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Synthesis of Glucosyl-sugar Alcohols Using Glycosyltransferases and Structural Identification of Glucosyl-maltitol

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Enzymatic synthesis of glucosyl-sugar alcohols using various transglycosylating enzymes, such as cyclodextrin glucanotransferase (CGTase), α-amylase, α-glucosidase, and pullulanase was investigated using various sugar alcohols, such as sorbitol, xylitol, inositol, maltitol, and lactitol as glucosyl acceptors. CGTase showed the highest transglycosylating activity to sugar alcohols compared to other transglycosylating enzymes, and inositol and maltitol were the most suitable glucosyl acceptors. Soluble starch, extruded starch, cyclodextrins, and maltooligosaccharides were also identified to be adequate glucosyl donors for transglycosylation reaction of CGTase to sugar alcohols. The synthesis of glucosyl-maltitol in the reaction system using extruded starch as the glucosyl donor and maltitol as the glucosyl acceptor showed the best results showing the highest transglycosylation yield. The transglycosylation products were purified by activated carbon column chromatography with ethanol gradient elution. Chemical structures of above transglucosylated products were analyzed by nuclear magnetic resonance spectroscopy, and two products were identified to be maltotritol and maltotetraitol, in which one or two glucose molecules attached to the parent maltitol molecule by a α-1,4-glucosidic bond, respectively.

Various sugar alcohols, sorbitol, xylitol, mannitol, inositol, and maltitol are widely distributed in nature in fruits and marine vegetation. They are produced commercially by hydrogenation of corresponding reducing sugars (7). Sugar alcohols show considerably high endothermic heat in solution when compared to conventional sugars, therefore they give a cooling sensation in the mouth. In addition, they do not participate in Maillard reactions, and show high heat stability as compared to corresponding mono- and di-saccharides because of the absence of free carbonyl groups. The sugar alcohols are also more resistant to microbial degradation than conventional sugars, and are very hygroscopic, furthermore they are recognized as being less cariogenic. As a result, sugar alcohols have been widely used in foods and pharmaceuticals, such as special dietary foods, dietary management of diabetes, humectants, sequesterants for certain multivalent metals, stabilizers, and viscosity control agents (2, 19).

Recently, the transglycosylation function of various starch related enzymes, such as α-glucosidase (5), α-amylase (11), cyclodextrin glucanotransferase (CGTase) (21), pullulanase (20) and sucrose phosphorylase (25), etc., has been attracting considerable attention, either for improvement of the physicochemical properties of sugars or for new effective method for synthesis of functional oligosaccharides. However, the transglycosylation reaction of above transglycosylation enzymes using sugar alcohols as the glucosyl acceptor has rarely been studied.

Sato *et al.* (23, 24) has reported the synthesis of oligoglucosyl-inositol from *myo*-inositol as an acceptor and β-cyclodextrin as a glucosyl donor using the transglycosylation reaction of CGTase from *Bacillus ohbensis*. Among the synthesized oligoglucosyl-inositols, maltosyl-inositol exhibited the most effective growth stimulation effect for *Bifidobacterium* (24). In addition, Kitao and Sekine (13) have synthesized glucosyl-xylitol using the transglycosylation reaction of sucrose phosphorylase from *Leuconostoc mesenteroides*, and identified it as a possible candidate for a preventive sweetener for dental caries because it can reduce the synthesis of water-insoluble glucan by *Streptococcus mutans* and maintain a neutral pH in the cell suspension. Above functionalities of glucosyl-sugar alcohols have lead us to investigate the development of a reaction system suitable for the effective production of glucosyl-sugar alcohols, including selection of an effective enzyme for the transglycosylation reaction, identification of the optimal enzyme reac-

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tion conditions, and purification of the produced glucosyl-sugar alcohols.

Previously, we developed a heterogeneous enzyme reaction system for the transglycosylation reaction of CGTase using various saccharides and glucosides as glucosyl acceptors and insoluble starch as a glucosyl donor (14-16, 18). Insoluble raw starch or swollen extruded starch was used as a glucosyl donor instead of conventional soluble starch. This reaction system showed a high transglycosylation yield and rate on various glucosyl acceptors, such as mono-, di-saccharides and glucosides. Furthermore, the separation and purification of transglucosylated products was significantly easier as compared to a conventional reaction system, because the residual starch remained in an insoluble state and the formation of oligosaccharides acting as by-products were minimized.

