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Isolation and characterization of a novel α-glucosidase with transglycosylation activity from *Arthrobacter* sp. DL001

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ABSTRACT

A strain of *Arthrobacter* sp. DL001 with high transglycosylation activity was successfully isolated from the Yellow Sea of China. To purify the extracellular enzyme responsible for transglycosylation, a four-step protocol was adopted and the enzyme with electrophoretical purity was obtained. The purified enzyme has a molecular mass of 210 kDa and displays a narrow hydrolysis specificity towards α-1,4-glucosidic bond. Its hydrolytic activity was identified as decreasing in the order of maltotriose > panose > maltose. Only 3.61% maltose activity occurs when *p*-nitrophenyl α-D-glycopyranoside serves as a substrate, suggesting that this enzyme belongs to the type II α-glucosidase. In addition, the enzyme was able to transfer glucosyl groups from the donors containing α-1,4-glucosidic bond specific to glucosides, xylosides and alkyl alcohols in α-1,4- or α-1,6-manners. A decreased order of activity was observed when maltose, maltotriose, panose, β-cyclodextrin and soluble starch served as glycosyl donors, respectively. When maltose was utilized as a donor and a series of *p*-nitrophenyl-glycosides as acceptors, the glucosidase was capable of transferring glucosyl groups to *p*-nitrophenyl-glucosides and *p*-nitrophenyl-xylosides in α-1,4- or α-1,6-manners. The yields of *p*-nitrophenyl-oligosaccharides could reach 42–60% in 2 h. When a series of alkyl alcohols were utilized as acceptors, the enzyme exhibited its transglycosylation activities not only to the primary alcohols but also to the secondary alcohols with carbon chain length 1–4. Therefore, all the results indicated that the purified α-glucosidase present a useful tool for the biosynthesis of oligosaccharides and alkyl glucosides.

1. Introduction

Carbohydrates play a pivotal role in regulating the processes of the embryogenesis, development and differentiation during the human lifespan [1]. Unfortunately, due to their complicated structures and difficulties in chemical synthesis, the role of carbohydrates in detail remain poorly understood [2,3].

Enzymatic-catalyzed transglycosylation is a promising method for synthesis of carbohydrates due to its mild reaction condition, high regio- and stereo-selectivity, less by-products and friendly to environment, especially when generally harsh conditions or toxic (heavy metals) catalysts are undesirable, such as food or cosmetics areas [4]. There are two major classes of enzymes used in glycosylation, the glycosyl transferases and the glycosidases [1,5]. Compared with glycosyltransferases, the glycosidases are synthetically attractive due to their accommodation of inexpensive glycosyl donors, availability and widespread occurrence in nature [6–8].

The α-glucosidases [EC 3.2.1.20, α-D-glucoside glucohydrolase] catalyze not only a cleavage of an α-glucosyl residue substrate, but also transglycosylation reaction to synthesize various α-glucosylated compounds. Based on their substrate specificity, enzymes are divided into three groups (type I, II, and III). Type I α-glucosidase hydrolyzes heterogeneous substrates, such as aryl α-glucosides or sucrose, more efficiently than maltose; type II enzymes prefer maltose and isomaltose to aryl glucosides while type III enzymes possess similar specificity to type II enzymes, but they are capable of attacking polysaccharides like amylose and starch [9]. Although α-glucosidases have been recognized as an ideal candidate for carbohydrate synthesis, challenges still exist for the scientists to explore more glycosidases with high transglycosylation activity and defined catalysis mechanism. Meanwhile, low yield of transglycosylation and hydrolysis of products is another bottleneck needed to be addressed to advance the application of glycosidases in the biosynthesis [3,8].

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In our previous study, an effective methodology of characterizing transglycosylation activity has been well established with the aim to find more glycosidases with high transglycosylation activity from the nature [10–12]. We have demonstrated that this analysis method could detect the transglycosylation activities more precisely and accurately even with the hydrolytic activity. In the present study, we described an isolation of the strain with transglycosylation activity using the modified analytical method and the purification of a new α-glucosidase from the supernatant of the strain culture. Specifically, the substrate specificities, including hydrolytic and donor/acceptor specificity, as well as the linkage types of the products were characterized.

2. Materials and methods

2.1. Chemicals and reagents

Uridine 5′-diphosphoglucose disodium salt (UDPG), 4-methylumbelliferyl α-D-glucoside (MUG), *p*-nitrophenyl α-D-glucopyranoside (pNP α-G), pNP β-D-glucopyranoside (pNP β-G), pNP α-D-xylopyranoside (pNP α-X), pNP β-D-xylopyranoside (pNP β-X), pNP β-D-maltoside, pNP α-L-arabinopyranoside, pNP β-D-cellobioside, pNP α-D-galactopyranoside, pNP β-D-galactopyranoside, pNP β-L-fucopyranoside, pNP α-L-fucopyranoside, pNP β-D-mannopyranoside, pNP α-D-mannopyranoside, pNP α-L-rhamnopyranoside and some saccharides (cellobiose, maltose, panose, trehalose, sucrose and lactose) were obtained from Sigma (St. Louis, MO) and used as enzyme substrates. Maltotriose is a product of Wako Pure Chemical Co., Japan. Glucose oxidase-base Kit was obtained from Rongsheng Biotech Co., Ltd, China. Sephacryl S-200 HR was from GE Healthcare. Toyopearl DEAE-650M and Toyopearl Butyl 650C are from TOSOH Corporation (Japan). Methanol and acetonitrile (HPLC grade) are from Tedia (Fairfield, USA) and millipore water (Millipore, Bedford, MA) was employed. Other general chemicals used were of AR grade.

2.2. Isolation of the strain with transglycosylation activity

Microorganisms were isolated from the seawater samples of the Yellow Sea (Dalian, China). The culture medium was composed of 20 g/l soluble starch, 1 g/l KNO_3, 0.5 g/l NaCl, 0.5 g/l K_2HPO_4, 0.5 g/l $MgSO_4{\cdot}7H_2O$, 0.01 g/l $FeSO_4{\cdot}7H_2O$ (pH 7.0). Seawater samples were added to 100 ml medium and cultured at 30 °C for 48 h to enrich bacteria. Then the culture was spread on 1.5% agar plates (mixed agar with culture medium previously described) and incubated at 30 °C for two days. The appearing colonies were picked and subcultured to get pure isolates. Each isolate was inoculated into 5 ml medium and cultivated aerobically at 30 °C for two days. Broth culture was centrifuged at 8000 × *g* for 10 min at 4 °C, and the cell-free supernatant was stored at −20 °C until use. The transglycosylation activity was determined by incubating the cell-free supernatant with 2.5 mM maltose and 0.5 mM pNP β-G in 50 mM citrate–phosphate buffer (pH 5.5) in a 200 μl reaction mixture at 30 °C for 30 min. The reaction was initiated by addition of the cell-free supernatant and terminated by adding 200 μl methanol. After centrifugation at 21,000 × *g* for 5 min at 4 °C, aliquots of the reaction supernatant were qualitatively and quantitatively analyzed by ultra-fast liquid chromatography/mass spectra (UFLC–MS) and high-performance liquid chromatography (HPLC). The colonies containing transglycosylation activity were re-streaked onto agar plates to obtain pure cultures for further study.

2.3. Strain identification

Cell morphology of the isolated strain was examined under light microscopy (Olympus CK40, Japan). Gram staining was carried out by using the standard Gram reaction [13].

