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Molecular Characterization of the α-Galactosidase SCO0284 from *Streptomyces coelicolor* A3(2), a Family 27 Glycosyl Hydrolase S

Uyangaa Temuujin#, Jae Seon Park, and Soon-Kwang Hong*

Department of Bioscience and Bioinformatics, Myongji University, Yongin 17058, Republic of Korea

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**Corresponding author*
Phone: +82-31-330-6198;
Fax: +82-31-335-8249;
E-mail: skhong@mju.ac.kr

#Present address: School of Veterinary Medicine, Mongolian University of Life Science, Khan-Uul District, Zaisan 17024, Ulaanbaatar, Mongolia

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The *SCO0284* gene of *Streptomyces coelicolor* A3(2) is predicted to encode an α-galactosidase (680 amino acids) belonging to glycoside hydrolase family 27. In this study, the *SCO0284* coding region was cloned and overexpressed in *Streptomyces lividans* TK24. The mature form of SCO0284 (641 amino acids, 68 kDa) was purified from culture broth by gel filtration chromatography, with 83.3-fold purification and a yield of 11.2%. Purified SCO0284 showed strong activity against *p*-nitrophenyl-α-D-galactopyranoside, melibiose, raffinose, and stachyose, and no activity toward lactose, agar (galactan), and neoagarooligosaccharides, indicating that it is an α-galactosidase. Optimal enzyme activity was observed at 40°C and pH 7.0. The addition of metal ions or EDTA did not affect the enzyme activity, indicating that no metal cofactor is required. The kinetic parameters V_{max} and K_m for *p*-nitrophenyl-α-D-galactopyranoside were 1.6 mg/ml (0.0053 M) and 71.4 U/mg, respectively. Thin-layer chromatography and mass spectrometry analysis of the hydrolyzed products of melibiose, raffinose, and stachyose showed perfect matches with the masses of the sodium adducts of the hydrolyzed products, galactose (M+Na, 203), melibiose (M+Na, 365), and raffinose (M+Na, 527), respectively, indicating that it specifically cleaves the α-1,6-glycosidic bond of the substrate, releasing the terminal D-galactose.

Keywords: *Streptomyces coelicolor*, SCO0284, α-galactosidase, GH 27 family, NPCBM

Introduction

Galactose is widely distributed in nature as monomers, oligosaccharides, and polysaccharides in plant seeds and cell walls, and it is an important source of renewable energy. Galactosidases (E.C. 3.2.1.) are a group of hydrolytic enzymes that degrade galactosides and are classified as either α- or β-galactosidases depending on the configuration of the anomeric carbon atom in the substrate.

α-Galactosidases (α-D-galactoside galactohydrolase; E.C. 3.2.1.22) catalyze the hydrolysis of α-1,6-linked galactose linkages and release terminal α-galactosyl residues from oligosaccharides/polysaccharides and liposaccharides [26]. They have been reported in microorganisms [15], plants [14], and animals, including humans [4]. α-Galactosidase is of great importance for many bioindustrial applications, such as the processing of food and feed, sugar, paper, and pulp, to enhance sweetness and remove raffinose and stachyose. They are also medically useful for blood group conversion and for the treatment of Fabry disease [26].

Streptomycetes, a soil bacterium, has been used to produce many secondary metabolites, including antibiotics, as well as many enzymes important for food production and other biotechnological applications. They can degrade the insoluble residues from the processing of other biomaterials, such as lignocellulose and chitin in the soil, and are regarded as the central organisms in carbon recycling. According to the genomic sequencing of *Streptomyces coelicolor* A3(2), there are at least 819 (10.5%) potentially secreted proteins, some of which are responsible for exploiting nutrients in the soil [2]. Among the species in the genus *Streptomyces*, *S. coelicolor* A3(2) has unique characteristics in that it can use agar, a galactan from red alga, as a sole carbon source for growth. Two secretory enzymes, DagA [27] and DagB [26], work cooperatively in the degradation of agar into neoagarobiose (3,6-anhydro-α-

