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One Pot Enzymatic Production of Nigerose from Common Sugar Resources Employing Nigerose Phosphorylase

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Abstract: One-pot enzymatic production of nigerose was demonstrated from abundantly available sugar resources, including maltose, cellobiose, sucrose and starch. (i) 319 mM nigerose was generated from 500 mM maltose by the combined actions of maltose phosphorylase and nigerose phosphorylase, which share the same β -D-glucose 1-phosphate, in the presence of phosphate. The yield was 62% based on the concentration of maltose as the starting material. (ii) 129 mM nigerose was produced from 250 mM cellobiose by cellobiose phosphorylase and nigerose phosphorylase in the presence of phosphate, in combination with the enzymatic pathway to convert α -D-glucose 1-phosphate to β -D-glucose 1-phosphate via D-glucose 6-phosphate by the combined actions of α -phosphoglucosyltransferase and β -phosphoglucosyltransferase, resulting in a yield of 52%. (iii) 350 mM nigerose was produced from 500 mM sucrose by substituting cellobiose phosphorylase with sucrose phosphorylase and adding xylose isomerase, giving a yield of 67%. (iv) 270 mM nigerose was generated from 100 mg/mL starch and 500 mM D-glucose by the concomitant actions of glycogen phosphorylase, isoamylase, α -phosphoglucosyltransferase, β -phosphoglucosyltransferase and nigerose phosphorylase, in the presence of phosphate. In addition, 280 mM 3-O- α -D-glucopyranosyl-D-galactose was produced by substituting D-glucose with D-galactose. Based on the concentrations of D-glucose and D-galactose as the starting materials, the yields were calculated to be 52 and 56%, respectively. These one-pot enzymatic approaches can be extended to include practical production of a variety of oligosaccharides by substituting nigerose phosphorylase with other β -D-glucose 1-phosphate-forming phosphorylases together with various carbohydrate acceptors.

Key words: nigerose, maltose, sucrose, soluble starch, nigerose phosphorylase, reversible phosphorolysis

INTRODUCTION

Phosphorylases are *exo*-lytic enzymes that catalyze phosphorolysis of particular oligosaccharides to produce sugar 1-phosphate with strict substrate specificity.^{1–3} The reaction is reversible, allowing synthesis of the oligosaccharides from the corresponding sugar 1-phosphate and suitable carbohydrate acceptors with strict regioselectivity.^{1–3} In addition, the reversibility enables practical production of the oligosaccharides from abundantly available natural sugars without the addition of costly sugar 1-phosphate, using single phosphorylases^{1,4,5} or by the combined actions of two phosphorylases that share the same sugar 1-phosphate.^{1,6–10} Recently we developed practical methods to produce β -D-galactosides, which include lacto-N-biose (Bifidus factor in human milk), from sucrose by the combined actions of sucrose phosphorylase (EC 2.4.1.7) and α -D-galactose 1-phosphate-forming phosphorylases

together with the enzymatic system to convert α -D-glucose 1-phosphate to α -D-galactose 1-phosphate, using UDP-glucose-hexose 1-phosphate uridylyltransferase (EC 2.7.7.12) and UDP-glucose 4-epimerase (EC 5.1.3.2).^{11–13} Furthermore, we succeeded in producing 4-O- β -D-mannopyranosyl-N-acetyl-D-glucosamine (core structure of N-glycans) from sucrose by the combined actions of sucrose phosphorylase and 4-O- β -mannosyl-N-acetyl-glucosamine phosphorylase (EC 2.4.1.-) together with the enzymes necessary to convert α -D-glucose 1-phosphate to α -D-mannose 1-phosphate [α -phosphoglucosyltransferase (EC 5.4.2.2), glucose 6-phosphate isomerase (EC 5.3.1.9), mannose 6-phosphate isomerase (EC 5.3.1.8) and phosphomannomutase (EC 5.4.2.8)].¹⁴ Thus, phosphorylases may be suitable as catalysts for the practical production of oligosaccharides.

