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Microbial α-L-Rhamnosidases of Glycosyl Hydrolase Families GH78 and GH106 Have Broad Substrate Specificities toward α-L-Rhamnosyl- and α-L-Mannosyl-Linkages

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Abstract: α-L-Rhamnosidases (α-L-Rha-ases, EC 3.2.1.40) are glycosyl hydrolases (GHs) that hydrolyze a terminal α-linked L-rhamnose residue from a wide spectrum of substrates such as heteropolysaccharides, glycosylated proteins, and natural flavonoids. As a result, they are considered catalysts of interest for various biotechnological applications. α-L-rhamnose (6-deoxy-L-mannose) is structurally similar to the rare sugar α-L-mannose. Here we have examined whether microbial α-L-Rha-ases possess α-L-mannosidase activity by synthesizing the substrate 4-nitrophenyl α-L-mannopyranoside. Four α-L-Rha-ases from GH78 and GH106 families were expressed and purified from *Escherichia coli* cells. All four enzymes exhibited both α-L-rhamnosyl-hydrolyzing activity and weak α-L-mannosyl-hydrolyzing activity. SpRhaM, a GH106 family α-L-Rha-ase from *Sphingomonas paucimobilis* FP2001, was found to have relatively higher α-L-mannosidase activity as compared with three GH78 α-L-Rha-ases. The α-L-mannosidase activity of SpRhaM showed pH dependence, with highest activity observed at pH 7.0. In summary, we have shown that α-L-Rha-ases also have α-L-mannosidase activity. Our findings will be useful in the identification and structural determination of α-L-mannose-containing polysaccharides from natural sources for use in the pharmaceutical and food industries.

Key words: glycosyl hydrolase family 78, glycosyl hydrolase family 106, α-L-rhamnosidase, α-L-mannosidase

INTRODUCTION

α-L-Rhamnosidase (α-L-Rha-ase; 3.2.1.40) is a glycosyl hydrolase (GH) that exists in a plethora of microorganisms, animals, plants, bacteria, and fungi. α-L-Rha-ases are responsible for cleaving the terminal α-L-rhamnose from natural flavonoids, glycosylated proteins, and heteropolysaccharides, as well as the artificial substrate 4 nitrophenyl-α-L-rhamnopyranoside (*p*NP-α-L-Rha).[1)2)] α-L-Rha-ase is a biotechnologically important enzyme that has been used for the derhamnosylation of natural products in various application such as debittering of citrus fruit juices and enhancement of wine aromas,[3)4)] in addition, it has been used for the synthesis of α-L-rhamnose-containing chemicals by reverse hydrolysis, suggesting an as-yet unexplored potential of α-L-Rha-ase enzymes in the chemical and pharmaceutical industry.[5)]

α-L-Rha-ases are classified into three different GH families (GH28, GH78, and GH106) based on sequence similarities in the Carbohydrate-Active enzyme (CAZy) database (http://www.cazy.org/).[6)7)8)] α-L-Rhamnose (α-L-Rha) is a hexose that is extensively distributed in the backbone of pectin in plant cell walls,[9)10)] bacterial heteropolysaccharides, and many natural products such as glycolipids and glycosides.[11)] Many L-hexoses are important components of biologically relevant compounds and thus play an important role in the pharmaceutical industry.[12)] For example, α-L-Rha acts as a chiral intermediate in the organic synthesis of pharmaceutically important agents and plant protective agents.[1)]

α-L-Rha (6-deoxy-L-mannose) is also structurally similar to the rare sugar α-L-mannose (Fig. 1). Rare sugars are considered to be monosaccharides that are present in limited quantities in nature. In their L-form, rare sugars have received much attention as potential starting materials for the synthesis of many active compounds in the food and pharmaceutical industries.[12)13)] However, only a few studies have reported the production of rare L-form monosaccharides from microorganisms.[14)15)16)17)] Furthermore, although the metabolism of rhamnose-containing glycoconjugates has been extensively studied, to our knowledge an α-L-

