

Optimized Substrate Concentrations for Production of Long-Chain Isomaltooligosaccharides Using Dextranucrase of *Leuconostoc mesenteroides* B-512F

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Isomaltooligosaccharide (IMO) is a promising dietary component with prebiotic effect, and the long-chain IMOs are preferred to short chain ones owing to the longer persistence in the colon. To establish the optimal process for synthesis of long-chain IMOs, we systematically examined the reaction condition of dextranucrase of *Leuconostoc mesenteroides* B-512F by changing the ratio of sucrose to maltose (varying as 1:4, 1:2, 1:1, and 2:1) and amount of each sugar (from 2% to 20%). As a result, a ratio of 2:1 (sucrose to maltose, 10:5% or 20:10%, w/v) was determined as an optimal condition for long-chain IMO synthesis (DP3–DP9) with relatively higher yields (70–90%, respectively).

Keywords: *Leuconostoc mesenteroides*, dextranucrase, isomaltooligosaccharide, prebiotic, acceptor reaction

Prebiotics are non-digestible carbohydrates that are used to improve the microbial balance of the human gut [19]. They are selectively fermented in the colon by beneficial bacterial species such as bifidobacteria and lactobacilli [6]. Isomaltooligosaccharide (IMO) is an α -(1→6)-linked glucooligosaccharide with degrees of polymerization (DP) ranging from 2 to 6 in the case of commercial products [1, 2]. Generally, IMO is a promising dietary constituent that gives rise to prebiotic effect together with high levels of butyrate formation [12]. Recently, IMO was registered as a functional food supplement by KFDA (Korea Food and Drug Administration) and its use in the food manufacturing process is expected to grow gradually.

With increasing public perception against oligosaccharides, the production processes for oligosaccharides have attracted strong commercial interest [5]. Commercial IMOs are

produced from starch by a two-step enzymatic hydrolysis process [2]. However, significant concentrations of digestible sugars such as glucose, maltose, and maltooligosaccharides still remain in the final product [9, 13, 20]. In addition, a problem with the prebiotic efficiency of the short-chain commercial IMOs is that they are partially absorbed in the small intestine [3, 6]. On the contrary, longer IMOs will be absorbed to a much lower degree and will persist longer in the colon, and hence the long-chain IMOs tend to be preferred to short ones [3, 6].

The dextranucrase (E.C. 2.4.1.5) excreted by *Leuconostoc* spp. transfers the glucose moiety of sucrose to form dextran and also catalyzes the transfer of glucose from sucrose (donor) to other carbohydrates (acceptors) by mainly linking an α -(1→6)-glucosyl bond [14, 18]. When the acceptor is a monosaccharide or disaccharide, a series of oligosaccharide acceptor-products is usually produced by successive transfers of the glucose residue of sucrose to the nonreducing end of the acceptor. In the case of experiments with dextranucrase of *Leuconostoc mesenteroides* NRRL B-512F, maltose was determined as the best acceptor molecule [16, 17] and it was used for the production of size-controlled dextrans and oligosaccharides [21, 22]. The acceptor reaction of dextranucrase has a potential advantage in the manufacturing process of IMO in its ability to control the chain length of oligosaccharides by balancing concentrations of donor (sucrose) and acceptor (maltose) compounds.

In this study, to establish an optimal process for the industrial synthesis of long-chain IMOs, we systematically examined the reaction condition of the dextranucrase of *Ln. mesenteroides* B-512F by changing the ratio of sucrose to maltose and amount of each sugar.

All chemicals used in this study, unless otherwise stated, were of analytical-grade purity supplied by Sigma Chemical Co. (St. Louis, MO, U.S.A.). Maltose was obtained from Duksan Pharmaceuticals Co. (Yongin, Korea). Lactobacilli

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MRS broth was purchased from Difco Laboratories (Detroit, MI, U.S.A.).

