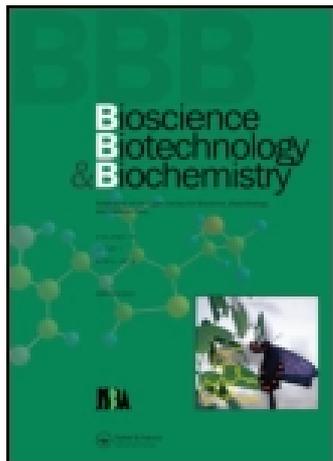


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## Purification and Characterization of Thermostable $\alpha$ -Galactosidase from *Ganoderma lucidum*

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$\alpha$ -Galactosidase was purified from a fresh fruiting body of *Ganoderma lucidum* by precipitation with ammonium sulfate and column chromatographies with DEAE-Sephadex and Con A-Sepharose. The purified enzyme was homogeneous on polyacrylamide gel electrophoresis. Its N-terminal amino acid sequence was similar to that of *Mortierella vinacea*  $\alpha$ -galactosidase. The molecular mass of the enzyme was about 56 kDa by SDS-polyacrylamide gel electrophoresis, and about 249 kDa by gel filtration column chromatography. The optimum pH and temperature were 6.0 and 70°C, respectively. The enzyme was fully stable to heating at 70°C for 30 min. It hydrolyzed *p*-nitrophenyl- $\alpha$ -D-galactopyranoside ( $K_m = 0.4$  mM) but hydrolyzed little *o*-nitrophenyl- $\alpha$ -D-galactopyranoside. It also hydrolyzed melibiose, raffinose, and stachyose. The enzyme catalyzed the transgalactosylation reaction which synthesized melibiose. The product was confirmed by various analyses.

**Key words:**  $\alpha$ -galactosidase; *Ganoderma lucidum*; thermostable enzyme; transglycosylation; galactooligosaccharide

*Ganoderma lucidum* is a mushroom traditionally used in oriental medicine because of its complex polysaccharides which are known to stimulate the immune response. Several polysaccharides obtained from fruiting bodies of *G. lucidum* were reported to have anti-tumor activity and consist of glucose, galactose, mannose, and fucose.<sup>1,2</sup> This mushroom is known as the producer of ganoderan C, which is a peptidoglycan with hypoglycemic potency. Its glycan is composed of D-glucose (69.6% of peptidoglycan) and D-galactose (2.9% of peptidoglycan), and physico-chemical and chemical studies demonstrated that the backbone and side chains of ganoderan C contained D-glucopyranosyl  $\beta$ -1 $\rightarrow$ 3 and  $\beta$ -1 $\rightarrow$ 6 linkages and a D-galactopyranosyl  $\alpha$ -1 $\rightarrow$ 6 linkage.<sup>3</sup>

Previous studies of *G. lucidum* have mainly concentrated on the medicinal properties of this mushroom. Limited information is available on the activities of the enzymes which modify oligo- and polysaccharides present in the mushroom, though preliminary reports<sup>4-7</sup> have shown that *G. lucidum* used galactose as the best carbon source for producing potentialized ganoderan having anti-tumor and anti-complementary activity. We found that cultivated *G. lucidum* produced a wide variety of glycosidases, and had strong  $\alpha$ -galactosidase activity.

$\alpha$ -Galactosidase (EC 3.2.1.22) is known to catalyze the hydrolysis of the terminal  $\alpha$ -linked galactoside residues from  $\alpha$ -O-galactosides including galacto-containing polysaccharides such as galactomannans and galacto(gluco)mannans.<sup>8,9</sup> Despite the hydrolytic activity,  $\alpha$ -galactosidase is powerful for oligosaccharide synthesis by transglycosylation, and also by reverse hydrolysis.<sup>10-13</sup> Therefore, the mechanism for producing polysaccharides of *G. lucidum* might be related to both the hydrolytic activity and galactosyl transfer activity of  $\alpha$ -galactosidase. We isolated and characterized *G. lucidum*  $\alpha$ -galactosidase and found that this enzyme was relatively thermally stable and had the transfer activity for oligosaccharide synthesis.