In this work, the feasibility of transglucosylation of various transglycosylating enzymes catalyzing starch hydrolysis, such as CGTase, α-glucosidase, α-amylase, and pullulanase, using sugar alcohols as the glucosyl acceptor is compared, and the most suitable enzyme system identified. The suitable sugar alcohols that can be used for transglycosylation of CGTase were also identified. The optimal reaction conditions using maltitol as the glucosyl acceptor and CGTase as the enzyme system were determined, including the specificities of the glucosyl donors, the amount of CGTase applied, and the optimal mixing ratio for glucosyl donor and acceptor. The transglycosylated products were purified using chromatographic methods with maltitol as the glucosyl acceptor, and the structural nature of transglycosylated maltitol was analyzed using NMR spectroscopy.

MATERIALS AND METHODS

Transglycosylating Enzymes

α-Amylase from *Bacillus licheniformis* (EC 3.2.1.1., Sigma Chemical Co., U.S.A.), α-glucosidase from *Rhizopus* mold (EC 3.2.1.3., Sigma Chemical Co., U.S.A.), cyclodextrin glucanotransferase from *Bacillus macerans* (EC 2.4.1.19, Amano Pharmaceutical Co., Ltd., Japan), and pullulanase from *Enterobacter aerogenes* (EC 3.2.1.41, Sigma Chemical Co., U.S.A.) were used as the transglycosylating enzymes.

Measurement of Enzyme Activities

The activity of α-amylase was determined by the reducing sugar determination method (22) using 1% (w/v) soluble starch in 20 mM Tris-Malate-NaOH buffer (pH 6.0) as a substrate, and incubated at 50°C for 10 min. One unit of enzyme was defined as the amount of enzyme producing 1 μM of maltose per min, and the specific activity of α-amylase used was determined to be 1,900 units/mg protein.

The activity of α-glucosidase was determined by the maltose-hydrolyzing method (1) using maltose as the substrate at 37°C for 30 min. One unit of enzyme was defined as the amount of enzyme hydrolyzing 1 μM of maltose per min, and the specific activity of α-glucosidase used was determined to be 820 units/mg protein.

The activity of CGTase was determined by Kitahata's methods (10), using 1% (w/v) soluble starch in 0.02 M Tris-Malate-NaOH buffer (pH 6.0) as substrate at 37°C for 30 min. One unit of activity was defined as the amount of CGTase corresponding to a 1% increase in transmittance at 660 nm per min, and the specific activity of used CGTase was 513.5 units/mg protein.

The activity of pullulanase was determined by the pullulan hydrolyzing method (8), with 1% (w/v) pullulan in 0.02 M phosphate buffer (pH 4.5) as substrate, at 37°C for 30 min. One unit of activity was defined as the amount of enzyme producing 1 μM maltotriose per min, and the specific activity of pullulanase used was 30 units/mg protein.

Sugar Alcohols Used as the Glucosyl Acceptors

Sorbitol (MW 182.2), xylitol (MW 152.1), *myo*-inositol (MW 180.2), maltitol (MW 344.3), and lactitol (MW 344.3) were used as glucosyl acceptors, and purchased from Sigma Chemical Co., U.S.A.

Carbohydrates Used as the Glucosyl Donors

Maltooligosaccharides (G_3~G_7, Sigma Chemical Co., U.S.A.), α-, β-, and γ- cyclodextrins (CycloLab, Ltd., Hungary), glycogen, dextrin, and soluble starch (Sigma Chemical Co., U.S.A.) were used as glucosyl donors. Extruded starch prepared with a single screw extruder as described previously (17) was also used as the glucosyl donor.

Transglyosylation Reaction

50 g of the various carbohydrates used as glucosyl donors and 50 g of the sugar alcohol used as the glucosyl acceptor were suspended in 1.0 liter of 0.02 M Tris-Malate-NaOH buffer (pH 6.0), and then different amount of each transglycosylating enzyme were added. The transglycosylation reaction was carried out at 50°C, pH 6.0 and 200 rpm. The amount of transglycosylating enzymes and the mixing ratio of glucosyl acceptor and donor was changed in some cases.

