Enzymatic Synthesis of α-1,3-Glucosyl Disaccharides by Novel Nigerose Phosphorylase from *Clostridium phytofermentans*

Takanori NIHIRA[1,2], Hiroyuki NAKAI[1,2,3*] and Motomitsu KITAOKA[3]

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Summary

A novel phosphorylase (Cphy1874) belonging to glycoside hydrolase family 65 was first characterized from *Clostridium phytofermentans*. Recombinant Cphy1874 protein produced in Escherichia coli showed phosphorolytic activity toward nigerose in the presence of inorganic phosphate, resulting in release of D-glucose and β-D-glucose 1-phosphate with inversion of the anomeric configuration. Kinetic parameters of the phosphorolytic activity on nigerose were k_{cat} 67 s-1 and K_m 1.7 mM. Additionally Cphy1874 showed the highest synthetic activity with D-glucose as the acceptor in the reverse reaction with β-D-glucose 1-phosphate as the donor. The synthesized product was mostly nigerose at the early-stage of the reaction. The enzyme also showed synthetic activity with D-xylose, 1,5-anhydro-D-glucitol, D-galactose, and methyl-α-D-glucoside in this order. All the major products were α-1,3-glucosyl disaccharides. We proposed nigerose phosphorylase as the short name of the enzyme.

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Key words :nigerose phosphorylase, glycoside hydrolase family 65, *Clostridium phytofermentans*

Phosphorylases are exo-lytic enzymes catalyzing phosphorolysis to produce monosaccharide 1-phosphate with strict substrate specificity. The reaction is reversible, enabling practical syntheses of oligosaccharides from the monosaccharide 1-phosphate as donor and several carbohydrate acceptors with strict regioselectivity (Kitaoka and Hayashi, 2002; Luley-Goedl and Nidetzky, 2010). However, only 15 phosphorylases have been identified (Kitaoka and Hayashi, 2002), the variety of oligosaccharides synthesized by the reported phosphorylases has been limited. Therefore it would be beneficial to find novel phosphorylases that possess unreported substrate specificities in reversible phosphorolysis, leading to increase structural diversity of synthetic oligosaccharides.

Clostridium phytofermentans is an anaerobic mesophilic cellulolytic bacterium found in forest soil (Warnick *et al*., 2002). Its whole genome sequence (GenBankTM accession number CP000885) revealed that it possesses many glycoside hydrolases that is capable of fermenting all major carbohydrate components of biomass. We noticed that *C. phytofermentans* also possesses a number of genes encoding putative phosphorylases belonging to glycoside hydrolase family (GH) 65, 94, and 112 classified based on the amino acid sequences (Cantarel *et al*., 2009). Our recent report on the GH112 proteins has revealed that two of them are 3-*O*-β-D-galactopyranosyl-*N*-acetyl-D-hexosamine phosphorylase (Cphy0577 and Cphy3030, EC 2.4.1.211), which are often found in commensal or pathogenic bacteria of human (Kitaoka *et al*., 2005; Nakajima and Kitaoka, 2008; Nakajima *et al*., 2008; Nakajima *et al*., 2009a), and the other one is 4-*O*-β-D-galactopyranosyl-L-rhamnose phosphorylase (cphy1920, EC 2.4.1.247) (Nakajima *et al*., 2009b). In this study, we characterized the phosphorylase homologs (cphy1874) belonging to GH65 from *C. phytofermentans* ISDg and synthesized several α-1,3-glucosyl disaccharides through the reverse reaction catalysed by the Cphy1874 with suitable carbohydrate acceptors and β-D-glucose 1-phosphate (β-G1P) as the donor. We here report the first identification of nigerose phosphorylase.

MATERIALS AND METHODS

Materials

Trehalose, kojibiose, nigerose, maltose monohydrate, isomaltose, and 1,5-anhydro-D-glucitol, D-galactose, D-glucose, and D-xylose were purchased from Wako Pure Chemicals (Osaka, Japan). β-G1P bis(cyclohexylammonium) salt and D-glucose 6-phosphate disodium salt hydrate was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cloning, expression, and purification

A GH65 gene (cphy1874; GenBankTM accession number is ABX42243.1) was amplified by PCR from genomic DNA of *C. phytofermentans* ATCC700394 as the template using KOD plus DNA polymerase (Toyobo, Osaka, Japan) with primer pairs constructed based on the genomic sequence: forward primer containing NdeI site (underline) 5'-gatatacatatgaattgga

