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Novel Transglucosylating Reaction of Sucrose Phosphorylase to Carboxylic Compounds Such as Benzoic Acid

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We examined the synthesis of benzoyl glucoside using the transglucosylation reaction of sucrose phosphorylase. Sucrose phosphorylase from *Streptococcus mutans* showed marked transglucosylating activity, particularly under acidic conditions. On the other hand, sucrose phosphorylase from *Leuconostoc mesenteroides* showed very weak transglucosylating activity. Three main products were detected from the reaction mixture using benzoic acid as an acceptor molecule and sucrose as a donor molecule. These compounds were identified as 1-*O*-benzoyl α-D-glucopyranoside, 2-*O*-benzoyl α-D-glucopyranose and 2-*O*-benzoyl β-D-glucopyranose on the basis of their isolation and the isolation of their acetylated products and subsequent analysis using 1D- and 2D-NMR analyses. From the results of the time-course analyses of the enzyme reaction and the degradation of 1-*O*-benzoyl α-D-glucopyranoside, 1-*O*-benzoyl α-D-glucopyranoside was considered to be initially produced by the transglucosylation reaction of the enzyme, and 2-*O*-benzoyl α-D-glucopyranose and 2-*O*-benzoyl β-D-glucopyranose were produced from 1-*O*-benzoyl α-D-glucopyranoside by intramolecular acyl migration reaction. The acceptor specificity in the glucosylation reaction of *S. mutans* sucrose phosphorylase was also investigated. This sucrose phosphorylase could transglucosylate toward various carboxylic compounds. Short-chain fatty acids, hydroxy acids and dicarboxylic acids were also glucosylated with this sucrose phosphorylase.

[**Key words:** sucrose phosphorylase, transglucosylation, carboxylic group, carboxylic compounds, sucrose, benzoic acid, intramolecular acyl migration]

Many types of biologically active compound are used in food and cosmetic materials, and many of these compounds include carboxylic groups in their structures. For example, benzoic acid has been commonly used as a food preservative, butyric acid has anti-inflammatory effects in distal ulcerative colitis (1) and proliferative effects on human colonic mucosa (2), and glycolic acid promotes cell proliferation and collagen production in cultured human skin fibroblasts (3). However, some of these compounds have a strong smell or acidity, or have low solubility, particularly at low pH. Thus, it is very important to improve these characteristics to enhance their usefulness as food and cosmetic ingredients.

Glycosylation is an important method of the structural modification of bioactive compounds. It can be used to make water-insoluble compounds water soluble. Furthermore, the difference between the α- and β-configurations of the glycosidic linkage plays a very important role in biological activities. In our previous studies, we synthesized hydroquinone-α-D-glucopyranoside, that is, α-arbutin (4, 5), α-arbutin-α-glycosides (6) and arbutin-α-glycosides (7), and compared their inhibitory activities on human tyrosinase with those of hydroquinone-β-D-glucopyranoside, that is, arbutin. α-Arbutin showed stronger inhibitory activity than arbutin (7) and inhibited the melanin biosynthesis of cultured human melanoma cells and a three-dimensional human skin model under noncytotoxic conditions (8). There are two methods that are thought to be used for glucosylation: chemical and biochemical methods. The chemical synthesis of glucosides is a multiplestep reaction, and results in the production of a mixture of glucosides with α- and β-configurations, and is always accompanied by the formation of some byproducts. On the other hand, the enzymatic synthesis of glucosides is a one-step stereospecific reaction under mild conditions. There are many reports on α-anomer-selective glucosylation to saccharides and aglycones with alcoholic OH groups and phenolic OH groups using α-glucosidase (9), α-amylase (10, 11), neopullulanase (12, 13), cyclodextrin glucosyltransferase (14), and sucrose phosphorylase (15). However, there have been no reports on transglucosylation toward carboxylic groups in acceptor molecules using an enzyme reaction. We describe here the glucosylation of carboxylic compounds by sucrose phosphorylase, focusing on benzoic acid and the structure of benzoyl glucose produced by transfer action. Furthermore, we also report the

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acceptor specificity of sucrose phosphorylase toward various carboxylic compounds.

