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Catalytic reversibility of *Pyrococcus horikoshii* trehalose synthase: Efficient synthesis of several nucleoside diphosphate glucoses with enzyme recycling

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ABSTRACT

The trehalose synthase (TreT) from *Pyrococcus horikoshii* represented reversible catalysis in alternative synthesis of trehalose and nucleoside 5′-diphosphate-glucose (NDP-Glc), depending on the substrates involved. TreT from *P. horikoshii* had differential preferences on NDP-Glc as a donor for trehalose synthesis, in which guanosine 5′-diphosphate (GDP)-Glc was the most favored in terms of reaction specificity, k_{cat}/K_m. Uridine 5′-diphosphate (UDP)- and adenosine 5′-diphosphate (ADP)-Glcs were employed with less preferences. This enzyme reversely cleaved trehalose to transfer the glucosyl moiety to various NDPs, efficiently producing NDP-Glcs. Although ADP-Glc was the least favorable donor, the counterpart, ADP, was the most favorable acceptor for the reverse synthesis of NDP-Glc in k_{cat}/K_m. GDP and UDP were less preferred, compared to ADP. In a batch reaction of 12 h, the molar yield of NDP-Glc per NDP used was decreased approximately in the order of ADP-Glc > GDP-Glc > cytidine 5′-diphosphate (CDP)-Glc or UDP-Glc. The overall productivity of the enzyme was largely improved in a gram scale for NDP-Glcs using repetitive batch reactions with enzyme recycling. Thus, it is suggested that TreT from *P. horikoshii* may be useful for the regeneration of NDP-Glc from NDP, especially for ADP-Glc from ADP, with trehalose as a glucose resource.

1. Introduction

The oligosaccharides of numerous glycoconjugates play important biological roles in intra- and intercellular communication processes and tumor development [1,2]. This makes them worthwhile targets for pharmaceutical applications [3]. Recently, the sugar nucleotide-dependent glycosyltransferases have been recognized to be valuable tools for the synthesis of the oligosaccharides in glycoconjugates [4]. The enzymatic approaches make it possible to evade the extensive protection and deprotection manipulations required in the chemical synthesis of carbohydrates [5]. Generally, the glycosyltransferases, like Leloir-glycosyltransferases, catalyze the sequential transfer of a monosaccharide moiety from a nucleotide-activated sugar donor to a growing oligosaccharide chain in a stereospecific manner [6–8]. Therefore, the availability of the nucleotide-activated sugars as glycosyl donor substrates is a prerequisite for the enzymatic synthesis of the oligosaccharides at low cost [9].

Nucleotide-activated sugars (or nucleotide sugars) are ubiquitous metabolites for the biosynthesis of carbohydrates in nature. Reportedly, nine nucleotide sugars are mainly utilized in mammalian cells [7]. Because of the high price of the nucleotide sugars, several approaches for the synthesis have been probed to increase access to these crucial compounds. Chemical syntheses have been developed, but they have some limitations of low yields and the use of organic solvents, which are not economical for scaled-up processes [10]. In contrast, enzymatic syntheses of nucleotide sugars have been more attractive to attain high yields in a relatively short time, while being easy to scale up and carry out in aqueous solutions [7]. Particularly, multiple enzyme systems with regeneration strategies of nucleoside 5′-diphosphate (NDP) have been preferred to increase the yields and eliminate the possible inhibition of NDP against the glycosyltransferase [11]. NDP is released as a by-product from NDP-sugar donors in the glycosyl transfer reaction of glycosyltransferases. Pyrophosphorylase (EC 2.7.7.9) has been applied to regenerate NDP-sugar from the released NDP with kinase-mediated phosphorylation in a two-step enzymatic process [9,12]. However, this requires a continuous supply of phosphoenolpyruvate (PEP) as a phosphoryl donor to convert NDP to nucleoside 5′-triphosphate (NTP). The enzymatic process also requires a continuous supply of sugar-1-phosphate as a sugar donor to react with NTP, producing NDP-sugar. Finally, pyrophosphatase (EC 3.6.1.1) is needed to remove the inorganic pyrophosphate generated, thereby driving the reaction forward. As an advanced method, NDP-sugars have been regenerated in a one-step reaction by plant glycosyltransferase, a sucrose synthase (EC 2.4.1.13, accession number Q84T18) [13–15]. This system does not involve PEP as the donor substrate and phosphate, a possible enzyme inhibitor, as

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the side product. Sucrose synthase catalyzes the reversible cleavage of sucrose with NDP to generate NDP-glucose (NDP-Glc) and D-fructose. That is, this enzyme regenerates NDP-Glc from NDP using sucrose as a cheap and renewable energy source.

Recently, we have just established a novel process by efficiently regenerating nucleotide sugars using another renewable sugar, trehalose, with microbial glycosyltransferase. Previously, the trehalose synthase from *Pyrococcus horikoshii* (O58762) has been reported to synthesize trehalose or trehalose analogues with NDP-Glc donors and monosaccharide acceptors such as glucose and galactose [16,17]. Generally, the catalytic direction of nucleotide sugar-dependent glycosyltransferases is perceived to be unidirectional [18]. However, trehalose glycosyltransferring synthase (TreT, Q7LYW5) from *Thermococcus litoralis* has been already reported to be reversible in the catalysis [19]. Similarly, the trehalose synthase from *P. horikoshii* (hereafter abbreviated as *Pyrococcus* TreT) catalyzed the reversible cleavage of trehalose with NDP to generate NDP-Glc and D-glucose. In this study, the reversibility of this *Pyrococcus* TreT was described for the alternative synthesis of trehalose and NDP-Glc in both catalytic directions. The reaction progress was compared for various NDP-Glc and NDP substrates in the enzyme catalysis. Regeneration yields of several NDP-Glc sugars were also investigated. In addition, the enzymatic productions of NDP-Glcs were evaluated in a repetitive batch mode with enzyme recycling system.