### Materials and Methods

**Enzyme source.** *Ganoderma lucidum* was collected at the campus of Chiang Mai University. The fruiting bodies of the mushrooms were used as a source of the enzyme.

**Preparation of crude extract of mushroom.** The mushroom fruiting bodies were extracted using 100 mM sodium phosphate buffer (pH 6.0), mixed in a blender for 1 min and then filtered through cotton cloth. The filtrate was centrifuged at 7,500  $\times$  g for

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15 min at 4°C. The supernatant obtained was used as the starting crude enzyme preparation for purification.

**Enzyme assay.** The activity of  $\alpha$ -galactosidase was assayed by measuring the amount of *p*-nitrophenol released from *p*-nitrophenyl (*p*NP)  $\alpha$ -D-galactopyranoside as a substrate. The standard reaction mixture contained 4 mM *p*NP- $\alpha$ -D-galactopyranoside, 200 mM sodium phosphate buffer (pH 6.0), and the enzyme in a final volume of 0.6 ml. After incubation at 40°C for 10 min, 2.7 ml of 200 mM borate buffer (pH 10.0) was added to the mixture to stop the reaction. Thereafter, the absorbance of the mixture was measured at 400 nm.

One unit of  $\alpha$ -galactosidase was defined as the amount of enzyme which released 1  $\mu$ mol of *p*-nitrophenol from *p*NP- $\alpha$ -D-galactopyranoside per min under the standard assay conditions. The specific activity was expressed as units per mg protein.

**Protein measurement.** The protein concentration was measured by the method of Bradford using bovine serum albumin as a standard<sup>14</sup>) or by measuring the optical density at 280 nm. The latter method was used for monitoring protein on column chromatographies.

**Purification of the enzyme.** All purification procedures were done at 4°C. Unless otherwise indicated, 10 mM potassium phosphate buffer (pH 6.0) was used throughout the purification.

To the crude extract (160 ml), solid ammonium sulfate was added to the final 80% saturation. After centrifugation (12,500  $\times$  *g*) for 30 min, the precipitate was dissolved in the buffer and dialyzed against the same buffer for 12 h. The dialyzed solution was put to a column of DEAE-Sephadex A-50 (2.2  $\times$  36 cm) previously equilibrated with the buffer. A stepwise elution was done by increasing the NaCl concentration in the buffer from 0 to 0.4 M. The  $\alpha$ -galactosidase was mainly eluted with the buffer containing 0.2 M NaCl. The active fractions were pooled, concentrated by ultrafiltration, and dialyzed against the same buffer. Next, the dialyzed solution was put on a column of Con A-Sepharose (1.2  $\times$  5 cm) previously equilibrated with 20 mM Tris-HCl buffer (pH 7.4) containing 1 mM each of MnCl<sub>2</sub>, MgCl<sub>2</sub>, and CaCl<sub>2</sub>, and 0.5 M NaCl. A stepwise elution was done by increasing the concentration of methyl  $\alpha$ -D-mannoside in 20 mM Tris-HCl buffer (pH 7.4) containing 0.5 M NaCl from 0 to 1.0 M. The  $\alpha$ -galactosidase was eluted with the above buffer containing 0.2 M methyl  $\alpha$ -D-mannoside. The active fractions were pooled, concentrated by ultrafiltration, and dialyzed against 10 mM potassium phosphate buffer (pH 6.0). The concentrated enzyme solution was used as the purified enzyme.

**Molecular mass measurement.** The molecular mass of the purified enzyme was estimated by gel filtration with a Superdex 200HR column (Pharmacia-LKB). The column was calibrated with high molecular weight markers (Pharmacia-LKB). The column was pre-equilibrated with 50 mM sodium phosphate buffer (pH 7.0) containing 0.15 M NaCl, and eluted with the same buffer at a flow rate of 0.5 ml/min.

**Polyacrylamide gel electrophoresis.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was done in a 12% acrylamide gel at pH 8.8 using 25 mM Tris-glycine buffer containing 0.1% (w/v) SDS as described by Laemmli.<sup>15</sup>) A low molecular mass calibration mixture (Pharmacia-LKB) was used as the standard marker. The gel was stained with Coomassie Brilliant Blue R-250 (Nacalai Tesque).

