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Purification and Properties of Non-Cariogenicity Sugar Produced by Alkalophilic *Bacillus* sp. S-1013

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Abstract The NCS(Non-Cariogenicity Sugar) from *Bacillus* sp. S-1013 was purified by cold acetone and methanol precipitation, and DEAE- cellulose ion-exchange and Sephadex G-100 column chromatographies, to yield an amorphous yellow syrup. The melting point and $[\alpha]_D^{20}$ were 155- 157°C and +53, respectively. Instrumental analyses such as FT-IR, ^{1}H-NMR, and ^{13}C-NMR showed that the NCS contained an O-H group, C-H, C=O, NH_2, anomeric carbon, anomeric proton, N-acetylgalactose, fucose, and neuramic acid, thus, the NCS was determined to be a trisaccharide of Fuc(1→4)GalNAc(2→6)NeuAc.

Key words: *Bacillus* sp. S-1013, Fuc(1 →4)GalNAc(2→6) NeuAc

The Committee on Medical Aspects of Food Policy (COMA) stated in 1989 that the frequent intake of sugar was the most important cause of oral diseases [9]. Therefore, many researchers have attempted to develop sugar substitutes that have features of sweeteners but with low cariogenicity, however, the substitutes developed are not efficient in the metabolism of oral microbes in which sugars are converted to acids [20]. The sugar substitutes developed should be innocuous to teeth and greatly contribute to the prevention of dental caries [3, 4, 21, 25, 27]. Oligosaccharides, such as lactose and galactose [23], and most polyols have been shown to have low cariogenicity. Among monosaccharides and disaccharides, galactose has lower fermentability by oral microorganisms than that of sucrose or glucose [30], but is important in the determination of plaque baseline pH and plaque microorganism. As a main component of saliva glycoprotein, galactose may be hydrolyzed by mucin and oral microorganisms, or as a component of other sugars [8, 17]. Therefore, one of the most effective ways to prevent dental caries is the substitution of sucrose with other sugars. Some researchers investigated the preventive effect of dental caries [2, 19, 13, 15, 24, 26, 28, 32], however, no studies on the sugars produced by microorganisms have yet been carried out.

We recently reported the production of Non-Cariogenicity Sugar (NCS) by novel alkalophilic *Bacillus* sp. S-1013. [29] This NCS was shown to inhibit the growth of *Streptococcus mutans* JC-2 that cause tooth decay and periodontal diseases, and synthesis of D-glucans from sucrose by glucosyltransferase (GTase) that is associated with the formation of plaque. Thus, this NCS may be useful for protection from the development of dental caries. In the present study, therefore, we purified the NCS to elucidate its chemical structure and characteristics.

Materials and Methods

Strain

The strain used in this study was an alkalophilic one with high productivity of NCS that was identified in the previous study and named as *Bacillus* sp. S-1013. The isolated strain was kept at 4°C. GPY agar medium (glucose 10 g/l, peptone 5 g/l, yeast extract 5 g/l, KH_2PO_4 1 g/l, $MgSO_4 \cdot 7H_2O$ 0.2 g/l, Na_2CO_3 10 g/l) was used for the growth of the strain, and the initial pH of the media was adjusted to 11.0 by varying the concentration of Na_2CO_3, depending on the purpose of the examination. Cell growth and GTase activity were measured by the methods described in our recent report [29].

Purification of Non-Cariogenicity Sugar

After addition of two volumes of cold acetone to the supernatant obtained by centrifuging the culture, the

