

*Article*

Purification and characterization of  $\alpha$ -galactosidase I from  
*Bifidobacterium longum* subsp. *longum* JCM 7052

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Received November 18, 2009; Accepted January 18, 2010.

$\alpha$ -Galactosidase I was purified and characterized from *Bifidobacterium longum* subsp. *longum* JCM 7052 grown on glucose. It had a molecular mass of 162 kDa and consisted of two same subunits. The molecular mass of the subunit was 85 kDa. MALDI-TOF-MS showed that this enzyme is a homologue of BLD1483 and BL1518 found in the genomic sequences of *B. longum* subsp. *longum* strains DJO10A and NCC 2705, respectively.  $\alpha$ -Galactosidase I showed optimal activity at pH 7.5 and 45°C. Thermal stability was lost during 5 h incubation at 50°C, and was partly protected in the presence of 0.15 M NaCl. The  $K_m$  values for 4-nitrophenyl- $\alpha$ -D-galactopyranoside, raffinose, and stachyose were 0.34, 15.5, and 39.3 mM, respectively. The enzyme activity was inhibited competitively by Tris (hydroxymethyl) aminomethane ( $K_i = 24$  mM). Transglycosylation activity was also observed from 4-nitrophenyl- $\alpha$ -D-galactopyranoside to galactose, arabinose, glucose, mannose, sucrose, melibiose, and galactinol.

**Keywords:**  $\alpha$ -galactosidase, purification, *Bifidobacterium longum* subsp. *longum*

**Introduction**

$\alpha$ -Galactosidases (EC 3.2.1.22) catalyze the hydrolysis of the terminal  $\alpha$ -linked galactoside residues from  $\alpha$ -galactosides including melibiose, raffinose, stachyose, galactomannanes, and galactanes [1].  $\alpha$ -Galactosidases are widely distributed in microorganisms, plants, animals, and humans [2-7]. The enzymes are known to have transglycosylation activities, which are expected to be powerful tools for oligosaccharide synthesis [8].

Bifidobacteria, predominant members of intestinal microbiota in humans [9], have  $\alpha$ -galactosidase activities to use the variety of  $\alpha$ -galactosides as energy source.  $\alpha$ -Galactosidases have been isolated either as native or recombinant enzymes from *Bifidobacterium adolescentis* DMS 20083 [8, 10, 11], *Bifidobacterium breve* 203 [12], *Bifidobacterium bifidum* NCIBM 41171 [13], and *Bifidobacterium longum* subsp. *longum* JCM 7052 [14]. These enzymes evidently showed transgalactosylation activities able to synthesize galacto-oligosaccharides.

*B. longum* subsp. *longum* JCM 7052 has been found to grow on gum arabic [14]. A high activity of  $\alpha$ -galactosidase is observed in cells

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grown on gum arabic, and the purified enzyme,  $\alpha$ -galactosidase X, shows optimal activity at pH 8.0. The cells grown on glucose also contain  $\alpha$ -galactosidase but its activity is one tenth of that obtained in the cells grown on gum arabic. To learn whether these activities could be attributed to different enzymes, we partially purified  $\alpha$ -galactosidase from the cells grown on glucose. This enzyme,  $\alpha$ -galactosidase I, showed weak binding affinity for Q-Sepharose compared to  $\alpha$ -galactosidase X, suggesting the two enzymes are different proteins. In the present study  $\alpha$ -galactosidase I was purified and characterized from *B. longum* subsp. *longum* JCM 7052 grown on glucose. Identification of the gene encoding for each enzyme was attempted by means of MALDI-TOF-MS.

### Materials and Methods

**Chemicals** 4-Nitro-phenyl (4-NP) glycosides, melibiose, stachyose, and 3,5-dinitrosalicylic acid were purchased from Sigma-Aldrich Co. (St. Louis, USA). Other chemicals were reagent grade products of Wako Pure Chemical Industries (Osaka, Japan).

