Supporting information

Engineering the Specificity of Trehalose Phosphorylase as a General Strategy for the Production of Glycosyl Phosphates

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Methods

Enzyme engineering: The recombinant expression of the His_6-tagged TP from *C. subterraneus* in *E. coli* was described earlier, together with the homology modeling and ligand docking.[1] Mutant libraries with NNK degeneracy were created and screened following established protocols.[2] The mutagenic primers and antiprimers are listed in Table S1. The PCR cycling conditions were as follows: 95°C (3 min); 5 cycles of 95°C (30 s), 58°C (1 min) and 72°C (1 min/kb megaprimer); 20 cycles of 95°C (30 s), 68°C (2 min/kb template); 68°C (2 min/kb template). Each reaction contained 50 ng of plasmid DNA, 2.5 U *Pfu*Ultra DNA polymerase (Stratagene, USA), 10x *Pfu*Ultra HF AD buffer, 0.2 mM of dNTP mix and 0.1 μM of primer pairs in a total volume of 50 μL. After the PCR reactions, the mixture was incubated overnight with DpnI (10 U) at 37°C to completely digest parental DNA. The PCR mixtures were purified with the QIAquick PCR Kit before transformation into *E. coli* XL10-Gold. Cultures were plated out on LB medium supplemented with 0.1 g/L ampicillin, after which individual colonies were inoculated in the wells of microtiter plates containing 200 μL liquid medium using a QPix2 colony-picker (Genetix, UK). After 12h incubation at 37°C and 250 rpm, the cultures were collected by centrifugation at 4500 rpm and 4°C for 10 min, and pellets were frozen at -20°C. The pellets were resuspended in 100 μL of lysis buffer composed of 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 4 mM $MgCl_2$, 50 mM NaCl and 1 mg/mL lysozyme to extract the intracellular enzymes. Lysis was carried out for 30 min at 37°C followed by centrifugation at 4500 rpm and 4°C for 10 min. The supernatants were used for enzyme screening by mixing crude cell extract with 100 mM lactotrehalose in 100 mM phosphate buffer (final concentrations) at pH 7 and 60°C. At appropriate times, samples were taken to measure glucose and galactose with the glucose oxidase-peroxidase (GOD-POD) assay[3] and the lactose/galactose assay kit from Megazyme (Ireland), respectively.

Production of β-Gal-1P: The purified enzyme variant (0.3 mg/mL) was mixed with 100 mM lactotrehalose in 500 mM phosphate buffer at pH 7 and 60°C. After 16h incubation, the remaining inorganic phosphate was selectively precipitated as struvite[4] and removed by filtration. The filtrate was then loaded onto a Dowex 1X8 column in the acetate form (Alfa Aesar), followed by washing with two bed volumes of deionized water and elution with 250 mM potassium acetate buffer at pH 4.5. Fractions containing the glycosyl phosphate were concentrated in a rotavapor at 50°C, and then precipitated by the addition of ethanol (85% final concentration). After resuspension in distilled water, the product was concentrated in vacuo to remove traces of ethanol. Its purity was analyzed by HPLC on a CarboPac PA20 column coupled to a PAD detector (Dionex). Elution was performed at room temperature with 100 mM NaOH and a gradient of NaOAc (from 100 to 175 mM in 8 min) at a flow rate of 0.5 mL min^{-1}.

Enzyme characterization: The purification of TP on a Ni-NTA resin has been described previously.[1] The kinetic parameters for Gal and Glc were determined in the presence of 30mM β-Glc-1P[5] and those for β-Gal-1P and β-Glc-1P in the presence of 100 mM Glc (in 30 mM MES buffer at pH 6 and 60°C). The release of P_i was monitored with a colorimetric assay, based on its complexation with molybdate and reduction by ascorbate.[2] Protein stability was examined by incubating the purified enzymes for 1 hour in a gradient thermocycler (Biometra), and then measuring their residual activity as described above.

Figures and Tables

Table S1 Lists of primers used for saturation mutagenesis.

Primer*	Sequence
CsTP_L649_NNK	5'-ACCGATCACCACCCACGACTCTTCT**NNK**TCTCCGGCTATCT-3'
CsTP_A693_NNK	5'-GACGGTATCCACGCT**NNK**GCTATGGCTGGTTCTTGG-3'
CsTP_W371NNK	5'-GGTTACGAAGGTCACTACTTC**NNK**GACTCTGACATCTACATCCTG-3'
CsTP_D372NNK	5'-CGGTGAAGGTTACGAAGGTCACTACTTCTAT**NNK**TCTGACATCTACATCC-3'
CsTP_A693TAA_Fw	5'-ACGGTATCCACGCTTAAGCTATGGCTGGTTCTTG-3'
CsTP_Sanch_REV	5'-TGATGCCTGGCAGTTTATGG-3'

* The mutagenic bases are in bold

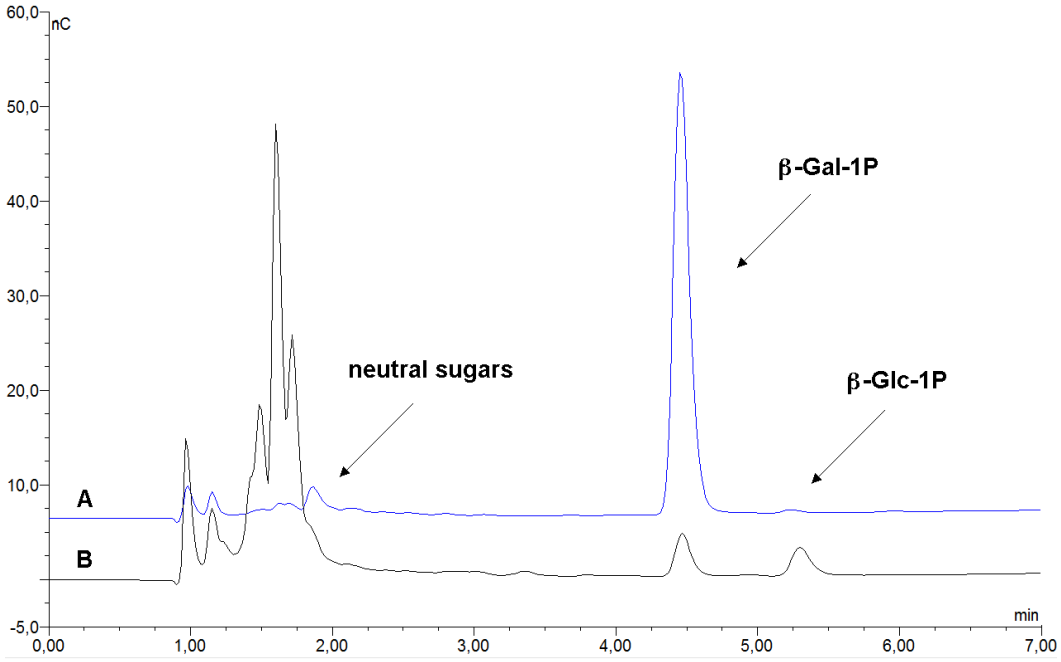

Fig. S1 HPLC chromatograms. (A) The purified product β-Gal-1P, and (B) the mixture of β-Gal-1P, β-Glc-1P and neutral sugars involved in the reaction. The sensitivity of the PAD detector for reducing sugars is about 20 times higher than that for glycosyl phosphates.

References

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