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mixture was left in dark for 24 h in order to precipitate for insoluble substance. Then, the precipitated substance was separated from acetone, and a small amount of water was added to suspend the precipitate and the suspension was concentrated by evaporation. After dissolving in a small amount of water by heating, an equal amount of methanol was added to the concentrate in order to separate the methanol insoluble and soluble fractions. Each fraction was concentrated by rotary evaporator, and lyophilized to obtain an active fraction, methanol soluble fraction (MSF). After dissolving in distilled water, the MSF preparation was applied onto DEAE-cellulose column (3×50 cm: Cl^- form) that was preequilibrated with distilled water, and the column was eluted at a flow rate of 48 ml/h with 4 ml each fraction to provide MSF-1. Meanwhile, MSF-2 and MSF-3 were obtained by performing NaCl (0-0.4 M) gradient elution. The MSF-1 fraction with inhibitory activity of GTase was swollen in distilled water for four days to process on Sephadex G-100: column (3×50 cm) and eluted with distilled water at 40 ml/h flow rate of 4 ml each fraction. GTase inhibitory activity in each fraction was measured, whereas sugar and protein were analyzed by the phenol-sulfuric acid method and Lowry's method, respectively. The active fraction showing GTase inhibitory activity in gel chromatography was named as NCS, and its purity was determined by two-dimensional TLC: First, 1 g/ml of sample solution was spotted on TLC plates (silica gel 60 F254, Merck Co.) to be developed in solvent system (ethylacetate:acetic acid:water=2:1:1) for six hours. Then, the plates were sufficiently dried for two-dimensional analysis by using solvent system (n-butanol:ethanol:water =9:7:4). Ethanol solution containing 5% sulfuric acid was sprayed, and the plates were treated at 110°C for five minutes in order to develop the spots. The purification procedure is summarized in Fig. 1.

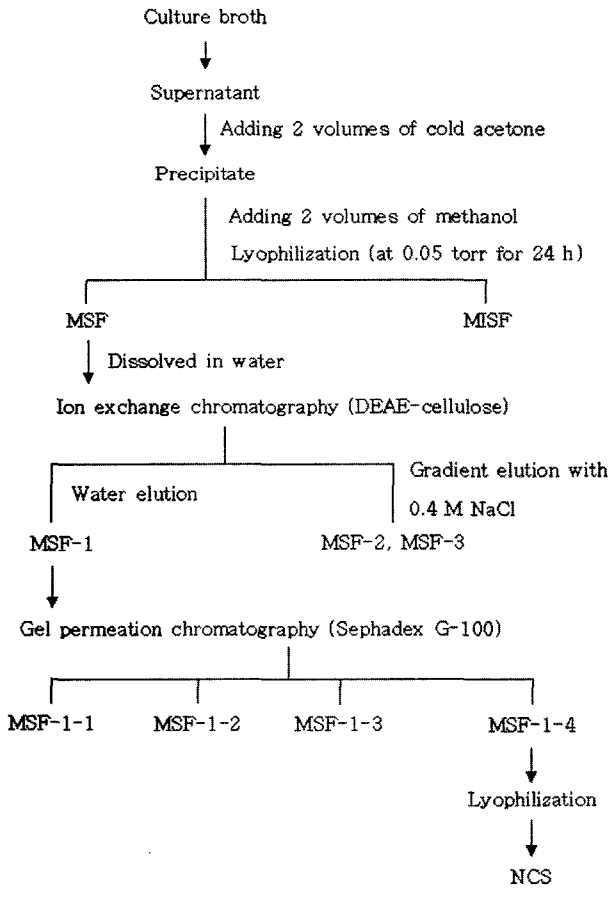

Fig. 1. Purification procedure of non-cariogenicity sugar.

Color Reaction

After NCS was prepared as 0.1% (w/v) solution, the color of the above samples was developed by Anthron, Fehling, carbazole-sulfuric, iodine-starch, ninhydrin, Elson-morgan, biuret, and Seliwanoff reactions according to the methods described previously [12].

Quantification of Components

The quantification of amino nitrogen was carried out by the Kjeldahl method [5], while total carbohydrate was colorimetrically analyzed based on the phenol-sulfuric acid method [5]. In order to obtain the amount of sugar from the standard curve, optical density was measured at 490 nm after 5 ml of concentrated H_2SO_4 was added to the tubes in which 1 ml each of NCS sample and 5% phenol were added, and was spun for 30 min.