2. Materials and methods

2.1. Materials

Nucleoside 5′-diphosphates (NDPs) such as uridine 5′-diphosphate (UDP), adenosine 5′-diphosphate (ADP), guanosine 5′-diphosphate (GDP), and NDP-sugars such as UDP-glucose (UDP-Glc), ADP-glucose (ADP-Glc), and GDP-glucose (GDP-Glc) were obtained from Sigma Chemical Co (St. Louis, MO, USA). ADP was also obtained from Amresco Inc. (Solon, OH, USA) and Genechem Inc. (Daejeon, Korea). Cytidine 5′-diphosphate (CDP), thymidine 5′-diphosphate (TDP), CDP-glucose (CDP-Glc), and TDP-glucose (TDP-Glc) were purchased from Genechem Inc. UDP-Glc was also obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). α,α-Trehalose and porcine kidney trehalase were also obtained from Sigma. All other chemicals used were of reagent grade.

2.2. Enzyme preparation

Pyrococcus TreT was prepared and purified as previously reported with a slight modification [16]. *Escherichia coli* strain MC1061 [F⁻ *araD139 recA13 Δ(araABC-leu)7696 galU galK ΔlacX74 rpsL thi hsdR2 mcrB*] was used as a host for the constitutive expression of the recombinant enzyme. The *E. coli* transformant was cultured at 37 °C in Luria–Bertani (LB) medium supplemented with ampicillin (100 μg/mL). The purification of the expressed enzyme with a hexahistidine-tag was performed by nickel-nitrilotriacetic acid (Ni-NTA) affinity column chromatography (Qiagen, Hilden, Germany), followed by Q-Sepharose column chromatography (Pharmacia) pre-equilibrated with 50 mM Tris–HCl buffer (pH 7.0). The enzyme was eluted with a NaCl gradient of 0–0.5 M in the same buffer at a flow rate of 1 mL/min. Active fractions were pooled, desalted, and concentrated by ultrafiltration.

2.3. Enzyme reaction

For synthesis of trehalose, the enzyme (0.67 mg/mL) was reacted with 0.5% (w/v, 7.9–8.3 mM) NDP-Glc as a glucosyl donor (UDP-Glc, ADP-Glc, and GDP-Glc) and 5% (w/v, 278 mM) glucose as an acceptor in 50 mM sodium acetate buffer (pH 6.0) at 37 °C and 60 °C for 24 h. Appropriate aliquots were taken periodically from the reaction mixture during reaction, and the reaction was then immediately quenched by adding 0.1 M HCl solution. The reaction mixture was centrifuged at 12,000 × *g* for 10 min, and filtered using 0.45-μm membrane filters for further experiments. The formation of trehalose was confirmed by thin-layer chromatography (TLC), and the amount of trehalose produced was determined using high performance anionic exchange chromatography (HPAEC) as described in the section below. For reverse synthesis of NDP-Glc, the enzyme (0.67 mg/mL) was incubated with 5% (w/v, 140 mM) trehalose as glucosyl donor and 0.5% (w/v, 11.3–12.4 mM) NDP (UDP, ADP, GDP, CDP, and TDP) as acceptor in the same conditions for 24 h. The reaction samples were taken periodically from the reaction mixture and the reaction was stopped by the addition of 0.1 M HCl. After the filtration, the formation of NDP-Glc was quantitatively analyzed using high performance liquid chromatography (HPLC) as described below.

2.4. TLC, HPAEC, and HPLC analyses of reaction products

The reaction products for the synthesis of trehalose or NDP-Glc were analyzed using TLC on Whatman K5F silica gel plates (Whatman, Kent, UK) with both solvent systems of isopropyl alcohol:ethyl acetate:water (3/1/1, v/v/v) and *n*-butanol:pyridine:water (7/3/1, v/v/v) [16]. Carbohydrates on the TLC plate were visualized by dipping method as previously reported. The time-dependent change in the composition of the reaction mixture was periodically investigated with analytical instruments. HPAEC was employed to analyze trehalose as the reaction product on a Dionex CarboPac PA10 column (0.4 cm × 25 cm, Sunnyvale, CA, USA) with an isocratic gradient of 150 mM sodium hydroxide at a flow rate of 1.0 mL/min [20]. The standard curve for the determination of trehalose production had a slope of 15.93 (mV s/mM, peak area/trehalose concentration) with a correlation coefficient of 0.995. The identity of the synthesized trehalose was further confirmed by hydrolysis with trehalase (EC 3.2.1.28) [17]. HPLC analysis was carried out to qualitatively and quantitatively determine the synthesis of NDP-Glc from NDP and trehalose using a Prevail Carbohydrate ES column (250 mm × 4.6 mm, Alltech Associates, Inc., Deerfield, IL, USA) connected to a Waters Model 510 system with 996 photodiode array detector at 254 nm. A linear HPLC gradient was composed of 80% acetonitrile in water (solvent A) and 0.5 M ammonium carbonate or 0.5 M NaCl in water (solvent B). After a 20-μL injection of each sample, solvent B was either increased from 0 to 30% in 20 min and then held at 30% for 5 min, followed by reduction to 0% in 5 min for 0.5 M ammonium carbonate, or increased from 0 to 100% in 40 min and then held at 100% for 5 min, followed by a reduction to 0% in 10 min for 0.5 M NaCl. The analysis was performed at 25 °C and a flow rate of 0.8 mL/min. The standard curves for HPLC analysis of the respective NDP-Glc were used in the range of 0–9 mM to determine the concentration in the reaction sample, in which the slopes were 7.57 (for UDP-Glc), 7.74 (for ADP-Glc), and 7.62 (for GDP-Glc) (V s/mM, peak area/NDP-Glc concentration) with the correlation coefficient of 0.99 for the gradient elution of 0.5 M ammonium carbonate. The NDP-Glc synthesized was further confirmed to be utilized as the glucosyl donor for the formation of trehalose by the enzymatic reaction with glucose. The detailed glycosidic structure of UDP-Glc synthesized was representatively confirmed by ^{13}C NMR. The ^{13}C NMR spectra of the product was recorded with a JEOL JNM LA-400 MHz NMR spectrometer (Tokyo, Japan). The sample was dissolved in DMSO-d_6 at 24 °C with tetramethylsilane (TMS) as the chemical shift reference.

