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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 (Tre6P) with**

inversion of the anomeric configuration to produce  $\beta$ -D-glucose 1-phosphate ( $\beta$ -Glc1P) and D-glucose 6-phosphate (Glc6P). TrePP in *Lactococcus lactis* ssp. *lactis* (LITrePP) is, alongside the phosphotransferase system, involved in the metabolism of trehalose. In this study, recombinant LITrePP 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 Tre6P proceeded through a sequential bi bi mechanism involving the formation of a ternary complex of the enzyme,  $\beta$ -Glc1P, and Glc6P. Suitable acceptor substrates were Glc6P, and, at a low level, D-mannose 6-phosphate (Man6P). From  $\beta$ -Glc1P and Man6P, a novel sugar phosphate,  $\alpha$ -D-Glcp-(1 $\leftrightarrow$ 1)- $\alpha$ -D-Manp6P, was synthesized with 51% yield.

*Abbreviations:* BN-PAGE, blue native polyacrylamide gel electrophoresis; COSY, correlated spectroscopy; ESI, electrospray ionization; GH, glycoside hydrolase family;  $\beta$ -Glc1P,  $\beta$ -D-glucose 1-phosphate; Glc6P, 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; LITrePP, trehalose 6-phosphate phosphorylase from *Lactococcus lactis* ssp. *lactis*; Man6P, D-mannose 6-phosphate; MES, 2-morpholinoethanesulfonic acid; NMR, nuclear magnetic resonance; TrePP, trehalose 6-phosphate phosphorylase; Tre6P, trehalose 6-phosphate.

## 1 Introduction

2 Trehalose 6-phosphate phosphorylase (EC 2.4.1.216, TrePP) catalyzes the phosphorolysis  
3 of trehalose 6-phosphate (Tre6P:  $\alpha$ -D-Glcp-(1 $\leftrightarrow$ 1)- $\alpha$ -D-Glcp6P) to produce  $\beta$ -glucose 1-phosphate  
4 ( $\beta$ -Glc1P) and glucose 6-phosphate (Glc6P).<sup>1)</sup> In *Lactococcus lactis* ssp. *lactis*, TrePP (LTrePP) is  
5 involved in trehalose metabolism. Trehalose is taken up into cells and phosphorylated to Tre6P  
6 through the phosphotransferase system. Tre6P is then phosphorolyzed by LTrePP into  $\beta$ -Glc1P and  
7 Glc6P, which are further metabolized through glycolysis. TrePP seems to be exclusively responsible  
8 for catabolizing Tre6P in *L. lactis*, because of the absence of genes coding for any other trehalose-  
9 metabolizing enzymes such as trehalose-phosphatase (EC 3.1.3.12),  $\alpha,\alpha$ -trehalase (EC 3.2.1.28), or  
10  $\alpha,\alpha$ -phosphotrehalase (EC 3.2.1.93).

11 Glycoside phosphorylases are classified into glycoside hydrolase families (GHs) 13, 65,  
12 94, 112, and 130, and glycosyltransferase families 4 and 35 in the Carbohydrate-Active Enzymes  
13 database (<http://www.cazy.org/>) based on amino acid sequence similarity.<sup>2)</sup> TrePP is found only in  
14 GH65. GH65 is mainly composed of  $\alpha$ -glucoside phosphorylases including TrePP, maltose  
15 phosphorylase (EC 2.4.1.8),  $\alpha,\alpha$ -trehalose phosphorylase (EC 2.4.1.64), kojibiose phosphorylase (EC  
16 2.4.1.230), nigerose phosphorylase (EC 2.4.1.279), 3-O- $\alpha$ -glucopyranosyl-L-rhamnose  
17 phosphorylase (EC 2.4.1.282), 1,2- $\alpha$ -glucosylglycerol phosphorylase (EC 2.4.1.332), and 1,3- $\alpha$ -  
18 oligoglucan phosphorylase (EC 2.4.1.334). GH65 enzymes have an ( $\alpha/\alpha$ )<sub>6</sub>-barrel fold catalytic  
19 domain, similar to those of GH15 and GH94 enzymes.<sup>3,4)</sup> These GH families form clan GH-L based  
20 on the similarity of their tertiary structures.<sup>2)</sup>

