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Cooperative Action of α-Glucanotransferase and Maltogenic Amylase for an Improved Process of Isomaltooligosaccharide (IMO) Production

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Maltogenic amylase and α-glucanotransferase (α-GTase) were employed in an effort to develop an efficient process for the production of isomaltooligosaccharides (IMOs). *Bacillus stearothermophilus* maltogenic amylase (BSMA) and α-GTase from *Thermotoga maritima* were overexpressed in *Escherichia coli* using overexpression vectors. An IMO mixture containing 58% of various IMOs was produced from liquefied corn syrup by the hydrolyzing and transglycosylation activities of BSMA alone. When BSMA and α-GTase were reacted simultaneously, the IMO content increased to 68% and contained relatively larger IMOs compared with the products obtained by the reaction without α-GTase. Time course analysis of the IMO production suggested that BSMA hydrolyzed maltopentaose and maltohexaose most favorably into maltose and maltotriose and transferred the resulting molecules simultaneously to acceptor molecules to form IMOs. α-GTase transferred donor sugar molecules to the hydrolysis products such as maltose and maltotriose to form maltopentaose, which was then rehydrolyzed by BSMA as a favorable substrate.

KEYWORDS: ***Bacillus stearothermophilus*** **maltogenic amylase; isomaltooligosaccharides (IMOs);** ***Thermotoga maritima*****; α-glucanotransferase (α-GTase)**

INTRODUCTION

Production of isomaltosaccharides (IMOs) with various compositions and useful properties is in great demand in the starch industry. High-IMO syrups are characterized by low viscosity, resistance to crystallization, and reduced sweetness (*1*−*3*). They have been developed to prevent dental caries, as substitute sugars for diabetics, or to improve the intestinal microflora. Chemically, IMOs are glucosyl saccharides consisting solely of α-(1,6)-glycosidic linkages, but IMOs available commercially include all glucosyl saccharides with α-(1,6)-glycosidic linkages such as isomaltose, panose and isopanose.

Transglycosylation catalyzed by amylases and their related enzymes has been utilized in the industry for the production of various oligosaccharides (*4*). Many bacterial saccharifying α-amylases catalyze α-(1,4)-transglycosylation in addition to the hydrolysis of α-(1,4)-glycosidic linkages. Maltogenic amylases (EC 3.2.1.133) from various bacteria exhibited both α-(1,6)-transglycosylation and α-(1,4)-hydrolysis activities (*5*−*8*). Action modes of maltogenic amylases have been studied in detail, which were mainly composed of three steps: maltosyl transfer, glucosyl transfer, and condensation (*9*−*12*). The coupled transglycosylation and hydrolysis activities of maltogenic amylases (*13*, *14*) were used to produce IMOs from liquefied starch in a more efficient way than the traditional process, which usually required more enzymes and longer time. The maximum concentration of IMOs accumulated in the final reaction mixture was 58% using maltogenic amylase, whereas it was only 40% in the traditional process.

Enzymic synthesis of a wide variety of oligosaccharides has been attained in vitro using the transfer reactions between a segment of donor and various acceptors (*13*, *15*). Usually the transfer takes place from a specific donor to a relatively large number of structurally different acceptors. 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 often plays a role in determining the position of transfer for the formation of a glycosidic bond.

