Enzymatic synthesis of branched α-D-glucosyl trisaccharides by novel maltose phosphorylase from *Bacillus selenitireducens* MLS10

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Summary

We discovered an inverting maltose phosphorylase (Bsel2056) belonging to glycoside hydrolase family 65 from *Bacillus selenitireducens* MLS10, which possesses synthetic ability for α-D-glucosyl disaccharides and trisaccharides through the reverse phosphorolysis with β-D-glucose 1-phosphate as the donor. Bsel2056 showed the preference for monosaccharide acceptors with alternative C2 substituent (2-amino-2-deoxy-D-glucose, 2-deoxy-D-*arabino*-hexose, 2-acetamido-2-deoxy-D-glucose, D-mannose), resulting in production of 1,4-α-D-glucosyl disaccharides with strict regioselectivity. In addition, Bsel2056 synthesized two maltose derivatives possessing additional D-glucosyl residue bound to C2 position of the D-glucose residue at the reducing end, 1,4-α-D-glucopyranosyl-[1,2-α-D-glucopyranosyl]-D-glucose and 1,4-α-D-glucopyranosyl-[1,2-β-D-glucopyranosyl]-D-glucose, from 1,2-α-D-glucopyranosyl-D-glucose (kojibiose) and 1,2-β-D-glucopyranosyl-D-glucose (sophorose), respectively, as the acceptors. Modeling structure analysis using *Lactobacillus brevis* GH65 maltose phosphorylase as the template and superimposition of an inhibitory pseudomaltotetrasacchaide acarbose from a complex with *Aspergillus awamori* GH15 glucoamylase suggested that Bsel2056 possessed a binding space to accommodate the bulky C2 substituent of D-glucose.

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Key words : branched α-D-glucosyl trisaccharide; glycoside hydrolase family 65; maltose phosphorylase.

Various oligosaccharides possessing multiple functional bioactive properties such as prebiotic effects (Gibson, 1995), antibacterial effects (Hickey, 2012), and immunopotentiative action (Murosaki *et al*., 1999) have been reported and widely used as food additive and pharmaceutical materials (Patel and Goyal, 2011). The functions depend on their constituent carbohydrates and linkage types (Sela and Mills, 2010). Therefore, it would be valuable to produce a wide variety of oligosaccharides efficiently and specifically by suitable synthetic methods. Disaccharide phosphorylases catalyze phosphorolysis of particular disaccharide to produce monosaccharide 1-phosphate (Luley-Goedl and Nidetzky, 2010 ; Kitaoka and Hayashi, 2002). The phosphorolysis is reversible, making the phosphorylases suitable catalysts for efficient syntheses of particular disaccharides. Various oligosaccharides have been synthesized using the reverse phosphorolysis from the corresponding sugar 1-phosphate as donor substrate and suitable acceptors (Kitaoka *et al*., 1991). In addition, the reversibility makes it possible to synthesize oligosaccharides practically from abundantly available natural sugar by using single phosphorylase (Kitaoka and Hayashi, 2002 ; Sawangwan *et al*., 2009 ; Nakai *et al*., 2010a) or by the combined reaction of two phosphorylases that share the same monosaccharide 1-phosphate (Daurat-Larroque *et al*., 1982 ; Murao *et al*., 1985 ; Kitaoka *et al*., 1992a ; Kitaoka *et al*., 1993 ; Ohdan *et al*., 2007 ; Nishimoto and Kitaoka, 2007 and 2009 ; Suzuki *et al*., 2009 ; Nakajima *et al*., 2010). Maltose phosphorylase (EC 2.4.1.8) are characterized from several bacteria (Inoue *et al*., 2002b ; Nakai *et al*., 2009 ; Kamogawa *et al*., 1973 ; Hidata *et al*., 2005) and found to catalyze reversible phosphorolysis of maltose with inversion of the anomeric configuration at the C1 atom. Sequence analysis has assigned the maltose phosphorylase to glycoside hydrolase family (GH) 65, together with trehalose phosphorylase (EC 2.4.1.20) (Inoue *et al*., 2002a ; Maruta *et al*., 2002), kojibiose phosphorylase (EC 2.4.1.230) (Chaen *et al*., 1999 ; Yamanoto *et al*., 2004), nigerose phosphorylase (EC 2.4.1.279) (Nihira *et al*., 2012a), and α,α-trehalase (EC 3.2.1.28) (Destruelle *et al*., 1995) in CAZy database (http://www.cazy.org : Cantarel *et al*., 2009). The three-dimensional structure of GH65 maltose phosphorylase from *Lactobacillus brevis* has been determined (Egloff *et al*., 2001) and shows similarities with the $(\alpha/\alpha)_6$-barrel fold of GH15 glucoamylase (EC 3.2.1.3) (Aleshin *et al*., 1994), and the GH94 cellobiose phosphorylase (EC 2.4.1.20) (Hidaka *et al*., 2006) and *N,N'*-diacetylchitobiose phosphorylase (EC 2.4.1.280) (Honda *et al*., 2004). Previously we reported that the reverse phosphorolysis catalyzed by GH65 maltose phosphorylase from *Lactobacillus acidophilus* NCFM, which transferred from the glucosyl moiety of β-D-glucose 1-phosphate to several monosaccharide acceptors, was useful for specific production of α-(1,4)-D-glucosyl disaccharides (Nakai *et al*., 2009, 2010a, and 2010b). By contrast, none of di- and

