic were determined in *B. longum* JCM 7052. Hydrolytic activities for gum arabic were observed in whole cells harvested at an early stationary phase of growth on gum arabic and in crude extracts prepared from them, to be 1.02 ± 0.060 and 1.65 ± 0.21 μmol/60 min/mg of protein, respectively. However, no activity was detected in supernatant of the culture. When 4-NP-glycosides were used as substrate, activities of α- and β-galactosidases, α-glucosidase, α-arabinofuranosidase, and α-arabinopyranosidase, were observed in crude extracts (Table 1). Among these enzymes α- and β-galactosidase activities were at highest levels when cells were grown with gum arabic, about 10- and 4-times compared with those in glucose-grown cells, respectively. Hydrolytic activities for 4-NP-glycosides were determined in the extracts from cells harvested at several stages of cell growth on gum arabic (Fig. 1). α-Galactosidase activity was high in the stationary phase of growth, and activity levels were maintained at least for 12 h in the growth phase. The highest level of β-galactosidase activity was observed in the stationary growth

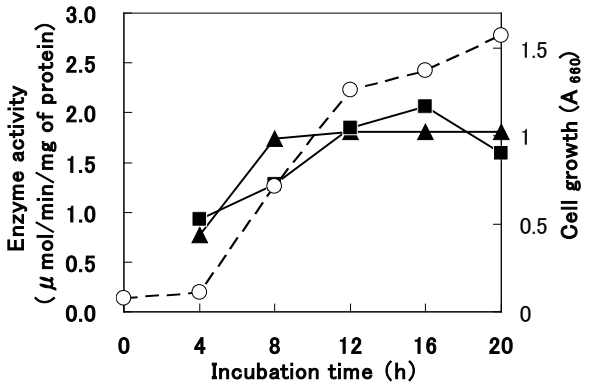

Fig 1. Enzyme activities during growth of *B. longum* JCM 7052. ○, Cell growth; ▲, 4-NP-α-D-galactosidase; and ■, 4-NP-β-D-galactosidase.

phase. Activities of the other enzymes were low during cell growth (data not shown). These findings suggested that gum arabic was hydrolyzed by unidentified enzymes on the cell surface and was further degraded by at least α- and β-galactosidases in the cytoplasm of *B. longum* JCM 7052. Some properties of crude α-galactosidase were reported in *B. longum* [22], while β-galactosidase was purified and characterized from *B. longum* CCRC 15708 [27].

Purification of α-galactosidase from *B. longum* JCM 7052

For purification of α-galactosidase (EC 3.2.1.22) from *B. longum*, the strain JCM 7052 grown on 1% gum arabic was used. The purification of α-galactosidase from *B. longum* JCM 7052 is summarized in Table 2. Q-Sepharose ion exchange chromatography showed a peak of α-galactosidase activity at 0.53 M

Table 2. Purification of α-galactosidase from *B. longum* grown on 1% gum arabic

Purification step	Protein (mg)	Total activity (units)	Specific activity (units/mg of protein)	Yield (%)
1. Crude extract	119	103	0.86	100
2. Ammonium sulfate fraction	26.4	74	2.80	72
3. Sepharose 4B	8.13	63	7.75	61
4. Q Sepharose	1.36	45	33.1	44
5. Butyl-S Sepharose	0.66	24	36.4	23
6. Hydroxyapatite	0.14	8	57.1	8

concentration of NaCl. In hydrophobic chromatography with Butyl-S Sepharose 6 Fast Flow the enzyme activity was released at 20% saturated concentration of $(NH_4)_2SO_4$ in Tris-HCl (pH 7.2). Finally, α-galactosidase was desorbed from hydroxyapatite by 0.1 M phosphate (pH 7.2). α-Galactosidase was purified 66-fold and showed a single protein as shown in Fig. 2.

Molecular mass of α-galactosidase

SDS-PAGE showed a molecular mass of α-galactosidase to be 79 kDa (Fig. 2A). Molecular mass was also determined to be 150 kDa by native PAGE (Fig. 2B). These results suggested that α-galactosidase of *B. longum* is active in dimeric form. The genome sequence of *B. longum* NCC 2705 revealed that this strain probably contains two genes encoding for α-galactosidase, BL0177 and BL1518 [11]. BL1518 consists of 768 amino acids and has molecular mass of 83 kDa. Another possible α-galactosidase BL0177 is composed of 469 amino acids and has molecular mass of 51 kDa. As far as we know, this is the first report of purification and characterization of α-galactosidase from *B. longum*. The purified α-galactosidase appeared to be a homologue of BL1518 enzyme if *B. longum* JCM 7052 had two kinds of α-galactosidase gene in its genome.