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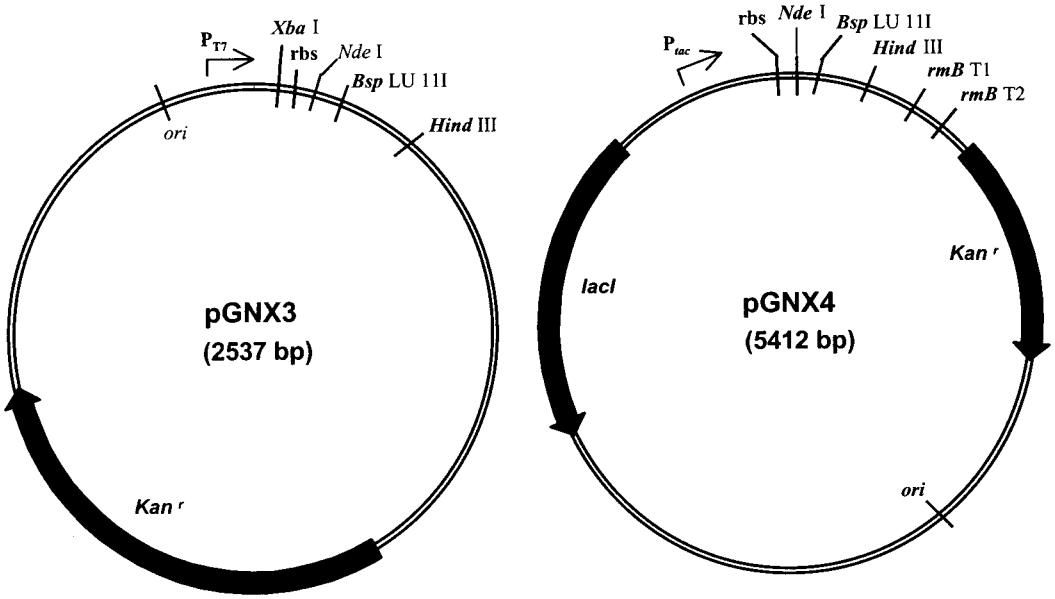

Figure 1. Restriction maps of pGNX3 and pGNX4. A 1.7 kb *Nco*I–*Hin*dIII fragment containing the BSMA gene was inserted at the *BspLU*11I/*Hin*dIII sites, and a 1.3 kb *Nde*I–*Hin*dIII fragment containing the α-GTase gene was inserted at the corresponding sites on the vectors.

In this study, α-glucanotransferase (α-GTase; EC 2.4.1.25) from *Thermotoga maritima* with α-(1,4)-transferring and liquefying activities was introduced in an effort to improve the IMO productivity using liquefied starch and maltogenic amylase from *Bacillus stearothermophilus* ET1 (BSMA). For that purpose, both enzymes were overexpressed in *Escherichia coli* from the corresponding genes subcloned on expression vectors, pGNX3 and pGNX4, and used to determine the most appropriate conditions for effective IMO production. The cooperative action mode of the two enzymes to enhance the production of IMOs from liquefied starch was also investigated.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. *E. coli* MC1061 [F$^-$, *araD*139, *recA*13, Δ(*araABC-leu*)7696, *gal*U, *gal*K, Δ*lac*X74, *rpsL*, *thi*, *hsdR*2, *mcrB*] and HB101 [F$^-$, *supE*44, *ara*-14, *proA*2, *gal*K2, *lacY*1, *rpsL*20, *xyl*-5, *mtl*-1, *hsdS*20(r_B^- m_B^-)] were used as hosts for DNA manipulation and transformation. *E. coli* TG1 [*supE*, *hsd*Δ5, *thi*, Δ(*lac-proAB*), F′(*traD36*, *proAB*$^+$, *lacI*^{q}, *lacZ*Δ*M15*)] and BL21 [F$^-$, *ompT*, *hsdSB*(r_B^- m_B^-), *gal*, *dcm*(DE3)] were used as hosts for overexpression of the clones constructed in this study. *E. coli* MC1061 and HB101 were grown in LB medium (1% Bacto-tryptone, 0.5% yeast extract, and 0.5% NaCl) at 37 °C. *E. coli* transformants were cultured in LB medium containing ampicillin (100 μg/mL) or kanamycin (100 μg/mL). *E. coli* vectors, pB*R*322, pUC119, and pBluescript II SK (Stratagene, La Jolla, CA), were used for cloning and subcloning (*16*).

Overexpression and Purification of Enzymes. To overexpress BSMA or α-GTase in *E. coli*, the corresponding genes were subcloned into pGNX3 and pGNX4 under the control of the T7 and *tac* promoters, respectively. The resulting transformants were screened for starch hydrolyzing activity by the iodine test after treatment with D-cycloserine (*5*).

