Production and Characterization of Branched Oligosaccharides from Liquefied Starch by the Action of *B. licheniformis* Amylase

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A branched oligosaccharides (BOS) mixture was produced from liquefied starch solution using a maltogenic amylase of *Bacillus licheniformis* (BLMA). The BOS mixture was produced by both α-1,4-bond hydrolyzing and α-1,6-transglycosylation activities of BLMA, and it contained 58.3% of various branched oligosaccharides. Small branched oligosaccharides such as isomaltose, isopanose, and panose were identified in the mixture by various analyses including high performance ion-chromatography (HPIC). Major branched DP4 and DP5 molecules in the mixture were determined as 6^2-O-α-maltosyl-maltose, 6^3-O-α-maltosyl-maltotriose and 6^2-O-α-maltotriosyl-maltose, respectively. Time course study of BOS production suggested that the hydrolysis and transglycosylation reactions catalyzed by BLMA were coupled. BLMA was likely to transfer a sugar moiety hydrolyzed from a non-reducing end of maltooligosaccharide, mainly maltose, to another moiety of sugar via the formation of α-1,6-linkage. Immobilization of BLMA was attempted as an effort to achieve a continuous process for BOS production. The immobilized enzyme showed improved thermal stability and slight loss of enzyme activity was observed during repeated usage.

Darstellung und Charakterisierung verzweigter Oligosaccharide aus verflüssigter Stärke durch die Wirkung von *B. licheniformis*-Amylase. Unter Verwendung einer maltogenen Amylase aus *Bacillus licheniformis* (BLMA) wurde eine Gemisch verzweigter Oligosaccharide (BOS) aus verflüssigter Stärkelösung hergestellt. Die BOS-Mischung wurde durch die α-1,4-Bindungen hydrolysierenden und α-1,6-Transglykosylierungs-Aktivitäten des BLMA erzeugt und enthielt 58,3% verschiedener verzweigter Oligosaccharide. Kleine verzweigte Oligosaccharide wie Isomaltose, Isopanose und Panose wurden in der Mischung durch verschiedene Analysen einschließlich der Hochleistungs-Ionenchromatographie (HPIC) identifiziert. Die hauptsächlich verzweigten DP4- und DP5-Moleküle in der Mischung wurden als 6^2-O-α-Maltosyl-maltose, 6^3-O-α-maltosyl-maltotriose und 6^2-O-α-Maltotriosyl-maltose bestimmt. Die zeitliche Verlaufsuntersuchung der BOS-Produktion weist darauf hin, daß die Hydrolyse- und Transglykosylierungsreaktionen durch BLMA miteinander gekoppelt waren. Wahrscheinlich übertrug BLMA einen hydrolysierten Zuckeranteil von einem nichtreduzierenden Ende eines Maltooligosaccharids, vorwiegend Maltose auf einen anderen Anteil Zucker über die Bildung einer α-1,6-Bindung. Die Immobilisierung von BLMA wurde versucht in dem Bestreben, um einen kontinuierlichen Prozeß zur BOS-Produktion zu entwickeln. Das immobilisierte Enzym zeigte eine verbesserte thermische Stabilität und eine leichte Verringerung der Enzymaktivität bei wiederholter Verwendung.

1 Introduction

Recently, the importance of various oligosaccharides has been increased due to their physicochemical properties and physiological effects on human health. BOS are oligomers of glucose in α-1,4-linkage, containing at least more than one α-1,6-glucosidic linkage. Its potential application to food industry is pretty diverse, depending on types of monomer and linkage in the molecule of BOS.

BOS are used as a new sweetener. They taste softer and milder than sucrose. They are also beneficial in preventing dental caries [1]. They are known to be effective for the growth of human intestinal bacteria, *Bifidobacteria* [2]. They are used as low-calorie sweetner because they are not digested readily in human body [3]. BOS have low water activity, thereby preventing the growth of spoilage microorganisms (*Kweon* et al., in press). Added as a food component, BOS could retard retrogradation of starchy foods. Due to these useful properties, production of BOS mixtures has been increased by 50–100% per year during last few years.

Industrial production of a BOS mixture is carried out by a two step saccharification system applying β-amylase and transglucosidase to starch solution liquefied by α-amylase [4]. We reported isolation of a novel branching enzyme, BLMA, which showed transglycosylation activity in the presence of excess amount of glucose in addition to hydrolyzing activity on pullulan, cyclodextrin, and starch [5]. From these, we proposed that various kinds of branched oligosaccharides would be synthesized by using these activities of BLMA. Recently, we succeeded in producing an anomalously linked oligosaccharide mixture from starch by carrying out the liquefaction and saccharification processes simultaneously in the presence of a thermostable α-amylase and BLMA [6]. Resulting product contained almost 50% of branched oligosaccharides including isomaltose. *Kuriki* et al. [7] reported a method for the production of an isomalto-oligosaccharide syrup from starch using neopullulanase of *B. stearothermophilus* and α-amylase of *B. subtilis* simultaneously. They characterized the composition of various isomaltooligosaccharides in the syrup, but failed to determine the ratio between isopanose and panose in it.

The purpose of this study was to optimize conditions for the production of a BOS mixture from liquefied starch using BLMA and characterize the mixture. The composition of the BOS mixture was determined by various analysis methods including HPIC and the action mode of the enzyme was proposed based on the investigations. Immobilization of the

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BLMA enzyme was attempted as an effort to develop a continuous process for BOS manufacture.

