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Note

One-pot Enzymatic Synthesis of Sophorose from Sucrose and Glucose

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Abstract: In this study, we developed a method to synthesize sophorose using three enzymes—sucrose phosphorylase from *Leuconostoc mesenteroides*, 1,2-β-oligoglucan phosphorylase from *Enterococcus italicus*, and exo β-1,2-glucooligosaccharide sophorohydrolase from *Parabacteroides distasonis*—in a one-pot reaction, employing inexpensive starting materials. After optimization, a reaction was carried out using 5 mM glucose, 250 mM sucrose, 10 mM inorganic phosphate, and enzyme concentrations of 5 μg/mL sucrose phosphorylase, 20 μg/mL 1,2-β-oligoglucan phosphorylase, and 50 μg/mL exo β-1,2-glucooligosaccharide sophorohydrolase at 30 ˚C for 48 h, yielding 108 mM sophorose. Following yeast treatment, sophorose was purified by size-exclusion chromatography with a final yield of 45 % based on the amount of sucrose used as the donor substrate.

Key words: 1,2-β-oligoglucan phosphorylase, exo β-1,2-glucooligosaccharide sophorohydrolase, sophorose, sucrose phosphorylase

Sophorose is a disaccharide composed of two glucose residues linked by a β-1,2-glycosidic bond. It is recognized for its prebiotic function and its role in inducing cellulase production in *Trichoderma reesei* [1, 2]. Sophorose is often used as a substrate for analyzing various carbohydrate-active enzymes. However, commercially available sophorose is expensive. One approach to synthesizing sophorose using inexpensive substrates involves the condensation of glucose by β-glucosidase [3, 4]. Nevertheless, because this method also produces various other disaccharides, the purification of sophorose remains challenging. Recently, an exo β-1,2-glucooligosaccharide sophorohydrolase (EC 3.2.1.214) was discovered in *Parabacteroides distasonis*, and a method for preparing pure sophorose from linear 1,2-β-glucan using this enzyme was developed [5, 6]. Linear 1,2-β-glucan can be synthesized from glucose and sucrose through the actions of 1,2-β-oligoglucan phosphorylase (EC 2.4.1.333) and sucrose phosphorylase (EC 2.4.1.7), and then purified by yeast treatment and ethanol precipitation [7, 8]. However, the experiments reported in the literature require a long enzyme reaction time (5–12 days) to obtain a sufficient amount of 1,2-β-glucan, and the ethanol precipitation process is somewhat inconvenient.

Here, we developed a simple one-pot method for synthesizing sophorose by simultaneously performing the reactions of three enzymes: sucrose phosphorylase from *Leuconostoc mesenteroides* subsp. *mesenteroides* ATCC 8293 (LmSP), 1,2-β-oligoglucan phosphorylase from *Enterococcus italicus* DSM 15952 (EiSOGP), and exo β-1,2-glucooligosaccharide sophorohydrolase from *P. distasonis* ATCC 8503 (BDI_3064), using glucose, sucrose, and inorganic phosphate as starting materials. An outline of this method is shown in Fig. 1. A significant advantage of this approach is that it omits the purification of 1,2-β-glucan, which was required in the previously established method for sophorose preparation [6]. Consequently, both experimental time and effort are considerably reduced. In this method, LmSP produces α-D-glucose 1-phosphate (α-Glc1*P*) from sucrose by phosphorolysis, and EiSOGP transfers a glucose residue via a β-1,2-linkage to glucose or sophorooligosaccharides by reverse phosphorolysis, using the resulting α-Glc1*P* as a donor substrate. In the early stages of the one-pot synthesis reaction, sophorose is likely produced by the action of EiSOGP using glucose as the acceptor; however, it is a poor substrate compared to sophorooligosaccharides [7, 8]. Subsequently, sophorose, a more favorable acceptor substrate, leads to the synthesis of sophorotriose, followed by sophorotetraose and sophoropentaose, through elongation reactions catalyzed by EiSOGP. The inorganic phosphate produced in the reverse phosphorolysis reaction catalyzed by EiSOGP is recycled and used for the phosphorolysis of sucrose by LmSP. Sophorotetraose and higher oligosaccharides are hydrolyzed by BDI_3064 into disaccharide units from the non-reducing end, primarily producing sophorose and a small amount of sophorotriose. It has been reported that sophorotriose is not a substrate for BDI_3064 [5]. Repeated cycles of sucrose phosphorolysis, sophorooligosaccharide elongation, and sophorooligosac-

