J. Appl. Glycosci., **67**, 129–135 (2020)
doi: 10.5458/jag.jag.JAG-2020_0009
©2020 The Japanese Society of Applied Glycoscience

Note

Enzymatic Synthesis and Structural Confirmation of Novel Oligosaccharide, D-Fructofuranose-linked Chitin Oligosaccharide

(Received August 27, 2020; Accepted November 2, 2020)

Hiroki Hosaka,[1] Sayaka Shirai,[1] Sora Fujita,[1] Mitsuru Tashiro,[2] Takako Hirano,[1,3] Wataru Hakamata,[1,3] and Toshiyuki Nishio[1,3,†, †]

[1] *Bioresource Utilization Science Course, Graduate School of Bioresource Sciences, Nihon University (1866 Kameino, Fujisawa, Kanagawa 252–0880, Japan)*
[2] *Department of Chemistry, College of Science and technology, Meisei University (2–1–1 Hodokubo, Hino, Tokyo 191–8506, Japan)*
[3] *Department of Chemistry and Life Science, College of Bioresource Sciences, Nihon University (1866 Kameino, Fujisawa, Kanagawa 252–0880, Japan)*

Abstract: Utilizing transglycosylation reaction catalyzed by β-*N*-acetylhexosaminidase of *Stenotrophomonas maltophilia*, β-D-fructofuranosyl-(2↔1)-α-*N*, *N*′diacetylchitobioside ($GlcNAc_2$-Fru) was synthesized from *N*-acetylsucrosamine and *N*, *N*′-diacetylchitobiose ($GlcNAc_2$), and β-D-fructofuranosyl-(2↔1)-α-*N*, *N*′, *N*′′-triacetylchitotrioside ($GlcNAc_3$-Fru) was synthesized from $GlcNAc_2$-Fru and $GlcNAc_2$. Through purification by charcoal column chromatography, pure $GlcNAc_2$-Fru and $GlcNAc_3$-Fru were obtained in molar yields of 33.0 % and 11.7 % from $GlcNAc_2$, respectively. The structures of these oligosaccharides were confirmed by comparing instrumental analysis data of fragments obtained by enzymatic hydrolysis and acid hydrolysis of them with known data of these fragments.

Key words: noble oligosaccharide, D-fructofuranose-linked chitin oligosaccharides, enzymatic synthesis, β-*N*-acetylhexosaminidase, structural confirmation

N-Acetyl-D-glucosamine (GlcNAc), a monosaccharide obtained by the hydrolysis of its β-1,4-polymer chitin, is being used as a supplement because evidence shows that intake of this monosaccharide brings physiologically beneficial effects.[1)2)3)4)] Several reports described that chitin oligosaccharides (COSs) of specific chain length exhibit properties beneficial in fields such as medicine[5)6)] and agriculture[7)8)9)], indicating that COSs are biofunctional molecules. Moreover, it is known that several hetero-oligosaccharides containing GlcNAc as a constituent monosaccharide exhibited physiologically beneficial functions in human body.[10)11)12)13)14)15)] We are particularly interested in lacto-*N*-biose, a disaccharide derived from human milk oligosaccharides, because this GlcNAc-containing heterodisaccharide functions as a bifidus factor.[14)15)] Based on these facts, we are trying to prepare various GlcNAc-containing oligosaccharides by exploiting enzyme reaction and develop them as functional oligosaccharides. Until now, we have succeeded in synthesizing COSs and several hetro-oligosaccharides containing GlcNAc, utilizing regio- and anomer-selective hydrolysis or transglycosylation reaction catalyzed by carbohydrate related enzymes. For example, we had established the methodology for large-scale production of *N*, *N*′-diacetylchitobiose ($GlcNAc_2$) from squid pen β-chitin, by the fermentation method using genetically engineered cells of *Escherichia coli* that secretes recombinant *Vibrio parahaemolyticus* chitinase (Fig. 1A).[16)17)] Subsequently, using $GlcNAc_2$ as a raw material, we had synthesized COSs with GlcNAc polymerization degree of 3 and 4 utilizing *Stenotrophomonas maltophilia* dried cells containing transglycosylation reaction-catalyzing β-*N*-acetylhexosaminidase (βNAHex) as a whole-cell catalyst (Fig. 1B).[18)] Also, we had synthesized *N*-acetylsucrosamine (SucNAc), a heterodisaccharide in which D-glucose residue of sucrose (Suc) was replaced with GlcNAc, using GlcNAc and Suc as raw materials and *Aspergillus oryzae* mycelia containing transglycosylation reaction-catalyzing β-fructofuranosidase (βFFase) as a whole-cell catalyst (Fig. 1C).[19)] With respect to SucNAc, we had established the methodology for the large-scale production by a bioreactor using dried *A. oryzae* mycelia containing this enzyme.[20)] We are currently evaluating the prebiotics function of this disaccharide and have recently found that SucNAc grows certain species

†Corresponding author (Tel. +08–466–84–3951, E-mail: nishio.toshiyuki@nihon-u.ac.jp).

