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Acceptor specificity of amylomaltase from *Corynebacterium glutamicum* and transglucosylation reaction to synthesize palatinose glucosides

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ABSTRACT

Acceptor specificity for intermolecular transglucosylation reaction of amylomaltase from *Corynebacterium glutamicum* was investigated using starch as glucosyl donor and various saccharide acceptors. Maltooligosaccharides (G1–G4), mannose, palatinose and sucrose were efficient acceptors; the best one was glucose. This amylomaltase preferred hexose sugar containing the same configuration of C2-, C4- and C6-hydroxyl groups as glucopyranose. Palatinose was chosen as suitable acceptor for the synthesis of palatinose glucosides (PGs). The optimal condition was to incubate 5 U/ml amylomaltase with 7.5 mM palatinose and 1.0% (w/v) soluble potato starch at 30 °C for 24 h. In addition to PGs, maltooligosaccharides were also produced as by product. The product yield was 67.9%, in which the ratio of PGs to maltooligosaccharides was 1:1. Then PGs were separated by Bio-Gel-P2 column chromatography and analyzed by HPAEC. PG1–PG13 were identified with PG1 and PG2 as major products. NMR analysis showed that the PGs produced are novel products, PG1 and PG2 were a tri- and tetra-saccharide with the structure [*O*-α-D-glucopyranosyl-(1→4)]$_n$-*O*-α-D-glucopyranosyl-(1→6)-D-fructofuranose, where n = 1–2. PG was less sweet than palatinose and sucrose, more hygroscopic with similar prebiotic activity as palatinose. PGs thus have potential to replace sucrose or palatinose in food products for health benefits.

1. Introduction

Amylomaltase (EC 2.4.1.25), an intracellular 4-α-glucanotransferase (4αGTase), was first found in *Escherichia coli* as an enzyme involved in maltose metabolism [1,2]. The enzyme was later reported in most thermophilic bacteria and archaea such as *Thermus aquaticus* [3], *Aquifex aeolicus* [4] and *Pyrobaculum aerophilum* IM2 [5]. The corresponding D-enzyme was reported in plants, e.g. potato [6] and cassava [7]. Amylomaltase catalyzes an intermolecular transglucosylation reaction, transferring glucosyl residues from donor to acceptor at free OH group of C4 to produce longer linear oligosaccharides. In addition, this enzyme possesses a unique intramolecular transglucosylation reaction to yield cycloamyloses or large-ring cyclodextrins (LR-CDs) with a degree of polymerization (DP) from 16 onwards. The enzyme has four different activities: disproportionation, cyclization, coupling and hydrolysis, with the first two as main [8,9]. Amylomaltase has many potential applications. Firstly, it is used in the production of LR-CDs via cyclization reaction. LR-CD can form inclusion complex with guest molecules by trapping them into hydrophobic cavity resulting in a change of solubility, stability and biological properties of guest molecules [8]. Secondly, amylomaltase is used to modify starch and produce thermo-reversible starch gel with gelatin-like property [5,10] and can be used as fat and cream alternatives in dairy products to improve creaminess in low-fat yogurt [11]. Lastly, the enzyme can be used in the production of linear oligosaccharides and glucoside products through intermolecular transglucosylation reaction. Short-chain isomaltooligosaccharides (IMOs) as DP2–DP6 with prebiotic activity synthesized by combination of amylomaltase and transglucosidase have been reported [12].

Palatinose (isomaltulose, 6-*O*-α-D-glucopyranosyl-D-fructofuranose) is a disaccharide consisting of glucose and fructose linked by an α-1,6 glycosidic bond. Naturally, palatinose is found in honey, sugar cane [13,14] and it can be synthesized by enzymatic method. Palatinose was first produced as a by-product in dextran production process [15]. It was also synthesized by sucrose isomerase from many organisms such as *Protaminobacter rubrum* [16], *Erwinia rhapontici* [17], and *Pantoea dispersa* [18] using sucrose as substrate. Sucrose isomerase catalyzes an isomerization of sucrose, converting α-1,2 glycosidic bond to α-1,6 glycosidic

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bond [19]. Various immobilized enzymes, such as immobilized α-glucosyltransferase from *Serratia plymuthica* NCIB No. 8285, have also been used in palatinose production [20,21]. Palatinose is hydrolyzed more slowly than sucrose so plasma glucose and insulin levels are gradually increased to reach the maximum level which is lower than that of sucrose [22,23]. For this reason, palatinose is suitable to be used as a sugar substitute [24] and sweetener for diabetics [22,25]. Moreover, palatinose reveals anticariogenic property by inhibition of glycosyltransferase (GTase) activity of *Streptococcus mutans*. Production of organic acids, plaque and insoluble glucan from sucrose that involved in causing dental caries are inhibited [26–28].

In recent year, the demand for glucoside products in food, drink and cosmetic industries increases, as glucosides usually have better properties, e.g. solubility, stability and bioactivity, than their parent compounds. Most glucoside products have been produced by transferase or hydrolase [29]. For example, 3-*O*-α-maltosyl-L-ascorbate with high stability under oxidative conditions was synthesized from α-maltosyl fluoride and L-ascorbic acid catalyzed by mutated cyclodextrin glycosyltransferase (CGTase) [30]. And isomaltooligosaccharides with prebiotic activity were produced by transglucosylation reaction of α-glucosidase from *Microbacterium* sp. using maltose as substrate [31]. However, for palatinose, only one report on the synthesis of palatinose glucosides has been found. The tri- and tetra-saccharide glucoside products of palatinose were synthesized by *Thermoanaerobacter brockii* kojibiose phosphorylase using β-D-glucose 1-phosphate and palatinose as substrates [32].

