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Note

Efficient Synthesis of β-Glucose 1-Phosphate through Enzymatic Phosphorolysis and Baker's Yeast Fermentation

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Abstract: β-Glucose 1-phosphate (βGlc1P) is a donor substrate in the synthesis of various α-glucosides by glycoside phosphorylases belonging to the glycoside hydrolase family 65. This study presents an efficient synthesis of βGlc1P combining enzymatic phosphorolysis of inexpensive maltose and baker's yeast fermentation to bias the equilibrium toward maltose phosphorolysis by removing released glucose. Mass production of βGlc1P was obtained in a 2 L reaction mixture initially containing 500 mM maltose and inorganic phosphate, with a yield of 76 %. βGlc1P was isolated from the reaction mixture by crystallization after electrodialysis to obtain 181 g of βGlc1P as a bis(cyclohexylammonium) salt.

Key words: β-glucose 1-phosphate, maltose phosphorylase, baker's yeast fermentation, phosphorolysis

Glycoside phosphorylases catalyze the phosphorolysis of specific oligosaccharides at the non-reducing end to produce sugar 1-phosphate [1−4]. The energy released during the cleavage of the glycosidic bond is similar to that released during the cleavage of the sugar phosphate ester, resulting in an equilibrium between the phosphorolysis and the reverse synthetic reaction. Reversible phosphorolysis makes phosphorylases suitable catalysts for efficient synthesis of specific oligosaccharides. Various oligosaccharides have been synthesized via reverse reactions with strict regioselectivity using the corresponding sugar 1-phosphate as the donor substrate and several suitable carbohydrate acceptors [1–4]. However, utilization of the reverse reaction to synthesize oligosaccharides practically is limited by the high cost of sugar 1-phosphate.

β-Glucose 1-phosphate (βGlc1P) serves as a glucosyl donor in the reverse reaction of phosphorylases belonging to the glycoside hydrolase family (GH) 65 [2, 5]. In addition to its role as a crucial substrate for the synthesis of oligosaccharides, βGlc1P is obtained via phosphorolysis, which involves the breakdown of α-glucosides. Previous studies have reported methods for βGlc1P production from inexpensive maltose and trehalose [6, 7]. However, the equilibrium constants for phosphorolyses of maltose and trehalose are approximately 0.23 and 0.17 at pH 7.0, respectively, indicating that they predominantly favor synthesis over degradation [7, 8]. Therefore, strategies for synthesizing βGlc1P are expected to exhibit potential for enhancing the reaction yield by favoring phosphorolysis and shifting the reaction equilibrium. Phosphorolysis equilibrium would be shifted in favor of phosphorolysis by removing the coproduct from the reaction system.

Here, we developed an efficient production system combining enzymatic phosphorolysis and baker's yeast (*Saccharomyces cerevisiae*) fermentation to generate βGlc1P practically from inexpensive maltose. Baker's yeast, a single-celled fungus commonly used in baking, was employed in this system to remove glucose as a coproduct. It is often used in purification steps to remove coproducts such as mono- and di-saccharides in carbohydrate preparations containing unfermentable product [7]. Glucose and maltose are completely removed by the corresponding transporters and metabolized in yeast [9, 10]. However, in the presence of both sugars, baker's yeast metabolizes maltose only after glucose is depleted, because glucose transcriptionally and post-translationally regulates α-glucoside transporters [9, 10]. In our system, maltose was initially subjected to phosphorolysis without being metabolized by baker's yeast. Only liberated glucose was removed, resulting in a shift in the reaction equilibrium and favoring phosphorolysis.

The reaction scheme for the production of βGlc1P from maltose is shown in Fig. 1. Maltose, prepared from starch on an industrial scale, is a cost-effective starting material. The mass production of βGlc1P was carried out in a reaction mixture (2 L) in 500 mM sodium phosphate buffer (pH 6.5) containing 500 mM maltose, 0.5 U/mL maltose phosphorylase, and 1 % (w/v) dried baker's yeast at 30 °C. The mixture

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Abbreviations: βGlc1P, β-glucose 1-phosphate; GH, glycoside hydrolase family; TLC, thin-layer chromatography.

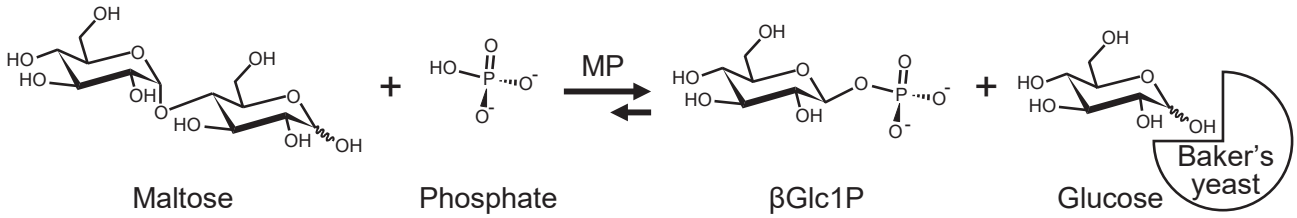

Fig. 1. The reaction scheme for the synthesis of βGlc1P combining enzymatic phosphorolysis by maltose phosphorylase (MP) and baker's yeast fermentation.