21 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  $\beta$ -Glc1P. This  $\beta$ -Glc1P was used as glycosyl donor substrate for reverse phosphorolysis of maltose phosphorylase, and disaccharides such as  $\alpha$ -Glc $p$ -(1 $\rightarrow$ 4)-GlcNAc and  $\alpha$ -Glc $p$ -(1 $\rightarrow$ 4)-L-Fuc $p$  were synthesized in the presence of high concentrations of GlcNAc and L-Fuc respectively.<sup>6)</sup> 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<sup>5)</sup>. GH65  $\alpha$ -glucoside phosphorylases are useful tools to produce  $\alpha$ -glucosyl residue-containing saccharides. Among them, TrePP is the sole enzyme that acts on sugar phosphate as the acceptor and produces Tre6P in the reverse phosphorolysis.<sup>1)</sup> Tre6P has been receiving increasing attention as a signaling molecule in plants. A mutant *Arabidopsis thaliana* that overaccumulated Tre6P showed early flowering<sup>7)</sup> and perturbed sugar metabolism.<sup>8)</sup> Tre6P synthesis from  $\beta$ -Glc1P and Glc6P has been achieved using reverse phosphorolysis by a recombinant LITrePP enzyme.<sup>1)</sup> Herein, we describe in detail the acceptor substrate preference of LITrePP, to investigate the possibility of synthesis of other sugars. D-Mannose 6-phosphate (Man6P), the sole acceptor substrate of LITrePP except for Glc6P, was used as an acceptor substrate, and a Tre6P analogue,  $\alpha$ -D-Glc $p$ -(1 $\leftrightarrow$ 1)- $\alpha$ -D-Man $p$ 6P, was synthesized in

good yield.

## Materials and methods

### Preparation of $\beta$ -glucose 1-phosphate

$\beta$ -Glc1P 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<sup>9)</sup> was incubated at 37°C for 96 h. The  $\beta$ -Glc1P produced was purified by anion exchange column chromatography with Amberjet 4400 (3.6 cm i.d.  $\times$  90 cm, acetate form; Organo, Tokyo, Japan) according to a method previously described.<sup>10)</sup>  $\beta$ -Glc1P 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<sup>+</sup> type; Wako Pure Chemical Industries, Osaka, Japan) to remove the counter ions of  $\beta$ -Glc1P. The pH was adjusted to 8.0 with KOH, and  $\beta$ -Glc1P was precipitated in 80% ethanol.  $\beta$ -Glc1P was filtered, dried *in vacuo*, and stored at  $-20^{\circ}\text{C}$ . From 0.6 mol maltose, 0.073 mol  $\beta$ -Glc1P 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 LITrePP

The LITrePP 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 LITrePP**

A transformant of *Escherichia coli* BL21 (DE3) harboring the expression plasmid for  
LITrePP 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 ( $10,000 \times g$ , 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 ( $6,000 \times g$ , 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.  $\times$  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,<sup>11)</sup> 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<sup>12)</sup> and the UV method,<sup>13)</sup> 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).<sup>14)</sup>

#### **Blue native PAGE**

The molecular mass of LITrePP without protein denaturation was determined by blue native PAGE (BN-PAGE).<sup>15)</sup> 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 Tre6P from  $\beta$ -Glc1P and Glc6P by LITrePP was determined by measuring the liberation of inorganic phosphate. A reaction mixture of 20  $\mu$ L, composed of an appropriate concentration of enzyme, 105 mM MES-NaOH (pH 5.5), 10 mM  $\beta$ -Glc1P, 10 mM Glc6P (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.<sup>16)</sup> One unit of enzyme activity was defined as the amount of enzyme producing 1  $\mu\text{mol}$  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 Tre6P. Phosphorolytic activity of LITrePP was measured as described,<sup>1)</sup> but Thio-NADP<sup>+</sup> (Nacalai Tesque) was used instead of NADP<sup>+</sup>.

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 Tre6P 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, LITrePP (0.14  $\mu\text{g/mL}$ ) was incubated at various temperatures for 15 min at pH 5.5. To determine pH stability, LITrePP (6.9 and 0.46  $\mu\text{g/mL}$ ) 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  $\geq 90\%$  of the original activity were regarded as the stable ranges.

