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Structural Characteristics of Novel Branched Oligosaccharides Synthesized by a Maltose Acceptor Reaction with Dextransucrase from *Leuconostoc mesenteroides* M-12

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Leuconostoc mesenteroides M-12 덱스트란수크라제의 말토스 억셉터 반응으로 합성된 새로운 분지 올리고당의 구조 특성

서 현 창

신구전문대학 식품영양과

요 약

효소반응과 부분 산가수분해 결과를 해석하여 *Leuconostoc mesenteriodes* M-12 덱스트란수크라제 억셉터 반응 산물인 새로운 분지올리고당의 구조를 확인하였다. 분지올리고당 B_4의 구조는 6^2-*O*-α-D-kojibiosylmaltose인 것으로 확인되었으며, 분지올리고당 B_5의 구조는 6^3-*O*-α-D-kojibiosylpanose였다. 억셉터 반응산물을 덱스트라나제로 분해한 결과 새로운 올리고당인 D_4를 확인할 수 있었다. 억셉터 반응산물을 억셉터로 이용한 두번째 억셉터 반응의 생성물을 덱스트라나제 처리하여 D_4를 얻었는데 덱스트라나제와 글루코아밀라제에 의해 분해되지 않았다. 그 구조는 6^2-*O*-α-D-kojibiosylisomaltose로 확인되었다. 직선상 또는 분지 결합을 가진 d.p. 6 이하의 억셉터 반응산물의 생성 패턴도 확인하였다.

주요어 : *Leuconostoc mesenteroides*, 덱스트란수크라제, 분지올리고당, 억셉터반응

INTRODUCTION

There is an increasing interest in the application of dextransucrase for the synthesis of novel sugars and oligosaccharides that have special linkages or branched structures[1]. Robyt and Eklund showed that when the acceptor is a monosaccharide there usally is produced a series of oligosaccharide acceptor products[2]. Many acceptor products using various acceptors were produced but acceptor products containing α-(1→2) branched linkages are not well known[3]. Among the acceptors maltose was the best acceptor in the dextransucrase reaction[2]. Remaud-Simeon et. al.[4] reported on the synthesis of oligosaccharides containing α-(1→2) branched linkages. They reported the synthesis of branched oligosaccharide using acceptor reaction catalyzed by the dextransucrase from *Leuconostoc mesenteroides* B-1299. They proposed the structure of branched oligosaccharide B_4 (d.p. 4) in accordance with the results obtained from enzymatic hydrolyses and ^{13}C-NMR. But they could not propose the structure of branched oligosaccharide B_5(d.p. 5) and the formation pattern of acceptor reaction products.

Novel maltose acceptor products containing a α-(1→2) branched linkage synthesized by a reaction with dextransucrase from *Leuconostoc mesenteroides* M-12 were examined in this paper. The main purpose of this research was to elucidate the structure and formation pattern of novel branched oligosaccharides synthesized by acceptor reaction. The structures of three branched oligosaccharides, B_4, B_5, and D_4(endodextranase digestion product, d.p. 4) were proposed and the formation pattern of acceptor reaction products was also

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examined.

MATERIALS AND METHODS

1. Dextransucrase production

Medium for the production of dextransucrase from *Leuconostoc mesenteroides* M-12 consisted of 5g of Bacto-peptone, 5g of yeast extract, 20g of K_2HPO_4, 20g of sucrose, and 10mL of salt solution per liter. Salt solution consisted of 20g of $MgSO_4 \cdot 7H_2O$, 1g of NaCl, 1g of $FeSO_4 \cdot 7H_2O$, 1g of $MnSO_4 \cdot H_2O$, 1.3g of $CaCl_2 \cdot 2H_2O$ per liter. The culture media were sterilized by autoclaving, sugar and salts were autoclaved separately from other medium components and added asceptically after sterilization. Inoculated culture was incubated at 30℃ without shaking and the culture was stopped at the end of the exponential growth phase. The culture supernatant was recovered by centrifugation and the enzyme was precipitated by 80% $(NH_4)_2SO_4$. The precipitant was then harvested and dialyzed against 20mM acetate buffer, pH 5.2, 1mM $CaCl_2$, 0.02% NaN_3 at 4℃. The activity of the enzyme was determined by radiochemical assay method[5]. Assay was conducted using [U-^{14}C] sucrose at 25℃ and pH 5.2 with 20mM acetate buffer containing 1mM $CaCl_2$. The amount of radioactive glucose incorporated into methanol insoluble dextran was determined by liquid scintillation spectrometry. One IU of dextransucrase was determined by the amount of enzyme necessary to incorporate 1μmole of D-glucose into dextran for 1 min.