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trisaccharides served as the acceptors (Nakai *et al*., 2009). We here report about the first identification of a GH65 maltose phosphorylase possessing synthetic ability for branched α-D-glucosyl trisaccharides through the reverse phosphorolysis.

MATERIALS AND METHODS

Sequence analysis

ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) was used for the phylogenetic analysis using full-length amino acid sequences of functionally characterized GH65 members (http://www.cazy.org/GH65.html). A rectangular cladogram tree was generated using treeview version 1.6.6 software and a bootstrap test based on 1000 resamplings (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

Construction of expression plasmid

The *bsel2056* (GenBank ID: ADH99560) was amplified by PCR from genomic DNA of *B. selenitireducens* MLS10 as the template using KOD-plus DNA polymerase (Toyobo, Osaka, Japan) with the following oligonucleotides based on the genomic sequence (GenBank ID: CP001791): 5'-atggctagcaaacc ttatttaaaagaagatcc -3' as the forward primer containing an *Nhe*I site (underlined) and 5'- tttctcgagttaatgattaatggatagttc -3' as the reverse primer containing a *Xho*I site (underlined). The amplified *bsel2056* was purified with a High Pure PCR Purification Kit (Roche Applied Science, Mannheim, Germany), digested by *Nhe*I and *Xho*I (New England Biolabs, Beverly, MA, USA), and inserted into pET28a (+) (Novagen, Madison, WI, USA) to add a His_6-tag at the N-terminal of the recombinant protein. The expression plasmid *Bsel2056*/pET28a was propagated in *E. coli* DH5a (Toyobo, Osaka, Japan), purified by High Pure Plasmid Isolation Kit (Roche Applied Science), and verified by sequencing (Operon Biotechnologies, Tokyo, Japan).

Recombinant Bsel2056 preparation

An *E. coli* BL21 (DE3) (Novagen) transformant harboring *bsel2056*/pET28a was grown at 37℃ in 200 mL Luria-Bertani medium (1% tryptone, 0.5% yeast extract, and 0.5% NaCl) containing 50 μg/mL of kanamycin up to an absorbance of 0.6 at 600 nm. Expression was induced by 0.1 mM isopropyl β-D-thiogalactopyranoside and continued at 18℃ for 24 h. Wet cells harvested by centrifugation at 20,000g for 20 min was suspended in 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-NaOH (pH 7.5) containing 500 mM NaCl (buffer A). The suspended cells were disrupted by sonication (Branson sonifier 250A , Branson Ultrasonics, Emerson Japan, Ltd., Kanagawa, Japan), and the supernatant collected by centrifugation at 20,000g for 20 min was applied to a HisTrap HP column (GE Healthcare, Buckinghamshire, UK) equilibrated with buffer A containing 10 mM imidazole using a ÄKTA prime (GE Healthcare). After a wash with buffer A containing 22 mM imidazole, followed by elution using a 22-400 mM imidazole linear gradient in buffer A, fractions containing Bsel2056 were pooled, dialyzed against 10 mM HEPES-NaOH (pH 7.0), and concentrated (AMICON Ultra; Millipore Co., Billerica, MA, USA). The protein concentration was determined spectrophotometrically at 280 nm using a theoretical extinction coefficient of ε = 163,070 $cm^{-1}M^{-1}$, based on the amino acid sequence (Pace *et al*., 1995). SDS polyacrylamide-gel electrophoresis was performed using Mini-PROTEAN Tetra electrophoresis system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) according to the manufacture's protocol.

