Running title: Acceptor selectivity of trehalose 6-phosphate phosphorylase

Evaluation of acceptor selectivity of *Lactococcus lactis* ssp. *lactis* trehalose 6-phosphate phosphorylase in the reverse phosphorolysis and synthesis of a new sugar phosphate

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Abstract

Trehalose 6-phosphate phosphorylase (TrePP), a member of glycoside hydrolase family 65, catalyzes the reversible phosphorolysis of trehalose 6-phosphate (Tre6*P*) with

inversion of the anomeric configuration to produce β-D-glucose 1-phosphate (β-Glc1*P*) and D-glucose 6-phosphate (Glc6*P*). TrePP in *Lactococcus lactis* ssp. *lactis* (LlTrePP) is, alongside the phosphotransferase system, involved in the metabolism of trehalose. In this study, recombinant LlTrePP was produced and characterized. It showed its highest reverse phosphorolytic activity at pH 4.8 and 40°C, and was stable in the pH range 5.0–8.0 and at up to 30°C. Kinetic analyses indicated that reverse phosphorolysis of Tre6*P* proceeded through a sequential bi bi mechanism involving the formation of a ternary complex of the enzyme, β-Glc1*P*, and Glc6*P*. Suitable acceptor substrates were Glc6*P*, and, at a low level, D-mannose 6-phosphate (Man6*P*). From β-Glc1*P* and Man6*P*, a novel sugar phosphate, α-D-Glc*p*-(1↔1)-α-D-Man*p*6*P*, was synthesized with 51% yield.

Abbreviations: BN-PAGE, blue native polyacrylamide gel electrophoresis; COSY, correlated spectroscopy; ESI, electrospray ionization; GH, glycoside hydrolase family; β-Glc1*P*, β-D-glucose 1-phosphate; Glc6*P*, D-glucose 6-phosphate; HEPES, 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid; 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* ssp. *lactis*; Man6*P*, D-mannose 6-phosphate; MES, 2-morpholinoethanesulfonic acid; NMR, nuclear magnetic resonance; TrePP, trehalose 6-phosphate phosphorylase; Tre6*P*, trehalose 6-phosphate.

Introduction

Trehalose 6-phosphate phosphorylase (EC 2.4.1.216, TrePP) catalyzes the phosphorolysis of trehalose 6-phosphate (Tre6*P*: α-D-Glc*p*-(1↔1)-α-D-Glc*p*6*P*) to produce β-glucose 1-phosphate (β-Glc1*P*) and glucose 6-phosphate (Glc6*P*).[1)] In *Lactococcus lactis* ssp. *lactis*, TrePP (LlTrePP) is involved in trehalose metabolism. Trehalose is taken up into cells and phosphorylated to Tre6*P* through the phosphotransferase system. Tre6*P* is then phosphorolyzed by LlTrePP into β-Glc1*P* and Glc6*P*, which are further metabolized through glycolysis. TrePP seems to be exclusively responsible for catabolizing Tre6*P* in *L. lactis*, because of the absence of genes coding for any other trehalose-metabolizing enzymes such as trehalose-phosphatase (EC 3.1.3.12), α,α-trehalase (EC 3.2.1.28), or α,α-phosphotrehalase (EC 3.2.1.93).

Glycoside phosphorylases are classified into glycoside hydrolase families (GHs) 13, 65, 94, 112, and 130, and glycosyltransferase families 4 and 35 in the Carbohydrate-Active Enzymes database (http://www.cazy.org/) based on amino acid sequence similarity.[2)] TrePP is found only in GH65. GH65 is mainly composed of α-glucoside phosphorylases including TrePP, maltose phosphorylase (EC 2.4.1.8), α,α-trehalose phosphorylase (EC 2.4.1.64), kojibiose phosphorylase (EC 2.4.1.230), nigerose phosphorylase (EC 2.4.1.279), 3-*O*-α-glucopyranosyl-L-rhamnose phosphorylase (EC 2.4.1.282), 1,2-α-glucosylglycerol phosphorylase (EC 2.4.1.332), and 1,3-α-oligoglucan phosphorylase (EC 2.4.1.334). GH65 enzymes have an $(\alpha/\alpha)_6$-barrel fold catalytic domain, similar to those of GH15 and GH94 enzymes.[3,4)] These GH families form clan GH-L based on the similarity of their tertiary structures.[2)]

Phosphorolysis catalyzed by phosphorylases is often reversible, and, therefore,

oligosaccharides can be synthesized through the reverse reaction. Sugar phosphate is used as a glycosyl donor, and the glycosyl group is transferred to an acceptor molecule. Because the acceptor specificity of phosphorylases is not always strict, products of the reverse phosphorolysis are not limited to the original substrate for phosphorolysis. Sugar phosphate is not required when phosphorolysis occurs in the presence of substrate and inorganic phosphate. For instance, maltose phosphorylase catalyzed the phosphorolysis of maltose and produced β-Glc1*P*. This β-Glc1*P* was used as glycosyl donor substrate for reverse phosphorolysis of maltose phosphorylase, and disaccharides such as α-Glc*p*-(1→4)-GlcNAc and α-Glc*p*-(1→4)-L-Fuc*p* were synthesized in the presence of high concentrations of GlcNAc and L-Fuc respectively.[6] It is also possible to include two different phosphorylases, distinguishably catalyzing phosphorolysis and reverse phosphorolysis, respectively, in a one-pot reaction, as shown for example in trehalose production using two GH65 enzymes, maltose phosphorylase and trehalose phosphorylase[5]. GH65 α-glucoside phosphorylases are useful tools to produce α-glucosyl residue-containing saccharides. Among them, TrePP is the sole enzyme that acts on sugar phosphate as the acceptor and produces Tre6*P* in the reverse phosphorolysis.[1] Tre6*P* has been receiving increasing attention as a signaling molecule in plants. A mutant *Arabidopsis thaliana* that overaccumulated Tre6*P* showed early flowering[7] and perturbed sugar metabolism.[8] Tre6*P* synthesis from β-Glc1*P* and Glc6*P* has been achieved using reverse phosphorolysis by a recombinant LlTrePP enzyme.[1] Herein, we describe in detail the acceptor substrate preference of LlTrePP, to investigate the possibility of synthesis of other sugars. D-Mannose 6-phosphate (Man6*P*), the sole accepter substrate of LlTrePP except for Glc6*P*, was used as an acceptor substrate, and a Tre6*P* analogue, α-D-Glc*p*-(1↔1)-α-D-Man*p*6*P*, was synthesized in

good yield.