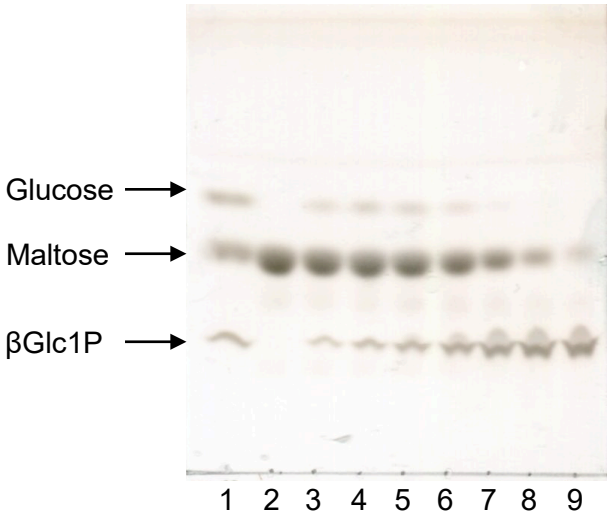

Fig. 2. TLC analysis of βGlc1P production from maltose.
βGlc1P production was carried out in 500 mM sodium phosphate buffer (pH 6.5) containing 500 mM maltose, 0.5 U/mL maltose phosphorylase and 1 % (w/v) dried baker's yeast at 30 ˚C. The sugars were developed by acetonitrile/water solvent (v/v, 80/20). Lane 1; marker solution (1 μL) containing 10 mM each glucose, maltose, and βGlc1P. Lanes 2–9; 20-fold-diluted reaction mixtures (1 μL) after 0, 1, 5, 10, 24, 48, 72, and 96 h.

in a loosely sealed bottle was stirred with a magnetic bar during the reaction. Maltose was converted into βGlc1P and glucose by maltose phosphorylase, with glucose selectively consumed by the baker's yeast. The production of βGlc1P reached a plateau after 72 h under the condition (Fig. 2). After a 96-hour reaction (76 % reaction yield), the mixture was centrifuged at 4 ˚C (4,000 G for 20 min) to remove baker's yeast. The yield was over twice the theoretical maximum yield achieved through enzymatic production of βGlc1P from maltose without fermentation by baker's yeast [6, 8]. The resultant supernatant was heat-treated at 65 ˚C for 30 min to stop the enzyme reaction, centrifuged (8,000 G for 20 min), filtered through a 0.45 μm filter, and then electrodialyzed. The first electrodialysis step, used a 100 Da molecular mass cutoff membrane to effectively separate inorganic phosphate from the mixture. Following the second electrodialysis with a 300 Da molecular mass cutoff membrane, βGlc1P was recovered in the dialysate solution. The resulting solution was adjusted to pH 7.0 by the addition of 5 M NaOH. The yield of βGlc1P was 74 % after the two electrodialysis. After the first crystallization, 177 g of βGlc1P as a bis(cyclohexylammonium) salt was recovered by filtration, followed by freeze drying. Additionally, 4 g of crystalline βGlc1P was recovered from the filtrate after concentration and crystallization under the same condition. Consequently, we obtained 181 g of crystalline βGlc1P as bis(cyclohexylammonium) salts (52 % isolation yield). The yield of βGlc1P is about 3 times higher than the previously reported method [6]. The abundance of βGlc1P will significantly contribute to advance research on GH65 phosphorylases and facilitate the synthesis of diverse α-glucosides by these enzymes.

EXPERIMENTAL

Carbohydrates. Glucose and maltose monohydrate were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Glucose 1,6-bisphosphate potassium salt hydrate was obtained from Merck KGaA (Darmstadt, Germany).

Enzymes and microorganism. Maltose phosphorylase from bacteria and glucose-6-phosphate dehydrogenase from *Leuconostoc* sp. were purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). β-Phosphoglucomutase from *Lactococcus* sp. was acquired from Merck KGaA. Dried baker's yeast was procured from Oriental Yeast Co., Ltd.

βGlc1P assay. The βGlc1P concentration was determined using the β-phosphoglucomutase/glucose-6-phosphate dehydrogenase method as previously described [11, 12].

Electrodialysis. Electrodialysis was performed using the Micro Acilyzer S3 (Astom Co., Tokyo, Japan). First, the reaction mixture was electrodialyzed through the electrodialysis cartridge AC-110-550 (molecular mass cutoff 100 Da, NEOSEPTA cartridge, Astom Co.) against 200 mL of deionized water until the current was lower than 0.01 A at 3.5 V. Then, the mixture was electrodialyzed through the electrodialysis cartridge AC-220-550 (molecular mass cutoff 300 Da, NEOSEPTA cartridge, Astom Co.) against 200 mL of deionized water until the current was lower than 0.01 A at 3.5 V.

TLC analysis. The 1 μL samples were spotted onto a 7.5 × 5.0 cm TLC plate (silica gel 60 F254, Merck KGaA) and developed with acetonitrile:water solvent (v/v, 80:20). The TLC plate was soaked in a 5 % sulfuric acid-methanol solution and heated in an oven until carbohydrates were clearly visible.

Crystallization. The dialysate containing 738 mmol of βGlc1P was treated with 370 mL of Amberlite IR120B (H+ type, Organo Co., Tokyo, Japan) for 10 min by a batch method to remove cations, the pH was adjusted to 8.6 with cyclohexylamine. The solution was then concentrated to 300 mL using a rotary evaporator, followed by dropwise addition of 600 mL of acetone to crystallize βGlc1P as a bis(cyclohexylammonium) salt. The resulting crystals were separated by filtration, and the filtrate was further concentrated to 70 mL using a rotary evaporator. Finally, 140 mL acetone was added dropwise to recrystallize βGlc1P as a bis(cyclohexylammonium) salt, and the resulting crystals were collected by filtration. The ^{1}H-NMR spectrum shown in Fig. S1 (see J.

Appl. Glycosci. Web site) indicates a single compound.

CONFLICTS OF INTEREST

The authors declare no conflict of interests.

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