2. Oligosaccharides synthesis and separation

Acceptor reaction was conducted at 37℃ in 50mM sodium acetate buffer, pH 5.2, 100g of sucrose, 100g of maltose per liter, 20mM $CaCl_2$, and 0.2IU/mL of *Leuconostoc mesenteroides* M-12 dextransucrase. The reaction was stopped when sucrose was consumed completely. Purification of branched oligosaccharide B_4, D_4 and B_5 was done with silica gel column chromatography using 70:30(v/v) acetonitrile-water and confirmed with glucoamylase and endodextranase digestion. For the production of D_4 second acceptor reaction was carried out using acceptor products as acceptors. The second reaction was done after removing acetone precipitant and glucose from the first acceptor products. Glucose component was completely eliminated by yeast fermentation using *Saccharomyces cerevisiae*.

3. Oligosaccharide hydrolysis

The endodextranase (E.C. 3.2.1.11) from *Penicillium* sp. which hydrolyze only α-(1→6) linkages and the glucoamylase from *Rhizopus niveus* (Seikagaku Kogyo Co., Ltd.) which hydrolyze α-(1→4) and α-(1→6) linkages from the nonreducing end of linear glucooligosaccharides were used to hydrolyze linear oligosaccharides synthesized. Partial acid hydrolysis of oligosaccharides was conducted by adding HCl to final 0.3N followed by boiling in water at 100℃ for 30min in a sealed glass vial.

4. Chromatography

Thin-layer chromatography was conducted on Whatman K_5 plates using two different solvent systems: four ascents of solvent 1, 85:15(v/v) acetonitrile-water; two ascents of solvent 2, 1:2:3:4:5(v/v/v/v/v) nitroethane-nitromethane-ethanol-water-1-propanol. The carbohydrates were visualized by dipping the plates into 5% (v/v) H_2SO_4 in methanol containing 0.5%(w/v) α-naphtol, followed by drying and baking at 120℃ for 15min. The densities of the carbohydrate spots on the TLC plate were determined by using an Uniscan densitometer(Analtech, Inc., Newark, DE).

RESULTS AND DISCUSSION

1. Synthesis of branched oligosaccharide

Using dextransucrase from *Leuconostoc mesenteroides* B-512FM, homologous Ln (Ln is an oligosaccharide acceptor product of d.p.=n composed of an isomaltodextrin chain linked α-(1→6) and a maltose residue at the reducing end of the oligosaccharide) series of acceptor reaction products

were known to be produced[6]. Oligosaccharides different from Ln series were produced as a result of acceptor reaction with dextransucrase from *Leuconostoc mesenteroides* M-12 (Fig. 1). The products were thought to be branched oligosaccharides in that acceptor reaction pattern was similar to the pattern with dextransucrase from *Leuconostoc mesenteroides* B-742 and B-1299[4,6]. By increasing maltose to sucrose ratio of the acceptor reaction the synthesis of oligosaccharide with d.p. higher than 5 was decreased (Fig. 2). When the acceptor reaction was conducted using maltose to sucrose ratio of 1, followed by recovering supernatant from 2 volume of acetone precipitation, oligosaccharides smaller than d.p. 9 could be obtained. It was also shown on Fig. 3 that when the reaction temperature of dextran synthesis was increased from 10℃ to 35℃ using 0.5IU/mL of the dextransucrase the total amount of synthesized dextran was decreased by 50%. From the results the optimum temperature and the reaction conditions for the acceptor reaction in the production of branched oligosaccharide were thought to be 37℃ and maltose to sucrose ratio of 1. It was also shown in Fig. 5 a novel branched oligosaccharide, D_4 could be produced as a result of the digestion of second maltose ac-