Abbreviations: COS, chitin oligosaccharide; ESI, electrospray ionization; FCOS, D-fructofuranose-linked chitin oligosaccharide; Fru, D-fructofuranose; βFFase, β-fructofuranosidase; GlcNAc, *N*-acetyl-D-glucosamine; $GlcNAc_2$; *N*, *N*′-diacetylchitobiose; GlcNAc3, *N*, *N*′, *N*′′-triacetylchitotriose; $GlcNAc_2$,/-Fru, β-D-fructofuranosyl-(2↔1)-α-*N*, *N*′-diacetylchitobioside; $GlcNAc_3$-Fru, β-D-fructofuranosyl- (2↔1)-α-*N*, *N*′, *N*′′-triacetylchitotrioside; HPLC, high performance liquid chromatography; MS, mass spectrometry; βNAHex, β-*N*-acetylhexosaminidase; NMR, nuclear magnetic resonance; Suc, sucrose; SucNAc, *N*-acetylsucrosamine; TLC, thin layer chromatography.

This is an open-access paper distributed under the terms of the Creative Commons Attribution Non-Commercial (by-nc) License (CC-BY-NC4.0: https://creativecommons.org/licenses/by-nc/4.0/).

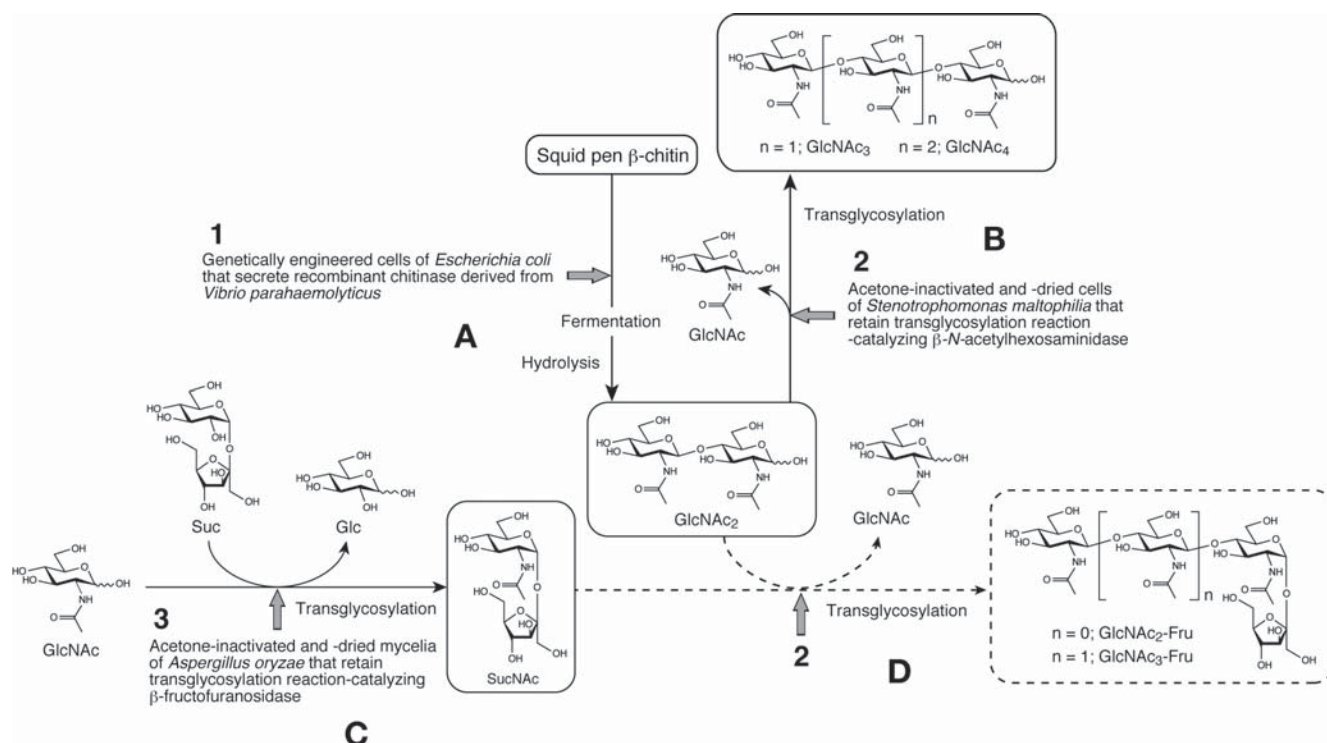

Fig. 1. Schematic representation of the production of COSs and SucNAc as already reported (A, B, and C) and plan for producing FCOSs (D).

of bifidobacteria well.[21] Applying the results obtained so far, we next decided to try synthesizing D-fructofuranose (Fru) linked COSs (FCOSs), by exploiting transglycosylation action of glycosidase. We intend to develop FCOSs as a functional oligosaccharide.