Separation of Transglycosylated Products

The reaction mixture of maltitol and extrusion starch after 24 h of reaction was centrifuged at 3,000 g for 15 min, and the supernatant was concentrated to 5 fold with a vacuum evaporator. The glucosyl-maltitols were separated by activated carbon column chromatography in which the activated carbon and celite (Sigma. Co., U.S.A.) were mixed at a 2 : 1 ratio, washed thoroughly with distilled water, and then packed on a glass column (3 cm diameter and 15 cm height). 5 ml of the concentrated reaction mixture was applied to the column prepared and

washed with 100 ml of distilled water. Thereafter the column was eluted with an ethanol solution gradating from 0 to 35% (v/v), with the flow rate maintained at 15 ml/h, and then fractionated by 2 ml. The amount of total carbohydrate in each fraction was analyzed by the phenol-sulfuric method (17), and the sugar composition in the carbohydrate containing fraction was analyzed by HPLC.

^{13}C-Nuclear Magnetic Resonance (NMR) Spectroscopy

The structural nature of the purified fraction of glucosyl-maltitol was analyzed by ^{13}C-NMR. Before structural analysis, the purified fraction was lyophilized in a freeze drier and then dissolved in D_2O. ^{13}C-NMR spectra of the purified products was recorded at 25°C on a Varian Unity Plus Spectrometer (Varian Co., U.S.A.) operating at 75 MHz using trimethylsilane (TMS) as an external reference.

Analytical Methods

Sugar alcohols and transglycosylated products were analyzed by HPLC (Gilson Medical Electronics, Inc., France); Cosmosil packed column 5NH_2 (Nacalai Tesque, Inc., Japan), acetonitrile/water (65/35), 1 ml/min, and RI detector.

RESULTS AND DISCUSSION

Comparison of Various Enzymes for Transglycosylation of Sugar Alcohols

Table 1 compares transglycosylation efficiencies of various enzymes belonging to the catalyzing starch hydrolysis, such as α-amylase, α-glucosidase, cyclodextrin glucanotransferase (CGTase), and pullulanase on the transglycosylation yields of various sugar alcohols, including sorbitol, *myo*-inositol, maltitol, xylitol, and lactitol, after 24 h. CGTase showed the highest transglycosylation activity on sugar alcohols compared to other enzymes. Especially, CGTase showed the high specificity for transglycosylation on *myo*-inositol and maltitol, and the transglycosylation yields reached up to 48.5 and 56.4%, respectively. CGTase also showed transglycosylation activity against sorbitol and xylitol, but relatively to a lower extent.

Table 1. Comparison of transglycosylation yield by various glycosyltransferases using sugar alcohols as the glucosyl acceptor.

Sugar alcohols	Glycosyltransferases			
	CGTase	α-Glucosidase	α-Amylase	Pullulanase
Erythritol	N.D.	N.D.	N.D.	N.D.
Xylitol	27.3	6.6	N.D.	N.D.
Ribitol	N.D.	N.D.	N.D.	N.D.
Sorbitol	15.6	N.D.	16.3	N.D.
myo-Inositol	46.5	N.D.	2.2	N.D.
Mannitol	N.D.	N.D.	N.D.	N.D.
Maltitol	49.9	14.3	N.D.	N.D.
Lactitol	N.D.	N.D.	N.D.	N.D.

1) N.D., not detected.
2) Transglycosylation yield (%) after 24 h.
Reaction condition: 50 g/l of each sugar alcohol, 50 g/l of donor, pH 6.0, 50°C.
Amount of enzyme: α-glucosidase, 420 units/ml; CGTase, 1,800 units/ml; α-amylase 350 units/ml; pullulanase 120 units/ml.
Kinds of glycosyl donor: soluble starch for CGTase and α-amylase, maltose for α-glucosidase, and pullulan for pullulanase.

α-Amylase, α-glucosidase, and pullulanase showed relatively low transglycosylation yields on various sugar alcohols as compared to CGTase. Each transglycosylating enzyme showed quite different glucosyl acceptor specificities on sugar alcohols in contrast to CGTase. For example, α-amylase showed only transglycosylation activity on *myo*-inositol and sorbitol, α-glucosidase on xylitol and maltitol, and pullulanase on sorbitol only.