Identification of the isolated strain was done by sequencing the 16S rDNA, which was conducted by Takara Biotechnology (Dalian) Co., Ltd. In brief, the 16S rDNA sequence was amplified by TaKaRa 16S rDNA Bacterial Identification PCR Kit (Code No. D310, TaKaRa, Dalian Co., Ltd). The PCR amplification was conducted in a total volume of 50 μl, containing 1 μl DNA template, 0.2 mM forward primer, 0.2 mM reverse primer and required H_2O. We conducted the PCR amplification as followings: an initial denaturation at 94 °C for 5 min, then 30 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C 1.5 min, followed by a final extension at 72 °C for 5 min. For each reaction, we take 5 μl of purified resultant PCR products to conduct agarose (1%) gel electrophoresis.

The 16S rDNA gene sequence was subjected to BLAST searches of the National Center for Biotechnology Information (NCBI) GenBank database for identification. Related sequences were obtained from the GenBank database. Alignments of sequences were carried out with Clustal W [14]. A phylogenetic tree was constructed using the neighbor-joining method and evaluated by bootstrap sampling (1000 replicates) using the MEGA 4 program [15].

2.4. Purification of extracellular α-glucosidase

Chromatography was performed at room temperature, and fractions were stored at 4 °C upon collection. The following buffers were used in the purification process: buffer A, 50 mM citrate–phosphate buffer (pH 5.5); buffer B, buffer A containing 1.0 M ammonium sulfate.

Two liters of culture was prepared as described in Section 2.2. The bacterial cells were removed by centrifugation at 8000 × *g*, 4 °C for 10 min. The supernatant fraction was subjected to 70–90% saturation ammonium sulfate precipitation. The resulting precipitate was collected and dissolved in buffer B. Then the sample was applied to a Toyopearl Butyl 650C column (Φ 1.2 cm × 5 cm), which was pre-treated with buffer B. Proteins were eluted with a 200-ml linear gradient of 1.0-0 M $(NH_4)_2SO_4$. The α-glucosidase active fractions between 0.6 and 0.45 M $(NH_4)_2SO_4$ were pooled, dialyzed extensively against buffer A at 4 °C overnight, and loaded onto a Toyopearl DEAE-650M column (Φ 1.2 cm × 6 cm) previously equilibrated with the buffer A. A stepwise elution with the buffer over a range of NaCl concentrations (0–0.4 M) was undertaken. The fractions eluted by 0.08 M NaCl were collected. The active fractions eluted by 0.08 M NaCl were combined and concentrated to 0.6 ml by ultrafiltration using a 3 kDa membrane (Microcon YM-3, Millipore). The concentrated sample was loaded on a Sephacryl S-200 HR column (Φ 1.0 cm × 80 cm) which was pre-equilibrated with buffer A. Then we eluted proteins in the same buffer at a flow rate of 0.5 ml/min and collected the fractions with transglycosylation activity.

2.5. Enzyme and protein assay

2.5.1. Transglycosylation activity assay

The transglycosylation activity was determined by using maltose as a sugar donor and pNP β-G as a sugar acceptor. The reaction was conducted by incubating the enzyme solution with 2.5 mM maltose and 0.5 mM pNP β-G in 50 mM citrate–phosphate buffer (pH 5.5) in a 200 μl reaction mixture at 30 °C for 30 min. All the reactions were initiated by the addition of the enzyme and terminated by adding 200 μl methanol. After centrifugation at 21,000 × *g* for 5 min at 4 °C, aliquots of the reaction supernatant (30 μl) were quantitatively analyzed by HPLC. One unit of transglycosylation

activity was defined as the amount of enzyme catalyzes transglycosylation of 1 μmol of pNP β-G per minute.

2.5.2. Hydrolysis activity assay

The α-glucosidase activity was measured by incubating the enzyme solution with 10 mM maltose in 50 mM citrate–phosphate buffer (pH 5.5) at 30 °C for 10 min. The reaction was stopped by heat inactivation at 100 °C for 5 min. The liberated glucose was measured by the glucose oxidase–peroxidase method (GOD-POD) using a glucose oxidase-base kit (Rongsheng Biotech Co., Ltd, China) [16]. One unit of α-glucosidase activity was defined as the amount of enzymes that catalyze hydrolysis of 1 mmol of maltose per minute.

2.5.3. Protein assay

Protein was quantified according to the Bradford method [17], using bovine serum albumin (BSA) as a standard. The fractions eluted from all chromatographic runs were monitored for protein by measuring the absorbance at 280 nm.

2.6. Electrophoresis and determination of molecular weight

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 8% polyacrylamide gel in a Mini-Protein III dual-slab cell electrophoresis unit (Bio-Rad) [18], and proteins were visualized with Coomassie Brilliant Blue R250. Native-PAGE was performed with 8% polyacrylamide gels without SDS. Molecular mass of enzymes was estimated by both SDS-PAGE and gel-filtration on a column of Sephacryl S-200 HR (Φ 1.0 cm × 80 cm). The gel filtration column was equilibrated with 50 mM citrate–phosphate buffer (pH 5.5) and calibrated by elution of standard protein markers, which included catalase (250 kDa), BSA (66 kDa), ovalbumin (44 kDa) and cytochrome C (12 kDa).

2.7. Identification of transglycosylation activity of the protein band

The purified enzyme band without staining on the polyacrylamide gel was excised and dissolved in 50 mM citrate–phosphate buffer (pH 5.5). The extracted protein band was then subjected to the transglycosylation analysis. The reaction was conducted by incubating the band with 2.5 mM maltose and 0.5 mM pNP β-G in 50 mM citrate–phosphate buffer (pH 5.5) at 30 °C for 1 h. The transglycosylation products were analyzed by HPLC.

2.8. Donor/acceptor specificity of transglycosylation

To characterize the synthetic abilities of the purified enzyme, a 200 μl reaction system containing 5 μl purified enzyme, a variety of acceptors and donors in a 50 mM citrate–phosphate buffer (pH 5.5), was incubated at 30 °C for 1 h. When we used pNP β-G (0.5 mM) as an acceptor for the purified enzyme, the synthesis of pNP-oligosaccharides was achieved. To evaluate its donor specificity, pNP β-G (0.5 mM) was used as an acceptor and the reactions were carried out using maltose (0.5, 1, 2.5, 12.5 mM), isomaltose (0.5 mM), maltrotriose (0.5 mM), panose (0.5 mM), UDPG (0.5 mM), sucrose (0.5 mM), β-cyclodextrin (β-CD, 0.5 mM), trehalose (0.5 mM), lactose (0.5 mM), cellobiose (0.5 mM), pNP α-G (0.5 mM), pNP β-G (0.5 mM) or soluble starch (0.5 g/l) as donors. To evaluate the sugar-acceptor specificity, reactions were carried out by using maltose (2.5 mM) as a donor and a series of pNP-glycosides (0.5 mM) as acceptors. All the reactions were stopped by the addition of 200 μl methanol. After centrifugation at 21,000 × *g* for 5 min at 4 °C, aliquots of the reaction supernatants (5 μl) were qualitatively analyzed by UFLC–MS. In the assays with pNP-substrates, if glycosylation occurred, other 30 μl of the supernatants were quantitatively analyzed by HPLC.