Measurement of enzymatic activity

Phosphorolytic activity was routinely determined by quantifying the D-glucose released during the reaction in 50 mM 2-morpholinoethanesulfonic acid (MES)-NaOH (pH 6.5), 10 mM substrates, and 10 mM phosphate at 30 ℃ by glucose oxidase-peroxidase method (Bauminge, 1974), as described previously (Nihira *et al*., 2012a). Reverse phosphorolytic activity was routinely determined by measuring the increase in phosphate in the reaction mixture containing 10 mM β-D-glucose 1-phosphate and 10 mM D-glucose in 50 mM sodium acetate (pH 5.5) at 30 ℃ by following the method of Lowry and Lopez (Lowry and Lopez, 1946) as described previously (Nihira *et al*., 2012a).

Substrate specificity analysis

Phosphorolytic activities on α-linked glucobioses (trehalose, kojibiose, nigerose, maltose, and isomaltose) were determined under the standard conditions using 87 nM Bsel2056 by quantifying the liberated D-glucose. The phosphorolytic activities on maltooligosaccharides of DP 3-6 under the standard conditions using 87 nM Bsel2056 were examined by thin layer chromatography (TLC). The reaction mixture was spotted on a TLC plate (Kieselgel 60 F_{254}; Merck, Darmstadt, Germany), and the sample was developed with a solution of acetonitrile-water (7:3, v/v). The TLC plates were soaked in 5% sulfuric acid-methanol solution and heated in an oven until bands were sufficiently visible.

Acceptor specificity analysis

Reverse phosphorolysis to investigate the acceptor specificity of Bsel2056 (17 μM) was performed under the standard conditions by substituting D-glucose into putative carbohydrate acceptors (D-mannose, D-allose, D-galactose, D-xylose, D-arabinose, L-arabinose, D-lyxose, L-rhamnose, D-fructose, 2-deoxy-D-*arabino*-hexose, D-glucal, 2-amino-2-deoxy-D-glucose, 2-amino-2-deoxy-D-galactose, 2-amino-2-deoxy-D-mannose, α-D-glucose 1-phosphate, β-D-glucose 1-phosphate, D-glucose 6-phosphate, D-glucuronic acid, D-galacturonic acid, methyl α-D-glucoside, methyl β-D-glucoside, 1,5-anhydro-D-glucitol, 3-*O*-methyl-D-glucose, 2-acetamido-2-deoxy-D-glucose, 2-acetamido-2-deoxy-D-galactose, 2-acetamido-2-deoxy-D-mannose, trehalose, kojibiose, nigerose, maltose, isomaltose, sophorose, laminaribiose, cellobiose, gentiobiose, xylobiose, melibiose, lactose, lacturose, 1,4-β-D-mannobiose, sucrose, and *N,N'*-diacetylchitobiose) for 2 h. The reaction mixture was spotted on a TLC plate, and

the sample was developed with a solution of acetonitrile-water (4:1, v/v). The TLC plates were soaked in 5% sulfuric acid-methanol solution and heated in an oven until bands were sufficiently visible.

Temperature and pH profile

The effects of pH on the enzymatic activity for phosphorolysis and synthesis of maltose using 87 nM Bsel2056 was measured under the standard conditions by substituting 50 mM MES-NaOH (pH 6.5) and 50 mM sodium acetate buffer (pH 5.5) into the following 40 mM buffers: sodium citrate (pH 3.0-5.5), bis(2-hydroxyethyl) iminotris(hydroxymethyl)methane-HCl (pH 5.5-7.0), HEPES-NaOH (pH 7.0-8.5), and glycine-NaOH (pH 8.5-10.5). The thermal and pH stabilities were evaluated by measuring the residual phosphorolytic activity on maltose under the standard conditions after incubation of Bsel2056 (435 nM) at the temperature range 30-90 ℃ for 15 min in 50 mM MES-NaOH (pH 6.5) and in the various pH values at 4 ℃ for 24 h, respectively.