Bond Pattern of Component Sugar

As for the bond pattern of component sugar, the consumption of periodate (IO^{-4}) was spectrophotometrically measured in the samples collected at various time periods after 10 mg of NCS was added to 100 ml of 10 mM sodium metaperiodate to be oxidized in the dark at 4°C for seven days. The production of formic acid was measured by titrating with 1 mM NaOH and 0.5% phenolphthalein [33]. After 12 h of reaction with 0.1 g of sodium borohydride, the excess reduced reagent was neutralized by 0.1 N HCl and dialyzed for 24 h. The formic acid produced was calculated according to the formula below:

$$\text{The production of formic acid (Mole)} = \frac{A \times B \times C}{D \times 1,000}$$

A: The amount of consumed alkali (ml)
B: The concentration of alkali used (N)
C: Total amount of sample (ml)
D: The amount of sample used (ml)

Interpretation of UV Spectrum

The UV spectrum was obtained with a UV/VIS spectrometer (Secoman S1000, U.S.A.) at the wavelength range of 190-800 nm with distilled water as solvent.

Interpretation of Infrared Spectrum

The IR spectrum was obtained with a spectrophotometer (JASCO, IR-700, Japan), and the reagent was prepared according to the KBr purification method. KBr powder without reagent was pressure-purified to be used as the reference substance, in order to determine the partial structure by spectrum after eliminating the loss due to infrared scatter or absorption of impurity.

Interpretation of NMR (Nuclear Magnetic Resonance)

The NMR spectrum was measured with a using JEOL FX-100 NMR, based on the pulse Fourier transform method, as TMS [tetramethylsilane: $(CH_3)_4Si$)] serving as reference substance, and 7-10 mg of the reagent were dissolved in D_2O at the ratio of 5-20% (w/v).

Melting Point and $[\alpha]_D^{20}$

Melting point was measured with a melting point apparatus (AX-80, Hitachi, Japan) with 1-2 mg of the reagent, and $[\alpha]_D^{20}$ was measured with a polarimeter (JASCO, DIP-370, Japan) after 2-5 mg of the reagent were dissolved in 95% ethanol, and calculated according to the formula below, when methanol served as the reference substance.

$$[\alpha]_D^{20}=\frac{100\times A}{C\times L}(^\circ)$$

A : Specific rotation
C : The amount of reagent in 1 ml (for instance, if 10 mg/ml, c=1)
L : The length of metering tube (mm)

Determination of Physical Characteristics and Solubility

The appearance of NCS was measured by gross observaton in order to investigate the physical characteristics. For the measurement of solubility in polar and nonpolar solvents, 1 mg of the reagent was placed in 1 ml of solvent and the mixture was vortexed for 10 min. The remaining reagent was filtered through a filter paper that was previously metered, and the weight of the reagent on the paper was determined after the solvent was completely removed. The NCS was considered to be 'soluble' when the amount of reagent was reduced by 50% and more, whereas 'slightly soluble' when the amount was reduced by 20-40%.

pH and Temperature Stability of NCS

One mM NCS in 0.05 M sodium citrate-HCl buffer (pH 3-4), 0.05 M potassium phosphate buffer (pH 5-8), and 0.05 M ammonium hydroxide-ammonium chloride buffer (pH 9-10) was processed for two hours. The GTase inhibitory activity of the pH-processed NCS solution was compared with that of the control to which NCS was not added.

One mM NCS was treated at 30°C, 40°C, 50°C, 60°C, 70°C, 80°C, and 90°C, and the GTase inhibitory activity of the heat-treated NCS solution was compared with that of the control to which NCS was not added.