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Abbreviations: α-L-Rha-ase, α-L-rhamnosidase; GH, glycoside hydrolase; *p*NP, para-nitrophenyl.
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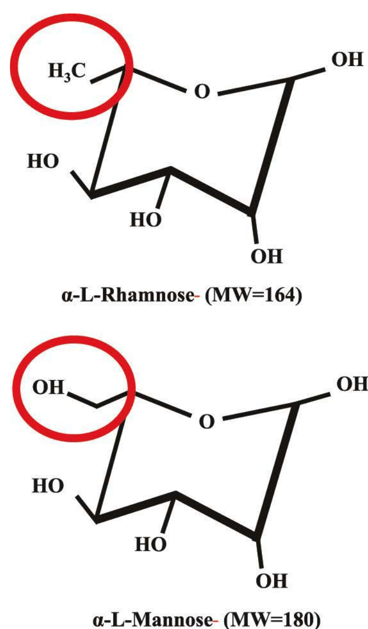


Fig. 1. Schematic diagram of the neutral α-L-rhamnose and α-L-mannose.

mannosidase has not yet been reported. Therefore, it is vital to discover an exoglycosidase involved in the liberation of α-L-mannose residues from glycoconjugates.

In the present study, the genes *rhaA* and *rhaB* from *Bacillus* sp. GL1,[19)20)] and *koRha* from *Klebsiella oxytoca*[21)] encoding GH78 α-L-Rha-ases, and *rhaM* from *Sphingomonas paucimobilis* FP2001[22)23)] encoding a GH106 α-L-Rha-ase were cloned and expressed in *Escherichia coli* cells. By analyzing the hydrolysis of *p*NP-α-L-rhamnose and *p*NP-α-L-mannose substrates, we show that BsRhaA, BsRhaB, KoRha, and SpRhaM exhibit activity of both α-L-Rha-ase and α-L-mannosidase.

MATERIALS AND METHODS

Reagents. The 4-nitrophenyl-α-L-rhamnopyranose (*p*NP-α-L-Rha) substrate was obtained from Sigma. *E. coli* BL21(DE3) codon plus (Stratagene) and the cloning vector pET50b+ (Novagen) were used as, respectively, host cells and the expression plasmid for each target gene. Isopropyl β-D-thiogalactopyranoside (IPTG) and all other reagents were analytical grade. L-Mannose was purchased from Toyo Science Corp. (Tokyo, Japan). All chemicals used for the synthesis of *p*NP-α-L-Man were commercially available, and all organic solvents also used for the synthesis of *p*NP-α-L-Man were dried over appropriate drying agents and distilled prior to use.

Instrumental analysis for* p*NP-α-L-Man. Compounds were analyzed using the Varian 400 MHz systems at the Okayama University Collaboration Center. ^{1}H NMR (operating at 400 MHz) and ^{13}C NMR (operating at 100 MHz) spectra were measured in D_2O. NMR chemical shifts (δ) are provided in parts per million (ppm), and coupling constants (*J*) are listed in Hz. Residual peak of H_2O (4.63 ppm) was used as ^{1}H-NMR references. The melting points were obtained on Yamato melting point MP-21 apparatus. Optical rotations value was measured with a JASCO P-2200 apparatus (20 ºC, sodium discharge lamp). Elemental analyse was measured at the Division of Instrumental Analysis, Okayama University. TLC was carried out on Merck Kieselgel 60 PF_{254} (0.25 mm thickness). Column chromatography was performed using Merck Kieselgel 60 (0.063–0.200 mm).

Synthesis of 4-nitrophenyl α-L-mannopyranoside (*p*NP-L-Man). To a suspension of L-mannose (1.80 g) in acetic anhydride (50 mL) was added sodium acetate (0.41 g, 5.00 mmol) at 23 °C. The reaction mixture was stirred at 60 °C for 5 h and then at 23 °C for 24 h. The mixture was filtered through a Celite pad and the filtrate was concentrated *in vacuo*. The residue was diluted with EtOAc (100 mL), washed with sat. aq. $NaHCO_3$ solution (100 mL × 3) and brine (100 mL), dried over $MgSO_4$, and concentrated *in vacuo* to dryness, producing per acetylated L-mannose as a yellow syrup (3.90 g, quant).