Kinetic analysis

Kinetic analysis of the phosphorolytic reaction was performed by using the continuous glucose assay. Reaction mixtures were prepared in wells of a 96-well microtiter plate by adding various concentration of maltose (0.50, 1.0, 2.0, 3.0, and 5.0 mM), various concentrations of phosphate (0.50, 1.0, 2.0, 3.0 and 5.0 mM), and 8.7 nM Bsel2056 to the coloring reagent for the D-glucose assay prepared with 50 mM MES-NaOH buffer (pH 6.5). The reaction was carried out in a temperature-controlled microplate reader (Thermo Fisher Scientific Inc., Waltham, MA) at 30℃ , and the absorbance at 505 nm was continuously monitored without stop reaction at 30 sec intervals. The kinetic parameters were calculated by curve-fitting the experimental data to the theoretical equation (1) for sequential bi bi mechanism using GraFit version 7.0.2 (Erithacus Software Ltd., London, UK).

$$v = V_{max}[A][B]/(K_{iA}K_{mB} + K_{mB}[A] + K_{mA}[B] + [A][B]) \quad (1)$$

(A = maltose, B = phosphate)

Kinetic analysis of the reverse phosphorolysis was carried out under the standard conditions with 22-433 nM Bsel2056 and various concentrations (1.0, 2.0, 3.0, 5.0, 10, 20, and 70 mM) of the suitable acceptors. The kinetic parameters were calculated by curve fitting the experimental data with the Michaelis-Menten equation $\{v = k_{cat}[E]_0[S] / (K_m + [S])\}$ using GraFit version 7.0.2.

Structural Determination of branched trisaccharides

Products for structural determination were generated in reaction mixture containing 17 μM Bsel2056, 50 mM β-D-glucose 1-phosphate, and 50 mM kojibiose or sophorose in a final volume of 500 μL of 5 mM sodium acetate buffer (pH 5.5) incubated at 30 ℃ for 24 h. The reaction mixtures were desalted using Amberlite MB-3 (Organo, Tokyo, Japan), and the products were purified by a high performance liquid chromatography system (Prominence, Shimadzu, Kyoto, Japan) equipped with a Shodex Asahipak NH2P-50 4E column (4.6 mm φ × 250 mm; Showa Denko K.K., Tokyo, Japan) at 30℃ under a constant flow (1.0 mL/min) of mobile phase (acetonitrile/water v/v, 75/25). Fractions containing the reaction products were collected, and followed by lyophilization. The amounts of both products obtained were 2 mg. The one-dimensional (^{1}H and ^{13}C) and two-dimensional [double-quantum-filtered correlation spectroscopy (DQF-COSY), totally correlated spectroscopy (TOCSY), heteronuclear single-quantum coherence (HSQC), and heteronuclear multiple-bond correlation (HMBC)] nuclear magnetic resonance (NMR) spectra of the product was taken in D_2O with 2-methyl-2-propanol as an internal standard using a Bruker Avance 800 spectrometer (Bruker Biospin, Rheinstetten, Germany). Proton signals were assigned based on DQF-COSY spectra. ^{13}C signals were assigned with HSQC spectra, based on the assignment of proton signals. The linkage position in each trisaccharide was determined by detecting interring cross peaks in each HMBC spectrum.

Homology modeling of Bsel2056

The three-dimensional structure of Bsel2056 was modeled by swiss model (http://swissmodel.expasy.org/)[47] using the reported structure of *L. brevis* maltose phosphorylase (PDB: 1H54) (Egloff *et al.*, 2001) as a template. To illustrate substrate and acceptor binding interaction to Bsel2056, acarbose of the complex with *Aspergillus awamori* glucoamylase belonging to GH15 (PDB: 1AGM) (Aleshin *et al.*, 1994) was superimposed on to the modeled Bsel2056 structure using PyMOL Molecular Graphics System Version 1.5.0.4 (Schrödinger, LLC., San Francisco, CA).