The transglycosylation activity towards non-sugar acceptors was measured by using maltose as a donor and methanol, ethanol, 1-propanol, 2-propanol, 1-butanol, or 2-methyl-2-propanol as an acceptor respectively. A 100 μl reaction mixture containing 5 μl purified enzyme, 2.5 mM maltose and alcohol acceptor 10% (v/v) in a 50 mM citrate–phosphate buffer (pH 5.5), was incubated at 30 °C for 1 h. The reaction was stopped by the addition of 100 μl acetonitrile. After centrifugation at 21,000 × *g* for 5 min at 4 °C, 5 μl of the reaction supernatants were qualitatively analyzed by UFLC-ESI-MS and 100 μl of the reaction supernatants were also deposited on Silica gel 60 F 254 plate (Merck, Germany). The reaction products were visualized by soaking rapidly into methanol solution containing 10% (v/v) sulfuric acid and heating it at 110 °C for 10 min. The TLC solvent system was 1-butanol/ethyl acetate/isopropanol/acetic acid/water (1:3:2:1:1, v/v/v/v/v).

2.9. Substrate specificity of hydrolysis

Hydrolytic activity against saccharides (0.5 mM), pNP α/β-G (0.5 mM), β-CD (0.5 mM) and soluble starch (0.5 g/l) were determined by measuring the amount of released glucose with the glucose oxidase–peroxidase method (GOD-POD) using a glucose oxidase-base kit (Rongsheng Biotech Co., Ltd, China) [16].

Activities towards pNP-glycosides were routinely assayed by using a reaction mixture (total volume 300 μl) containing 0.5 mM of each substrate and 5 μl of the purified enzyme in 50 mM citrate–phosphate buffer (pH 5.5). After incubating at 30 °C for 30 min, the reaction was stopped by the addition of 0.25 M NaOH (1.5 ml), and then the absorbance was read at 405 nm with UV–vis spectrophotometer (JASCO V-530, Japan). The reference cuvette contained all reactants except the enzyme. One unit of enzyme activity was defined as the amount of enzyme liberating 1 mmol of *p*-nitrophenol per minute under the above-mentioned conditions.

2.10. Isolation, purification and NMR analysis of transfer products

We used the cell free supernatant to prepare sufficient quantities of the pNP β-G transglycosylation products for NMR analysis, and scaled up the incubation system to 100 ml. The pNP β-G (0.5 mM) was incubated with the cell free supernatant and the maltose (2.5 mM) for 60 min at 30 °C, respectively. Reaction mixture was extracted with 50% ethyl acetate, and the organic layer was separated after the reaction mixture centrifuging at 5000 × *g* for 10 min. The extraction was repeated three times, and the organic layers were combined and dried in a vacuum. The residues were dissolved in methanol (2.5 ml). The substrates and products were separated by HPLC, and the eluent containing the transglycosylation product p1-1 or p1-2 was collected and dried in a vacuum, respectively. The purity of product p1-1 and p1-2 was approximately 90% and 95% (HPLC), respectively.

The product p1-2 and the pNP β-G were dissolved in DMSO-d_6 for NMR analysis. All NMR data (^{1}H, ^{13}C, HMBC, HMQC, COSY and NOESY) were acquired on a Bruker AV-500 spectrometer (Bruker, Newark, DE) operating at 500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR.

2.11. Chromatography conditions

High-performance liquid chromatography (HPLC) was performed following the protocol provided by Okuyama et al. with minor modification [19]. Briefly, the HPLC system (Shimadzu, Kyoto, Japan) consisted of an SCL-10AVP system controller, one LC-10ADVP quaternary pump, a PGU-14A degasser, an SIL-10ADVP autoinjector, and an SPD-M10AVP diode array detector. An ODS column (4.6 mm × 200 mm × 5 μm, Kromasil) was adopted as solid phase, while the mobile phase consisted of H_2O (A) and

CH_3OH (B). Two gradient profiles were employed to analyze different samples containing various substrates: (i) pNP β-G and pNP α-G: 0.01–20.0 min, 15% A; 20.01–25.00 min, 15–90% A; 25.01–35.00 min, 90% A; 35.01–45.00 min, 15% A; (ii) for pNP β-X and pNP α-X, 0.01–20.0 min, 20–40% A; 20.01–25.00 min, 40–90% A; 25.01–35.00 min, 90% A; 35.01–45.00 min, 20% A. The flow rate was 0.7 ml/min, and the injection volume was 30 μl. The detection wavelength was set at 300 nm.

The Ultra-fast liquid chromatography/mass spectra (UFLC–MS) were utilized to qualitatively characterize the products. UFLC system (Shimadzu Kyoto, Japan) equipped with a CBM-20A communications bus module, an SIL-20ACHT auto sampler, two LC-20AD pumps, a DGU-20A3 vacuum degasser, a CTO-20AC column oven and an SPD-M 20A diode array detector. A Shim-pack XR-ODS (150 mm × 2.0 mm, 2.2 μm, Shimadzu) analytical column together with an ODS guard column (5 mm × 2.0 mm, 2.2 μm, Shimadzu) were used and kept at 50 °C. Mass detection was performed on a Shimadzu LCMS-2010EV instrument with an ESI interface in negative ion mode (ESI^-) for pNP-glycosides from *m/z* 200 to 850 and positive ion mode (ESI^+) for alkyl-glycosides from *m/z* 200 to 500. The detector voltage was −1.55 kV and 1.5 kV, respectively. The temperature of either curved desolvation line (CDL) or the block heater was 250 °C. All the other MS detection conditions were set as followings: interface voltage, 4 kV; CDL voltage, 40 V; nebulizing gas (N_2) flow, 1.5 l/min; drying gas (N_2) pressure, 0.06 MPa. For pNP-glycosides analysis, the mobile phase consisted of 0.2% formic acid in H_2O (A) and CH_3CN (B) and the related parameters were listed as followings: 0.01–2.00 min, 92% A; 2.01–10.00 min, 92–12% A; 10.01–12.50 min, 5% A; 12.51–16.00 min, 92% A. The flow rate was 0.4 ml/min, and the injection volume was 5 μl. DAD detection was performed in a range of 190–370 nm. The wavelength of measurement was 300 nm. Similarly, a mobile phase composed of 0.2% formic acid in H_2O (A) and CH_3OH (B) was used to determine alkyl-glycoside under following condition: 0.01–5.00 min, 100% A; 5.01–10.00 min, 100–20% A; 10.01–12.50 min, 10% A; 12.51–16.00 min, 100% A. The flow rate was 0.3 ml/min, and the injection volume was 5 μl.

3. Results

3.1. Identification and characterization of a strain with transglycosylation activity

Several colonies capable of transferring glucosyl groups to pNP β-G were isolated from the seawater. Among them, the isolate with highest transglycosylation yield (named DL001) was selected for the following study. The strain DL001 was an aerobic, gram-negative, motile and rod-shaped bacterium. The colonies appear circular, smooth and milky white with a diameter of 0.5–2.0 mm after culture for 2 days at 30 °C.

To determine the phylogenetic position, the 16S rDNA gene sequence of strain DL001 (comprising 1422 nt) was analyzed, and a dendrogram based on 1422 nucleotides was constructed (Fig. 1). The 16S rDNA gene sequence of strain DL001 has been submitted in the GenBank database with accession No. HM172535. The phylogenetic analysis indicated that the strain DL001 was closely related to the members of the genus *Arthrobacter* sp. (97–98% 16S rDNA gene sequence similarity). This isolated strain has been preserved in China center for type culture collection (CCTCC) with the preservation serial number CCTCC No. 2010107.