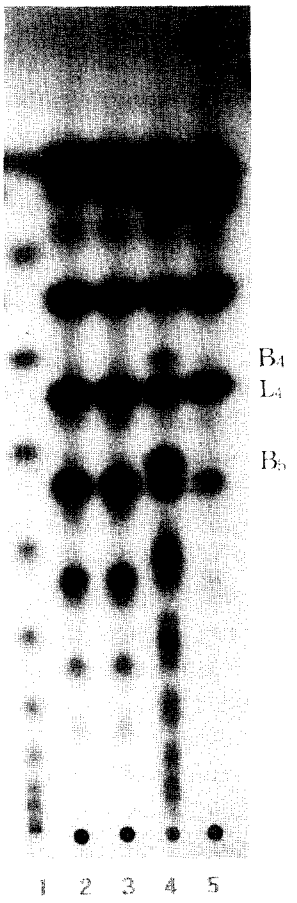

Fig. 1. Acceptor reaction products from various *Leuconostoc mesenteroides* strains. Lane 1: Isomaltodextrin standards (glucose, IM_2, IM_3, IM_4, IM_5, IM_6, IM_7, IM_8: from the top); lane 2: oligosaccharide products from *Leuconostoc mesenteroides* M-1; lane 3: oligosaccharide products from *Leuconostoc mesenteroides* M-8; lane 4: oligosaccharide products from *Leuconostoc mesenteroides* M-12; lane 5: oligosaccharide products from *Leuconostoc mesenteroides* B-512FM (glucose, sucrose, panose, L_4, L_5, L_6: from the top).

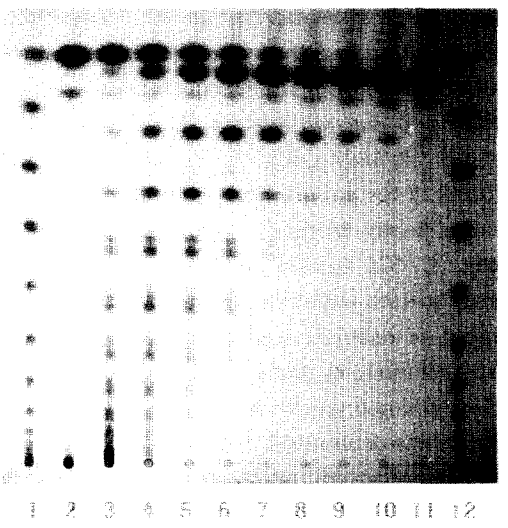

Fig. 2. Thin-layer chromatogram of sucrose acceptor reactions using different ratios of maltose to sucrose at 100mM constant total carbohydrate and 0.5 IU/mL dextransucrase from *Leuconostoc mesenteroides* M-12. The chromatography was conducted on Whatman K_5 plate using 3 ascents of 1:2:3:4:5(v/v/v/v/v) nitroethane-nitromethane-ethanol-water-1-propanol. Ln is an oligosaccharide acceptor product of d.p.=n composed of an isomaltodextrin chain linked α-(1→6) and a maltose residue at the reducing end of the oligosaccharide. Lane 1 and 12: isomaltodextrin standards (IMn); maltose to sucrose ratio of each lane, from lane 2 to lane 11, is 0, 1:5, 1:2, 1:1, 2:1, 5:1, 10:1, 20:1, 40:1, 100:1, respectively.

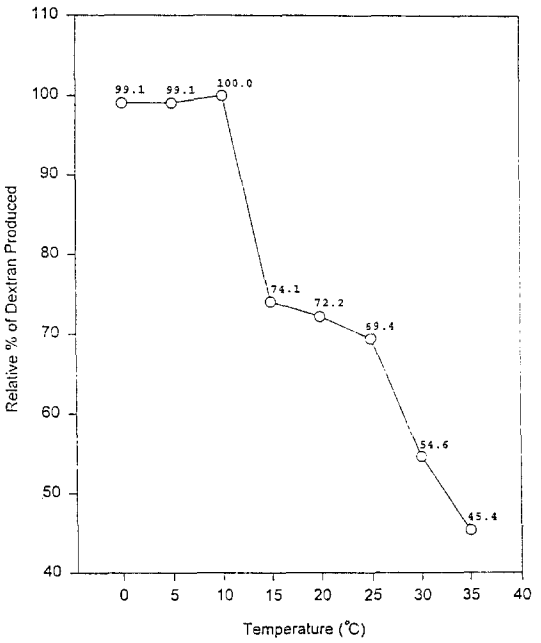

Fig. 3. Production of dextran from *Leuconostoc mesenteroides* M-12 as a function of temperature. 0.2 IU/mL of the dextransucrase was added to the same volume of 300mM sucrose.