The major role of α-amylase, α-glucosidase, and pullulanase is to hydrolyze the α-1,4- or α-1,6-glucosidic bond, therefore, above enzymes cannot carry out the transglycosylation reaction effectively except in unusual reaction conditions, such as the presence of unusually high substrate concentrations which induce the reverse reaction of the hydrolytic enzyme (4) or reaction in the presence of on organic solvent to reduce the water activity and to facilitate the reverse reaction or the glucosyl transfer reaction of hydrolases (26).

On the other hand, the major role of CGTase is to catalyze the intramolecular and intermolecular transglycosylation reactions, which produce cyclodextrin and glucosylated saccharides (24). As a consequence, the glycosylation reaction can be carried out in relatively mild reaction conditions provided suitable acceptor and donor molecules are present. It also shows wide acceptor specificity on various saccharides and glucosides (12) compared to other enzymes. Because of the nature of CGTase, it showed higher trasnglycosylation activity on sugar alcohols when compared to other amylases used in this experiments.

Kitahata *et al.* (12) suggested that saccharides suitably utilized as a glucosyl acceptor for the transglycosylation reaction of CGTase required the pyranose structure with one free hydroxyl group such as the C4-OH group in glucose and that the configuration of the hydroxyl group in C2 and C3 must be equatorial form. *myo*-Inositol and maltitol have the configurations both of the C4-OH group and of the C2 and the C3-OH group and are suitable as the glucosyl acceptor for CGTase, therefore they showed higher transglycosylation yields among various sugar alcohols. Maltitol and CGTase were selected as glucosyl acceptor and transglycosylating enzyme, respectively, to investigate the optimal reaction conditions and to study the nature of the structure of glucosylated sugar

alcohols.

Glucosyl Donor Specificity for the Transglycosylation Reaction of CGTase on Maltitol

Table 2 compares the donor specificities for the transglycosylation reaction of CGTase on maltitol as the glucosyl acceptor, among various α-1,4-glucans, such as maltooligosaccharides (G_3~G_7), α-, β-, γ-CDs, dextrin, soluble starch, glycogen, and extruded starch. Polysaccharides, including soluble starch and extruded starch showed much higher transglycosylation yields compared to oligosaccharides. Especially, extruded starch was found to be the most suitable glucosyl donor effectively used for the transglycosylation reaction of CGTase.

Among CDs used as the glucosyl donors, α-CD showed the highest transglycosylation yield, which should be figured out from the characteristics of the structural nature of the active sites of the CGTase molecule. CGTase from *Bacillus macerans* produces primarily α-type CD during the CD synthesis enzyme reaction from starch, and this indicates that it shows higher affinity to α-CD among the three CDs in the transglycosylation and hydrolysis reaction (23). Sato *et al.* (23) who used β-type CGTase from *B. ohbensis* for the synthesis of oligoglucosyl-inositols reported that β-CD was the most suitable of the glucosyl donors compared to the other CDs. The susceptibilities of the CDs for the transglycosylation reaction of CGTase are closely related with the type of CGTase used.

Soluble starch also showed higher transglycosylation yields, however, it may cause several technical problems, especially in the purification of transglycosylated products. These problems are probably mainly caused by the following two reasons, both the soluble starch and the transglycosylated products exist in soluble states, and a large amount of the reducing sugar is accumulated as by-products after the reaction. These problems will be reduced by using insoluble starch such as extruded starch as the glucosyl donor.

Extruded starch remains in an insoluble state after the reaction and could easily be separated out and minimize the accumulation of oligosaccharides, as reported in our previous work on CD synthesis using swollen extruded starch as substrate (6). It is expected that extruded starch will be as an effective glucosyl donor for the synthesis of glucosyl-sugar alcohols using CGTase, with a high transglycosylation yield and facilitated purification of the transglycosylated sugar alcohols, and further studies on the reaction system for the synthesis of glucosyl-sugar alcohols need to be conducted.