3.2. Enzyme purification, molecular mass determination

A novel α-glucosidase was purified through a four-step procedure as described in Table 1. Its transglycosylation activity was monitored throughout the process of purification. The purified enzyme exhibited approximately 108-fold increase in purity with a recovery of 19.2% relative to the amount of the crude enzyme in transglycosylation. The specific activity of the enzyme for the transglycosylation of pNP β-G was 27.7 U mg^{-1} protein. The hydrolysis activity of the purification was also shown in Table 1. The purified enzyme appeared as a single band on PAGE either in the presence (Fig. 2a, lane 4) or absence (Fig. 2b, lane 4) of SDS. Its subunit mass was estimated to be approximately 107 kDa on SDS-PAGE. Gel filtration of the enzyme in Sephacryl S-200 HR produced an apparent molecular mass of 210 kDa. Therefore, the enzyme was though to be a dimer.

In order to clarify whether the protein isolated on PAGE was responsible for the transglycosylation activity, the corresponding band of the enzyme on PAGE without staining was extracted and further incubated with the sugar donor and acceptor. Consequently, two transglycosylation products of pNP β-G were observed, indicating that the purified enzyme possess the transglycosylation activity.

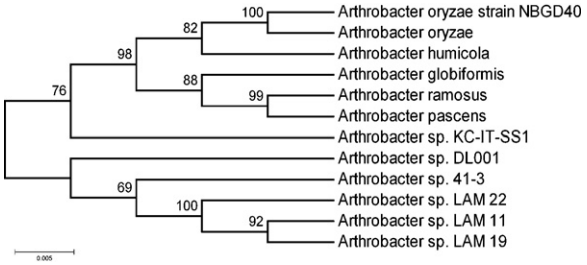

Fig. 1. A phylogenetic tree based on 16S rDNA gene sequences showing the positions of the strain DL001 among species of the genus *Arthrobacter*. Numbers at branch points refer to bootstrap percentages (from 1000 resamplings).

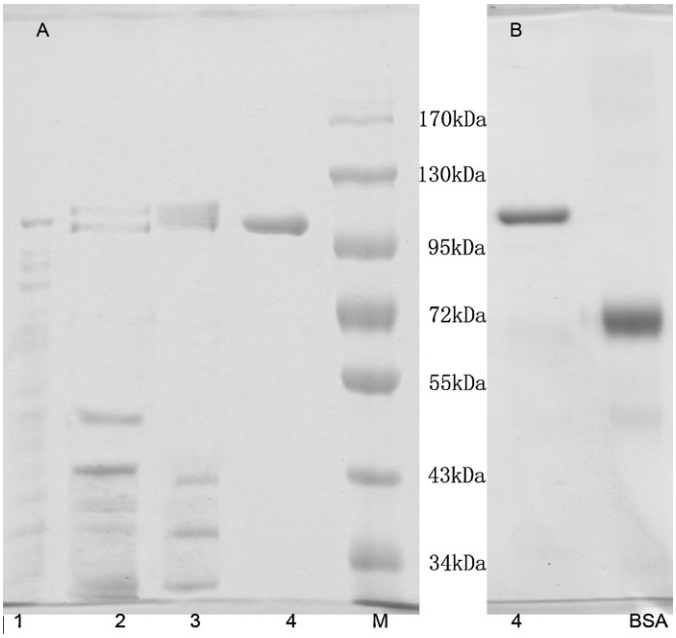

Fig. 2. SDS-PAGE and PAGE of the purified enzyme at various stages of purification. (A) The SDS-PAGE of the enzymes. Lane 1, active fraction after $(NH_4)_2SO_4$ precipitation; lane 2, active fraction after butyl 650C chromatography; lane 3, active fraction after Toyopearl DEAE 650M chromatography; lane 4, the sample after gel-filtration, purified enzyme AG-I; lane M, molecular weight marker. (B) The PAGE of the enzymes. Lane 4, the sample after gel-filtration, purified enzyme AG-I; lane BSA, the standard of BSA.

Table 1
Purification of the α-glucosidase from *Arthrobacter* sp.

Purification step	Total activity (U)[a]	Total protein (mg)[b]	Specificity activity (U/mg)[c]	Yield (%)	Purification (fold)
Hydrolysis activity					
$(NH_4)_2SO_4$ precipitation	24.8	125	0.20	100	1.00
HIC	2.88	6.13	0.47	11.6	2.36
Toyopeal DEAE 650M	1.71	1.40	1.23	6.91	6.16
Sephacryl S200 HR	0.41	0.22	1.86	1.66	9.32
Transglycosylation activity					
$(NH_4)_2SO_4$ precipitation	32.1	125	0.26	100	1.00
HIC	25.8	6.13	4.20	80.3	16.3
Toyopeal DEAE 650M	13.7	1.40	9.83	42.8	38.2
Sephacryl S200 HR	6.15	0.22	27.7	19.2	108

Two liters of cell free supernatants were as used for purification and assays were performed with substrates at pH 5.5 and 30 °C.

[a] Details of the protocol are described in Sections 2.5.1 and 2.5.2. One unit of α-glucosidase hydrolysis activity was defined as the amount of enzyme that catalyzes hydrolysis of 1 mmol of maltose per minute. One unit of transglycosylation activity was defined as the amount of enzyme that catalyzes the transglycosylation of 1 μmol of pNP β-G per minute.

[b] Protein was quantified according to the Bradford method, using bovine serum albumin as a standard.

[c] Specific activity represents the amount of enzyme activity in each milligram of protein.

3.3. Specificity of transglycosylation activity

Transglycosylation activity of the enzyme was evidenced by the fact that we could detect pNP-disaccharides when the enzyme was incubated with maltose and pNP β-G. To further clarify the substrate specificity for transglycosylation, a series of donors and acceptors were adopted. The donor specificity of transglycosylation reaction was investigated by various saccharides when pNP β-G served as an acceptor. As shown in Table 2, a highest donor activity was observed in maltose, which was followed by maltotriose, panose, β-CD and soluble starch. The UDPG and other substrates were considered as non-effective donors. Further analysis of the structures of donors indicated that the saccharides containing α-1,4-linked glucoside might be the effective donors. Therefore, maltose was selected as a standard donor for transglycosylation analysis. Its optimal concentration for standard tranglycosylation assay was further identified as 2.5 mM in the reaction mixture according to the linear range of the donor concentration and the transglycosylation products (data not shown).

The specificity of acceptor was investigated by a series of pNP-substrates and alkyl alcohols when maltose (2.5 mM in the reaction mixture) served as the donor. The constituents in the transglycosylation reactions of pNP-glycosides were separated and qualitatively analyzed by UFLC-ESI-MS.