Native PAGE was done at 4°C using a 4 to 30% linear gradient slab gel with the running buffer (pH 8.4) consisting of 90 mM Tris-HCl, 80 mM borate, and 2.5 mM EDTA by the method of Manabe *et al.*<sup>16</sup>) Protein bands in the gel were stained with a silver stain kit (Wako Chemicals). The activity staining of  $\alpha$ -galactosidase on the gel was done using 4-methylumbelliferyl- $\alpha$ -D-galactoside as substrate and the activity band was made visible under UV light at 365 nm.

**N-Terminal amino acid sequence analysis.** Purified enzyme (140  $\mu$ g) was put through SDS-PAGE and electroblotted onto an Immobilon-P<sup>5Q</sup> 0.45  $\mu$ m polyvinylidene fluoride membrane (Millipore). The membrane was stained with 0.025% Coomassie Brilliant Blue R-250 in 40% methanol and washed with 50% methanol. The protein band was then cut out. The strip of membrane holding the protein was put through automatic Edman degradation for sequencing of the N-terminal amino acids using an Applied Biosystems 477A protein sequencer (Perkin-Elmer).

**Kinetic studies.** Kinetic studies were done under various concentrations of structures such as *p*-nitrophenyl- $\alpha$ -D-galactopyranoside, *o*-nitrophenyl- $\alpha$ -D-galactopyranoside and *m*-nitrophenyl- $\alpha$ -D-galactopyranoside. The apparent Michaelis constant ( $K_m$ ) was calculated from the Lineweaver-Burk plot.

**Thin layer chromatography (TLC).** TLC was done on a Kiesel gel 60 alumina plate (Merck) with a solvent of *n*-propanol and water (80:20, v/v). The sugar compounds were visible after spraying with 0.2% orcinol in 1 M sulfuric acid, followed by heating at 140°C.

**Sugar analyses.** For the measurement of D-galactose released from natural oligosaccharides by the enzyme, the reaction mixture containing 200  $\mu$ l of

4 mM natural oligosaccharides (melibiose, raffinose, and stachyose), 200  $\mu$ l of 200 mM sodium phosphate buffer (pH 6.0), and 200  $\mu$ l of the enzyme solution was incubated at 40°C for 10 min. After boiling for 10 min, the released D-galactose was measured using a lactose/D-galactose kit (Boehringer Mannheim GmbH Biochemicals) by the UV method at 340 nm.

To examine the sugar composition of the compound, TMS (trimethylsilyl) derivatives were put through gas chromatography (GC-18A, Shimadzu, Japan) with a capillary column of HiCap-CBP5 (25 m; Shimadzu, Japan), programmed at 2°C/min from 150 to 230°C. To analyze the linkage of sugars, permethylation was done first, according to the method of Ciucanu and Kerek.<sup>17)</sup> The peaks on the gas chromatogram were identified by mass spectrometry (GCMS-QP 5050, Shimadzu, Japan) and by comparison of the retention times with those of partially methylated alditol acetates prepared from authentic standards as a reference.

**Chemicals.** *p*-Nitrophenyl- $\alpha$ -D-galactopyranoside, *o*-nitrophenyl- $\alpha$ -D-galactopyranoside, *m*-nitrophenyl- $\alpha$ -D-galactopyranoside, and other glycosides were purchased from Sigma Chemical Co. DEAE-Sephadex A-50 and Con A-Sepharose were products of Pharmacia.  $\alpha$ -Galactosidase of green coffee bean and  $\beta$ -galactosidase of jack bean were obtained from Sigma Chemical Co. and Seikagaku Kogyo Co., respectively. All other reagents used were of the highest purity available from commercial sources.

## Results

### *Glycosidase activities in the extract of fruiting bodies of Ganoderma lucidum*

The glycosidase activities in the extract of mushroom fruiting bodies were examined using various *p*-NP-glycosides as substrates. As shown in Table 1, various glycosidase activities were found. Among them, the  $\alpha$ -galactosidase activity was stronger than the other activities except for  $\beta$ -*N*-acetylhexosaminidase activity.