The kinetic parameters for the reverse phosphorolysis of Tre6P were calculated from the initial velocities toward various concentrations of  $\beta$ -Glc1P (0.13, 0.20, 0.26, 0.33, and 0.39 mM) and Glc6P (3.7, 5.5, 7.2, 9.2, and 11 mM) by fitting to the equation for a sequential bi bi mechanism.<sup>17)</sup> 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  $\mu\text{g/mL}$ , and various compounds in place of Glc6P 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  $\alpha$ -D-glucoside (Wako Pure Chemical Industries), methyl  $\beta$ -D-glucoside (Wako Pure Chemical Industries), D-glucitol (Nacalai Tesque), D-glucosamine (Tokyo Chemical Industry, Tokyo, Japan), Man6P (Sigma, St. Louis, MO, USA), and D-fructose 6-phosphate (Sigma).

## Structural analysis of synthesized oligosaccharide

A reaction mixture composed of 100 mM  $\beta$ -Glc1P, 100 mM Man6P, 100 U/mL LITrePP, 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  $\mu$ L 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  $\text{NaNO}_3$  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  $\text{H}_2\text{O}$  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  $\text{D}_2\text{O}$  (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  $\alpha$ -D-Glcp-(1↔1)- $\alpha$ -D-Manp6P were used as standards. The concentration of  $\alpha$ -D-Glcp-(1↔1)- $\alpha$ -D-Manp6P standards was measured using the phenol-sulfuric acid method.<sup>18)</sup>

## Results and discussion

### Production, purification and basic properties of recombinant LITrePP

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 LITrePP was purified. The specific activity, expressed as a reverse phosphorolytic reaction rate acting on 10 mM  $\beta$ -Glc1P and 10 mM Glc6P at 30°C, was 253 U/mg. SDS-PAGE analysis indicated that the molecular mass of LITrePP 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 LITrePP was reported to be a monomer in Native PAGE,<sup>1)</sup> these results indicate that LITrePP is a

homodimeric protein in non-denaturing conditions. Most GH65 enzymes analyzed are homodimers in solution, both native<sup>19–21)</sup> and recombinant enzymes.<sup>19–24)</sup> The reaction velocity of phosphorolysis of 0.670 mM Tre6P in the presence of 0.1 M inorganic phosphate at 35°C was 36.5  $\mu\text{mol}/\text{min}/\text{mg}$  protein, which was close to the reported velocity of the native enzyme, 32.4  $\mu\text{mol}/\text{min}/\text{mg}$ , in the same reaction conditions.

Because there is no N-terminal signal peptide in LITrePP, LITrePP is thought to be an intracellular enzyme<sup>1)</sup>. For the phosphorolysis of Tre6P, the optimum pH was 6.1, which is considered almost the same as previous study<sup>1)</sup> (Fig. 2A). This optimum pH close to neutral is suitable for the intracellular decomposition of Tre6P, since the intracellular pH of bacteria is generally neutral.<sup>25)</sup> However, the optimum pH of LITrePP for the synthesis of Tre6P 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.<sup>20,23,26,27)</sup> However, *Lactobacillus acidophilus* maltose phosphorylase had a considerably lower optimum pH for the synthetic reaction (pH 3.4) than for phosphorolysis (pH 6.2),<sup>22)</sup> as also observed here for LITrePP. A lower optimum pH for reverse phosphorolysis was also observed for some trehalose phosphorylases.<sup>21,24,28,29)</sup> On the basis of the proposed reaction mechanism for inverting phosphorylases,<sup>3,4)</sup> 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 LITrePP 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 LITrePP 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 Tre6P synthesis was outside the stable pH range; thus, further characterization was carried out at pH 5.5. The optimum temperature of LITrePP was 40°C in a 10-min reaction, and LITrePP retained  $\geq 90\%$  activity up to 30°C (Fig. 3).

#### Kinetic analysis of the reverse phosphorolysis of LITrePP

Initial reaction velocities of LITrePP were measured for the reverse phosphorolysis at various concentrations of  $\beta$ -Glc1P and Glc6P. Double reciprocal plots of  $1/[\beta\text{-Glc1P}]$  versus  $1/v$  at various concentrations of Glc6P 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 Tre6P.<sup>1)</sup> Kinetic parameters for the reverse phosphorolysis were:  $k_{\text{cat}}$   $759 \pm 108 \text{ s}^{-1}$ ,  $K_{\text{m Glc6P}}$   $1.85 \pm 0.918 \text{ mM}$ ,  $K_{\text{m } \beta\text{-Glc1P}}$   $0.413 \pm 0.0632 \text{ mM}$ , and  $K_{\text{i } \beta\text{-Glc1P}}$   $0.519 \pm 0.499 \text{ mM}$ .