In the MS chromatography of pNP-substrates, most components predominantly produced $[M+2H_2O-H]^-$ ions depending on their structural features. $[M-H]^-$ and $[M+HCOOH-H]^-$ ions were also observed. These ions provided reliable information for confirming molecular weight of the constituents. With respect to the acceptor pNP β-G (peak 1 in Fig. 3a) and pNP α-G (peak 3 in Fig. 3c), the dominant ion *m/z* 336 $[M+2H_2O-H]^-$ was observed, while the 498 $[M+2H_2O-H]^-$ ion was observed for their transglycosylation products p1-1, p1-2, p3-1 and p3-2 (Fig. 3a and c). Therefore the molecular weight of the products is 463, indicating that a glucosyl group was introduced. In the case of pNP β-X, the dominant ion *m/z* is 306 $[M+2H_2O-H]^-$ (peak 2, Fig. 3b), and their transglycosylation products are 432 $[M-H]^-$, 468 $[M+2H_2O-H]^-$ for p2-1 (Fig. 3g) and 630 $[M+2H_2O-H]^-$ for p2-2 and p2-3, respectively. These products were recognized as the monoglycosylated, diglycosylated and diglycosylated products, respectively. The case of pNP α-X was the same as pNP β-X. But for other pNP-glycosides, no product was detected out. Therefore, the glucosides and xylosides were regarded as the preferred acceptors. Further quantitative analysis of the transglycosylation reaction indicated that about 47% of the pNP β-G was converted into pNP-disaccharides derivatives (data not shown) within 2 h, including 34% pNP β-isomaltoside and 13% pNP β-maltoside. About 60% pNP β-X, 42% pNP α-G and 50% pNP α-X were converted into the corresponding pNP-oligosaccharide within 2 h, respectively (Fig. 3b–d).

The transglycosylation activity was also investigated by using maltose as a donor and six different alcohols as

Table 2
The substrate specificity of α-glucosidase.

Substrate	Hydrolysis specificity[a]	Sugar donor specificity[b]	Sugar acceptor specificity[c]
Maltose	100[1a]	100	N.A.[d]
Isomaltose	3.97[1a]	N.D.[d]	N.A.
Maltotriose	172[1a]	79.0	N.A.
Panose	126[1a]	49.8	N.A.
β-CD	15.2[1a]	23.5	N.A.
Soluble starch	14.4[1a]	16.8	N.A.
Sucrose	1.44[1a]	N.D.[d]	N.A.
Trehalose	1.08[1a]	N.D.	N.A.
Lactose	0.00[1a]	N.D.	N.A.
Cellobiose	0.36[1a]	N.D.	N.A.
Gentiobiose	13.7[1a]	N.D.	N.A.
MUG	0.72[1a]	N.D.	N.A.
UDPG	N.A.	N.D.	N.A.
pNP α-D-glucopyranoside	3.61[1a]	2.10	+
pNP β-D-glucopyranoside	1.44[1a]	N.D.	+
pNP α-D-xylopyranoside	0.00[2a]	N.D.	+
pNP β-D-xylopyranoside	0.00[2a]	N.D.	+
pNP α-L-fucopyranoside	0.00[2a]	N.D.	–
pNP β-L-fucopyranoside	0.00[2a]	N.D.	–
pNP α-D-galactopyranoside	0.00[2a]	N.D.	–
pNP β-D-galactopyranoside	0.00[2a]	N.D.	–
pNP α-D-mannopyranoside	0.00[2a]	N.D.	–
pNP β-D-mannopyranoside	0.00[2a]	N.D.	–
pNP α-L-arabinopyranoside	0.00[2a]	N.D.	–
pNP α-L-rhamnopyranoside	0.00[2a]	N.D.	–
pNP β-D-cellobioside	0.00[2a]	N.D.	+
pNP	N.A.	N.A.	–

The purified enzyme was assayed in the standard assay condition with various compounds. Each value represents the mean of triplicate measurements and varied from the mean by not more than 10%.

[a] The hydrolysis of the substrates in 50 mM citrate–phosphate buffer (pH 5.5) at 30 °C was measured. Activity expressed relative to activity measured on maltose (100%).

[1a] Hydrolysis activity was determined by measuring the amount of released glucose with the glucose oxidase–peroxidase method.

[2a] Hydrolytic activity was determined by measuring the absorbance at 405 nm with UV–vis spectrophotometer (JASCO V-530, Japan) due to the liberation of pNP.

[b] The sugar donor specificity was expressed relative to activity measured with maltose. A 100 μl reaction mixture, containing 5 μl purified enzyme, 0.5 mM pNP β-G as a sugar acceptor and various sugar donor in a 50 mM citrate–phosphate buffer (pH 5.5), was incubated at 30 °C for 30 min.

[c] Sugar acceptor specificity was qualitatively determined by UFLC–MS using maltose as sugar donor. Activity was noted as positive (+) or negative (–).

[d] N.A.: not assay; N.D.: not detected.