### *Purification of $\alpha$ -galactosidase from the extract of fruiting bodies of Ganoderma lucidum*

The  $\alpha$ -galactosidase was purified by ammonium sulfate precipitation and column chromatographies

on DEAE-Sephadex A-50 and Con A-Sepharose. The results of the enzyme purification are summarized in Table 2. The enzyme was purified about 12-fold with a yield of 1.7% of total activity.

### *Homogeneity and molecular mass of the enzyme*

The final enzyme preparation appeared to be homogeneous on native PAGE (Fig. 1A) and staining using 4-methylumbelliferyl- $\alpha$ -galactopyranoside (Fig. 1B) and presented as one major band on SDS-PAGE (data not shown).

The N-terminal amino acid sequence of the purified enzyme was identified as SNWGLAITPQ. To compare the amino acid sequence of the  $\alpha$ -galactosidase with the deduced amino acid sequences of other genes, the FASTA database<sup>18)</sup> was searched. The N-terminal amino acid sequence of *G. lucidum*  $\alpha$ -galactosidase was found to have the highest level of similarity with the sequence (SNNGLAITPQ) between positions 21 and 30 from the N-terminus of  $\alpha$ -galactosidase from *Mortierella vinacea*.

The molecular mass of the enzyme was estimated to be about 56 kDa by SDS-PAGE and about 249 kDa by gel filtration column chromatography. According to these results, the purified  $\alpha$ -galactosidase is composed of 4 subunits each having a molecular mass of 56 kDa.

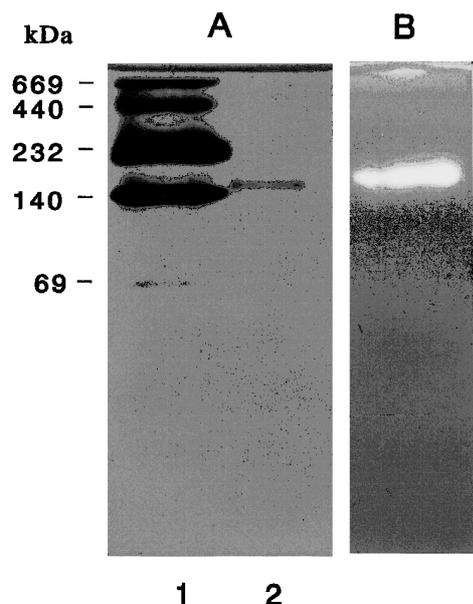
**Table 1.** Various Glycosidase Activities in the Extract of Fruiting Body of *Ganoderma lucidum*

Glycosidase	Hydrolyzing activity (U/mg)
$\alpha$ -D-glucosidase	9
$\alpha$ -D-glucosidase	188
$\alpha$ -D-galactosidase	400
$\alpha$ -D-galactosidase	12
$\alpha$ -D-mannosidase	8
$\alpha$ -D-mannosidase	12
$\alpha$ -D-fucosidase	1
$\alpha$ -D-fucosidase	5
$\alpha$ -D-arabinosidase	0
$\alpha$ -D-xylosidase	15
<i>N</i> -acetyl- $\beta$ -D-glucosaminidase	810
<i>N</i> -acetyl- $\beta$ -D-galactosaminidase	815

The enzyme activities were measured at pH 6.0 using various *p*-NP-glycosides (4 mM) as substrates. The reaction was done at 40°C and the released *p*-nitrophenol was measured.

**Table 2.** Purification of the  $\alpha$ -Galactosidase from Fruiting Body of *Ganoderma lucidum*

Purification steps	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Yield (%)
Crude extract	68.2	36.5	1.9	100
Ammonium sulfate precipitation (80%)	33.2	19.8	1.7	48.7
DEAE-Sephadex A-50 chromatography	15.0	1.8	8.4	22.0
Con A-Sepharose chromatography	1.16	0.05	23.2	1.7



**Fig. 1.** Native Gradient Polyacrylamide Gel Electrophoresis and the Activity Staining of *Ganoderma lucidum*  $\alpha$ -Galactosidase. (A) The native PAGE was done using 4 to 30% acrylamide. Lane 1: molecular size marker, Lane 2: the purified  $\alpha$ -galactosidase. (B) Activity staining of the purified  $\alpha$ -galactosidase was done using 4-methylumbelliferyl- $\alpha$ -galactopyranoside.