#### *Bacterial strain and growth conditions*

*Bifidobacterium longum* subsp. *longum* JCM 7052 was used in this study. The strain was anaerobically grown at 37°C on Bifidobacterium medium, which contained (g/l) Polypepton (Nihon Seiyaku, Japan), 10; extract from bonito (Wako Pure Chemical Industries), 5; yeast extract (Oriental Yeast, Japan), 5; glucose, 10; K<sub>2</sub>HPO<sub>4</sub>, 3; sodium ascorbate, 10; L-cysteine HCl, 0.5; and Tween 80, 1 ml/l. pH was adjusted to 6.8 with 2 N NaOH. Anaerobic growth conditions were attained by flashing CO<sub>2</sub> for 3-5 min to butyl rubber-stoppered tubes or by filling the neck of screw-capped bottles with the medium. The medium was inoculated with 2% volume of an overnight culture in the same medium.

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** Standard assay of  $\alpha$ -galactosidase activity was performed with 4-nitrophenyl (4-NP)- $\alpha$ -galactopyranoside as substrate. The reaction mixture (1 ml) contained an enzyme preparation, 80 mM K-phosphate (pH 7.0) and 1 mM 4-NP- $\alpha$ -galactopyranoside. The reactions were started by adding an enzyme preparation. After incubation at 30°C for 10 min the reactions were terminated by the addition of 0.5 ml of 0.2 M Na<sub>2</sub>CO<sub>3</sub>. The amount of 4-nitrophenol released was determined at 400 nm with an extinction coefficient of 18.3 mM<sup>-1</sup> cm<sup>-1</sup>. One unit of enzyme activity is defined as the amount of enzyme that releases 1  $\mu$ mol of 4-nitrophenol per min from 4-NP- $\alpha$ -galactopyranoside.

For assay of enzyme activities which hydrolyze oligosaccharides, reducing sugar produced from oligosaccharide was determined in the reaction mixtures containing 80 mM K-phosphate (pH 7.0), enzyme preparation, and 100 mM raffinose or stachyose. After incubation at 30°C for 60 min, 3,5-dinitrosalicylic acid was added to stop the reaction. The absorbance was measured at 540 nm. Galactose was used as the standard for calibration. One unit of enzyme activity is defined as the amount of enzyme that produces 1  $\mu$ mol of reducing sugar per min from the oligosaccharide.

Transglycosylation activity was assayed by detection of product in TLC with Silica gel 60 HPTLC plates (Merck Ltd., Germany). The reaction mixtures (1 ml) contained 80 mM K-phosphate buffer (pH 7.0), 15 mM 4-NP- $\alpha$ -D-galactopyranoside, 80 mM saccharide, and 0.2 units of purified  $\alpha$ -galactosidase I, and were incubated for 24 h. Saccharides were separated in *n*-butanol:acetic acid:water (5:4:1), and were detected with spray of 5% sulfuric acid in ethanol.

Thermal stability was assayed by incubating purified  $\alpha$ -galactosidase I at 40, 45, 50 and 60°C and drawing aliquots at intervals. The enzyme activity was then determined by the standard assay method described above.

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  $\alpha$ -galactosidase I** Wet cells (30 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  $\times g$  for 30 min at 4°C. The supernatant was used as crude extract (Step 1. Crude extract, 120 ml). The precipitate obtained between 30 and 70% saturation of ammonium sulfate was dissolved in 50 mM Tris-HCl, pH 7.2 (Step 2. Ammonium sulfate fraction, 7 ml). Proteins dissolved were loaded on a column (2.5  $\times$  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  $\alpha$ -galactosidase activity were pooled (Step 3. Sepharose 4B, 98 ml). The pooled fractions were loaded on a column (1.5  $\times$  7 cm) of Q-Sepharose (GE Healthcare, Sweden) equilibrated in 50 mM Tris-HCl (pH 7.2) containing 0.2 M NaCl. Proteins were eluted at a flow rate of 0.4 ml/min with a linear concentration gradient of NaCl (0.2-0.7 M). Fractions containing  $\alpha$ -galactosidase activity were pooled (Step 4. Q-Sepharose, 23 ml). The solution pooled was mixed with ammonium sulfate at a concentration of 1.84 M, then was loaded on a column (1.5  $\times$  7 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  $\alpha$ -galactosidase were pooled (Step 5. Butyl-S Sepharose, 4.9 ml). The enzyme pooled was desalted and concentrated by centrifugation with a filtration tube Vivaspin 4 (Sartorius, Germany). Concentrated proteins were loaded on 6% polyacrylamide gel electrophoresis (PAGE) without sodium dodecyl sulfate (SDS). An area stained with  $\alpha$ -galactosidase 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 6. Native PAGE, 1.3 ml).

