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# Gentiobiose Synthesis from Glucose Using Recombinant β-Glucosidase from *Thermus caldophilus* GK24

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**Abstract** Recombinant β-glucosidase from *Thermus caldophilus* GK24 was easily purified partially by a heat treatment procedure, resulting in 8-fold and recovery yield of 80% from crude enzyme. When the β-glucosidase was incubated with a 80% glucose solution (w/w), gentiobiose (β1,6-glucobiose) was the major product in the reaction mixture. The optimal conditions for producing gentiobiose (11% yields of total sugar) were pH 8-9 and 70°C for 72 h.

*Keywords*: gentiobiose, β-glucosidase, *Thermus caldophilus* GK24

β-Glucosidase(β-D-glucoside glucohydrolase, E.C. 3.2.1.21) catalyzes hydrolysis of glycosidic linkages in aryl and alkyl β-glucosides and cellobiose [1] and can be used for the synthesis of disaccharide and trisaccharide (by transglycosylation) [2]. Several *Thermus* strains produce a thermostable β-glucosidase, which shows broad substrate specificity for other β-glycosides such as cellobiose and lactose [3]. Such thermostable β-glucosidases catalyze biochemical reactions at high temperature and have a longer half-life in enzyme preparations, making them attractive for their extreme stability and resistance to organic solvents and detergents [4]. Practical applications of thermostable β-glucosidases include the hydrolysis of various biologically active oligosaccharides and synthesis of oligosaccharide [2,5], food processing, carbohydrate structure research, the flavor industry [6], and as a drug for Gaucher's disease [7]. Gentio-oligosaccharides ($Glu\beta1\text{-}6[Glu\beta1\text{-}6]_n$ where n = 1 ~ 5) are glucose polymers that can be used as prebiotics for human gut health [8]. Accordingly, the current study investigated the optimum conditions for producing gentiobiose (6-O-β-D-glucopyranoside-β-D-glucose; disaccharide) from glucose using the β-glucosidase.

The gene encoding β-glucosidase from *Thermus caldophilus* GK24 was cloned and expressed in pKK223-3/MV1184 [4]. The gene contains an open reading frame encoding 431 amino acids with a molecular weight of 48,658 Da. The recombinated *E. coli* was cultured at 37°C in an LB medium containing 100 μg ampicillin per mL. When the $A_{600}$ reached 0.5 ~ 0.7, 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG, Sigma Co, USA) was added to the culture medium and cultured for 7 h. The cells were then harvested by centrifugation and the pellet suspended in buffer A (50 mM potassium phosphate pH 7.2). Thereafter, the cells were disrupted using an ultrasonic homogenizer of Branson sonifier 450 (Branson Ultrasonics Co., USA) and centrifuged at 10,000×*g* for 30 min at 4°C.

The supernatant was heated at 80°C for 30 min, and then cooled in an ice bath for 1 h. Next, the heat-treated solution was centrifuged at 10,000×*g* for 45 min at 4°C and the supernatant filtered using a 0.22 μm syringe filter (Millipore, USA). The enzyme activity was assayed by hydrolyzing the *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG, Sigma Co, USA) and the protein amount checked using the Lowry method.

The heat-treated enzyme (40 U/mL) was then used to produce β-gentiobiose (6-O-β-D-glucopyranoside-β-D-glucose) from glucose (80%, 90% and 100% in buffer A, w/w). To determine the optimal conditions for producing β-gentiobiose, the reaction was performed with variable pHs (4-9), temperatures (4-90°C), and reaction times (1-72 h). To study the effect of each condition, one parameter was varied at a time, while keeping the other parameters fixed. The reaction products were analyzed by Bio-LC (Metrohm, Swiss) using Metrosep Carb 1 – 250 column (4.6 × 250 mm) eluted with 30 mM sodium acetate and 100 mM NaOH. Before injecting the reaction mixture, the solution was boiled for 5 min, centrifuged at 10,000×g for 10 min, and the supernatant filtered using a 0.22 μm syringe filter.