In this study, we report one-pot enzymatic production of nigerose (3-O- α -D-glucopyranosyl-D-glucose) from common sugar resources such as maltose, cellobiose, sucrose and soluble starch by nigerose phosphorylase (EC 2.4.1.279), a unique phosphorylase we recently discovered that acts reversibly on

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nigerose to produce β -D-glucose 1-phosphate and D-glucose,¹⁵⁾ together with the enzymatic pathway to convert α -D-glucose 1-phosphate to β -D-glucose 1-phosphate via D-glucose 6-phosphate by the combined actions of α -phosphoglucomutase (EC 5.4.2.2) and β -phosphoglucomutase (EC 5.4.2.6).

MATERIALS AND METHODS

Carbohydrates. D-Glucose, maltose monohydrate and soluble starch were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). α -D-Glucose 1,6-bisphosphate potassium salt hydrate and D-galactose were purchased from Sigma-Aldrich Corp. (St. Louis, USA). Cellobiose and sucrose were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan) and Wako Pure Chemical Industries, Ltd. (Osaka, Japan), respectively.

Enzymes. Nigerose phosphorylase (GenBank ID: ABX42243.1) from *Clostridium phytofermentans*, cellobiose phosphorylase (GenBank ID: BAA28631.1) from *Cellvibrio gilvus* and sucrose phosphorylase (GenBank ID: BAJ65776.1) from *Bifidobacterium longum* JCM1217 were prepared as previously described.^{11,15,16)} Maltose phosphorylase from bacteria, β -glucosidase from almond and glucoamylase from *Rhizopus* sp. were purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). β -Phosphoglucomutase from *Lactococcus* sp., isoamylase from *Pseudomonas* sp. and invertase from *Saccharomyces cerevisiae* were purchased from Sigma-Aldrich. Xylose isomerase from *Streptomyces rubiginosus* was purchased from Hampton Research Corp. (Aliso Viejo, USA). α -Phosphoglucomutase from *Thermococcus kodakarensis* KOD1 (GenBank ID: BAD85297.1) and glycogen phosphorylase from *Thermotoga maritima* MSB8 (GenBank ID: AGL50099.1) were prepared as described in previous papers^{17,18)} with several modifications. *Escherichia coli* BL21 (DE3) and *E. coli* Rosetta2 (DE3) were transformed with expression plasmids for α -phosphoglucomutase and glycogen phosphorylase genes, respectively, which were inserted into pET-30a and pET-24a, respectively, between NdeI and XhoI sites. Recombinant proteins were purified using previously described methods for His-tag fusion proteins.¹⁴⁾ The protein concentrations of α -phosphoglucomutase and glycogen phosphorylase were determined spectrophotometrically at 280 nm using theoretical extinction coefficients of $\epsilon = 26,930$ and $151,150 \text{ M}^{-1}\text{cm}^{-1}$, respectively, based on the amino acid sequences.¹⁹⁾

Enzyme activity. Reverse phosphorolytic activity of nigerose phosphorylase was determined by methods described previously.¹⁵⁾ Phosphorolytic activities of α -D-glucose 1-phosphate-forming phosphorylases were routinely determined by quantifying α -D-glucose 1-phosphate released from the corresponding substrate (10 mM cellobiose for cellobiose phosphorylase, 10 mM sucrose for sucrose phosphorylase or 10 mg/mL soluble starch for glycogen phosphorylase) in 40 mM HEPES-NaOH (pH 7.0) containing 10 mM phosphate at 30°C using the phosphoglucomutase-glucose 6-phosphate dehydrogenase method²⁰⁾ as described previously.^{11,21)} α -Phosphoglucomutase activity was determined by measuring the increase in D-glucose 6-phosphate²²⁾ from 10 mM α -D-glucose 1-phosphate in 40 mM HEPES-NaOH buffer (pH 7.0) containing 5.0 U/mL glucose 6-phosphate dehydrogenase, 0.25 mM thio-NAD⁺, 41 μM D-glucose 1,6-bisphosphate and 10 mM MgCl₂ at

30°C. One unit of enzyme was defined as the amount of enzyme that liberated 1 μmol product per min under the above conditions. The activities of all commercial enzymes were defined according to the manufacturer's protocol.