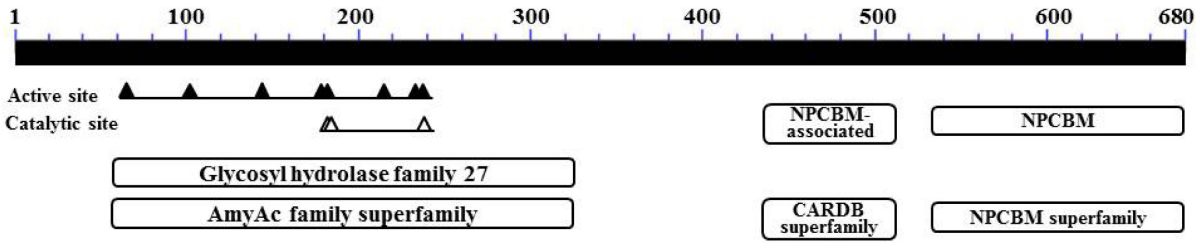

Fig. 1. The distribution of conserved domains in SCO0284.
SCO0284 (680 amino acids) contains a glycosyl hydrolase family 27 domain belonging to the AmyAc family superfamily in the N-terminal region (amino acids 58–324), a carbohydrate-binding module (NPCBM/NEW2) in the C-terminal region (amino acids 540–679), and an NPCBM-associated/NEW3 domain located between the GH27 and NPCBM domains (amino acids 435–512). The conserved amino acids in the active sites (W65, D102, D103, Y144, K180, D182, C214, R233, and D237) and catalytic sites (D182, K180, and D237) are depicted as filled (▲) and open (△) triangles, respectively.

L-galactopyranosyl-1,3-D-galactose). To identify the neoagarobiose hydrolase that can hydrolyze neoagarobiose to 3,6-L-anhydrogalactose and D-galactose, we examined several candidate genes predicted to encode α-galactosidase. Here, we report a functional validation of SCO0284 as an α-galactosidase belonging to glycoside hydrolase family 27 (Fig. 1).

Materials and Methods

Substrates and Chemicals

Chemicals were purchased from Sigma Chemical Co. (USA). DNA-modifying enzymes, including Taq DNA polymerase, were purchased from Roche (Switzerland). Primers for PCR were obtained from DyneBio Inc. (Korea). Silica gel 60 thin-layer chromatography (TLC) plates were purchased from E. Merck AG (Germany).

Bacterial Strains and Plasmids

S. coelicolor A3(2) and *S. lividans* TK24 were obtained from the John Innes Institute, UK. *Escherichia coli* DH5α and the cloning vector T&A were purchased from Promega Corp. (USA) and RBC (Taiwan), respectively. The *Streptomyces-E. coli* shuttle vector pUWL201PW [5] was used to express SCO0284 in *Streptomyces* cells.

Media and Culture Conditions

E. coli was maintained on LB agar and routinely cultured in LB broth at 37°C with agitation [10]. *Streptomyces* strains were grown in R2YE liquid broth at 28°C to prepare protoplasts and isolate genomic or plasmid DNA [13]. Media were supplemented with either ampicillin (50 μg/ml) or thiostrepton (25 μg/ml) when required.

Construction of Expression System

To clone the *SCO0284* gene, PCR was performed using a set of primers designed based on the nucleotide sequence [2]. A 770 bp fragment (SCO0284-N) of the amino-terminal coding region was amplified by PCR using forward primer F1, 5′-CATGCATATGCGTCACCTTCCCATGCG-3′ (NdeI site is underlined) and reverse primer R1, 5′-CTCGGCGAGCTCGAGGTTGCTCTTC-3′ (XhoI site is underlined). The 1,300 bp fragment (SCO0284-C) of the carboxyl-terminal coding region was amplified by PCR using the forward primer F2, 5′-GAAGAGCAACCTCGAGCTCGCCGAG-3′ (XhoI site is underlined), and the reverse primer R2, 5′-CGCGCGAATTCACTCAGGTGCAGGTGACC-3′ (EcoRI site is underlined). The PCR was performed as previously described [27], and the resulting DNA fragments were cloned into the T&A cloning vector to confirm the nucleotide sequence. SCO0284-N and SCO0284-C were double digested with NdeI/XhoI and XhoI/EcoRI, respectively, and ligated together into pUWL201PW digested with NdeI/EcoRI to generate pUWL201-0284.