RESULTS AND DISCUSSION

Gene cloning, expression, and purification of recombinant Bsel2056

The genomic sequence of *B. selenitireducens* MLS10 (GenBank ID: CP000896) has revealed that it possesses three genes encoding possible GH 65 phosphorylases (http://www.cazy.org/GH65_bacteria.html). A GH65 homologous protein (Bsel2056) encoded by *bsel2056* (GenBank ID: ABX81345.1) exhibits 67% sequence identity and 78% similarity with maltose phosphorylase from *Paenibacillus* sp. (Hidaka *et al.*, 2005) and the phylogenetic tree analysis also categorizes it as maltose phosphorylase. In this study, the *bsel2056* cloned from the genomic DNA of *B. selenitireducens* MLS10 was expressed as a His_6-tag fusion protein in *Escherichia coli* BL21 (DE3) to investigate its enzymatic properties, resulting that approximately 12 mg of purified protein was obtained from cell lysate of 200 mL culture. The purified Bsel2056 migrated in SDS polyacrylamide-gel electrophoresis as a single protein band with an estimated size of approximately 90 kDa in agreement with the theoretical molecular mass of 90,660.

Basic properties of Bsel2056

Bsel2056 showed highest apparent phosphorolytic

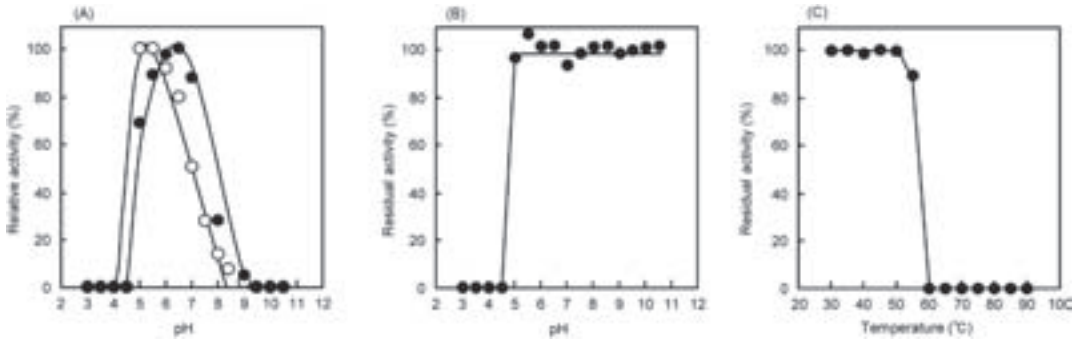

Fig 1. Effect of pH and temperature on the activity and stability of Bsel2056. (A) pH activity dependence for phosphorolysis and synthesis of maltose by 87 nM Bsel2056 in 40 mM sodium citrate (pH 3.0-5.5), bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane-HCl (pH 5.5-7.0), HEPES-NaOH (pH 7.0-8.5), and glycine-NaOH (pH 8.5-10.5). Closed and open symbols represent phosphorolytic and synthetic activities, respectively. (B) pH stability of 435 nM Bsel2056 at 4 ℃ for 24 h. (C) Stability of 435 nM Bsel2056 at the temperature range 30-90 ℃ for 15 min.

activity at pH 6.5, whereas the reverse phosphorolytic reaction has optimum at pH 5.5 (Fig. 1A). Bsel2056 was stable at 4 ℃ for 24 h in the range of pH 5.0 to 10.5 (Fig. 1B) and during 15 min incubation up to 50 ℃ (Fig. 1C).