From our previous study, a novel amylomaltase from *Corynebacterium glutamicum* ATCC 13032 with low amino acid sequence identity (20–25%) to amylomaltases from *Thermus* sp. was reported. The enzyme gave cycloamyloses or LR-CD products in the range of DP19-DP50 from its intramolecular transglucosylation activity [33]. Maltooligosylsucrose with anticariogenic property and the prebiotic isomaltooligosaccharides were successfully synthesized by the intermolecular transglucosylation reaction of this amylomaltase [34]. In this study, we here focus on investigation of acceptor specificity for saccharides of *C. glutamicum* amylomaltase and the synthesis of palatinose glucosides through the intermolecular transglucosylation activity of the enzyme.

2. Materials and Methods

2.1. Materials

Maltooligosaccharides: D(+)-glucose, G1 to maltoheptaose, G7; D(+)-mannose, D(+)-cellobiose, D(−)-fructose, L-fucose, L-rhamnose and palatinose were purchased from Sigma (USA). D(+)-galactose and sucrose were products from Bio Basic (Canada) while D(−)-arabinose was from BDH Chemical (England). Ribose was obtained from Wako (Japan) while D(+)-allose and D(+)-melibiose were products from Tokyo Chemical (Japan). Lactose and raffinose were purchased from Ajax Finechem (Australia). Soluble potato starch was from Scharlau (Spain) and Bio-Gel-P2 beads were from Bio-Rad Laboratories (USA). Ampicillin, glucoamylase from *Aspergillus niger*, isopropyl β-D-1-thiogalactopyranoside (IPTG) and rat intestinal acetone powder were purchased from Sigma (USA). All other chemicals were of analytical grade.

2.2. Preparation and purification of amylomaltase

Amylomaltase gene from *C. glutamicum* ATCC 13032 was expressed in *E. coli* BL21(DE3) using pET-19b expression vector as previously described [33]. A recombinant clone was cultured in LB medium containing 100 μg/ml ampicillin at 37 °C until O.D.$_{600}$ reached 0.4–0.6, then 0.4 mM IPTG was added to induce the enzyme production. Intracellular crude enzyme was obtained, after cell sonication the enzyme was purified by HisTrap affinity (1 ml, HisTrap FF^{TM}) column chromatography. Starch transglucosylation activity [33] was measured as described below and protein concentration was determined by Bradford method [35].

2.3. Determination of acceptor specificity and synthesis of glucoside products

Saccharides tested as acceptor substrates were classified into four groups: monosaccharides, which were divided into three subgroups: hexose (glucose, allose, mannose, galactose and fructose), deoxy hexose (fucose and rhamnose) and pentose (arabinose and ribose), disaccharides (maltose, cellobiose, lactose, melibiose, palatinose and sucrose), trisaccharides (maltotriose and raffinose) and oligosaccharides (G4–G7).

The acceptor specificity was determined by starch transglucosylation activity of amylomaltase which was assayed as follows. The 600 μl reaction mixture containing 0.2%, w/v soluble potato starch, 2.5 mM saccharide and 60 μl purified amylomaltase in 50 mM phosphate buffer, pH 6.0 was incubated at 30 °C for 10 min, after that the reaction was stopped by boiling. Then, 100 μl of sample was withdrawn to measure the decrease in starch by reacting with 1 ml of iodine solution (0.02% I_2 in 0.2% KI, w/v) and A_{600} was determined. The acceptor with the highest activity was set as 100% of relative activity. The reaction mixtures were analyzed by TLC and HPLC for glucoside products.

One unit of starch transglucosylation activity was defined as the amount of enzyme that produces 1% decrease in blue color of starch–iodine complex per minute.

2.3.1. Thin Layer Chromatography (TLC)

Sample was spotted on TLC plate (Silica gel 60, Merck) and twice developed in butanol:pyridine:water (5:4:1 by volume) [34]. Glucoside products were detected by spraying the mixture of concentrated sulfuric acid and absolute methanol (1:2 by volume), then heated at 110 °C for 20 min [36].

2.3.2. High Performance Liquid Chromatography (HPLC)

Analysis of glucoside products was performed by HPLC (Shimadzu 10AVP, Japan). The reaction mixture was centrifuged (18,000 × *g*, 4 °C, 45 min) to remove majority of the remain soluble potato starch before loading onto a Resex RSO-Oligosaccharide Ag^+ column (200 × 10.0 mM, Phenomenex, Inc., USA,). The glucoside products were eluted by ultrapure water at 80 °C with a flow rate of 0.2 ml/min and detected by refractive index detector (RID) [34]. The yield of glucoside products was calculated from the equation:

$$\text{Product yield}\,(\%) = \frac{\text{Peak area of product}}{\text{Peak area of acceptor at } t_0} \times 100$$

when peak area of product was determined from the difference between palatinose peak obtained when reaction mixture was treated by glucoamylase and that of untreated.

2.4. Optimization of the synthesis of glucoside products

Optimization for the highest glucoside product yield was performed in 50 mM phosphate buffer, pH 6.0 at 30 °C [33] by varying four parameters: palatinose concentration (0–15 mM), soluble potato starch concentration (0–5% w/v), enzyme concentration (0–12 U/ml) and incubation time (0–42 h). Analysis of glucoside products was performed by HPLC.