Quantification by high performance liquid chromatography (HPLC). The concentrations of reaction products were monitored during the reaction by HPLC (Prominence, Shimadzu, Kyoto, Japan) equipped with an RI detector and a Shodex Asahipak NH2P50-4E column (4.6 mm internal diameter $\times$ 250 mm, Showa Denko K.K., Tokyo, Japan) at 30°C under a constant flow (1 mL/min) of mobile phase (acetonitrile/water v/v, 75/25). The one-pot enzymatic reaction was terminated at specific intervals by adding 10 μL of the reaction mixture to 90 μL of 100 mM HCl, followed by pH adjustment by 1 M NaOH (pH 7.0, 5.0, 5.0 or 7.0 for maltose, cellobiose, sucrose and soluble starch, respectively) for enzymatic digestion of the remaining starting materials. The each maltose, cellobiose, sucrose and soluble starch was digested by addition of 85 μL of 115 $\mu\text{g/mL}$ (5 U/mL) glucoamylase, 100 μL of 10 mg/mL (100 U/mL) β -glucosidase, 3.3 μL of 10 mg/mL (3,000 U/mL) invertase and 85 μL of 115 $\mu\text{g/mL}$ (5 U/mL) glucoamylase, respectively, at 30°C overnight.

Purification by gel-filtration chromatography. After a 216 h reaction, 9 mL of 100 mM HCl was added to the reaction mixture to stop the one-pot enzymatic reaction, followed by pH adjustment by 1 M NaOH (pH 7.0, 5.0, 5.0 or 7.0 for maltose, cellobiose, sucrose and soluble starch, respectively). The remaining maltose, cellobiose, sucrose and soluble starch were digested by addition of 23 μL of 23 mg/mL (1,000 U/mL) glucoamylase, 100 μL of 505 mg/mL (5,050 U/mL) β -glucosidase, 3.3 μL of 10 mg/mL (3,000 U/mL) invertase and 23 μL of 23 mg/mL (1,000 U/mL) glucoamylase, respectively, at 30°C overnight. After heat treatment at 60°C for 30 min to inactivate the added glucoamylase, β -glucosidase or invertase, the supernatant collected by centrifugation at $10,000 \times G$ for 20 min was concentrated by a centrifugal evaporator (CVE-3100, Tokyo Rikakikai Co., Ltd, Tokyo, Japan). The concentrated sample was applied to a Toyopearl HW40S column (50 mm internal diameter $\times$ 90 cm; Tosoh Corp., Tokyo, Japan) equilibrated with Milli-Q water (Merck Millipore Corp., Billerica, USA), followed by elution at a flow rate of 1.0 mL/min using ÄKTA prime (GE Healthcare Limited, Buckinghamshire, UK). The elution patterns of the products in the gel filtration chromatography were monitored using thin layer chromatography (TLC) (Kieselgel 60 F₂₅₄, Merck Millipore Corp.), developed by acetonitrile/water (v/v, 80/20). The TLC plates were soaked in 5% sulfuric acid-methanol solution and heated in an oven until the bands were visible. Fractions containing nigerose or 3-O- α -D-glucopyranosyl-D-galactose were collected, followed by lyophilization. ¹H-NMR spectra of the products were acquired in D₂O using a Bruker DMX 600 spectrometer (Bruker Biospin GmbH, Rheinstetten, Germany). The structures of isolated products were confirmed by comparing ¹H-NMR data with that of authentic nigerose and 3-O- α -D-glucopyranosyl-D-galactose standards.¹⁵⁾