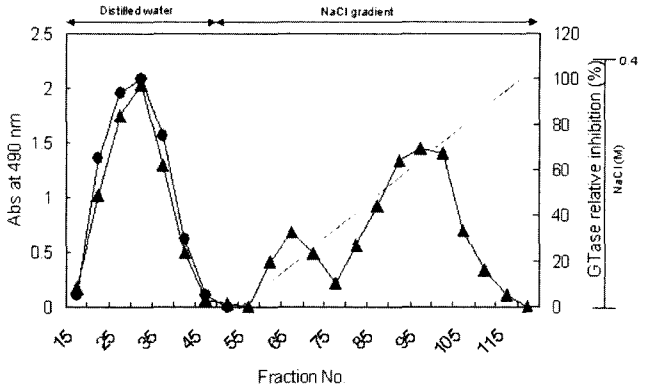

Fig. 2. DEAE cellulose ion-exchange chromatography of MSF.
-●-: NCS; -▲-: Dry cell weight.

RESULTS AND DISCUSSION

Purification of NCS

Precipitate obtained (53.7 g/l) from the supernatant of strain culture by adding two-times volumes of cold acetone was dissolved in a small amount of distilled water. After an equal amount of methanol was added to form the precipitate, 14.6 g/l methanol soluble fraction (MSF) was obtained. Figure 2 shows the result of DEAE-cellulose (Cl^- form) ion-exchange chromatography of the MSF. MSF-1 was obtained in the run-off fraction, while MSF-2 and MSF-3 were obtained from the adsorbed fraction. The production of MSF-1 (active fraction) was 4.27 g/l, and the yield was 7.95%. The MSF-2 and MSF-3 fractions, acid saccharides having the characteristics of polyvalent anion in general, did not show GTase inhibitory activity, while the neutral fraction eluted by distilled water showed the activity. The NCS is also considered to be a saccharide

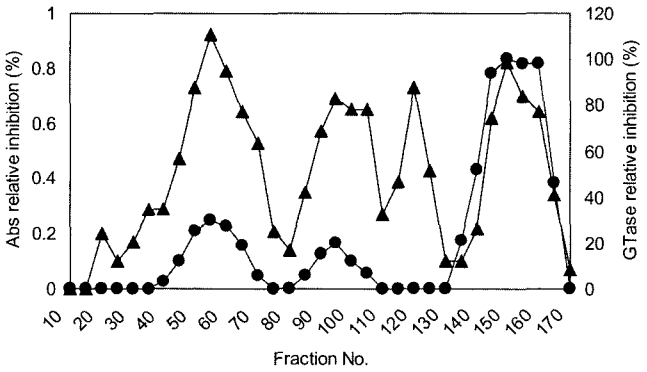

Fig. 3. Sephadex G-100 gel permeation chromatography of MSF-1.
-●-: NCS; -▲-: Dry cell weight.

Table 1. Summary of purification of NCS.

Purification step	NCS production (g/l)	Yield (%)	Specific inhibitory activity %/NCS g
Acetone precipitate	53.7	100	1.9
Methanol solution (MSF-1)	4.6	27.19	7.1
DEAE-cellulose (MSF-1-4)	4.27	7.19	24.4
Sephadex G-100 (NCS)	1.04	1.94	100

with an amino group, because it becomes light purple in ninhydrin reaction. As seen in Fig. 3, the Sephadex G-100 gel permeation chromatography of MSF-1, which was obtained from the DEAE-cellulose (Cl^- form) ion-exchange chromatography, showed four saccharide fractions (MSF-1-1, MSF-1-2, MSF-1-3, and MSF-1-4) with amino group, indicating that the MSF-1 was composed of four kinds of compound whose molecular weights were different from each other. Of these, MSF-1-4, having a strong GTase inhibitory activity, was isolated and lyophilized, and was named as NCS for further examinations. The production of NCS was 1.04 g/l, and the yield was 1.94%. The results of the above-mentioned purification are depicted in Table 1.

In order to examine the purity by two-dimensional TLC, the NCS was first resolved in the solvent system of ethylacetate:acetic acid:water (2:1:1), followed by the solvent system of n-butanol:ethanol:water (9:7:4) (see Fig. 4). The NCS showed a single spot (Rf: 0.53) in UV light and color reaction by iodine vapor after thin layer chromatography. Also, the NCS was positive in color reactions by iodine, 10% H_2SO_4, alkaline $KMnO_4$, and ferric chloride after resolution in the same solvent systems, indicating that the substance was a sugar. Furthermore, the NCS turned light purple when the ninhydrin reaction was carried out at 100°C.