2.5. Enzyme kinetics

The kinetic parameters for the enzyme-catalyzed synthesis of trehalose or NDP-Glc were determined with various concentrations of donor and acceptor substrates in 50 mM sodium acetate buffer (pH 6.0) at 60 °C. The donor substrates such as NDP-Glc were employed in the range of 0.16–10.0 mM with 55.6 mM glucose acceptor for the trehalose synthesis, and acceptor substrates such as NDP were in the range of 4.3–340 mM with 444 mM trehalose for the synthesis of NDP-Glc. After an aliquot (about 100 μL) was taken periodically from the reaction mixture, the reaction was immediately quenched by adding 0.1 M HCl solution and centrifuged, followed by analysis with HPAEC or HPLC, as described above. The kinetic constants were calculated by a non-linear regression program, DNRPEASY, derived by Duggleby and Leonard [21], producing K_m and k_{cat} values.

2.6. Repetitive batch production with enzyme recycling

In order to increase overall productivities of the enzyme, the reaction was carried out by repetitive batch technique with recycling of enzyme [22]. The batch productions of respective NDP-Glc were repeated by free enzyme retained above ultrafiltration membrane (Amicon Ultra-4 or 15 device, molecular weight cut-off of 10 kDa, Millipore Co., Bedford, MA, USA). For one cycle of the batch reaction in total volume of 4–10 mL, the enzyme (0.24 mg/mL) was incubated with 16% (w/v, 444 mM) trehalose as glucosyl donor and 5% (w/v, 113–124 mM) NDP (UDP, ADP, GDP, and CDP) as acceptor at 37 °C for 12 h, respectively, inside the reaction reservoir tube. Then, the solution of reaction product was collected through a centrifugation (5000 × *g*) as filtrate in the membrane retention method. The centrifugal filtration was done until an approximately 100 μL of the solution inside the membrane was remained. For the next turn of the batch reaction, the same amount of the substrate solution was newly added into the remaining solution containing the enzyme and the reaction was repeated at the same conditions, followed by the centrifugation. These repetitive batch processes (2 cycles per a day) were continuously performed for 2 or 3 weeks, depending on the substrate. The filtrate in each turn was analyzed using high performance liquid chromatography (HPLC) with the gradient elution of 0.5 M NaCl, pooled, and kept at 4 °C for the product purification. The standard curves for the HPLC analysis of NDP-Glcs with the gradient elution of 0.5 M NaCl were estimated to have the slopes of 5.7 (for UDP-Glc), 5.1 (for ADP-Glc), and 5.0 (for GDP-Glc), and 5.7 (for CDP-Glc) (V s/mM, peak area/NDP-Glc concentration) in the range of 0–16 mM with the correlation coefficient of 0.97. The NDP-Glc product was efficiently purified from unreacted substrates such as NDP and trehalose and glucose co-products by preparative HPLC on an Alltech Prevail Carbohydrate ES column (300 mm × 20 mm) connected to a Waters Delta Prep 4000 system with the gradient elution of 0.5 M ammonium carbonate up to 30% at a flow rate of 5.0 mL/min.

Table 1
Kinetic parameters for the synthesis of trehalose from various NDP-Glc donors and glucose acceptor.

Substrate	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (mM^{-1} min^{-1})
UDP-Glc	2.46 ± 0.57	31.72 ± 3.02	12.9
GDP-Glc	2.08 ± 0.59	32.48 ± 2.66	15.6
ADP-Glc	3.20 ± 0.53	26.28 ± 1.81	8.2

The transfer reaction was carried out with 0.16–10.0 mM NDP-Glc, 55.6 mM glucose, and 0.33 mg/mL (6.62 μM) enzyme in 50 mM sodium acetate buffer (pH 6.0) at 60 °C. The amount of trehalose produced was analyzed using HPAEC. The specific activity (V_{max}/mg protein) of the enzyme for GDP-Glc was typically evaluated to be approximately 0.7 units/mg protein in this condition.