To a solution of per-acetylated L-mannose (3.90 g, 10.0 mmol) with MS 4 Å in 1,2-dichloroethane (50 mL) was added 4-nitrophenol (6.96 g, 50.0 mmol) and boron trifluoride diethyl etherate (6.33 mL, 50.0 mmol) at 0 °C. The reaction mixture was stirred at 0 °C for 1 h and then at 23 °C for 24 h. Next, the mixture was filtered through a Celite pad and the filtrate was concentrated *in vacuo.* The residue was diluted with EtOAc (100 mL), washed with sat. aq. $NaHCO_3$ solution (100 mL × 3) and brine (100 mL), dried over $MgSO_4$, and concentrated *in vacuo* to dryness, producing a mixture of 4-nitrophenyl 2,3,4,6-tetra-*O*-acetyl-α-L-mannopyranoside and by-products as a yellow syrup. This mixture was used for deacetylation without any purification.

To a solution of 4-nitrophenyl 2,3,4,6-tetra-*O*-acetyl-α-L-mannopyranoside and by-products in CH_3OH (50 mL) was added 28 % aq. NH_3 solution (50 mL) at 0 °C. The resulting solution was stirred at 0 °C for 1 h and then at 23 °C for 24 h. The reaction solution was concentrated by using a rotary evaporator (35 °C) to produce 4-nitrophenyl α-L-mannopyranoside and by-products as a brown solid. The target material was purified twice by silica gel column chromatography ($CHCl_3$:CH_3OH, 4/1) to give 4-nitrophenyl α-L-mannopyranoside (*p*NP-L-Man) as a white solid (0.66 g, 2.19 mmol, 22 %).

Rf: 0.65 ($CHCl_3$-CH_3OH, 4:1); $[\alpha]_D$ -154 (c 0.1, H_2O); mp 178-180 °C; ^{1}H-NMR (D_2O): δ 8.26 (d, 2H, *J*o,m = 9.0 Hz, Ph(m)), 7.24 (d, 2H, *J*o,m = 9.0 Hz, Ph(o)), 5.78 (d, 1H, $J_{1,2}$ = 1.6 Hz, H-1), 5.00 (dd, 1H, $J_{1,2}$ = 1.6, $J_{2,3}$ = 3.8, H-2), 4.46 (m, 1H, H-4), 4.15(m, 1H, H-3), 3.93 (m, 1H, H-5), 3.87-4.18 (m, 2H, H-6a and 6b); ^{13}C-NMR (D_2O): δ 175.1, 162.2, 125.3, 116.4, 106.4, 84.2, 82.0, 76.7, 70.7, 62.7; Anal. Calc. for: $C_{12}H_{15}NO_8$: C, 47.84; H, 5.02; N, 4.65. Found: C, 47.66; H, 5.00; N, 4.68.

Gene cloning and sequence analysis. The recombinant expression plasmids of the four α-L-Rha-ase genes were amplified by PCR using DNA polymerase PrimeStar GXL (Takara) and the following primers pairs: pET50-F, CGGATCCTGG-TACCCGGGTCCCT; pET50-R, AATTCTGTA-CAGGCCTTGGCGCGCCC; BsRhaA-F:CCAGGATCC-

