Efficient one-pot enzymatic synthesis of trehalose 6-phosphate using GH65 α-glucoside phosphorylases

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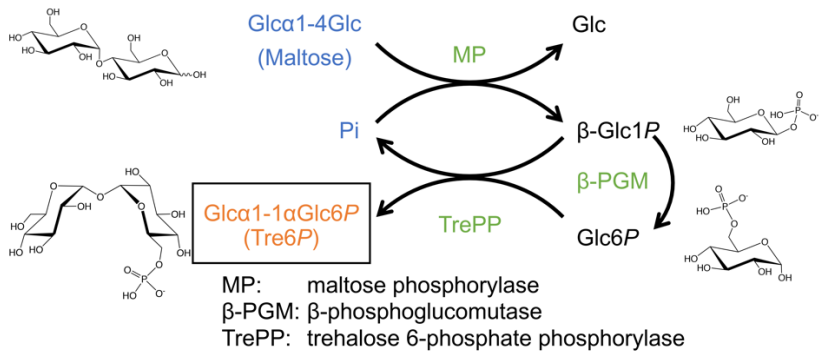
Abstract

Trehalose 6-phosphate (Tre6*P*) is an important intermediate for trehalose biosynthesis. Recent researches have revealed that Tre6*P* is an endogenous signaling molecule that regulates plant development and stress responses. The necessity of Tre6*P* in physiological studies is expected to be increasing. To achieve the cost-effective production of Tre6*P*, a novel approach is required. In this study, we utilized trehalose 6-phosphate phosphorylase (TrePP)

from *Lactococcus lactis* to produceTre6*P*. In the reverse phosphorolysis by the TrePP, 91.9 mM Tre6*P* was produced from 100 mM β-glucose 1-phosphate (β-Glc1*P*) and 100 mM glucose 6-phosphate (Glc6*P*). The one-pot reaction of TrePP and maltose phosphorylase (MP) enabled production of 65 mM Tre6*P* from 100 mM maltose, 100 mM Glc6*P*, and 20 mM inorganic phosphate. Addition of β-phosphoglucomutase to this reaction produced Glc6*P* from β-Glc1*P* and thus reduced requirement of Glc6*P* as a starting material. Within the range of 20–469 mM inorganic phosphate tested, the 54 mM concentration yielded the highest amount of Tre6*P* (33 mM). Addition of yeast increased the yield because of its glucose consumption. Finally, from 100 mmol maltose and 60 mmol inorganic phosphate, we successfully achieved production of 37.5 mmol Tre6*P* in a one-pot reaction (100 mL), and 9.4 g Tre6*P* dipotassium salt was obtained.

Graphical abstract



Highlights

Tre6*P* was formed from β-Glc1*P* and Glc6*P* by TrePP.

With two other enzymes, Tre6*P* was produced from only maltose in one-pot reactions.

Gram-scale synthesis of Tre6*P* from maltose and inorganic phosphate was established.

Keywords

Trehalose 6-phosphate; Trehalose 6-phosphate phosphorylase; Glycoside hydrolase family 65; Maltose phosphorylase; Phosphoglucomutase

Abbreviations: COSY, correlated spectroscopy; ESI, electrospray ionization; GH 65, glycoside hydrolase family 65; β-Glc1*P*, β-D-glucose 1-phosphate; Glc6*P*, D-glucose 6-phosphate; GP, glycoside phosphorylase; HMBC, heteronuclear multiple bond correlation; HPAEC-PAD, high-performance anion-exchange chromatography equipped with a pulsed amperometric detector; HSQC, heteronuclear single quantum coherence; HSQC-TOCSY, HSQC–total correlation spectroscopy; LlTrePP, trehalose 6-phosphate phosphorylase from *Lactococcus lactis* subsp. *lactis*; MS, mass spectrometry; MP, maltose phosphorylase; NMR, nuclear magnetic resonance; β-PGM, β-phosphoglucomutase; TPS, α,α-trehalose-phosphate synthase; TrePP, trehalose 6-phosphate phosphorylase; Tre6*P*, trehalose 6-phosphate.

1. Introduction

Trehalose (α-D-Glc*p*-(1↔1)-α-D-Glc*p*) is a non-reducing disaccharide, which is widely distributed in diverse organisms, including bacteria, fungi, insects, and plants. In these organisms, trehalose serves as an important compatible solute and stress protectant. Trehalose is synthesized through the Trehalose phosphate synthase–Trehalose phosphatase (TPS–TPP) pathway in various organisms.

The first enzyme in the pathway, α,α-trehalose phosphate synthase (EC 2.4.1.15; TPS), catalyzes the formation of trehalose 6-phosphate (α-D-Glc*p*-(1↔1)-α-D-Glc*p*6*P*; Tre6*P*) from UDP-glucose and D-glucose 6-phosphate (Glc6*P*), and the second enzyme, trehalose phosphatase (EC 3.1.3.12; TPP), dephosphorylates Tre6*P* [1]. Besides the occurrence of Tre6*P* as the intermediate in the TPS–TPP pathway, Tre6*P* is also produced as a bacterial trehalose metabolite. The bacterial phosphotransferase system transports extracellular trehalose into cells concomitant with its phosphorylation [2]. The Tre6*P* produced is then hydrolyzed by Tre6*P* hydrolase [3] or phosphorolyzed into β-D-glucose 1-phosphate (β-Glc1*P*) and Glc6*P* by trehalose 6-phosphate phosphorylase (EC 2.4.1.216; TrePP) [4]. The physiological importance of Tre6*P* as a signaling molecule in the regulation of development, stress tolerance, and carbohydrate metabolism in plants has been recognized in the last decade. The null mutant of *Arabidopsis thaliana tps1*, which is thought to cause Tre6*P* deficiency, is unable to complete embryogenesis, resulting in embryonic lethality [5,6]. The *AtTPS1* overexpressor in *A. thaliana* displays a dehydration tolerance phenotype [7]. Chemically modified Tre6*P*, which is permeable in plants, changes sucrose allocation and increases yield in wheat [8]. These findings indicate that Tre6*P* is important for plant development and shows potential for agricultural applications. Not only for the plant, but Tre6*P* is also good to target for antibiotic development. In *Mycobacterium tuberculosis*, the gene of TPP which degrade Tre6*P* is necessary for growth because of the Tre6*P*-associated toxicity [9]. Improved access to Tre6*P* expected to the development of new antibiotics.