The acceptor specificity of LITrePP was investigated by measuring its activities towards various acceptors (10 mM) in the presence of 10 mM  $\beta$ -Glc1P. LITrePP showed such strict acceptor specificity that Glc6P was the almost the only acceptor substrate. Aside from Glc6P, among the tested compounds, only Man6P served as an acceptor substrate of LITrePP, and it was a poor substrate (Table 1). The reaction velocity for Man6P was  $0.134 \text{ } \mu\text{mol/min/mg}$ , which was 0.053% of the value for Glc6P. 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.<sup>20,21,22)</sup>

However, LITrePP did not use D-xylose, L-arabinose, or D-glucose as acceptors in reverse phosphorolysis. These results, together with Man6P 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 LITrePP using 100 mM Man6P and 100 mM  $\beta$ -Glc1P, 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  $\mu$ mol. The product was purified through electrodialysis, and 126  $\mu$ mol 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  $^1H$ - and  $^{13}C$ -NMR analyses are summarized in Table 2. Correlation peaks between C1 of a Glc residue and H1 of a Man6P residue, and between H1 of a Glc residue and C1 of a Man6P residue, were observed in the HMBC spectrum (Fig. 6), indicating that the glycosidic linkage was formed between C1 of Glc and C1 of Man6P. Because the  $J_{H1, H2}$  value of the D-glucosyl residue was 3.7 Hz, and  $J_{C1, 'H1}$  of the Man6P residue obtained in the HMBC spectrum was 174 Hz (around 170 Hz for  $\alpha$ -hexopyranoses),<sup>30)</sup> both sugar residues were linked by an  $\alpha$ -linkage. Taken together, the reaction product was determined to be a new sugar phosphate,  $\alpha$ -D-Glcp-(1 $\leftrightarrow$ 1)- $\alpha$ -D-Manp6P.

1           This compound might have the potential to act as a signaling molecule in plants because it  
2   structurally mimics Tre6P. Recently, it has been reported that trehalose metabolism, including  
3   Tre6P, has a significant effect on plant development. Tre6P-deficient mutants of *A. thaliana* were  
4   defective in completing embryogenesis, resulting in embryonic lethality.<sup>31)</sup> Sugar metabolism and  
5   amino acid flux were changed when Tre6P levels were altered by genetic modification.<sup>32,33)</sup> In  
6   addition, application of plant-permeable analogues of Tre6P (1 mM) modulated Tre6P levels *in*  
7   *planta*, and increased resilience, and crop yield by 13–20%.<sup>34)</sup> These analogues were chemically-  
8   modified to mask charge of phosphate. These findings imply that other Tre6P analogues have  
9   potential as bioactive tools.  $\alpha$ -D-Glcp-(1 $\leftrightarrow$ 1)- $\alpha$ -D-Manp6P might have bioactivity and be applied for  
10   further research into Tre6P function.

## 12   **Author's contribution**

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

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## Figure legends

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

Purified LITrePP (1  $\mu$ g and 3  $\mu$ g) was analyzed by SDS-PAGE (A) and BN-PAGE (B), respectively. Lane M, size makers; lane S, purified LITrePP. The molecular masses of the standards are shown on the left. LITrePP 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 LITrePP.

(A) pH activity curve for the phosphorolytic activity. Glc6P-releasing activity was measured with 0.67 mM Tre6P 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  $\beta$ -Glc1P and 10 mM Glc6P 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 LITrePP (6.9  $\mu$ g/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. LITrePP (0.46  $\mu$ g/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 LITrePP.

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 LITrePP.