GAATTCTATGGCGGAGATCGAAGTATA; BsRhaA-R, GGCCTGTACAGAATTCGGCTACTTAACCG-TAAACCGATAC; BsRhaB-F, ACCAGGATCC-GAATTCTATGGCAGGCAGGAATTGGAA; BsRhaB-R, GCCTGTACAGAATTCGGTTAGCTCATATACT-TAACCTGCGT; KoRha-F, ACCAGGATCCGAATTC-TATGGGTAGCAGCCATCAT; KoRha-R, GGCCTGTA-CAGAATTCGGTTACAGACCATACTGACGAATA; SpRhaM-F, GGTACCAGGATCCGATGATTCGTAAAC; and SpRhaM-R, TAATTAAGCCTCGAGTTAACGAT-CACCTGTTTCGGCAATCAGTGTC. The amplified DNA fragments were cloned into the EcoRI/XhoI restricted site of the pET50b vector and ligated with an In-Fusin HD Cloning Kit (Takara) as recommended by the manufacturer. The resulting plasmids, named pET50b-BsRhaA, pET50b-BsRhaB, pET50b-KoRha, and pET50b-SpRhaM, respectively, were verified by DNA sequencing. Amino acid sequences retrieved from the NCBI database (accession numbers: BsRhaA, BAB62314; BsRhaB, BAB62315; KoRha, 4XHC_4; and SpRhaM, BAD12237) were used for sequence alignment. Pfam was used for domain predictions and CLUSTAL W (http://culstalw.ddbj.nig.ac.jp) was used to generate the phylogenetic tree with GH106 SpRhaM as the root.

Preparation of recombinant α-L-Rha-ase proteins. For the pre-culture, *E. coli* cells were grown overnight in a 50-mL falcon tube containing 10 mL of MMIKC (1.25 % triptone, 2.5 % yeast extract, 0.85 % NaCl, 0.4 % glycerol, 20 mM Tris-HCl (pH 7.2), 30 mg/L of kanamycin and chloramphenicol) with constant shaking at 37 °C to an $OD_{600\ nm}$ of 0.5–1.0. For the main culture, the pre-culture was inoculated into a 500-mL Erlenmeyer flask containing 250 mL of LBKC (2.5 % LB powder (Merck), 30 mg/L of kanamycin, and chloramphenicol), and growth was continued with constant shaking (150 × rpm) at 30 °C for 4–5 h to an $OD_{600\ nm}$ of 0.5–1.0. Expression of recombinant α-L-Rha-ase was induced with 100 mM IPTG, and the cells were incubated with constant shaking (150 × rpm) at 15 °C for 18 h. Bacterial cells were harvested by centrifugation (7,000 × rpm, 10 min, 4 °C), suspended in lysis buffer (20 mM sodium phosphate (pH 7.4), 0.5 M NaCl, containing EDTA protease inhibitor tablet (Roche)) and disrupted by ultrasound (Insonator Model 201 M; Kubota) on ice (9 kHz for ~20 min).

For protein purification, the supernatant was obtained by centrifugation (15,000 × rpm, 10 min, 4 °C) and passed through a 1-mL column His-TrapTM FF (GE Healthcare) in accordance with the manufacturer's instructions. Molecular mass was analyzed by using an 8 % SDS-polyacrylamide gel, stained with Coomassie Brilliant Blue (CBB) Ez-Stain Aqua (Atto). The protein concentration of the purified α-L-Rha-ases was measured by a BCA Protein Assay Kit (Takara), and subsequently used in an enzymatic assay to determine α-L-Rha-ase and α-L-mannosidase activity.

Enzymatic assay. The α-L-Rha-ase and α-L-mannosidase activities of recombinant purified BsRhaA, BsRhaB, and KoRha α-L-Rha-ases were determined by using 4 mM *p*NP-α-L-Rha and 4 mM *p*NP-α-L-Man as substrates, respectively. In a final volume of 160 μL, the reaction mixture (containing 10 μL of 100 mM sodium phosphate buffer (pH 7.0), 20 μL of 4 mM *p*NP-α-L-Rha or *p*NP-α-L-Man and 50 μL of diluted enzyme) was incubated at 30 °C for 0, 5, 10, 15, and 20 min. Reactions were terminated by the addition of 80 μL of 1 M Na_2CO_3. The activities of SpRhaM were determined by the assay of Miyake and colleagues[22] with slight modification. The reaction mixture containing 10 μL of 4 mM *p*NP-α-L-Rha or 4 mM *p*NP-α-L-Man in an appropriate enzyme solution in 100 mM Tris-HCl (pH 8.0) was incubated at 37 °C for 5-min intervals. The addition of 100 μL of 100 mM NaOH was used to stop the reaction. In all assays, the liberation of *p*-nitrophenol (*p*NP) was detected at 405 nm spectrophotometrically. One unit (U) of enzyme was defined as the amount of enzyme required to liberate 1 μmol of *p*NP per minute. All experiments were conducted in triplicate.