To better understand the physiological functions of Tre6*P*, especially in plants, efficient synthetic methods are required to produce a sufficient amount of Tre6*P* for analyses. To date, Tre6*P* has been synthesized by yeast fermentation [10] and organic synthesis [11,12]. In the former system, D-glucose provided as the carbon source is converted into Tre6*P* with 11% yield. The latter system produces yields higher than 50% but requires several reaction steps, including protection and

deprotection of hydroxy groups, and uses an organic solvent. In contrast, enzymatic synthesis is simpler and environmentally friendly because it is carried out in aqueous solution under mild conditions.

Glycoside phosphorylases (GPs) catalyze the reversible phosphorolysis of glycosides with strict substrate specificity. Thus, they are useful catalysts for the specific and efficient synthesis of oligosaccharides by reverse phosphorolysis. A variety of oligosaccharides have been produced by glycosyl transfer from glycosyl phosphate in the reverse phosphorolysis of GPs [13,14]. Because the sugar phosphate can be supplied through the phosphorolysis of abundant oligosaccharides by a specific GP, a sugar phosphate is not always necessary as a starting material to produce target oligosaccharides. For example, α,α-trehalose was produced with a high yield of 60% from the abundant oligosaccharide maltose and a catalytic amount of inorganic phosphate through phosphorolysis by maltose phosphorylase (EC 2.4.1.8; MP) and reverse phosphorolysis by trehalose phosphorylase (EC 2.4.1.64) in a one-pot reaction [15]. Some α-(1→4)-glucosyl disaccharides were also produced with very high yields (84%–91%) from maltose and a number of other sugars by MP alone [16].

The TrePP of *Lactococcus lactis* subsp. *lactis* is thought to be responsible for the degradation of Tre6*P* because this bacterium possesses the phosphotransferase system incorporating trehalose but lacks genes encoding Tre6*P*-metabolizing enzymes, namely TPP, α,α-trehalase (EC 3.2.1.28), or α,α-phosphotrehalase (EC 3.2.1.93) [4]. Its amino acid sequence indicates that TrePP is a member of the glycoside hydrolase family 65 (GH65), which comprises inverting α-glucoside phosphorylases, such as MP, α,α-trehalose phosphorylase, and kojibiose phosphorylase (EC 2.4.1.230) [17]. TrePP catalyzes the phosphorolysis and synthesis of Tre6*P* through a sequential bi-bi mechanism involving the formation of a ternary complex of the enzyme and two substrates [4,18]. The reverse phosphorolysis of TrePP was used to synthesize Tre6*P*. However, a large amount of

starting materials is difficult to use. In this study, we describe a simple and efficient enzymatic method for Tre6*P* synthesis from simple abundant carbohydrate material using TrePP in combination with some other enzymes.

2. Materials and methods

2.1. Chemicals and enzymes

As described previously [18], β-Glc1*P* was prepared from maltose using MP. Recombinant TrePP from *L. lactis* subsp. *lactis* (LlTrePP) was heterologously transformed into BL21 (DE3) competent *Escherichia coli* cells and purified by Ni-affinity column chromatography as described previously [18]. One unit of TrePP activity was defined as the amount of enzyme that produces 1 μmol inorganic phosphate from 10 mM β-Glc1*P* and 10 mM Glc6*P* per minute under the condition previously described [18]. MP was purchased from Oriental Yeast (Tokyo, Japan). As stated in the manufacturer's instructions, 1 unit of MP was defined as the amount of enzyme that produces 1 μmol D-glucose per minute from 100 mM maltose and 100 mM phosphate buffer (pH 5.6) at 30°C. β-Phosphoglucomutase (EC 5.4.2.6; β-PGM) was purchased from Sigma-Aldrich (St Louis, MO, USA) and used to obtain the results in section 3.3. For the other reactions, recombinant β-PGM from *L. lactis* subsp. *lactis* was used, which was heterologously transformed into BL21 (DE3) competent *E. coli* cells and purified by Ni-affinity column chromatography. The *pgmB* gene encoding a β-PGM (GenBank accession no. AE005176.1) was amplified by PCR using genomic DNA of *L. lactis* subsp. *lactis* JCM 5805 as the template, primers (sense, 5'-AAAGAAGGTAAAAAGATGTTTAAAGCAGT -3'; antisense, 5'-TTATTAATTACGAATTATTTTTGCTTTTG -3'), and PrimeSTAR® HS DNA polymerase (Takara Bio, Otsu, Japan). Amplified DNA was ligated into the pET23a expression vector (Novagen, Darmstadt, Germany) at the sites between *Nde*I and the His-tag coding sequence

(CACCACCACCACCACCACTGA) using the In-Fusion® HD Cloning Kit (Takara Bio). One unit of β-PGM was defined as the amount of enzyme that converts 1 μmol β-Glc1*P* to Glc6*P* per minute at 37°C and pH 7.0 [19]. A hexose phosphatase, YidA, from *E. coli* (EC3.1.3.23; accession no. NP_418152.1 [20,21]) was prepared as described previously [20]. One unit of YidA is defined as the amount of enzyme that releases 1 μmol inorganic phosphate from 20 mM Glc6*P* per minute at 37°C and pH 7.0.