RESULTS AND DISCUSSION

The reaction scheme for production of nigerose from maltose is shown in Fig. 1 (A). In the reaction, maltose is phosphorylated by maltose phosphorylase into β -D-glucose

1-phosphate and D-glucose. The resulting products are continuously converted by nigerose phosphorylase to nigerose and phosphate. Because the phosphate is recycled during the reaction, the overall reaction is described as the transformation of maltose to nigerose in the presence of catalytic amounts of phosphate by the combined actions of the two phosphorylases that share the same sugar 1-phosphate. The one-pot enzymatic synthesis of nigerose was demonstrated in a reaction mixture (1 mL) containing 500 mM maltose, various concentrations (1–100 mM) of sodium phosphate buffer (pH 7.0), 27 $\mu\text{g/mL}$ (0.30 U/mL) maltose phosphorylase and 20 $\mu\text{g/mL}$ (0.62 U/mL) nigerose phosphorylase at 30°C. We examined the optimum concentration of the phosphate in the reaction mixture (pH 7.0: the optimum pH of reverse phosphorolysis using β -D-glucose 1-phosphate and D-glucose by nigerose phosphorylase), resulting that no significant effect

Table 1. One-pot enzymatic syntheses of nigerose and its derivative.

Starting material	Acceptor	Product	Yield (%)
500 mM Maltose	—	Nigerose	62 ^a
250 mM Cellobiose	—	Nigerose	52 ^a
500 mM Sucrose	—	Nigerose	67 ^a
100 mg/mL Soluble starch	500 mM D-Glucose	Nigerose	52 ^b
100 mg/mL Soluble starch	500 mM D-Galactose	3-O- α -D-Glucosyl-D-galactose	56 ^b

^aThe yields were calculated based on the concentrations of the starting materials. ^bThe yields were calculated based on the concentrations of the acceptors.

Fig. 1. Production of nigerose from maltose.

(A) The reaction scheme for one-pot enzymatic production of nigerose from maltose. MP, maltose phosphorylase; NP, nigerose phosphorylase. (B) Time course of nigerose production from maltose.

on the concentration of nigerose were observed with increase in that of phosphate. As shown in Fig. 1(B), the concentration of nigerose in the reaction mixture reached 310 mM at 72 h. The reaction yield was 62% based on the concentration of maltose used as the starting material (Table 1). The produced nigerose was purified by gel-filtration column chromatography, and it yielded 109 mg lyophilized nigerose.

We also produced nigerose from cellobiose by cellobiose phosphorylase and nigerose phosphorylase together with the enzymatic system to convert α -D-glucose 1-phosphate to β -D-glucose 1-phosphate (Fig. 2(A)). The one-pot enzymatic synthesis of nigerose was demonstrated in a reaction mixture (1 mL) containing 250 mM cellobiose, 25 mM sodium phosphate buffer (pH 7.0), 10 mM MgCl_2 , 41 μM α -D-glucose 1,6-bisphosphate, 2.3 mg/mL (0.63 U/mL) cellobiose phosphorylase, 420 $\mu\text{g/mL}$ (0.25 U/mL) α -phosphoglucomutase, 210 $\mu\text{g/mL}$ (5.0 U/mL) β -phosphoglucomutase and 20 $\mu\text{g/mL}$ (0.62 U/mL) nigerose phosphorylase at 30°C. In the reaction, cellobiose is first phosphorylated by cellobiose phosphorylase into α -D-glucose 1-phosphate and D-glucose. The resulting α -D-glucose 1-phosphate is continuously converted into β -D-glucose 1-phosphate via D-glucose 6-phosphate by the concomitant actions of α -phosphoglucomutase and β -phosphoglucomutase. Finally, nigerose is synthesized from β -D-glucose 1-phosphate

Fig. 2. Production of nigerose from cellobiose.