Substrate specificities of α-galactosidase

Substrate specificity of the purified α-galactosidase was investigated with 4-NP-glycoside compounds. Hydrolytic activity was observed with 4-NP-α-D-galactopyranoside, but

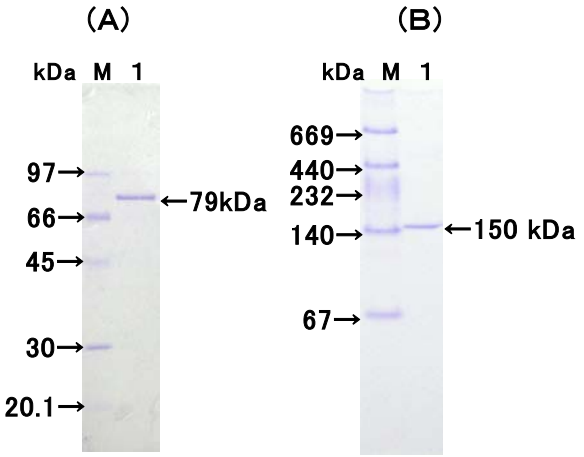

Fig 2. Molecular mass delermination of α-galactosidase by SDS-PAGE **(A)** and native gradient PAGE **(B)**. Lane M, marker proteins; lane 1, purified enzyme. Proteins were stained by Coomassie blue R-250.

no activity with other 4-NP-α-glycosides, 4-NP-α-D-glucopyranoside, 4-NP-α-D-mannopyranoside, 4-NP-α-L-rhamnopyranoside, 4-NP-α-L-fucopyranoside, 4-NP-α-L-arabinopyranoside, and 4-NP-α-L-arabinofuranoside. 4-NP-β-D-galactopyranoside and 4-NP-β-D-fucopyranoside gave no enzyme activity. Michaelis constant K_m was determined by double reciprocal plots to be 0.15 mM for 4-NP-α-D-galactopyranoside. Maximal velocity (V_{max}) was also shown to be 5.1 μmol/min/mg of protein.

Raffinose and stachyose were also hydrolyzed by α-galactosidase. K_m values were determined to be 88.3 and 126 mM for raffinose and stachyose, respectively. Values of V_{max} were 45.1 and 60.3 μmol/min/mg of protein in the

reaction with raffinose and stachyose, respectively. This enzyme hydrolyzed melibiose to galactose and glucose, those products were detected by HPTLC (data not shown). No reducing sugar was produced from 0.5% gum arabic during 1 h incubation with the enzyme (7.2 mU of 4-NP-α-galactopyranoside hydrolytic activity) at 37°C and pH 7.0.

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

The optimal pH was assayed by incubating the purified enzyme with 4-NP-α-galactopyranoside in 80 mM buffers ranging from pH 5.0 to pH 9.5. The highest activity of α-galactosidase was obtained at pH 8.0 in 80 mM glycine-NaOH buffer (Fig. 3). pH dependency of α-galactosidase activity was also investigated in extracts prepared from the other species of *B. longum*. All strains tested JCM 1217, JCM 7055, and 105-A contained α-galactosidase activity and showed maximal activity at pH 8.0 (data not shown). These findings indicated that *B. longum* had an α-galactosidase operating optimally in semialkaline environments, and this enzyme is distinct from the α-galactosidase active at pH 5.8 [22]. α-Galactosidases have been purified from the other three species of *Bifidobacterium* [23-25], and their optimal activities are found at pH 5.5-6.0. When α-galactosidase activity was assayed in Tris-HCl buffers, activities observed at pH 6.5–9.0 were lower than the activities observed in the other buffers (Fig. 3). Double reciprocal plots showed that the enzyme was inhibited competitively by tris (hydroxymethyl) aminomethane (Fig. 4), and its inhibition constant K_i was estimated to be 32 mM in Dixon plot.

The optimal temperature for α-galactosidase was 40–45°C at pH 7.5 in a 5 min reaction (Fig. 5A), but a remarkable decline in activity was observed during 5 h incubation at 40–45°C and its instability was protected partly by 0.15 and 0.3 M NaCl (Fig. 5B). However, the enzyme activity was decreased by 30% after 30 min of incubation at 50°C and was not maintained even in the presence of 0.15 M NaCl. The enzyme activity in the presence of 0.15 M NaCl was

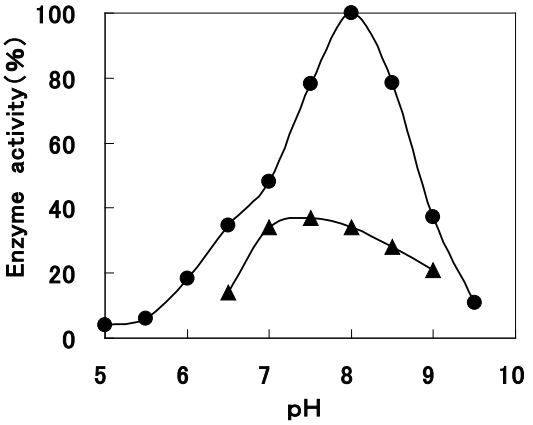

Fig 3. Effects of pH on activity of α-galactosidase purified from *B. longum* JCM 7052. ●, Buffers used were 80 mM acetate (pH 5.0–5.5), phosphate (pH 6.0–7.0), and glycine-NaOH (pH 7.5–9.5). ▲, 80mM Tris-HCl (pH 6.5–9.0)