Two buffer systems (0.1 M sodium phosphate buffer (pH 6.0–7.0) and 0.1 M Tris-HCl (pH 7.0–9.0)) were used to evaluate the optimal pH for SpRhaM activity toward 4 mM *p*NP-α-L-Rha or 4 mM *p*NP-α-L-Man substrate. For analysis, the highest activity for each pH tested was set as 100 %, and relative activities were reported.

RESULTS

Subcloning, expression, and purification of recombinant α-L-Rha-ases.

The purification, characterization, and properties, including structural and molecular biological properties, of various α-L-Rha-ases from different sources have been reported;[1] however, it is not clear whether these enzymes possess α-L-mannosidase activity. Owing to limited information on α-L-Rha-ases from the GH28 family, which are pectin hydrolases, three GH78 and one GH106 α-L-Rha-ases were selected for the present study. BsRhaA and BsRhaB from *Bacillus* sp. GL1 are well-documented α-L-Rha-ases of the GH78 family, and the structure of BsRhaB is known. KoRha from *K. oxytoca* is an α-L-Rha-ase from the GH78 family reported to have a smaller structure, while SpRhaM from *S. paucimobilis* FP2001 was characterized as a representative from the GH106 family.

The sequence identity among the three GH78 proteins was found to be relatively low: for example, BsRhaA and BsRhaA from *Bacillus* sp. GL1 share 23 % identity, while BsRhaB and KoRha share 27 % identity over 383 amino acids.[21)26]

Next, we used the Pfam database to predict the presence of functional domains in each α-L-Rha-ase from their amino acid sequences (Fig. 2B). We found that BsRhaA and BsRhaB have a bacterial α-L-Rha-ase domain at their N-terminus, a C-terminal domain, a concanavalin-like domain, and a 6-hairpin glycosidase domain. By contrast, KoRha and SpRhaM (GH106) do not have any recognizable conserved domains other than the catalytic domain of α-L-Rha-ase (Fig. 2C).

We expressed the four NusA-tagged recombinant α-L-Rha-ases by using an *E. coli* strain, and purified the recombinant enzymes from the cell-free extracts by using His-affinity column chromatography, followed by gel fil-

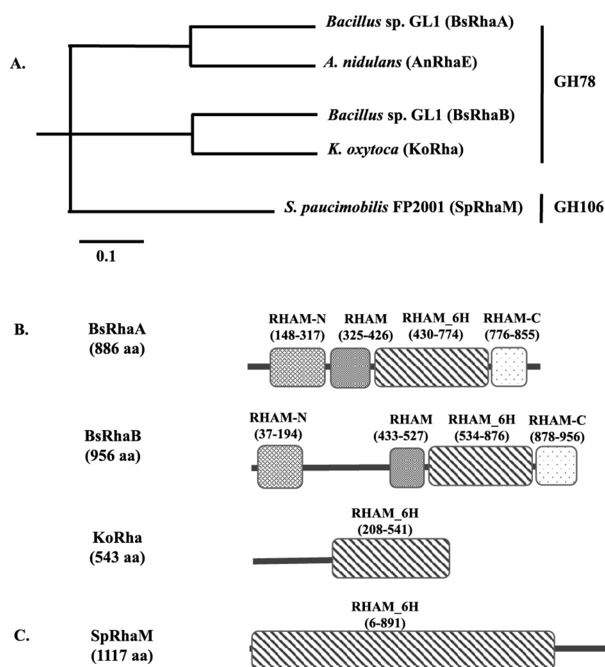


Fig. 2. Phylogenetic relationships between α-L-Rha-ases and their domain structures.