E. coli transformants harboring the genes were cultured in LB broth containing kanamycin. Expression of the gene cloned on pGNX4 was induced by the addition of IPTG (1 μmol/mL) when the culture reached mid-log phase. The cells harvested by centrifugation (5000*g*, 4 °C, 20 min) were resuspended in 4 mL of 50 mM Tris-HCl buffer (pH 7.5) per gram of wet cells. Cells were homogenized (Microfluidizer; Microfluidics Co., Newton, MA), and polyethylenimine (PEI) was carefully added to a final concentration of 0.5% (w/v) to remove nucleic acids. To purify BSMA, the precipitate was removed by centrifugation (12000*g*, 4 °C, 30 min) and washed once more. The supernatant was dialyzed against 50 mM Tris-HCl buffer (pH 7.5) and concentrated using a Pellicon ultrafiltration kit (Millipore Co., Bedford, MA; MW cutoff 10000). Other proteins were precipitated in 20% (w/v) ammonium sulfate at 4 °C for 2 h in an ice bath. Proteins in the supernatant were precipitated in saturated ammonium sulfate (60%, w/v) at 4 °C for 2 h. The pellet harvested by centrifugation (10000*g*, 4 °C, 15 min) was resuspended in 50 mM Tris-HCl buffer (pH 7.5) and dialyzed against the same buffer (pH 7.5) before being concentrated using a Pellicon ultrafiltration kit (Millipore Co.; MW cutoff 10000). The partially purified BSMA solution was used for enzymatic reactions in this study.

To purify α-GTase, the cells were incubated at 80 °C for 20 min in a water bath, and then PEI was carefully added to a final concentration of 0.5% (w/v). The precipitate was removed by centrifugation (12000*g*, 4 °C, 30 min) and washed once more. The supernatant was dialyzed against 50 mM Tris-HCl buffer (pH 7.5) and concentrated using a Pellicon ultrafiltration kit (Millipore Co.; MW cutoff 10000).

Enzyme Assay. BSMA activity was assayed according to the dinitrosalicylic acid (DNS) method (*17*) by determining the amount of reducing sugar produced by the enzyme. The enzyme reaction mixture was composed of 250 μL of 1% (w/v) substrate solution (β-CD or soluble starch) in 50 mM sodium acetate buffer (pH 6.0), 160∼220 μL of reaction buffer (50 mM sodium acetate buffer, pH 6.0), and 30∼90 μL of enzyme solution. A reaction mixture was preincubated at 55 °C for 5 min before diluted enzyme solution was added and incubation continued for 30 min. The reaction mixture was stopped by adding 1.5 mL of DNS and boiling for 5 min. The reaction was cooled immediately by placing the tube under running water. Absorbance was measured at 575 nm using a spectrophotometer (UV-1601, Shimadzu, Kyoto, Japan). One unit of β-CD hydrolyzing activity (CU) was defined as the amount of BSMA that formed reducing sugars to give 1.0 unit of ΔAbs_{575}.

The activity of α-GTase was measured by the change in iodine-staining properties during the conversion of amylose in the presence of maltose (*18*). The assay mixture containing 0.05% amylose, 0.05% maltose, 50 mM Tris-HCl buffer (pH adjusted to 7.5 at 60 °C), and the enzyme was incubated at 60 °C. Samples (0.1 mL) taken at 0 and 15 min were mixed with 1 mL of 0.02% iodine/potassium iodide solution (Lugol's solution diluted 1:50 in 50 mM Tris/HCl buffer, pH 7.5), and absorbance at 620 nm was measured immediately with a spectrophotometer. The absorbance at 0 min was ∼1.0. The difference between the absorbances of each sample taken at 0 and 15 min was used to estimate the enzyme unit. One unit of α-GTase activity (AU) was arbitrarily defined as the amount of enzyme that caused a change of absorbance by 1 in 15 min under the conditions described above. The assay was reproducible and linear for the range of enzyme concentrations causing absorbance differences up to 0.5. Protein

Table 1. Expression Levels of BSMA and α-GTase from Various Constructs

	BSMA				α-GTase	
constructs	pSG18	pUC BSBL119	pGNX3-BSMA	pGNX4-BSMA	pGNX3-α-GT	pGNX4-α-GT
host	TG1	TG1	BL21 (DE3)	TG1	BL21 (DE3)	TG1
plasmids	pUC18	pUC119	pGNX3	pGNX4	pGNX3	pGNX4
promoters	BSMA	BLMA	T7	tac	T7	tac
expression level[a]	<5%	10%	20%	20%	20%	20%

[a] Determined by densitometry of the protein bands appeared on SDS-PAGE gel.

concentrations were measured using a Bio-Rad Dc protein assay kit (Bio-Rad, Hercules, CA) and bovine serum albumin (Sigma, St. Louis, MO) as a standard.