Materials and methods

Preparation of β-glucose 1-phosphate

β-Glc1*P* was prepared by phosphorolysis of maltose using maltose phosphorylase. A reaction mixture of 2 L composed of 0.3 M maltose (Nacalai Tesque, Kyoto, Japan), 0.3 M potassium phosphate buffer (pH 8.0), and 25 mg/L maltose phosphorylase from *Bacillus* sp. AHU2001[9] was incubated at 37°C for 96 h. The β-Glc1*P* produced was purified by anion exchange column chromatography with Amberjet 4400 (3.6 cm i.d. × 90 cm, acetate form; Organo, Tokyo, Japan) according to a method previously described.[10] β-Glc1*P* was concentrated *in vacuo*, and precipitated in 80% ethanol. The precipitate was dissolved in water and passed through a column of cation exchange resin (Dowex 50, H^+ type; Wako Pure Chemical Industries, Osaka, Japan) to remove the counter ions of β-Glc1*P*. The pH was adjusted to 8.0 with KOH, and β-Glc1*P* was precipitated in 80% ethanol. β-Glc1*P* was filtered, dried *in vacuo*, and stored at −20°C. From 0.6 mol maltose, 0.073 mol β-Glc1*P* was obtained, and the yield was 12%. The purity was judged to be 96.3% or more by high-performance anion-exchange chromatography equipped with a pulsed amperometric detector (HPAEC-PAD).

Construction of the expression plasmid for LlTrePP

The LlTrePP gene (GenBank accession no. Y18267.1 ORF3) was obtained by PCR with genomic DNA of *L. lactis* ssp. *lactis* JCM 5805 as the template, primers (sense, 5′-

CATATGACTGAAAAAGATTGGATAATCCA-3′; antisense, 5′-GCGGCCGCTTTTAAATCAAATTTAGTCTG-3′), and PrimeSTAR® HS DNA polymerase (Takara Bio, Otsu, Japan). The amplified DNA fragment was ligated into vector pBluescript II SK (+) (Stratagene, La Jolla, CA, USA) and cloned. To construct the expression plasmid, cloned DNA was ligated into vector pET23a (Novagen, Darmstadt, Germany) at *Nde*I and *Not*I sites introduced by the PCR. The DNA sequence was confirmed using an Applied Biosystems 3130 Genetic Analyzer (Life Technologies, Carlsbad, CA, USA).

Production and purification of recombinant LlTrePP

A transformant of *Escherichia coli* BL21 (DE3) harboring the expression plasmid for LlTrePP was cultured in 1.0 L of Luria–Bertani medium containing 100 μg/mL ampicillin at 37°C until A_{600} reached 0.5. Protein production was induced by the addition of isopropyl β-D-1-thiogalactoside (Wako Pure Chemical Industries) at a final concentration of 0.1 mM, and the incubation was continued at 18°C for 23 h with vigorous shaking. The *E. coli* cells were harvested by centrifugation (10,000 × *g*, 4°C, 10 min), and suspended in 30 mL of 10 mM 2-morpholinoethanesulfonic acid (MES)-NaOH buffer (pH 6.5) containing 0.5 M NaCl and 25 mM imidazole (buffer A). The bacterial cells were disrupted by sonication using a Sonifier 450 (Bronson, Danbury, CT, USA), and the supernatant obtained by centrifugation (6,000 × *g*, 4°C, 10 min) was regarded as the cell extract. It was loaded onto a Ni-chelating Sepharose Fast Flow column (1.5 cm i.d. × 10 cm; GE Healthcare, Uppsala, Sweden) equilibrated with buffer A. After thorough washing of the column with buffer A, a linear gradient from 25 to 500 mM imidazole was applied, and the

adsorbed protein was eluted. Proteins in fractions were analyzed by SDS-PAGE,[11] and highly purified fractions were pooled. The collected sample was dialyzed against 10 mM MES-NaOH buffer (pH 6.5), and stored at 4°C. The protein concentrations of the cell extract and fractions from the column chromatography were determined by the Bradford method[12] and the UV method,[13] respectively. The concentration of the purified recombinant enzyme was calculated based on the molar quantities of each amino acid measured using a JLC-500/V (JEOL, Tokyo, Japan) after complete acid hydrolysis of the enzyme (6 M HCl, 110°C, 24 h).[14]

Blue native PAGE

The molecular mass of LlTrePP without protein denaturation was determined by blue native PAGE (BN-PAGE).[15] The analytical sample was prepared using a NativePAGE™ Sample Prep Kit (Life Technologies). Native PAGE Novex 4–16% Bio-Tris Gels (Life Technologies) were used and the electrophoresis was carried out at a constant 150 V for 115 min on ice. Native Mark Unstained Standard (Life Technologies) was used to provide protein molecular size standards.