For preparative purification, silica gel column chromatography (30 × 300 mm) with solvent A (ethylacetate : methanol : water = 6 : 4 : 1; v/v/v) was applied to the reaction mixture. The eluate was purified (recovery yield was 60%) and loaded onto a silica TLC plate (20 × 20 cm). The developing solution in the TLC chamber was 6 : 4 : 1 of ethylacetate : methanol : water. The collected β-gentiobiose part was then resuspended in distilled water. Using the product solution, the NMR and mass spectra were determined by tandem mass spectrometry and 300 MHz-NMR at the Korea Basic Science Institute

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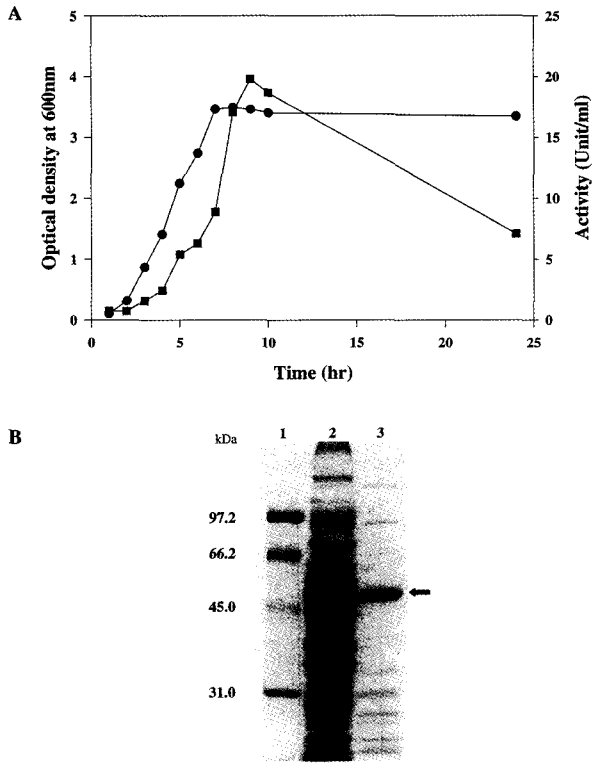


**Fig. 1.** Time course of β-glucosidase production and cell growth under optimal culture conditions in flask (A) and SDS-PAGE (12%) gel of the β-glucosidase (B). Symbols of (A): (●) cell growth, (■) enzyme activity and lanes of (B): lane 1. Molecular marker 79.2, 66.2, 45.0, 31.0, Lane 2. crude extract, Lane 3. heat treatment solution.

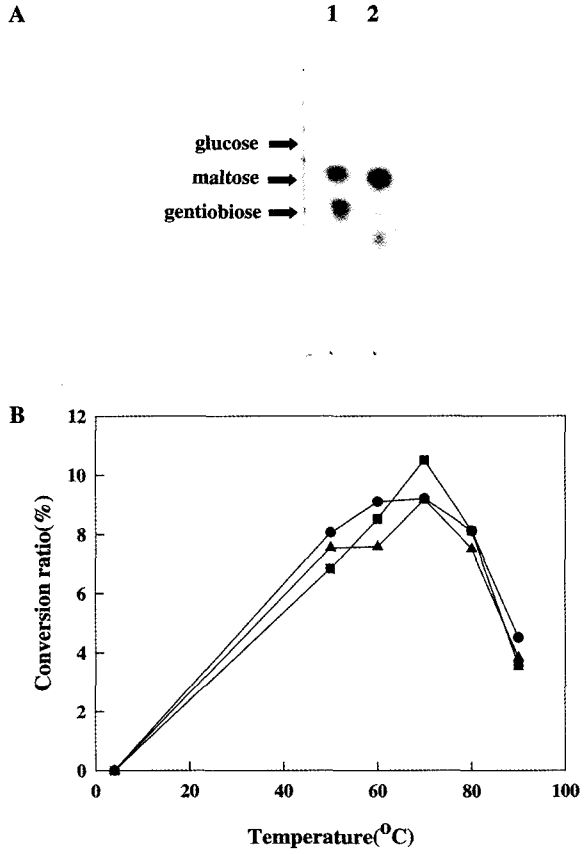


**Fig. 2.** Identification of reaction mixture by TLC (A) and production of β-gentiobiose using β-glucosidase with D-glucose (B). (A)lane 1: glucose, maltose, gentiobiose (top to bottom) and lane 2: products of enzyme reaction. (B) enzyme loading (40 U/mL) and conversion ratio=gentiobiose/initial glucose Symbols: (●) 100% glucose, (▲) 90% glucose, (■) 80% glucose.

(KBSI) and Korea Research Institute of Bioscience and Biotechnology (KRIBB), respectively.

The time course of β-glucosidase production by the recombinant *E. coli* (pKK223-3/MV1184) is shown in Fig. 1. Enzyme activity was detected in the culture with the LB medium at 37°C, the highest concentration was reached 8 h after inoculation, and the maximum activity was 20 U/mL at 9 h. Although the crude extracts included many *E. coli* enzymes, the thermostable enzyme, β-glucosidase, was partially purified 8-fold with recovery yield of 80% from crud enzyme by heat treatment procedure. Several other purified β-glucosidases have also been reported from other thermospecies: *Thermoascus aunantiacus* (specific activity of 206 U/mg) and *Thermotoga neapolitana* (specific activity of 255 U/mg) [9,10]. However, in the current study, the specific activity of the recombinant heat-treated β-glucosidase used to prepare gentiobiose from glucose was 2 U/mg.