#### Various properties of the enzyme

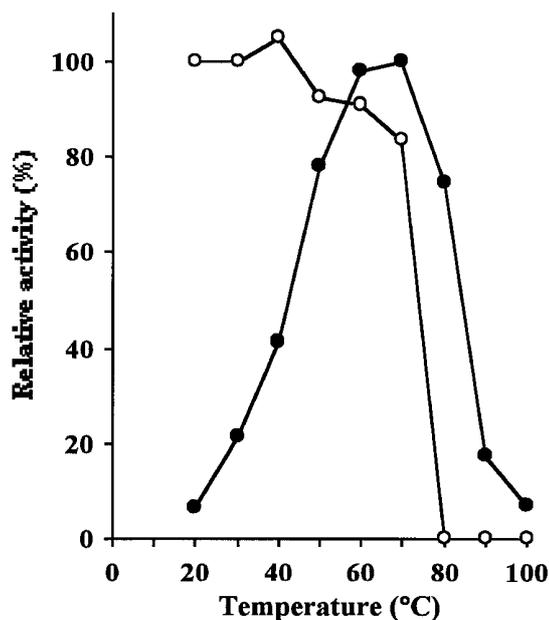
The optimum pH for enzymic activity was measured over a pH range from 1.2 to 8.0 using either 0.1 M glycine buffer (pH 1.2 to 3.2) or 0.1 M McIlvaine buffer (pH 3.2 to 8.0) under standard assay conditions. The enzyme was most active at pH 6.0.

The pH stability of the enzyme was measured by the residual activity after incubation in 0.1 M McIlvaine buffer (pH 3.2 to 8.0) for 1 h at 40°C. The enzyme was stable at pH 5.0 to 7.0.

The optimum temperature for enzymic activity was measured by a standard assay at various temperatures ranging from 20 to 100°C and the thermal stability of the enzyme was measured by assaying residual activity after incubation of the enzyme at 20 to 100°C for 1 h. As shown in Fig. 2, the enzyme was most active at 60 to 70°C and was stable below 70°C. The effect of heat treatment on the enzyme was investigated by heating the enzyme at 70, 80, and 100°C for 30 min and the remaining activity was assayed. As shown in Fig. 3, the enzyme was fully stable at 70°C and about 30% of the original activity remained after heating at 80°C for 30 min.

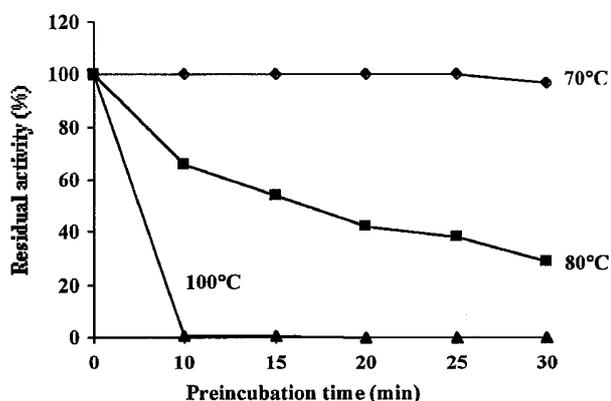
#### Substrate specificity of the enzyme

Synthetic substrates of glycosides and natural oligosaccharides were used to investigate the substrate specificity of the enzyme. As shown in Table 3, *p*-NP- $\alpha$ -D-galactopyranoside was the most susceptible substrate for this enzyme and *o*-NP- $\alpha$ -D-galac-



**Fig. 2.** Effects of Temperature on the Activity (A) and Stability (B) of  $\alpha$ -Galactosidase.

The optimum temperature of the enzyme (○) was measured at pH 6.0 at various temperatures (20 to 100°C). The thermal stability of the enzyme (●) was measured by assaying the remaining activity under standard conditions after incubating the enzyme at pH 6.0 at various temperatures for 1 h.



