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Synthesis of long-chain isomaltooligosaccharides from tapioca starch and an *in vitro* investigation of their prebiotic properties

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ABSTRACT

The recombinant wild-type (WT) or Y101S-mutated amylomaltase prepared from the gene screened from soil DNA was used in combination with *Aspergillus niger* transglucosidase to produce isomaltooligosaccharides (IMOs) from tapioca starch. The highest IMO yield was obtained when 30% (w/v) soluble tapioca starch was incubated with 120 Units of both amylomaltases and 6 Units of transglucosidase at 40 °C for 30 min with WT or for 1 h with Y101S-mutated enzymes. Mass spectrometry and ^{1}H NMR analysis of the IMOs synthesized from WT and Y101S-mutated enzymes showed the presence of α-1,4 and α-1,6 glycosidic bonds with a polymerization degree of ≤9. HPAEC-PAD analysis showed that the sizes of the IMOs synthesized from both enzymes were significantly larger than those of commercial IMOs. A smaller proportion of long-chain IMOs was observed when Y101S-amylomaltase was used. As for physical and biological properties, the degree of sweetness of both synthesized IMOs was lower than those of sucrose and commercial IMOs, while the brown color from the Maillard reaction, the viscosity and the hygroscopicity were higher than those of commercial IMOs. The prebiotic properties of both synthesized IMOs showed that they were able to tolerate acidic conditions, heat, and human digestive enzymes. In addition, these IMOs could stimulate the growth of *Lactobacillus casei* and resulted in a decrease of the culture pH, similar to the effect of commercial IMOs.

1. Introduction

Amylomaltase (EC. 2.4.1.25), a 4α-glucanotransferase of bacterial cells, is an intracellular enzyme involved in maltose metabolism [1,2]. This enzyme catalyses the transglucosylation reaction of α-1,4-glucan, in which the transfer reaction of the glucosyl moiety may be through an intermolecular or intramolecular transglucosylation reaction. The intramolecular transglucosylation, also called cyclization, produces cycloamylose, while the intermolecular transglucosylation or disproportionation reaction produces a longer linear oligosaccharide [2]. One interesting application of amylomaltase is its use in the production of prebiotic isomaltooligosaccharides (IMOs) *via* the intermolecular transglucosylation reaction. IMOs are non-digestible prebiotic oligosaccharides with health benefits for the host. They selectively stimulate the growth or activity of non-pathogenic bacteria while limit a number of pathogenic bacteria in the colon [3–6]. The production of long-chain IMOs is increasingly becoming a subject of interest in the health food industry. Accordingly, several research groups have investigated a new process to produce IMOs in a more efficient way. Usually, the production of IMOs from starch has required a multi-stage process [7–10]. Starch is first hydrolysed into α-1,4-linked dextrins, with α-amylase (E.C. 3.2.1.1), pullulanase (EC 3.2.1.41) and β-amylase (E.C. 3.2.1.2) normally used. The α-1,4-linked dextrins are then converted to α-1,6-linked oligosaccharides using α-D-glucosidase (E.C. 3.2.1.20). Principally, the enzymatic synthesis of a wide variety of oligosaccharides has been attained *in vitro* using the transfer reactions that occur between a segment of the donor and various acceptors [11,12]. The specificity of the transfer is dependent on the enzyme used, which usually determines the configuration of the glycosidic bond that is formed. The structure of the acceptor also plays a role in determining the trans-

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fer position for the formation of a glycosidic bond. Other enzymatic routes for IMO production have been explored; the main example is the use of α-glucanotransferase from *Thermotoga maritima* in combination with maltogenic amylase from *Bacillus stearothermophilus* to produce IMOs from corn starch with a 67.4% yield [8]. In addition, IMOs are being industrially produced from maltose and maltodextrins through the action of *Aspergillus* sp. α-transglucosidase (EC 2.4.1.24), yielding a commercial IMO product with a low DP in the range of IMO2-IMO6 [13]. The limitation of short-chain IMOs such as isomaltose (IMO2), isomaltotriose (IMO3), panose, and isomaltotetraose (IMO4) is that they can be partially digested and absorbed in the small intestine [14,15]. As reported, long-chain IMOs are more effective than the short-chain varieties in increasing microflora such as *Bifidobacteria* and *Lactobacillus* [14]. We have previously shown that the p17AMY recombinant amylomaltase could be efficiently used in combination with *Aspergillus niger* transglucosidase (or α-glucosidase, EC 3.2.1.20) for the synthesis of IMOs (IMO2-IMO6) from maltotriose substrate [16]. However, the IMOs obtained have the same chain length as the commercially available IMOs.

Therefore, the purpose of this study was to explore the newly developed use of WT or Y101S-mutated amylomaltase in cooperation with *A. niger* transglucosidase for synthesizing IMO products from tapioca starch. This novel use has not, to date, been reported. Moreover, the IMOs produced from the co-action of these two different enzymes were characterized and compared with the commercial IMO products. We also report the physical and biological properties of IMOs to assess the value of their use in processed foods.