Transformation Procedure

E. coli was transformed by the $CaCl_2$ method [10]. *Streptomyces* protoplasts were prepared as described and transformed using the polyethylene glycol-mediated transformation method [13]. Transformants were selected by overlaying 1 ml of distilled water containing 500 μg of thiostrepton.

Purification of SCO0284 Protein

S. lividans TK24/pUWL201-0284 was cultured in R2YE broth for 1 day at 28°C with agitation. All purification procedures were carried out at 4°C unless otherwise stated. Culture fluid (1 L) was centrifuged at 6,000 ×*g* for 15 min, and the total protein in the cell-free supernatant was concentrated by ammonium sulfate precipitation (75% saturation). The precipitate was dissolved in buffer A (20 mM Tris-Cl (pH 7.0)) and dialyzed twice against 1 L of buffer A for 16 h. The dialyzed product was concentrated 30-fold by an Amicon Ultra-30 centrifugal filter unit (30 kDa cut-off), and 100 μl of the concentrate was applied to a Superdex 200 HR 10/30 gel filtration column (Amersham Pharmacia Biotech, Sweden) equilibrated with 3× PBS buffer (0.8 g NaCl, 0.02 g KCl, 0.14 g

Na_2HPO_4, and 0.02 g KH_2PO_4 per 100 ml, pH 7.4) containing glycerol (10%). The protein was eluted with equilibration buffer at a flow rate of 0.5 ml/min.

Protein Analysis

The protein concentration was measured by the Bradford method [3] using bovine serum albumin as the standard. Proteins were resolved by 0.1% sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis (SDS-PAGE) as previously described [17].

Enzyme Assay with Chromogenic Substrate

Galactosidase activity was measured by using the artificial chromogenic substrate *p*-nitrophenyl α-D-galactopyranoside (*p*NP-α-Gal). The reaction mixture consisted of 500 µl of reaction buffer (100 mM potassium phosphate (pH 7.0), 10 mM KCl, and 1 mM $MgSO_4$) and 200 µl of *p*NP-α-Gal (4 mg/ml). The mixture was prewarmed at 40°C for 5 min, rapidly mixed with 500 µl of enzyme solution, and incubated at 40°C for 15 min. The reaction was stopped by adding 500 µl of 1 M Na_2CO_3 stop solution, and the absorbance at 420 nm (OD_{420}) was recorded. One unit of enzyme was defined as the amount that produced an OD_{420} of 0.001 after 15 min of incubation.

Biochemical Properties of SCO0284

The temperature profile of SCO0284-mediated hydrolysis was studied by conducting the reaction at 4–70°C and pH 7.0 for 15 min. Thermal stability was determined by pre-incubating the enzyme solution at various temperatures (20–70°C) and pH 7.0 for 1 h and then measuring the residual α-galactosidase activity at 40°C for 15 min. Relative activity was defined as a percentage of the maximum enzyme activity.

The optimum pH was determined in 50 mM buffer solutions with different pH values (pH 4.0–11.0) at 40°C for 15 min. Sodium citrate buffer was used for pH 4.0–5.0, artificial substrate buffer (100 mM potassium phosphate (pH 7.0), 10 mM KCl, and 1 mM $MgSO_4$) was used for pH 7.0, sodium phosphate buffer was used for pH 6.0 and pH 8.0, and glycine-NaOH buffer was used for pH 9.0–11.0.

The purified enzyme was incubated with various metal ions (4 mM), such as $CaCl_2$, $CoCl_2$, $CuCl_2$, NaCl, $NiCl_2$, $MnCl_2$, and $MgCl_2$, as well as EDTA, and then the enzyme activity was measured at pH 7.0 and 40°C for 15 min.