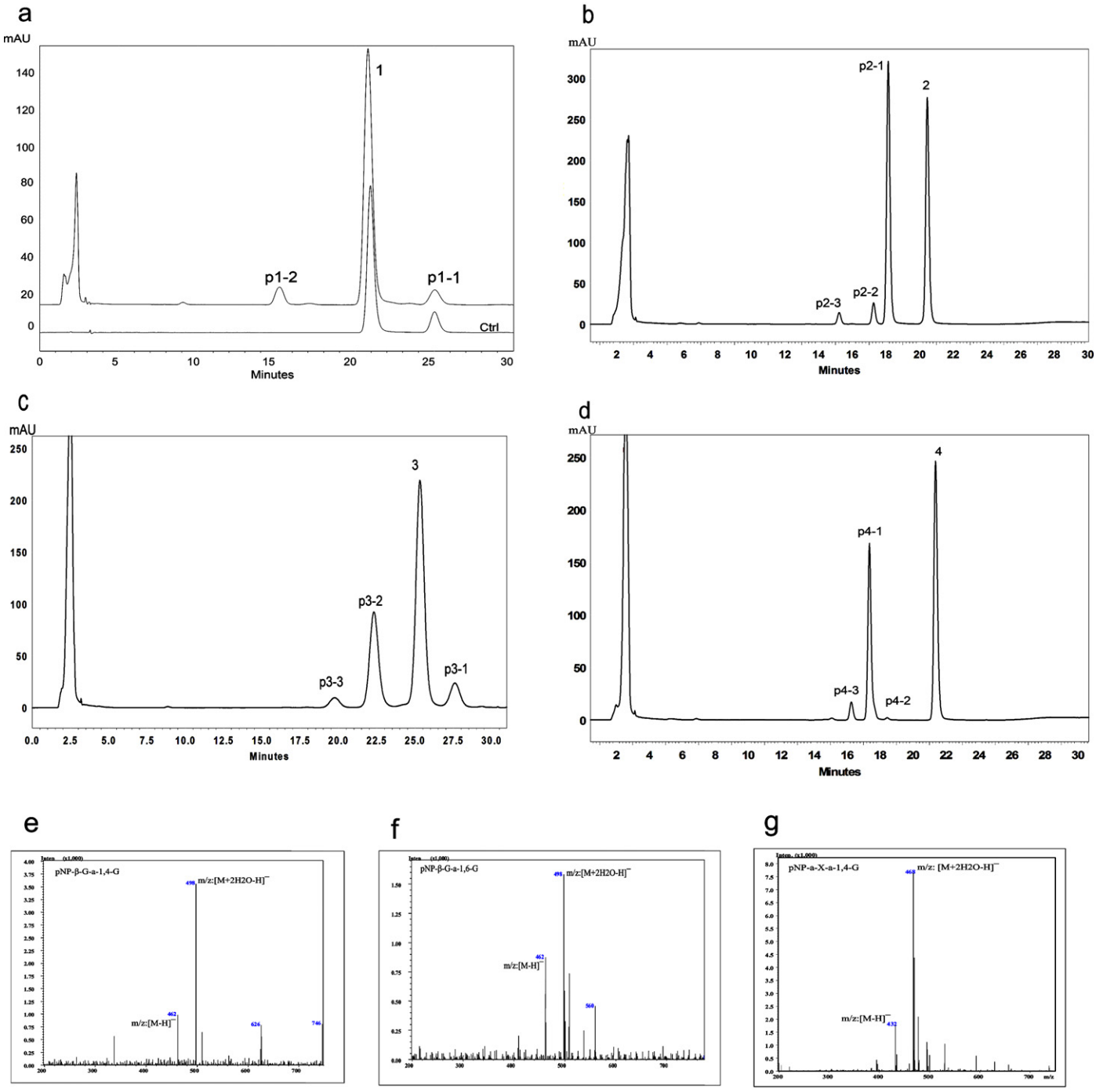

Fig. 3. The chromatography profile of transglycosylation of pNP-glycosides with the purified enzyme. The activity was assayed by incubating the enzyme solution with 2.5 mM maltose and 0.5 mM each substrate (pNP α/β-G or pNP α/β-X) in 50 mM citrate–phosphate buffer (pH 5.5) in a 200 μl reaction mixture at 30 °C for 30 min. (a) The HPLC chromatogram of the transglycosylation of pNP β-G. Ctrl: The standard pNP β-G and pNP β-maltoside. p1, The peak of pNP β-G; peak 1-2, the peak of pNP β-maltoside; peak 1-2, the peak of pNP β-isomaltoside. (b) The HPLC chromatogram of the transglycosylation of pNP β-X; p2, the peak of pNP β-X; p2-1, the peak of monoglucosylation product of pNP β-X; p2-2 and p2-3, the peak of diglucosylation products of pNP β-X. (c) The HPLC chromatogram of the transglycosylation of pNP α-G. p3, The peak of pNP α-G; p3-1 and p3-2, the peak of monoglucosylation product of pNP α-G; p3-3, the peak of diglucosylation products of pNP α-G. (d) The HPLC chromatogram of the transglycosylation of pNP α-X. p4, The peak of pNP α-X; p4-1, the peak of monoglucosylation product of pNP α-X; p4-2 and p4-3, the peak of diglucosylation product of pNP α-X. (e) The mass spectrum (MS) chromatogram of the transglycosylation product pNP β-maltoside (p1-1), m/z = 462 $[M-H]^-$ and 498 $[M+2H_2O-H]^-$ were the main ions observed for pNP β-maltoside. (f) The MS chromatogram of the transglycosylation product pNP β-isomaltosides (p1-2), m/z = 462 $[M-H]^-$ and 498 $[M+2H_2O-H]^-$ were the main ions observed for pNP β-isomaltoside. (g) The MS chromatogram of the transglycosylation product pNP β-X-G (p2-1), m/z = 432 $[M-H]^-$ and 468 $[M+2H_2O-H]^-$ were the main ions observed for the transglycosylation product of pNP β-X.

acceptors (methanol, ethanol, 1-propanol, 2-propanol, 1-butanol and 2-methyl-2-propanol). TLC and MS analysis were applied to determine the transglycosylation products. An extra band considered to be the corresponding transglycosylation product was detected in TLC in the reactions containing alcohols except 2-methyl-2-propanol (Fig. 4, lanes 1–6). The MS analysis revealed that all the transglycosylation products formed the $[M+Na]^+$ ions. Moreover, $[M+K]^+$, $[M+H_2O+Na]^+$, $[M+3H_2O+H]^+$ ions were detected out.

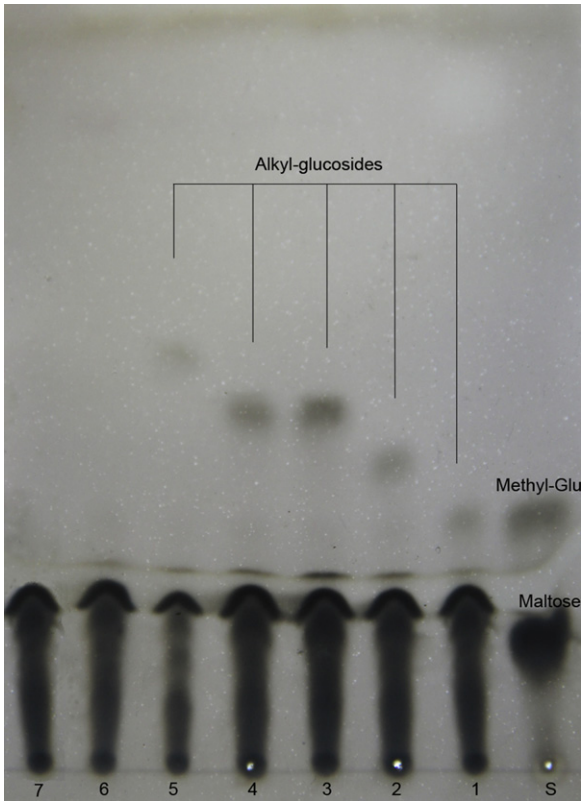

Fig. 4. TLC analysis of the transglycosylation products catalyzed by the purified enzyme. Enzyme reactions were performed as described in Section 2.8 with or without various organic solvents. Reaction products produced with maltose and alcohols (final concentration of 10%). Lane S, standards (methyl-glucoside, maltose); lane 1, methanol; lane 2, ethanol; lane 3, 1-propanol; lane 4, 2-propanol; lane 5, 1-butanol; lane 6, 2-methyl-2-propanol; lane 7, no alcohol. In all reactions containing alcohols, besides the bands of hydrolysis product and substrates, an additional band was detected and was considered as transfer product (alkyl glycoside) because it was not detected in the reaction without alcohol.

The molecular weight of ion $[M+Na]^+$ is m/z = 217, 231, 245, 245 and 259, which are corresponding to methyl glucoside, ethyl glucoside, propyl glucoside, isopropyl glucoside and butyl glucoside (Fig. 5).

3.4. Hydrolytic specificity

No transglycosylation activity was detected when UDPG served as a sugar donor, which indicated that the enzyme involved in the transglycosylation was not transferase but hydrolase. Therefore, we further explored the hydrolysis ability of the enzyme. According to the data in Table 2, the enzyme displayed a narrow hydrolytic specificity towards α-1,4-linked homogeneous substrate. The optimal hydrolytic substrate was maltotriose, followed by panose and maltose. The enzyme displayed 12–15% of maltose activity when gentibiose, β-CD and soluble starch were used as the substrates. Other substrates listed in Table 2 showed only less than 4% of the maltose activity. The hydrolytic substrate specificity of the enzyme suggests the enzyme belongs to the type II glucosidase.

Since the purified enzyme exhibited hydrolysis activity as described above, the data of the hydrolysis activity of the enzyme solutions throughout the purification process was also provided in Table 1. The hydrolysis activity/transglycosylation activity ratios throughout purification steps are not constant, which is probably due to the fact that the culture broth of DL001 was a mixture with multiple-glucosidase.

Table 3
^{13}C data (500 MHz, DMSO, 30 °C) and ^{1}H data (125 MHz, DMSO, 30 °C) for pNP β-G and its transfer product (chemical shifts [ppm], coupling constant [Hz]).