Determination of the Optimal Reaction Conditions for Transglycosylation of Maltitol by Cyclodextrin Glucanotransferase

Fig. 1 shows the effect of CGTase on the transglycosylation yield of maltitol using extruded starch as the glucosyl donor. The amount of enzyme was changed from 100 to 1,500 units/g of starch, and the reaction was carried out at 50°C for 24 h. The transglycosylation yield was increased proportionally as the amount of CGTase increased up to 800 units/g of starch, and thereafter, remained at a similar level. The excess amount of CGTase would induce unnecessary side reactions, either the hydrolysis of CDs and a disproportionation reaction (14), therefore it did not contribute to the transglycosylation of maltitol any futher. The optimal amount of CGTase was around 800 units/g of extruded starch.

Table 2. Donor specificity of transglycosylation reaction of CGTase from *B. macerans* using maltitol as the glucosyl acceptor.

Kinds of donors	Transglycosylation yield (%)
α-Cyclodextrin	48.4
β-Cyclodextrin	32.5
γ-Cyclodextrin	28.7
Maltotriose (G_3)	10.5
Maltotetraose (G_4)	22.3
Maltopentaose (G_5)	38.6
Maltohexaose (G_6)	40.2
Maltoheptaose (G_7)	41.2
Dextrin	46.9
Soluble starch	49.9
Glycogen	46.8
Extruded starch	52.6

Reaction condition: 50 g/l of maltitol, 50 g/l of each donors, 1,800 units of CGTase/ml, 50°C, 200 rpm, and 24 h.

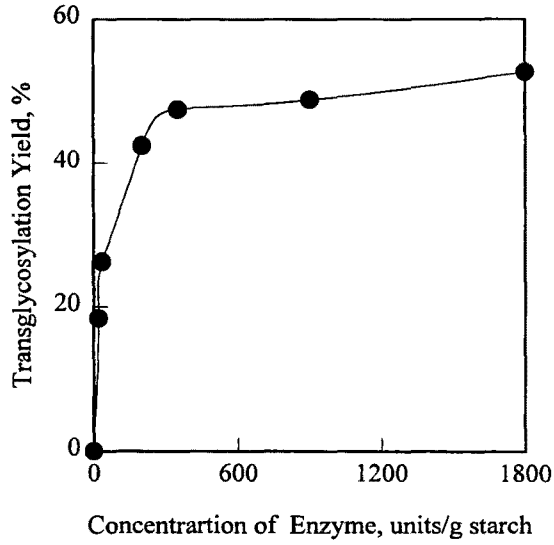

Fig. 1. Effect of the amount of CGTase on the transglycosylation yield of maltitol using extruded starch as the glucosyl donor.

Reaction condition: 50 g/l of maltitol, 50 g/l of extruded starch, pH 6.0, 200 rpm, 50°C and 24 h.

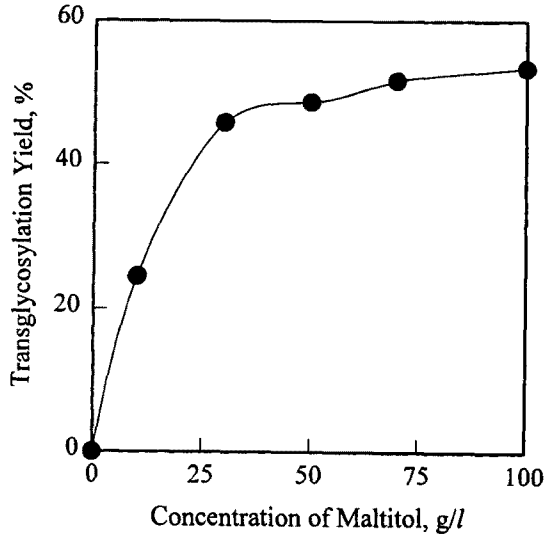

Fig. 2. Effect of the mixing ratio between maltitol and extruded starch on the transglycosylation yield of maltitol.
Reaction condition: 50 g/l of extruded starch, 10~100 g/l of maltitol, pH 6.0, 200 rpm, 50°C and 24 h.

Fig. 2 shows the effects of the mixing ratio of maltitol and extruded starch, in which the extruded starch was fixed at 50 g/l and maltitol was changed from 10 to 100 g/l. The transglycosylation yield decreased as the concentration of maltitol increased and the maximum value was obtained at a mixing ratio of 1 : 1 (g of maltitol : g of extruded starch).