**Molecular mass determination** The molecular mass of the native enzyme was determined by concentration gradient PAGE without SDS. The concentration gradient of polyacrylamide gel was 5-15% (w/v). A molecular weight marker set (NativeMark Unstained Protein Standard, Invitrogen, USA) was used for calibration. SDS-PAGE was carried out with 12.5% (w/v) polyacrylamide gel according to Laemmli [15]. Molecular weight marker proteins for SDS-PAGE (GE Healthcare, UK) were used for calibration. Protein bands in gels were stained with Coomassie blue R-250 (Bio-Rad Laboratories, USA).

**Protein identification by mass spectrometry** The proteins in the SDS-PAGE gel were digested using sequence-grade modified trypsin (Promega, Madison, USA) using the procedures of Tanaka *et al.* [16], with slight modifications. The identification of each protein was carried out by peptide mass fingerprinting (PMF) analysis using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) and tandem mass spectrometry (MS/MS), using an Ultraflex MS system (Bruker Daltonik GmbH, Bremen, Germany). The PMF results were used to search the NCBI protein database using the Mascot Search engine. The PMF result and one MS/MS result were combined to make the results of the search reliable.

## Results

### Purification of $\alpha$ -galactosidase I

The cells grown on glucose of *Bifidobacterium longum* subsp. *longum* JCM 7052 possess  $\alpha$ -galactosidase activity [14]. Q-Sepharose ion exchange chromatography showed two peaks of  $\alpha$ -galactosidase activity in extracts of the cells which were grown in a stationary phase of 12 h incubation (Fig. 1A).

**Table 1.** Purification of  $\alpha$ -galactosidase I from *B. longum* subsp. *longum* JCM 7052 grown on 1% glucose

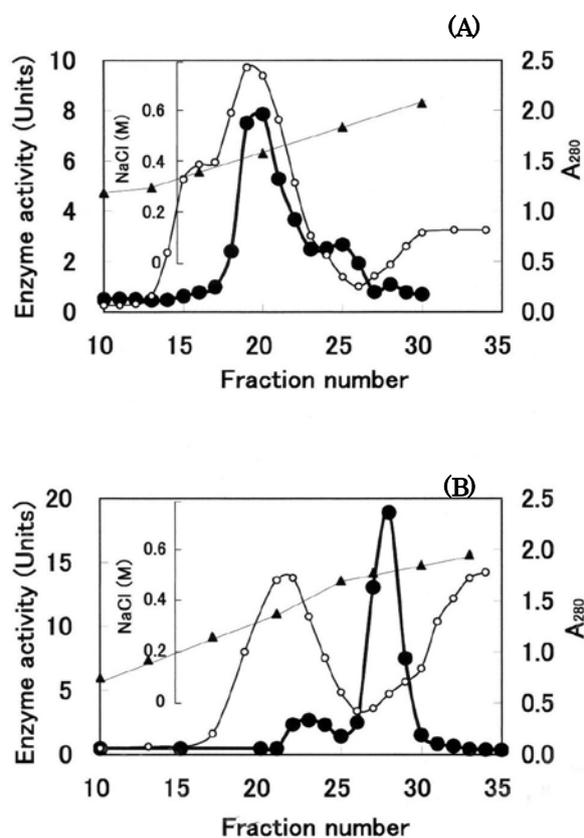
Purification step	Protein (mg)	Total activity (units)	Specific activity (units/mg of protein)	Yield (%)
1. Crude extract	310	40.2	0.130	100
2. Ammonium sulfate fraction	198	30.5	0.154	76
3. Sepharose 4B	150	26.9	0.180	67
4. Q Sepharose	41.9	18.5	0.442	46
5. Butyl-S Sepharose	3.90	4.8	1.22	12
6. Native PAGE	0.18	3.1	17.8	8

The major and minor peaks were eluted at 0.43 and 0.53 M NaCl, respectively. The latter peak of the activity seemed to be the same as the enzyme of  $\alpha$ -galactosidase which was inducible by gum arabic (Fig. 1B). The inducible  $\alpha$ -galactosidase has been purified and characterized from the same strain grown on gum arabic [14]. We, therefore, attempted to purify another  $\alpha$ -galactosidase from the cells grown on 1% glucose. We call the enzyme purified in this study  $\alpha$ -galactosidase I, and the previously purified enzyme  $\alpha$ -galactosidase X.