Carbon	pNP β-isomaltose		pNP β-G	
	C	H	C	H
H,C-aromatic				
1	162.4 s	–	162.3 s	–
2,6	125.8 d	8.30	116.5 d	8.20
3,5	116.8 d	7.27	99.8 d	7.22
4	141.8 s	–	141.6 s	–
H,C-glucose				
1′	100.3 d	4.98 (d, J = 7.5 Hz)	99.8 d	5.08 (d, J = 7.3 Hz)
2′	73.2 d	3.43 m	73.0 d	3.27 m
3′	76.6 d	3.30 m	77.2 d	3.38 m
4′	69.9 d	3.16 m	69.4 d	3.19 m
5′	74.9 d	3.66 m	76.4 d	3.29 m
6′	66.4 t	3.73 (d, J = 9.65 Hz)	60.5 t	3.70 m
		3.61 m		3.48 m
1″	98.1 d	4.69 (d, J = 3.6 Hz)		
2″	71.8 d	3.22 m		
3″	73.0 d	3.31 m		
4″	70.1 d	3.08 m		
5″	72.6 d	3.37 m		
6″	60.7 t	3.51 m		
		3.38 m		

3.5. Characterization of the pNP β-G transglycosylation products

Although both pNP β-G and pNP β-X were demonstrated to be effective acceptors, compared to the only one product for pNP β-X, two transglycosylation products were identified when using pNP β-G as acceptor. Therefore, pNP β-G was chosen as the acceptor for the structure analysis of transglycosylation products. In addition, cell-free supernatant was used for preparation of the product because the transglycosylation products catalyzed by either the cell-free supernatant or the purified enzyme were similar (data not shown) according to the LC–MS analysis. According to the transglycosylation analysis, two new peaks (designated as p1-1 and p1-2, Fig. 3a) were detected and further identified as mono-glycosylated products by UFLC–MS (Fig. 3e and f). The peak of p1-1 was identified as pNP β-maltoside evidenced by the m/z $[M+2H_2O-H]^-$ 498 and the retention time exhibited in the UV spectra when compared with the authentic pNP β-maltoside. Due to the limited standard, product of p1-2 was purified by HPLC and further identified by NMR (Table 3). Compared with the substrate, ^{13}C NMR spectrum of transformed product exhibited additional carbon signals of δ60.7, δ72.6, δ70.1, δ73.0, δ71.8 and δ98.1. This suggests the presence of an additional sugar residue in the chemical molecule. The anomeric proton signal of this sugar (δ4.69, J = 3.6 Hz) was also observed. All the data supported the involvement of the α-D-glucopyranosyl residue. In the HMBC spectrum, the proton signal of δ 4.69 (H-1″) exhibited long-range correlation with the carbon signals of δ66.4, δ72.6 and δ73.0, respectively, suggesting the substitution pattern of the glycosyl moiety. Thus, this transformed product was identified as pNP β-D-isomaltoside.

4. Discussion

Glycosidases are increasingly becoming more attractable in the biosynthesis due to their transglycosylation activities. Nearly 3000 glycosidases in 130 clans has been recorded in CAZy database (http://www.cazy.org/Home.html) so far. However, only a few of them were reported to possess the transglycosylation activity. Moreover, among those glycosidases with transglycosylation activity, over 90% of them were failed to be utilized for the biosynthesis due to their pretty low transglycosylation activity [20].

Shortage of the effective methods for product analysis has become a major bottleneck that severely hinders the isolation

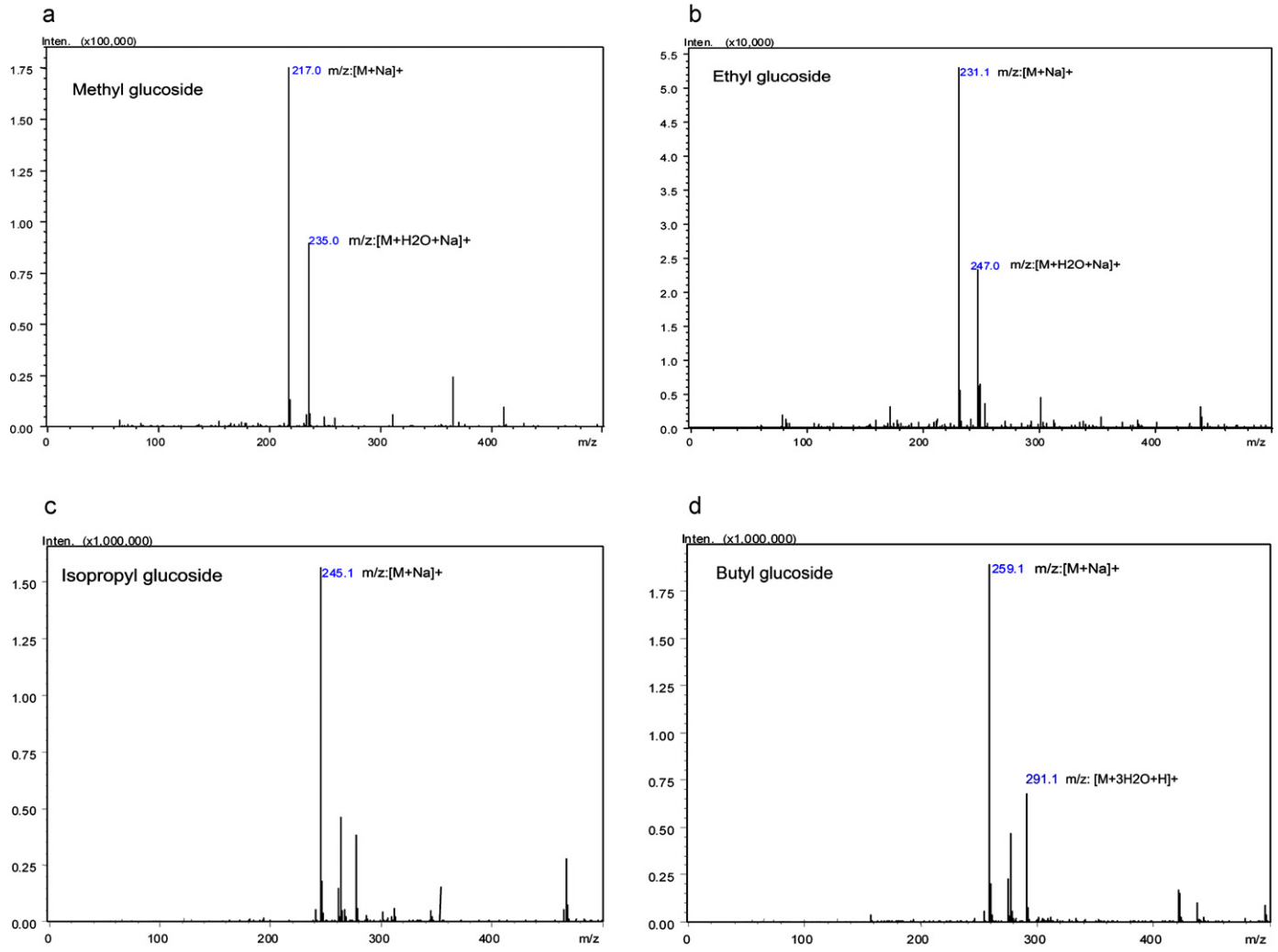

Fig. 5. The representative mass spectrum (MS) chromatogram of transglycosylation products of alkyl alcohols. The transglycosylation reactions were conducted in a 100 μl reaction mixture containing 50 μl enzyme, 2.5 mM maltose and alkyl alcohol acceptors (10%, V/V) in a 50 mM citrate–phosphate buffer (pH 5.5). (a) MS chromatogram of methyl glucoside, $m/z = 217$ $[M+Na]^+$. (b) MS chromatogram of ethyl glucoside, $m/z = 231$ $[M+Na]^+$. (c) MS chromatogram of isopropyl glucoside, $m/z = 245$ $[M+Na]^+$. (d) MS chromatography of butyl glucoside, $m/z = 259$ $[M+Na]^+$.