ceptor reaction products with endodextranase. The major digestion products of the second acceptor reaction were D_4 and B_5. Secondary acceptor reaction using acceptor products smaller than d.p. 6 followed by an endodextranase treatment revealed that the branched oligosaccharides, B_5 and D_4 could be overproduced. After removing glucose from the second acceptor products the composition of D_4 and B_5 was 15.36% and 16.87%, respectively. After treatment with endodextranase the resistant products to the enzyme was not shown as a homologous series.

2. Structural analysis of branched oligosaccharides

Structural analysis was carried out using partial acid hydrolysis and enzymatic hydrolyses pattern of acceptor reaction products (Fig. 4, Fig. 5, Fig. 6, Fig. 7, Fig. 8). Kojibiose standard was used to elucidate α-(1→2) linkage. Acceptor products from maltose to sucrose ratio of 1 were digested with glucoamylase and endodextranase and the digestion products were analyzed. As shown in Fig. 4, Bn (Bn is a branched oligosaccharide acceptor product of d.p.=n, linking glucosyl unit as α(1→2) to the nonreducing end of oligosaccharide L_{n-1}) series of branched oligosaccharides was remained by digestion with glucoamylase. These series of oligosaccharides was shown to have a branched linkage at the nonreducing end in that they were resistant to glucoamylase which digest α-(1→4) and α-(1→6) linkages. B_5 was a major product (65.99% after removing glucose) of all branched oligosaccharides produced by glucoam-

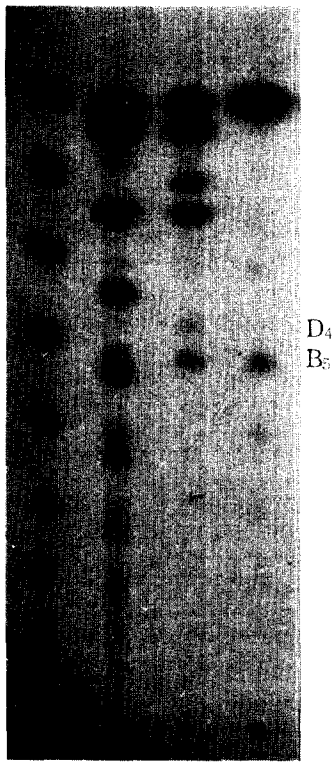

Fig. 4. Digestion of acceptor products with enzymes. Acceptor reaction was carried out using the same maltose to sucrose ratio and dextransucrase from *Leuconostoc mesenteroides* M-12. Lane 1: Isomaltodextrin standards (IMn); lane 2: acceptor reaction products using dextransucrase from *Leuconostoc mesenteroides* M-12; lane 3: acceptor reaction products digested with endodextranase; lane 4: acceptor reaction products digested with glucoamylase.

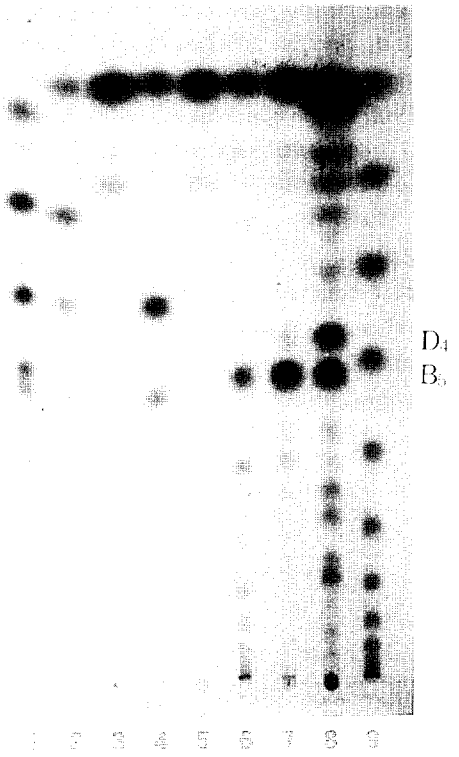

Fig. 5. Enzymatic digestion pattern of oligosaccharide acceptor products. Lane 1: maltose acceptor reaction products of dextransucrase from *Leuconostoc mesenteroides* M-12; lane 2: maltose acceptor products of panose; lane 3: maltose acceptor products of panose digested with glucoamylase; lane 4: maltose acceptor products of L_4; lane 5: maltose acceptor products of L_4 digested with glucoamylase; lane 6: maltose acceptor products of B_5; lane 7: maltose acceptor products of B_5 digested with glucoamylase; lane 8: second maltose acceptor reaction products using lane 1 products as acceptors followed by a digestion with dextranase; lane 9: isomaltodextrin standards (IMn).