**Fig. 3.** Effects of Heat Treatment on the Stability of  $\alpha$ -Galactosidase.

The enzyme was heated at 70°C, 80°C, and 100°C for the indicated time, respectively, and the remaining activity was assayed under standard conditions.

topyranoside was slightly susceptible, though the enzyme can scarcely act on *m*-NP- $\alpha$ -D-galactopyranoside. Natural substrates, such as melibiose, raffinose, and stachyose, were good substrates for this enzyme. On the other hand, *p*-NP- $\alpha$ -D-glucopyranoside, *p*-NP-*N*-acetyl- $\alpha$ -D-galactopyranoside, and *p*-NP- $\beta$ -D-galactopyranoside were not hydrolyzed by the enzyme. The  $K_m$  values of the enzyme for *p*-NP- $\alpha$ -D-galactopyranoside, *o*-NP- $\alpha$ -D-galactopyranoside, and *m*-NP- $\alpha$ -D-galactopyranoside were 0.4 mM, 0.67 mM, and 16.7 mM, respectively.

**Table 3.** Substrate Specificity of  $\alpha$ -Galactosidase from *Ganoderma lucidum*

Substrate	Hydrolytic activity (%)
<i>p</i> NP- $\alpha$ -D-galactoside	100.0
<i>o</i> NP- $\alpha$ -D-galactoside	27.6
<i>m</i> NP- $\alpha$ -D-galactoside	0.3
<i>p</i> NP- $\alpha$ -D-glucoside	0.3
<i>p</i> NP- <i>N</i> -acetyl- $\alpha$ -D-galactosaminide	0
<i>p</i> NP- <i>N</i> -acetyl- $\alpha$ -D-glucosaminide	0.1
<i>p</i> NP- $\beta$ -D-galactoside	0.1
<i>p</i> NP- $\beta$ -D-glucoside	0
<i>p</i> NP- <i>N</i> -acetyl- $\beta$ -D-galactosaminide	0
<i>p</i> NP- <i>N</i> -acetyl- $\beta$ -D-glucosaminide	0
Melibiose	42.1
Raffinose	52.4
Stachyose	69.7

The enzyme activities were measured at pH 6.0 using various nitrophenylglycosides (4 mM) or oligosaccharides (4 mM) as substrates. The reaction was done at 40°C and released nitrophenol or galactose was measured by a colorimetric method.

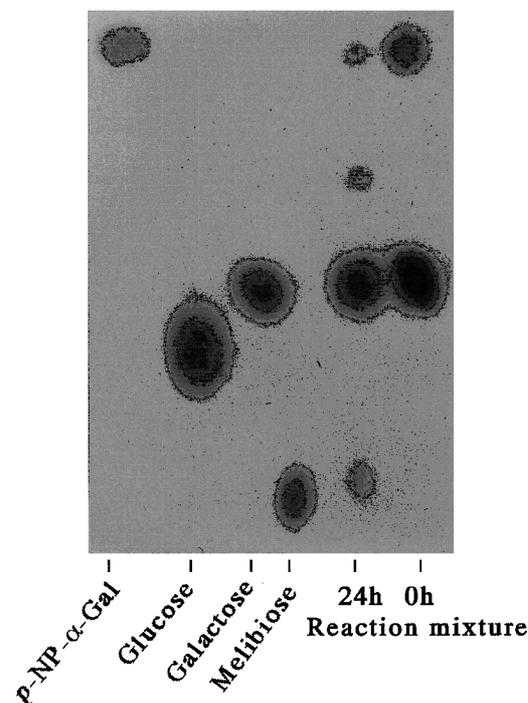
#### Effects of various compounds and metal ions on the enzymic activity

The effects of various metal ions (1 mM) and other reagents (1 mM) on the activity of the enzyme were examined under the standard enzyme assay conditions. The enzyme activity intensely decreased to 32% and 9% of the original activity by addition of  $\text{Ag}^+$  and  $\text{Hg}^{2+}$ , respectively. Other metal ions such as  $\text{Al}^{3+}$ ,  $\text{Ba}^{2+}$ ,  $\text{Ca}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{K}^+$ , and  $\text{Na}^+$  and reagents such as  $\beta$ -mercaptoethanol and EDTA did not affect the activity. The enzyme activity seemed to be slightly stimulated by  $\text{Cu}^{2+}$  (about 1.2-fold).