[1] Faculty of Agriculture, Niigata University, Niigata 950-2181, Japan

[2] National Food Research Institute, National Agriculture and Food Research Organization, Ibaraki 305-8642, Japan

[3] Graduate School of Science and Technology, Niigata University, Niigata 950-2181, Japan

cattaacaaat-3' and reverse primer containing XhoI site (underline) 5'-ggtgctcgagcatttcaattttactaatag-3'. Amplified *cphy1874* gene was purified by MinElute Reaction Cleanup Kit (Qiagen, Hilden, Germany), digested by NdeI and XhoI (New England Biolabs, Beverly, MA, USA), and inserted into pET-24a (+) (Novagen, Madison, WI) to add a hexahistidine tag at the C-terminal of the recombinant protein. *Escherichia coli* BL21 (DE3) (Novagen) transformant harboring the expression plasmid was grown in Luria-Bertani medium (1% tryptone, 0.5% yeast extract, 1% NaCl) containing 33 μg/mL of kanamycin at 37℃ up to an absorbance of 0.6 at 660 nm. Expression was induced by 0.1 mM isopropyl-β-D-thiogalactopyranoside and continued at 25℃ for 6 h. Wet cells collected by centrifugation at 4000 × g for 5 min were suspended in 50 mM 3-(*N*-morpholino) propanesulfonic acid (MOPS)-NaOH (pH 7.5) containing 500 mM NaCl (buffer A). The suspended cells were sonicated and centrifuged at 10,000 ×g for 20 min, and the supernatant was used as a crude enzyme solution.

Purification of the recombinant Cphy1874 was carried out with a Profinia protein purification system (Bio-Rad Laboratories, Inc., Hercules, CA) by an immobilized metal affinity chromatography (IMAC) and a Bio-gel P-6 gel filtration chromatography according to the supplier's protocol. The sample was then loaded on an IMAC column equilibrated with buffer A containing 5 mM imidazole. The column was washed with buffer A containing 5 mM and 10 mM imidazole until almost all unspecific-bound components were removed, and the Cphy1874 was eluted with 500 mM imidazole at a flow rate of 2 mL/min. The purified enzyme was finally eluted in 10 mM MOPS-NaOH (pH 7.5) containing 100 mM NaCl by desalting column (Bio-gel P-6). Approximately 7 mg purified protein was obtained from 200 mL of culture medium. Protein concentration was determined by neasuring Abs280 with the thoretical extinction coefficient of $E^{0.1\%}$ = 1.74 based on the amino acid sequence (Pace *et al.*, 1995). The SDS polyacrylamide-gel electrophoresis was performed using PhastSystem (GE Healthcare UK Ltd., Little Chalfont, Buckinghamshire, UK) according to the supplier's protocol. Molecular mass of the native protein was measured by the gel-filtration method using Superose 12 HR10/30 (GE Healthcare UK Ltd.) with 25 mM bis(2-hydroxyethyl)imino-tris(hydroxymethyl)methane-HCl (pH 6.8) containing 150 mM NaCl as the solvent at the flow rate of 0.5 mL/min with MW-Marker (HPLC) (Oriental Yeast Co., Ltd., Tokyo, Japan) as the standards.

Measurement of phosphorolytic and synthetic activity

Phosphorolytic activity was determined by quantifying the D-glucose released from nigerose as follows. The reaction mixtures (200 μL) containing 25 mM MOPS-NaOH (pH 7.0), 10 mM phosphate, 10 mM nigerose, and enzyme were incubated at 30℃. After the certain time intervals, 25 μL of the reaction mixtures were added to 25 μL of DMSO to stop the enzymatic reaction. The D-glucose produced was quantified by glucose oxidase/peroxidase method (Bauminger, 1974). The absorbance of the reaction mixture was measured at 505 nm, after incubating the sample with 100 μL of Glucose C-II Test Wako solution (Wako Pure Chemicals) with the substitution of the phosphate buffer into 200 mM MOPS-NaOH (pH 7.0). Phosphorolytic activity was also measured routinely as described below. The above-mentioned reagent was mixed with half volume of solution containing various concentrations of substrate and enzyme. Then this mixture was incubated at 30℃ in a temperature-controlled microplate reader (Tecan Sunrise Rainbow Thermo, Tecan, Männedorf, Switzerland) and the increase in absorbance at 505 nm was continuously monitored without stopping the reaction. Phosphorolytic activities on other substrates were measured by the same method with substituting nigerose into them. One unit of the phosphorolytic activity was defined as the amount of the enzyme that generated 1 μmol of glucose per minute under the conditions described above.