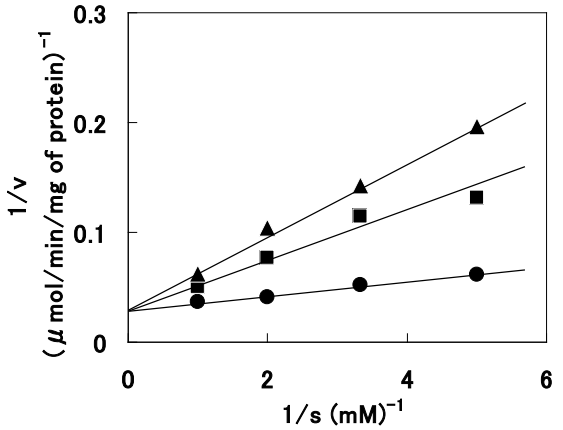

Fig 4. Inhibitory effects of Tris on activity of the purified α-galactosidase. Double reciprocal plots of α-galactosidase activity against 4-NP-α-D-galactopyranoside in the presence of Tris-HCl. For the enzyme activity assay, K-phosphate buffer (pH7.5) was used. The concentrations of Tris were 0 (●), 60 (■), and 120 mM (▲).

inhibited about 10% (data not shown). Though no attempt has been done to examine effects of NaCl on thermostability of α-galactosidase as far as we know, the stabilization of enzyme would endow it with great industrial potential.

To determine the effects of chemicals, enzyme activities were measured in the presence of 1 mM metal chloride salts or EDTA. α-Galactosidase activity was inhibited about 40% by Cu^{2+} and about 10–15% by Fe^{2+}, Mg^{2+}, Mn^{2+}, and Co^{2+}.

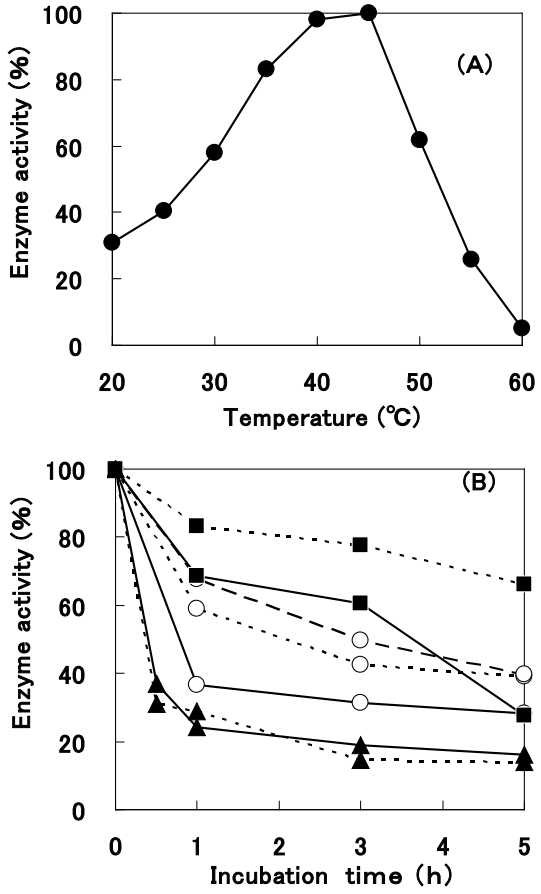

Fig 5. Effects of temperature on the activity (A) and stability (B) of the purified α-galactosidase. In (B), ■, 40℃; ○, 45℃; and ▲, 50℃. Solid lines indicate activities in the absence of NaCl, dotted lines in the presence of 0.15 M NaCl, broken line in the presence of 0.3 M NaCl.

EDTA was less active as a chelating reagent for α-galactosidase activity. Contrary to our results, Garro et al. [22] reported that Mn^{2+} showed a positive effect on activity of the acidic α-galactosidase in crude extracts from *B. longum* CRL 849.

Transgalactosylation activity of the enzyme

α-Galactosidase is known to have transgalactosylation activity [24, 25, 28, 29]. The purified α-galactosidase was briefly tested to have galactosyl transferring activity. HPTLC showed a new saccharide in the reaction mixture containing 4-NP-α-D-galactopyranoside and galactose (data not shown). A similar result was also obtained by mixing 4-NP-α-D-galctopyranoside and melibiose. However, we could not identify products of transgalactosylation. The evaluation of the reaction conditions will be the subject of further investigation.

In this study *B. longum* JCM 7052 was found to be able to grow on gum arabic as a carbon and energy source. α- and β-Galactosidase activities were observed at higher levels in extracts from cells grown on gum arabic compared with those in cells grown on glucose. This probably suggests that α- and β-galactosidases play some roles in degradation of gum arabic. The subunit of the α-galactosidase had the molecular mass of 79 kDa, suggesting that the purified α-galactosidase appeared to be a homologue of BL1518 enzyme in *B. longum* NCC 2705 [11]. The purified enzyme seemed to be a semialkaline α-galactosidase which showed an optimal activity at pH 8.0, though acid α-galactosidases have been found in the other species of *Bifidobacterium* [22-25]. It is necessary to find a gene encoding the semialkaline α-galactosidase and to clarify the role of the enzyme in metabolism of gum arabic in *B. longum* JCM 7052.

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