2.2. Quantification of carbohydrates

Carbohydrates were quantified using high-performance anion-exchange chromatography equipped with a pulsed amperometric detector (HPAEC-PAD ICS 5000$^+$; Thermo Fisher Scientific, Waltham, MA, USA) on a CarboPac PA1 column (4 mm i.d. × 250 mm; Thermo Fisher Scientific). The mobile phase was 200 mM NaOH with a linear gradient of sodium acetate (0–250 mM) in 40 min (flow rate, 0.8 mL/min). The sample injection volume was 10 μL. The standards were 25–200 μM Tre6*P* (Sigma Aldrich), 25–200 μM Glc6*P* (Oriental Yeast), 25–200 μM β-Glc1*P*, 50–400 μM maltose (Nacalai Tesque, Kyoto, Japan), and 50–400 μM glucose (Nacalai Tesque). The retention times of glucose, maltose, Tre6*P*, β-Glc1*P*, and Glc6*P* were 4, 9, 19, 20, and 26 min, respectively.

2.3. Tre6*P* synthesis from β-Glc1*P* and Glc6*P*

The reaction mixture (10 mL) containing 0.2 U/mL LlTrePP, 100 mM β-Glc1*P*, 100 mM Glc6*P*, and 250 mM sodium acetate buffer (pH 5.0) was incubated at 30°C for 36 h. The reaction was stopped by heating the sample at 80°C for 5 min. Inorganic phosphate was measured following the method of Lowry and Lopez [22]. The concentration of Tre6*P* after the reaction was determined by HPAEC-PAD. Remaining β-Glc1*P*, Glc6*P*, inorganic phosphate, and sodium acetate were removed from the sample by electrodialysis with a Microacylizer G1 instrument (Asahi Kasei, Tokyo, Japan) as

described previously [18]. Electrospray ionization (ESI)–mass spectrometry (MS) of the oligosaccharide produced was carried out using an Executive mass spectrometer (Thermo Scientific, San Jose, CA, USA). The sample was introduced by flow injection. Methanol was used as the mobile phase solvent. The negative ion was detected under the following conditions: tube lens voltage, 80 V; skimmer voltage, 30 V. Nuclear magnetic resonance (NMR) spectra were recorded in D_2O (99.9%; Sigma) using a Bruker AMX500 instrument (500 MHz, Bruker, Billerica, MA, USA). A series of two-dimensional homo- and heteronuclear correlated spectroscopy (COSY), heteronuclear single quantum coherence (HSQC), HSQC–total correlation spectroscopy (HSQC-TOCSY), and heteronuclear multiple bond correlation (HMBC) spectra were obtained.

2.4. Tre6*P* synthesis from maltose and Glc6*P* using TrePP and MP

The reaction mixture (1 mL) containing 1.2 U/mL LlTrePP, 3.0 U/mL MP, 100 mM maltose, 100 mM Glc6*P*, and 20 mM sodium phosphate buffer (pH 6.8) was incubated at 30°C. Aliquots (20 μL) were taken at indicated times and heated at 80°C for 5 min to stop the reaction. The concentration of Tre6*P* after the reaction was determined by HPAEC-PAD.

2.5. Tre6*P* synthesis from maltose and inorganic phosphate using TrePP, MP, and β-PGM

The reaction mixture (1 mL) containing 1.2 U/mL LlTrePP, 3.0 U/mL MP, 1.0 U/mL β-PGM, 100–120 mM maltose, 20, 54, 110, 224, or 469 mM sodium phosphate, 1 mM magnesium acetate, and 0.1 μM α-D-glucose 1,6-bisphosphate (Sigma) was incubated at 30°C for 120 h. Aliquots (100 μL) were taken and heated at 80°C for 5 min. β-Glc1*P*, Glc6*P*, Tre6*P*, glucose, and maltose were quantified by HPAEC-PAD, and inorganic phosphate was measured following the method of Lowry and Lopez [22].

Synthesis of Tre6*P* from high concentrations of the substrates (1 M maltose and 0.6 M

sodium phosphate buffer, pH 7.0) was performed in 1 mL reaction mixture. This reaction mixture contained the same concentrations of enzyme and magnesium acetate but did not contain α-D-glucose 1,6-bisphosphate. Efficiency improvement was achieved by addition of 1% (w/v) bakery yeast [23,24]. The reaction proceeded under the same condition. Compounds were quantified as described above. On day 4 of the reaction, 20.6 μL of the enzyme mixture containing 1.2 U LlTrePP, 3.0 U MP, and 3.0 U β-PGM was added, and the incubation was continued.

For Tre6*P* preparation, 100 mL of the reaction mixture containing 1 M maltose, 0.6 M sodium phosphate buffer (pH 7.0), 1 mM magnesium acetate, and 3 U/mL MP was incubated at 30°C for 5 days. At 48 h, 2 mL of the enzyme mixture containing 320 U LlTrePP, 500 U MP, and 590 U β-PGM was added. The reaction was terminated on day 5 by heating at 80°C for 5 min. To remove Glc6*P* and β-Glc1*P*, 2.0 U/mL YidA was added and incubated at 30°C for 8 h followed by heating at 80°C for 5 min. The reaction mixture was filtered and glycine (Wako Pure Chemical Industries, Osaka, Japan) was added to a final concentration of 1 M. This mixture was concentrated at 60°C in a rotary evaporator for the Maillard reaction. The sample was loaded onto an anion exchange column of Amberjet® 4400 resin (4.8 cm i.d. × 30 cm, 300 mL, hydroxy form, Organo, Tokyo, Japan) in accordance with a previously described method [25]. Fractions containing Tre6*P* were collected, and magnesium acetate and ammonia were added (12 mmol, three times the inorganic phosphate content) for struvite precipitation [26,27]. The pH was adjusted to 10 with 6 M KOH, and the mixture was concentrated in a rotary evaporator. The mixture was subjected to gel filtration chromatography on a TOYOPEARL® HW-40S column (5.0 cm i.d. × 100 cm, 1770 mL; Tosoh, Tokyo, Japan) using water as the eluent (flow rate: 0.9 mL/min). Fractions containing Tre6*P* were collected, and the pooled sample was passed through a column of cation exchange resin (Dowex 50, H^+type; Wako Pure Chemical Industries) to remove the counterions of Tre6*P*. The pH was adjusted to 8.5 with 6 M KOH and concentrated in a rotary evaporator. Then Tre6*P* was

precipitated in 96% ethanol. The purity was confirmed by HPAEC-PAD and NMR.