Synthetic activity was determined by measuring the increase in phosphate in the reaction mixture (Lowry and Lopez, 1946) as described below. The reaction mixtures (200 μL) containing 10 mM β-G1P and 10 mM acceptor in 25 mM MOPS-NaOH (pH 7.0), and enzyme were incubated at 30℃. After the certain time intervals, 25 μL of the reaction mixtures were added to 100 μL of 0.2 M sodium acetate (pH 4.0) to stop the enzymatic reaction. Then 12.5 μL of 1% ammonium molybdate containing 25 mM sulfate and 12.5 μL of 1% ascorbic acid containing 0.05% potassium bisulfate were mixed with the samples. The mixture was incubated at 37℃ for 1 h, and the absorbance was measured at 700 nm. One unit of the synthetic activity was defined as the amount of the enzyme that generated 1 μmol of inorganic phosphate per minute under the conditions described above.

Temperature and pH profile

Effect of pH on the activity was measured on the phosphorolysis and synthesis of nigerose with the standard conditions by substituting the 25 mM MOPS-NaOH (pH 7.0) into the following 100 mM buffers: sodium citrate, pH 3.0-5.5; 4-morpholine-ethanesulfonic acid-NaOH, pH 5.5-7.0; 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-NaOH, pH 7.0-8.5; glycine-NaOH, pH 8.5-10.5. Effect of temperature on the activity was measured on the phosphorolysis of nigerose under the standard conditions at various temperatures for 10 min. On the other hand, pH and thermal stabilities were defined as remaining activity after incubation of enzyme (175 μg/mL) at various temperatures for 10 min in 25 mM MOPS-NaOH (pH 7.0) and in various pHs at 30℃ for 30 min, respectively. Remaining activities were determined by measuring phosphorolytic activities under the standard conditions.

Kinetic analysis

A kinetic analysis of the phosphorolytic reaction was performed by using the continuous glucose assay. Reaction mixtures (150 μL) were prepared in wells of a 96-well microtiter plate by adding various concentrations of nigerose,

various concentrations of phosphate, and 583 ng/mL of Cphy1874 enzyme to the coloring reagent for the D-glucose assay prepared with 25 mM MOPS-NaOH (pH 7.0). The reaction was carried out in a temperature-controlled microplate reader (Sunrise Rainbow Thermo) 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 with the theoretical equation using Grafit version 4 (Erithacus Software, Middlesex, United Kingdom).

Kinetic analyses of the synthetic reactions were carried out under the standard condition with different concentrations of acceptors. The kinetic parameters were calculated by curve fitting the experimental data with the theoretical equation using Grafit version 4.

Structural analysis of the synthesized product

The reaction mixture (500 μL) containing 50 mM β-G1P and 50 mM acceptor (D-glucose, D-xylose, 1,5-anhydro-D-glucitol, D-galactose, or methyl-α-D-glucoside) and Cphy1874 protein (22, 44, 44, 44, or 350 μg/mL for acceptors, respectively) in 100 mM MOPS-NaOH (pH 7.0) was incubated at 30℃ for 20 h. After desalting with Amberlite MB-3 (Organo, Tokyo, Japan), the reaction products were separated on a Toyopearl HW-40F column (26 mm φ×320 mm; TOSOH, Tokyo, Japan) equilibrated with distilled water at a flow rate of 0.5 mL/min. Fractions containing the reaction products were collected and desalted again with Amberlite MB-3, followed by lyophilization. The amounts of products obtained were 4.7, 5.6, 6.1, 6.0, and 8.1 mg, respectively. The 1D (1H and 13C) and 2D [double-quantum-filtered correlation spectroscopy (DQF-COSY), heteronuclear single-quantum-coherence (HSQC), and heteronuclear multiple-bond correlation (HMBC)] nuclear magnetic resonance (NMR) spectra of the products were taken in D2O with 2-methyl-2-propanol as an internal standard using a Bruker Avance 800 or Avance 500 spectrometer (Bruker Biospin, Rheinstetten, Germany). Proton signals were assigned based on DQF-COSY spectra; 13C signals were assigned with HSQC spectra, based on assignment of proton signals. The position of linkage in each disaccharide was determined by detecting inter-ring cross peaks in each HMBC spectrum.