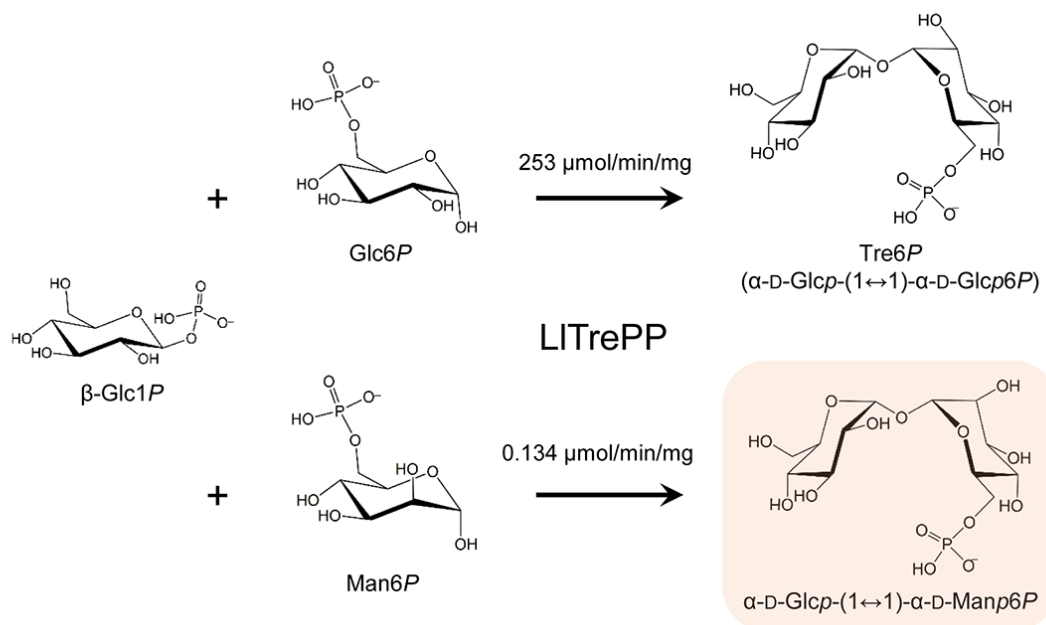
Double reciprocal plots for synthesis of Tre6P. The concentrations of Glc6P were: open circle 3.7 mM, filled circle 5.5 mM, open square 7.2 mM, filled square 9.2 mM, and open triangle 11 mM. The lines were obtained by fitting the data to an equation for a sequential bi bi mechanism. The data are presented as the means of triplicate experiments with standard deviations.

Fig. 5. Time course of product formation by LITrePP from 100 mM Man6P and 100 mM  $\beta$ -Glc1P.

Liberated inorganic phosphate, which was produced at an equimolar concentration with the product in reverse phosphorolysis by LITrePP, was measured. The data are presented as the means of triplicate experiments with standard deviations.

Fig. 6. HMBC analysis of the product from  $\beta$ -Glc1P and Man6P in the reverse phosphorolysis.

1 The signals for the anomeric protons and carbons of the product in the HMBC analysis are shown.  
2 Dotted lines indicate correlations between Glc H1 and Man6P C1, and between Man6P H1 and Glc  
3 C1. The coupling constants of Glc H1  $J_{H1, H2}$  and Man6P H1  $J_{H1, C1}$  were 3.7 Hz and 174 Hz,  
4 respectively.  
5  
6 Supplementary Fig. 1. HSQC-spectrum of  $\alpha$ -D-Glcp-(1 $\leftrightarrow$ 1)- $\alpha$ -D-Manp6P.  
7 Two-dimensional homo- and heteronuclear correlated spectra (HSQC) of a novel sugar phosphate,  $\alpha$ -  
8 D-Glcp-(1 $\leftrightarrow$ 1)- $\alpha$ -D-Manp6P. Each number shown after the residue indicates the number of carbon  
9 and proton. The dotted lines show the correlation between carbon and proton.