Fig. 4. Thin layer chromatogram of NCS.
1st; ethylacetate:acetic acid:water (2:1:1). 2nd; n-BuOH:MeOH:water (9:7:4).

Color Reaction

The results of the color reaction of NCS are given in Table 2. The NCS was positive to the anthron, Fehling, Benedict, biuret, and ninhydrin reactions. It was determined to be an amino sugar, because it was positive to the biuret and ninhydrin reactions; however, unlike fructose it did not contain ketose, because it was negative to the Seliwanoff reaction.

Components

The contents of amino nitrogen and carbohydrate in the NCS were 0.19% and 99%, respectively. Such results are considered to be due to the amino group in the sugar structure, and are similar to the composition of 1-deoxynojirimycin, an amino sugar produced by *Bacillus subtilus* var *niger* (ATCC 9372) that was reported by Hardick and Hutchinson [11].

Bond Pattern of Component Sugar

As seen in Fig. 5, the results of the measurement of the bond pattern of the NCS by periodic oxidation showed that the NCS consumed IO^{4-} while simultaneously producing formic acid, and reached a certain value on the second day of the reaction. The conversion of IO^{4-} consumption and formic acid production to molar ration was 5:5:1. According to Kang [16], Lee *et al.* [18] and McAnally and Manna [22], the molar ratio of IO^{4-} consumption and HCOOH production is 2:1, when the oxide is 1,3-glucan and 1,6-glucan. In the case where there is no branch, the nonreducing and reducing terminals are oxidized to provide

Table 2. Color reaction of the NCS.

Test	NCS
Anthron	+
Lowry-Folin	+
Ninhydrin	+
Fehling	+
Carbazol-sulfate	-
Elson-Morgan	-
Seliwanoff	-
Biuret	+
Benedict	+

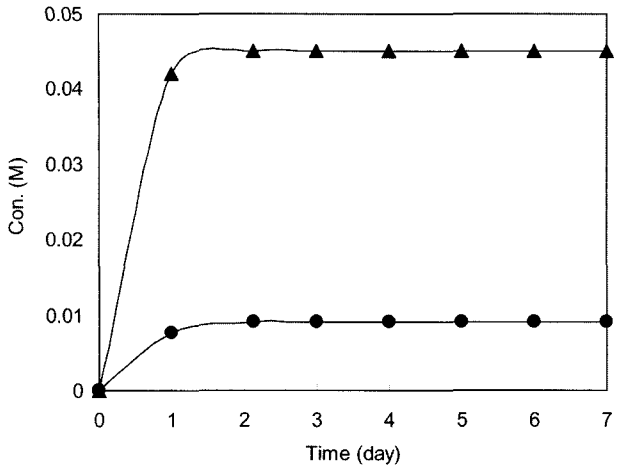

Fig. 5. Oxidation of NCS with periodate.
-●-: Formic acid production; -▲-: IO_4^- consumption.

IO^{4-} consumption and HCOOH production at the molar ratio of 5:3, while the main chain is not oxidized. As for 1,4-glucan, the nonreducing and reducing terminals are oxidized to consume IO^{4-} and produce HCOOH at the same time, while the backbone is oxidized to consume IO^{4-} but not to produce HCOOH. In the reducing terminus, 3 M IO^{4-} is consumed and 2 M HCOOH is produced. The 1,4-glucan, having a 1,6 residue, is oxidized to consume IO^{4-} but not to produce HCOOH. Therefore, the NCS was considered to be α-1,4 glucan containing an α-1,6 branch. However, the NCS could be a sugar consisting of only α-1,4 glucan because glycerol is produced in α-1,4 glucan that does not contain an α-1,6 branch, or could be composed of α-1,4 glucan containing an α-2,6 branch.