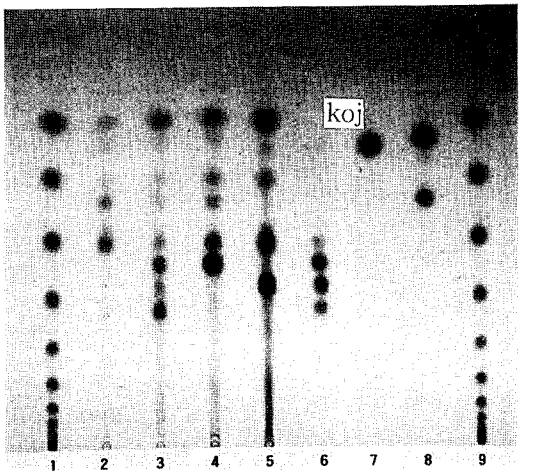

Fig. 6. Thin-layer chromatogram of partial acid hydrolysis products of purified oligosaccharides, B_4, B_5, L_4, and D_4 conducted on Whatman K_5 plate using 4 ascents of 85:15 acetonitrile-water. The condition of final 0.3N HCl followed by boiling for 30min at 100℃ was used to partially hydrolyze the oligosaccharide. Lane 1 and 9: standard saccharides (glucose, IM_2, IM_3, IM_4, IM_5: from the top); lane 2: partial acid hydrolysis products of branched oligosaccharide B_4 (B_4, 6-*O*-*α*-D-kojibiosylglucose, panose, isomaltose(IM_2), kojibiose, maltose, glucose: from the bottom); lane 3: partial acid hydrolysis products of branched oligosaccharide B_5 (B_5, D_4, L_4, IM_3, 6 *O*-*α*-D-kojibiosyl glucose, panose, IM_2, kojibiose, maltose, glucose: from the bottom); lane 4: partial acid hydrolysis product of oligosaccharide L_4 (L_4, IM_3, panose, IM_2, maltose, glucose: from the bottom); lane 5: partial acid hydrolysis product of branched oligosaccharide D_4 (D_4, IM_3, 6-*O*-*α*-D-kojibiosylglucose, IM_2, kojibiose, glucose: from the bottom); lane 6: oligosaccharide standards, B_4, L_4, D_4, and B_5 (from the top); lane 7: D-kojibiose; lane 8: maltose and panose standard.

ylase hydrolysis of the acceptor products. As shown in Fig. 5, B_5, L_4 and panose were good maltose acceptors and acceptor reaction products using purified L_4 and panose as acceptors were completely digested with glucoamylase. But glucoamylase could not completely digest the acceptor products of maltose acceptor reaction using purified B_5 as acceptors. Therefore, the glucoamylase resistant portions were thought to be branched oligosaccharides. Purification for structural analysis was done with branched oligosccharides, B_4, D_4, and B_5. All the three branched oligosaccharides were resistant to endodextranase and glucoamylase digestion.

Purified oligosaccharide products, B_4, B_5, L_4, and D_4 were partially hydrolyzed with HCl and the hydrolysis pattern was analyzed (Fig. 6, Fig. 7, Fig. 8). From the partial hydrolysis pattern it could be suggested that B_4, B_5, and D_4 contained kojibiosyl unit. As shown in Fig. 6 branched oligosaccharide B_4 contained 6-*O*-*α*-D-kojibiosylgluc-