The effects of addition of various sugars (10 mM) on the enzymic activity were also examined. The activity was inhibited completely by galactose and slightly by xylose (88% of the original activity). Lactose, maltose, and sucrose did not affect the enzymic activity.

#### Transglycosylation activity of $\alpha$ -galactosidase

The enzyme was found to have transglycosylation activity in addition to hydrolyzing activity. The transglycosylation reaction was done in a 200 mM sodium phosphate buffer (pH 6.0) in the presence of 0.1 unit of  $\alpha$ -galactosidase per ml, 25 mM *p*NP- $\alpha$ -D-galactopyranoside as a glycoside donor, and 50 mM glucose as an acceptor. The incubation was done at 40°C for 24 hr, and the reaction was stopped by boiling for 5 min. After cooling, the reaction products were analyzed by TLC. As shown in Fig. 4, the transgalactosylation product was found to be produced by transferring a galactosyl residue from *p*NP- $\alpha$ -D-galactopyranoside to glucose. The reaction mixture was separated into *p*NP- $\alpha$ -D-galactopyranoside, galactose, glucose, and the transgalactosylation product by TLC. The product was found in the same position as the authentic disaccharide, melibiose.



**Fig. 4.** Thin Layer Chromatography of the Transgalactosylation Reaction Mixture.

The transgalactosylation reaction was done using *p*NP- $\alpha$ -D-galactopyranoside as the glycoside donor and glucose as the acceptor. The reaction was done at 40°C for 24 h.

Additionally, the reaction mixture with galactose as an acceptor also showed the transgalactosylation product on TLC, which was found in the same position as the authentic disaccharide, melibiose (data not shown).

#### Analysis of the transgalactosylation product

To isolate the transgalactosylation product (Gal-Glc), the reaction mixture was put on a column (1 × 76 cm) of Iatrobeds 6RS-8060 (Iatron Lab. Inc., Tokyo, Japan) equilibrated with chloroform-methanol-water at a ratio of 60:30:7 (v/v). The fractions containing the transgalactosylation product was detected with phenol- $\text{H}_2\text{SO}_4$ , pooled, and evaporated. The purified product was methanolized, and the methanolysates were converted to trimethylsilyl (TMS) derivatives for analysis by gas chromatography (GC). The gas chromatograms of the product defined the sugar component, of which appropriate molar ratios were found after correction for their relative molar responses as glucose and galactose (molar ratio, 1:1) as shown in Fig. 5A. Subsequently, to identify the sugar linkage, partially methylated alditol acetate derivatives of the transgalactosylation product were prepared and analyzed by GC and GC-MS. The identification was accomplished using the data of Björndal *et al.*<sup>19)</sup> and Stellner *et al.*<sup>20)</sup> The methylation analysis demonstrated the presence of 1-substituted galactose (1,5-di-(*o*-

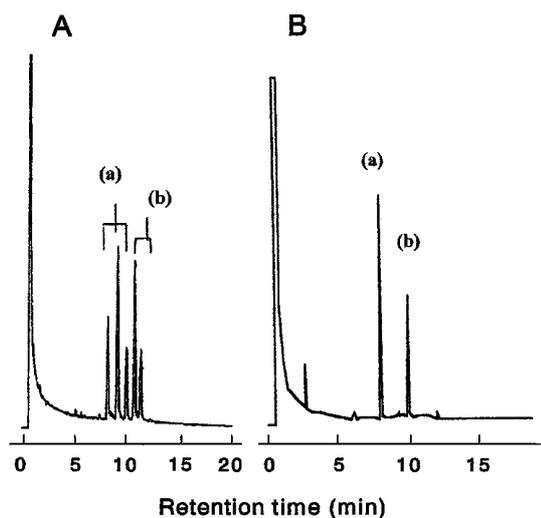


Fig. 5. Sugar Composition and Linkage Analyses of the Transgalactosylation Product.