UV/VIS Spectrum

In order to estimate the structure, the absorption spectrum of the NCS dissolved in distilled water was investigated at 190–800 nm wavelength by UV/VIS spectrometer (see Fig. 6). The absorption of hydroxy group and amino group was observed at 190 nm and 210 nm, respectively, indicating

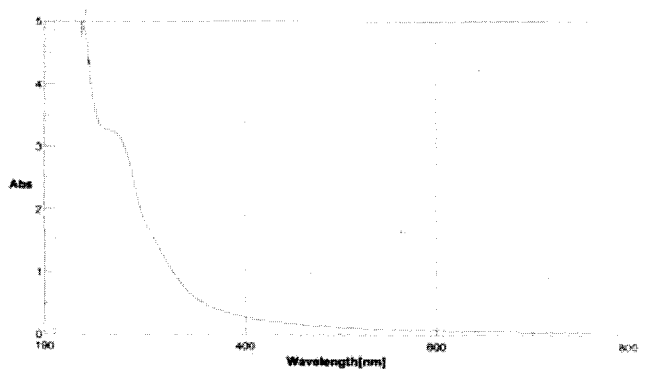

Fig. 6. UV/VIS spectrum of NCS.

Fig. 7. FT-IR spectrum of NCS.

the electronic transition of n→λ* band or →π* band. There was the typical absorption wavelength of C-O between 250 nm and 260 nm, therefore, the NCS was estimated to be an amino sugar derivative containing an amino group.

Interpretation of Infrared Spectrum

In order to estimate the structural component of the NCS, the absorption spectrum was investigated by the KBr pellet method (see Fig. 7). In the spectrum, the O-H group was shown at 3,400 cm^{-1}, the specific absorption band of sugars, C-H group at 2,900 cm^{-1}, C=O stretching band and C-O stretching band at 1,633 and 1,055 cm^{-1}, respectively, and amino group at 1,532 cm^{-1}. Therefore, the NCS was considered to be an amino sugar compound.

Interpretation of Nuclear Magnetic Resonance (NMR)

Figures 8 and 9 show the ^{1}H-NMR and ^{13}C-NMR of the NCS produced by *Bacillus* sp. S-1013. As seen in Fig. 8, the ^{1}H-NMR values were shown at δ-93.59, 58.41, 72.37, 89.68, 74.80, and 58.55 ppm, respectively, compared with standard data of reporter groups [1, 6, 14, 19, 31]. The H-1 signal of GalNAc was assessed based on the phenomenon of GalNAc (H-1–H-4) equal spin-coupling

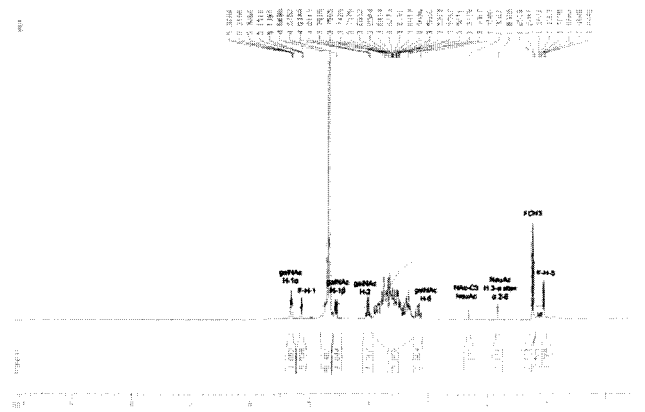

Fig. 8. ^{1}H-NMR spectrum of NCS.