MATERIALS AND METHODS

Chemicals and reagents Glucose-1-phosphate (G-1-P) was purchased from Sigma (St. Louis, MO, USA). Benzoic acid and sucrose were purchased from Wako Pure Chemical Industries (Osaka). All other chemicals used were obtained commercially and were chemically pure grade.

Enzymes Recombinant *Streptococcus mutans* sucrose phosphorylase was prepared and purified as described by Fujii *et al.* (16). Sucrose phosphorylase from *Leuconostoc mesenteroides* (Oriental Yeast, Tokyo) was purified by chromatography with Phenyl-Toyopearl 650 M (Tosoh, Tokyo). α-Glucosidase from *Saccharomyces* sp. was purchased from Toyobo (Osaka).

General experimental procedures Mass spectra were recorded on a KRATOS Compact-Maldiseq instrument (Kratos Analytical, Manchester, UK). ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) spectra were obtained using a JEOL JNM-A500 spectrometer (JEOL, Tokyo) in DMSO-d_6 and $CDCl_3$ containing tetramethylsilane as an internal standard.

Enzyme assay Sucrose phosphorylase activity was measured as described by Silverstein *et al.* (17). Sucrose phosphorylase was assayed in 50 µl of a reaction mixture containing 5% (w/v) sucrose, 100 mM sodium phosphate buffer (pH 7.0), and the enzyme. The reaction mixture was incubated at 37°C for 20 min, and the reaction was terminated by heating at 100°C for 5 min. The amount of G-1-P produced was coupled to the reduction of NAD in the presence of phosphoglucomutase and glucose-6-phosphate dehydrogenase. One unit of sucrose phosphorylase was defined as the amount of enzyme that caused the reduction of 1 µmol of NAD per minute under these assay conditions.

Determination of glucosylation of benzoic acid A solution (1 ml) containing 0.4% benzoic acid and 20% sucrose in distilled water was used and its pH was adjusted to 4.2 with 5 N NaOH. Sucrose phosphorylase from *S. mutans* (100 U) was added to the solution, and incubated at 37°C for 16 h. The production of glucosylated products was detected as new peaks and new spots by high-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC). HPLC was carried out under the following conditions: column, LiChrospher RP-18 (4.0×250 mm; Merck, Frankfurt, Germany); solvent, acetonitrile-water (1:3, v/v); flow rate, 0.8 ml/min; pump, Shimadzu LC-6AD (Shimadzu, Kyoto); detector, Shimadzu SPD-6A (at 280 nm). TLC was carried out by the ascending method using silica gel (Merck) and a solvent system of acetonitrile–water (85:15, v/v). In the TLC, spots were first detected by irradiating with UV-254 nm, and then visualized by spraying with H_2SO_4-methanol (1:1, v/v) followed by heating at 130°C.

Effect of pH on efficiency of glucosylation by sucrose phosphorylase A substrate mixture (1 ml) containing 0.8% benzoic acid and 30% sucrose in distilled water was used and its pH was adjusted to several pHs with NaOH, and sucrose phosphorylase from *S. mutans* or *L. mesenteroides* (100 U) was added to the substrate mixture. The reaction mixture was incubated at 40°C for 16 h. The amounts of benzoic acid and its glucoside were measured as benzoic acid by HPLC. The conversion ratio of glucosylation was expressed as the percentage of the peak area of the transfer product against the total peak area of the transfer product and benzoic acid.

Time-course analysis of production of benzoyl glucoses A solution (20 ml) containing 2% sodium benzoate and 30% sucrose in distilled water was used and its pH was adjusted to 4.6 with 5 N HCl. Sucrose phosphorylase from *S. mutans* (3200 U) was added to the solution, and the solution was incubated at 40°C for 48 h. During the reaction, the pH of the solution was adjusted between 4.6–4.8 with 5 N HCl. The reaction mixture was analyzed by HPLC using a LiChrospher RP-18 (4.0×250 mm) column with a mixture of methanol-water-phosphoric acid (20:80:0.3, v/v).