3. Results and discussion

3.1. Tre6*P* synthesis from β-Glc1*P* and Glc6*P* by reverse phosphorolysis of LlTrePP

LlTrePP was incubated with 100 mM β-Glc1*P* and 100 mM Glc6*P* at 30°C, and released inorganic phosphate was monitored (Fig. 1). The concentration of inorganic phosphate was identical to that of the produced Tre6*P*. Tre6*P* did not significantly change after 24 h. Tre6*P* attained 91.9 mM in 36 h. From this result, the equilibrium constant of the phosphorolysis of Tre6*P* —K_{eq} = [β-Glc1*P*][Glc6*P*]/[Tre6*P*][inorganic phosphate]— was determined to be 0.0078. The reaction product was purified by electrodialysis and analyzed by ESI-MS and NMR. The product gave a signal at 421.07 *m*/*z* $[M]^-$. The chemical shifts detected in the ^{1}H- and ^{13}C-NMR analyses are summarized in Table 1. Correlation peaks between C1 of Glc and H1 of Glc6*P*, and between H1 of Glc and C1 of Glc6*P*, were observed in the HMBC spectrum, which indicated that double glucosidic linkages had formed between Glc and Glc6*P*. The $J_{H1, H2}$ values of Glc and Glc6*P* were 3.75 Hz and 3.85 Hz, respectively, which indicated that both pyranoses were in an α-anomeric configuration. The chemical shifts obtained from the ^{1}H- and ^{13}C-NMR matched those previously reported for Tre6*P* [11,12]. Consequently, the reaction product of LlTrePP from β-Glc1*P* and Glc6*P* was confirmed to be Tre6*P*.

3.2. One-pot synthesis of Tre6*P* from maltose and Glc6*P* by LlTrePP and MP

Instead of using β-Glc1*P* as a starting material, β-Glc1*P* synthesis from maltose, which is a readily available substrate, was investigated. Maltose and Glc6*P* were used as starting materials for Tre6*P* synthesis. It was expected that β-Glc1*P* was supplied by phosphorolysis of maltose by MP and used for Tre6*P* production by LlTrePP (Fig. 2A). The inorganic phosphate used as a substrate in the first MP reaction is released in the second reverse phosphorolysis. Using 100 mM maltose and 100 mM

Glc6*P* in the presence of 20 mM sodium phosphate buffer (pH 6.8) as initial concentrations, Tre6*P* was produced and accumulated to 65 mM in 28 h (Fig. 2B). Extending the reaction time after 8 h did not significantly change the Tre6*P* concentration. The yield was 65% based on the maltose concentration (100 mM) and comparable to those of trehalose production from maltose (60%) by the action of two α-glucoside phosphorylases, trehalose phosphorylase and MP [15].

3.3. One-pot synthesis of Tre6*P* from only maltose and its dependence on inorganic phosphate concentration

Tre6*P* was produced using maltose as a sole carbohydrate source. The expected reaction scheme is shown in Fig. 3A. Compared with the reaction shown in Fig. 2A, Glc6*P* was not added, but the addition of β-PGM was expected to compensate by isomerization of β-Glc1*P*. This enzyme has strict anomeric selectivity and it did not produce α-glucose 1-phosphate. Tre6*P* was produced from 100–120 mM maltose and 20–469 mM inorganic phosphate. The progress of other reaction products was monitored (Fig. 3B–F). During all the reactions, the change in the amounts of total glucosyl groups and total phosphate in the reaction mixtures was within 12%. In the reaction with the initial inorganic phosphate concentration of 20 mM (Fig. 3B), the products attached a plateau rapidly (in 6 h). The concentration of Tre6*P* was 20 mM, which was comparable to the initial concentration of inorganic phosphate, and those of β-Glc1*P* and Glc6*P* were low, which indicated that almost all the inorganic phosphate was incorporated into Tre6*P*. The Tre6*P* yield based on the initial concentration of maltose (100 mM) was 20%; that is, 40% of maltose molecules were used for Tre6*P* synthesis. Hereinafter, the percentage of maltose molecules that was used for its synthesis was used as the yield. The K_{eq} ($K_{eq} = K_{TrePP} \times K_{MP}^2 \times K_{\beta\text{-PGM}} = [\text{Tre6}P][\text{glucose}]^2/([\text{maltose}]^2[\text{inorganic phosphate}])$ calculated at 12 h was 3.87 ± 0.18, which is close to the theoretical K_{eq} (4.73), calculated from K_{TrePP} (31.3 at 35°C in pH 7.0 [4]), K_{MP} (0.071 at 30°C in pH 7.0 [28]), $K_{\beta\text{-PGM}}$ (30 at 30°C in pH 7.0; data

not shown). Higher initial inorganic phosphate concentrations up to 224 mM resulted in higher consumption of maltose up to 93% consumption (Fig. 3E). The maximum Tre6*P* concentration (33 mM) was attained with 48 h reaction with an initial phosphate concentration of 54 mM (Fig. 3C). The yield was 55%. Initial phosphate concentrations higher than 54 mM resulted in lower Tre6*P* production but significant accumulation of Glc6*P*. The ratio of produced Tre6*P* and Glc6*P* was not changed by decreasing β-PGM concentration, which only delayed the reaction attaining the plateau (data not shown). It is preferable for efficient Tre6*P* production to set the concentration of inorganic phosphate lower than that of maltose. In the 120 h reaction with 469 mM inorganic phosphate, lower consumption of maltose (59% consumption), a higher concentration of β-Glc1*P* than Glc6*P*, and lower production of Tre6*P* (15 mM) were observed (Fig. 3F). In the early stage of all reactions, maltose decreased quickly. Concomitantly, β-Glc1*P* was accumulated first and replaced by Tre6*P* and Glc6*P*. The reduction of β-Glc1*P* was delayed depending on inorganic phosphate concentration. Unlike other reactions, β-Glc1*P* was accumulated more than Glc6*P* under 469 mM inorganic phosphate. The concentrations continued to change after 80 h and seemed not to attain equilibrium. We confirmed that under a high concentration of inorganic phosphate, the β-PGM reaction took longer to attain equilibrium than at a low concentration of inorganic phosphate (data not shown). The β-PGM reaction is considered to be inhibited by inorganic phosphate, which is consistent with the finding that β-PGM activity of *Euglena gracilis* was inhibited by 20% in the presence of 10 mM inorganic phosphate [29]. Therefore, the production of Glc6*P* and Tre6*P* did not proceed until the concentration of inorganic phosphate decreased and the β-PGM reaction started. That was the reason why all reactions took a long time despite the high enzyme concentration.