Production and Analysis of IMO. BSMA (100 or 200 CU/g of substrate) was added to 30% (w/v) liquefied corn starch (Genedex, Samyang Genex, Taejeon, Korea; 33 °Brix, DE 22) in 50 mM phosphate buffer (pH 6.5) and incubated at 50 °C for 50 h. For further investigation, the reactant was boiled for 5 min after 20 h of reaction to inactivate BSMA, and then α-GTase (6000 AU/g of substrate) or additional BSMA (100 CU/g of substrate) was added to the reactant after it was cooled to 50 °C. The reaction was then allowed to continue for 30 h at the same temperature. To determine the effect of α-GTase on the IMO production, α-GTase and BSMA were added to liquefied corn starch (33 °Brix, DE 22, pH 6.5) simultaneously and incubated at 50 °C for 50 h.

The reaction products were analyzed by high-performance anion exchange chromatography (HPAEC) using a Carbopac PA1 column (0.4 × 25 cm, Dionex, Sunnyvale, CA) and an electrochemical detector (ECD40, Dionex). 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 of buffer B at a flow rate of 1.0 mL/min. Twenty microliters of sample was injected to the column. Some of the standards (glucose, maltose, isomaltose, and panose) used for calibration were purchased from Sigma Co. (reagent grade), and isopanose was a gift from Prof. Y. Sakano at Tokyo Noko University (Japan). Others were prepared, and their structures and purities were confirmed by thin-layer chromatography, methylation studies, and NMR previously in our laboratory (*7*, *13*, *14*).

RESULT AND DISCUSSION

Overexpression of BSMA and α-GTase. To overexpress BSMA in *E. coli*, the corresponding gene that had been cloned in pSG18 previously (unpublished data of S. Hong and H. Lee) was subcloned into pGNX3 and pGNX4 expression vectors on which the gene was expressed under the control of the T7 and *tac* promoters, respectively (**Figure 1**). The resulting recombinants were designated pGNX3-BSMA and pGNX4-BSMA, respectively, and transformed into *E. coli*. The expression level of BSMA was determined by densitometry of the protein bands appearing on an SDS-PAGE gel. The *E. coli* clone carrying pGNX3-BSMA or pGNX4-BSMA produced BSMA as much as 20% of the total protein concentration (**Table 1**), which was 4 and 2 times higher than the amount of enzyme produced by pSG18 and pUCBSBL119, respectively. The difference in the expression level among the original clones and newly constructed ones was likely to be due to the type and strength of the promoters. The promoters for the BSMA and *B. licheniformis* maltogenic amylase (BLMA) genes were relatively weak compared to the T7 and *tac* promoters, which are well-known as strong promoters for *E. coli* expression systems.

The α-GTase gene was amplified from the *T. maritima* chromosomal DNA by Polymerase Chain Reaction based on the genome sequencing data (*19*) and subsequently cloned on the overexpression vectors in *E. coli*. The resulting clones were designated pGNX3-α-GT and pGNX4-α-GT, respectively. Both clones produced α-GTase as much as 20% of the total proteins produced in small-scale cultures (**Table 1**). The *E. coli* TG1 clone harboring pGNX4-BSMA or pGNX4-α-GT was used for further experiments because the clones showed relatively higher expression levels than other clones and were stable during successive culture.