Dextranucrase was produced by the previous procedure [10] by batch fermentation of *Ln. mesenteroides* B-512F in S-medium for 24 h at 28°C. The S-medium was composed of 24.7 g sucrose, 4.2 g peptone, 4.2 g yeast extract, 20 g K_2HPO_4 , 0.2 g $MgSO_4 \cdot 2H_2O$, 0.1 g NaCl, 0.1 g $FeSO_4 \cdot 7H_2O$, 0.1 g $MnSO_4 \cdot H_2O$, and 0.13 g $CaCl_2 \cdot 2H_2O$ per liter of distilled water [8], where 0.5–1 mg/ml Tween 80 was added for enzyme stabilization during separation. The cells were separated from supernatant by centrifugation at $7,484 \times g$ for 10 min at 4°C. To digest dextran for lyophilization, dextranase (Sigma, U.S.A.) was added to the culture supernatant, which was then dialyzed overnight against 0.02 M sodium acetate (pH 5.2), 0.05 M NaCl, in a regenerated cellulose dialysis membrane tubing (3.5 kDa molecular mass cutoff, Spectra/Por; Spectrum Laboratories Inc., CA, U.S.A.) at 4°C with two changes of buffer. Dialyzed solution was concentrated by dehydration with polyethylene glycol 6,000 at 4°C overnight. The dextranucrase was filtered through a 0.22- μm filter and stored at -70°C until use. Dextranucrase activities were measured by assaying changes in the fructose concentration [11] after modification [15] in 20 mM Na-acetate buffer solution (pH 5.2) containing 100 mM sucrose, 1 mM $CaCl_2$, and 0.02% NaN_3 . The fructose concentration was measured using a high-pressure liquid chromatography system (HPLC, Younglin, Daejeon, Korea) with an Asahipak NH2P-50 4E column (25 cm \times 4.6 mm I.D.; Shodex, Kawasaki, Japan) with an RI detector. One unit of dextranucrase was defined as the amount of enzyme used to produce 1 μ mole of fructose per minute at 25°C [7].

To confirm the synthesis of oligosaccharide by the acceptor reaction of dextranucrase, the enzyme solution retaining 13.5 units/ml of activity was mixed with substrate solution (10% sucrose and 10% maltose) in 20 mM of Na-acetate buffer (pH 5.2) with 1 mM $CaCl_2$, and incubated by shaking at 30°C. From monitoring of the sugar contents using TLC analysis along with enzyme reaction, the acceptor reaction by dextranucrase was found to occur after 1 h (Figs. 1A and 1B), transferring the glucose residue from sucrose to maltose, thereby producing panose (6²- α -D-glucopyranosylmaltose, DP3), isomaltosyl maltose (DP4), isomaltotriosyl maltose (DP5), and isomaltotetraosyl maltose (DP6). Almost the entire sucrose had disappeared within 4 h, whereas about half of maltose was used as acceptor molecules. After 7 h reaction, concentrations of panose and DP4 reached the highest level (7% total), and even longer oligosaccharides like DP5 and DP6 were detected on the TLC plate when a different developing solution (nitromethane/distilled water/1-propanol=2:3:5) was used (Fig. 1B). When the same sample was analyzed using a high-pressure ion-exchange chromatography (HPIC; Dionex Corp., Sunnyvale, CA, U.S.A.), the concentrations of maltose,