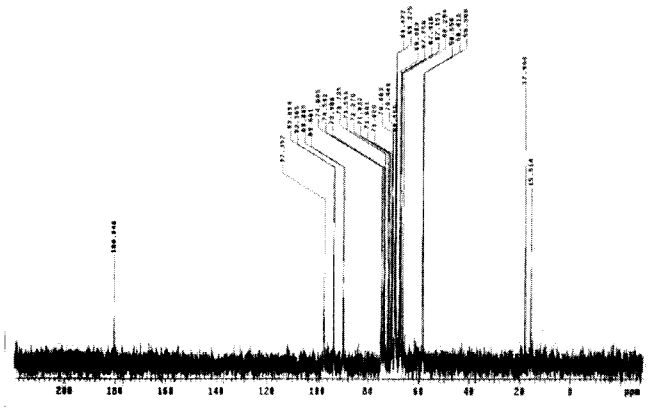

Fig. 9. ^{13}C-NMR spectrum of NCS.

signal observed in the HONANA spectrum. The GalNAc H-1, H-2, H-3, H-4, H-5, H-6, H-6', and Nac were observed at 4.254, 3.496, 3.654, 3.890, 3.595, 3,740, 3.731, and 2.069 ppm, respectively, while two spin systems, H-1, 2, 3, 4 and H-5, 6, 6', were traced. The H-1, H-5, CH_3 proton signals of Fuc residue formed the starting point, and the signals of Fuc H-1, H-2, H-3, H-4, H-5, and CH_3 were shown at 5.107, 3.681, 3.907, 4.856, and 1.173 ppm, respectively. The α-2,6 linked NeuAc residue was characterized by acetamido. The H-3a, H-3e, and NAc signals were observed at 1.724, 2.694, and 2.033, respectively. As seen in the ^{13}C-NMR in Fig. 9, NeuAc C-1, 2, 3, 4, 5, 6, 7, 8, and 9 signals were observed at 180.24, 93.69, 69.09, 73.73, 17.90, and 58.30 ppm, respectively, Fuc C-1, 2, 3, 4, 5, and 6 signals at 97.35, 71.83, 73.98, 69.55, 73.55, and 66.29 ppm, and GalNAc C-1, 2, 3, 4, 5, and 6 signals at 93.59, 58.41, 72.37, 89.68, 74.80, and 58.55 ppm, respectively. The NCS that was purified in this study was concluded to be an amino sugar containing a carboxyl group, and trisaccharide

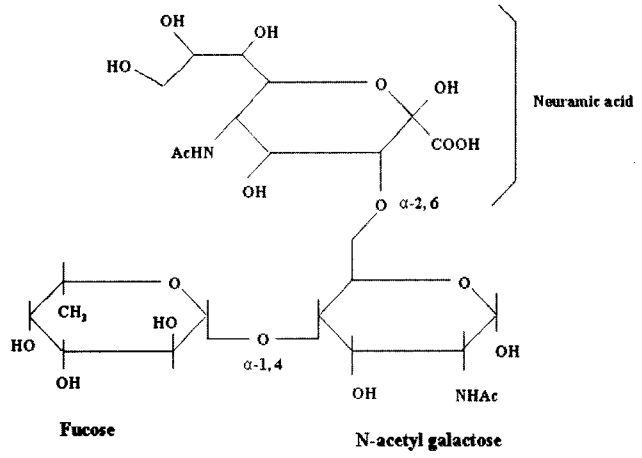

Fig. 10. Structure of NCS.

Table 3. Physical properties of NCS.

Melting point		155- 157°C
(1) $[\alpha]_D^{20}$		+53°
Appearance		Yellow syrup
Solubility	Water	Soluble
	Methanol	Soluble
	Ethanol	Soluble
	Isopropanol	Slightly Soluble
	Isoamyl alcohol	Slightly Soluble
	Hexane	Insoluble
	Benzene	Insoluble
	Ether	Insoluble
	Chloroform	Insoluble
	Acetone	Insoluble

with the structure of Fuc(1→4)GalNAc(2→6)NeuAc. The structure of the NCS purified in this study is given in Fig. 10.

Melting Point and $[\alpha]_D^{20}$

The melting point with 1- 2 mg of the NCS was measured with a melting point apparatus (AX-80, Hitachi, Japan), while $[\alpha]_D^{20}$ was measured with 2- 5 mg of the compound dissolved in 95% ethanol using a polarimeter (JASCO, DIP-370, Japan). The melting point and $[\alpha]_D^{20}$ of the NCS were between 155- 157°C and +53, respectively.

