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原 著

Synthesis of *p*-Nitrophenyl β-D-Gentiobioside by *Streptomyces* β-Glucosidase

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Streptomyces β-glucosidase was found to catalyze the transglucosylation from *p*-nitrophenyl β-D-glucoside (PNPG). A main product formed was purified by HP-20 column chromatogrphy follwing to TOYOPEARL HW-40F column chromatography. The transfer product obtained was identified as *p*-nitrophenyl β-D-gentiobioside (PNPGen) with the enzymatic and acid hydrolysis, and methylation analysis. This result was supported by MS and ^{13}C-NMR.

Streptomyces sp. W 19-1 produced both β-1, 3-glucanase and β-glucosidase using curdlan (β-1, 3-glucan) as a carbon source. The cooperative action of both enzymes resulted in the formation of gentiobiose from curdlan. The β-1, 3-glucanase hydrolyzed curdlan to produce glucose and laminaribiose, and the β-glucosidase formed gentiobiose by transglucosylation from the resultant laminarioligosaccharides, especially laminaribiose.[5]

We showed the purification of *Streptomyces* β-glucosidase and the properties of the enzyme, especially the mechanism of transfer reaction and some phenomena of transfer on various substrates by the enzyme. The enzyme acted on phenolic β-D-glucosides to produce unknown transfer products.[6]

In order to identify the main transfer product (TP) from *p*-nitrophenyl β-D-glucoside by *Streptomyces* β-glucosidase, the main TP was purified and structural

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studies were carried out in the present paper.

MATERIALS AND METHODS

Chemicals. *p*-Nitrophenyl β-D-glucoside (PNPG) was purchased from Nakarai Chemical Co. All other chemicals were obtained commercially and of analytical grade. Sophorose (β-1, 2-glucobiose) was prepared from a partial acid hydrolysate of stevioside (13-O-β-sophorosyl 19-O-β-glucosylsteviol).[3] Laminaribiose (β-1, 3-glucobiose) and gentiobiose (β-1, 6-glucobiose) were prepared from hydrolysates of curdlan by the β-1, 3-glucanase systems from *Streptomyces* sp. K27-4 [7] and W19-1,[4] respectively. Cellobiose (β-1, 4-glucobiose) was purchased from Wakô Pure Chemical Industries.

Chromatography. Thin-layer chromatography (TLC) was done on a Kieselgel 60 plate (Merck Co.) with a solvent system of chloroform-methanol-water (30:20:1, v/v). The sugars on the TLC plate were shown by heating at 110℃ for 10 min after spraying with 50% sulfuric acid (methanol). Diaion HP-20 was from Mitubishi Kasei Co. TOYOPEARL HW-40F was from Tosoh Co.

Enzyme assay. PNPG-hydrolyzing activity was assayed by spectrophotometric measurement of *p*-nitrophenol released from PNPG as described in our previous paper.[5] One unit of the β-glucosidase activity was defined as the amount of the enzyme that released 1 μmol of *p*-nitrophenol per min under the optimum conditions.

Enzymes. *Streptomyces* β-glucosidase was purified as described in our previous paper.[5] *Streptomyces* β-glucosidase crude fraction was used as *Streptomyces* β-glucosidase.

Almond emulsin β-glucosidase (11.5 units/mg) was purchased from Tôyôbô Co. The enzyme preparation did not hydrolyze maltose or isomaltose.

Determination of sugar. The amount of sugar was determined by the method of phenol-H_2SO_4.[8]

Methylation analysis. Sugar was methylated by the method of Ciucanu *et al.*[1] The methylated sugar was hydrolyzed in 10% trifluoroacetic acid, hydrogenated with sodium borohydride, and acetylated with an equal mixture of pyridine and acetic anhydride. The resulting alditol-acetate was analyzed by gas-liquid chromatography on stainless steel column packed with 3 % ECNSS-M on Gas Chrom Q (Nippon Kuromato Kogyo) [2] at 155℃.

Mass spectrum. The mass spectrum (MS) was recorded on a Hitachi M-80 at 70 eV (ionization voltage) using a data processor M-003.