Conversion of nigerose into kojibiose

The reaction mixtures (20 μL) containing 25 mM MOPS-NaOH (pH 7.0), 10 mM phosphate, 70 mM nigerose, and the enzyme (260 μg/mL) were incubated at 30℃ . After the certain time intervals, 1 μL of the reaction mixtures were added to 9 μL of 0.2 M sodium acetate (pH 4.0) to stop the enzymatic reaction. The nigerose and kojibiose formation were quantified by a high performance liquid chromatography (HPLC; LC-20A Shimadzu, Kyoto, Japan) system equipped with a Corona charged aerosol detector (ESA Biosciences, Inc., Chelmsford, MA) under the following conditions. After mixing with 90 μL of distilled water, the mixture was desalted by using Amberlite MB-3 (Organo Co., Tokyo, Japan). The sample was loaded onto a Shodex Asahipak NH2P-50 4E column (Showa Denko K.K., Tokyo, Japan) and eluted with acetonitrile-water (75:25 by vol.) as the solvent at a flow rate of 1.0 mL/min at room temperature.

Anomeric specificity of Cphy1874 protein

The substrate solution was prepared by mixing 5 μL of 500 mM acceptors (D-glucose or D-galactose) and 25 μL of 100 mM β-G1P and keeping the mixture at room temperature (24℃). The reaction was started by adding 20 μL of enzyme solution in 10 mM MOPS-NaOH (pH 7.5) containing 100 mM NaCl (1.75 mg/mL enzyme for D-glucose and 18.5 mg/mL enzyme for D-galactose), which was stored at room temperature, to the substrate solution (final concentration of acceptors and β-G1P was 50 mM). The anomeric forms of each acceptor and product were quantified using HPLC equipped with a refractive index detector, RI model 504 (GL Science, Tokyo, Japan) under the following conditions. After 1 min incubation, 10 μL of the reaction mixture was injected onto a TSK amide-80 column (4.6 mm φ×250 mm; Tosoh, Tokyo, Japan) equilibrated with acetonitrile-water (82:18 by vol.). Samples were eluted with the same solvent at a flow rate of 1.5 mL/min. Peaks of both anomers of the disaccharides were identified based on the existence ratio of the α-anomer over the β-anomer at the equilibrium determined by NMR.

RESULTS

Cloning, expression, and purification of recombinant Cphy1874

The amino acid sequence deduced from the *cphy*1874 has no predicted N-terminal signal sequence based on an analysis using SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP/), suggesting that this protein is cytosolic. Cphy1874 has been annotated as kojibiose phosphorylase on the basis of sequence similarity so far, because it shows the highest amino acid identity (33%) with the kojibiose phosphorylase from *Thermoanaerobacter brockii* (Chaen *et al*., 1999) among the GH65 enzymes. Additionally the Cphy1874 exhibits 31%, 26% and 26% sequence identities with trehalose phosphorylase from *T. brockii* (Maruta *et al*., 2002), maltose phosphorylase from *Bacillus stearothermophilus* (Inoue *et al*., 2002) and trehalose 6-phosphate phosphorylase from *Lactococcus lactis* (Andersson *et al*., 2001), respectively. The *cphy1874* gene was expressed in *E. coli* to investigate the enzymatic properties. Approximately 7 mg of purified protein was obtained from 200 mL of culture medium. The purified Cphy1874 protein showed single protein band approximately at 90 kDa. This native molecular mass was determined to be 203 kDa, suggesting that it was homodimeric enzyme.

Phosphorolytic and synthetic activities of recombinant Cphy1874

Phosphorolytic activity of the recombinant Cphy1874 protein toward several α-linked glucobioses such as trehalose, kojibiose, nigerose, maltose, and isomaltose, was

Table 1. Substrate specificity of Cphy1874 protein

Reaction	Substrate	Specific activity (unit/mg)	Relative activity (%)
Phosphorolysis	Trehalose	–	–
	Kojibiose	0.17	0.50
	Nigerose	32	100
	Maltose	–	–
	Isomaltose	–	–
	Sucrose	–	–
Synthesis*	D-Glucose	54	100
	D-Galactose	4.6	8.5
	D-Xylose	9.3	17
	1,5-Anhydro-D-glucitol	8.1	15
	D-Glucuronic acid	0.08	0.15
	Methyl α-D-glucoside	0.17	0.33

Following compounds did not act as the acceptor: D-mannose, D-allose, L-arabinose, D-lyxose, L-fucose, L-rhamnose, D-fructose, 2-deoxy-D-glucose, D-glucal, D-glucosamine, α-D-glucose 1-phosphate, β-D-glucose 1-phosphate, D-glucose 6-phosphate, methyl β-D-glucoside, 3-methyl-D-glucose, N-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine, trehalose, kojibiose, nigerose, maltose, isomaltose, sophorose, laminaribiose, cellobiose, gentiobiose, xylobiose, lactose, and sucrose.