Physical Characteristics

The appearance of the NCS was macroscopically observed in order to investigate the physical characteristics. The results of measurement of solubility in polar and nonpolar solvents are shown in Table 3. The NCS, yellow viscid syrup, was easily dissolved in polar solvents such as water or methanol, while hardly dissolved in nonpolar solvents, including ethyl ether and chloroform, indicating the characteristics of the NCS were similar to those of general sugars including glucose.

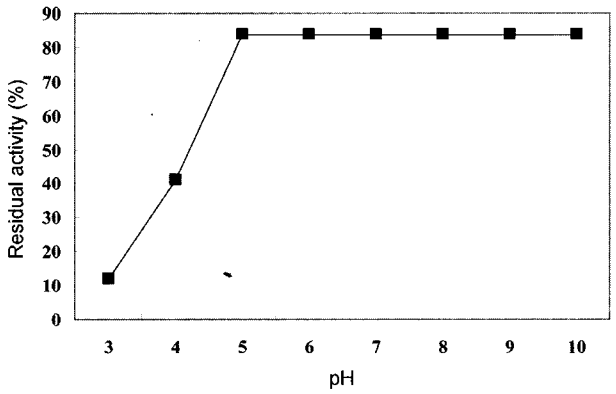

Fig. 11. pH stability of NCS activity on GTase of *S. mutans* JC-2.

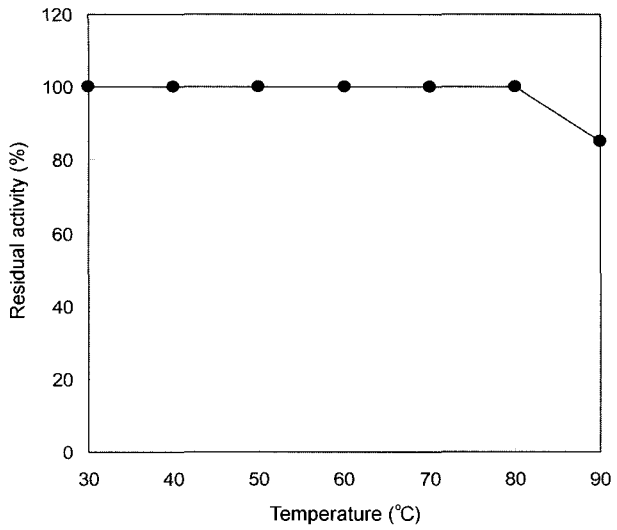

Fig. 12. Temperature stability of NCS activity on GTase of *S. mutans* JC-2.

pH Stability

The NCS was dissolved in 0.05 M sodium citrate-HCl buffer (pH 3-4), 0.05 M potassium phosphate buffer (pH 5-8), and 0.05 M ammonium hydroxide-ammonium chloride buffer (pH 9-10) to a final 1 M concentration, and then was left at room temperature for two hours. The GTase inhibitory activities of the NCS solutions processed above in different pHs were measured and compared with that of the control group in which the NCS was not added. As seen in Fig. 11, the inhibitory activity was relatively stable at pH 6, 7, 8, 9, and 10, while drastically reduced at pH 5.0 and below.

The results indicate that the isolated NCS was unstable in acidic medium, but stable in alkali, conforming to the fact that glycosides in monosaccharides are unstable in acid but stable in neutral or alkaline pH, and that the glycoside bond in disaccharides is unstable in acid [7]. Therefore, the NCS in this study, a sugar consisting of glycoside bonds, was inactivated by acid, and the GTase inhibitory activity was reduced.

Temperature Stability

The NCS was dissolved in distilled water at 1 M concentration, pretreated at 30-90°C for an hour, and the remaining GTase inhibitory activity was measured. As seen in Fig. 12, the inhibitory activity remained stable up to 80°C treatment, when compared to that of the control in which the NCS was not added. Therefore, the NCS in this study was relatively temperature stable.

Acknowledgment

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