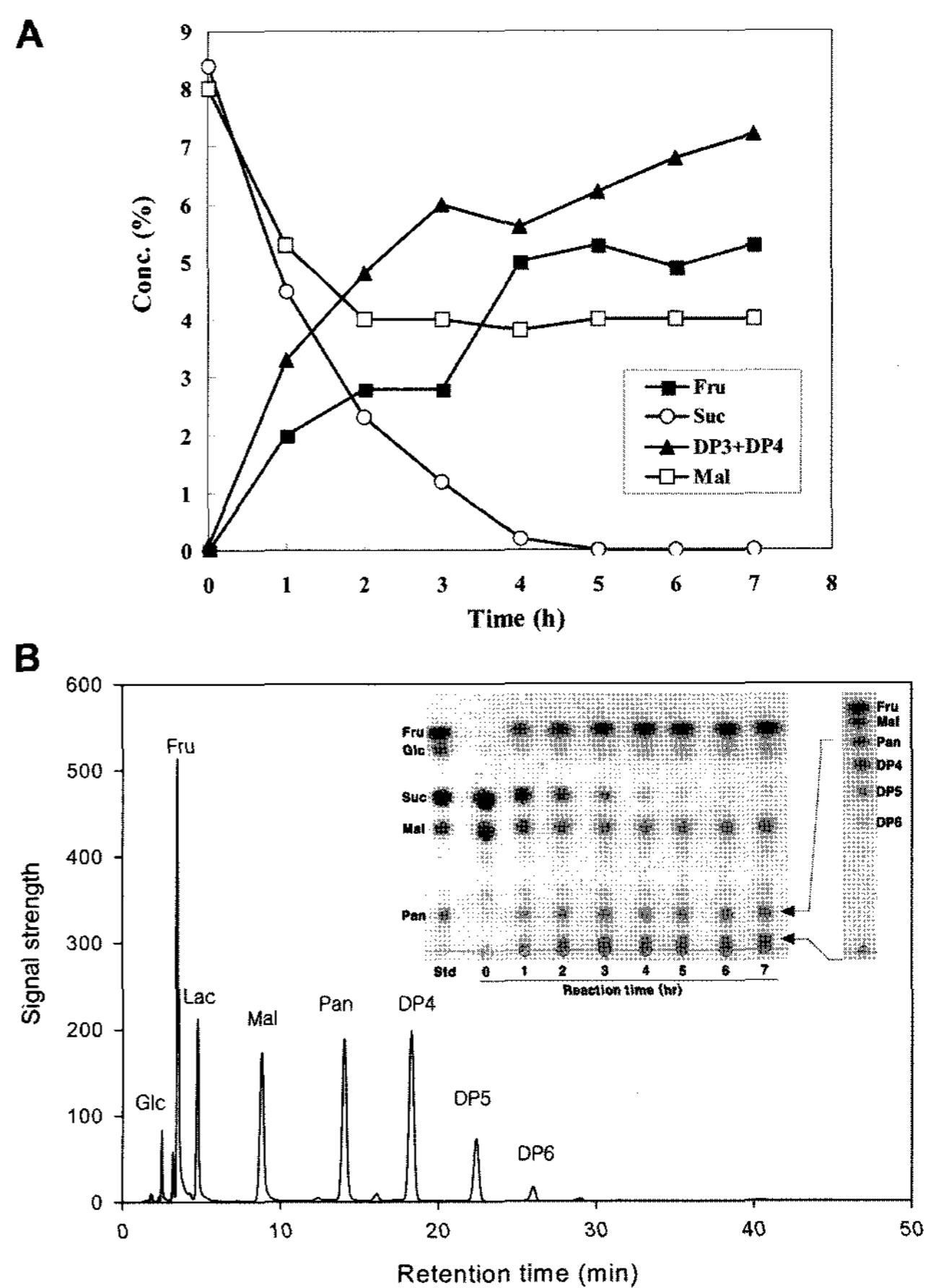


Fig. 1. Time course of individual sugar contents along with dextranucrase reaction with 10% sucrose and 10% maltose.

A. Sugar profile from assaying sugar contents with a densitometer. Sucrose (■), maltose (●), fructose (◆), Pan+DP4 (▲). **B.** After 7 h reaction, sugar contents were analyzed using HPIC; lactose (Lac) was used as an internal standard. The contents of maltose, panose, IM4, IM5 were 3.5%, 4.5%, 5.22%, and 1.54%, respectively. Inset, sugar contents were also monitored by TLC (developing solution, acetonitrile:H₂O=85:15, v/v). Fru, fructose; Glc, glucose; Suc, sucrose; Mal, maltose; Pan, panose; Std, standard molecules. Oligosaccharides synthesized after 7 h was also separated on a TLC plate using nitromethane/distilled water/1-propanol (2:3:5) developing solution. DP4, isomaltosyl maltose; DP5, isomaltotriosyl maltose; DP6, isomaltotetraosyl maltose.

panose, DP4, and DP5 were 3.5%, 4.5%, 5.22%, and 1.54%, respectively (Fig. 1B). By the reaction, 11.26% of total oligosaccharide was synthesized from 10% each of sucrose and maltose, and thus the conversion yield was 75.07% based on the glucose equivalent. Quantitative sugar analysis was carried out using HPIC with a CarboPac PA-1 column and an electrochemical detector as the pulsed amperometric detector (PAD). For a quantitative and qualitative analysis of peaks, the software Chromate Window v.3.0 (Interface Engineering Inc., U.S.A.) was used. At the same time, TLC analysis was used for monitoring oligosaccharide synthesis along with enzyme reaction. For analysis of mono- or di-saccharides, the TLC plate (Whatman K5 TLC plates; Merck, Darmstadt, Germany) was developed three