3. Results and discussion

3.1. Reversible reaction of Pyrococcus TreT

It has been previously revealed that TreT (named as PhGT in previous report) from an archaeon *P. horikoshii* catalyzes the transfer of glucose from UDP-glucose donor to D-glucose acceptor, rather than D-glucose-6-phosphate (G-6-P), directly forming a trehalose [16]. This acceptor specificity makes the enzyme unique, because the most widely distributed pathway for trehalose synthesis takes trehalose-phosphate synthase (EC 2.4.1.15) that utilizes a G-6-P acceptor to produce trehalose-6-P, followed by phosphorolysis with phosphatase [23]. Additionally, *Pyrococcus* TreT was able to employ other NDP-Glcs such as ADP-Glc, GDP-Glc, and CDP-Glc for the synthesis of trehalose. In this study, we found that there was a tendency for the enzyme to use NDP-Glc as the donor substrate. As shown in Table 1, the kinetic preference of the enzyme was compared with NDP-Glc donors. The enzyme preferred GDP-Glc most among the NDP-Glcs used in terms of k_{cat}/K_m. The reaction progresses of the enzyme for trehalose synthesis were also compared with NDP-Glc donors in the presence of 35 times higher concentration of glucose acceptor along with 12–24 h incubation in Fig. 1. The much higher concentration of glucose than that of NDP-Glc may keep in driving the reaction going forward. Here, the reaction of the trehalose production reached the plateau of the production curve approximately at 12 h incubation. It was also observed that the GDP-Glc was preferred most for trehalose production among the NDP-Glcs used at this point. The product formation with GDP-Glc by the enzyme was approximately 3.5 times larger than that with ADP-Glc at 37 °C and 60 °C. The enzyme employed UDP-Glc and ADP-Glc as donors with approximately 88 and 30% efficiencies for 12 h incubation at 60 °C, respectively, compared to that (100%) for GDP-Glc. In fact, these trehalose-synthesizing glycosyltransferases have also been discovered in *T. litoralis* and *Thermoproteus tenax*, which show the broad substrate specificities on NDP-Glc donors [19,24]. However, these enzymes differed in that TreT from *T. litoralis* favored ADP-Glc exclusively as the donor substrate with relatively low levels (5–6%) of efficiency for UDP-Glc and GDP-Glc, whereas TreT from *T. tenax* (Q703X2) preferred UDP-Glc, with 10% efficiency for ADP-Glc. The search of a conserved domain database (http://pfam.sanger.ac.uk/search) revealed that the C-terminal regions of TreT from *P. horikoshii* (220–393 residues), TreT from *T. litoralis* (218–391 residues), and TreT from *T. tenax* (200–373 residues) were very homologous with the glycosyl transferases group 1 (Pfam 00534), which is generally known to catalyze the transfer of sugar moieties from NDP-sugars such as UDP, ADP, or GDP-linked sugars to a variety of specific acceptor molecules, forming glycosidic bonds [25]. Multiple sequence alignment (http://www.ncbi.nlm.nih.gov/BLAST) also showed that TreT from *P. horikoshii* shared overall sequence identity of 84 and 48% with TreTs from *T. litoralis* and *T. tenax* in the amino acid residues of the C-terminal regions, respectively. From the sequence analyses, it was then suggested that the C-terminal domains of the enzymes would be responsible for binding NDP-Glc donors and the sequence discrepancy among them affected the preferences of the enzymes for the NDP species for trehalose synthesis due to significant differences in the binding site architectures of the enzymes [8].

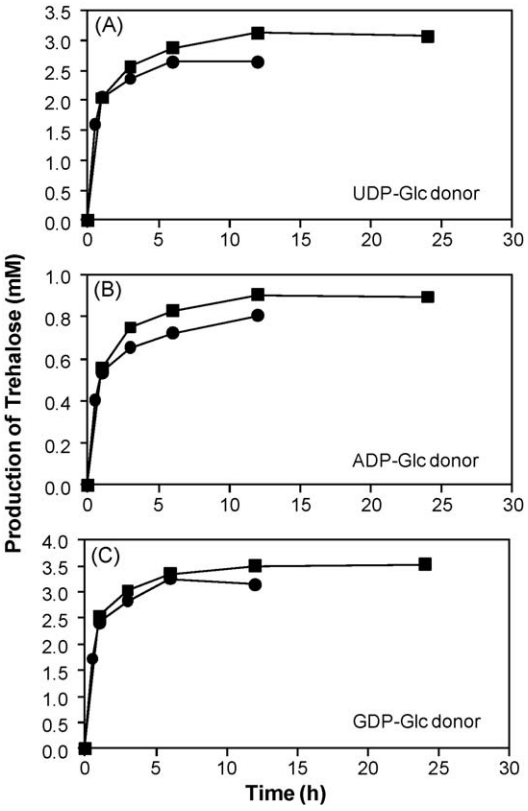

Fig. 1. Reaction progresses for the enzymatic synthesis of trehalose using various NDP-Glcs and glucose according to time at 37 °C (●) and 60 °C (■). (A) UDP-Glc was used as donor (8.2 mM). (B) ADP-Glc was used donor (7.9 mM). (C) GDP-Glc was used donor (8.3 mM). The final concentration of the enzyme was 0.67 mg/mL. The amount of trehalose was duplicately determined using HPAEC.

Pyrococcus TreT has also catalytic activity for hydrolyzing trehalose slowly and partially to yield D-glucose. Interestingly, when we added UDP into the reaction mixture, it was observed that the activity of the hydrolysis increased as the concentration of UDP increased (data not shown). As a result, we have found that *Pyrococcus* TreT reversely catalyzes the glucosyl-transfer of trehalose in the presence of UDP to produce UDP-Glc and D-glucose (Fig. 2A). After purification of the UDP-Glc produced, the glycosidic structure was confirmed by ^{13}C NMR, as was the glucosyl-transfer reaction that utilized the synthesized UDP-Glc as a donor to produce the trehalose (Fig. 2B). The resulting trehalose was evidently hydrolyzed to D-glucose by the trehalase (lane 4 in Fig. 2B). As shown in Fig. 3, the enzyme also had the capability to transfer the glucosyl moiety of trehalose to other NDPs such as ADP, GDP, CDP, and TDP, synthesizing their NDP-Glcs efficiently. All the NDP-Glcs synthesized were able to be used as glucosyl donor substrates for the trehalose synthesis. Consequently, the results indicate that the catalysis of *Pyrococcus* TreT is clearly reversible and the initial direction of the catalyzed reaction is dependent on the substrates involved in the reaction system. The reaction scheme for the catalytic reversibility of *Pyrococcus* TreT can be summarized in the forward and the reverse directions, as shown in Fig. 4. Previously, TreT from *T. litoralis* has been already reported to have reversible catalysis, even while being assumed to favor the degradation of trehalose after the sugar is imported through ABC transporter into cell [19]. Sucrose