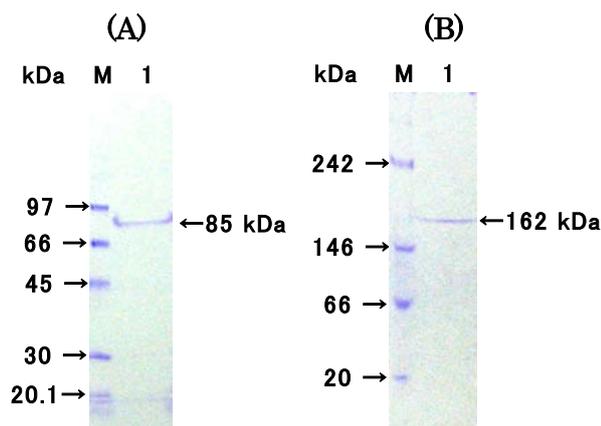
$\alpha$ -Galactosidase I was purified to an electrophoretically single band, and an overall yield of 8% was obtained (Table 1). The enzyme, which was eluted at 0.43 M NaCl from Q-Sepharose, was released by 0.5 M ammonium sulfate in hydrophobic chromatography with Butyl-S Sepharose 6 Fast Flow. In the last step we used native PAGE for separation of the enzyme, and the obtained preparation contained only a single protein, which was confirmed by SDS-PAGE.

#### Determination of the molecular mass of $\alpha$ -galactosidase I

Molecular mass of  $\alpha$ -galactosidase I was determined by PAGE with and without SDS. SDS-PAGE showed the molecular mass of  $\alpha$ -galactosidase I to be 85 kDa (Fig. 2A). The molecular mass was also determined to be 162 kDa by native PAGE (Fig. 2B). These results indicate that  $\alpha$ -galactosidase I consists of two same subunits. These values of molecular mass are obviously different from those of the



**Fig. 1.** Ion exchange chromatography on Q Sepharose of  $\alpha$ -galactosidases from *B. longum* subsp. *longum* JCM 7052. Enzyme preparations of  $\alpha$ -galactosidase obtained by gel filtration with Sepharose 4B were applied on columns (1.5  $\times$  5 cm) of Q Sepharose and eluted with an NaCl gradient in 50 mM Tris-HCl, pH 7.2. (A), an enzyme preparation obtained from cells grown on 1% glucose; (B), from cells grown on 1% gum arabic. ●,  $\alpha$ -galactosidase activity; ○, absorbance at 280 nm; △, concentrations of NaCl. Each fraction contained 2.4 ml.



**Fig. 2.** Molecular mass determination of  $\alpha$ -galactosidase I by SDS-PAGE (A) and native gradient PAGE (B). Lane M shows marker proteins, and lane 1 purified enzyme. Proteins were stained by Coomassie blue R-250.

previously purified enzyme  $\alpha$ -galactosidase X (79 and 150 kDas [14]).