2 Materials and Methods

2.1 Enzymes and substrates

BLMA was purified from *E. coli* HB101 transformed with pIJ322 as described previously [5]. A thermostable α-amylase (Termamyl 120 KNU/g) and glucoamylase (AMG 300L; 300 AG/ml) were purchased from Novo Nordisk Co. (Denmark). Pullulanase of *Enterobacter aerogenes* was purchased from Sigma Chemical Co. (St. Louis, USA.). Anhydrous dextrose was purchased from Junsei Chemical Co., Ltd. (Japan) and isopanose was kindly supplied by Dr. *Y. Sakano* (Tokyo Noko University, Japan). Other maltooligosaccharide standards and pullulan were purchased from Sigma Chemical Co..

2.2 Analysis of enzyme activities

The activity of BLMA was determined as described previously [5]. The activities of Termamyl and pullulanase were determined as described by *Takasaki* [8] and *Sakano* et al. [9], respectively. One unit of cyclodextrin hydrolyzing activity (CU) of BLMA was defined as the amount of enzyme producing reducing sugar equivalent to one unit change of absorbance at 575nm. One unit of Termamyl was defined as the amount of enzyme that produced reducing sugar corresponding to 1μmole of glucose from soluble starch per minute. One unit of pullulanase was defined as the amount of enzyme that liberated 1μmole of maltotriose from pullulan per minute.

2.3 Liquefaction and saccharification of starch by α-amylase

Rice or corn flour suspension (30%, w/v) was prepared by dissolving flour in 50mM maleate-NaOH buffer, and the liquefaction process was carried out by adding $4.4\text{-}7.2 \times 10^8$ units of α-amylase. Then the reaction was initiated by placing the solution in a boiling pot for 10min and the reaction was stopped by autoclaving it at 121°C for 10min. The sample (500μl) was mixed with same volume of acetonitrile, filtered through a membrane with 0.45μm pores after centrifugation, and then subjected to high performance liquid chromatography or HPIC.
More than 200CU of BLMA was added per gram of starch, because BLMA showed transferring activity above the concentration. EDTA was added to the reaction mixture at the enzyme final concentration of 5mM in order to activate BLMA. The reaction was stopped by boiling for 5min after incubating at 50°C for 15h.

2.4 Purification of the BOS mixture

The reaction mixture containing branched oligosaccharides was incubated for 2h, with stirring in the presence of 1% (w/v) activated charcoal (18-20 mesh, Sigma). Charcoal was removed by vacuum filtration using Whatman No.5 filter paper. This step was repeated twice. The reaction mixture was then run through a mixed bed ion-exchanger. The solution was frozen at −60°C for 3h after it was concentrated by a rotary vacuum evaporator at 70-80°C. White powder of BOS was obtained after freeze drying.

2.5 High performance liquid chromatography (HPLC)

A HPLC apparatus (Waters 600E, USA) was used with a Lichrosorb NH_2 column (4.6×250mm, Merck) and a line-filter. Elution of carbohydrate was detected with a differential refractometer (Waters R400). Same volume of acetonitrile (L.C. grade, Merck) was added to the sample in order to prevent precipitation. Then it was centrifuged and filtered through a membrane with 0.45μm pores. The column was eluted with acetonitrile-deionized water usually at the ratio of 70 to 30 (v/v) at room temperature. Samples (5-20μl) were injected into the column and eluted at a velocity of 1-1.6ml/min. All the solvents were degassed by vacuum stirring and filtered through a polyvinylidene difluoride membrane filter with 0.45μm pores (Gelman Sciences, Inc.). An isocratic chromatographic system consisted of a Delta prep 4000 pump (Waters), a preparative differential refractometer detector (Waters, Model 403), and an μ-Bondapak NH_2 column (19×150mm) were used for preparative HPLC. One to five ml of samples were injected into the column and eluted with acetonitrile-deionized water at a velocity of 10ml/min.

2.6 Paper electrophoresis / Thin layer chromatography (TLC)

Paper electrophoresis and TLC analyses were carried out as described previously [5].

2.7 High performance ion chromatography (HPIC)

High performance ion chromatography was carried out using a CarboPac PA1 column (Dionex Bio LC 4500i) and a pulsed amperometric detector (PAD, Dionex). All solvents were degassed and filtered as described above. The samples (30% liquefied starch treated with BLMA) were eluted at a flow rate of 1.0ml/min with 150mM NaOH during first 40min of the chromatography and then eluted with gradients of 150mM NaOH (100% to 0%) and 600mM sodium acetate (0% to 100%) during the rest of the chromatography, which spanned about 30min. Twenty microliters of 0.02% (w/v) sample solution was injected into the column for analysis.

2.8 Immobilization of BLMA

BLMA was immobilized on silane coated CPC silica beads (Fluka Chemie, Switzland) which were derivatized with an aminopropyl group, 3-aminopropyl-triethoxysilane. The silica beads had pore size of 375Å and diameter about 1.0mm. The silica beads were activated by mixing with 2.5% glutaraldehyde solution at room temperature for 2h [10]. After the beads were washed completely with distilled water, BLMA in 50mM maleic buffer containing 5mM of EDTA (ME buffer; pH 6.8) was coupled to the beads for 8-12h at room temperature with gentle shaking. Immobilized BLMA was washed with the ME buffer and used for the production of BOS.