Table 2. Kinetic parameters of acceptors in the synthetic reaction of Cphy1874 protein

Acceptor	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($M^{-1}s^{-1}$)	Δ ΔG (kcal/mol)
D-Glucose[a]	110 ± 3	3.3 ± 0.3	33,000	0
D-Xylose[b]	118 ± 6	86 ± 10	1,400	1.9
1,5-Anhydro-D-glucitol[c]	120 ± 12	79 ± 13	1,500	1.9
D-Galactose[d]	69 ± 2	104 ± 8	700	2.3
Methyl α-D-glucoside[e]	ND	ND	50	3.9

Values were calculated by regressing the experimental data with the following equation: $v = k_{cat}[E]_0[S]/(K_m + [S])$, where S is the acceptor. The Δ ΔG is the Gibbs energy of activation for k_{cat}/K_m for the enzymatic D-glucosyl transfer to an acceptor, relative to transfer to D-glucose. It was calculated according to Δ ΔG = RTln[(k_{cat}/K_m)D-glucose/(k_{cat}/K_m)acceptor], whereby R is 1.987 cal・mol^{-1}・K^{-1} and T is 303.15 K.
The range of substrate concentrations were: a, 0.5-20 mM; b, 20-200 mM; c, 6-60 mM; d, 33-400 mM; e, 40-400 mM.

examined. Under the condition with inorganic phosphate, it showed the highest activity on nigerose, a faint activity on kojibiose, and no activity on the others (Table 1). On the other hand, this protein did not cleave nigerose in the absence of phosphate. These results indicate that this protein phosphorolyzed nigerose.

Double reciprocal plots of initial velocities against various initial concentrations of nigerose and phosphate gave a series of lines intersecting at a point. This indicates that the phosphorolytic reaction of Cphy1874 protein follows a sequential bi-bi mechanism, as do inverting phosphorylases (Kitaoka and Hayashi, 2002). Kinetic parameters of Cphy1874 protein determined by regressing data against the rate equation for the sequential bi bi reaction are shown in the legend of Fig.1. The values of k_{cat}, K_{mA} and K_{mB} were in the same range as other phosphorylases (Nakajima and Kitaoka, 2008; Nakajima *et al*., 2009a), suggesting that nigerose is the true substrate of the enzyme.

In the synthetic reaction, Cphy1874 protein utilized D-glucose, D-xylose, 1,5-anhydro-D-glucitol, D-galactose, methyl-α-D-glucoside, and D-glucuronic acid, in this order, as the acceptors in the presence of β-G1P as the donor (Table 1). The enzyme produced only corresponding disaccharides from all the acceptors. No significant preferences of anomers of the acceptors were detected by the anomeric analysis in the synthetic reaction with either D-glucose or D-galactose. Additionally none of disaccharides examined (listed in Table 1) were acted as the acceptors.

Kinetic parameters of Cphy1874 protein on the effective acceptors were determined. The K_m value on D-glucose was in the millimolar range and was 24-32 times smaller than those on the other acceptors (Table 2). On the contrary, the k_{cat} value on D-glucose was in the same range as those on the other acceptors. This result indicates that k_{cat}/K_m values mainly depend on K_m values. The fact that the k_{cat}/K_m value on D-glucose was 22-660 times larger than those on the other acceptors indicates that D-glucose is the most effective acceptor for Cphy1874 protein.

Products of the synthetic reaction

The reaction products using D-glucose, 1,5-anhydro-D-glucitol, and D-galactose as the acceptors were determined to