^{13}C-NMR spectrum. A ^{13}C-NMR spectrum was taken on a Bruker AM-500 spectrometer at 125 MHz.

RESULTS

Effect of Substrate Concentration on Transfer Reaction

In order to confirm transfer products (TPs), the experiment on the effect of substrate concentration was performed under the conditions described in the legend of Fig. 1. The result (shown in Fig. 1) indicated that the increase in the yield of

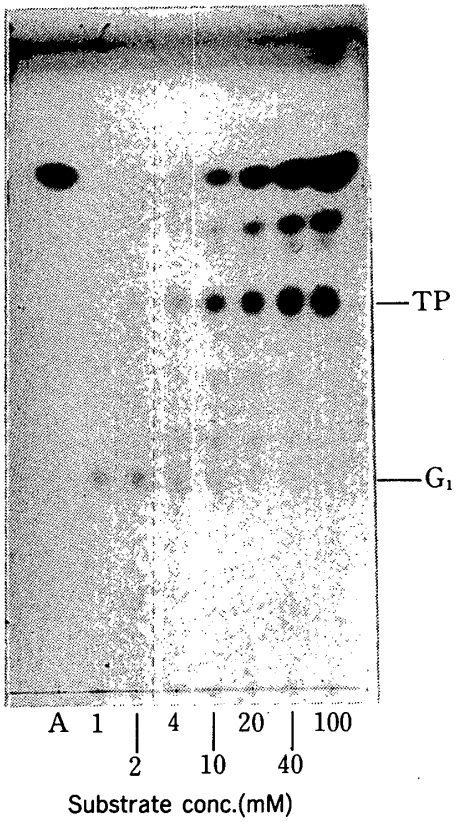


Fig. 1. Effect of Substrate Concentration on Transfer Reaction.

A, Authentic *p*-nitrophenyl β-D-glucoside.

Each reaction mixture contained 0.5 ml of McIlvaine buffer (pH 6.0), 0.4 ml of water and 0.1 ml (1 unit) of enzyme solution except substrate (PNPG). Concentrations of PNPG in the reaction mixtures were as follows; 1, 1 mM; 2, 2 mM; 4, 4 mM; 10, 10 mM; 20, 20 mM; 40, 40 mM; 100, 100 mM. The enzyme reactions were carried out at 55℃ for 30 min. After 30 min, the reaction mixtures were heated at 100℃ for 5 min to inactivate the enzyme. Each 4μl of the mixtures was applied to TLC.

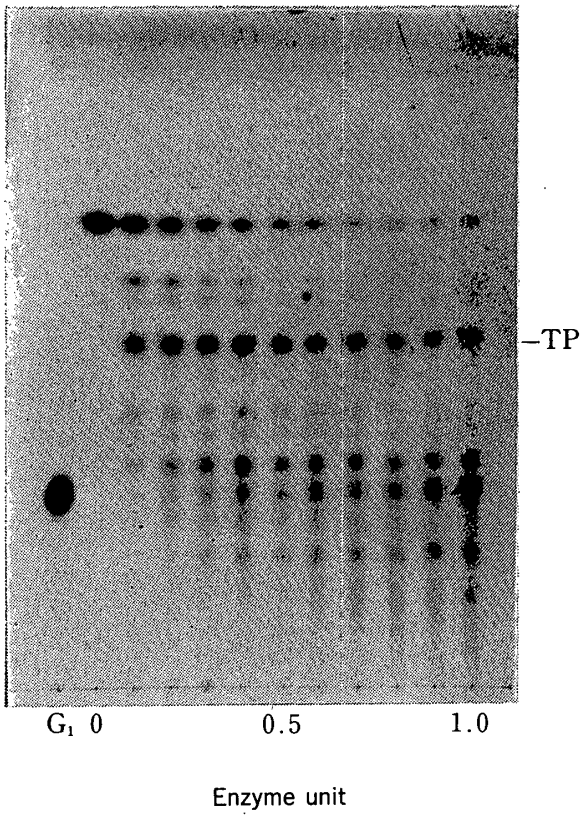


Fig. 2. Effect of Enzyme Concentration on Transfer Reaction.