3 Results and Discussion

3.1 Conditions for the production of a BOS mixture

For the production of BOS, it was desirable to obtain liquefied starch solution with a large fraction of linear maltotriose, maltotetraose, maltopentaose, and a small fraction of glucose and maltose. Maltooligosaccharides larger than maltose were likely to be converted to branched forms by the action of BLMA. The largest amount of DP3, DP4, DP5 molecules was obtained when $4.8\text{-}7.2 \times 10^8$ units of α-amylase were added to 30% (w/v) rice starch solution (data not shown).
Effect of various buffers on the activity of BLMA was also tested to optimize the saccharification and transglycosylation pro-

cess. Fifty millimoles of buffers containing 5mM EDTA and in the range of pH 6.8–8.0 were tested; Bis-Tris [bis-(2-hydroxyethyl)immino]-tris[(hydroxymethyl)methane, pH 7.0], Tris-HCl [Tris (hydroxy-methyl)aminomethane-HCl, pH 8.0], phosphate (pH 8.0), maleate-NaOH (pH 6.8). The relative activity of BLMA was highest in maleate-NaOH buffer (pH 6.8). BLMA showed 25%, 30%, and 70% of the activity in maleate buffer when it was in Bis-Tris buffer, Tris buffer, and phosphate buffer, respectively.

Two different amounts of BLMA were used for the production of branched oligosaccharides and the resulting mixtures were analyzed by HPLC. The contents of the mixtures are compared in Table 1. The mixture treated with 300CU of BLMA per gram of starch contained more branched DP3, DP4, DP5 (57.4%) than the one treated with 200CU of the enzyme (41.9%).

Table 1.
Compositions of the BOS Mixtures Produced from 30% Liquefied Starch Using Different Amounts of BLMA.

Sugar	Composition (%) 200CU[a)]	300CU[a)]
Glucose	19.6	16.7
Maltose and Isomaltose	29.1	25.9
Maltotriose	7.2	ND[b)]
Branched DP3	13.2	22.6
Maltotetraose	2.0	ND[b)]
Branched DP4	25.0	27.3
Branched DP5	3.7	7.5
Sum of BOS[c)]	≧41.9	≧57.4

[a)] amount of BLMA activity/g starch
[b)] not detected
[c)] isomaltose not included

From the results, it was concluded that the highest yield of branched oligosaccharides could be obtained when 30% (w/v) starch solution liquefied with $4.8\text{–}7.2\times10^8$ units of α-amylase was treated with more than 300CU of BLMA per gram of starch in 50mM maleate-NaOH buffer (pH 6.8) containing 5mM EDTA.

3.2 Transferring action of BLMA on various mono- and maltooligosaccharides

We have shown previously that BLMA was capable of hydrolyzing α-1,4-glucosidic linkages in starch, maltooligosaccharides, and cyclodextrin mainly to maltose [5]. However, the enzyme could not hydrolyze maltose further to glucose in solutions containing maltose at low (0.5%, w/v) or high concentration (5.0%, w/v). When various maltooligosaccharide solutions (G4, G5, G6, and G7) of low concentration (0.3%) were treated with BLMA, they were hydrolyzed to glucose and maltose (Figure 1). However, the result of TLC indicated that additional products were present in the reaction mixtures when the solutions contained maltooligosaccharides at a higher concentration (3%, w/v) (Figure 1). Therefore, BLMA might be capable of cleaving certain α-1,4-glucosidic linkage in maltooligosaccharides and transfer the product onto the cleavage site of another moiety of sugar, thus forming branched products with α-1,6-glucosidic linkages. The enzyme did not hydrolyze none of isomaltose, panose, isopanose at either concentration in 24h of reaction (data not shown). These suggested that BLMA could not hydrolyze α-1,6-glucosidic linkage of di- and trisaccharides and these molecules would be the final products in the reaction mixtures treated with the enzyme. In order to test the transferring activity of BLMA further, 5%(w/v) glucose solutions containing 1% (w/v) maltotriose or 1% (w/v) maltotetraose were treated with BLMA (400 CU per one gram of starch) at 50°C for 4h. The reaction mixtures were analyzed by HPLC after the reactions were stopped by boiling for 5min. Isomaltose and branched DP3 molecules were produced by BLMA in both reactions (data not shown) and the result correlated with that of the TLC analysis shown in Figure 1. This indicated that glucose or maltose produced by BLMA was transferred to glucose by forming α-1,6-linkage. The branched DP3 molecule eluted between maltotriose and maltotetraose was considered to be isopanose based on our working hypothesis [5]. From the formation of isopanose, we learned that maltose as well as glucose was utilized as a donor molecule by BLMA.

Other monosaccharides such as fructose, xylose, galactose, and mannose were tested if they were used as donor mole-

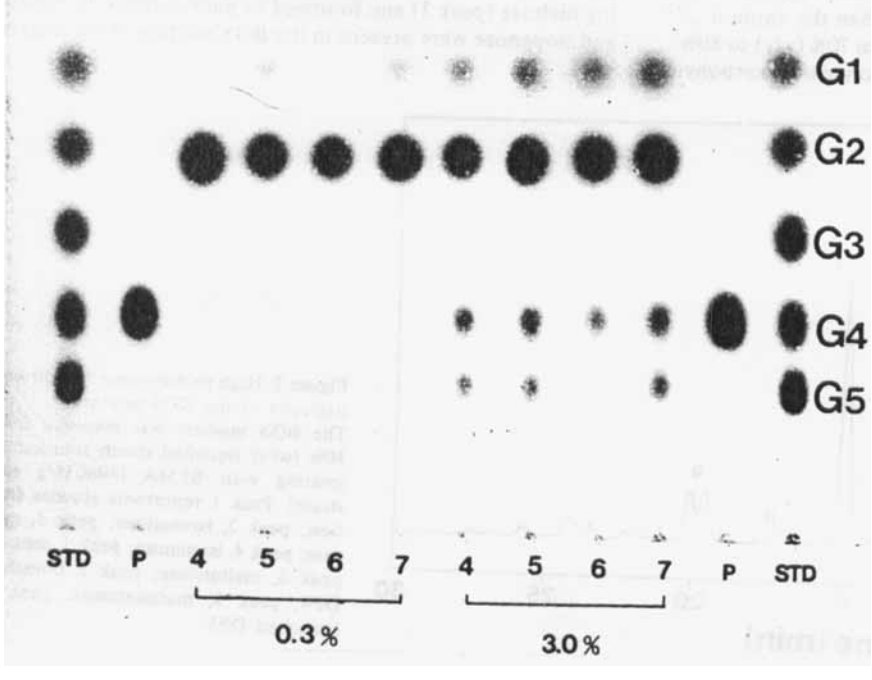

Figure 1. Thin layer chromatography of maltooligosaccharide solutions treated with BLMA.
Maltotetraose (4), maltopentaose (5), maltohexaose (6), and maltoheptaose (7) solutions at two different concentrations were treated with BLMA. The standard markers used are G1-G5 and Panose (P).