We at first tried synthesizing FCOSs from COSs we produced and Suc utilizing transfructosylation action of βFFases of *A. oryzae*[19] and *Microbacterium saccharophilum,*[22)23] because the raw material cost is low in this method. However, FCOSs were not synthesized at all by this methodology (data not shown). We think that these facts attribute to the substrate specificity of the enzymes used. In consideration of these facts, we next decided to try synthesizing FCOSs from SucNAc and $GlcNAc_2$ for which mass production methods were established by our research. Referring to our previous paper reporting the synthesis of medium-chain-length COSs,[18] we used dried *S. maltophila* NYT501 cells, which contain βNAHex showing high transglycosylation activity, as a catalyst for FCOS synthesis (Fig. 1D). However, only a few transglycosylation products were produced by this method (data not shown). This method using the enzyme retained in dried bacterial cells as a catalyst may not be suitable for FCOS synthesis from SucNAc and $GlcNAc_2$. Then, we next tried to use βNAHex crude preparation extracted from *S. maltophila* cells for the synthesis of FCOSs. This crude enzyme was prepared according to the procedure described in our previously paper.[18]

A 0.4 mL of 20 mM sodium phosphate buffer (pH 7.0) containing 0.11 unit of crude βNAHex of *S. maltophila*, 40 mg of $GlcNAc_2$, and 72.3 mg SucNAc (molar ratio of $GlcNAc_2$ to SucNAc; 1:2) was incubated at 30 °C for 8 h with gently shaking. The activity measurement and unit definition of this βNAHex were performed according to the procedure described in our previous paper.[24] At a designated time points during reaction, 40 µL-aliquot of the reaction mixture was withdrawn and heated at 95 °C for 5 min in hot dry bath to stop the enzyme reaction. Qualitative and quantitative analysis of saccharides contained in collected reaction mixtures was confirmed by Silica gel thin-layer chromatography (TLC) and high performance liquid chromatography (HPLC), respectively. From the TLC analysis results (Fig. 2A-1), it was confirmed that the spots presumed to be a transglycosylation product (indicated as **I**) appeared as $GlcNAc_2$ spots disappeared. From the HPLC analysis results (Fig. 2A-2), compound **I** was found to make up approximately 20 % of the total saccharides after 8-h reaction. To purify compound **I**, the above reaction was performed for 8 h on a 12.5-fold scale, and then this compound was isolated from the reaction mixture by charcoal column chromatography. Solvent of the solution containing purified compound **I** was removed by evaporation, resulted residue was lyophilized after dissolved in a small amount of H_2O, and then the weight of the dried sample was measured. Next, structure of this compound was analyzed. At first, we investigated mass of this compound by mass spectrometry (MS) under positive direct electrospray ionization (ESI) condition. The spectra of ESI-MS of compound **I** corresponded to $[M+H]^+$ and $[M+Na]^+$ species at *m/z* 587 and 609 (Fig. 2C-1), respectively, indicating that compound **I** is trisaccharide consists of 2 GlcNAc and Fru. Next, we planned confirming the structure of this compound directly by a nuclear magnetic resonance (NMR) spectroscopy. However, since many overlapping ^1H signals were observed in the ^1H NMR spectra of compound **I**, we thought it is difficult to obtain reliable information about its structural by NMR analysis. Furthermore, as the degree of polymerization of the monosaccharide increases,