#### Identification of $\alpha$ -galactosidase I by mass spectrometry

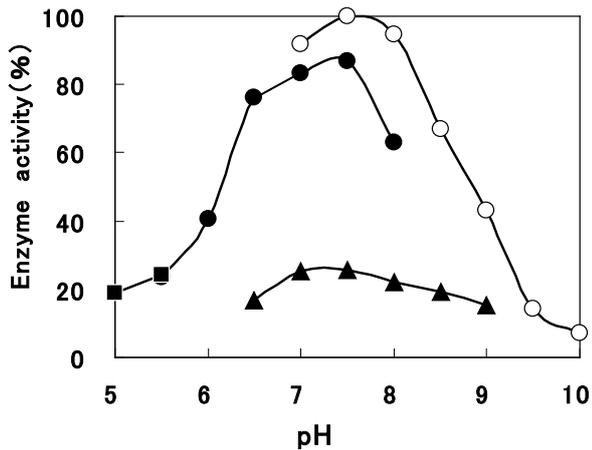
We have identified the purified enzyme by means of MALDI-TOF-MS as described in the Materials and Methods. The PMF result was used to search the NCBI protein database using the Mascot Search engine. The PMF result (Accession number, gi|46190700; Sequence coverage, 33.1%; score, 153) and two MS/MS results (1714.891 (535-551), TAHPGLEIESCSSG GGR; score, 111) and (1917.171 (747-763), YGIRPPSLHPQQAVLLK; score, 97) were combined and searched again. Both amino acid sequences were coincided with those in  $\alpha$ -galactosidase (Accession number, gi|46190700; combined score, 303), which has been found and given the locus tag BLD 1483 in the genomic sequence of *B. longum* subsp. *longum* DJO10A [17].  $\alpha$ -Galactosidase I was, therefore, identified as a homologue of BLD 1483 found in *B. longum* subsp. *longum* DJO10A. This  $\alpha$ -galactosidase consists of 763 amino acids and has the molecular mass of 83,309 Da. This value of the molecular mass is consistent with that of 85 kDa determined by SDS-PAGE. The BLD 1483  $\alpha$ -galactosidase belongs to the glycosyl hydrolase (GH) 31 family.

#### Substrate specificities of $\alpha$ -galactosidase I

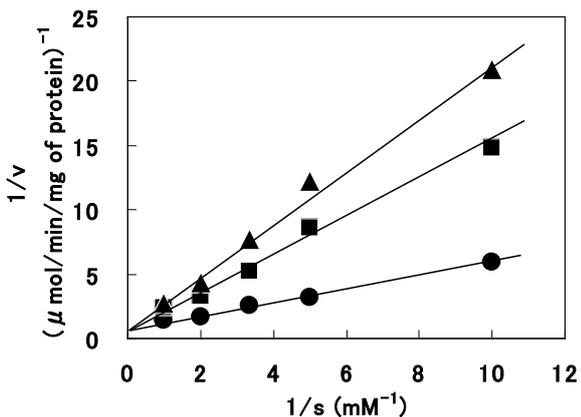
Substrate specificity of the purified  $\alpha$ -galactosidase I was investigated with 4-NP-glycoside compounds. Hydrolytic activity was observed with 4-NP- $\alpha$ -D-galactopyranoside, but no activity with other 4-NP- $\alpha$ -glycosides, 4-NP- $\alpha$ -D-glucopyranoside, 4-NP- $\alpha$ -D-mannopyranoside, 4-NP- $\alpha$ -L-rhamnopyranoside, 4-NP- $\alpha$ -L-fucopyranoside, 4-NP- $\alpha$ -L-arabinopyranoside, and 4-NP- $\alpha$ -L-arabinofuranoside. 4-NP- $\beta$ -D-galactopyranoside and 4-NP- $\beta$ -D-fucopyranoside gave no enzyme activity. The  $K_m$  and  $V_{max}$  values for 4-NP- $\alpha$ -D-galactopyranoside were determined by double reciprocal plots to be 0.34 mM and 75.8  $\mu$ mol/min/mg of protein, respectively. Raffinose and stachyose were also hydrolyzed by  $\alpha$ -galactosidase I. The  $K_m$  values for raffinose and stachyose were determined to be 15.5 and 39.3 mM, respectively. The  $V_{max}$  values were 22.8 and 28.8  $\mu$ mol/min/mg of protein in the reaction with raffinose and stachyose, respectively. In previous study,  $\alpha$ -galactosidase X showed the  $K_m$  values higher than 80 mM for raffinose and stachyose [14]. The affinity of  $\alpha$ -galactosidase I for these  $\alpha$ -galactosides appeared to be higher than that of  $\alpha$ -galactosidase X.