Graphical abstract

**Table 1.** Synthetic activity toward various acceptor substrates.

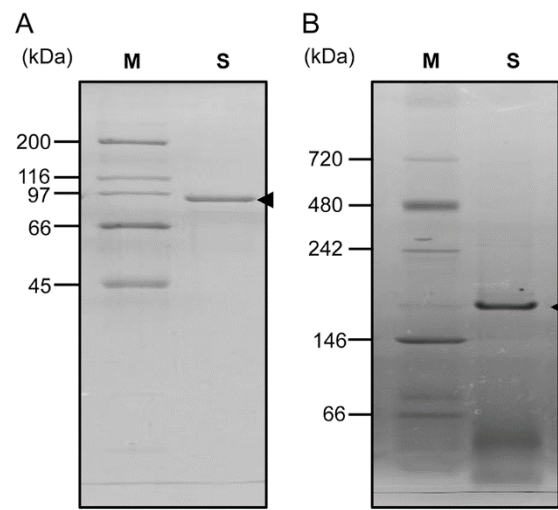
| Substrate            | Velocity<br>( $\mu\text{mol}/\text{min}/\text{mg}$ ) | Relative activity<br>(%) |
|----------------------|------------------------------------------------------|--------------------------|
| Glucose 6-phosphate* | 253                                                  | 100                      |
| Mannose 6-phosphate* | 0.134                                                | 0.0528                   |
| Other substrates*    | <0.0300                                              | <0.0119                  |
| None                 | <0.0300                                              | <0.0119                  |

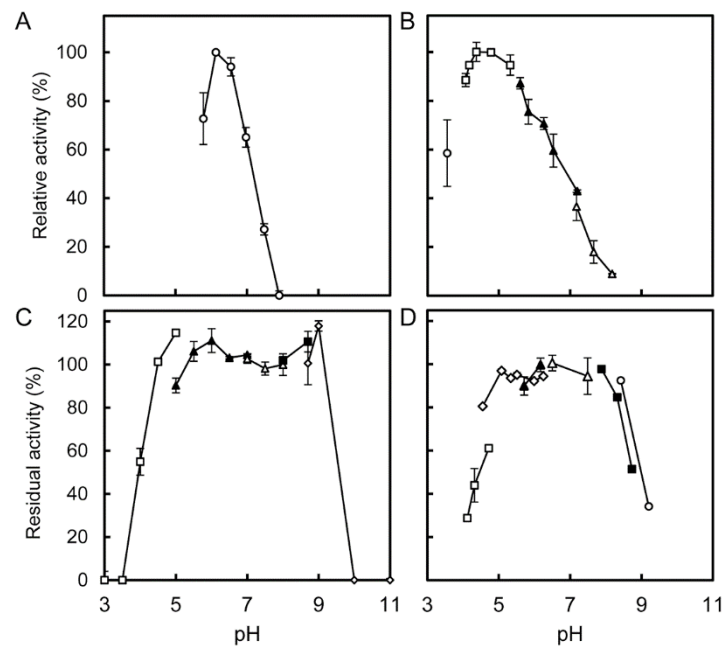
As donor substrate, 10 mM  $\beta$ -Glc1P was used.