3.4. Efficiency improvement of Tre6*P* synthesis using yeast

The reaction from a higher concentration of starting materials, 1 M maltose and 0.6 M inorganic

phosphate, was examined (Fig. 4A). During the reaction, the change in the amounts of total glucosyl groups and total phosphate was less than 10%. Maltose concentration decreased up to 800 mM and β-Glc1*P* was accumulated in the early stage of the reaction in the presence of remaining inorganic phosphate (Fig. 4A). The maltose and β-Glc1*P* concentrations did not change after 2 days. That was because, in the presence of a high concentration of inorganic phosphate, the activity of β-PGM was inhibited by remained inorganic phosphate, and β-PGM might be deactivated during incubation. Therefore, we added three enzymes, LlTrePP, MP, and β-PGM on day 4. After the supplemental addition of LlTrePP, MP, and β-PGM, maltose and β-Glc1*P* concentrations decreased, and Tre6*P* and Glc6*P* concentrations increased. At day 9, 0.7 M maltose was consumed, and 0.7 M glucose and 0.3 M Tre6*P* were produced. The reaction progressed more quickly with addition of yeast in the reaction mixture for reduction of glucose, which is a product of maltose phosphorolysis (Fig. 4B). The amounts of total glucosyl groups were decreased to 50% in the 9-days reaction because yeast consumed liberated glucose. The amount of total phosphate in the reaction mixture was within a variation of 10%. In this case, maltose and inorganic phosphate concentrations decreased more quickly and β-Glc1*P* accumulated to 0.5 M at day 2. After the addition of LlTrePP, MP, and β-PGM, the Tre6*P* concentration attained 0.4 M in the reaction mixture (the yield was 80%). In either case, Glc6*P* and Tre6*P* were considered not to be produced before the additional supplementation of enzymes. This is because of the inactivation of β-PGM during the reaction.

For preparation of Tre6*P*, the reaction mixture was up-scaled to 100 mL, which contained 100 mmol maltose, 60 mmol inorganic phosphate, and MP in the presence of yeast (Fig. 5, Table 2). Because only MP reaction proceeded for 2 days (Fig. 5), the other two enzymes were not added. At day 2 of incubation, LlTrePP, MP, and β-PGM were added. At day 5, Tre6*P* attained 370 mM (38 mmol). For purification of Tre6*P*, Glc6*P* and β-Glc1*P* were dephosphorylated by a hexose phosphatase, EcYidA. Remaining glucose, Glc6*P* were removed by a Maillard reaction followed by

anion exchange column chromatography. Glc6*P* and Tre6*P* were difficult to separate by anion exchange column chromatography, but anion exchange column chromatography could remove the product of Maillard reaction. After this purification procedure, β-Glc1*P* was not detected. Remaining inorganic phosphate was removed by struvite precipitation. Then, Tre6*P* was further purified by gel filtration and cation exchange column chromatography, and 19 mmol (9.4 g) Tre6*P* was obtained as dipotassium salt with a yield of 38%. The purity was higher than 98%. We confirmed the NMR spectra of purified Tre6*P* (Fig.6B). These spectra were well-matched with the Tre6*P* synthesized from β-Glc1*P* and Glc6*P* (Fig.6A).

The Tre6*P* yield of this enzymatic method was higher than that of the yeast fermentation method (11%) and lower than that of organic synthesis (55%) [10–12]. However, the primary advantage of the enzymatic synthesis method established here is the simple synthesis of Tre6*P* at gram-scale in one-pot reactions from abundantly available starting materials, i.e. maltose and inorganic phosphate. This synthesis pathway breaks the barrier of Tre6*P* usage and is expected to facilitate functional analysis of Tre6*P*. Tre6*P* metabolism is a good target for antibiotics for bacteria, especially *M. tuberculosis* [9].

In plants, Tre6*P* is recognized as an essential signaling molecule in the regulation of growth and development. Analyses are required for an improved understanding of the functions of Tre6*P*. The simple enzymatic synthesis of Tre6*P* established in this study provides large amounts of Tre6*P* for such analyses. Although it may be challenging, isotopically labeled Tre6*P*, which would be helpful for tracing metabolites, might be produced by using ^{13}C-labeled maltose and ^{32}P-labeled inorganic phosphate, for instance, as substrates. Tre6*P* analogs, such as Glcα1-α1Man6*P* [18], also could be supplied.

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Declaration of interest

No potential conflict of interest was reported by the authors.

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Figure legends

Fig. 1. Time course of Tre6*P* formation from 100 mM β-Glc1*P* and 100 mM Glc6*P* by LlTrePP.

(A) Expected reaction scheme. LlTrePP, Tre6*P* phosphorylase from *L. lactis* subsp. *lactis*. (B) Time course of accumulation of Tre6*P*. Starting materials were Glc6*P* (100 mM), β-Glc1*P* (100 mM). Liberated inorganic phosphate was measured following the method of Lowry and Lopez [22].

At 36 h, Tre6*P* was quantified by HPAEC-PAD. The concentration of inorganic phosphate was identical to that of the produced Tre6*P*. Data are means of triplicate experiments with standard deviations in three independent reactions.

Fig. 2. One-pot synthesis of Tre6*P* from maltose and Glc6*P* by LlTrePP and MP.