Enzyme assay

The synthetic activity of Tre6*P* from β-Glc1*P* and Glc6*P* by LlTrePP was determined by measuring the liberation of inorganic phosphate. A reaction mixture of 20 μL, composed of an appropriate concentration of enzyme, 105 mM MES-NaOH (pH 5.5), 10 mM β-Glc1*P*, 10 mM Glc6*P* (Oriental Yeast, Tokyo, Japan), and 0.5 mg/mL bovine serum albumin, was incubated at 30°C for 10 min. The reaction was terminated by incubating the sample at 80°C for 5 min. The initial

reaction rates of inorganic phosphate liberation were measured.[16] One unit of enzyme activity was defined as the amount of enzyme producing 1 μmol of inorganic phosphate in 1 min in these conditions. The velocity of liberation of inorganic phosphate was regarded as the same as that of liberation of Tre6*P*. Phosphorolytic activity of LlTrePP was measured as described, [1] but Thio-$NADP^+$ (Nacalai Tesque) was used instead of $NADP^+$.

The optimum temperature was determined in the reaction condition for the enzyme assay, but reaction temperature changed in the range of 20–50°C. The optimum pH for the synthetic activity of Tre6*P* was determined in the reaction conditions for the enzyme assay but using the following buffers (100 mM): glycine-HCl buffer (pH 3.5), sodium acetate buffer (pH 4.0–5.3), MES-NaOH buffer (pH 5.6–7.2), and (2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES)-NaOH (pH 7.2–8.2). For determination of the pH optimum for the phosphorolytic activity, the pH of the potassium phosphate buffer was varied in the range from 5.5–8.0. To assess temperature stability, LlTrePP (0.14 μg/mL) was incubated at various temperatures for 15 min at pH 5.5. To determine pH stability, LlTrePP (6.9 and 0.46 μg/mL) was respectively kept with 0.5 and 0.9 mg/mL bovine serum albumin at various pHs at 4°C for 24 h or at 30°C for 15 min. The pH of the enzyme solution was adjusted with the following buffers: sodium acetate buffer (pH 3.0–5.0), MES-NaOH buffer (pH 5.0–7.0), HEPES-NaOH buffer (pH 7.0–8.0), Tricine-NaOH buffer (pH 8.0–8.7), and glycine-NaOH buffer (pH 8.7–11.0) in the 4°C assay, using buffer concentrations of 100 mM; and sodium acetate buffer (pH 4.1–4.7), sodium citrate buffer (pH 4.5–6.3), MES-NaOH buffer (pH 5.7–6.2), HEPES-NaOH buffer (pH 6.5–7.5), Bicine-NaOH buffer (pH 7.9–8.7), and *N*-cyclohexyl-2-aminoethanesulfonic acid-NaOH buffer (pH 8.4–9.2) in the 30°C assay, using buffer concentrations

of 20 mM. Residual activities were measured in the standard conditions, and the ranges of pH and temperature in which the enzyme retained ≥90% of the original activity were regarded as the stable ranges.

The kinetic parameters for the reverse phosphorolysis of Tre6*P* were calculated from the initial velocities toward various concentrations of β-Glc1*P* (0.13, 0.20, 0.26, 0.33, and 0.39 mM) and Glc6*P* (3.7, 5.5, 7.2, 9.2, and 11 mM) by fitting to the equation for a sequential bi bi mechanism.[17] Non-linear regression was performed with Grafit version 7.0.2 (Erithacus Software, West Sussex, UK).

Acceptor analysis

Acceptor substrates were screened by measuring enzymatic activities in the standard assay for synthetic activity, but the enzyme concentration was 345 μg/mL, and various compounds in place of Glc6*P* were tested as acceptor substrates as follows: D-Glucose (Nacalai Tesque), D-galactose (Wako Pure Chemical Industries), D-mannose (Wako Pure Chemical Industries), D-fructose (Nacalai Tesque), L-arabinose (Nacalai Tesque), D-xylose (Wako Pure Chemical Industries), D-sorbose (Nacalai Tesque), maltose (Nacalai Tesque), trehalose (Hayashibara, Okayama, Japan), lactose (Nacalai Tesque), sucrose (Nacalai Tesque), methyl α-D-glucoside (Wako Pure Chemical Industries), methyl β-D-glucoside (Wako Pure Chemical Industries), D-glucitol (Nacalai Tesque), D-glucosamine (Tokyo Chemical Industry, Tokyo, Japan), Man6*P* (Sigma, St. Louis, MO, USA), and D-fructose 6-phosphate (Sigma).

Structural analysis of synthesized oligosaccharide

A reaction mixture composed of 100 mM β-Glc1*P*, 100 mM Man6*P*, 100 U/mL LlTrePP, 5 mM MES-NaOH (pH 6.5) and 0.5 mg/mL bovine serum albumin was incubated at 30°C. Aliquots were taken at 0.5, 1, 2, 4, 8, 16, 24, 36 and 48 h, and kept at 80°C for 5 min to stop the reaction. Inorganic phosphate concentrations were measured, and regarded as the same as the product saccharide concentrations. From 2460 μL of the 48-h reaction mixture, the product saccharide was purified by electrodialysis with a Microacylizer G1 (Asahi Kasei Co., Tokyo, Japan). Firstly, inorganic phosphate was removed from the sample by electrodialysis against 30 mL of 0.5 M $NaNO_3$ solution with cartridge AC-110 (molecular mass cutoff 100 Da; Astom, Tokyo Japan) until the current was <0.02 A. The sample was then electrodialyzed against 30 mL of H_2O through cartridge AC-220 (molecular mass cutoff 300 Da; Astom) for 1 h to obtain the sugar phosphate in the dialysate.

Electrospray ionization (ESI)-MS was performed with an Executive Mass Spectrometer (Thermo Scientific, San Jose, CA, USA). The sample was dissolved in methanol, and injected for analysis by flow injection. Methanol was used as the mobile phase solvent. The negative ion was detected in 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 (500 MHz; Bruker, Billerica, MA, USA). A series of two-dimensional homo- and heteronuclear correlated spectra, i.e. correlated spectroscopy (COSY), heteronuclear single quantum coherence (HSQC), HSQC-total correlation spectroscopy (HSQC-TOCSY), and heteronuclear multiple bond correlation (HMBC), were obtained.