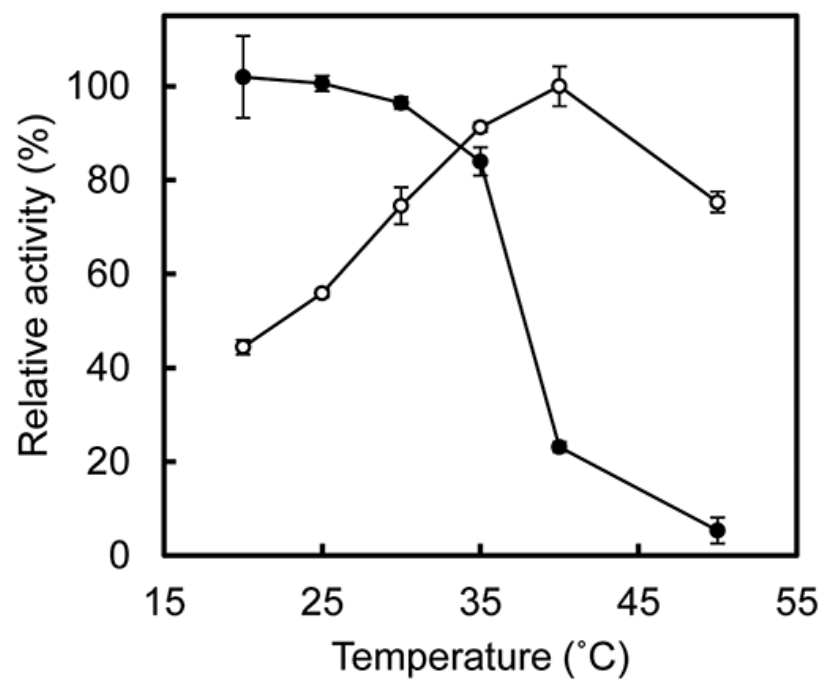
\*10 mM

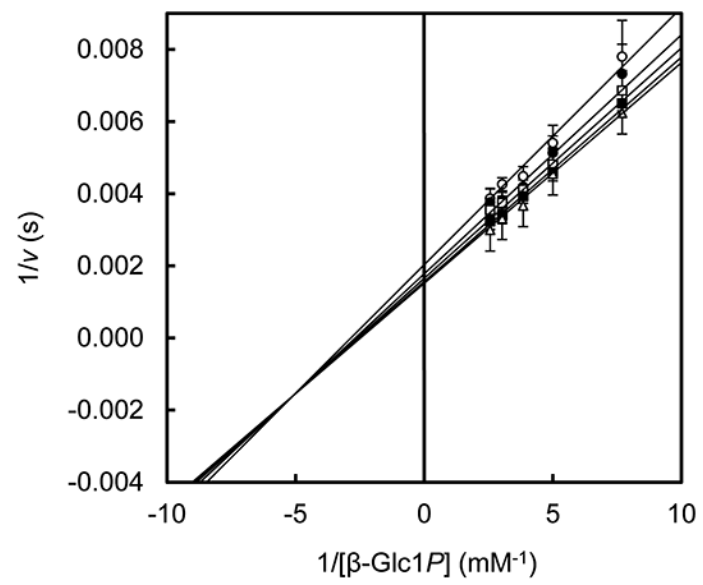
**Table 2.** Chemical shifts of  $\alpha$ -D-Glcp-(1 $\leftrightarrow$ 1)- $\alpha$ -D-Manp6P in  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra.

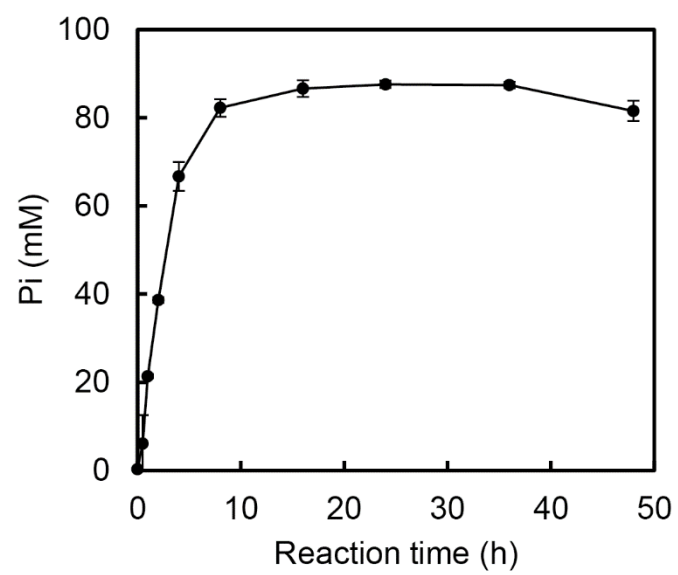
| Residue          | Number | $\delta\text{C}$ (ppm) | $\delta\text{H}$ (ppm) | $J$ (Hz) |      |
|------------------|--------|------------------------|------------------------|----------|------|
| $\alpha$ -Glc    | 1      | 94.3                   | 5.11                   | d        | 3.70 |
|                  | 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        |      |
| $\alpha$ -Manp6P |        |                        | 3.76                   | m        |      |
|                  | 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        |      |