The K_m and V_{max} values for SCO0284 (0.49 µg) acting on *p*NP-α-Gal (final concentration, 0–8 mg/ml) were calculated by linear regression analysis of Lineweaver-Burk double-reciprocal plots of initial velocity data [19] at pH 7.0 and 40°C for 5 min. The values for k_{cat} (turnover number) and k_{cat}/K_m (catalytic efficiency) were calculated based on the K_m, V_{max}, and [E] (enzyme concentration).

Substrate Specificity of SCO0284

Various galactose-containing oligosaccharides, including raffinose, melibiose, and stachyose, were used as substrates. The reaction mixtures, containing 20 µl of purified SCO0284 (8.52 µg), 40 µl of 20 mM Tris-Cl (pH 7.0) buffer, and 40 µl of 1% substrate (w/v) were incubated for 24 h at 40°C, and then analyzed by TLC (Silica Gel 60). The reaction products were developed with *n*-butanol:glacial acetic acid:water (2:1:1 by volume). The sugars on the plate were detected by spraying with sulfuric acid and heating at 90°C for 10 min. A portion of the reaction sample was dried in vacuo, extracted with methanol, and then dried again in vacuo. The molecular mass distribution of the reaction products was determined using direct MALDI TOF mass spectrometry (Autoflex III; USA).

Results

Heterologous Expression of SCO0284 in *S. lividans* TK24 and its Purification

S. lividans TK24/pUWL201-0284 was cultivated in R2YE medium at 28°C for 7 days, and agarase activity and cell growth were assessed during the cultivation. *S. lividans* TK24/pUWL201-0284 showed faster and higher growth than the control (24.4 g vs. 11.6 g of wet cell weight/l after 3 days of cultivation). Galactosidase activity toward *p*NP-α-Gal in *S. lividans* TK24/pUWL201-0284 cells consistently reached a maximum (A_{420} = 2.3) on day 6, which was 6 times higher than the maximum activity (A_{420} = 0.41) of the control (Fig. 2A). SDS-PAGE analysis of the total extracellular protein revealed a protein with the approximate molecular weight (Mw) of SCO0284 (68 kDa) that was produced at high levels in the culture broth of *S. lividans* TK24/pUWL201-0284 cells from the first day of cultivation, but not in the culture broth of control cells (Fig. 2B).

Recombinant SCO0284 protein was purified as described in the Materials and Methods. The purified protein showed a purification fold of 83.3 and a yield of 11.2%, and the specific activity toward *p*NP-α-Gal was 27.5 U/mg. SDS-PAGE analysis of the purified protein also revealed a single band with an approximate Mw of 68 kDa (Fig. 2B). The Signal-P program [23] predicted that SCO0284 is cleaved between Ala-39 and Ala-40, yielding a mature form of 641 amino acids (theoretical pI/Mw: 4.90/68,161 Da). Therefore, the Mw of SCO0284 on SDS-PAGE showed good agreement with the Mw of the mature protein deduced from the nucleotide sequence.

Enzymatic Properties of Recombinant SCO0284 as an α-Galactosidase

Stereospecificity. When the galactosidase activity was measured using artificial chromogenic substrates, SCO0284 showed much stronger activity toward *p*NP-α-Gal (OD_{420} = 2.19) than toward *p*NP-β-Gal (OD_{420} = 0.09). This result clearly implies that SCO0284 is an α-galactosidase that specifically