Analysis of the synthetic reaction product

Quantification of products was performed by HPAEC-PAD (Thermo Fisher Scientific, Waltham, MA, USA) on a CarboPac PA1 column (4 × 250 mm; Thermo Fisher Scientific). The mobile phase was 200 mM sodium hydroxide with a liner gradient of sodium acetate (0–250 mM) over 40 min. The flow rate was 0.8 mL/min, and sample injection volume was 10 μL. To quantify the product, 30–120 μM α-D-Glc*p*-(1↔1)-α-D-Man*p*6*P* were used as standards. The concentration of α-D-Glc*p*-(1↔1)-α-D-Man*p*6*P* standards was measured using the phenol-sulfuric acid method.[18)]

Results and discussion

Production, purification and basic properties of recombinant LlTrePP

The TrePP-encoding gene (GenBank accession no. Y18267.1 ORF3) of *L. lactis* ssp. *lactis* JCM 5805, the same strain as *L. lactis* ssp. *lactis* ATCC 19435, was obtained from the genomic DNA by PCR. The recombinant enzyme, of which the primary structure was the same as the gene product with an 11-residue C-terminal extension AAALDHHHHHH, was produced in *E. coli* and purified by Ni-chelating column chromatography. From 1.0 L of culture broth of the *E. coli* transformant, 19 mg of LlTrePP was purified. The specific activity, expressed as a reverse phosphorolytic reaction rate acting on 10 mM β-Glc1*P* and 10 mM Glc6*P* at 30°C, was 253 U/mg. SDS-PAGE analysis indicated that the molecular mass of LlTrePP was 90 kDa (Fig. 1A), which coincided well with the theoretical mass (88,387 Da). The mass determined by BN-PAGE was 184 kDa (Fig. 1B). Although native LlTrePP was reported to be a monomer in Native PAGE,[1)] these results indicate that LlTrePP is a

homodimeric protein in non-denaturing conditions. Most GH65 enzymes analyzed are homodimers in solution, both native[19–21] and recombinant enzymes.[19–24] The reaction velocity of phosphorolysis of 0.670 mM Tre6*P* in the presence of 0.1 M inorganic phosphate at 35°C was 36.5 μmol/min/mg protein, which was close to the reported velocity of the native enzyme, 32.4 μmol/min/mg, in the same reaction conditions.

Because there is no N-terminal signal peptide in LlTrePP, LlTrePP is thought to be an intracellular enzyme[1]. For the phosphorolysis of Tre6*P*, the optimum pH was 6.1, which is considered almost the same as previous study[1] (Fig. 2A). This optimum pH close to neutral is suitable for the intracellular decomposition of Tre6*P*, since the intracellular pH of bacteria is generally neutral.[25] However, the optimum pH of LlTrePP for the synthesis of Tre6*P* was 4.8 (Fig. 2B), 1.3 pH units lower than that for phosphorolysis. Some GH65 enzymes exhibit similar optimum pHs for both phosphorolysis and reverse phosphorolysis.[20,23,26,27] However, *Lactobacillus acidophilus* maltose phosphorylase had a considerably lower optimum pH for the synthetic reaction (pH 3.4) than for phosphorolysis (pH 6.2),[22] as also observed here for LlTrePP. A lower optimum pH for reverse phosphorolysis was also observed for some trehalose phosphorylases.[21,24,28,29] On the basis of the proposed reaction mechanism for inverting phosphorylases,[3,4] the catalytic acidic residue, acting as a general acid in the phosphorolysis, should be dissociated in the reverse phosphorolysis to act as a general base catalyst that takes the proton from the hydroxy group of the acceptor substrate. Lower pH is, however, unfavorable for the dissociation of the catalytic residue. The fact that LlTrePP showed a lower optimum pH for reverse phosphorolysis might be attributed to some amino acid residue near the active-site and the state of phosphate released.

Activity of LlTrePP was retained after incubation at pH 4.5–9.0 at 4°C for 24 h (Fig. 2C) or pH 5.0–8.0 at 30°C for 15 min (Fig. 2D). The optimum pH (pH 4.8) for Tre6*P* synthesis was outside the stable pH range; thus, further characterization was carried out at pH 5.5. The optimum temperature of LlTrePP was 40°C in a 10-min reaction, and LlTrePP retained ≥90% activity up to 30°C (Fig. 3).

Kinetic analysis of the reverse phosphorolysis of LlTrePP

Initial reaction velocities of LlTrePP were measured for the reverse phosphorolysis at various concentrations of β-Glc1*P* and Glc6*P*. Double reciprocal plots of 1/[β-Glc1*P*] versus 1/*v* at various concentrations of Glc6*P* were linear and crossed at a single point (Fig. 4). Thus, this enzyme catalyzes the reactions through a sequential bi bi mechanism involving the formation of a ternary complex, as observed in the phosphorolysis of Tre6*P*.[1)] Kinetic parameters for the reverse phosphorolysis were: k_{cat} 759±108 s^{-1}, $K_{m\ Glc6P}$ 1.85±0.918 mM, $K_{m\ \beta\text{-}Glc1P}$ 0.413±0.0632 mM, and $K_{i\ \beta\text{-}Glc1P}$ 0.519±0.499 mM.