Chromatographic Fractionation of Glucosyl-maltitols

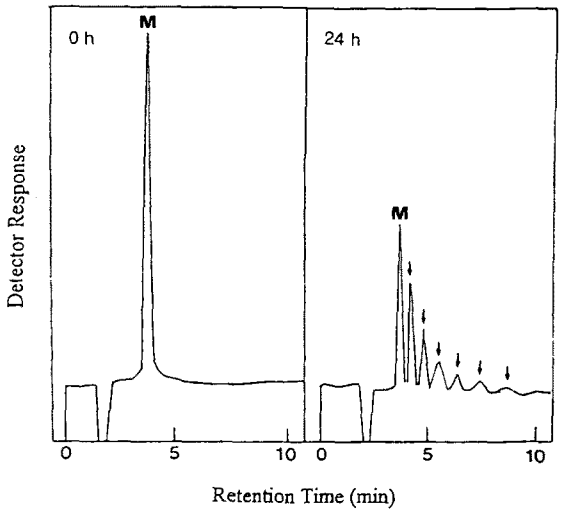

Fig. 3. HPLC chromatogram of the reaction mixture obtained from transglycosylation reaction of CGTase using matitol as the glucosyl accpetor and extruded starch as the glucosyl donor.
M and arrows indicate maltitol and transglycosylated products, respectively.

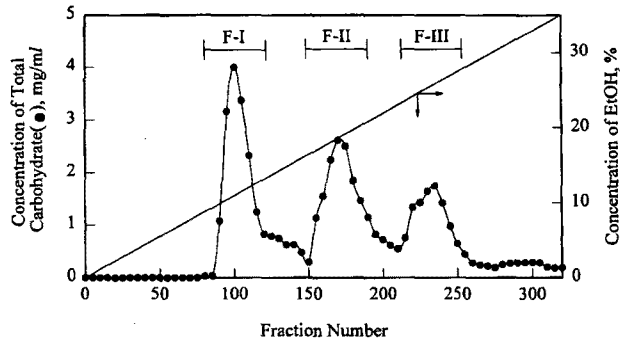

Fig. 4. Purification profiles of each transglycosylated products on activated carbon column chromatography.
Packing glass column, 3 cm diameter and 15 cm height; ethanol gradient elution from 0 to 35%(v/v); flow rate, 15 ml/h; fraction volume, 2 ml.

Fig. 3 shows the HPLC chromatogram of various compounds existing in the reaction mixtures at 24 h after the transglycosylation reaction using maltitol as the glucosyl acceptor and extruded starch as the glucosyl donor. The HPLC chromatogram shows that there are at least 5 different kinds of transglycosylated products, each product was purified by activated carbon column chromatography with ethanol gradient elution for identification of the number of glucosyl residues and the binding sites of the glucosyl group.

Fig. 4 shows the amount of total carbohydrate measured by the phenol-sulfuric acid method in each fraction obtained from chromatographic separation with an ethanol gradient elution up to 35% (v/v). Three peaks were separated by the column chromatographic method, and named F-I, F-II, and F-III corresponding the ethanol concentration of 12, 18, and 24% (v/v), respectively. The fractions on the peaks were analyzed by HPLC for iden-

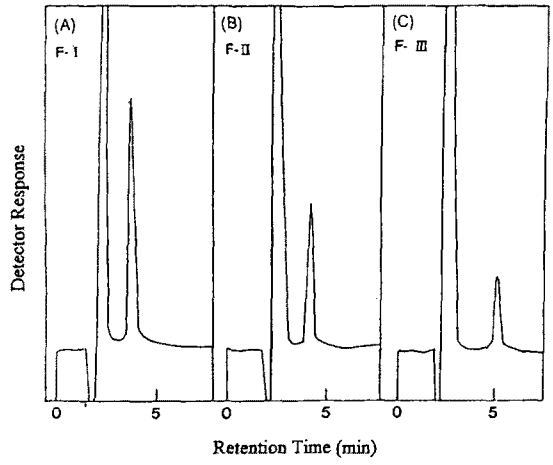

Fig. 5. HPLC chromatogram of purified fraction of glucosyl maltitols, F-I (A), F-II (B), and F-III (C).

tification of the number of glucosyl residues. The HPLC chromatogram of the separated fractions were identified as shown in Fig. 5, and it was found that peak F-I was maltitol (Fig. 5A) and other two were mono- and di-glucosyl maltitol (Fig. 5B and C), respectively.