cules by BLMA. Monosaccharide solutions (20%, w/v) were tested with BLMA in the presence of 1% (w/v) maltotriose at 50°C for 4h. None of the reaction mixtures produced BOS (data not shown). So far, glucose and maltose were identified as donor molecules which were transferred by BLMA.

3.3 Characterization of BOS fractions produced from liquefied starch by BLMA

Previously, we proposed a model for the reactions catalyzed by BLMA in producing a BOS mixture [6]. BLMA is likely to synthesize a BOS mixture in a complicated manner: it hydrolyzes liquefied starch further to glucose, maltose, maltotriose, etc., and simultaneously transfers the molecules produced at the non-reducing end side onto another moiety of sugar by forming α-1,6-linkages. The whole reaction is completed by repeats of the coupled hydrolysis and transfer reactions, thereby creating a new population of branched oligosaccharide molecules in the reaction mixture. In order to elucidate the reaction mechanism more precisely, the BOS mixture prepared from 30%, (w/v) liquefied starch solution was characterized in detail after treated with 400 CU of BLMA/g of starch at 50°C for 24h. The mixture was analyzed by HPLC. It contained glucose, maltose, branched DP3, branched DP4, and branched DP5 molecules etc. The composition was similar to those of the BOS mixtures shown in Table 1.

We showed that isomaltose and isopanose were formed by the transferring activity of BLMA in solutions containing maltooligosaccharide and excessive glucose. However, isomaltose and isopanose were not separated from maltose and panose, respectively, when 30% of liquefied starch solution treated with BLMA was analyzed by HPLC. *Rocklin* and *Pohl* [11] introduced a new method of carbohydrate separation using anion-exchange chromatography. By the method, carbohydrates are ionized at a high pH and they are separated in the alkaline mobile phase as the result of variation in pK_a values. Recently *Tsang* et al. [12] reported qualitative and quantitative analysis of sweeteners in various soft drinks including maltose and isomaltose. They could separate traces of maltose and isomaltose by HPIC. An isomaltose peak was detected after big glucose-fructose peaks and was followed by the peak of maltose. The maltose peak of the BOS mixture exhibited a small shoulder, suggesting the presence of isomaltose when analyzed by HPLC [6]. The resolution was improved when the amount of acetonitrile in the eluent was increased from 70% (v/v) to 80% (v/v), but still not fully separated. In order to separate carbohydrates with similar structure such as maltose and isomaltose, or panose and isopanose, we tried HPIC of the BOS mixture. Maltose and isomaltose were separated by HPIC as shown in Figure 2. Isomaltose (peak 2) was eluted earlier than maltose (peak 3) by 150mM NaOH. Maltose and isomaltose were present in the BOS mixture at the ratio of 3.4:1.

Branched DP3 molecules in the BOS mixture were fractionated by preparative HPLC and subjected to paper electrophoresis in order to determine their chemical structures. The result is shown in Figure 3. The spots derived from the branched

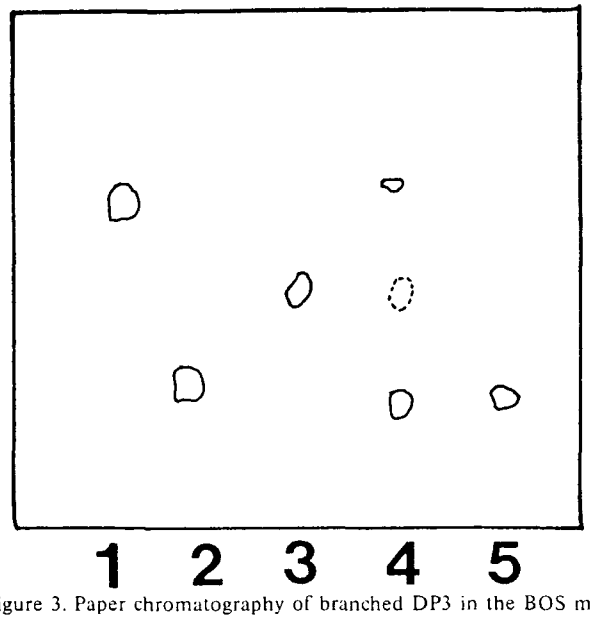

Figure 3. Paper chromatography of branched DP3 in the BOS mixture.
The chromatogram was copied onto a tracing paper for better resolution. Lane 1 was spotted with glucose; lane 2, panose; lane 3, isopanose; lane 4, branched DP3 in the BOS mixture; lane 5, cyclodextrin.

DP3 fraction had Rf values corresponding to those of panose or isopanose. Therefore, the branched DP3 fraction was considered to be mainly consisted of panose and isopanose. The presence of panose and isopanose in the DP3 fraction was also proved by HPIC (Fig. 2). Isopanose (peak 4) was eluted following maltose (peak 3) and followed by panose (peak 5). Panose and isopanose were present in the BOS mixture at the ratio of 3.2:1.