The acceptor specificity of LlTrePP was investigated by measuring its activities towards various acceptors (10 mM) in the presence of 10 mM β-Glc1*P*. LlTrePP showed such strict acceptor specificity that Glc6*P* was the almost the only acceptor substrate. Aside from Glc6*P*, among the tested compounds, only Man6*P* served as an acceptor substrate of LlTrePP, and it was a poor substrate (Table 1). The reaction velocity for Man6*P* was 0.134 μmol/min/mg, which was 0.053% of the value for Glc6*P*. Some GH65 enzymes act on D-xylose and even L-fucose as acceptors, i.e. the recognition of the hydroxymethylene group of D-glucose as acceptor substrate is not strict.[20,21,22)]

However, LlTrePP did not use D-xylose, L-arabinose, or D-glucose as acceptors in reverse phosphorolysis. These results, together with Man6*P* serving as a poor substrate, suggest that the phosphate group at the C6 position is essential in the acceptor substrate, and the spatial configuration of the other hydroxy groups might be structurally important for recognition by the enzyme.

Synthesis and structural analysis of a novel disaccharide phosphate

In the synthetic reaction of LlTrePP using 100 mM Man6*P* and 100 mM β-Glc1*P*, release of inorganic phosphate was monitored (Fig. 5). At 16 h, the reaction almost reached equilibrium. The concentration of the inorganic phosphate released was 86.6 mM, and the equilibrium constant was 41.8. At 48 h, the inorganic phosphate concentration was 81.6 mM. The saccharide product concentration measured by HPAEC-PAD was 83.2 mM, and the total content was 205 μmol. The product was purified through electrodialysis, and 126 μmol product was obtained, with a yield of 51.1% based on substrate. This product was analyzed by ESI-MS and NMR. The product gave a signal at 421.08 *m*/*z* $[M]^-$. The chemical shifts of ^{1}H- and ^{13}C-NMR analyses are summarized in Table 2. Correlation peaks between C1 of a Glc residue and H1 of a Man6*P* residue, and between H1 of a Glc residue and C1 of a Man6*P* residue, were observed in the HMBC spectrum (Fig. 6), indicating that the glycosidic linkage was formed between C1 of Glc and C1 of Man6*P*. Because the $J_{H1, H2}$ value of the D-glucosyl residue was 3.7 Hz, and $J_{'C1,'H1}$ of the Man6*P* residue obtained in the HMBC spectrum was 174 Hz (around 170 Hz for α-hexopyranoses),[30] both sugar residues were linked by an α-linkage. Taken together, the reaction product was determined to be a new sugar phosphate, α-D-Glc*p*-(1↔1)-α-D-Man*p*6*P*.

This compound might have the potential to act as a signaling molecule in plants because it structurally mimics Tre6*P*. Recently, it has been reported that trehalose metabolism, including Tre6*P*, has a significant effect on plant development. Tre6*P*-deficient mutants of *A. thaliana* were defective in completing embryogenesis, resulting in embryonic lethality.[31] Sugar metabolism and amino acid flux were changed when Tre6*P* levels were altered by genetic modification.[32,33] In addition, application of plant-permeable analogues of Tre6*P* (1 mM) modulated Tre6*P* levels *in planta*, and increased resilience, and crop yield by 13–20%.[34] These analogues were chemically-modified to mask charge of phosphate. These findings imply that other Tre6*P* analogues have potential as bioactive tools. α-D-Glc*p*-(1↔1)-α-D-Man*p*6*P* might have bioactivity and be applied for further research into Tre6*P* function.

Author's contribution

Y. T. conducted the experiments and wrote the manuscript. W. S., R. I., and H. M. analyzed the data and wrote the manuscript.

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Figure legends

Fig. 1. SDS-PAGE and BN-PAGE of purified recombinant LlTrePP.

Purified LlTrePP (1 μg and 3 μg) was analyzed by SDS-PAGE (A) and BN-PAGE (B), respectively. Lane M, size makers; lane S, purified LlTrePP. The molecular masses of the standards are shown on the left. LlTrePP was estimated to be a 90 kDa- and 184 kDa-protein by SDS-PAGE and BN-PAGE, respectively.

Fig. 2. Effects of pH on activity and stability of recombinant LlTrePP.

(A) pH activity curve for the phosphorolytic activity. Glc6*P*-releasing activity was measured with 0.67 mM Tre6*P* in 200 mM potassium phosphate buffer at the indicated pHs at 35°C. (B) pH activity curve. Inorganic phosphate-releasing activity was measured with 10 mM β-Glc1*P* and 10 mM Glc6*P* as substrates at the indicated pHs at 30°C. Open circle, glycine-HCl buffer (pH 3.5); open square, sodium acetate buffer (pH 4.0–5.3); filled triangle, MES-NaOH buffer (pH 5.6–7.2); open triangle, HEPES-NaOH buffer (pH 7.2–8.2). (C) pH stability at 4°C. Residual activities after LlTrePP (6.9 μg/mL) was incubated at various pHs at 4°C for 24 h are shown. Open square, sodium acetate buffer (pH 3.0–5.0); filled triangle, MES-NaOH buffer (pH 5.0–7.0); open triangle, HEPES-NaOH buffer (pH 7.0–8.0); filled square, Tricine-NaOH buffer (pH 8.0–8.7); and open diamond, glycine-NaOH buffer (pH 8.7–11.0). (D) pH stability at 30°C. LlTrePP (0.46 μg/mL) was incubated at various pHs at 30°C for 15 min. Open square, sodium acetate buffer (pH 4.1–4.7); open diamond, sodium citrate buffer (pH 4.5–6.3); filled triangle, MES-NaOH buffer (pH 5.7–6.2); open triangle, HEPES-NaOH

buffer (pH 6.5–7.5); filled square, Bicine-NaOH buffer (pH 7.9–8.7); and open circle, *N*-cyclohexyl-2-aminoethanesulfonic acid-NaOH buffer (pH 8.4–9.2). The data are presented as the means of triplicate experiments with standard deviations.

Fig. 3. Effects of temperature on activity and stability of recombinant LlTrePP.
Open circles show activities at the indicated temperatures at pH 5.5 in a 10-min reaction. Filled circles show residual activity after incubation at the indicated temperatures at pH 5.5 for 15 min. The data are presented as the means of triplicate experiments with standard deviations.