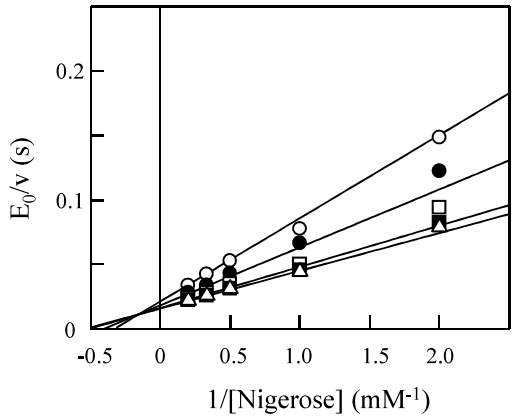

Fig 1. Double-reciprocal plot of the phosphorolytic reaction of Cphy1874 protein under different concentrations of inorganic phosphate. Concentration of phosphate are: Open circle, 0.5 mM; closed circle,1 mM; open square,2 mM; closed square, 3 mM; open triangle, 5 mM. Kinetic parameters were calculated to be k_{cat}= 67 ± 2.4 (s-1), K_{mA}= 1.7 ± 0.2 (mM), K_{mB} = 0.2 ± 0.06 (mM), K_{iA} = 6.4 ± 2.6 (mM), and k_{cat}/K_{mA}=39 ($s^{-1}mM^{-1}$), where A represents nigerose and B is phosphate. Values were determined by regressing data with the following equation, using Grafit version 4.0.10: $v = k_{cat}[E_0][A][B]/(K_{iA}K_{mB} + K_{mA}[B] + K_{mB}[A] + [A][B])$.

be nigerose, 3-*O*-α-D-glucopyranosyl-1,5-anhydro-D-glucitol, and 3-*O*-α-D-glucopyranosyl-D-galactose, respectively, indicating that α-1,3-D-glucosidic linkages were formed in the synthetic reaction. The reaction product from D-xylose contained two compounds, 3-*O*-α-D-glucopyranosyl-D-xylose (63%) and 2-*O*- α -D-glucopyranosyl-D-xylose (37%). Similarly, that from methyl-α-D-glucoside contained methyl-3-*O*-α -D-glucopyranosyl-α-D-glucoside (71%) and methyl-2-*O*-α -D-glucopyranosyl-α-D-glucoside (29%). In both cases, α -1,3-glucosides were the major products and the α -1,2-glucosides were the minor. It should be noted that a faint amount of kojibiose (1.6%) was detectable in the ^{1}H-NMR spectrum of the product from D-glucose. When nigerose was continuously incubated with the enzyme in the presence of phosphate, nigerose was decreased gradually with the increase in kojibiose (Fig. 2), suggesting that the ratio of kojibiose in the synthetic reaction with D-glucose might increase in long-term incubation.

Basic properties of recombinant Cphy1874.

Cphy1874 was stable up to 40℃, and the optimum temperature for phosphorolysis was 40℃. These temperatures corresponded with the optimum growth temperature of mesophilic *C. phytofermentans* (35℃). The enzyme was stable from pH 5.5 to 9.0, and the optimum pH for both phosphorylsis and synthetic reaction was around pH 7.0. These properties suggest that Cphy1874 protein can function in the cytosol of *C. phytofermentans* as far as temperature and pH profiles are concerned.

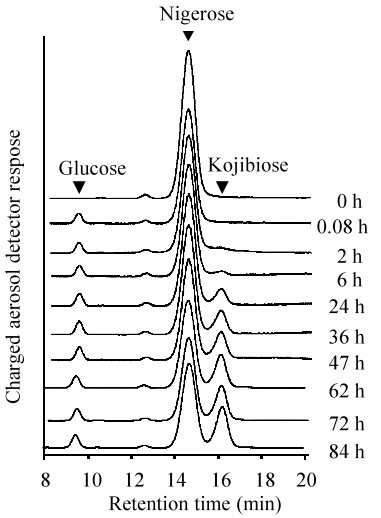

Fig 2. HPLC profiles during the conversion of nigeros into kojibiose.

DISCUSSION

Classification and Specificity of Cphy1874 Protein

Though *cphy1874* had been predicted to encode kojibiose phosphorylase, the active Cphy1874 actually showed only a faint phosphorolytic activity on kojibiose, but strong activity on nigerose. It was not active on either trehalose, maltose, or trehalose 6-phosphate (evidenced by the fact that G6P did not act as the acceptor), clearly indicating that the activity was completely different from other GH65 enzymes characterized. No protein has been reported to possess the phosphorolytic activity on nigerose. Cphy1874 protein did not utilize any disaccharides as the acceptors in the synthetic reaction with β-G1P to produce trisaccharides, indicating that the enzyme is a disaccharide-specific phosphorylase. It is a clear difference with kojibiose phosphorylases, which phosphorolyzes kojibiose as well as kojioligosaccharides of DP 3 and higher (Chaen *et al*., 1999). We here propose the systematic name of 3- α -D-glucosyl-D-glucose:phosphate β -D-glucosyltransferase and the short name of nigerose phosphorylase for Cphy1874 protein. In the synthetic reaction, the enzyme initially forms nigerose (and a faint amount of kojibiose) from β-G1P and D-glucose as the kinetic-controlled products, but long-term incubation causes equilibrated mixture of nigerose and kojibiose as the thermodynamic-controlled products.