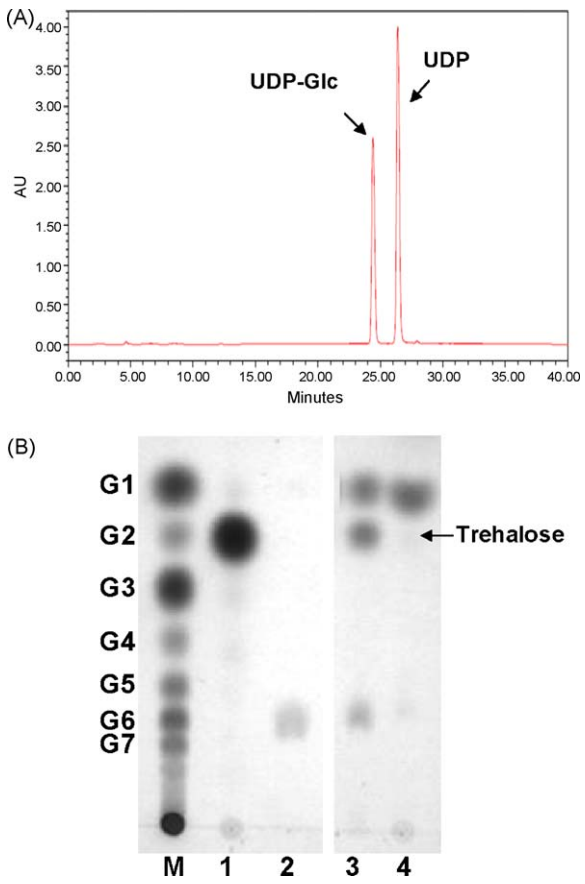

Fig. 2. HPLC and TLC analysis of *Pyrococcus* TreT-catalyzed reaction with UDP and trehalose. (A) HPLC of reaction products on a Prevail Carbohydrate ES column (250 mm × 4.6 mm) with the linear gradient of 0.5 M ammonium carbonate at 0.8 mL/min. UDP-Glc indicates the reaction product. (B) TLC for the confirmation of trehalose synthesis using the synthesized UDP-Glc. Lane M represents maltooligosaccharides (G1–G7); lane 1, trehalose; lane 2, synthesized and purified UDP-Glc; lane 3, enzymatic synthesis of trehalose from the synthesized UDP-Glc and glucose; lane 4, the hydrolysis of the reaction product by kidney trehalase.

synthase (SuSy), even if it comes from plant sources, has been well demonstrated to catalyze reversible and bidirectional reactions, in which the enzyme has been extensively investigated to produce several nucleotide sugars from NDP and sucrose [13–15]. Actually, there is an additional evidence of the reaction reversibility in the glycosyltransferase-mediated catalyses [26], even though the catalysis of nucleotide sugar-dependent glycosyltransferases is generally assumed to be unidirectional [18]. Meanwhile, TreT from *T. tenax* is proposed to have unidirectional catalysis, resulting in no degradation of trehalose [24]. Therefore, the reaction reversibility and the efficient ability for producing NDP-Glcs by *Pyrococcus* TreT suggest that this enzyme may be very useful for practical application in the group of nucleotide sugar-dependent glycosyltransferases, as well as sucrose synthase.

3.2. Enzymatic regeneration of NDP-Glc from NDP with trehalose resource

The catalytic efficiency (k_{cat}/K_m) of *Pyrococcus* TreT in the reverse reaction for the synthesis of NDP-Glcs was also significantly dependent on the species of NDP acceptors. The preference of the nucleotide moiety exerted by *Pyrococcus* TreT for the direction of NDP-Glc synthesis was not the same for that of trehalose synthesis, as shown in Table 2. *Pyrococcus* TreT preferred the synthesis of ADP-Glc most among NDP-Glcs. Although ADP-Glc was the least favorable donor substrate for the trehalose synthesis among the