Glucosidase treatment To elucidate the structure of the glucoside formed, an aqueous solution containing 0.04% benzoyl glucoses (200 µl) was treated with 10 U of α-glucosidase at 40°C for 15 h. After the hydrolysis, benzoic acid and benzoyl glucoses were analyzed by HPLC on an ODS column. The released glucose was analyzed by TLC.

Isolation of 1-*O*-benzoyl α-D-glucopyranoside (compound 1) A solution (100 ml) containing 0.4% benzoic acid and 20% sucrose in distilled water was used and its pH was adjusted to 4.2 with 5 N NaOH. Sucrose phosphorylase from *S. mutans* (10,000 U) was added to the solution, and the solution was incubated at 40°C for 27 h. The reaction mixture was applied to an amberlite XAD7 column (Organo, Tokyo) eluted with H_2O and 100% MeOH. The fraction eluted with 100% MeOH was chromatographed on a silica gel column (Wakogel C-200; Wako Pure Chemical Industries) eluted with 100% acetonitrile. The fraction containing mainly compound **1** was rechromatographed on the same column eluted with 100% acetonitrile to give the target compound (17 mg).

Isolation of 2-*O*-benzoyl α-D-glucopyranose (compound 2) A solution (20 ml) containing 2.0% sodium benzoate and 30% sucrose in distilled water was used and its pH was adjusted to 4.6 with 5 N NaOH. Sucrose phosphorylase from *S. mutans* (3200 U) was added to the solution, and the mixture was incubated at 40°C for 48 h. The reaction mixture was applied to a Wakogel 100C18 column (Wako Pure Chemical Industries), and eluted with H_2O and 100% MeOH. The fraction eluted with 100% MeOH was chromatographed on a Wakogel C-200 column eluted with 100% hexane and EtOAc–MeOH (20:1). The fraction that contained mainly compound **2** was chromatographed on a mightysil Si60 HPLC column (4.6×250 mm; Kanto Chemical, Tokyo) with ethyl acetate–methanol (25:1, v/v) to give the target compound (12 mg).

Acetylation of benzoyl glucoses A reaction mixture was used and its pH was adjusted to 2.5 with 5 N HCl and treated with diethylether to extract benzoic acid. The aqueous layer was applied to a Wakogel 100C18 column and washed with H_2O to remove free saccharides, and the absorbed fraction was eluted with methanol. The elute was evaporated to dryness, treated with a mixture of acetic anhydride-pyridine, and left to stand for 24 h at room temperature. Acetic anhydride and pyridine in this solution were evaporated and the acetylated benzoyl glucoses were extracted with chloroform. The acetylated benzoyl glucoses were further applied to a Wakogel C-200 column equilibrated with *n*-hexane and eluted with *n*-hexane, *n*-hexane–ethyl acetate (85:15, v/v) and ethyl acetate. The fraction that contained the acetylated benzoyl glucoses was evaporated and dissolved in *n*-hexane–ethyl acetate (1:1, v/v), which was finally purified by preparative HPLC using a mightysil Si60 column (20×250 mm) with *n*-hexane–ethyl acetate (6:4, v/v) to give 2,3,4,6-tetra-*O*-acetyl-1-*O*-benzoyl α-D-glucopyranoside (compound **4**), 1,3,4,6-tetra-*O*-acetyl-2-*O*-benzoyl α-D-glucopyranoside (compound **5**) and 1,3,4,6-tetra-*O*-acetyl-2-*O*-benzoyl β-D-glucopyranoside (compound **6**).

Degradation of 1-*O*-benzoyl α-D-glucopyranoside The pH of aqueous solutions of 1-*O*-benzoyl α-D-glucopyranoside (0.03%) was adjusted to 4.5 and 6.0 with 1 N HCl and NaOH. These solutions were incubated at 40°C for 23 h and then analyzed by HPLC.

Acceptor specificity of *S. mutans* sucrose phosphorylase towards various carboxylic compounds A reaction mixture (1 ml) containing sucrose phosphorylase (50 U), sucrose (40%, w/v) and an acceptor compound (1.0%, w/v) was used and its pH was adjusted to 4.2 with HCl and the mixture was incubated at 37°C for 16 h. The conversion ratio of glucosylation was measured by the same method as described above. In analyzing acetic acid, formic