(A) Gas chromatograms of TMS derivatives of the product. (a) 1-*O*-methyl-2,3,4,6-tetra-*O*-TMS-galactose; (b) 1-*O*-methyl-2,3,4,6-tetra-*O*-TMS-glucose. (B) Gas chromatogram of partially methylated alditol acetate derivatives of the product. (a) 1,5-di-(*o*-acetyl)-2,3,4-tri-(*o*-methyl) galactitol; (b) 1,5,6-tri-(*o*-acetyl)-2,3,4-tri-(*o*-methyl) glucitol.

acetyl)-2,3,4-tri-(*o*-methyl) galactitol) and 1,6-substituted glucose (1,5,6-tri-(*o*-acetyl)-2,3,4-tri-(*o*-methyl) glucitol) in the transgalactosylation product (Fig. 5B). Therefore, the product would be galactose 1→6 glucose disaccharide, and since it was degraded by the  $\alpha$ -galactosidase of green coffee beans but not by jack-bean  $\beta$ -galactosidase, it would be melibiose (galactose  $\alpha$ 1→6 glucose). Moreover, the transgalactosylation product obtained from galactose as an acceptor was also analyzed and identified as galactose 1→6 galactose (data not shown). From these results, this enzyme could transfer the *p*NP-galactose to the C-6 position of the sugar acceptor.

## Discussion

The fruiting bodies of *Ganoderma lucidum* had relatively high  $\alpha$ -galactosidase activity and the enzyme was thermally stable. We were interested in such enzymatic properties. It is unusual for a mushroom to have  $\alpha$ -galactosidase activity that is stronger than other glycosidase activities. Moreover, the activity had a maximum at pH 6.0 and this value is higher than the optimum pHs of most mushroom and fungal  $\alpha$ -galactosidases (pH 2.5–5.5).<sup>21–26</sup> The distinctive characteristic of the *G. lucidum*  $\alpha$ -galactosidase was its extreme thermostability. The optimum temperature of this  $\alpha$ -galactosidase is 70°C and the enzyme was fully stable after heating at 70°C for 30 min. Even when it was heated at 80°C for 30 min, almost 30% of its original activity remained. These properties are advantageous for possible industrial

applications because a high temperature and moderate pH are required for processing. Although it is difficult to obtain large amounts of the enzyme from *G. lucidum*, we are attempting to clone the gene encoding this enzyme for large-scale production of the enzyme.

Many glycosidases are known to have transglycosylation activity, which is considered as a special reaction of hydrolysis in which the carbohydrate moiety of the substrate is transferred to the hydroxyl group of various compounds instead of water.<sup>27</sup> Transglycosylation activity has been well studied in many glycosidases. Although the activity is also well studied in plant<sup>28,29</sup> and fungus<sup>10,30,31</sup>  $\alpha$ -galactosidases, the activity is almost unknown in mushroom  $\alpha$ -galactosidases. We found that *G. lucidum*  $\alpha$ -galactosidase also had transgalactosylation activity. When *p*-nitrophenyl- $\alpha$ -D-galactopyranoside and glucose were incubated with this enzyme, melibiose (galactosyl  $\alpha$ -1,6 glucose) was mainly produced. We are interested in the fact that ganodran C contained a galactosyl  $\alpha$ -1,6 linkage although *G. lucidum* had strong  $\alpha$ -galactosidase activity. The transgalactosylation activity of this enzyme might involve the synthesis of ganoderan C though a glycoside donor is unknown.

The substrate specificity of this enzyme is also of interest. This enzyme is highly active toward *p*-NP- $\alpha$ -galactoside, and slightly active against *o*-NP- $\alpha$ -galactoside, but not active toward *m*-NP- $\alpha$ -galactoside. The results suggest that this enzyme clearly recognizes the conformation of aglycon, or enzymatic inhibition occurred *via* hindrance of aglycon. Further investigations are currently ongoing.

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