A: Phylogenetic tree of GH78 α-L-Rha-ases (α-L-RHAs) created by subjecting the amino acid sequences to the neighboring-joining method of CLUSTAL W. A GH106 α-L-RHA was used to root the tree. **B**: Schematic depiction of the conserved domains present in GH78 α-L-RHAs: BsRhaA from *Bacillus* sp. GL1 (886 aa), BsRhaB from *Bacillus* sp. GL1 (956 aa), KoRha from *K. oxytoca* (543 aa). **C.**: The GH106 SpRhaM from *S. paucimobilis* FP2001 (1117 aa) contains a single α-L-RHA domain. In **B** and **C**, the corresponding amino acid residue numbers for each domain are indicated in parentheses. The following domains were identified: RHAMNOSID_N (α-L-RHA N-terminal domain), RHAMNOSID (Bacterial α-L-RHA concanavalin-like domain), RHAMNOSID_6H (Bacterial α-L-RHA 6 hairpin glycosidase domain), and RHAMNOSID_C (α-L-RHA C-terminal domain).

tration. The purity and molecular size of the proteins were determined by using 8 % SDS-PAGE as follows: BsRhaA, 153 kDa; BsRhaB, 161 kDa; KoRha, 116 kDa; and SpRhaM, 167 kDa (Fig. 3).

Enzymatic activities of the recombinant α-L-Rha-ases.

Two substrates, *p*NP-α-L-rhamnose and *p*NP-α-L-mannose, were used to determine the ability of the four α-L-Rha-ases to liberate α-L-rhamnose and α-L-mannose, respectively, in 100 mM sodium phosphate (pH 7.0) at 30 °C. All four α-L-Rha-ases exhibited α-L-rhamnosyl-hydrolyzing and weak α-L-mannosyl-hydrolyzing activity (1.2–4.5 %). Notably, the α-L-mannosidase activity of the GH106 family α-L-Rha-ase SpRhaM was slightly higher than that of the GH78 family enzymes (Table 1).

Characterization of the L-mannosidase activity of SpRhaM.

As described above, the GH106 α-L-Rha-ase, SpRhaM, showed relatively higher α-L-mannosidase activity as compared with the GH78 α-L-Rha-ases. To characterize the α-L-mannosidase activity of SpRhaM in more detail, the influence of pH on its activity toward both substrates, *p*NP-α-L-rhamnose and *p*NP-α-L-mannose, was examined.

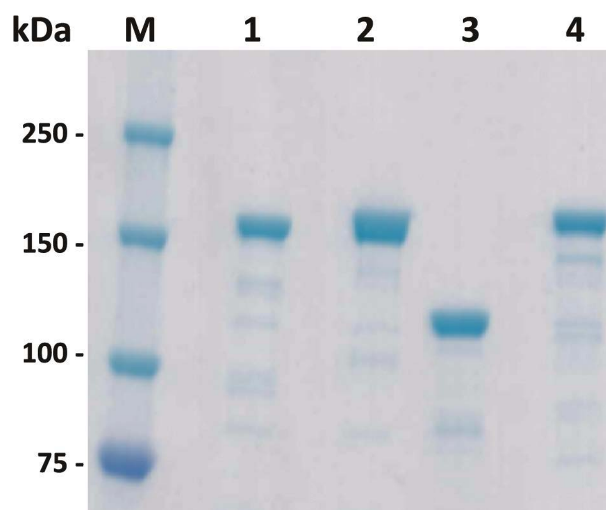


Fig. 3. Analysis of the purified recombinant enzymes by 8 % SDS-PAGE.

Lane M, molecular mass marker; lane 1, BsRhaA (153 kDa); lane 2, BsRhaB (161 kDa); lane 3, KoRha (116 kDa); and lane 4, SpRhaM (167 kDa).

Table 1. α-L-Rha-ases from the GH78 and GH106 families exhibit relatively low α-L-mannosidase activity.