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Abbreviations: α-Glc1*P*, α-D-glucose 1-phosphate; BDI_3064, exo β-1,2-glucooligosaccharide sophorohydrolase from *Parabacteroides distasonis*; EiSOGP, 1,2-β-oligoglucan phosphorylase from *Enterococcus italicus*; LmSP, sucrose phosphorylase from *Leuconostoc mesenteroides* subsp. *mesenteroides.*

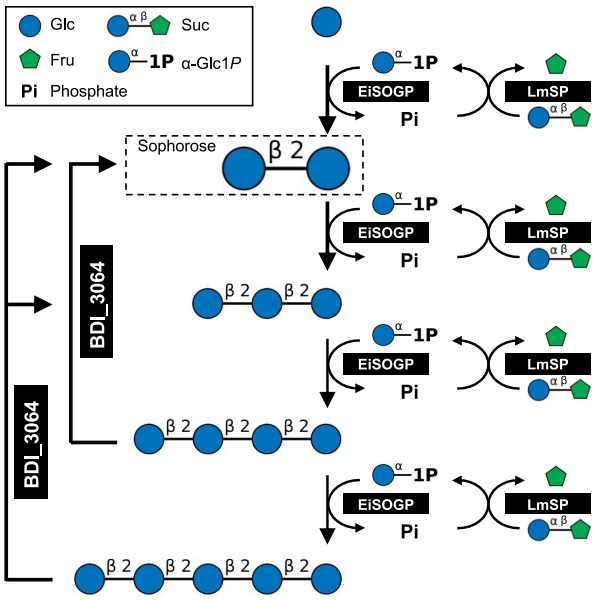

Fig. 1. Overview of one-pot synthesis of sophorose.

α-Glc1*P* is produced from sucrose by LmSP and used by EiSOGP to elongate sophorooligosaccharides. Sophorose is initially synthesized using glucose as the acceptor and further elongated into higher oligosaccharides, which are subsequently hydrolyzed by BDI_3064 to yield sophorose. This process is repeated, leading to sophorose accumulation.

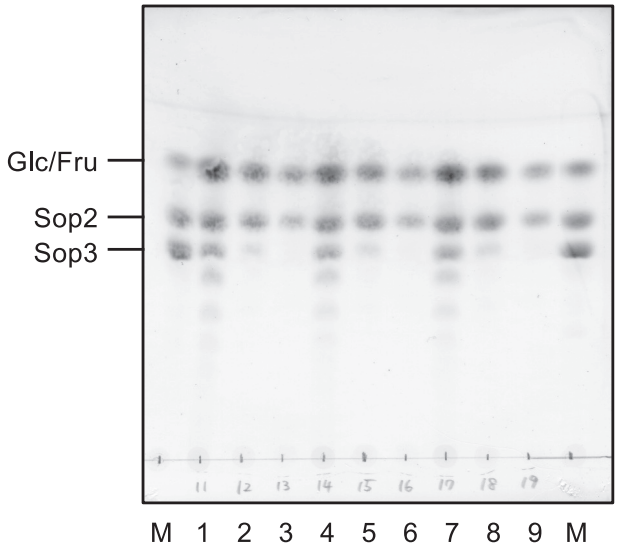

Fig. 2. TLC analysis of sugars produced from various concentrations of glucose and sucrose as substrates.

A reaction mixture containing 5–100 mM glucose, 100–250 mM sucrose, 20 mM sodium phosphate buffer (pH 7.0), 100 μg/mL LmSP, 100 μg/mL EiSOGP, and 100 μg/mL BDI_3064 was incubated at 30 ˚C for 48 h, followed by TLC analysis. Lanes (conditions) 1–3: 100 mM glucose; lanes 4–6: 25 mM glucose; lanes 7–9: 5 mM glucose. Lanes 1, 4, and 7: 500 mM sucrose; lanes 2, 5, and 8: 250 mM sucrose; lanes 3, 6, and 9: 100 mM sucrose. M, markers (Glc, glucose; Fru, fructose; Sop2, sophorose; Sop3, sophorotriose).

charide hydrolysis, as described above, lead to sophorose accumulation. The following section describes the results of several optimizations conducted for sophorose synthesis and its subsequent large-scale production based on these results.

First, we conducted a one-pot synthesis of sophorose using various combinations of glucose and sucrose concentrations. Glucose concentrations were set at 5, 25, and 100 mM, and sucrose concentrations were set at 100, 250, and 500 mM. Each enzyme concentration was 100 μg/mL, with 20 mM inorganic phosphate, and the reaction was carried out at 30 ˚C for 48 h. As expected, sophorose was successfully synthesized under all conditions (Fig. 2). Under Conditions 1, 4, and 7 (containing 500 mM sucrose), sophorotetraose and higher oligosaccharides were produced. In contrast, the main product was sophorose, with a small amount of sophorotriose under the other conditions. Notably, high concentrations of sophorose were obtained under Conditions 2 (116 mM), 5 (108 mM), and 8 (108 mM). Therefore, we selected Condition 8 for subsequent experiments, which had the lowest sugar concentrations (5 mM glucose and 250 mM sucrose) for sophorose synthesis.