Fig. 4. Double reciprocal plots for reverse phosphorolysis of LlTrePP.
Double reciprocal plots for synthesis of Tre6*P*. The concentrations of Glc6*P* were: open circle 3.7 mM, filled circle 5.5 mM, open square 7.2 mM, filled square 9.2 mM, and open triangle 11 mM. The lines were obtained by fitting the data to an equation for a sequential bi bi mechanism. The data are presented as the means of triplicate experiments with standard deviations.

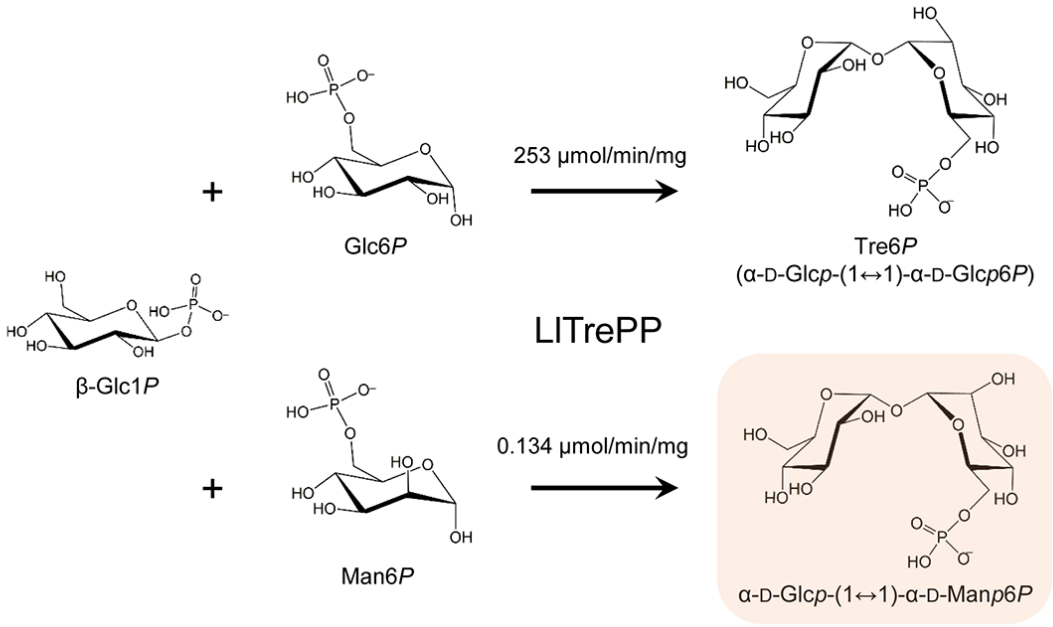
Fig. 5. Time course of product formation by LlTrePP from 100 mM Man6*P* and 100 mM β-Glc1*P*.
Liberated inorganic phosphate, which was produced at an equimolar concentration with the product in reverse phosphorolysis by LlTrePP, was measured. The data are presented as the means of triplicate experiments with standard deviations.

Fig. 6. HMBC analysis of the product from β-Glc1*P* and Man6*P* in the reverse phosphorolysis.

The signals for the anomeric protons and carbons of the product in the HMBC analysis are shown. Dotted lines indicate correlations between Glc H1 and Man6*P* C1, and between Man6*P* H1 and Glc C1. The coupling constants of Glc H1 $J_{H1, H2}$ and Man6*P* H1 $J_{H1, C1}$ were 3.7 Hz and 174 Hz, respectively.

Supplementary Fig. 1. HSQC-spectrum of α-D-Glc*p*-(1↔1)-α-D-Man*p*6*P*.

Two-dimensional homo- and heteronuclear correlated spectra (HSQC) of a novel sugar phosphate, α-D-Glc*p*-(1↔1)-α-D-Man*p*6*P*. Each number shown after the residue indicates the number of carbon and proton. The dotted lines show the correlation between carbon and proton.



Graphical abstract

Table 1. Synthetic activity toward various acceptor substrates.

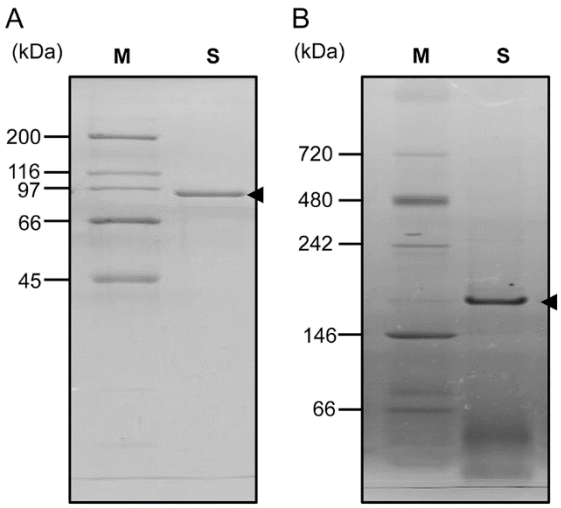
Substrate	Velocity (μmol/min/mg)	Relative activity (%)
Glucose 6-phosphate*	253	100
Mannose 6-phosphate*	0.134	0.0528
Other substrates*	<0.0300	<0.0119
None	<0.0300	<0.0119