#### Effects of pH, temperature, and chemicals on enzyme activity

The optimal pH was assayed by incubating the purified  $\alpha$ -galactosidase I with 4-NP- $\alpha$ -galactopyranoside in 80 mM buffers ranging from pH 5.0 to pH 10.0. The highest activity of  $\alpha$ -galactosidase was obtained at pH 7.5 in 80 mM glycine-NaOH buffer (Fig. 3). When  $\alpha$ -galactosidase activity was determined in Tris-HCl buffers (pH 6.5–9.0), the activities were lower than those observed in the other buffers (Fig. 3). The enzyme activities determined in 80 mM K-phosphate, pH 7.5 were lowered by 60% in the presence of 60 mM Tris-HCl, pH 7.5. 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 24 mM in Dixon plot.

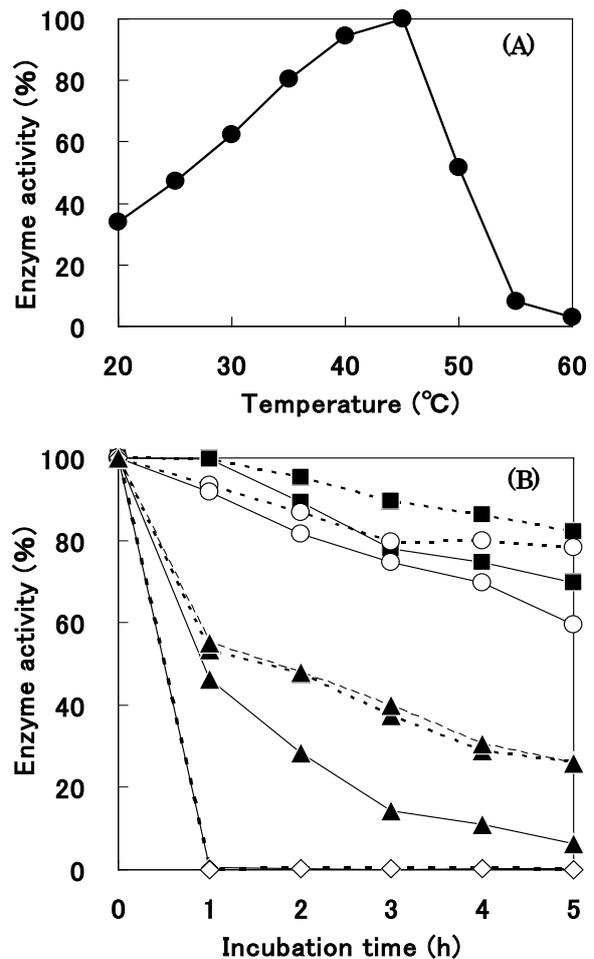


**Fig. 3.** Effects of pH on activity of the purified  $\alpha$ -galactosidase I. Buffers used were at 80 mM. ■, Na-acetate (pH 5.0–5.5); ●, K-phosphate (pH 5.5–8.0); ○, glycine-NaOH (pH 7.0–10.0); and ▲, Tris-HCl (pH 6.5–9.0).



**Fig. 4.** Inhibitory effects of Tris (hydroxymethyl) aminomethane on activity of the purified  $\alpha$ -galactosidase I. Double reciprocal plots of  $\alpha$ -galactosidase activity against 4-NP- $\alpha$ -D-galactopyranoside in the presence of Tris-HCl (pH 7.5). For the enzyme activity assay, K-phosphate buffer (pH 7.5) was used. The concentrations of Tris were 0 (●), 60 (■), and 120 mM (▲).

The optimal temperature for  $\alpha$ -galactosidase activity was 45°C at pH 7.5 in a 10 min reaction (Fig. 5A), but the enzyme activity was partly lost during 5 h incubation at 45°C. The enzyme was almost completely inactivated during 5 h incubation at 50°C and its stability was partly protected by 0.15 M NaCl (Fig. 5B). The enzyme



**Fig. 5.** Effects of temperature on the activity (A) and stability (B) of the purified  $\alpha$ -galactosidase I. In (B), ■, 40°C; ○, 45°C; ▲, 50°C; and ◇, 60°C. Solid lines indicate activities in the absence of NaCl, dotted lines in the presence of 0.15 M NaCl, and a broken line in the presence of 0.3 M NaCl.

activity was decreased by 30% after 30 min of incubation at 60°C and was not maintained even in the presence of 0.15 M NaCl. Thermostabilizing effects of NaCl on  $\alpha$ -galactosidase I would endow it with great industrial potential.