(A) The reaction scheme for one-pot enzymatic production of nigerose from cellobiose. NP, nigerose phosphorylase; α -PGM, α -phosphoglucomutase; β -PGM, β -phosphoglucomutase; CBP, cellobiose phosphorylase. (B) Time course of nigerose production from cellobiose.

and D-glucose by nigerose phosphorylase. Because phosphate is recycled during the reaction, the overall reaction is described as the transformation of cellobiose to nigerose. As shown in Fig. 2(B), the concentration of nigerose in the reaction mixture reached 129 mM at 168 h. The reaction yield was 52% based on the concentration of cellobiose used as the starting material (Table 1). Finally, 44 mg of purified nigerose was recovered by gel-filtration column chromatography and lyophilization.

Production of nigerose from sucrose was also performed using the above one-pot enzymatic approach by substituting cellobiose phosphorylase with sucrose phosphorylase and adding xylose isomerase to convert D-fructose liberated from sucrose by phosphorolysis of sucrose phosphorylase into D-glucose as the acceptor for nigerose synthesis (Fig. 3(A)). The production of nigerose was demonstrated in a reaction mixture (1 mL) containing 500 mM sucrose, 10 mM MgCl_2 , 41 μM α -D-glucose 1,6-bisphosphate, various concentrations (1–100 mM) of sodium phosphate buffer (pH 7.0), 9.1 or 91 $\mu\text{g/mL}$ (0.73 or 7.3 U/mL) sucrose phosphorylase, 33 or 330 $\mu\text{g/mL}$ (0.73 or 7.3 U/mL) xylose isomerase, 420 or 4,200 $\mu\text{g/mL}$ (0.25 or 2.5 U/mL) α -phosphoglucomutase, 21 or 210 $\mu\text{g/mL}$ (0.5 or 5.0 U/mL) β -phosphoglucomutase and 20 or 200 $\mu\text{g/mL}$ (0.62 or 6.2 U/mL) nigerose phosphorylase at 30°C. The optimum starting composition of the reaction mixture was determined to be 500 mM sucrose, 10 mM MgCl_2 , 41 μM α -D-glucose 1,6-bisphosphate, 25 mM sodium phosphate buffer (pH 7.0), 9.1 $\mu\text{g/mL}$ (0.73 U/mL) sucrose phosphorylase, 330 $\mu\text{g/mL}$ (7.3 U/mL) xylose isomerase, 420 $\mu\text{g/mL}$ (0.25 U/mL) α -phosphoglucomutase, 210 $\mu\text{g/mL}$ (5.0 U/mL) β -phosphoglucomutase and 20 $\mu\text{g/mL}$ (0.62 U/mL) nigerose phosphorylase. Under the optimum conditions, the concentration of nigerose in the reaction mixture reached 335 mM at 72 h, indicating a reaction yield of 67% based on the concentration of sucrose used as the starting material (Fig. 3 (B), Table 1). Finally, 120 mg of purified nigerose was recovered by gel-filtration column chromatography and lyophilization.