Structural Nature of Glucosyl-maltitols Analyzed by ^{13}C-NMR Spectroscopy

The structural nature of the glucosyl-maltitols, including the binding position of the glucosyl residues on maltitol, and the number of glucosyl residues, was analyzed by ^{13}C-NMR spectroscopy. Fig. 6 shows the ^{13}C-NMR spectrum of the separated fractions F-II and F-III, showing the chemical shifts of each carbon atoms in the glucosyl residues. Table 3 also describes the chemical shift values of the carbon atoms in maltitol, glucosyl maltitols (F-II, F-III), and standard samples including α-D-glucose, sorbitol, and maltitol, assigned on the basis of comparison of the relative intensities and the references (3).

The chemical shifts of C-1 atoms in two glucosyl residues of the glucosylated product F-II were identified at δ100.3 (D-Glu) and δ99.7 (α-1,4-D-Glu), and those in the three glucosyl residues of glucosylated product F-III were identified at δ100.3 (D-Glu), δ99.6 (α-1,4-D-Glu), and δ99.8 (α-1,4-D-Glu). These resonances of C-1 atoms were assigned to the linkage position to other glucosyl residues, because the signal of C-1 in non-linked α-D-glucose (δ93.0) was shifted downfield to the corresponding values.

The chemical shifts of C-4 atoms in the glucosyl residue and sorbitol residue in F-II were identified at δ76.6 (α-1,4-D-Glu) and δ81.9 (α-1,4-D-Sor), respectively. Meanwhile, the chemical shifts of the C-4 atoms in F-III