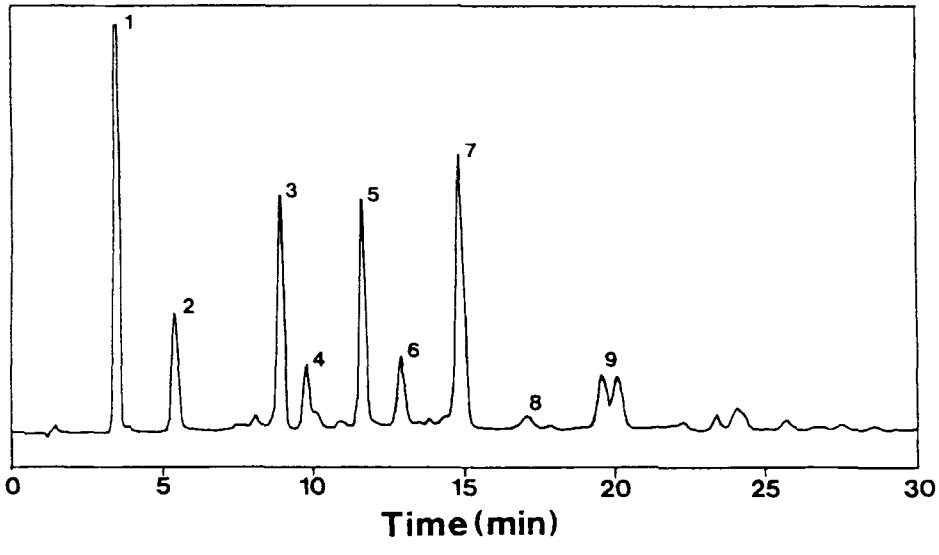

Figure 2. High performance ion chromatography of the BOS mixture.
The BOS mixture was prepared from 30% (w/v) liquefied starch solution by treating with BLMA (400CU/g substrate). Peak 1 represents glucose fraction; peak 2, isomaltose; peak 3, maltose; peak 4, isopanose; peak 5, panose; peak 6, maltotriose; peak 7, branched DP4; peak 8, maltotetraose; peak 9, branched DP5.

The branched DP4 and DP5 fractions of the BOS mixture were collected separately from a μBondapak NH_2 HPLC column and used for further characterization using pullulanase. Pullulanase hydrolyzes α-1,6-glucosidic linkages in pullulan to produce maltotriose [13]. Over 82% of the branched DP4 fraction was degraded to maltose by the action of pullulanase (Fig. 4). Therefore, the fraction was likely to be consisted mainly of 6^2-O-α-maltosyl-maltose. The rest of the fraction might be 6^3-O-α-glucosyl-maltotriose, which might eventually be hydrolyzed to panose and glucose as the reaction proceeds. Previously, a branched DP4 fraction was prepared from 1% (w/v) pullulan solution containing glucose (5%, w/v) by the action of BLMA. Pullulanase was incubated with the 1% (w/v) branched DP4 solution at 25°C for 4h, but no hydrolysis were detected by HPLC analysis (data not shown). Judging from the pullulanase action pattern, the DP4 fraction was considered to be 6^1-O-α-(6^2-O-α-glucosyl-maltosyl)-glucose [14]. The result suggested that BLMA transferred panose moiety generated from pullulan to glucose and this supported our hypothesis on BLMA action pattern. Also, this molecule was likely to be present in the BOS mixture.

The branched DP5 fraction was treated with either pullulanase or glucoamylase and then subjected to TLC and the result is shown in Figure 5. The branched DP5 molecules were hydrolyzed to maltose and maltotriose by pullulanase, and they were hydrolyzed solely to glucose when they were treated with glucoamylase. Pullulanase is known to hydrolyze α-1,6-glucosidic linkages in branched oligosaccharides such as 6^3-O-α-maltosyl-maltotriose and 6^2-O-α-maltotriosyl-maltose, but not the α-1,6-glucosidic linkages in isopanose and various glucosyl stubbed oligosaccharides [15]. Glucoamylase cleaves α-1,6-glucosidic linkages in such oligosaccharides as panose, 6^3-O-glucosyl-maltotriose, but does not act on saccharides containing only α-1,6-glucosidic linkages or those with two or more repetition of α-1,6-glucosidic linkages, such as isomaltose and isomaltotriose [16]. The result obtained by HPLC analysis of the reaction mixtures using a Licrosorb NH_2 column was in good correlation with the TLC analysis (chromatogram not shown). However, the HPIC exhibited two peaks which were likely to represent isomers of branched DP5 molecules (Fig. 2, peaks 9). They appeared in almost equal amount at the elution time of 19.5 and 20.5min. From this, the branched DP5 fraction was likely to be a mixture of two isomers, 6^3-O-α-maltosyl-maltotriose and 6^2-O-α-maltotriosylmaltose. Composition of the BOS mixture determined by HPIC is shown in Table 2. Total amount of BOS in the mixture was