(A) Expected reaction scheme. LlTrePP, Tre6*P* phosphorylase from *L. lactis* subsp. *lactis*; MP, maltose phosphorylase. (B) Time course of accumulation of Tre6*P*. Starting materials were Glc6*P* (100 mM), maltose (100 mM), and 20 mM inorganic phosphate. Tre6*P* was quantified by HPAEC-PAD. Data are means of triplicate experiments with standard deviations in three independent reactions.

Fig. 3. Production of Tre6*P* using maltose as a single carbohydrate source.

(A) Expected reaction scheme. An additional enzyme, β-phosphoglucomutase (β-PGM), was added. (B–F) Time course of accumulation of Tre6*P* synthesized from 100–120 mM maltose and inorganic phosphate (B: 20 mM, C: 54 mM, D: 110 mM, E: 224 mM, F: 469 mM). Tre6*P* and the other carbohydrates were quantified. Maltose (filled square), inorganic phosphate (open square), Tre6*P* (filled circle), glucose (open circle), Glc6*P* (open triangle), and β-Glc1*P* (filled triangle). [Pi]: right side axis in E and F. Data are means of triplicate experiments with standard deviations in three independent reactions.

Fig. 4. Time course of Tre6*P* synthesis from 1 M maltose and 0.6 M inorganic phosphate

Time course of Tre6*P* synthesis reactions without yeast (A) and with yeast (B) are shown. The enzymes were supplemented at day 4. Symbols are the same as shown in Fig. 3B. Data are means of triplicate experiments with standard deviations in three independent reactions.

Fig. 5. Time course of large-scale (100 mL) synthesis of Tre6*P*

Tre6*P* was synthesized in a 100-mL reaction mixture containing 1 M (100 mmol) maltose, 0.6 M (60 mmol) sodium phosphate was monitored. The enzymes were supplemented at day 2. Symbols are the same as shown in Fig. 3B.

Fig.6. ^{1}H- and ^{13}C-NMR spectra of synthesized Tre6*P*

The synthesized Tre6*P* from (A) β-Glc1*P* and Glc6*P*, and (B) maltose and inorganic phosphate were analyzed by ^{1}H- and ^{13}C- NMR.

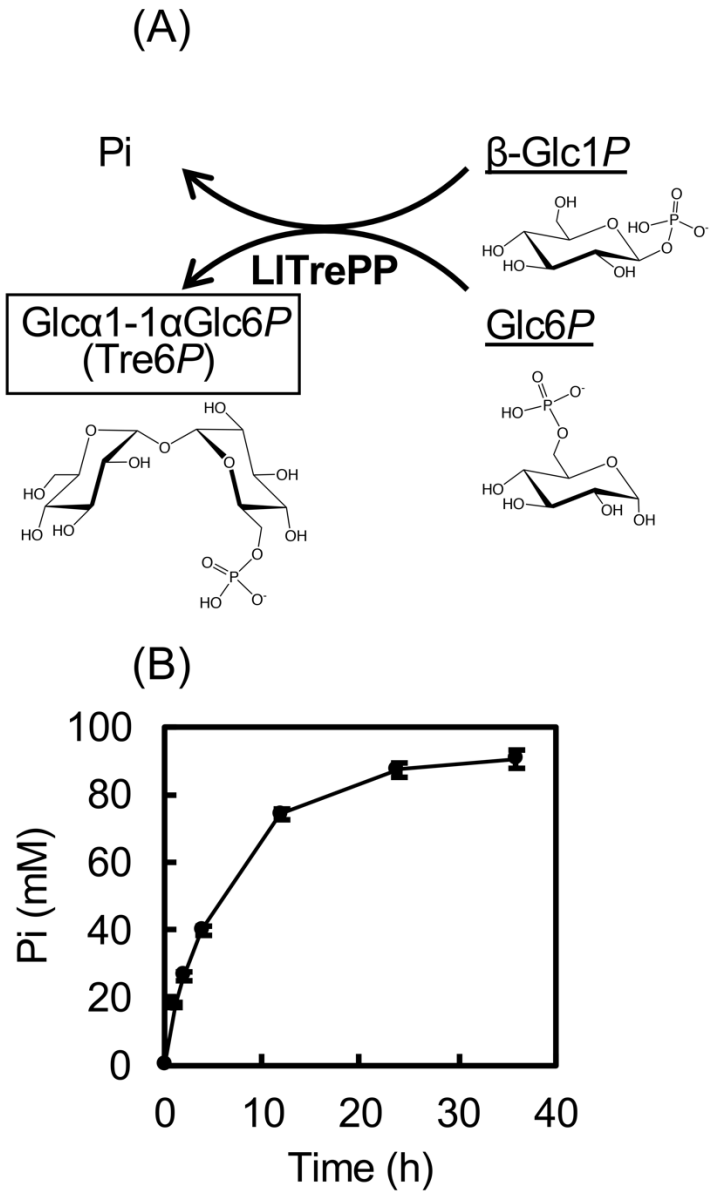
Table 1 Chemical shifts of α-D-Glc*p*-(1↔1)-α-D-Glc*p6P* in the ^{1}H- and ^{13}C-NMR spectra.

Residue	Number	δC (ppm)	δH (ppm)	*J* (Hz)	
α-Glc*p*	1	94.3	5.27	d	3.75
	2	71.9	3.70	m	
	3	73.4	3.93	m	
	4	70.6	3.51	t	9.43
	5	73.1	3.88	m	
	6	61.5	3.93	m	
			3.83	m	
α-Glc*p6P*	1	94.4	5.25	d	3.85
	2	72.1	3.76	m	
	3	73.2	3.90	m	
	4	70.1	3.66	t	9.60
	5	72.5	3.97	m	
	6	63.9	4.08	m	