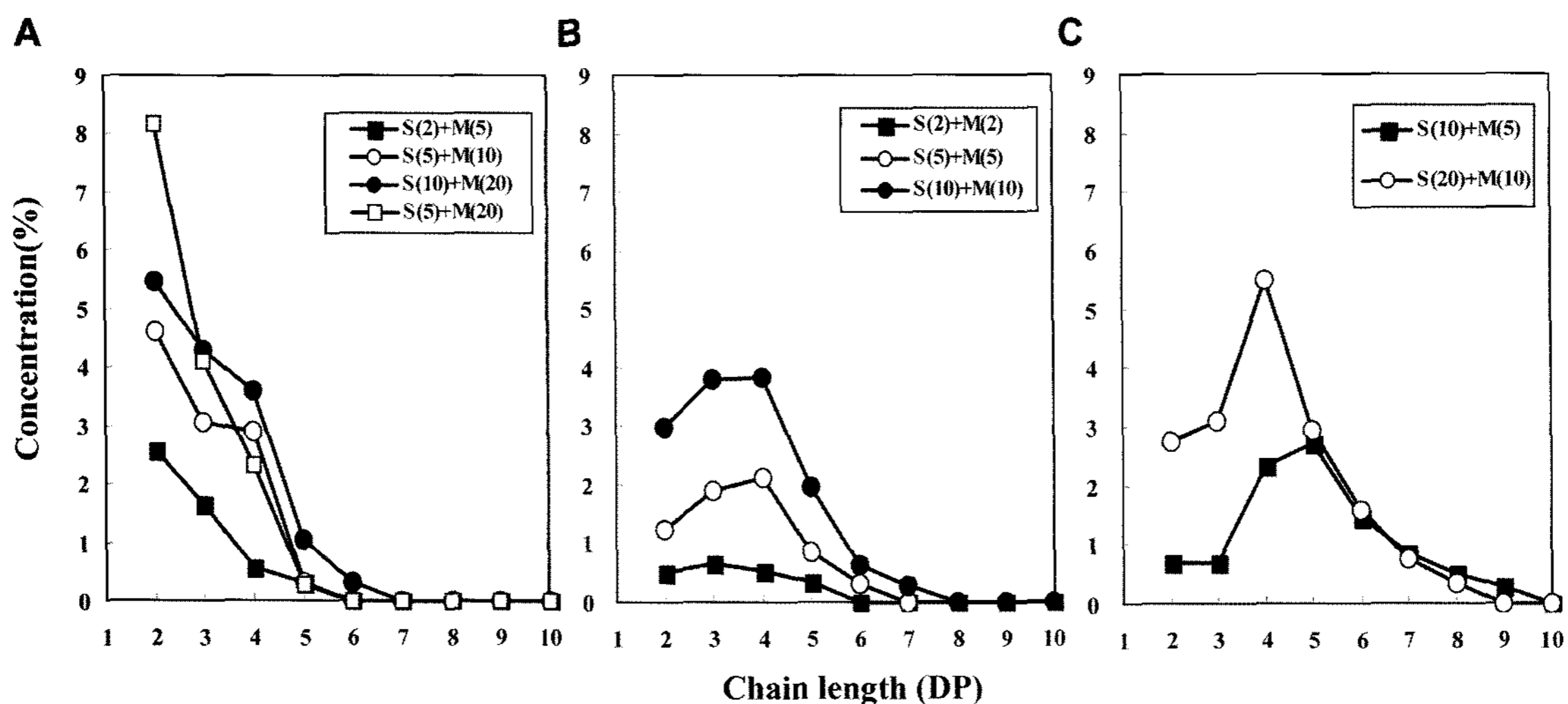


Fig. 2. Distribution of chain lengths of isomaltooligosaccharides synthesized by dextransucrase of *Ln. mesenteroides* B-512F with various ratios and concentrations (% w/v) of sucrose and maltose. A. Sucrose < maltose; B. Sucrose = maltose; C. Sucrose > maltose.

times with acetonitrile/distilled water (85:15, v/v), and for oligosaccharide analysis, the plate was developed twice with nitromethane/distilled water/1-propanol (2:3:5, v/v/v). The separated sugars were detected by dipping the plate in ethanol containing 0.5% (w/v) α -naphthol and 5% (v/v) sulfuric acid followed by heating at 110°C for 5 min. The quantity of each sugar on TLC plates was measured by an image scanner (Epson Perfection 1200U Scanner, Long Beach, CA, U.S.A.) and calculated by SigmaGel (Sigma Inc., U.S.A.) [15].