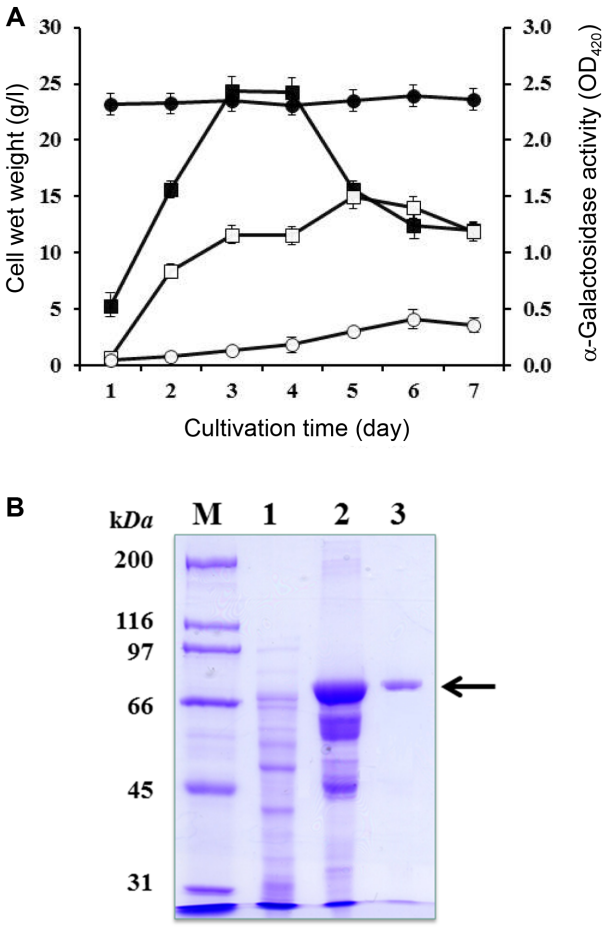

Fig. 2. Heterologous expression and purification of SCO0284 in *S. lividans* TK24.

(**A**) Cell growth as wet cell weight (g/l) and α-galactosidase activity (OD_{420}) of *S. lividans* TK24/pUWL201-0284 cells as a function of cultivation time. Cell growth (□) and α-galactosidase activity (○) of the control cells (*S. lividans* TK24/pUWL201PW), and cell growth (■) and α-galactosidase activity (●) of *S. lividans* TK24/pUWL201-0284. (**B**) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the proteins expressed by *S. lividans* TK24. SCO0284 overexpressed in *S. lividans* TK24 was purified by gel filtration chromatography using a Superdex 200 HR 10/30 column and fractionated by 0.1% SDS-10% PAGE. Lane M: molecular weight standards; lane 1: total extracellular protein from *S. lividans* TK24/pUWL201PW (control); lane 2: total extracellular protein from *S. lividans* TK24/pUWL201-0284; and lane 3: purified SCO0284 after gel filtration chromatography. The SCO0284 protein is indicated by a thick arrow.

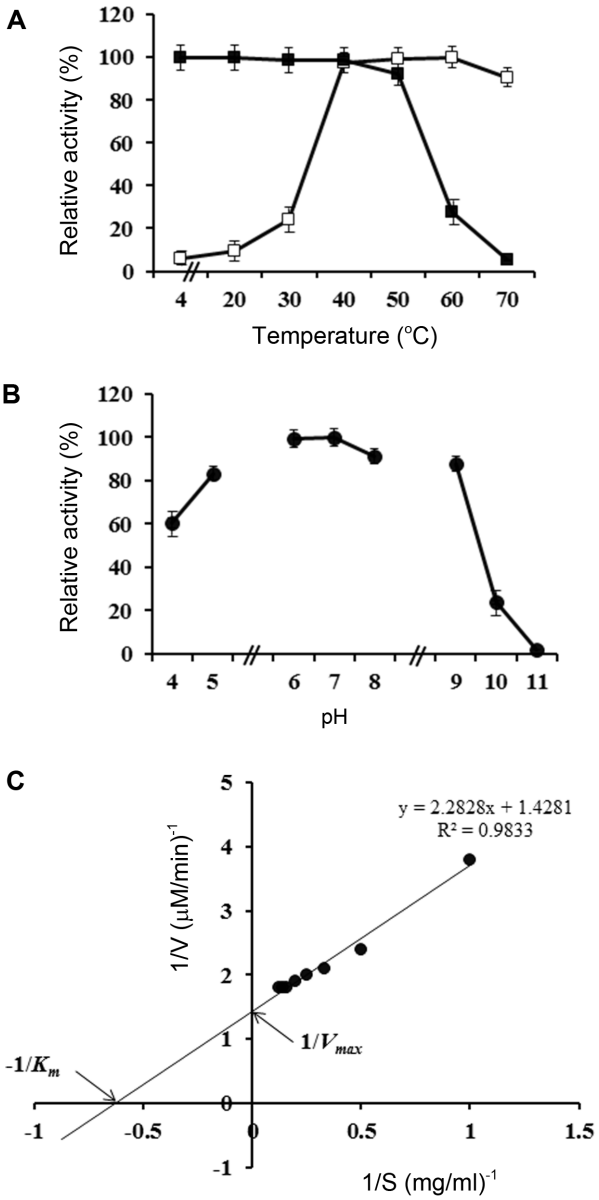

Fig. 3. Biochemical properties of SCO0284 toward *p*-nitrophenyl α-D-galactopyranoside.