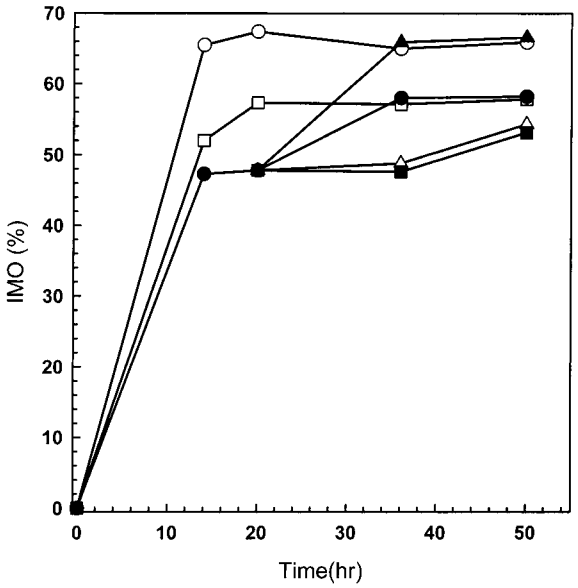

Figure 2. IMO production under various conditions. Liquefied starch solution (30%) was treated with BSMA and/or α-GTase in various combinations: (■) profile of IMO production by BSMA (100 CU/g of substrate); (□) BSMA (200 CU/g of substrate); (●) BSMA (100 CU/g of substrate) and subsequent addition of the same amount of the enzyme at 20 h of reaction; (○) BSMA (200 CU/g of substrate) and α-GTase (6000 AU/g of substrate); (▲) BSMA (100 CU/g of substrate) and subsequent addition of α-GTase (6000 AU/g of substrate) at 20 h of reaction; (△) α-GTase (6000 AU/g of substrate) after inactivation of BSMA (200 CU/g of substrate) at 20 h of reaction.

Production of IMOs Using BSMA and α-GTase. As an effort to improve the production of IMO, BSMA and α-GTase were added to liquefied corn syrup in various combinations: individually, simultaneously, or sequentially. The effect of BSMA and α-GTase activities on the IMO production was investigated by a series of reactions using liquefied corn syrup, in which maltopentaose was the main component. Prior to the experiments, the most appropriate amount of each enzyme for the highest yield of IMOs was determined as 200 CU/g of substrate for BSMA and 6000 AU/g of substrate for α-GTase (data not shown). First, BSMA (200 CU/g of substrate) was incubated with liquefied corn syrup (33 °Brix, DE 22, pH 6.5) at 50 °C for 50 h (**Figure 2**). The proportion of IMOs in the reactant produced by BSMA alone reached 58% in 20 h of incubation and remained almost constant during further incubation for 30 h. A similar result was observed when the reaction was carried out for 20 h with BSMA (100 CU/g of substrate), and then the reaction was continued for another 30 h by adding a second aliquot of BSMA. IMO production reached a plateau

Table 2. Compositions[a] of Isomaltooligosaccharides (IMOs) Produced in This Study and in Commercial Products

components		BSMA[b]	BSMA + α-GTase[c]	Sunoligo[d]	Isomalto GH[e]
DP1	glucose	9.7	10.8	42.1	22.0
DP2	maltose	14.1	11.4	4.6	21.0[g]
	BDP2[f]	3.0	8.2	24.4	
DP3	maltotriose	6.2	2.7	0.0	24.0[a]
	BDP3	16.0	23.8	15.7	
DP4	maltotetraose	2.4	1.3	0.0	33.0[g,h]
	BDP4	23.8	25.0	9.0	
≥ DP5	≥ maltopentaose	10.0	6.4	3.2	
	≥ BDP5	14.7	10.4	1.0	
Total amount of IMO		57.5	67.4	50.1	65.0

[a] Weight percentage. [b] The concentration of BSMA was 200 CU/g of substrate. [c] Enzymatic reaction was carried out using BSMA (200 CU/g of substrate) and α-GTase (6000 AU/g of substrate) simultaneously. [d] Product of Samyang Genex, Korea (declared composition). [e] Product of Showa Inc., Japan (declared composition). [f] Branched oligosaccharide with a degree of polymerization. [g] The product includes maltooligosaccharide and branched oligosaccharide. [h] Proportions of maltooligosaccharides larger than maltotetraose.