The incubation of the β-glucosidase with glucose gave various transglycosylation products (identified by TLC: Fig. 2A). The TLC data showed fractions of maltose and gentiobiose, which were also confirmed by Bio-LC (data not shown). Among the different variables, temperature was identified as the major factor for the synthetic reaction, while the pH only had a minor impact (data not shown). The gentiobiose synthesis by β-glucosidase from almond (16% after 50 h) and (75% after 20 days) Thai rosewood β-glucosidase has been reported by Park *et al.* [11] and Srisomsap *et al.* [2]. Our studies also indicated that β-glucosidase using 80% (w/w) glucose as the substrate produced gentiobiose of the maximum conversion yield (11% of total sugar) as the major product at 70°C after 3 days (Figure 2B). Despite many experimental works upon transglycosylation reactions, quantitative theoretical analyses have not been carried out except in a few cases, due to complexity of the mechanism and many adjustable kinetic parameters.

The β-gentiobiose was purified by silica gel column chromatography and preparative TLC. Finally, 30 mg of β-gentiobiose was obtained from 1 mL of the enzyme reaction mixture. To identify the structure, the purified β-gentiobiose was analyzed using a mass and NMR spectrometer. The mass spectrum revealed a molecular weight of 342 for the β-gentiobiose (Fig. 3). Meanwhile, the FT-

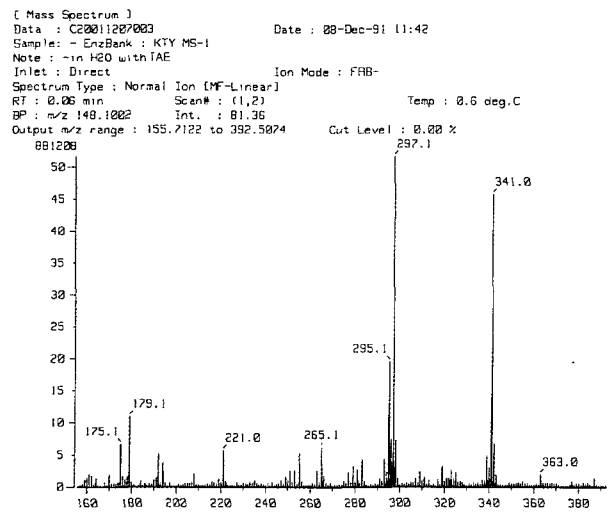


**Fig. 3.** Mass spectrum of β-gentiobiose (product from enzyme reaction) using tandem mass spectrometer. (Spectrum type: Normal Ion [MF-Linear], Ion mode: FAB) The main peak shows a molecular weight of 342, while the solvent peak is 297.1

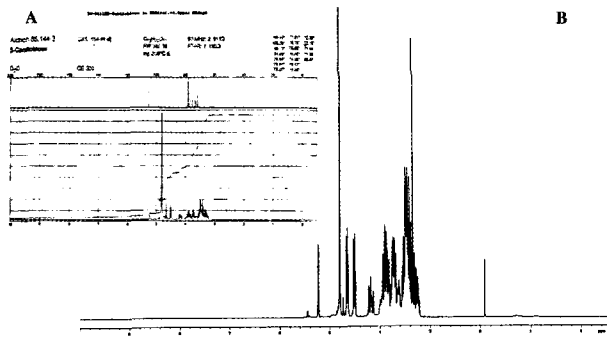


**Fig. 4.** NMR spectrum of β-gentiobiose. Standard spectrum of gentiobiose in Aldrich library of $^{13}C$ and $^{1}H$ FT-NMR spectrum (A) and FT-NMR spectrum of purified gentiobiose (B).

NMR spectrum of the β-gentiobiose was the same as that of the Aldrich library NMR spectra (Fig. 4). In general, gentiobiose has both a bitter and the sweet taste. In addition, since it is not hydrolyzed in the stomach or small intestine, gentiobiose reaches the colon intact, making it a good prebiotic candidate [12].

Accordingly, β-glucosidase is clearly an asset for the production of gentiobiose, due to its operational stability under different temperatures, organic solvents, and metal ions. Further work on scaling-up enzymatic gentiobiose production is currently in progress.

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