(**A**) Effect of temperature on the α-galactosidase activity of SCO0284. □: temperature; ■: stability. (**B**) Effect of pH on the α-galactosidase activity of SCO0284. The reaction was performed in 50 mM buffer solutions with different pH values as described in the Materials and Methods. The highest xylanase activity was set to 100% to calculate the relative activity. (**C**) Enzyme kinetics of SCO0284 α-galactosidase. Lineweaver-Burk plots were used to determine the kinetic parameters of SCO0284 acting on *p*-nitrophenyl α-D-galactopyranoside. All the data are the mean values of three replicate experiments.

recognizes the α-glycosidic bond of D-galactose. Therefore, all subsequent experiments were performed using *p*NP-α-Gal as the substrate.

Effect of temperature. SCO0284 showed maximum activity at 60°C and retained 97.6% and 99.5% of its maximum activity at 40°C and 50°C, respectively (Fig. 3A). The remaining activity after heat treatment at 40°C and

50°C for 1 h was 100% and 90%, respectively, whereas that after treatment at 60°C was 5% (Figs. 3A and S1).

Effect of pH. SCO0284 exhibited the highest activity at pH 7.0, and 99.3% and 91.3% of maximum activity was observed at pH 6.0 and pH 8.0, respectively (Fig. 3B). The enzyme retained approximately 80% of its maximum activity at pH 5.0 and pH 9.0. Therefore, we concluded that the optimum conditions for the α-galactosidase reaction catalyzed by SCO0284 were pH 7.0 and 40°C.

Effects of metal ions and a chelator. Metal ions and a chelator (4 mM), including Na^{+}, Ca^{2+}, Mg^{2+}, Cu^{2+}, Ni^{2+}, Mn^{2+}, Co^{2+}, and EDTA, did not affect the α-galactosidase activity of SCO0284 (Fig. S2). These results indicate that SCO0284 does not require a metal ion as a cofactor.

Enzyme kinetics. The K_m and V_{max} values toward *p*NP-α-Gal were 1.6 mg/ml (0.0053 M) and 71.4 U/mg, respectively (Fig. 3C). The k_{cat} and catalytic efficiency (k_{cat}/K_m) were 0.83 s^{-1} and 1.5 × 10^2 $s^{-1}{\cdot}M^{-1}$, respectively.

Substrate Specificity

The substrate specificity of SCO0284 α-galactosidase was studied by using various galactosides, such as melibiose, raffinose, and stachyose, as well as lactose and neoagarooligosaccharides as substrates (Fig. S3). When the hydrolysates of melibiose, raffinose, and stachyose generated by SCO0284 were analyzed by TLC, the hydrolyzed products, D-galactose and shortened oligosaccharides, were clearly detected (Fig. 4A). MALDI TOF mass spectral analysis revealed that SCO0284 could hydrolyze raffinose (*m/z* 527 $[M+Na]^{+}$) into galactose (*m/z* 203 $[M+Na]^{+}$) and sucrose (*m/z* 365 $[M+Na]^{+}$) (Fig. 4B). It also hydrolyzed melibiose (*m/z* 365 $[M+Na]^{+}$) into galactose (*m/z* 203 $[M+Na]^{+}$) and glucose (*m/z* 203 $[M+Na]^{+}$) and hydrolyzed stachyose (*m/z* 689 $[M+Na]^{+}$) into galactose (*m/z* 203 $[M+Na]^{+}$), sucrose (*m/z* 365 $[M+Na]^{+}$), and raffinose (*m/z* 527 $[M+Na]^{+}$) (Figs. S4 and S5). SCO0284 could not hydrolyze lactose, which has β(1,4)-linkages of D-galactose, or agar, neoagarotetraose, and neoagarobiose, which have α(1,3)-linkages of D-galactose (data not shown). Based on these data, we concluded that SCO0284 is an α-galactosidase that specifically recognizes and hydrolyzes α(1,6)-glycosidic bonds and releases terminal D-galactose.