Next, we determined the optimal concentration of inorganic phosphate for sophorose synthesis. Although a higher concentration of inorganic phosphate promotes the breakdown of sucrose, it may also facilitate the phosphorolysis of EiSOGP, potentially hindering the elongation of sophorooligosaccharides. We tested inorganic phosphate concentrations of 10 mM, 20 mM, 50 mM, and 100 mM. At 100 mM phosphate, the amount of sophorose produced was slightly lower (95 mM), whereas at 10–50 mM phosphate, the sophorose concentration ranged from 120 mM to 130 mM, with no considerable differences observed among these conditions. Therefore, 10 mM inorganic phosphate was selected for subsequent experiments.

We then evaluated the enzyme concentrations for the one-pot synthesis. Each enzyme concentration was set in the 5–100 μg/mL range. When BDI_3064 was used at concentrations below 20 μg/mL, only up to 23 mM of sophorose was produced. Under the conditions listed in Table S1 (see J. Appl. Glycosci. Website), sophorose production ranged from 80 mM to 129 mM. From the perspective of enzyme conservation, condition No. 13 (5 μg/mL LmSP, 20 μg/mL EiSOGP, and 50 μg/mL BDI_3064), which produced 106 mM sophorose, was considered suitable for one-pot synthesis.

Based on the above results, a mixture (100 mL) containing 5 mM glucose, 250 mM sucrose, 10 mM sodium phosphate buffer (pH 7.0), 5 μg/mL LmSP, 20 μg/mL EiSOGP, and 50 μg/mL BDI_3064 was incubated at 30 ˚C for 48 h; this yielded 108 mM sophorose (3.7 g), and a small amount of sophorotriose was also produced as a by-product. As it is believed that almost all of the glucose residues in sophorose synthesized by this method are derived from sucrose, a maximum of 0.5 mol sophorose can be theoretically synthesized from 1 mol sucrose. Therefore, the yield of sophorose in this reaction, based on the sucrose (25 mmol) used as the donor substrate, was estimated to be 86 %. The enzymes were inactivated by heating at 95 ˚C for 10 min. To reduce the concentrations of glucose and fructose, 2 g of dry yeast (Nisshin Seifun Welna Inc., Japan) was added, and the mixture was incubated at 30 ˚C for 2 h. The supernatant obtained by centrifugation was heated again at 95 ˚C for 10 min, concentrated using a rotary evaporator, and the sample was subjected to size-exclusion chromatography using Toyopearl HW-40S columns (2.6 cm i.d. × 100 cm × 3; Tosoh Co., Japan) with water as the mobile phase. The fractions containing high-purity sophorose were collected and lyophilized. Finally, 1.9 g of sophorose (5.6 mmol) was obtained in a 45 % yield based on the amount of donor substrate (25 mmol sucrose). The molecular mass of the

product analyzed by ESI-MS was as expected (*m/z* [M + Na]$^+$ 365). The ^{1}H and ^{13}C NMR spectra of the product were identical to those of commercial sophorose. Thus, this study demonstrates that sophorose can be easily and efficiently produced in a short time using a one-pot method with inexpensive substrates.

EXPERIMENTAL

Preparation of recombinant enzymes. *Escherichia coli* BL21(DE3) harboring a pET-23a (Merck KGaA, Darmstadt, Germany) derivative encoding LmSP (https://doi.org/10.17632/jycz6mrdcd.1) was cultured in 800 mL of LB medium containing 100 μg/mL ampicillin at 37 ˚C. Similarly, *E. coli* BL21(DE3) harboring a pET-30a (Merck KGaA) derivative encoding EiSOGP [8] was cultured in 400 mL of LB medium containing 50 μg/mL kanamycin at 37 ˚C. When the OD_{600} of these cultures reached 0.7, IPTG was added to the medium to a final concentration of 0.4 mM, and the incubation was continued for an additional 5 h. Moreover, *E. coli* BL21(DE3) harboring a pET-30a derivative encoding BDI_3064 [5] was cultured in 800 mL of autoinduction medium (#AIMLB0101, Formedium Ltd., Norfolk, UK) containing 50 μg/mL kanamycin at 28 ˚C for 20 h. Cells were harvested separately by centrifugation, resuspended in 20 mM MOPS-NaOH buffer (pH 7.5) containing 150 mM sodium chloride, and disrupted by sonication. After centrifugation, the supernatant was applied to Ni-NTA columns (5 mL; FUJIFILM Wako Pure Chemical Co., Osaka, Japan), and the target enzymes were eluted by increasing the imidazole concentration. The purified enzymes were concentrated, and the buffer was exchanged with 20 mM MOPS-NaOH buffer (pH 7.5), 1 mM EDTA, and 1 mM DTT, as previously described [9]. The concentration of the purified proteins was determined by measuring A_{280} using the molar extinction coefficient calculated using EMBOSS Pepstats [10].