Substrate specificity of Bsel2056 in phosphorolysis

The phosphorolytic activity of Bsel2056 on several α-linked glucobioses such as trehalose (α1, α1), kojibiose (α-1,2), nigerose (α-1,3), maltose (α-1,4), and isomaltose (α-1,6) was examined. In the presence of inorganic phosphate, Bsel2056 phosphorolyzed only maltose with inversion of the anomeric configuration releasing β-D-glucose 1-phosphate and D-glucose. Bsel2056 did not cleave the maltose in the absence of inorganic phosphate. In addition, maltooligosaccharides of degree of polymerization (DP) 3-6 were not substrate for Bsel2056. These results indicate that Bsel2056 is typical maltose phosphorylase, as with those from *L. acidophilus* (Nakai *et al*., 2009), *L. brevis* (Kamogawa *et al*., 1973) and *Paenibacillus* sp. (Hidaka *et al*. 2005). The double reciprocal plots of initial velocities against various initial concentrations of maltose and phosphate gave a series of lines intersecting at a point. This indicates that the phosphorolytic reaction follows a sequential bi bi mechanism, as reported for other inverting phosphorylases (Kitaoka and Hayashi, 2002 ; Nihira *et al*., 2012a and 2012b ; Tsumuraya *et al*., 1990 ; Nakajima and Kitaoka, 2008 ; Nakajima *et al*., 2009 ; Kitaoka *et al*., 1992b ; Schwarz *et al*., 2007 ; Derensy-Dron *et al*., 1999) including maltose phosphorylases from *L. brevis* (Tsumuraya *et al*., 1990). Kinetic parameters that appear in Equation 1 for the sequential bi bi mechanism (see Kinetic analysis in MATERIALS AND METHODS) were calculated to be k_{cat} = 12.3 s^{-1}, K_{mA} = 0.6 mM, K_{mB} = 0.1 mM, and K_{iA} = 24 mM, where A and B represent maltose and phosphate, respectively. The kinetic parameters are in the same range as other inverting phosphorylases (Kitaoka and Hayashi, 2002 ; Nihira *et al*., 2012a and 2012b ; Tsumuraya *et al*., 1990 ; Nakajima and Kitaoka, 2008 ; Nakajima *et al*., 2009 ; Kitaoka *et al*., 1992b ; Schwarz *et al*., 2007 ; Derensy-Dron *et al*., 1999), suggesting that maltose is the true substrate for Bsel2056.

Acceptor specificity of Bsel2056 in reverse phosphorolysis

The acceptor specificity in reverse phosphorolysis was examined by using various putative carbohydrate acceptors (see Acceptor specificity analysis in MATERIALS AND METHODS) together with β-D-glucose 1-phosphate as the donor, resulting that 8 monosaccharide [2-amino-2-deoxy-D-glucose, 2-deoxy-D-*arabino*-hexopyranose, D-glucose, D-xylose, 2-acetamido-2-deoxy-D-glucose, D-mannose, 1,5-anhydro-D-glucitol, and D-glucal] were acted as the acceptors, as with maltose phosphorylases from *L. acidophilus* (Nakai *et al*., 2009). Kinetic parameters on the suitable acceptors were summarized in Table 1. The acceptor recognition was illustrated by superimposing an inhibitory pseudomaltotetrasacchaide acarbose in a complex with *Aspergillus awamori* glucoamylase (PDB: 1AGM) of GH15 (Aleshin *et al*., 1994), constituting clan GH-L together with GH65, onto the modeled Bsel2056 structure constructed by using the reported structure of *L. brevis* maltose phosphorylase (PDB: 1H54) (Egloff *et al*., 2001) as a template (Fig. 2). The structural similarity coupled with the function of GH15 glucoamylase catalyzing *exo*-hydrolysis of α-1,4-linked maltooligosaccharides by an inverting mechanism supports that the environment at the active site of GH65 maltose phosphorylase is similar to that of GH15 glucoamylase. The recognition of equatorial C3 hydroxyl group of quinovose unit situated at subsite +1 by hydrogen bond interactions with Lys598 and Glu486 of Bsel2056 is in agreement with the acceptor specificity. Thus Bsel2056 did not utilize D-glucose derivatives at the C3 position (allose and 3-*O*-methyl-D-glucose), clearly suggesting that the equatorial C3 hydroxyl group of D-glucose is essential for acceptor binding at subsite +1. In addition, His415 and Glu417 hydrogen bond interactions with the α-anomeric C1 hydroxyl group support the low k_{cat}/K_m values of 1,5-anhydro-D-glucitol and D-glucal substituted