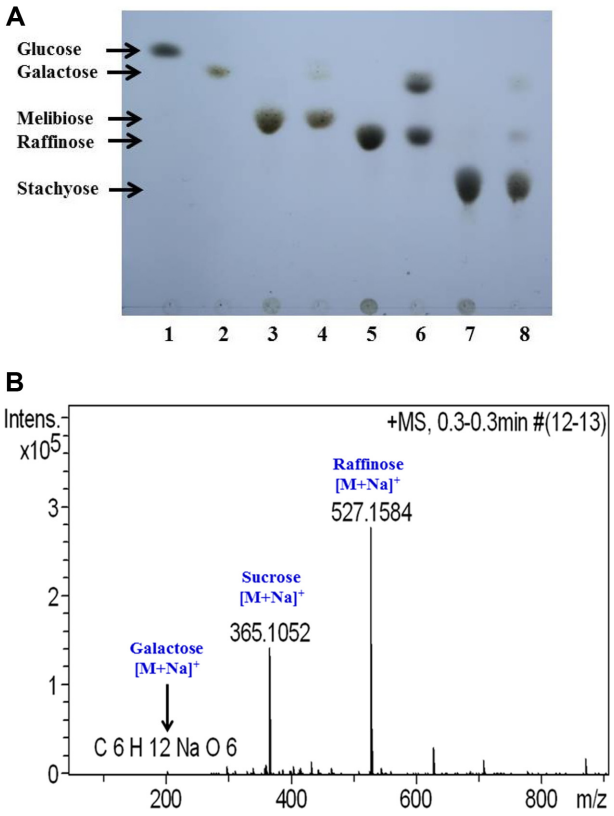

Fig. 4. Analysis of the substrate specificity of SCO0284.
(**A**) Thin-layer chromatography (TLC). Hydrolysis reactions using various substrates (1%) were carried out at 40°C for 24 h, and then the hydrolysates were separated on a Silica Gel 60 TLC plate. 1: glucose; 2: galactose; 3: melibiose; 5: raffinose; 7: stachyose; 4, 6, and 8: hydrolysates of melibiose, raffinose, and stachyose generated by SCO0284, respectively. (**B**) MALDI TOF mass spectrogram. The total hydrolysate from a reaction using raffinose as the substrate was dried in vacuo and extracted with methanol. The molecular mass distribution was then determined using direct MALDI TOF mass spectrometry. Spectra were recorded in reflector positive-ion mode, and the peaks for raffinose (*m/z* 527 $[M+Na]^{+}$) and its hydrolyzed products, galactose (*m/z* 203 $[M+Na]^{+}$) and sucrose (*m/z* 365 $[M+Na]^{+}$), are shown. The MALDI-TOF mass spectrograms for two other substrates, melibiose and stachyose, are presented in Figs. S2 and S3, respectively.

Discussion

SCO0284 was selected as a putative α-galactosidase in *S. coelicolor* A3(2). As shown in Fig. 1, SCO0284 contains a GH 27 and α-amylase catalytic superfamily domain (CD 14792) in the N-terminal region (amino acids 58–324, e-value 8.21 × e^{-158}) with a triad of catalytic residues (Asp-182, Glu-180, and Asp-237) [8]. GH 27 enzymes, which are found in both eukaryotes and prokaryotes, including Archaea, have a wide range of hydrolytic activities, including α-glucosidase and 3-α-isomalto-dextranase, and hydrolyze α-(1,4) and α-(1,6) glycosidic linkages with retention of the anomeric center. It contains a novel putative carbohydrate-binding module (NPCBM/NEW2) domain (pfam08305) in the C-terminal region (amino acids 540–679) with an e-value of

$9.09 \times e^{-67}$, which is typically found in the N-terminal region of GH family 98 proteins [22]. An NPCBM-associated/NEW3 domain (pfam10633) is also located between the GH27 and NPCBM domains (amino acids 435–512, e-value $1.74 \times e^{-18}$). This domain is associated with the NPCBM family (pfam08305) and is found in bacterial galactosidases; however, its exact function is still unknown.