Synthesis of sophorose. Based on the standard conditions of 5 mM glucose, 250 mM sucrose, 10 mM sodium phosphate buffer (pH 7.0), 100 μg/mL LmSP, 100 μg/mL EiSOGP, and 100 μg/mL BDI_3064, reactions were performed by varying the substrate and enzyme concentrations. The reaction mixture was incubated at 30 ˚C for 48 h. Further details are provided in the main text.

Analysis and purification of sugars. TLC, HPLC, size-exclusion chromatography, NMR, and ESI-MS were performed as previously described [9, 11]. Sophorose purchased from SERVA Electrophoresis GmbH (Heidelberg, Germany) was used as the standard. The sophorose concentration was calculated from the peak area obtained by HPLC.

CONFLICTS OF INTERESTS

The authors declare no conflict of interest.

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REFERENCES

[1] Zeng M, van Pijkeren JP, Pan X. Gluco-oligosaccharides as potential prebiotics: Synthesis, purification, structural characterization, and evaluation of prebiotic effect. Compr Rev Food Sci Food Saf. 2023; 22: 2611−51.

[2] Suto M, Tomita F. Induction and catabolite repression mechanisms of cellulase in fungi. J Biosci Bioeng. 2001; 92: 305−11.

[3] Semenova MV, Okunev ON, Gusakov AV, Sinitsyn AP. Disaccharide synthesis by enzymatic condensation of glucose: glycoside linkage patterns for different fungal species. Open Glycosci. 2009; 2: 20−4.

[4] Gao L, Gao F, Jiang X, Zhang C, Zhang D, Wang L, et al. Biochemical characterization of a new β-glucosidase (Cel3E) from *Penicillium piceum* and its application in boosting lignocelluloses bioconversion and forming disaccharide inducers: New insights into the role of β-glucosidase. Process Biochem. 2014; 49: 768−74.

[5] Shimizu H, Nakajima M, Miyanaga A, Takahashi Y, Tanaka N, Kobayashi K, et al. Characterization and structural analysis of a novel exo-type enzyme acting on β-1,2-glucooligosaccharides from *Parabacteroides distasonis*. Biochemistry. 2018; 57: 3849−60.

[6] Nakajima M, Taguchi H, Shimizu H, Nakai H. Method for producing exo-β-1,2-glucanase and sophorose. Worldwide patent application WO/2018/194092, 2018.

[7] Abe K, Nakajima M, Kitaoka M, Toyoizumi H, Takahashi Y, Sugimoto N, et al. Large-scale preparation of 1,2-β-glucan using 1,2-β-oligoglucan phosphorylase. J Appl Glycosci. 2015; 62: 47−52.

[8] Nakajima M, Tanaka N, Kobayashi K, Nakai H, Kimura S, Iwata T, et al. Enzymatic control and evaluation of degrees of polymerization of β-(1→2)-glucans. Anal Biochem. 2021; 632: 114366.

[9] Isono N, Yagura S, Yamanaka K, Masuda Y, Mukai K, Katsuzaki H. Enzymatic synthesis of β-D-fructofuranosyl α-D-glucopyranosyl-(1→2)-α-D-glucopyranoside using *Escherichia coli* glycoside phosphorylase YcjT. Biosci Biotechnol Biochem. 2023; 87: 1249−53.

[10] Madeira F, Park YM, Lee J, Buso N, Gur T, Madhusoodanan N, et al. The EMBL-EBI search and sequence analysis tools APIs in 2019. Nucleic Acids Res. 2019; 47: W636−41.

[11] Isono N, Mizutani E, Hayashida H, Katsuzaki H, Saburi W. Functional characterization of a novel GH94 glycoside phosphorylase, 3-*O*-β-D-glucopyranosyl β-D-glucuronide phosphorylase, and implication of the metabolic pathway of acidic carbohydrates in *Paenibacillus borealis*. Biochem Biophys Res Commun. 2022; 625: 60−5.