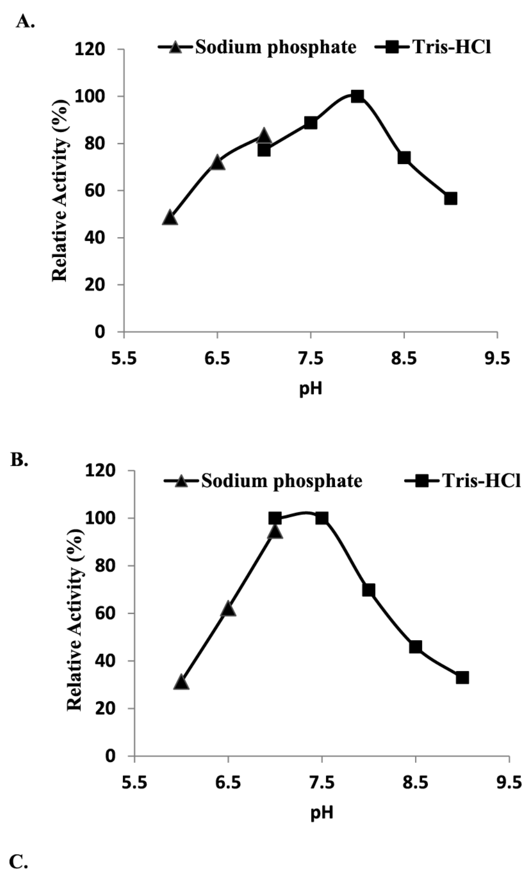
Source	Protein	GH	Relative L-Mannosidase Activity (%)
Bacillus sp. GL1	BsRhaA	78	1.2
Bacillus sp. GL1	BsRhaB	78	2.7
Klebsiella oxytoca	KoRha	78	1.2
Sphingomonas paucimobilis FP2001	SpRhaM	106	4.5

The enzyme exhibited an optimum pH of 7.8–8.0 toward *p*NP-α-L-rhamnose[22] with highest activity observed at pH 8.0 (Fig. 4A). By contrast, the highest activity toward *p*NP-α-L-mannose was observed at pH 7.0 and the α-L-mannosidase activity of SpRhaM decreased by 30 % at pH 8.0 (Fig. 4B). Thus, the α-L-mannosidase activity of SpRhaM depends on pH, and is highest (6.5 %) at pH 7.0 (Tris-HCl buffer) (Fig. 4C). A substrate-dependent shift of optimum pH was also observed for several glycosidases such as mammalian α-amylases and *Bacillus* endo-1,4-β-glucanase.[23)24)] In the presence of larger substrate L-mannose, C-6 position of mannose induces the conformation change of amino acids residues, and this change may affect the *p*Ka of general base catalyst (E594 of SpRhaM). Structural analysis of SpRhaM complex with L-mannose or L-rhamnose might help in revealing the underlying mechanism.

The K_m and V_{max} of the activity toward *p*NP-α-L-rhamnose were previously reported as 1.18 mM and 92.40 µM/min, respectively (Miake *et al*., 2000). By contrast, we found that the K_m and V_{max} of the activity toward *p*NP-α-L-mannose was 2.44 mM and 57.1 µM/min, respectively.

DISCUSSION

Rare sugars have recently been drawing attention owing to their potential application and high demand in the food and pharmaceutical industries. All hexoses are con-



C.

Buffer	0.1 M Sodium Phosphate			0.1 M Tris-HCl		
pH	6	6.5	7	7	7.5	8
Man/Rha activity (%)	2.1	2.5	4.5	6.5	3.9	2.5

Fig. 4. pH dependence of SpRhaM activity on *p*NP-α-L-rhamnose and *p*NP-α-L-mannose.