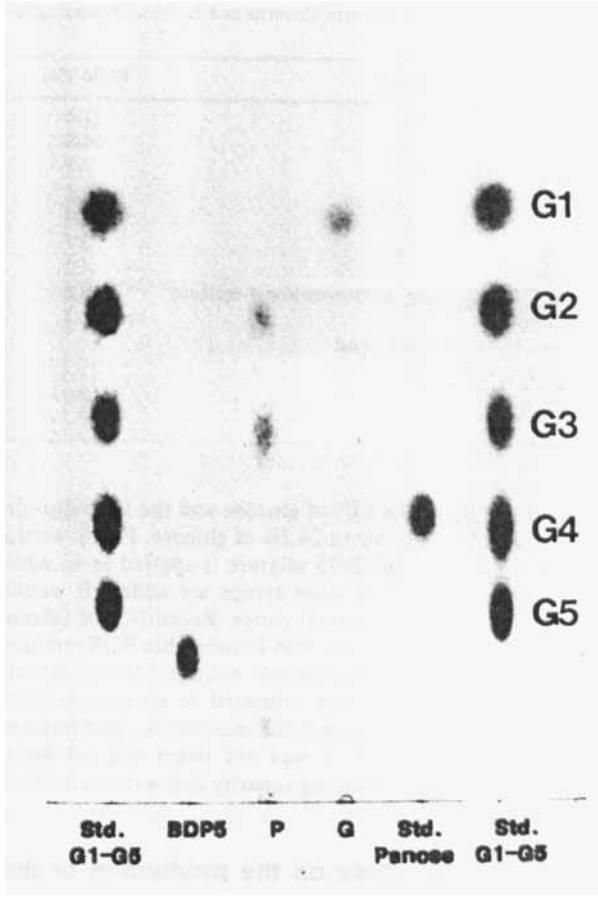

Figure 5. Thin layer chromatography of branched DP5 in the BOS mixture.
The branched DP5 molecules (BDP5) were hydrolyzed to maltose and maltotriose by pullulanase (P); to glucose by glucoamylase (G). The molecules used as standard markers (Std.) were G1-G5 and panose.

58.3%, which was higher than that of an anomalously linked oligosaccharide (Alo) mixture on market (45%) [4], and close to that of an isomalto-oligosaccharide syrup reported by *Kuriki* et al. (60%) [7]. The BOS mixture produced in the laboratory contained much less glucose (11.4%) than other mixtures: the

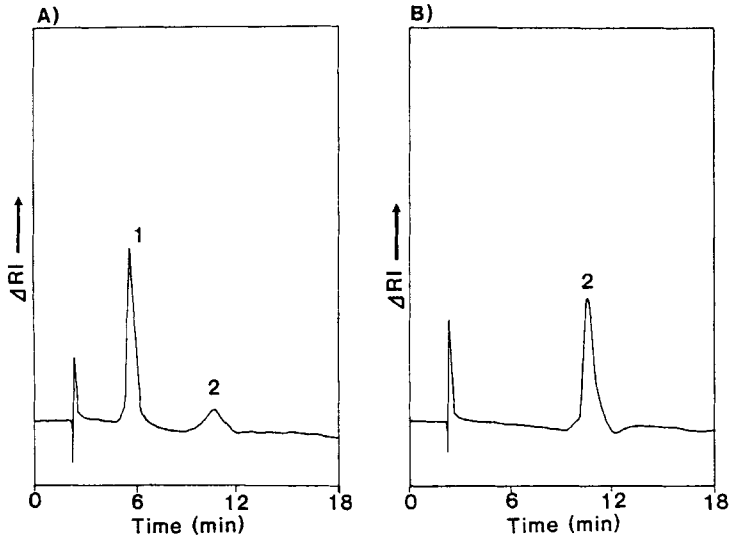

Figure 4. High performance liquid chromatography of branched DP4 in the BOS mixture.
Chromatogram A shows peaks resulted from pullulanase hydrolysis of branched DP4; chromatogram B, a peak of branched DP4 without pullulanase treatment. Peak 1 represents maltose; peak 2, branched DP4 in the BOS mixture.

Table 2.
Composition of the BOS Mixture Determined by Ion-chromatography Analysis.

Sugar	Ratio (%)
Glucose	11.4
Maltose	19.6
Isomaltose	5.7
Maltotriose	5.1
Panose	12.0
Isopanose	3.7
Maltotetraose	4.2
Branched DP4 including 6^2-O-α-maltosyl-maltose	18.6
Maltopentaose	1.4
6^2-O-α-maltosyl-maltotriose and 6^3-O-α-maltotriosyl-maltose	13.7
> Branched DP5	4.6
Sum of BOS	58.3

Alo mixture contained 40.5% of glucose and the isomalto-oligosaccharide syrup contained 24.3% of glucose. Foods would taste less sweet when the BOS mixture is applied as an additive than when sucrose or other syrups are added. It would also decrease casualty of dental caries. Recently, our laboratory succeeded in developing a non-fermentable BOS mixture which contained neither glucose nor maltose (manuscript in press). The BOS mixture was subjected to an immobilized yeast column to remove glucose and maltose by yeast fermentation. The resulting mixture was not sweet but exhibited same level of moisture retaining capacity and water activity as the BOS mixture.

3.4 Time-course study on the production of the BOS mixture

Change of the composition in the BOS mixture as a function of reaction time was monitored over 100h (Fig. 6). The reaction was carried out by incubating 30% (w/v) liquefied starch solution with 200CU BLMA per gram of starch at 40°C in order to slow down the reaction. The liquefied starch solution contained 8% glucose, 17% G2, 17% G3, 8% G4, 16% G5, 16% G6, and 5% G7 (Fig. 6B). The amount of glucose increased gradually as the reaction time increased until the final concentration reached 20%: G2 concentration showed a little change at the beginning of the reaction, but decreased steady fo the rest of the reaction. The concentrations of other oligosaccharides decreased as the reaction time increased. The amount of branched DP3 and DP4 in the mixture increased as the reaction proceeded (Fig. 6A). On the other hand, the amount of branched DP5, 6, and 7 began to decrease gradually when the molecules were accumulated to a certain amount. From these, it was likely that G3, G4, and G5 molecules were hydrolyzed by BLMA mainly to glucose and maltose, and a little bit of maltotriose. At the same time, they were transferred onto the cleavage site of other molecules by the formation of α-1,6-glucosidic linkage. Branched DP5 and DP6 molecules seemed to be hydrolyzed to smaller branched molecules and glucose (or maltose etc.) when G3, G4, and G5 molecules became scarce in amount for the hydrolysis reaction of BLMA. This might indicate that the hydrolysis and the transglycosylation reactions carried out by BLMA are coupled.
A more refined model of BLMA action pattern than the one described previously [6] is proposed in Figure 7. In a major reaction, BLMA hydrolyzes maltooligosaccharides by maltose unit from nonreducing ends of the molecules (Fig. 7B, b). Maltose produced from a nonreducing end serves as a donor molecule which is transferred by BLMA to an acceptor mole-