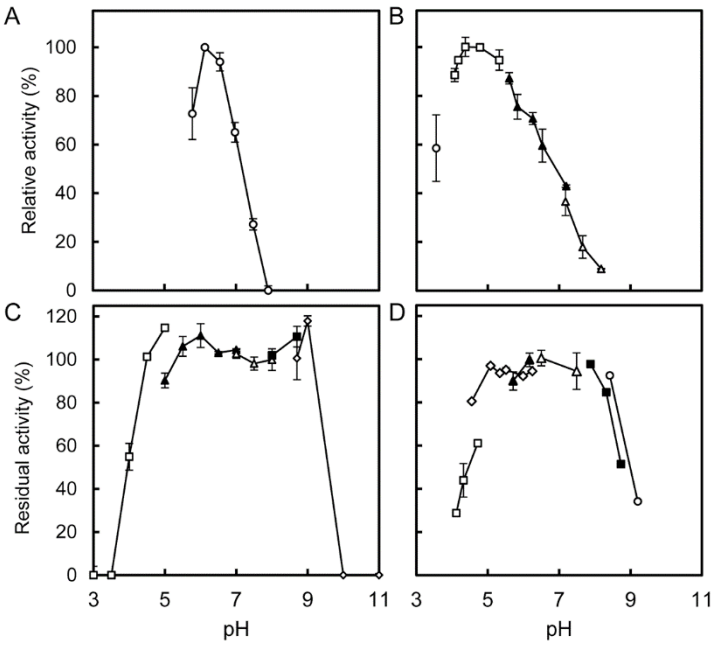
As donor substrate, 10 mM β-Glc1*P* was used.

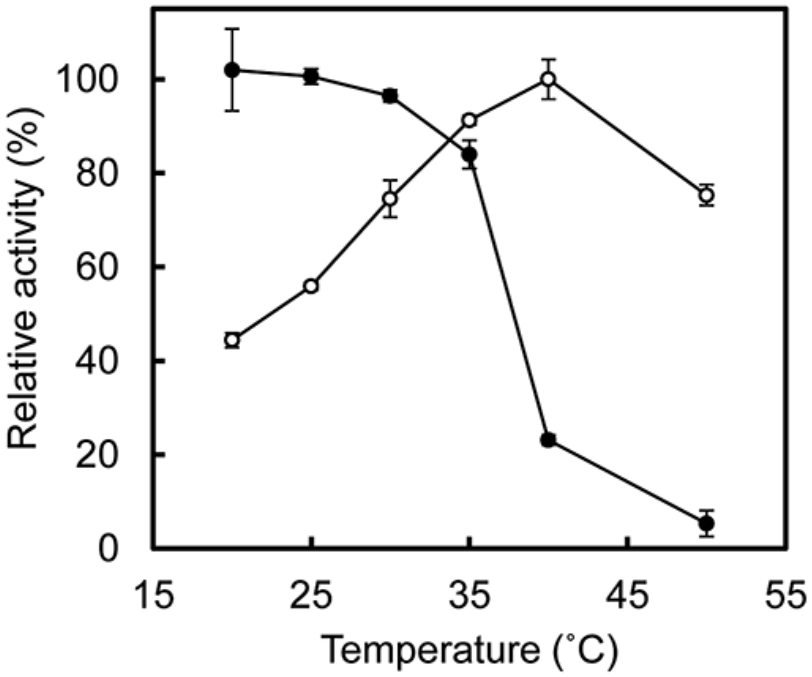
*10 mM

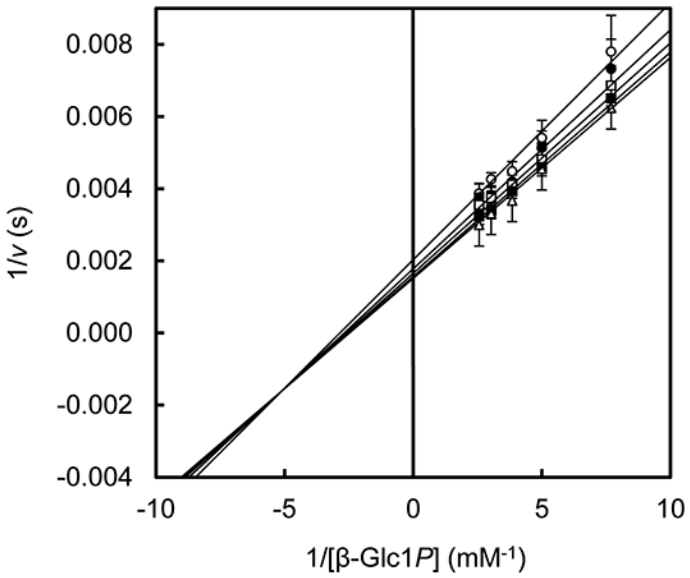
Table 2. Chemical shifts of α-D-Glc*p*-(1↔1)-α-D-Man*p*6*P* in ^{1}H and ^{13}C NMR spectra.

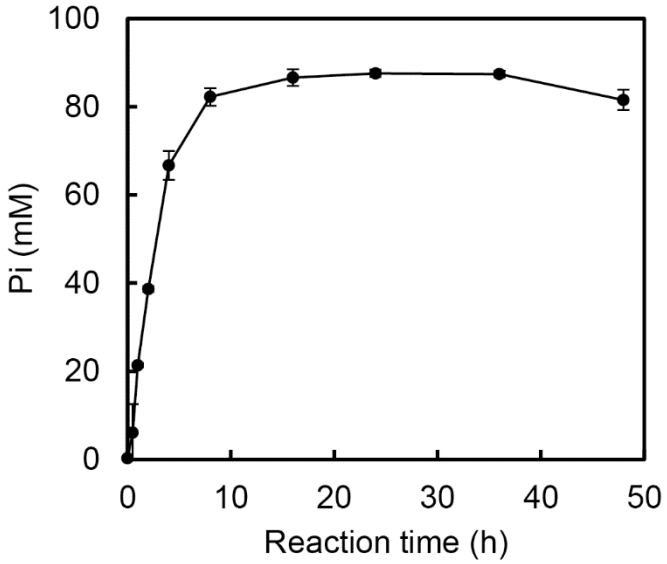
Residue	Number	δC (ppm)	δH (ppm)		*J* (Hz)
α-Glc	1	94.3	5.11	d	3.70
	2	71.6	3.55	m	
	3	73.3	3.65	m	
	4	70.4	3.35	t	9.55
	5	73.3	3.59	m	
	6	61.3	3.67	m	
			3.76	m	
α-Man*p*6*P*	1	96.0	5.03	d	1.10
	2	70.9	3.91	m	
	3	70.8	3.88	m	
	4	66.9	3.78	m	
	5	73.5	3.76	m	
	6	63.5	3.87	m	
			3.92	m	