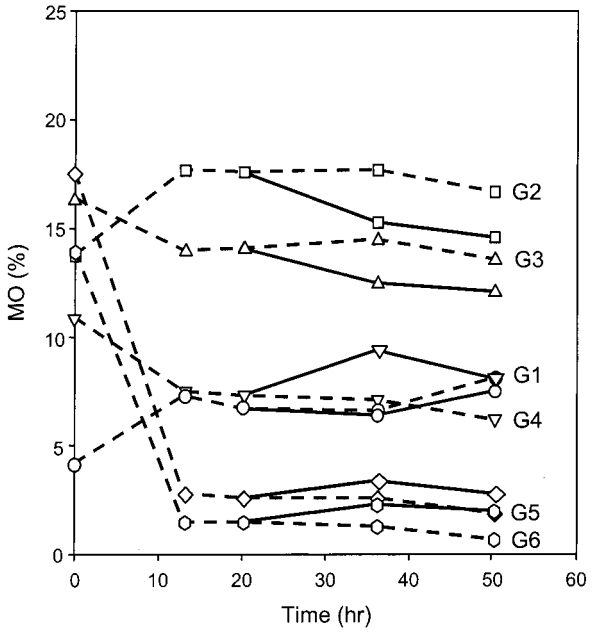

Figure 3. Maltooligosaccharide production during the two-step reaction: (○) glucose; (□) maltose; (△) maltotriose; (▽) maltopentaose; (◇) maltopentaose; (⬡) maltohexaose. Solid and dashed lines represent the profiles of change in maltoolisosaccharides during the reaction with and without the addition of α-GTase, respectively. Liquefied starch solution (30%) was treated with BSMA (200 CU/g of substrate) for 20 h, and the reaction was continued further for 30 h with or without the addition of α-GTase (6000 AU/g of substrate). Aliquots were taken during the reaction, and the composition of maltooligosaccharides was analyzed by HPAEC.

in 14 h in the proportion of 47.3% but began to increase at 20 h until it reached 58%. However, if BSMA (100 CU/g of substrate) was inactivated after 20 h of the reaction and α-GTase (6000 AU/g of substrate) was added subsequently, an increase of IMO production was observed only after 16 h. In 14 h of further reaction, the proportion of IMO reached 54.5%. This indicated that α-GTase alone did not contribute significantly to the formation of IMOs during the rest of the reaction. IMO production was enhanced greatly if α-GTase was added without inactivating BSMA after 20 h of the reaction. IMOs began to be produced more upon the addition of the second enzyme in the highest proportion of 68% at 16 h and remained almost

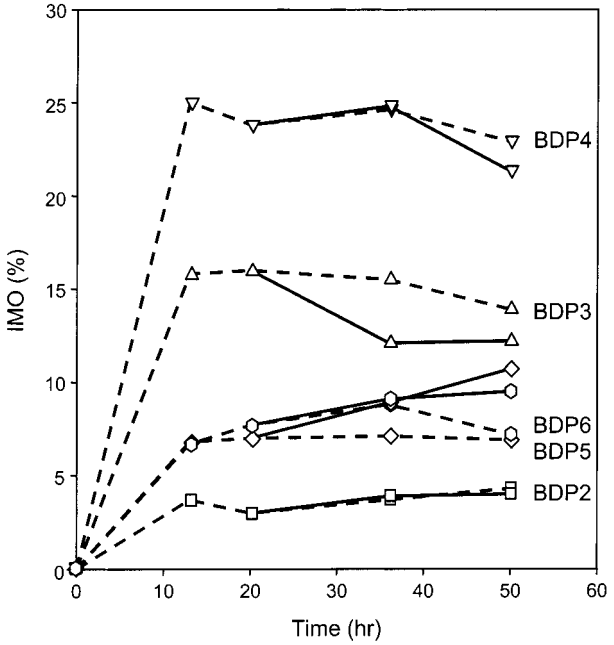

Figure 4. IMO production during the two-step reaction: (□) isomaltose; (△) isomaltotriose; (▽) isomaltotetraose; (◇) isomaltopentaose; (⬡) isomaltohexaose. Solid and dashed lines represent the profiles of change in IMO during the reaction with and without the addition of α-GTase, respectively. The reaction was carried out under the same conditions as described in **Figure 3**.

constant during the rest of the reaction. This result indicated that the residual substrate that could not be converted to IMOs by BSMA was turned to IMOs at the saturated level in the second step reaction employing α-GTase. Simultaneous reaction of BSMA (200 CU/g of substrate) and α-GTase (6000 AU/g of substrate) from the beginning increased the IMO content to 68% in 20 h. HPAEC analysis of the IMO mixtures demonstrated that cooperative reaction of BSMA and α-GTase certainly had a synergistic effect on the IMO production, especially for the production of branched oligosaccharide with a degree of polymerization of 2 (BDP2; isomaltose) and BDP3 (**Table 2**).