G_1, Authentic glucose.

Each reaction mixture contained 10 mM PNPG, 0.5 ml of McIlvaine buffer (pH 6.0), 0.4 ml of water and 0.1 ml of enzyme solution. Enzyme concentrations were as follows; 0, 0 unit; 0.5, 0.5 unit; 1.0, 1.0 unit per 1 ml (reaction mixture). The enzyme reactions were carried out at 55℃ for 1 hr. After 1 hr, the reaction mixtures were heated at 100℃ for 5 min to inactivate the enzyme. Each 4μl of the mixtures was applied to TLC.

TPs from PNPG was related with substrate concentration, and that the amount of TPs increased in proportion to substrate concentration. But, in more than 20 mM PNPG, original PNPG was mainly remained, and other TPs were produced. In the concentration of 10 mM PNPG (0.6 %), the percentage of formation of main TP was much higher.

Effect of Enzyme Concentration on Transfer Reaction

In order to determine suitable *Streptomyces* β-glucosidase concentration, the experiment on the effect of enzyme concentration was performed under the conditions described in the legend of Fig. 2. The result (shown in Fig. 2) indicated that the amounts of the main TP was much higher in the lower enzyme concentration.

Purification of TP

In preliminary experiments, we decided the optimum condition for the formation of TPs as follows; PNPG 10 mM, *Streptomyces* β-glucosidase 0.1 unit/ml,

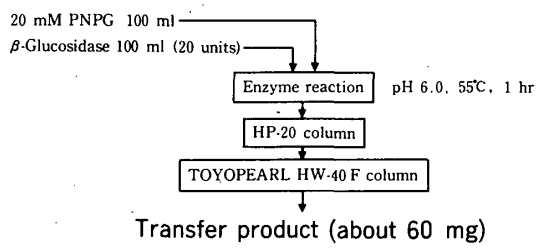


Fig. 3. Flow Chart of Purification of Transfer Product.

pH 6.0 (McIlvaine buffer), 55℃, 1 hr. After the enzyme reaction, the reaction mixture (200 ml) was heated at 100℃ for 5 min to inactivate the enzyme. The resultant mixture was put on a column (2×10 cm) of Diaion HP-20. After washing with distilled water to remove salt, glucose and oligosaccharides, phenolic sugars were eluted with 50% methanol. The resultant eluate was concentrated and put on a column (1.5×90 cm) of TOYOPEARL HW-40F, and then main TP and original PNPG were eluted with distilled water. Main TP was lyophilized to give white powder (about 60 mg). Flow chart of purification procedure of TP was shown in Fig. 3. Pattern of gel filtration on TOYOPEARL HW-40F was shown in Fig. 4.

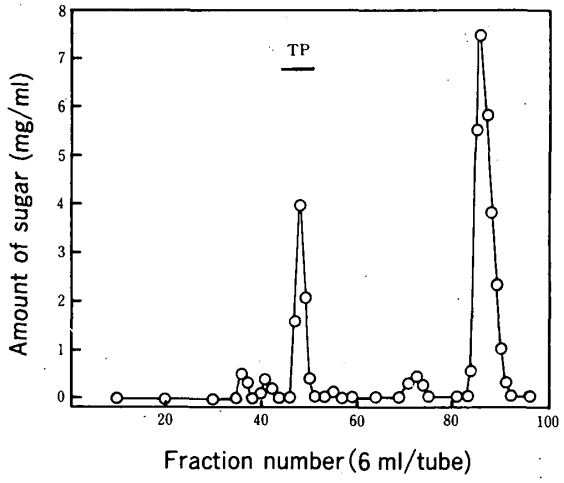


Fig. 4. Pattern of Gel Filtration on TOYOPEARL HW-40F.