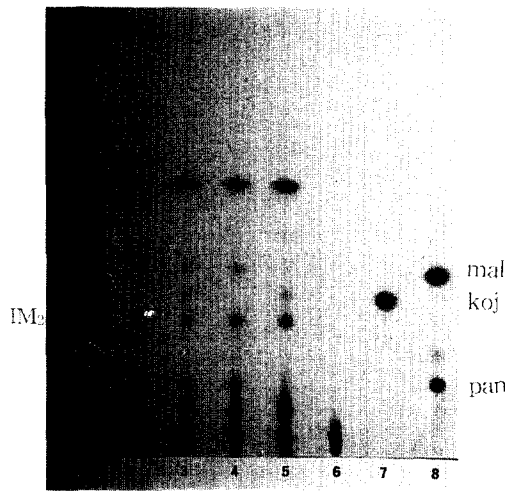

Fig. 7. Thin-layer chromatogram of partial acid hydrolysis products of purified oligosaccharides, B_4, B_5, L_4, and D_4 conducted on Whatman K_5 plate using 2 ascents of 1:2:3:4:5(v/v/v/v/v) nitroethane-nitromethane-ethanol-water-1-propanol. The condition of final 0.3N HCl followed by boiling for 30min at 100℃ was used to partially hydrolyze the oligosaccharide. Lane 1: standard saccharides (glucose, IM_2, IM_3, IM_4: from the top), lane 2: partial acid hydrolysis products of branched oligosaccharide B_4 (B_4, 6-*O*-α-D-kojibiosylglucose, panose, isomaltose(IM_2), kojibiose, maltose, glucose: from the bottom); lane 3: partial acid hydrolysis products of branched oligosaccharide B_5 (B_5, D_4, L_4, IM_3, 6-*O*-α-D-kojibiosylglucose, panose, IM_2, kojibiose, maltose, glucose: from the bottom); lane 4: partial acid hydrolysis product of oligosaccharide L_4 (L_4, IM_3, panose, IM_2, maltose, glucose: from the bottom); lane 5: partial acid hydrolysis product of branched oligosaccharide D_4 (D_4, IM_3, 6-*O*-α-D-kojibiosylglucose, IM_2, kojibiose, glucose: from the bottom); lane 6: oligosaccharide standards, B_4, L_4, D_4, and B_5 (from the top); lane 7: D-kojibiose; lane 8: maltose and panose standard.

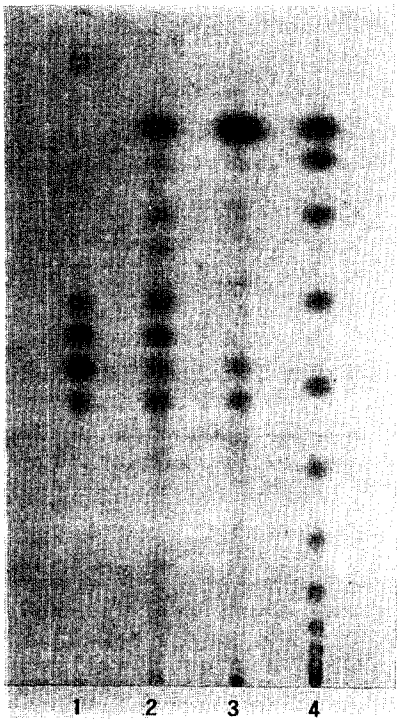

Fig. 8. Glucoamylase treatment of partial acid hydrolysis products of purified oligosaccharides B_5. The condition of final 0.3N HCl followed by boiling for 30min at 100℃ was used to partially hydrolyze the oligosaccharide. The chromatography was conducted on Whatman K_5 plates using 2 ascents of 1:2:3:4:5(v/v/v/v/v) nitroethane-nitromethane-ethanol-water-1-propanol. Lane 1: oligosaccharide standards, B_4, L_4, D_4, B_5 (from the top); lane 2: partial acid hydrolysis products of branched oligosaccharide B_5 (glucose, maltose, kojibiose, isomaltose (IM_2), panose, 6-*O*-α-D-kojibiosylglucose, IM_3, L_4, D_4, B_5: from the top); lane 3: glucoamylase treated partial hydrolysis products of branched oligosaccharide B_5 (glucose, kojibiose, 6-*O*-α-D-kojibiosylglucose, D_4, B_5: from the top); lane 4: standard saccharides (glucose, maltose, IM_2, IM_3, IM_4, IM_5, IM_6).