Purified SpRhaM was incubated with *p*NP-α-L-Rha (**A**) and *p*NP-α-L-Man (**B**) at 37 °C at the indicated pH. Sodium phosphate data are shown as shaded diamonds, and Tris-HCl as haded squares. (**C**) Summary of the mannose/rhamnose activity.

sidered rare sugars, because they are not common in nature and are thus very expensive.[12)28)] Although the rare sugar α-L-mannose is structurally similar to α-L-rhamnose, there have been few previous studies of whether α-L-Rha-ases possess α-L-mannosidase activity. Commercial naringinase contains multiple enzymes such as α-L-rhamnosidase and β-D-glucosidase activities. Esaki et al. reported that the commercial naringinase hydrolyzed several L-mannose-containing disaccharides such as 1,2-, 1,3-, and 1,5-linked methyl α-L-mannopyranosyl-D-glucosides and 1,6-linked α-L-mannopyranosyl-D-galactose.[29)] This result suggested that α-L-Rha-ase from fungi possess α-L-mannosidase activity.

The proteins of GH78 family of α-L-Rha-ases have been well studied owing to their potency for removing α-L-rhamnose from α-L-rhamnose-containing substrates as compared with the GH28 and GH106 families. In this study, we revealed that BsRhaA, BsRhaB, KoRha from the GH78 family, and SpRhaM from the GH106 family have both α-L-rhamnosidase and α-L-mannosidase activities. The GH106 SpRhaM α-L-Rha-ase from *S. paucimobilis* showed relatively higher α-L-mannosidase activity as compared with the GH78 α-L-Rha-ases. However, bacterial α-L-Rha-ases from the GH106 family remain poorly characterized relative to the GH78 family. The AnRhaE from *Aspergillus nidulans* was depicted to be the first eukaryotic α-L-Rha-ase that is phylogenetically distant to any reported filamentous fungal origin but more closely related to bacterial α-L-Rha-ases of class A BsRhaA from *Bacillus* sp. GL1[5)] with similar α-L-mannosidase activity (data not shown).

Recent studies have reported that heteropolysaccharides isolated from the fruiting bodies of fungus *Lactarius delicious* Gray (α-L-mannose and D-xylose; 3:1) and *Boletus speciosus Forst* (L-mannose and D-galactose; 2:1) have significant anti-tumor activities and antioxidant activities, respectively.[30)31)] Furthermore, structural characterization showed that MC-1 and MC-2 polysaccharides extracted from *Lepidium meyenii* (Maca) contained 11.8 % and 4.5 % α-L-mannose, respectively.[32)33)] These findings suggest that some natural substrates such as these fungal polysaccharides have a backbone α-1,4-linked L-mannopyranose residues, and that α-L-mannose-containing polysaccharides may be widely distributed in nature. Therefore, α-L-mannosidases will be useful to identify and determine the structure of α-L-mannose-containing polysaccharides from natural sources.

Hydrolytic activities of jack bean and almond α-mannosidases against α-D-rhamnopyranoside were investigated.[34)] These α-mannosidases hydrolyzed *p*NP-α-D-rhamnose as fast as *p*NP-α-D-mannose, indicating that hydroxyl group at C-6 does not have a crucial role in substrate discrimination.[34)] In this study, we showed that microbial α-L-rhamnosidases have broad substrate specificities toward α-L-rhamnosyl- and α-L-mannosyl-linkages. However, these α-L-rhamnosidases exhibited weak α-L-mannosidase activity due to the steric hindrance posed by the hydroxyl group at C-6 of L-mannose. The results presented in this study have great potential for enhancing the L-mannosidase activity of SpRhaM GH106. Only a few structures of GH106 family α-L-Rha-ases have been reported, including the crystal structures of protein BT0986 from *Bacteroides thetaiotaomicron*[35)] and RHA-P from *Novosphingobium* sp. PP1.[36)] Homology modeling of SpRhaM and these two GH106 proteins is undoubtedly needed to explore and evaluate the α-L-mannose-hydrolyzing activity of SpRhaM (GH106). Lastly, site-directed mutagenesis experiments around the C-6 position of the L-mannose residue may increase the α-L-mannosidase activity of SpRhaM.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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