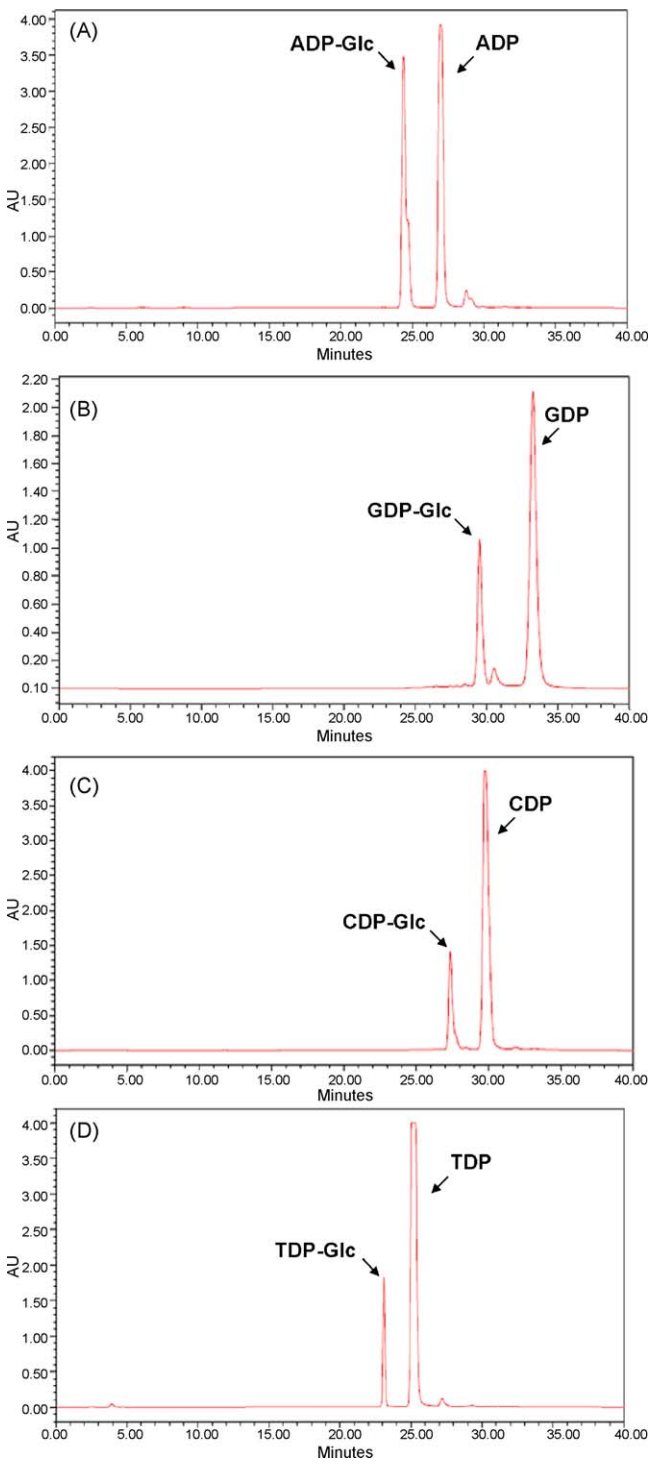

Fig. 3. HPLC analysis of *Pyrococcus* TreT-catalyzed reaction with various NDPs and trehalose. (A) Synthesis of ADP-Glc from ADP. (B) Synthesis of GDP-Glc from GDP. (C) Synthesis of CDP-Glc from CDP. (D) Synthesis of TDP-Glc from TDP. The linear gradient elution in HPLC was performed using 0.5 M ammonium carbonate at 0.8 mL/min.

NDP-Glcs used, its counterpart, ADP, was the most favorable acceptor substrate for the hydrolysis and concomitant glucosyl-transfer reaction of trehalose, yielding the corresponding nucleotide sugar. The reaction progresses of the enzyme for NDP-Glc synthesis were also compared with NDP acceptors in the presence of approximately 12 times higher concentration of trehalose donor along with 12–24 h incubation in Fig. 5. The higher concentration of trehalose

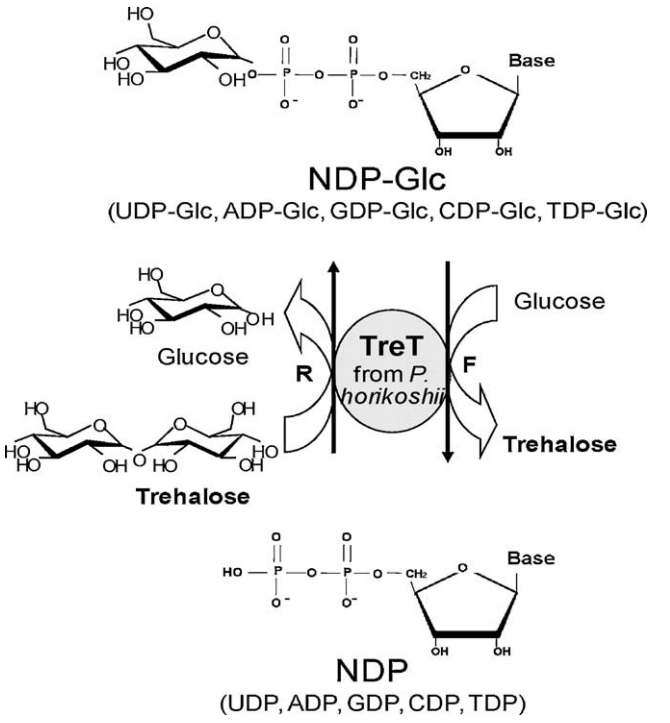

Fig. 4. Schematic diagram of reversible reaction of *Pyrococcus* TreT. F indicates the forward direction for the synthesis of trehalose from NDP-Glc and glucose. R indicates the reverse direction for the synthesis of NDP-Glc from NDP and trehalose.

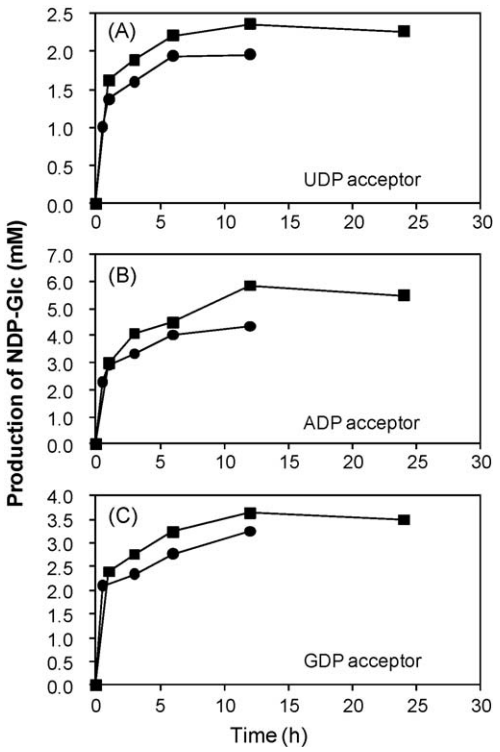

Fig. 5. Reaction progresses for the enzymatic synthesis of NDP-Glcs using various NDPs and trehalose according to time at 37 °C (●) and 60 °C (■). (A) Production of UDP-Glc from 0.5% UDP (12.4 mM). (B) Production of ADP-Glc from 0.5% ADP (11.7 mM). (C) Production of GDP-Glc from 0.5% GDP (11.3 mM). The final concentration of the enzyme was 0.67 mg/mL. The amount of NDP-Glc was duplicately determined using HPLC with the linear gradient of 0.5 M ammonium carbonate.

than that of NDP may also keep in driving the reaction to the synthesis of NDP-Glc. The reaction of the NDP-Glc production also reached the plateau of the production curve approximately at 12 h incubation. At this point, the formation of ADP-Glc by the enzyme was more than two times larger than that of UDP-Glc at 37 °C and 60 °C. The enzyme showed 62 and 40% efficiencies in the product formation for GDP and UDP, respectively, compared to that (100%) for ADP at 12 h incubation and 60 °C. ADP-Glc is generally the donor substrate in plant starch and bacterial glycogen synthesis [14].