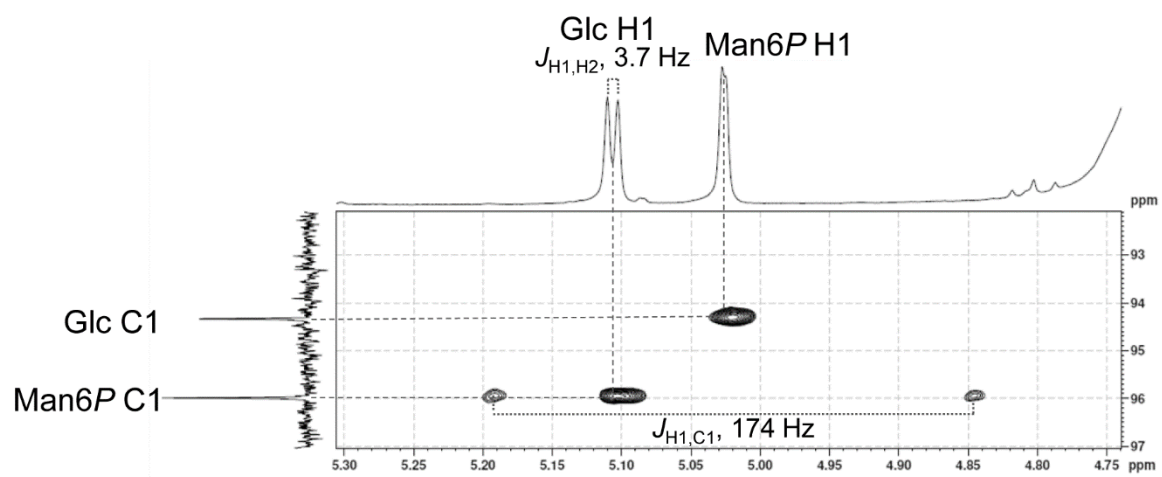


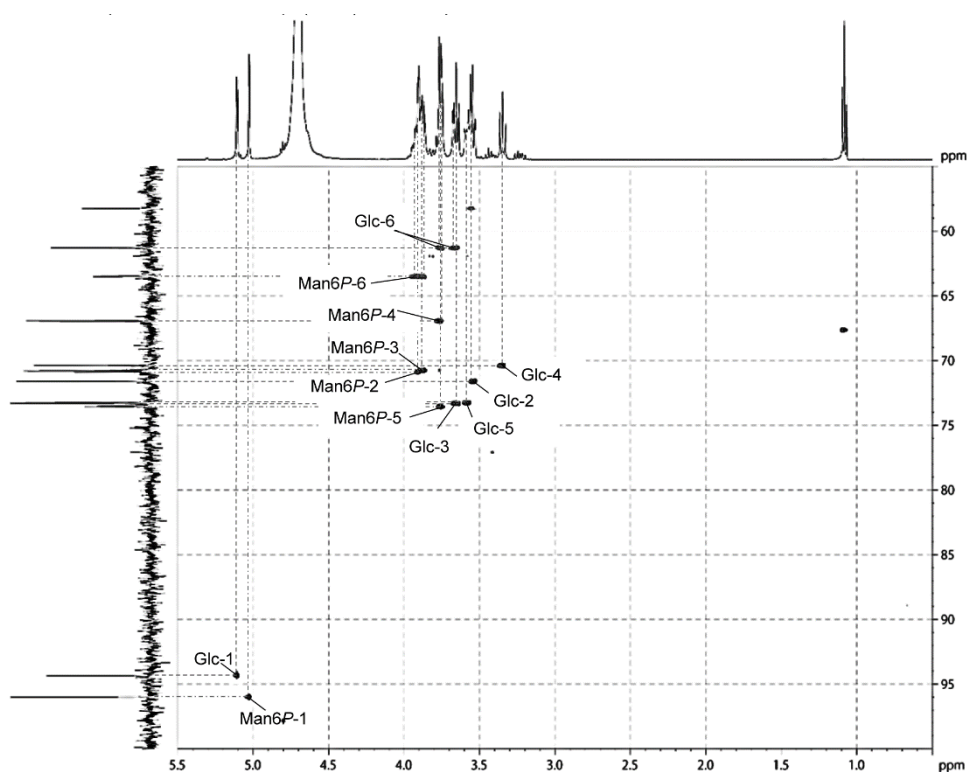












**Supplementary Fig. 1. HSQC-spectrum of  $\alpha$ -D-Glcp-(1 $\leftrightarrow$ 1)- $\alpha$ -D-Manp6P.**

Two-dimensional homo- and heteronuclear correlated spectra (HSQC) of a novel sugar phosphate,  $\alpha$ -D-Glcp-(1 $\leftrightarrow$ 1)- $\alpha$ -D-Manp6P. Each number shown after the residue indicates the number of carbon and proton. The dotted lines show the correlation between carbon and proton.