Table 2 Purification of Tre6*P*

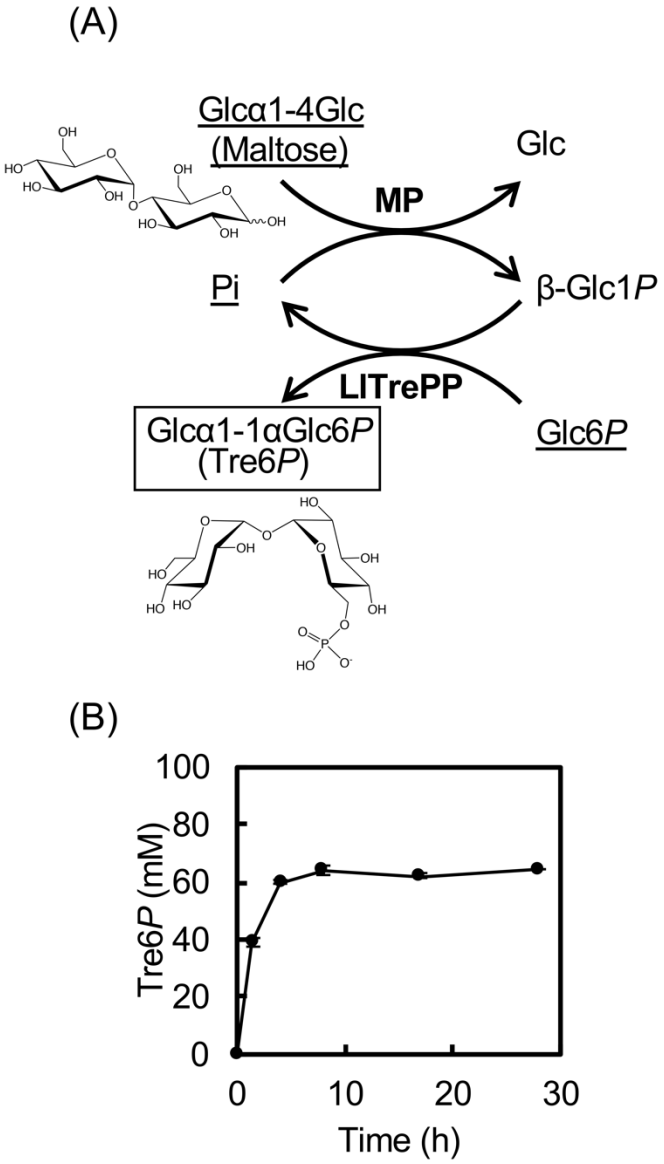
Process	Tre6*P* mmol	Glc6*P* mmol	β-Glc1*P* mmol	Purity %	Yield %
Reaction	38	12	0.23	64	75
EcYidA	36	8.5	0.19	73	72
Anion-exchange column chromatography	25	-	-	86	50
Gel-filtration column chromatography	19	-	-	98	38

*The yield was calculated based on the maltose molecules used for Tre6*P* synthesis.


(A)
Pi
β-Glc1P
LITrePP
Glcα1-1αGlc6P
(Tre6P)
Glc6P
(B)
Pi (mM)
Time (h)
0
20
40
60
80
100
0
10
20
30
40

1

2 **Fig. 1**


(A)
Glcα1-4Glc
(Maltose)
Glc
MP
Pi
β-Glc1P
LlTrePP
Glcα1-1αGlc6P
(Tre6P)
Glc6P
(B)
Tre6P (mM)
Time (h)
0
20
40
60
80
100
0
10
20
30

1

2 **Fig. 2**

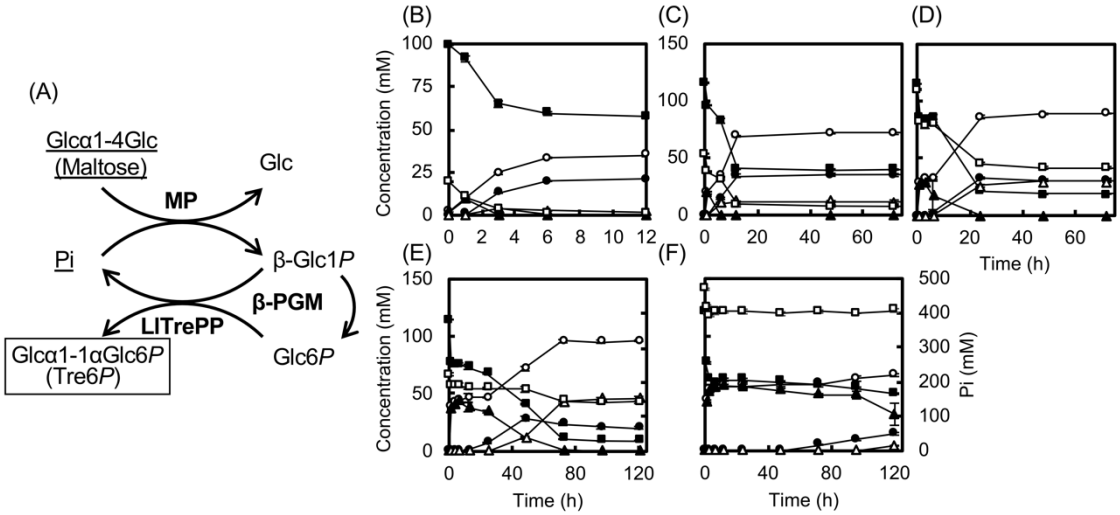

(A)
Glcα1-4Glc
(Maltose)
Glc
MP
Pi
β-Glc1P
β-PGM
LlTrePP
Glcα1-1αGlc6P
(Tre6P)
Glc6P
(B)
(C)
(D)
(E)
(F)
Concentration (mM)
Pi (mM)
Time (h)