Reportedly, SuSy has a high flexibility for NDP acceptors in the cleavage reaction of sucrose, but SuSy favors UDP, with poor efficiencies of 12 and 5% for ADP and GDP, respectively [13]. In this respect, *Pyrococcus* TreT was considered to be particularly suitable for the regeneration of ADP-Glc from ADP, using trehalose as the glucosyl donor. *Pyrococcus* TreT also showed reasonable productivity for GDP- and UDP-Glcs from the corresponding NDP molecules in the reverse direction. The formation of the products was approximately close to the saturation for 12 h of incubation and did not change significantly thereafter in the reaction conditions. The enzyme activity was stable for the entire incubation of each production, and was not affected significantly by the presence of 5 mM mono- and di-valent cations such as Na^+, K^+, Ca^{2+}, and Mg^{2+}. The percent production yields for the respective ADP-, GDP-, and UDP-Glcs (mM) per the corresponding NDP (mM) were calculated to be approximately 58%, 32%, and 22% at 60 °C. The results suggest that *Pyrococcus* TreT is useful for the regeneration of NDP-Glc from NDP, especially for the regeneration of ADP-Glc from ADP. This efficient enzyme system only needs trehalose as sugar fuel for regeneration, which is now a relatively cheap sugar and often used industrially as a food ingredient. These features of the *Pyrococcus* TreT system are some distinguished from those of the plant enzyme, SuSy, with overall 89 and 39% conversions for UDP- and ADP-Glc syntheses (SuSy from rice), respectively, and 55% conversion for ADP-Glc synthesis (SuSy from potato) [13–15].

Table 2
Kinetic parameters for the synthesis of NDP-Glc products from various NDP acceptors and trehalose donor.

Substrate	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (mM^{-1} min^{-1})
UDP	29.54 ± 2.80	89.73 ± 2.29	3.04
GDP	23.77 ± 2.17	102.69 ± 2.39	4.32
ADP	14.19 ± 1.04	102.69 ± 1.69	7.24
Trehalose[a]	25.24 ± 2.66	71.29 ± 1.87	2.8

The transfer reaction was carried out with 4.3–340 mM NDP, 444 mM trehalose, and 0.5 mg/mL enzyme (10.03 μM) in 50 mM sodium acetate buffer (pH 6.0) at 60 °C. The amount of NDP-Glcs produced was analyzed using HPLC with the gradient elution of 0.5 M ammonium carbonate. The specific activity (V_{max}/mg protein) of the enzyme for ADP was typically evaluated to be approximately 2.1 units/mg protein in this condition.

[a] The reaction was carried out with 324 mM UDP and 12–317 mM trehalose in the above conditions.

3.3. Repetitive batch-type production with retained free enzyme

In an attempt to increase the overall enzyme productivity of each NDP-Glc product, we tried to reuse the enzyme in subsequent batch reactions at 37 °C, as the free enzyme retained within the ultrafiltration membrane cell [22]. In the production of ADP-Glc with the recycling of the free enzyme over ultrafiltration membrane, which was shown as the most preferable product, the batch reaction was able to be stably repeated for 13 cycles with a supply of 5% ADP (117 mM) substrate per each batch (Fig. 6A). Then, the batch reaction was also continued for further 17 cycles with other company's ADP (Genechem). The results indicated that the enzyme retained in the membrane was stable without any significant inactivation for whole repetitive batches of 30 cycles, and also expected to be stably reused for further batches. In the 13th batches (6.5 days), the sum amount of ADP-Glc produced was calculated to be 1.67 g (2.7 mmol) from 3.25 g (7.6 mmol) ADP consumed.

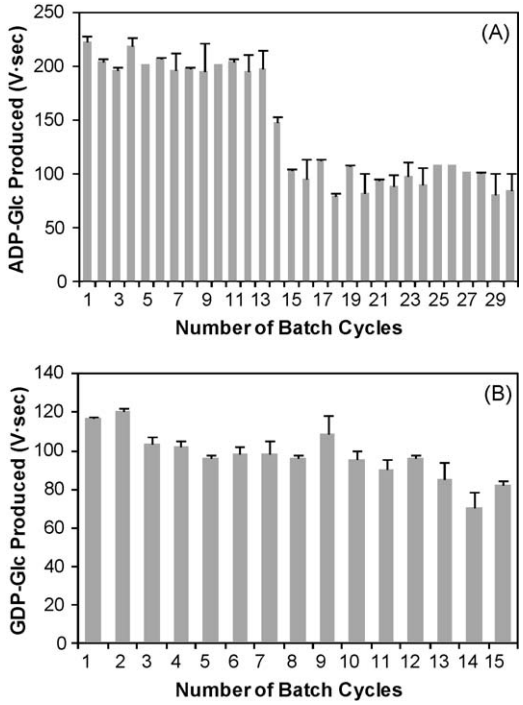

Fig. 6. Preparative synthesis of purine base NDP-Glcs, ADP-Glc (A) and GDP-Glc (B), using repetitive batch reaction with free enzyme. For ADP-Glc production, ADP was used in the concentration of 117 mM per a batch. For GDP-Glc production, GDP was used in the concentration of 113 mM per a batch. The reaction was carried out with the enzyme (0.24 mg/mL) and trehalose acceptor (420 mM) at 37 °C for 12 h. HPLC analysis for a batch sample was duplicately performed with the linear gradient of 0.5 M NaCl. The ordinate indicates the peak area (V s) of product on HPLC.