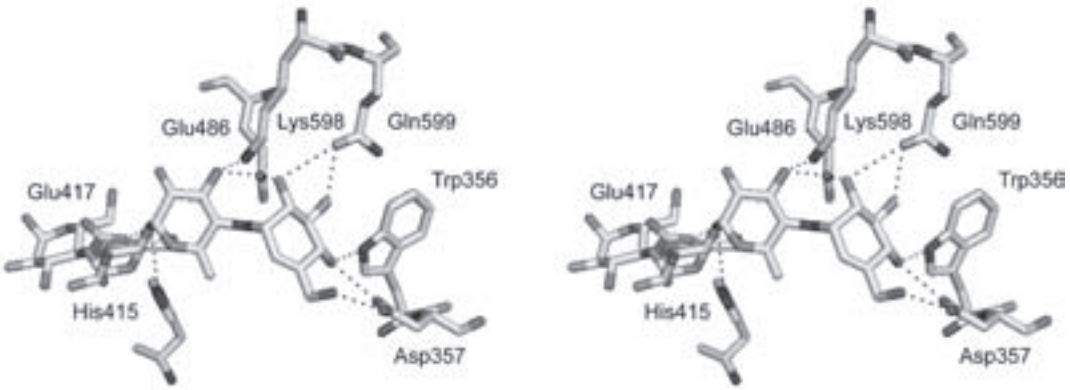

Fig 2. Stereo representation of the predicted substrate recognition of Bsel2056 at the active site. Acarbose from the complex with *A. awamori* GH15 glucoamylase (PDB: 1AGM) was superimposed onto the Bsel2056 structure modeled by using the structure of *L. brevis* GH65 maltose phosphorylase (PDB: 1H54) as a template. Suggested hydrogen bonds are shown as dotted lines.

Table 1. Kinetic parameters for the reverse phosphorolysis catalyzed by Bsel2056

Acceptor	K_m (mM)	k_{cat} (s^{-1})	k_{cat} / K_m (mM^{-1} s^{-1})
2-amino-2-deoxy-D-glucose	9.8	120	12
2-deoxy-D-*arabino*-hexose	12	136	12
D-glucose	10	107	11
D-xylose	13	122	9.4
2-acetamido-2-deoxy-D-glucose	40	159	4.0
D-mannose	12	31.9	2.6
1,5-anhydro-D-glucitol	ND	ND	0.060
D-glucal	ND	ND	0.060
kojibiose	24	40.0	1.7
sophorose	4.6	6.55	1.4

Calculated by curve-fitting with the curve of Michaelis-Menten Equation

C1 position (Table 1), as reported that maltose phosphorylase recognized α-maltose as the substrate (Tsumuraya *et al.*, 1990). Moreover the Bsel2056/acarbose model suggests that C2 hydroxyl group at subsite +1 would be solvent-exposed (Fig. 3), in accordance with accommodation of the C2 substituent of D-glucose (2-amino-2-deoxy-D-glucose and 2-acetamido-2-deoxy-D-glucose). Noticeably, Bsel2056 also utilized 1,2-α-D-glucopyranosyl-D-glucose (kojibiose) and 1,2-β-D-glucopyranosyl-D-glucose (sophorose) as the acceptors, which were not substrates for other disaccharide-specific maltose phosphorylases reported (Inoue *et al.*, 2002b ; Nakai *et al.*, 2009 ; Kamogawa *et al.*, 1973, Hidaka *et al.*, 2005 ; Tsumuraya *et al.*, 1990). The single trisaccharide products (Fig. 4) from kojibiose and sophorose were identified by ^{1}H- and ^{13}C-NMR spectroscopic analysis to be the corresponding 1,4-α-D-glucopyranosyl-[1,2-α-D-glucopyranosyl]-D-glucose and 1,4-α-D-glucopyranosyl-[1,2-β-D-glucopyranosyl]-D-glucose, respectively. Each branched structure was evidenced by the inter-ring HMBC cross peaks observed in the two positions (C2 and C4) of each reducing end D-glucose unit.