Fig. 3

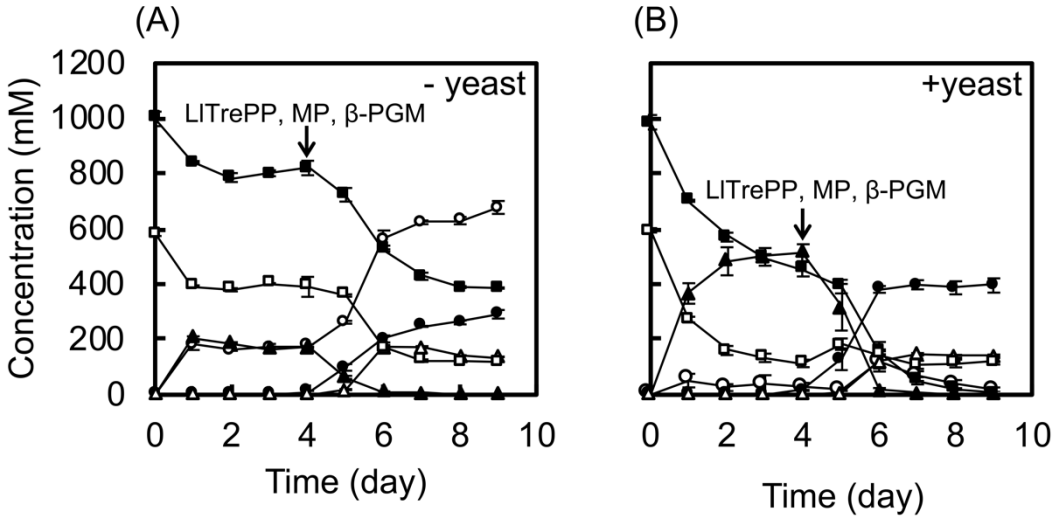

(A)
(B)
- yeast
+yeast
LITrePP, MP, β-PGM
LITrePP, MP, β-PGM
Concentration (mM)
1200
1000
800
600
400
200
0
0
2
4
6
8
10
Time (day)
Time (day)

Fig. 4

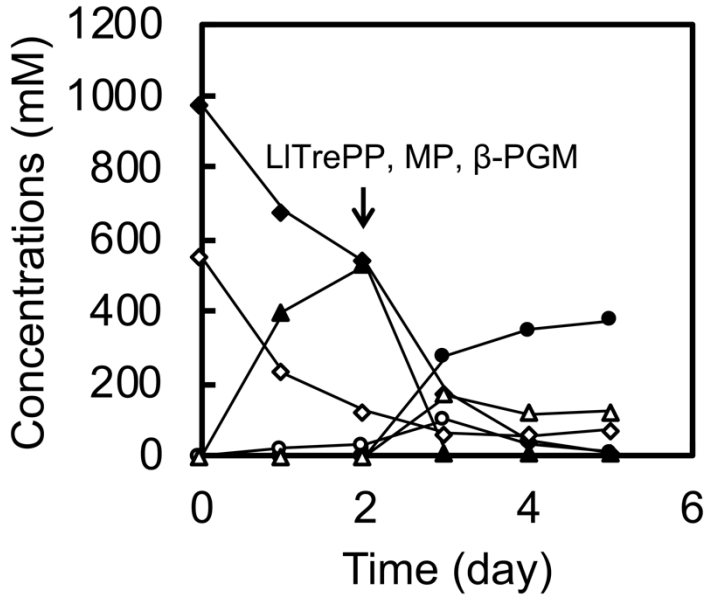

1200
1000
800
600
400
200
0
Concentrations (mM)
LlTrePP, MP, β-PGM
0
2
4
6
Time (day)

Fig. 5

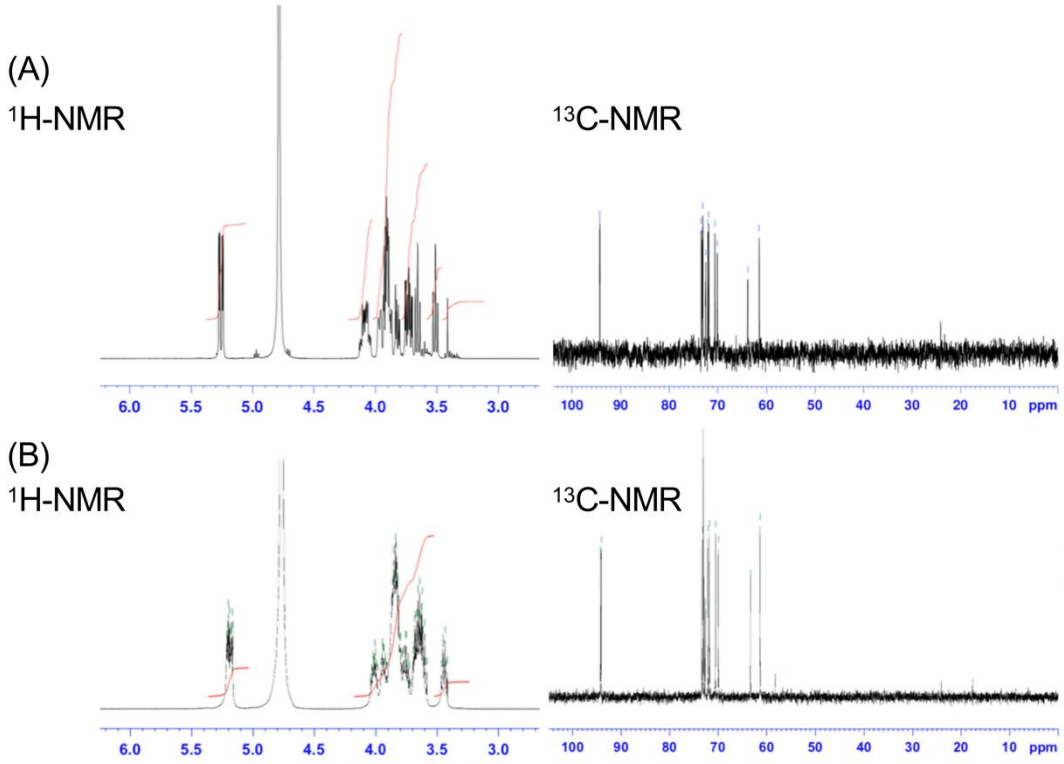

(A)
1H-NMR
13C-NMR
6.0 5.5 5.0 4.5 4.0 3.5 3.0
100 90 80 70 60 50 40 30 20 10 ppm
(B)
1H-NMR
13C-NMR
6.0 5.5 5.0 4.5 4.0 3.5 3.0
100 90 80 70 60 50 40 30 20 10 ppm

Fig. 6