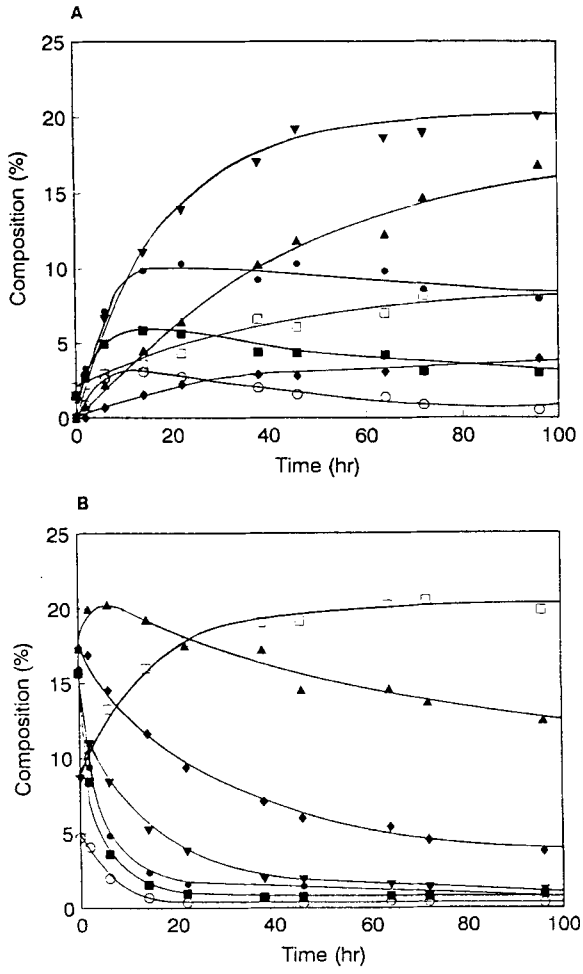

Figure 6. Time course assay of BOS production.
Panel A shows change in the concentration of glucose (□), maltose (▲), maltotriose (◆), maltotetraose (▼), maltopentaose (●), maltohexaose (■), and maltoheptaose (○) in the liquefied starch solution as BOS is synthesized by the action of BLMA. Panel B shows increase of isomaltose (□), isopanose (◆), panose (▲), BDP4 (▼), BDP5 (●), BDP6 (■), and BDP7 (○) as the reaction proceeded.

cule such as glucose, maltose, maltotriose etc. This would give rise to products such as isopanose, 6^2-O-α-maltosyl-maltose, and 6^3-O-α-maltosyl-maltotriose. 6^3-O-α-maltosyl-maltotriose may be hydrolyzed by BLMA further to glucose and 6^3-O-α-glucosyl-maltotriose as the concentration of maltooligosaccharides becomes low in the reaction mixture. In minor reactions, glucose or maltotriose is produced from nonreducing end of maltooligosaccharide, and they serve as donor molecules (Fig. 7B, a and c). When they are transferred to acceptor molecules, branched oligosaccharides such as isomaltose, panose, 6^3-O-α-glucosyl-maltotriose, 6^1-O-α-maltotriosyl-glucose, 6^2-O-α-maltotriosyl-maltose, and 6^3-O-α-maltotriosyl-maltotriose are formed. 6^1-O-α-maltotriosyl-glucose, 6^2-O-α-maltotriosylmaltose, and 6^3-O-α-maltotriosyl-maltotriose may be hydrolyzed further to maltose, isopanose, panose, so on, and used again as donor molecules for transglycosylation reactions. The reaction mode may resemble a two-site insertion mechanism proposed for the synthesis of dextran by dextransucrase [17], being different in that the product is released from the enzyme after every transglycosylation reaction.
Fogarty et al. [18] reported a mold α-amylase which produced a high level of maltose upon hydrolysis of starch and related substances. They concluded that both unimolecular and multi-