Acceptor recognition of nigerose phosphorlyase

Nigerose phosphorylase did not utilize any derivatives of D-glucose at C2 position (D-mannose, 2-deoxy-D-glucose, D-glucosamine, *N*-acetyl-D-glucosamine, and kojibiose), clearly suggesting that the C2 equatorial hydroxyl group of D-glucose is essential for the acceptor recognition in the synthetic raction. Aditionally kinetic parameters on the synthetic reaction with several acceptors suggest that the enzyme recognizes D-glucose derivatives at C1 (1,5-anhydro-

D-glucitol and methyl-α-D-glucopyranoside), C4 (D-galactose), and C5 (D-xylose) positions. It also accepts glucuronic acid much weaker than D-glucose and D-xylose, suggesting that the carboxyl group at C6 position causes either electro repulsion with an acidic amino acid residue of the enzyme or steric hindrance. The binding effect of each hydroxyl group can be estimated from the Gibbs energy of activation difference (the ΔΔG values) of each derivative of D-glucose (Nidetzky *et al*., 2000; Hidaka *et al*., 2006). The values of 1,5-anhydro-D-glucitol and D-galactose (around 2 kcal・mol^{-1}) suggest that the enzyme recognizes hydroxyl groups at positions 1 and 4 via single hydrogen bond. Though the enzyme is considered to recognize anomeric hydroxyl group via a hydrogen bond, no significant anomeric preference for the acceptor was observed in the anomeric analysis. The enzyme may recognize the α- and β-hydroxyl groups by different amino acid residues. The reaction with each D-xylose and methyl- α -D-glucoside as the acceptor caused the decrease in the α-1,3 specificity and the increase in the production of the corresponding α-1,2-glucoside. The k_{cat} /K_m value for the α-1,2-D-glucosyl transfer [(the overall k_{cat} /K_m value) × (the ratio of the α-1,2- D-glucosyl product)] on D-xylose (510 $s^{-1}M^{-1}$) is comparable with than on D-glucose (530 $s^{-1}M^{-1}$), indicating that the hydroxymethyl group at C5 is important for the substrate binding pattern to form α-1,3 linkage but not significantly affect the binding pattern to form α-1,2 linkage. On the other hand, the k_{cat} /K_m value for the α-1,2-D-glucosyl transfer on methyl-α-D-glucoside (15 $s^{-1}M^{-1}$) was much smaller than that on D-glucose, suggesting that the methyl substitution causes deficiency on forming of α-1,2 linkage as well as that of α-1,3 linkages.

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REFFERENCES

Andersson, U., F. Levander, P. Rådström. 2001. Trehalose-6-phosphate phosphorylase is part of a novel metabolic pathway for trehalose utilization in *Lactococcus lactis*. *J. Biol. Chem*., **276**: 42707-42713.

Bauminger, B.B. 1974. Micro method for manual analysis of true glucose in plasma without deproteinization. *J. Clin. Pathol.,* **27**: 1015-1017.

Cantarel, B.L., P.M. Coutinho, C. Rancurel, T. Bernard, V. Lombard, B. Henrissat. 2009. The Carbohydrate-Active EnZymes database (CAZy): an expert resource for Glycogenomics. *Nucl. Acids Res.,* **37**: D233-238.

Chaen, H., T. Yamamoto, T. Nishimoto, T. Nakada, S. Fukuda, T. Sugimoto, M. Kurimto, Y. Tsujisaka. 1999. Purification and charactereization of a novel phosphorylase, kojibiose phosphorylase, from *Thermoanaerobacter brockii*. *J. Appl. Glycosci.,* **46**: 423-429.

Hidaka, M., M. Kitaoka, K. Hayashi, T. Wakagi, H. Shoun, S. Fushinobu. 2006. Structural dissection of the reaction mechanism of cellobiose phosphorylase. *Biochem. J.,* **398**: 37-43.

Inoue, Y., N. Yasutake, Y. Oshima, Y. Yamamoto, T. Tomita, S. Miyoshi, T. Yatake. 2002. Cloning of the maltose phosphorylase gene from *Bacillus* sp. strain RK-1 and efficient production of the cloned gene and the trehalose phosphorylase gene from *Bacillus stearothermophilus* SK-1 in Bacillus subtilis. *Biosci. Biotechnol. Biochem.*, **66**: 2594-2599.