Structural Studies of TP

i) *Methylation analysis.* On the methylation analysis, TP gave 2,3,4,6-tetra and 2,3,4-tri-O-Me-D-glucoses in the approximate molar ratio of 1 : 1 (Table I). The result was similar in the case of gentiobiose.

ii) *Mild Acid Hydrolysis.* TP was hydrolyzed at 100℃ in 0.1 N hydrochloric acid, and the progress of hydrolysis was followed by TLC (as shown in Fig. 5). After partial acid hydrolysis, TP gave glucose, gentiobiose and PNPG.

iii) *Hydrolysis by Almond Emulsin β-Glucosidase.* The enzyme hydrolysis

Table I. Methylation Analysis of Transfer Product

	Alditol acetates of				
	3,4,6-Tri-O-Me-D-Glc	2,4,6-Tri-O-Me-D-Glc	2,3,6-Tri-O-Me-D-Glc	2,3,4-Tri-O-Me-D-Glc	2,3,4,6-Tetra-O-Me-D-Glc
Retention time(min)	11.2	11.6	14.9	14.5	5.4
Authentic saccharides					
Sophorose	+				+
Laminaribiose		+			+
Cellobiose			+		+
Gentobiose				+	+
Transfer product				+	+

Me, methyl; Glc, glucopyranose; +, 1 mol.

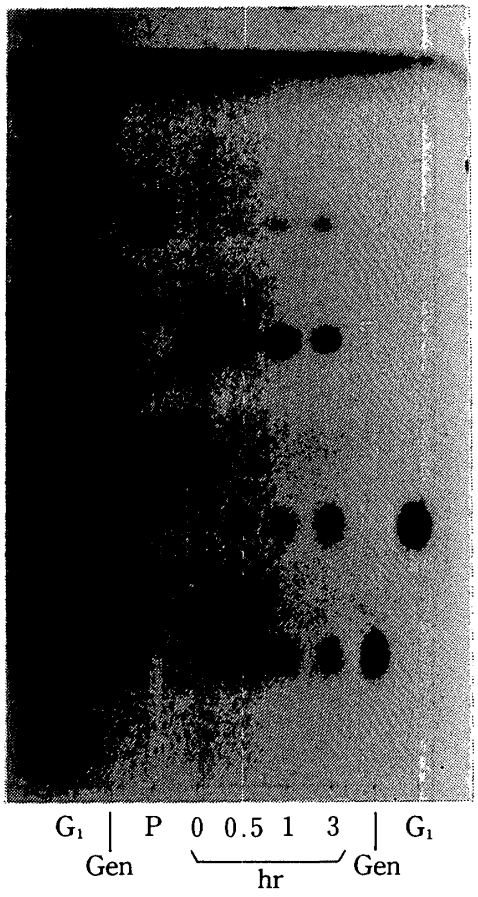


Fig. 5. Acid Hydrolysis of Transfer Product.
G_1, Authentic glucose;
Gen, Authentic gentiobiose;
P, PNPG.

was done at 55℃, pH 5.5 for 30 min in a 0.5% sample solution containing 1 mg of the enzyme. The enzyme hydrolyzed TP to produce glucose, PNPG and *p*-nitrophenol.

From structural studies described above, the proposed structure of TP is seemed to be *p*-nitrophenyl *β*-D-gentiobioside (PNPGen).

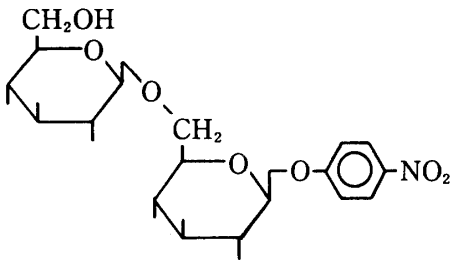
iv) *MS and* ^{13}C*-NMR.* The structure of TP was also supported by the EI-MS and ^{13}C-NMR spectra. The EI-MS spectrum of TP showed the sodium additive ion at m/z 486 and potassium additive ion at m/z 502. The molecular weight of TP is theoretically 463.

The ^{13}C-NMR spectrum of TP had two *β*-glucosyl anomeric carbon signals at *δ*99.0 and *δ*102.2, also had phenolic compound signals.

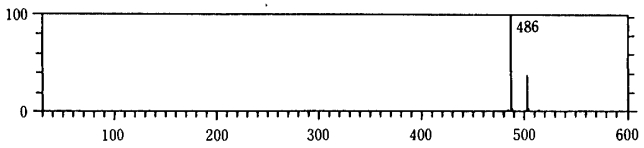
These spectra and the structure of PNPGen were shown in Fig. 6.