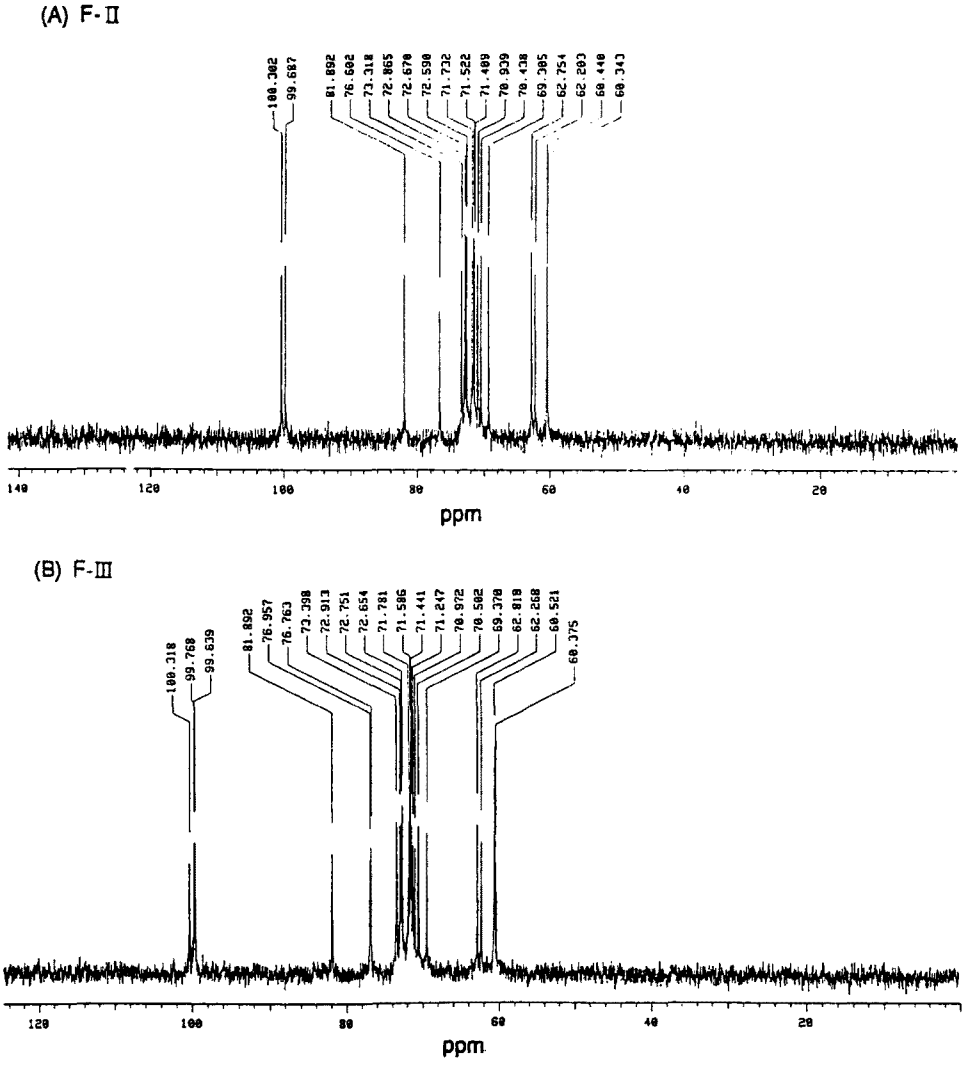

Fig. 6. ^{13}C-NMR spectra of purified fraction of glucosyl maltitols, F-II (A) and F-III (B).

Table 3. Chemical shift of D-Sorbitol, α-D-Glucose, maltitol, separated glucosylmaltitols F-II and F-III in ^{13}C-NMR spectra.

Glucosylmaltitols and standards	Residues	C-1	C-2	C-3	C-4	C-5	C-6
D-Sorbitol		63.8[a]	74.3	71.0	72.6	72.5	64.2
α-D-Glucose		93.0	72.5	73.8	70.6	72.3	61.8
Maltitol	D-Glu[b]	100.6	71.7	72.7	69.4	70.5	60.5
	α-1,4-D-Sor[c]	62.3	72.9	71.6	81.9	72.5	62.3
F-II	D-Glu	100.3	71.5	72.7	69.3	70.4	60.4
	α-1,4-D-Glu	99.7	70.9	72.9	76.6	71.7	60.3
	α-1,4-D-Sor	62.2	73.3	71.4	81.9	72.6	62.8
F-III	D-Glu	100.3	71.6	72.8	69.4	70.7	60.5
	α-1,4-D-Glu	99.6	70.9	72.9	76.9	71.8	60.4
	α-1,4-D-Glu	99.8	71.2	72.9	76.8	71.8	60.4
	α-1,4-D-Sor	62.3	73.4	71.4	81.9	72.7	62.8

[a]Chemical shift (δ) in ppm downfield from tetramethylsilane. [b]D-glucose residue. [c]D-sorbitol residue.

were identified at δ76.9 (α-1,4-D-Glu), δ76.8 (α-1,4-D-Glu), and δ81.9 (α-1,4-D-Sor). These values were also shifted downfield when compared to that of δ72.6 in non-linked D-sorbitol and δ70.6 in non-linked α-D-glucose. This observation indicates that the chemical bonds between glucosyl-residues in glucosyl maltitols were linked by α-1,4-glucosic bond. Therefore, the purified glucosyl-maltitol F-II was identified as maltotritol, α-D-Glu-[1→4]-α-D-Glu-[1→4]-D-sorbitol, and F-III as maltotetraitol, α-D-Glu-[1→4]-α-D-Glu-[1→4]-α-D-Glu-[1→4]-D-sobitol. The transglycosylation reaction of CGTase was carried out only by the α-1,4-glucosidic linkage (12), therefore, the transferring reaction of CGTase to maltitol was also found to occur on the 4-OH group of glucose residues in the nonreducing end of the parent maltitol molecule.

Glucosyl-sugar alcohols could be synthesized using various glucosyltransferases, especially CGTase, and synthesized glucosylated products can be useful as functional oligosaccharides which are low cariogenic, calorie free, and stimulating to the growth of *Bifidobacterim.* Further studies and examination of the physicochemical properties and functionalities of the produced glucosyl-sugar alcohols and development of the enzyme reaction system for the production of glucosyl-sugar alcohols need to be conducted.

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