Next, a systematic study of the effects of parameters on the acceptor reaction was made for *Ln. mesenteroides* B-512F dextransucrase using maltose as the acceptor. The amount and distribution of acceptor products were studied as a function of (1) the ratio of sucrose to maltose and (2) the concentration of sucrose and maltose (Fig. 2). The ratio of sucrose to maltose was varied as 1:4, 1:2, 1:1, and 2:1, and the concentration of sucrose and maltose was varied from 2% to 20%. As shown in Fig. 2A, when sucrose concentrations were lower than maltose, various sizes of oligosaccharides were synthesized and the amount of each saccharide product in the series decreased as the degree of polymerization increased. Keeping the ratio constant at 1:2 or 1:2.5 and increasing the concentration of sucrose from 2% to 10% also showed a linear increase in the amount of acceptor products. The number of acceptor products in this reaction remained relatively constant at three or four. When the same ratio (1:1) of sucrose and maltose was reacted, panose (DP3) and isomaltosyl maltose (DP4) were made as the major products and the saccharide products got longer, to DP6 or DP7 (Fig. 2B). The amount of saccharide products increased linearly depending on the concentration of sucrose from 2% to 10%, with the same pattern of DP distribution. In the same manner, when the

sucrose content increased to 2:1 ratio (Fig. 2C), the major products were isomaltosyl maltose (DP4) or isomaltotriosyl maltose (DP5), and the longest saccharide was DP8 or DP9. From these results, we obtained a general rule that, as the sucrose fraction increases, the chain-length distribution of oligosaccharides gets broader and the length of major product gets longer. Against each reaction, yields of IMO production were calculated based on the glucose equivalents, and the results are presented in Table 1. In the case of a low sucrose fraction (sucrose < maltose), yields were generally low at between 30% and 50%, since most of the glucose equivalents were retained in the maltose fraction. Keeping the constant ratio (1:1) of sucrose and maltose resulted in a

Table 1. Production yield of isomaltooligosaccharides depending on the ratio and concentrations of sucrose and maltose.^a

Ratio of Suc:Mal	Substrates concentration	IMO (% w/v)	Yield ^b
1:4	Suc 5%+Mal 20%	6.7	30.1
1:2	Suc 2%+Mal 5% ^c	2.5	42.4
	Suc 5%+Mal 10%	6.3	50.7
	Suc 10%+Mal 20%	9.3	37.5
1:1	Suc 2%+Mal 2%	1.5	51.5
	Suc 5%+Mal 5%	5.2	70.6
	Suc 10%+Mal 10%	10.5	71.3
	Suc 20%+Mal 20%	11.9	40.5
2:1	Suc 10%+Mal 5%	8.9	91.5
	Suc 20%+Mal 10%	14.2	73.2

^aSucrose (Suc) and maltose (Mal) were reacted with dextransucrase in Na-acetate buffer at 30°C and, after depletion of sucrose, contents of isomaltooligosaccharides were measured.

^bYield of oligosaccharide was calculated based on the glucose equivalents; Yield=IMO/0.47×(sucrose+maltose).

^cRatio of sucrose and maltose was 1:2.5.

moderate increase in yields at between 40 and 70%. In addition, when sucrose fraction was doubled against maltose, the yield increased dramatically up to 70–90%, consuming sucrose almost completely in synthesizing oligosaccharides. According to the results, long-chain IMO was obtained with highest yield (91.5%) when sucrose and maltose were reacted at 10% and 5%, respectively. In addition, when sucrose and maltose were 20% and 10%, respectively, the production yield was to some extent lowered to 73.2%. For industrial production of IMO, an enzyme reaction at high substrate concentration is generally recommended with its simpler purification process; hence, reaction at 20% and 10% concentrations of sucrose and maltose could be another choice for bulk processes with reasonable yield.

The conventional method for the production of IMOs from starch is to first hydrolyze starch into α -(1,4) linked dextrans using α -amylase (E.C. 3.2.1.1), pullulanase (E.C. 3.2.1.41), and β -amylase (E.C. 3.2.1.2), and then convert to α -(1,6)-linked oligosaccharides using α -D-glucosidase (E.C. 3.2.1.20). The α -D-glucosidase with transglucosylation activity is usually called transglucosidase or D-glucosyltransferase (E.C. 2.4.1.24). This process undoubtedly has a cost benefit, using starch as a raw substrate, rather than the system used in this study. However, the commercially available IMO, which is made by the above process, usually includes short-chain saccharides like isomaltose (DP2), panose (DP3), and isomaltotriose (DP3), and thus synthesis of long-chain IMO is unattainable. In contrast, as shown in this study, the acceptor reaction of dextransucrase using sucrose and maltose as donor and acceptor compounds, respectively, proved to be a suitable method to synthesize long-chain IMO. This method has an advantage to control the chain lengths of IMO simply by changing the balance of sucrose and maltose, and a 2:1 ratio of the substrates was determined to be the best condition for long-chain IMO. Additionally, a 10% sucrose concentration resulted in the highest conversion yield, and 20% sucrose, which is more feasible to the industrial process, still gave a reasonable yield. The current results will provide fundamental information for the alternative industrial production of IMO by using the acceptor reaction of dextransucrase.

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