Cooperative Action Mode of the Two Enzymes. To investigate the role of α-GTase in improving the production of IMOs, compositions of maltooligosaccharides (MOs) and IMOs were analyzed during the two-step reaction, in which BSMA (200 CU/g of substrate) was added at the beginning of the reaction, and the reaction was allowed to continue further for 30 h after the addition of α-GTase (6000 AU/g substrate) at 20 h (**Figure 3**). During the period in which the reaction was carried out by BSMA alone, longer MOs such as maltopentaose (G5) and maltohexaose (G6) were most readily hydrolyzed during the first 14 h, indicating that the enzyme preferred those molecules as substrate to others. Maltotriose (G3) and maltotetraose (G4) were hydrolyzed by the enzyme throughout the reaction but only at slower rates than G5 and G6. On the other hand, the content of glucose (G1) and maltose (G2) increased as the reaction proceeded. The results correlated with the known function of BSMA hydrolyzing liquefied starch to free sugars. Introducing α-GTase at 20 h of the reaction resulted in an increase of longer MOs including G4, G5, and G6 but a decrease of G2 and G3. The content of glucose increased for 16 h upon the addition of α-GTase but then began to decrease during the rest of the reaction. The results indicated that α-GTase produced longer MOs (G4, G5, and G6) through α-(1,4)-transglycosyla-

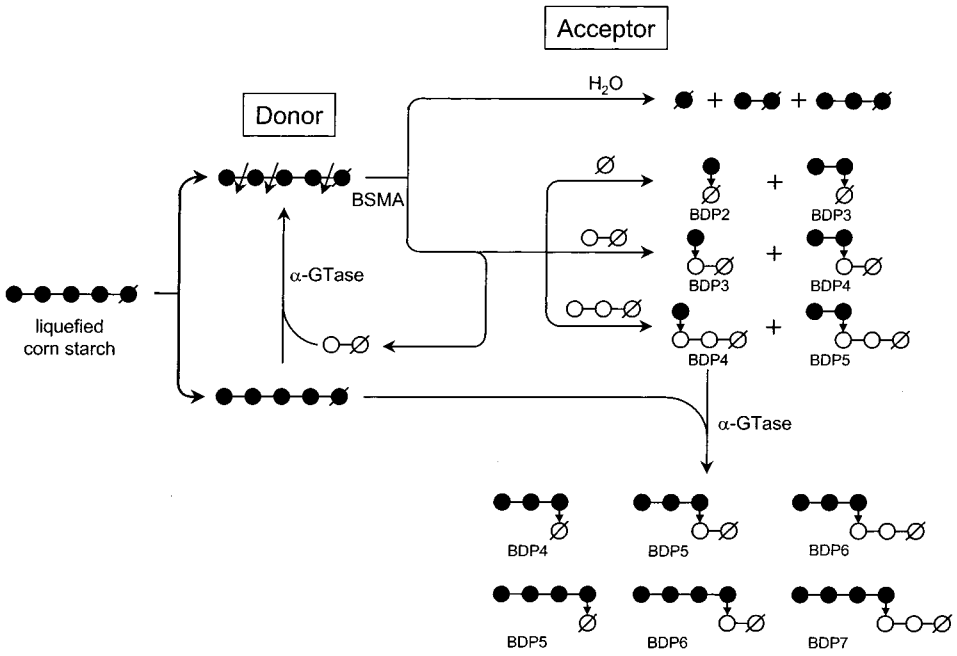

Figure 5. Cooperative action mode of BSMA and α-GTase for the production of IMOs. ○ and ● denote nonreducing glucopyranosyl residue in acceptor and donor molecules, respectively; ∅, reducing glucose residues. Hydrolyzing and transglycosylation activities of BSMA and disproportionation activity of α-GTase promote the production of IMOs with various lengths.

tion of shorter MOs (G1, G2, and G3) as acceptors and liquefied corn starch as a donor.