of enzyme with transglycosylation activity and further clarification of the related mechanism. The major problem for the current transglycosylation analysis methodologies, including qualitatively TLC and quantitatively HPAEC method, is the difficulty in discriminating the products with only difference of linkage manners. In addition, for some transglycosylation reactions, especially the transglycosylation of monosaccharide using maltose as a donor, the presence of excessive amounts of donor also interfered with the detection and analysis of transglycosylation products (disaccharides) [21]. It has been demonstrated that the pyranosidic structures possessing several hydroxyl groups often performed as an most interesting acceptors for the transglycosylation reactions catalyzed by glycosidases [22]. Therefore, in this research, a transglycosylation activity-oriented method was utilized for glucosidase discovery. Using the pNP-derivative of pyranose as an acceptor and maltose as a donor, the glucosdiase-catalyzed transglycosylation reaction was analyzed by qualitative UFLC–MS and quantitative HPLC method. Specifically, the utilization of pNP-derivative of pyranose as the acceptor could successfully avoid the interference between the glycosyl donors and the transglycosylation products. Meanwhile, the transglycosylation products with the different linkage manner could also be separated effectively. Using this method, a strain with transglycosylation activity was successfully isolated, and its enzyme was also evaluated in respect to substrate specificity.

The ocean is an underdeveloped biosystem distinct from the earth. A number of studies have indicated that the ocean environment furnished different sources of glycosyl hydrolases [23]. Thus, further explorating these enzymes and their transglycosylation activity are essential for the rational exploitation of the ocean resources. In the present study, the Yellow Sea of China is considered as a attractable resource for enzyme isolation. A strain with transglycosylation activity was isolated and a new α-glucosidase contributed to transglycosylation activity was purified through a four-step process. Our results demonstrated that this enzyme was type II glucosidase with narrow hydrolytic specificity toward α-1,4-glucosidic bond. The hydrolytic activity decreased in an order of maltotriose > panose > maltose, and only 3.61% maltose activity occurred when pNP α-G served as the substrate. These results were different from most of other ocean original α-glucosidase. For the α-glucosidase from *Aplysia fasciata* or strain *Geobacillus*, the K_{cat}/K_m for pNP α-G is 8 times or 57 times higher than that for maltose [24,25].

The comprehensive study of the sugar donor and acceptor specificity of the purified α-glucosidase was essential for the related mechanism study and its application. Our results showed that this

enzyme can transfer glucosyl groups from the donors containing α-1,4-glucosidic bond to their acceptors. The best transglycosylation activity occurs when maltose served as a donor, followed by maltotriose, panose, β-CD and starch. The transglycosylation activity decreased with the increasing length of the sugar chain. The sugar acceptor specificity researches considered the glucosides and xylosides as efficacious acceptors. From the comparison of structures of the substrates that could or could not act as acceptor, we concluded that an effective acceptor should possess pyranose structure with a similar configuration of the free C2-, C3-, and C4-hydroxyl groups to D-glucopyranose, while the configuration of C1-hydroxyl groups is less important (both pNP β-G and pNP α-G could be transglycosylated). Due to the exclusively structural difference between glucoside and xyloside is a hydroxyl methyl group on C-6 position, we speculate that C-6 position of the acceptor has little correlation with the transglycosylation. Several investigators have reported the formation of oligosaccharides from maltose catalyzed by α-glucosidase or other enzymes [24,26–28]. For example, Malaĭ and colleagues compared the substrate specificity of α-glucosidase from *Bacillus stearothermophilus* and Brewer's yeast [29]. They found that when xylose, mannose, galactose and sorbose were used as the acceptors, the α-glucosidase from *B. stearothermophilus* exhibited specific transglycosylation activity towards xylose only, and the yield was about 11%. In contrast, α-glucosidase from Brewer's yeast, which had a relative wider hydrolytic specificity, exhibited transglycosylation activity toward all the test acceptors with different yields varying from 7% to 15%. Based on their substrate specificity, it was hypothesized that the enzyme with high hydrolytic specificity usually has narrow acceptor specificity. Our results reported here are somewhat consistent with this speculation.

The specificity of the transglycosylation activity towards non-sugar acceptors (alkyl alcohols) was also qualitatively analyzed by TLC and MS. Our results supported that the enzyme was able to transfer glucosyl groups from maltose to primary or secondary alcohol with different length of the carbon chain (C1–C4). It has been well documented that most of the glucosidases exhibit acceptable transglycosylation activity towards alkyl alcohols only when using a donor with an easy leaving group, such as pNP-G [20,30]. The fact that the enzyme could catalyze the transglycosylation of alkyl alcohols using maltose as a donor suggests the exclusive advantage of this enzyme during the biosynthesis of the alkyl glucoside.

In addition, the enzyme displayed α-1,4 (minor) or α-1,6 (major) regioselectivity in transglycosylation and could convert 42–60% of pNP-monoglycosides to pNP-oligoglycosides even at a relatively low concentration of glycosyl donor (maltose, 2.5 mM) (Fig. 1b–d). These transglycosylation yields are significantly higher than those in the previous reports in which only 20–40% yields could be achieved at a relatively high sugar donor concentration (>200 mM) [25,29,31–33], and even comparable to some mutant glucosynthases [1].

It can be concluded that the enzyme be able to transfer glucosyl group from donors containing α-1,4-glucosidic bond specifically to glucosides, xylosides and alkyl alcohols in α-1,4- or α-1,6-manners. Combined with the identification of transglycosylation products, the specificity study of sugar acceptors and donors provided some hints to the mechanism of the transglycosylation reaction. Firstly, the fact that the enzyme accepted substrates containing α-1,4-glucosidic bond as sugar donors suggested that hydrolysis of an α-1,4-glucosidic linkage of the sugar donor might be the first step of the transglycosylation reaction. The donor specificity was in accordance with its hydrolytic specificity. Secondly, based on the structure analysis of the products, α-D-glucopyranosyl moiety was transferred to the acceptor. This indicated that sugar acceptors could compete with water molecules in the process of the product releasing, if a retaining mechanism is assumed for our enzyme. Finally, the identification of diglycosylation products indicated that the glycosylated products could serve as or become better sugar acceptors for the following glycosylation reaction. Thus, the enzyme could be a robust tool for multi-glycosylation.

Taken together, a new strain named *Arthrobacter* sp. DL001 with high transglycosylation activity was isolated from the Yellow Sea of China by using the transglycosylation activity-oriented method. Enzymatic studies of the enzyme purified from the strain culture indicated it had a narrow hydrolytic specificity and high specific for sugar donor. The facts that the α-glucosidase could transfer 42–60% pNP-monoglycosides to pNP-oligoglycosides and the glycosylated products could serve as glycosyl acceptors for further glycosylation indicate that the enzyme is of great biosynthesis potential. This work provides not only an improved strategy for transglycosylation study but also a robust tool for the biosynthesis of oligosaccharides and alkyl glucosides.

Acknowledgments

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