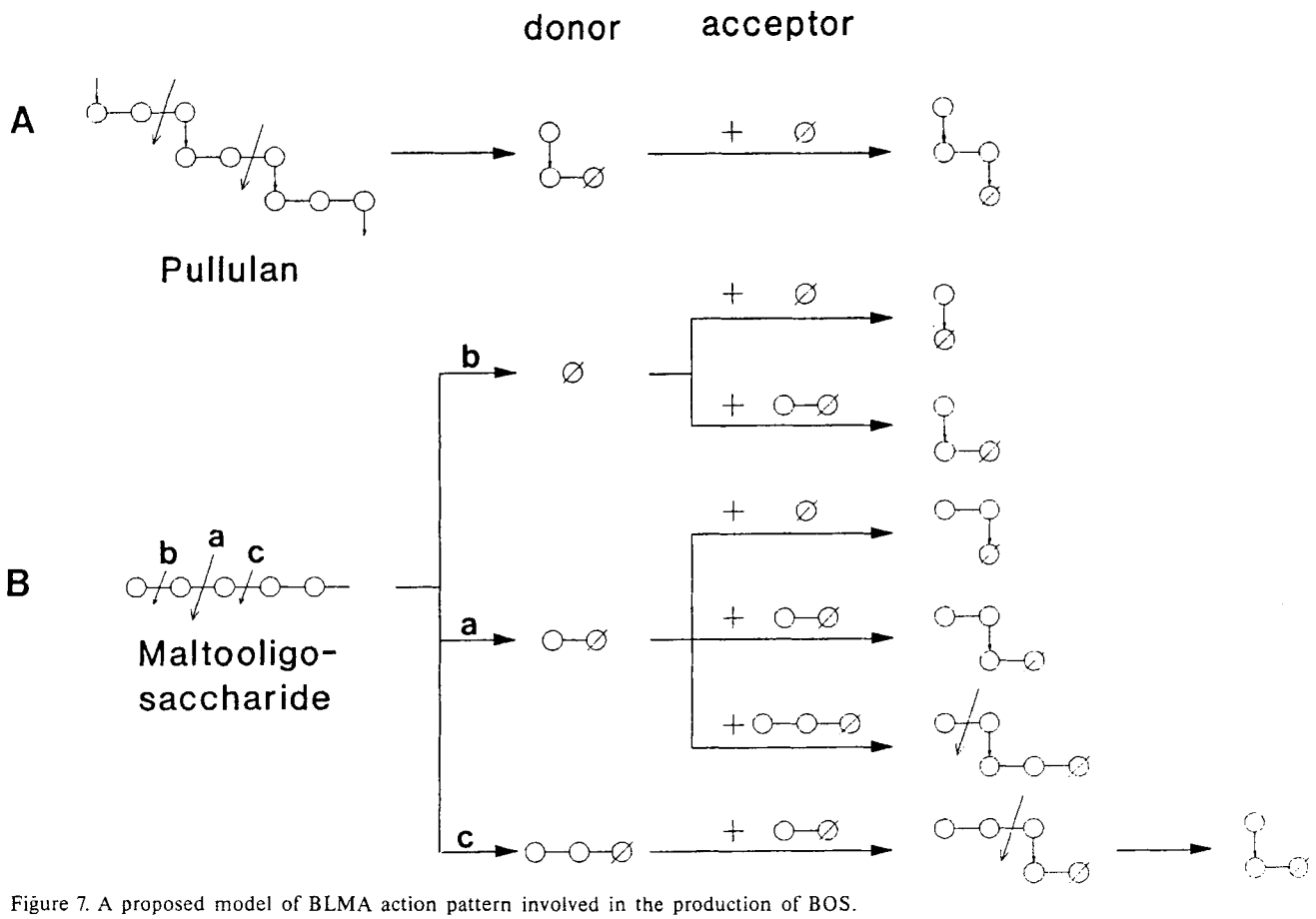

Figure 7. A proposed model of BLMA action pattern involved in the production of BOS.
(A) BLMA hydrolyzes pullulan at the α-1,4-linkage close to α-1,6-linkage and transfer the resulting panose moiety to glucose present in excessive amount by forming α-1,6-linkage, simultaneously. As the result, 6^1-O-α-(6^2-O-α-glucosyl-maltosyl)-glucose is produced. (B) BLMA hydrolyzes maltooligosaccharides mainly by maltose unit (b), but also to smaller maltooligosaccharides, for example glucose and maltotriose (a & c). Simultaneously, these are transfered to glucose, maltose, or maltotriose by the enzyme to produce isomaltose, panose, isopanose, 6^2-O-α-maltosyl-maltose, 6^3-O-α-maltosyl-maltotriose, and 6^2-O-α-maltotriosyl-maltose. Longer branched oligosaccharides are likely to be hydrolyzed further by BLMA.

molecular mechanisms occurred during the concentration-dependent degradation of maltotriose and maltotetraose. They also discussed nonhydrolytic transfer reactions during maltooligosaccharide degradation by several α-amylases.
From the time course study, we concluded that the highest yield of branched oligosaccharides could be obtained in 22-50h of reaction under the conditions. Production of a BOS mixture could be controlled selectively by changing reaction time and the composition of liquefied starch mixture.

3.5 Immobilization of BLMA

In order to enhance thermal stability of the enzyme, BLMA was immobilized on CPC Silica carrier by covalent linkage and used for the production of BOS. About 64CU of BLMA was coupled to 1g of the matrix, which corresponded to 10% coupling yield. Even though the coupling yield was relatively low, thermostability of the enzyme was increased remarkably. Decimal reduction time of the immobilized BLMA at 55°C was 96.8min, while that of BLMA at the same temperature was only 20.5min. An autoprecipitating matrix (AS-HF) and chitosan beads were tested as the matrix for BLMA immobilization, but they were not useful due to low pH range for precipitation and poor binding strength, respectively. The immobilized enzyme was mixed with 30% (w/v) liquefied starch in the final volume of 5ml and incubated at 45°C for 24h. The resulting reaction mixture was analyzed by HPLC and showed the same pattern as the previous analyses repeated several times (data not shown). The content of the BOS mixture produced by the continuous process using immobilized BLMA was constant for 400h. The result indicated that immobilized BLMA could be used repeatedly without considerable loss of enzyme activity. Liquefied starch solution was retained in the column for about 2.5h and the resulting product contained fairly good amount of BOS. The reaction temperature could be raised to 55°C using the column. By using immobilized BLMA packed in a column and immobilized *Saccharomyces cerevisiae*, we are trying to achieve continuous process of highly concentrated BOS manufacture that is free of glucose and maltose (manuscript in press J. Food Sci.).

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