ose, panose, isomaltose(IM_2), kojibiose, maltose, and glucose units. And it proved that B_5 contained D_4, L_4, IM_3, 6-*O*-α-D-kojibiosylglucose, panose, isomaltose(IM_2), kojibiose, maltose, and glucose units. It seemed like that IM_3 and B_4 had the same Rf value but from the glucoamylase digestion results (Fig. 8) it proved that B_5 contained IM_3 not B_4. Therefore it was clear that B_5 was not derived from B_4. For the clear verification of the presence of kojibiosyl unit, another solvent condition for the chromatogram was applied (Fig. 7) to separate the acid hydrolysis products. From Fig. 7 and glucoamylase digestion results(Fig. 4 and Fig. 8) it could be clearly verified that branched oligosaccharides, B_4, B_5, and D_4 were containing one kojibiosyl unit at the non reducing end of the oligosaccharides.

From these results the proposed structures of B_4, B_5, and D_4 were postulated. The structure of

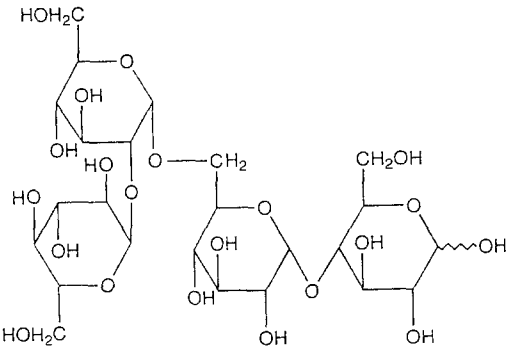

Fig. 9. Structure proposed for the branched oligosaccharide B_4, 6^2-*O*-α-D-kojibiosylmaltose.

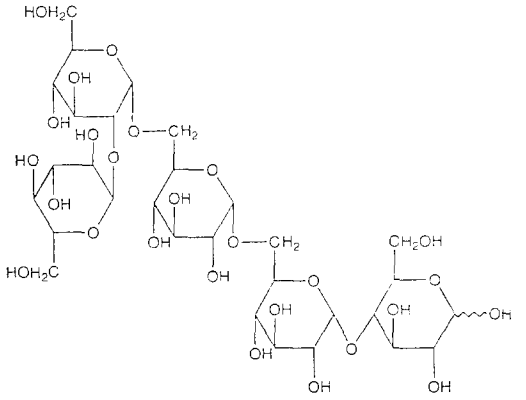

Fig. 10. Structure proposed for the branched oligosaccharide B_5, 6^2-*O*-α-D-kojibiosylpanose.

B_4 was shown to be 6^2-*O*-α-D-kojibiosylmaltose (Fig. 9), B_5 was shown to be 6^3-*O*-α-D-kojibiosylpanose (Fig. 10), and the structure of D_4 was 6^2-*O*-α-D-kojibiosylisomaltose (Fig. 11).

3. Synthesis pattern of oligosaccharide in the acceptor reaction

When using maltose as a glucosyl acceptor with sucrose as a donors, it was known that the first product appeared in the reaction mixture was panose, which became itself an acceptor to form 6^2-*O*-α-D-isomaltosylmaltose, and so on[7]. Formation pattern of branched linkage in the acceptor products was analyzed based on the results of structural analysis of branched oligosaccharides through enzymatic hydrolyses and acid hydrolysis pattern of the products. The postulated pattern of oligosaccharide synthesis was shown in Fig. 12. As shown in Fig. 12 oligosaccharides of d.p. 5 consisted of L_5, B_5, and B_5'. B_5'

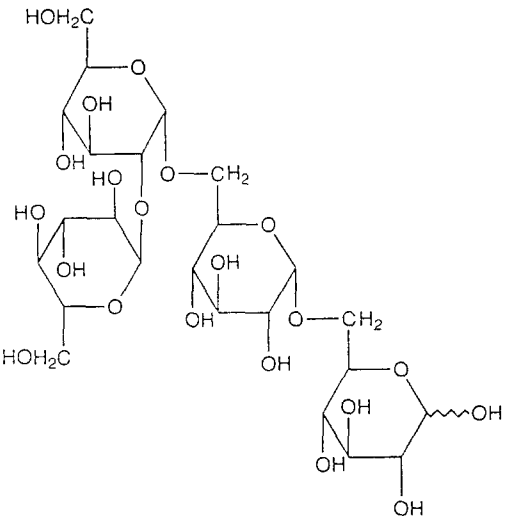

Fig. 11. Structure proposed for the branched oligosaccharide D_4, 6^3-*O*-α-D-kojibiosylisomaltose.