Kitaoka, M., K. Hayashi. 2002. Carbohydrate processing phosphorolytic enzymes. *Trends Glycosci. Glycotechnol*., **14**: 35-50.

Kitaoka, M., J. Tian, M. Nishimoto. 2005. Novel putative galactose operon involving lacto-*N*-biose phosphorylase in *Bifidobacterium longum*. *Appl. Environ. Microbiol*., **71**: 3158-3162.

Lowry, O.H., J.A. Lopez. 1946. The determination of inorganic phosphate in the presence of labile phosphate esters. *J. Biol. Chem.,* **162**: 421-428.

Luley-Goedl, C., B. Nidetzky. 2010. Carbohydrate synthesis by disaccharide phosphorylases: reactions, catalytic mechanisms and application in the glycosciences. *Biotechnol. J.*, **5**: 1324-1338.

Maruta, K., K. Mukai, H. Yamashita, M. Kubota, H. Chaen, S. Fukuda, M. Kurimoto. 2002. Gene encoding a trehalose phosphorylase from *Thermoanaerobacter brockii* ATCC 35047. *Biosci. Biotechnol. Biochem.*, **66**: 1976-1980.

Nakajima, M., M. Kitaoka. 2008. Identification of lacto-N-biose I phosphorylase from *Vibrio vulnificus* CMCP6. A*ppl. Environ. Microbiol*., **74**: 6333-6337.

Nakajima, M., T. Nihira, M. Nishimoto, M. Kitaoka. 2008. Identification of galacto-N-biose phosphorylase from *Clostridium perfringens* ATCC13124. *Appl. Microbiol. Biotechnol.,* **78**: 465-471.

Nakajima, M., M. Nishimoto, M. Kitaoka. 2009a. Characterization of β-1,3-galactosyl-N-acetylhexosamine phosphorylase from *Propionibacterium acnes*. *Appl. Microbiol. Biotechnol*., **83**: 109-115.

Nakajima, M., M. Nishimoto, M. Kitaoka. 2009b. Characterization of three β-galactoside phosphorylases from *Clostridium phytofermentans*: discovery of D-galactosyl- β 1-4-L-rhamnose phosphorylase. *J. Biol. Chem*., **284**: 19220-19227.

Nidetzky, B., C. Eis, M. Albert. 2000. Role of non-covalent enzyme-substrate interactions in the reaction catalysed by cellobiose phosphorylase from *Cellulomonas uda*. *Biochem. J.,* **351**: 649-659.

Pace, C.N., F. Vajdos, L. Fee, G. Grimsley, T. Gray. 1995. How to measure and predict the molar absorption coefficient of a protein. *Protein Sci.,* **4**: 2411-2423.

Warnick, T.A., B.A. Methé, S.B. Leschine. 2002. *Clostridium phytofermentans* sp. nov., a cellulolytic mesophile from forest soil. *Int. J. Syst. Evol. Microbiol*., **52**: 1155-1160.

新規ニゲロースホスホリラーゼを用いたα-1,3-グルコ2糖の生産

仁平高則[1,2]・中井博之[1,2,3]*・北岡本光[2]

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要　約

糖質加リン酸分解酵素（ホスホリラーゼ）は、その厳密な基質特異性と反応の可逆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15種類のみで、新たな基質特異性を有する新規ホスホリラーゼの発見が強く望まれている。そこで、これまで数種のホスホリラーゼが単離されている *Clostridium phytofermentans* が有する Glycoside Hydrolase Family 65 に属する遺伝子（*cphy1874*）をクローニングし、組換え酵素の酵素化学的性質を調査した。結果、Cphy1874はリン酸存在下でニゲロースに対し高い加リン酸分解活性を示すことが明らかになった。β-グルコース1リン酸と各種糖を作用させたところ，グルコースをアクセプターとした際に高い合成活性を示した。キシロース、1,5-アンヒドログルシトール、ガラクトース、メチル-α-グルコシドにおいてもアクセプターとなり、主生成物はいずれもα-1,3結合であった。このことから本酵素は，これまで報告の無かった新規加リン酸分解酵素ニゲロースホスホリラーゼであることが判明した。

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[1] 新潟大学農学部

[2] 農研機構　食品総合研究所

[3] 新潟大学大学院自然科学研究科