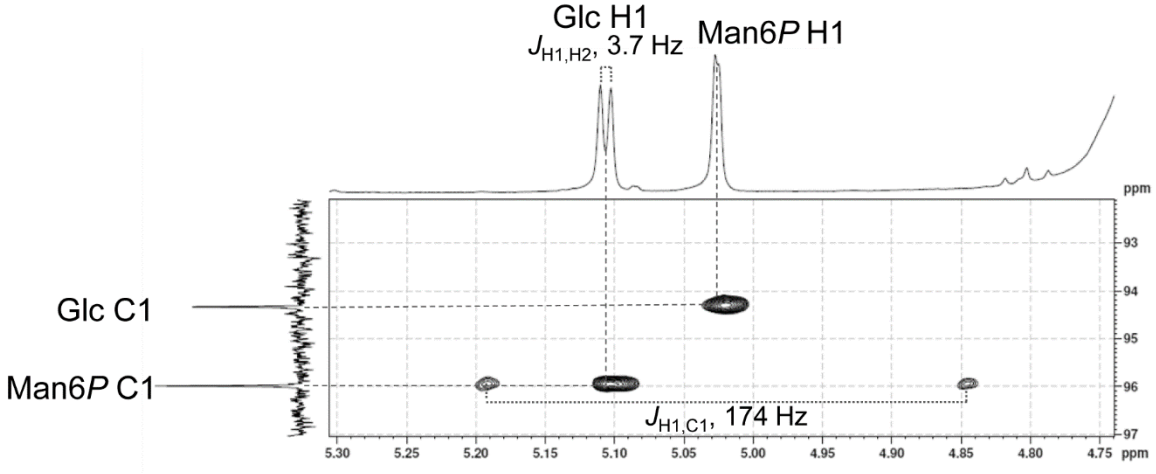

A
(kDa)
M
S
200
116
97
66
45
B
(kDa)
M
S
720
480
242
146
66

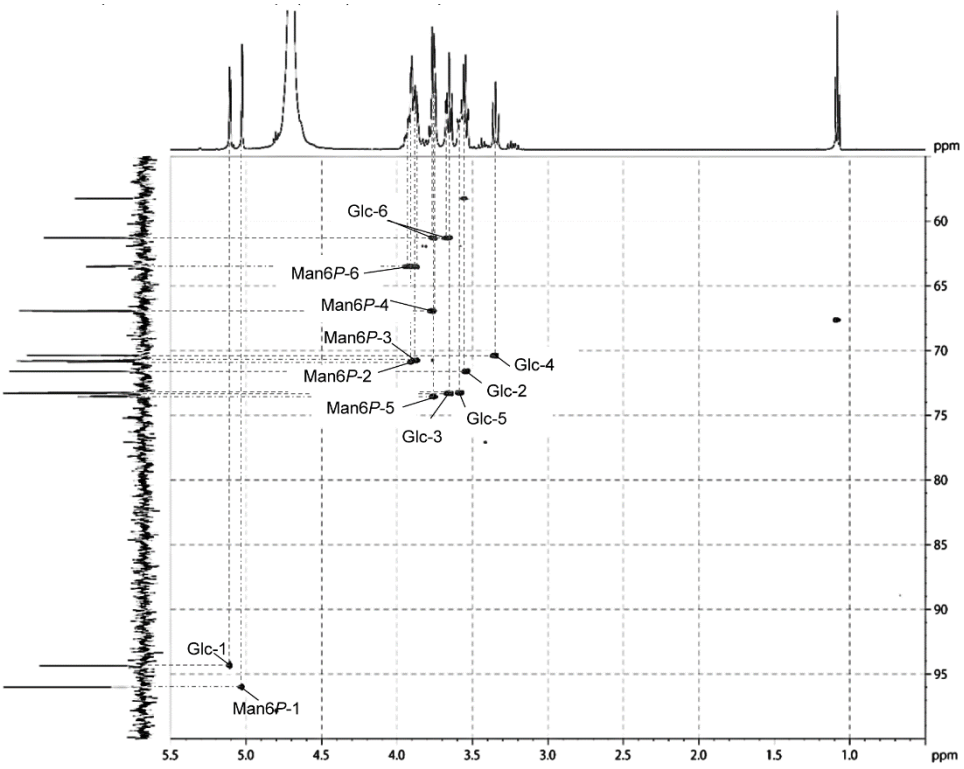

A
B
C
D
Relative activity (%)
Residual activity (%)
0
20
40
60
80
100
120
3
5
7
9
11
pH
pH


Relative activity (%)
100
80
60
40
20
0
15
25
35
45
55
Temperature (˚C)


0.008
0.006
0.004
0.002
0.000
-0.002
-0.004
1/v (s)
-10
-5
0
5
10
1/[β-Glc1P] (mM-1)


100
80
60
40
20
0
Pi (mM)
0
10
20
30
40
50
Reaction time (h)


Glc H1
$J_{H1,H2}$, 3.7 Hz
Man6*P* H1
Glc C1
Man6*P* C1
$J_{H1,C1}$, 174 Hz
ppm
93
94
95
96
97
5.30
5.25
5.20
5.15
5.10
5.05
5.00
4.95
4.90
4.85
4.80
4.75
ppm



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