The effects of chemicals on the enzyme activity were also determined in the presence of 1 mM metal chloride salts or EDTA (Table 2).  $\alpha$ -Galactosidase I activity was inhibited about 20% by  $\text{Cu}^{2+}$ . However, other metal ions  $\text{Fe}^{3+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Mn}^{2+}$ , and  $\text{Co}^{2+}$  showed almost no effects on the enzyme. EDTA also worked less actively as a chelating reagent for  $\alpha$ -galactosidase activity. These results are different from those observed

**Table 2.** Effects of some reagents on the activity of  $\alpha$ -galactosidase I from *B. longum* subsp. *longum* JCM 7052.

Reagents (1 mM)	Relative activity (%)
None	100
BaCl <sub>2</sub>	98
CaCl <sub>2</sub>	103
CoCl <sub>2</sub>	104
CuCl <sub>2</sub>	83
FeCl <sub>3</sub>	105
MgCl <sub>2</sub>	105
MnCl <sub>2</sub>	106
NiCl <sub>2</sub>	105
ZnCl <sub>2</sub>	109
EDTA	98

in  $\alpha$ -galactosidase X, which was slightly inhibited about 40% and 10-15% by Cu<sup>2+</sup> and the other ions, respectively [14]. No activation by Mn<sup>2+</sup> was observed with our enzyme, although Garro *et al.* [19] reported that Mn<sup>2+</sup> showed a positive effect on activity of the acidic  $\alpha$ -galactosidase in crude extracts from *B. longum* CRL 849.

#### Transgalactosylation activity of $\alpha$ -galactosidase I

Most  $\alpha$ -galactosidases often have transgalactosylation activity [1, 8]. The purified  $\alpha$ -galactosidase I was briefly tested to have galactosyl transferring activity. TLC showed a new saccharide in the reaction mixture containing 4-NP- $\alpha$ -D-galactopyranoside and galactose (data not shown). Similar results were also obtained by mixing 4-NP- $\alpha$ -D-galactopyranoside with arabinose, glucose, mannose, sucrose, melibiose, and galactinol. However, we could not identify products of transgalactosylation.

#### Discussion

In this study *B. longum* subsp. *longum* JCM 7052 was found to possess at least two kinds of  $\alpha$ -galactoside hydrolase,  $\alpha$ -galactosidases I and X. MALDI-TOF-MS analysis showed that  $\alpha$ -galactosidase I is a homologue of BLD1483,

which is found in the genome of *B. longum* subsp. *longum* strains DJO10A [17]. Almost the same amino acid sequence in BLD 1483 has been found in the deduced gene product BL1518 in the genome of *B. longum* subsp. *longum* NCC2705 [18].  $\alpha$ -Galactosidase I seemed to serve in degradation of galactosides having  $\alpha$ -1,6-linkage because this enzyme showed apparently high affinity for raffinose and stachyose. Moreover, BL1518 has been shown to be induced when *B. longum* subsp. *longum* NCC 2705 was grown on raffinose [19]. On the other hand,  $\alpha$ -galactosidase X was synthesized at high levels when *B. longum* subsp. *longum* JCM 7052 was grown on gum arabic as a carbon source [14]. However, physiological roles of  $\alpha$ -galactosidase X are not clear because  $\alpha$ -1,4- and  $\alpha$ -1,6-linked galactoses are not found in gum arabic. No homologue of  $\alpha$ -galactosidase X was found in genomic data compiled so far (unpublished results). This may suggest that *B. longum* subsp. *longum* JCM 7052 would have some additional genes specific for degradation of gum arabic and  $\alpha$ -galactosidase X would be one of products of those genes. Isolation of genes encoding some enzymes and proteins involved in degradation of gum arabic is now in process with probes designated on the basis of amino acid sequences found in  $\alpha$ -galactosidase X.