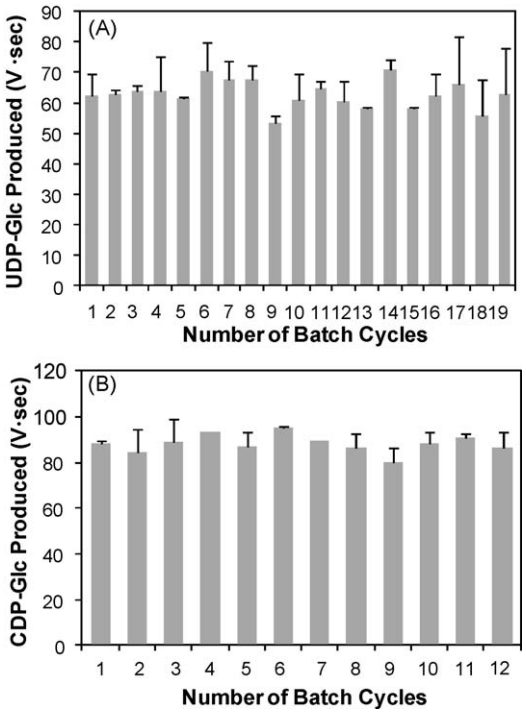

Fig. 7. Preparative synthesis of pyrimidine base NDP-Glcs, UDP-Glc (A) and CDP-Glc (B), using repetitive batch reaction with free enzyme. Both UDP and CDP were reacted in the concentration of 124 mM per a batch with the enzyme (0.24 mg/mL) and trehalose acceptor (420 mM) at 37 °C for 12 h. HPLC analysis for a batch sample was duplicately performed with the linear gradient of 0.5 M NaCl. The ordinate indicates the peak area (V s) of product on HPLC.

The average yield of ADP-Glc for every batch was estimated to be approximately 35% (mmol ADP-Glc/mmol ADP). The productivity of NDP-Glc may be increased at higher temperature, but the maintenance for thermal stability of the enzyme and NDP substrates in the repetitive reaction should be investigated. The enzyme reaction in every batch started with 444 mM (16%, w/v) trehalose. At the end of one batch reaction, trehalose was consumed to produce ADP-Glc, and more than 300 mM (11.3%, w/v) of trehalose was expected to still remain in the solution. Therefore, the high stability of the enzyme was considered mainly due to the stabilizing effect of trehalose. Generally, trehalose is known as the most effective sugar to stabilize protein even in 5–10% concentration against denaturing conditions such as heat, drying, freeze–thawing, and so on [27].

For GDP-Glc production, the same type of repetitive batch reaction was carried out for 15 repeated cycles with fresh enzyme (Fig. 6B). For the productions of UDP-Glc and CDP-Glc, the repetitive batches were performed in the same manner for 19 and 12 repeated cycles, respectively (Fig. 7A and B). The enzyme stability was approximately maintained for all the batch reactions employed in the experiment and also expected to be constant for more batches. The average yields of every batch for the repetitive productions of GDP-Glc, CDP-Glc, and UDP-Glc were evaluated to be about 17%, 12%, and 10% (mmol NDP-Glc produced per mmol NDP used), respectively. In fact, NDP-Glcs were produced in a gram scale with appropriate reaction volume (about 10 mL) by more or less 10 cycles of repetitive batch production with free enzyme entrapped in the ultrafiltration membrane. A purification of one batch solution was easily processed in a time by preparative HPLC. After the enzyme reaction with NDP (about 100–200 mg) and trehalose (800–1600 mg) in a batch, the whole reaction mixture was purified and eluted sequentially through the preparative column in the order of D-glucose and trehalose, NDP-Glc, and NDP, enabling us to separately collect the fractions of NDP-Glc and NDP. The purified compound, NDP, could be recycled after lyophilization for further use. In next experiment, repetitive batch reaction with immobilized enzyme would be investigated in the productions of NDP-Glcs. Generally, the immobilized enzyme is considered to have advantages of easier handling, reusability, keeping in long life, and improved kinetics [9]. However, due to the high stability of the enzyme and availability of trehalose in the present condition of batch reaction, this recycling system with free enzyme would be practically applicable in the gram-scale production of NDP-Glcs or its regeneration in the NDP-Glc dependent transglucosylation reaction. This enzyme system is proposed to be very effective to enhance the amount of products by modulating the size or the batch cycle of the reaction according to the need.

4. Conclusion

In the present study, we have shown that *Pyrococcus* TreT alternatively catalyzes the synthetic reaction of trehalose or NDP-Glc. Particularly, this enzyme is an useful microbial enzyme that effectively converts various NDPs into the corresponding NDP-Glcs, such as ADP-, GDP-, UDP-, and CDP-Glcs, in one step with the use of trehalose. The enzyme is favorable to the regeneration of ADP-Glc with greater conversion yield. In the repetitive batch-type reactions, the enzyme was highly stable and utilized for gram-scale productions of NDP-Glcs with extensive recycling. Further studies are needed to provide detailed structural insights that may explain the differences in the preferences of the enzymes to nucleotides as donors or acceptors. Furthermore, the unique property of the enzyme can make it applicable for the regeneration of highly expensive NDP-Glcs from NDP, by-product, in the nucleotide sugar-dependent glycosyltransferase-catalyzed reaction.

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