Nigerose was also produced from starch as the starting material (Fig. 4(A)). In the reaction, soluble starch is phosphorolytically hydrolyzed by glycogen phosphorylase together with hydrolysis of α -1,6-glucosidic linkages by isoamylase to liberate α -D-glucose 1-phosphate, which is continuously converted into β -D-glucose 1-phosphate via glucose 6-phosphate by the concomitant actions of α -phosphoglucomutase and β -phosphoglucomutase. The resulting β -D-glucose 1-phosphate is consumed together with D-glucose by nigerose phosphorylase to produce nigerose and phosphate, which is recycled for phosphorolysis of soluble starch during the reaction. The one-pot enzymatic synthesis of nigerose was demonstrated in a reaction mixture (1 mL) containing 100 mg/mL soluble starch, 500 mM D-glucose, various concentrations (1–100 mM) of sodium phosphate buffer (pH 7.0), 10 mM MgCl_2 , 41 μM α -D-glucose 1,6-bisphosphate, 960 $\mu\text{g/mL}$ (0.026 U/mL) glycogen phosphorylase, 0.33 $\mu\text{g/mL}$ (17 U/mL) isoamylase (EC 3.2.1.68), 420 $\mu\text{g/mL}$ (0.3 U/mL) α -phosphoglucomutase, 210 $\mu\text{g/mL}$ (5.0 U/mL) β -phosphoglucomutase and 20 $\mu\text{g/mL}$ (0.62 U/mL) nigerose phosphorylase at 30°C. The optimum concentration of the phosphate was determined to be 75 mM, resulting that the concentration of nigerose in the reaction mixture reached 259 mM at 192 h (Fig. 4(B)). The reaction yield was 52% based on the concentration of D-glucose used

(Table 1). Finally, 90 mg of purified nigerose was recovered by gel-filtration column chromatography and lyophilization. In addition, several nigerose derivatives can be synthesized by reverse phosphorolysis of nigerose phosphorylase by substituting D-glucose with suitable monosaccharide acceptors.¹⁵⁾ Therefore, the one-pot enzymatic approach using soluble starch as the starting material was applied to synthesize a nigerose derivative, 3-O- α -D-glucopyranosyl-D-galactose, by substituting 500 mM D-glucose with 500 mM D-galactose, resulting that the concentration of 3-O- α -D-glucopyranosyl-D-galactose in the reaction mixture reached 280 mM at 168 h in a yield of 56% based on the concentration of D-galactose used. Finally, 96 mg of purified 3-O- α -D-glucopyranosyl-D-galactose was obtained.

Nigerose is a constitutional unit of α -1,3-polysaccharides such as nigeran, pseudonigeran and isolichenin produced by filamentous fungi such as the genus *Aspergillus*.²³⁾ It has been also found in beer and Japanese rice wine²⁴⁾ and has been reported to show anticaries²⁵⁾ and immunostimulatory activity.²⁶⁾ It would therefore be beneficial to develop efficient production methods of nigerose as a functional oligosaccharide. Nigerose can be enzymatically synthesized from sucrose and D-glucose by transglycosylation of sucrose phosphorylase.²⁷⁾ In addition, nigerooligosaccharides including nigerose, nigerosylglucose (α -D-glucopyranosyl-1,3- α -D-glucopyranosyl-1,4- α -D-

Fig. 3. Production of nigerose from sucrose.

(A) The reaction scheme for one-pot enzymatic production of nigerose from sucrose. NP, nigerose phosphorylase; α -PGM, α -phosphoglucomutase; β -PGM, β -phosphoglucomutase; SP, sucrose phosphorylase; XI, xylose isomerase. (B) Time course of nigerose production from sucrose.

Fig. 4. Production of nigerose from soluble starch.

(A) The reaction scheme for one-pot enzymatic production of nigerose from soluble starch. GP, glycogen phosphorylase; IAm, isoamylase; NP, nigerose phosphorylase; α -PGM, α -phosphoglucomutase; β -PGM, β -phosphoglucomutase. (B) Time course of nigerose production from soluble starch.

glucose) and nigerosylmaltose (α -D-glucopyranosyl-1,3- α -D-glucopyranosyl-1,4- α -D-glucopyranosyl-1,4- α -D-glucose) are produced from maltose by transglycosylation of *Acremonium* sp. α -glucosidase (EC 3.2.1.20).²⁸⁾ The one-pot enzymatic approach developed in this study is an alternative method for production of nigerose from inexpensive sugars. It should be noted that the synthetic method can be extended to include the practical production of a variety of oligosaccharides by substituting nigerose phosphorylase with other β -D-glucose 1-phosphate-forming phosphorylases together with various carbohydrate acceptors.

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