In the genomic sequence of *S. coelicolor*, at least 20 open reading frames were annotated as putative galactosidases. In this study, we demonstrated that SCO0284 is a GH family 27 α-galactosidase that specifically cleaves the α(1,6)-glycosidic bonds of melibiose, raffinose, and stachyose, releasing D-galactose. In silico analysis revealed that SCO0284 shared 99% identity with putative α-galactosidases of *Streptomyces violaceoruber* (Sequence ID: WP030862282.1, 680 amino acids) and *Streptomyces lividans* TK24 (Sequence ID: EFD71674.1, 675 amino acids) and 89% identity with the putative α-galactosidase of *Streptomyces* sp. NRRL WC-3753 (Sequence ID: WP054101716.1, 683 amino acids). However, none of the encoding proteins was functionally validated. Therefore, we expect that at least the two proteins with the highest identity, WP030862282.1 and EFD71674.1, have properties similar to that of SCO0284.

Until now, one α-galactosidase (SCO0274), belonging to the GH 36 family, was reported in *S. coelicolor* [15]. It had optimal activity at 40°C and pH 7, a Mw of 57 kDa, and specifically hydrolyzed raffinose and stachyose, but not melibiose. In contrast to SCO0274, SCO0284 hydrolyzed the α-(1,6)-glycosidic bonds of melibiose, raffinose, and stachyose, releasing galactose. Several α-galactosidases from *Penicillium purpurogenum* [25], *Bacteroides ovatus* [9], *Saccharopolyspora erythraea* [24] were reported to have similar substrate specificities as SCO0284.

Recently, we found that SCO3841 had neoagarobiose hydrolase activity, and thus the complete agar degradation system into monomer by DagA-DagB-SCO3481 will be reported soon. Moreover, *S. coelicolor* produces various enzymes that hydrolyze hemicellulosic biomass such as xylan [6], xyloglucan [7], and cellulose [18]. This hydrolyzing ability of a broad range of biomass conferred this strain to be placed on a high position in efficient utilization of biomass. For example, red algal polysaccharides consist of agar (80%) and cellulose (80%), which can be completely monomerized by the hydrolytic enzymes produced by *S. coelicolor*.

α-Galactosidases have become applicable in the food, feed, and medical industries. They are useful for producing α-galactooligosaccharides and new transglycosylation products, and degrading antinutritive raffinose family oligosaccharides such as raffinose and stachyose sugars, which can relieve gastric distress in humans and animals caused by the ingestion of soybeans and other leguminous plant seeds [16]. Furthermore, it can enhance the crystallization efficiency of beet sugar through the enzymatic hydrolysis of raffinose from beet molasses [21, 25]. α-Galactosidases are also useful for the treatment of Fabry disease, as they hydrolyze terminal glycolipid α-galactosyl residues [20]. More importantly, it can be used for the conversion of blood groups, creating universal donor blood by cleaving a variety of terminal α-galactosyl residues on the erythrocyte surface [1].

In bioindustrial fields, microbial production of α-galactosidase is preferred to plant/animal production owing to its high yields, lower costs, and easy optimization of the processes. In addition, diverse microbial enzymes with different biochemical properties, especially enzymes with the ability to withstand and function efficiently in the acidic gastric environment and at high temperatures, are highly desired for industrial applications [11, 28]. Our system can efficiently produce SCO0284 α-galactosidase in a soluble secreted form with a yield of 34.65 $U \cdot h^{-1} \cdot l^{-1}$ within 24 h. Moreover, SCO0284 is stable over a wide range of pH values (pH 4–9) and is resistant to heat treatment at 50°C, which will be advantageous for biotechnological applications.

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