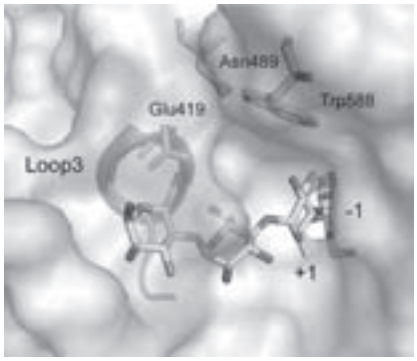

Fig. 3. Surface representation of the predicted binding site to accommodate C2 substituent of D-glucose derivatives. Acarbose was superimposed onto the modeled structure of Bsel2056. Amino acid residues (Glu419, Asn489, and Trp588) predicted to be involved in the acceptor recognition at the subsite +2 and loop 3 (His415-Glu423) preventing the binding of maltose as acceptor is indicated by stick and ribbon representation, respectively.

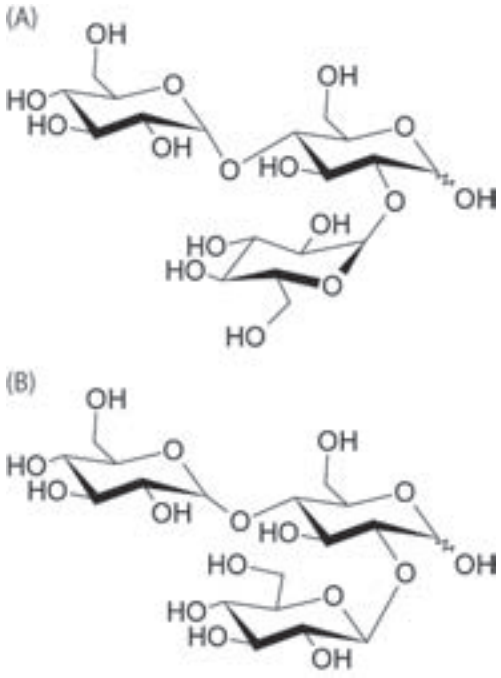

Fig. 4. Structures of branched trisaccharides produced by reverse phosphorolysis catalyzed by Bsel2056. (A), 1,4-α-D-glucopyranosyl-[1,2-α-D-glucopyranosyl]-D-glucose; (B), 1,4-α-D-glucopyranosyl-[1,2-β-D-glucopyranosyl]-D-glucose.

These results indicate that the C2 substituted D-glucose unit at the reducing end is required for binding of disaccharide acceptor to Bsel2056 at subsite +1 and formation of branched trisaccharides. The Bsel2056/acarbose model also suggests that Bsel2056 would possess a binding space enough to accommodate the bulky D-glucose unit at non-reducing end of kojibiose and sophorose (Fig. 3), which is predicted to be constructed by stacking interaction with Trp588 and hydrogen bond interactions with Glu419 and Asn489. In addition, it should be noted that the synthetic reaction of Bsel2056 for branched trisaccharides is clearly different from the continuous extension reaction for linear-chained oligosaccharides catalyzed by kojibiose phosphorylase (Charen *et al*., 2001). The Bsel2056/acarbose model supports that a loop 3 (His415-Glu423) between α3 and α4 of the $(\alpha/\alpha)_6$-barrel domain forms a barrier that prevents binding of maltose as the acceptor and production of liner-chained maltooligosaccharides (Fig. 3).

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マルトースホスホリラーゼを用いた分岐グルコ３糖の生産

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要　約

糖質加リン酸分解酵素（ホスホリラーゼ）は、その反応の可逆性と厳密な反応位置特異性からオリゴ糖合成に利用可能である。しかし既知のホスホリラーゼは16種類のみ報告されており、新たな特異性を有するホスホリラーゼの発見が望まれている。そこで今回 *Bacillus selenitireducens* MLS10が有する Glycoside Hydrolase Family 65に属するマルトースホスホリラーゼ遺伝子（*bsel2056*）をクローニングし、組換え酵素の糖受容体特異性を調査した。結果、グルコースの２位にアミノ基を有するグルコサミンなど８種の単糖類を糖受容体とし、α-1,4- グルコ２糖を生産することが明らかになった。さらに既知のマルトースホスホリラーゼとは異なり、本酵素は１、２結合を有するグルコ２糖すなわちコウジビオースおよびソフォロースを糖受容体として認識し、分岐グルコ３糖を生産した。また3D モデリング解析の結果から、糖受容体として結合するグルコースの２位の水酸基周辺に本酵素特有の嵩高い置換基（アミノ基やグルコース残基）を収納するための結合スペースが認められた。

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