$\alpha$ -Galactosidase I showed the optimal activity around neutral pH of 7.5 as  $\alpha$ -galactosidase X had done so at pH 8.0 [14]. Such a property of each enzyme is different from that of an  $\alpha$ -galactosidase partially purified from *B. longum* CRL 849 showing highest activity at pH 5.8 [20].  $\alpha$ -Galactosidases so far purified from the other species of *Bifidobacterium* are most active at pH 5.5-6.0 [8, 10-13], though the highest transgalactosylation activity of the *B. adolescentis* enzyme is obtained at pH 7.0-8.0 [8, 11]. In the CaZy database  $\alpha$ -galactosidases cloned from these *Bifidobacterium* species [11-13] including BL1518/BLD1483 [17, 18] are classified into the GH31 family (<http://www.cazy.org/fam/GH31.html>). Alignment of the amino acid sequences of  $\alpha$ -galactosidases from four species of *Bifidobacterium* shows that the amino acid sequences are homologous with 65-69% identity [12]. The catalytic mechanism of

the GH31 family is less clear, though two aspartic residues are confirmed to be involved in the catalytic site of  $\alpha$ -galactosidase from *Thermotoga maritima* [21]. Catalytic and structural studies are necessary to identify the amino acid residues which cause the alteration of pH optima.

$\alpha$ -Galactosidase I activity was competitively inhibited by Tris as was  $\alpha$ -galactosidase X [14]. Actions of Tris have been observed in some enzymes. Sucrase activity in intestinal brush border is also competitively inhibited by Tris [22]. Vesseur et al [22] have interpreted the inhibitory effect in terms of a mechanistic model in which Tris binds at two distinct sites in the enzyme's active center. Binding at the glucosyl sub-site occurs through the free hydroxyl groups of Tris. This positioning facilitates the interaction between the amino group of Tris and a proton donor in the enzyme's active center. In this way the competitive inhibition of sucrose is achieved. Tris has been recently suggested to be a competitive inhibitor of starch hydrolyzing activity of *Bacillus licheniformis*  $\alpha$ -amylase, and to have a high tendency to bind the enzyme active site [23].  $\beta$ -Galactosidase partially purified from *B. longum* subsp. *longum* JCM 7052 also showed low activities in Tris-HCl buffer compared with those observed in phosphate buffer (unpublished results). These findings suggest that Tris (hydroxymethyl) amino-methane would be effective as a competitive inhibitor in catalysis of some glycosidases.

$\alpha$ -Galactosidase I showed transgalactosylation activity from 4-NP- $\alpha$ -D-galctopyranoside to glucose, mannose, sucrose, melibiose, and galactinol.  $\alpha$ -Galactosidases have been known to hydrolyze  $\alpha$ -1,6-linkage of galactosides, and to produce new  $\alpha$ -1,6-galactosides by means of transgalactosylation activity [1, 8]. Recently, Zhao et al [12] showed that  $\alpha$ -galactosidase purified from *Bifidobacterium breve* 203 synthesizes galactosyl- $\alpha$ -1,4-linked compound, galactosyl- $\alpha$ -1,4-galactosyl- $\alpha$ -1,6-glucose from melibiose and 4-NP- $\alpha$ -D-galctopyranoside. However, we could not identify the products of transgalactosylation. The evaluation of reaction conditions and product identification will be the

subject of further investigation.

Due to enhanced solubility and reaction rates, most industrial enzymatic processes are carried out at elevated temperatures. Thermal inactivation is, therefore, the most frequently encountered cause of enzyme inactivation. We showed that  $\alpha$ -galactosidase I could be stabilized against heat inactivation by adding 0.15-0.3 M NaCl (Fig. 5B). NaCl is one of salts which have effects to stabilize proteins by reducing the solubility of hydrophobic residues on the protein molecule and by enhancing water clusters around the protein [24]. However, such properties of NaCl appeared not to be effective on stabilization of proteins at temperatures higher than 50°C.

#### Acknowledgement

The authors would like to thank Ann B. Cary for her critical reading of this paper. This research was supported partly by a grant (No. kr51421702) from Yukiyoishi Institute.

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Communicated by Ito Susumu