The composition of IMOs produced during the two-step reaction was determined by HPAEC (**Figure 4**). Addition of α-GTase after inactivation of BSMA caused an increase in the proportions of isomaltopentaose (BDP5) and isomaltohexaose (BDP6) but decreased those of isomaltotriose (BDP3) and isomaltotetraose (BDP4). The content of isomaltose (BDP2) was almost the same as in the IMO mixture produced by BSMA alone. The results indicated that α-GTase utilized BDP3 and BDP4 as acceptors and liquefied starch as donor to produce longer IMOs such as BDP5 and BDP6. Liebl et al. (*18*) proposed that the α-(1,4)-transglycosylation activity of α-GTase resulted in the disproportionation of α-(1,4)-glucans. The enzyme yielded linear maltohomologues using low molecular mass MOs (G4−G7) as sole substrate. However, maltose and maltotriose were not disproportionated by α-GTase, although both molecules were good acceptors for glucanosyl transfer. Glucose neither functioned as an acceptor in transfer reaction nor appeared as a reaction product. The chain length of glucanosyl segments transferred ranged from two to probably far more than six glucose residues.

On the basis of the changes in the MO and IMO compositions during the two-step reaction, α-GTase was likely to play a role in producing both longer MOs and IMOs. In the simultaneous reaction of BSMA and α-GTase, the concentration of donor sugars was increased because α-GTase produced donor sugars from acceptor sugars. Therefore, the production of donor sugars by the action of α-GTase increased the substrate concentration for BSMA. Addition of α-GTase converted shorter MOs to longer MOs such as G5 and G6. The longer MOs were utilized easily by BSMA, thereby increasing the content of IMOs. Another role of α-GTase could be explained by the conversion of shorter IMOs to longer ones. α-GTase elongated branched oligosaccharides produced by BSMA reaction by α-(1,4)-transglycosylation and caused the change in the IMO composition during the reaction. The synergistic effect between BSMA and α-GTase is summarized in **Figure 5**.

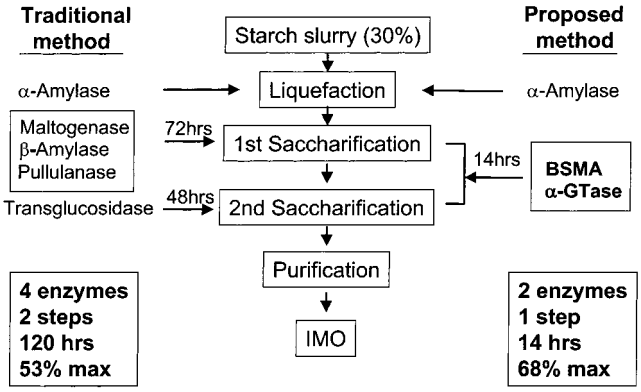

Figure 6. Comparison of traditional and newly improved processes for IMO production. The new method developed in this study improves the process of IMO production by employing fewer enzymes, taking shorter process time, and yielding more IMOs.

Improved Process for IMO Production. From the results described above, an improved process for IMO production was suggested (**Figure 6**). In the traditional method for the production of IMOs, α-amylase was added to starch slurry (30%), and then maltogenase, β-amylase, and pullulanase were employed for the first saccharification (*20*). For the second saccharification, transglucosidase was added and the resulting IMOs were purified. It takes ∼120 h to produce 53% of IMOs by the traditional method. However, the new method developed in this study employs BSMA and α-GTase simultaneously to process the starch solution that had been liquefied by α-amylase. In 14 h of reaction, 68% of IMOs was produced. Therefore, the improved process makes it possible to produce IMOs at a significant level in one step by employing only two enzymes. In addition, the IMO mixture with a high degree of branched oligosaccharides elongated by α-GTase is expected to be most effective in promoting the growth of bifidobacteria in the human intestine, because growth of the bacteria is known to be enhanced in proportion to the degree of glucosidic polymerization of the IMO components. Kaneko et al. (*21*) reported that

the IMO mixture containing trisaccharides and tetrasaccharides was more effective in enhancing the microflora than the IMO mixture of disaccharides.

In conclusion, two types of transferring activities exerted by BSMA and α-GTase on liquefied starch solution stimulated the formation of IMOs in a cooperative mode, thus providing a new possibility to improve the production for various oligosaccharide mixtures.

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