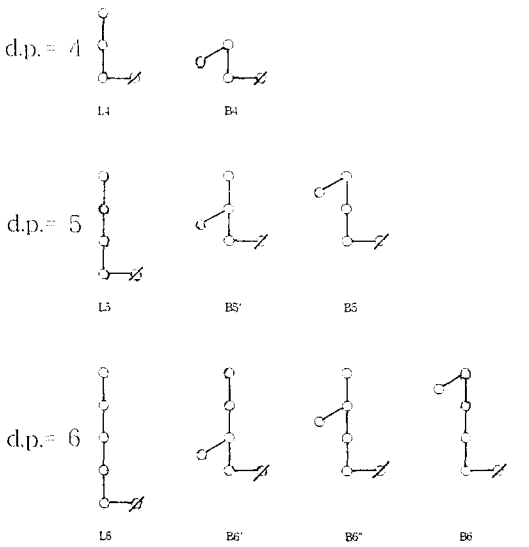

Fig. 12. Postulated acceptor reaction products synthesized by reaction with dextransucrase from *Leuconostoc mesenteroides* M-12. ϕ ;reducing end, ○;glucose residue, −;α-(1→4) linkage, |;α-(1→6) linkage, /;α(1→2) linkage.

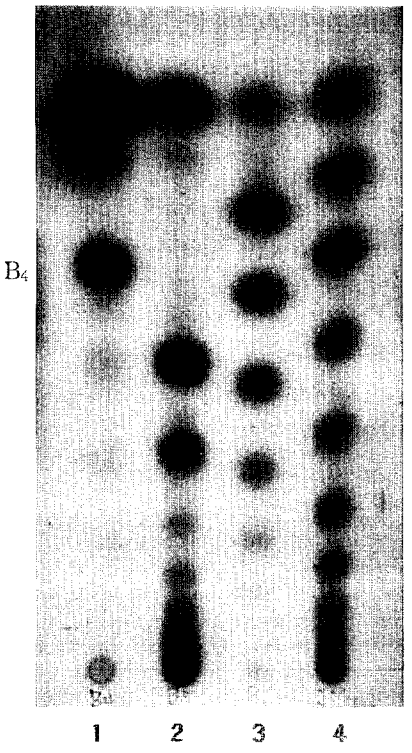

Fig. 13. Maltose acceptor reaction with oligosaccharides, B_4, B_5, and panose. Lane 1: maltose acceptor products of B_4; lane 2: maltose acceptor products of B_5; lane 3: maltose acceptor products of panose; and lane 4: isomaltodextrin standards (IMn).

was produced only a smear amount because B_4 was proved to be a poor acceptor (Fig. 13) and was digested with glucoamylase to produce glucose and B_4. Therefore the major products of d.p. 5 in the acceptor reaction were L_6 and the 6^3-*O*-α-D-kojibiosylpanose(B_5).

Further research will be focused on the structural analysis of branched oligosaccharides higher than d.p. 7 and the mass production of branched oligosaccharides, D_4, B_5, B_6, and their application to food industry.

ABSTRACT

The structures of novel branched oligosaccharides synthesized by the acceptor reaction with dextransucrase from *Leuconostoc mesenteriodes* M-12 were proposed in accordance with the results obtained from enzymatic hydrolyses and a partial acid hydrolysis. The structure of branched oligosaccharide B_4 was shown to be 6^2-*O*-α-D-kojibiosylmaltose. Branched oligosaccharide B_5 was shown to be 6^3-*O*-α-D-kojibiosylpanose. By reacting the acceptor reaction products with endodextranase a novel branched oligosaccharide(D_4) could be produced. D_4 was derived from the result of endodextranase hydrolysis of oligosaccharides synthesized by the second acceptor reaction with dextransucrase and was resistant to endodextranase and glucoamylase. The proposed structure of D_4 was 6^2-*O*-α-D-kojibiosylisomaltose. Formation pattern of the acceptor reaction products smaller than d.p. 6 with linear or branched linkage was also shown.

Key words : *Leuconostoc mesenteroides* dextransucrase, branched oligosaccharides, acceptor reaction.

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