2. Materials and methods

2.1. Materials

D(+)-Glucose, D(+)-sucrose, maltooligosaccharides (MOSs) with a degree of polymerization (DP) of 2–7, panose (P), rat intestinal acetone powder and bovine serum albumin (BSA) were obtained from Sigma–Aldrich (USA). Isomaltooligosaccharide standard (IMO2-IMO9) and 2-*O*-α-isomaltosyl-D-glucose (I-K2) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Bio-Gel® P-2 Gel was obtained from Bio-Rad Laboratories (USA). *Rhizopus* sp. glucoamylase (EC 3.2.1.3) was purchased from Sorachim (Genas, France) and *A. niger* transglucosidase (or α-glucosidase, EC 3.2.1.20) was obtained from Amano Enzyme Inc., (Nagoya, Japan). Tapioca starch (Flomax®) was a gift from the National Starch and Chemical Co., (USA). All other chemicals used were of analytical grade.

2.2. Amylomaltase preparation

Escherichia coli cells harboring the p19bAMY or the Y101S recombinant plasmid were grown in an LB medium containing 100 μg/ml ampicillin at 37 °C for 24 h. The pET19b-based plasmids in both recombinant cells contained the original amylomaltase gene (isolated directly from soil DNA) and the Y101S-mutated amylomaltase gene. This isolated gene had a high amino acid sequence identity to amylomaltase of *Thermus thermophilus* [17]. The expression of both recombinant enzymes was induced by 0.8 mM IPTG when A_{600} of the culture reached 0.5. After 4 h, cells were harvested and sonicated using a Vibra Cell™ VCX130 (tip diameter of 5 mm, Sonics, USA) in an ice bath with 50% amplitude for 3 cycles of a 5 min pulse and a 5 min pause. Bacterial cell debris was removed by centrifugation at 12,000 × *g* for 2 h at 4 °C. The obtained supernatant was used as a crude enzyme. It was then concentrated by AQUASORB™ before loading onto the HisTrap FF™ column. The column (0.7 × 2.5 cm) was equilibrated with at least 5 column volumes of 20 mM phosphate buffer (pH = 7.4) containing 0.5 M NaCl and 20 mM imidazole at a flow rate of 1 ml/min. Then, the crude enzyme from the WT or Y101S cells was applied to the column and washed with the same buffer until the A_{280} of eluent decreased to a steady baseline. The bound proteins were eluted by 500 mM imidazole in the same buffer. The active fractions were pooled and assayed for their enzyme activity by a starch transglycosylation assay [17], and the protein concentration was determined by the Bradford method [18] using BSA as the standard.

2.3. Optimization of the production of long-chain isomaltooligosaccharides (IMOs)

The optimum conditions for the production of IMOs were considered and defined in terms of obtaining the highest percentage yield of IMO products, as determined from the HPAEC-PAD results. The effects of varying the 10% (v/v) pullulanase-treated tapioca starch concentration (15–35% (w/v)), WT- or Y101S- amylomaltase (40–120 U/ml, starch transglycosylation activity), transglucosidase (3–15 U/ml, starch transglycosylation activity), temperature (40–80 °C), pH (5.0–8.0) and incubation time (0.5–7 h) were all investigated. After completion of the incubation period, all tested reactions were inactivated by boiling. Subsequently, 2 U of glucoamylase was added to the reaction mixture, and then it was incubated at 40 °C for 3 h prior to HPAEC-PAD analysis.

2.4. Larger scale production of long-chain IMOs

To produce a greater quantity of IMO products for further characterization, a larger scale production was undertaken in a reaction volume of 100 ml using the optimized reaction conditions. Pullulanase-treated tapioca starch (30% (w/v)) was incubated with 6 U/ml (starch transglycosylation unit) of transglucosidase and 120 U/ml (starch transglycosylation unit) of WT- or Y101S-amylomaltase in 20 mM phosphate buffer with a pH of 7.0 at 40 °C for 0.5 h with WT enzyme or 1 h with Y101S enzyme. After completion, the reaction mixture was brought to a halt by boiling for 10 min. The reaction products were dried by a lyophilizer (Biotechnologies Inc., Germany) before being applied to a Bio-Gel® P-2 Gel column.

2.5. Purification and characterization of the long-chain IMOs

2.5.1. Purification of the long-chain IMOs

The mixture of dried IMO products was dissolved with distilled water and clarified by centrifugation before being applied to a Bio-Gel® P-2 Gel column (1.2 × 97 cm). The column was equilibrated and eluted with distilled water at a flow rate of 8 ml/h with 1-ml fractions being collected. The fractions were measured for the presence of carbohydrates by the DNS assay [19]. The positive fractions were then analysed by HPAEC-PAD, and each pure fraction was collected and lyophilized for product characterization.

2.5.2. HPAEC-PAD analysis [20]

The long-chain IMO products were analysed by HPAEC-PAD using a CarboPac PA1 column (0.4 × 25 cm, Dionex Corp., USA) and an electrical detector (ECD40, Dionex Corp., USA). Two buffers, buffer A (150 mM NaOH in water) and buffer B (600 mM sodium acetate in buffer A), were used for the elution with a 0–30% (v/v) gradient (0–60 min) of buffer B at a flow rate of 1 ml/min. Twenty-five microliters of sample were injected into the column. Oligosaccharide standards (G1–G7, IMO2-IMO9, panose and 2-*O*-α-isomaltosyl-D-glucose) were used for calibration. The structures of the long-chain IMO products were confirmed by ESI-TOF MS and ^{1}H NMR.