DISCUSSION

Streptomyces *β*-glucosidase is active against PNPG. Under the condition (1 mM



p-nitrophenyl *β*-D-gentiobioside



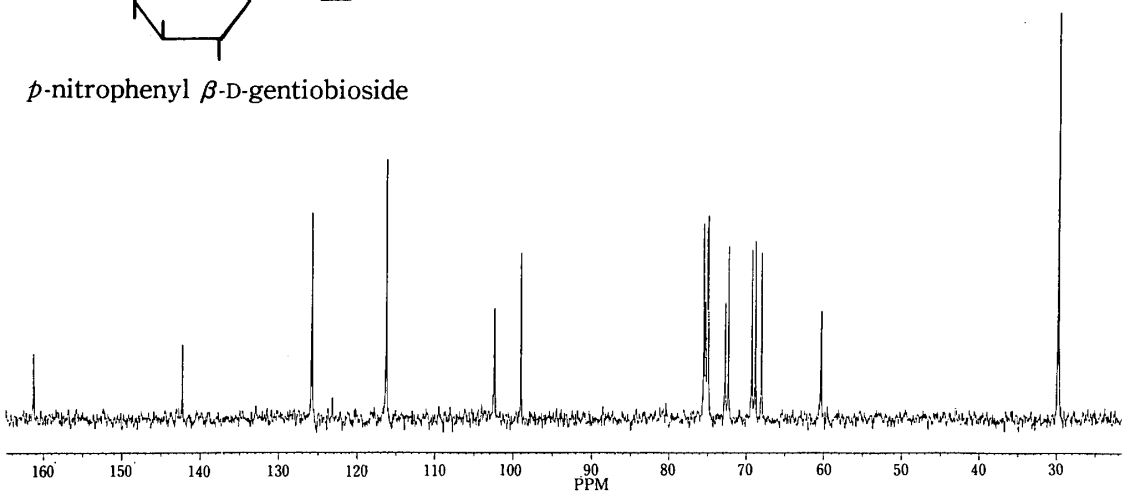


Fig. 6. Identification of Transfer Product.

PNPG) of the measurement for β-glucosidase activity, hydrolysis of PNPG is only found. However, under the higher substrate concentration (more than 10 mM, about 0.6%), we can easily find some TPs. This β-glucosidase has so strong transfer action that the enzyme seems to be a transglucosidase.

Main TP was purified and identified as *p*-nitrophenyl β-D-gentiobioside. This TP is a new substance that we could not find in the literature. The yield of TP was almost 10% against original PNPG.

The mechanism of formation of TP is seemed to be similar to that of formation of gentiobiose from laminaribiose by *Streptomyces* β-glucosidase.[6] That is as follows; A PNPG-enzyme complex is at first formed between PNPG and enzyme. *p*-Nitrophenol of PNPG is removed form the PNPG-enzyme complex by its hydrolytic action, but the nonreducing end glucosyl residue is allowed to form a glucosyl-enzyme complex. The glucosyl residue of the glucosyl-enzyme complex is transferred to the OH group at C-6 of free PNPG, and *p*-nitrophenyl β-D-gentiobiosde (PNPGen) is produced via the formation of a PNPGen-enzyme complex.

The position of transfer is favorable for the C-6 of free PNPG, because PNPGen is a main TP. This PNPGen is seemed to be used as a substrate for gentiobiohydrolase (which is not found yet) or β-1, 6-glucanase.

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放線菌β-グルコシダーゼによるp-ニトロフェニルβ-D-ゲンチオビオシドの合成

——渡辺　悟、田崎　弘之、日下部　功、村上　和雄——

放線菌β-グルコシダーゼはその基質であるp-ニトロフェニルβ-D-グルコシドより転移物を生成した。主要な転移物をHP-20カラムクロマトに続くTOYOPEARL HW-40Fカラムクロマトグラフィーにより精製し、その構造研究を行ったところ、p-ニトロフェニルβ-D-ゲンチオビオシドと同定された。