it becomes more difficult to assign all signals obtaind by NMR of those oligosaccharides. In the previous studies (Fig. 1A,B, and C),[17)18)19)] we had already analyzed the structure of SucNAc and short-chain COSs by NMR spectroscopy and have signal assignment data of them. In the context of these circumstances, to confirm the structure of compound **I**, we decided to use existing NMR spectra by devising a method for enzymatic or chemical fragmentation of this compound. After decomposing compound **I** into SucNAc and GlcNAc or $GlcNAc_2$ and Fru, we decided to confirm the structure of obtained disaccharides by comparing their ^{1}H NMR signals with those previously obtained for them. At first, we tried decomposing compound **I** using hydrolytic action of pure grade βNAHex of *Streptococcus pneumonia* (New England Biolabs, Ipswich, MA, USA) that release GlcNAc from non-reducing end of COSs. The activity measurement and unit definition of this βNAHex were performed according to the procedure described in the attached instructions. A 100 µL of 50 mM sodium citrate buffer (pH 4.5) containing 1 % (*w*/*v*) compound **I** and 10 units of this enzyme was incubated at 37 °C with shaking. At each designated time points, saccharides in the reaction mixture were analyzed by TLC. As the result, it was confirmed that compound **I** was decomposed into 2 fragments (indicated as **Ia** and **Ib** in Fig. 2B-1) with Rf values corresponding to GlcNAc and SucNAc, respectively. Compounds **Ib** was purified from the reaction mixture after 2-h incubation by charcoal column chromatography. We previously confirmed that β,α-2,1 glycosidic bond between Fru and GlcNAc is more susceptible to hydrolysis in acidic solution as compared to β-1,4 glycosidic bond between GlcNAc. Thereupon, we tried to decompose compound **I** to 2 fragments in acidic buffer solution. A 100 µL of 100 mM sodium *p*-toluensulfonate buffer (pH 1.5) containing 1 % (*w*/*v*) compound **I** was incubated at 70 °C for 4 h. At each designated time points, degradation products in the reaction mixture were analyzed by TLC. As the result, it was confirmed that compound **I** was decomposed into 2 fragments (indicated as **Ic** and **Id** in Fig. 2B-2) with Rf values corresponding to Fru and $GlcNAc_2$, respectively. Compounds **Id** was purified from the reaction mixture after 4-h incubation by charcoal column chromatography. Using the purified samples of compound **Ib** and **Id**, their structure was investigated by ESI-MS and NMR. The spectra of ESI-MS of compounds **Ib** and **Id** corresponded to $[M+Na]^+$ species at *m*/*z* 406 and 447 (Fig. 2C-2 and 3), respectively, indicating that compound **Ib** is disaccharide consists of GlcNAc and Fru and compound **Id** is disaccharide of GlcNAc. Here, we show only non-overlapping ^{1}H NMR signals that can be indicators of these compounds. In ^{1}H NMR spectroscopy analysis, signals of anomeric H (δ 5.39, d) and H of acetyl group (δ 2.06, s) of GlcNAc residue of SucNAc were observed in the spectra of compound **Ib**. Moreover, signals of H3 (δ 4.23, d) and H4 (δ 4.04, t) of Fru residue of SucNAc were also observed in ^{1}H NMR of this compound. Signals of anomeric H of reducing end αGlcNAc residue (δ 5.19, d) and βGlcNAc residue (δ 4.70, d) of $GlcNAc_2$ were observed in ^{1}H NMR spectra of compound **Id**. Moreover, signals of anomeric H of non-reducing end βGlcNAc residues (δ 4.58–4.62, 2 d) of $GlcNAc_2$ diastereomers were also observed in ^{1}H NMR spectra of this compound. The other ^{1}H NMR signals of compounds **Ib** and **Id** were corresponded to those of SucNAc and $GlcNAc_2$, respectively. From the above facts, it became clear that compound **I** produced from SucNAc and $GlcNAc_2$ by the transglycosylation action of *S. maltophilia* βNAHex is β-D-fructofuranosyl-(2↔1)-α-*N*, *N′*-diacetylchitobioside ($GlcNAc_2$-Fru). The above reaction and purification process yielded 228 mg of $GlcNAc_2$-Fru (molar yield from $GlcNAc_2$; 33.0 %).

FCOS having a higher polymerization degree of GlcNAc could not be obtained by the above reaction. Therefore, we next tried synthesizing FCOS with higher GlcNAc polymerization degree using $GlcNAc_2$ and $GlcNAc_2$-Fru as raw materials. The procedures for synthesizing, purifying, and confirming the structure of the target FCOSs were basically carried out according to those of $GlcNAc_2$-Fru. A 0.4 mL of 20 mM sodium phosphate buffer (pH 7.0) containing 0.11 unit of crude βNAHex of *S. maltophila*, 40 mg of $GlcNAc_2$, and 111 mg $GlcNAc_2$-Fru (molar ratio of $GlcNAc_2$ to $GlcNAc_2$-Fru; 1:2) was incubated at 30 °C for 8 h with gently shaking. From the TLC analysis results (Fig. 3A-1), it was confirmed that the spots presumed to be a transglycosylation product (indicated as **II**) appeared as $GlcNAc_2$ spots disappeared. From the HPLC analysis results (Fig. 3A-2), compound **II** was found to make up approximately 10 % of the total saccharides after 8-h reaction. To obtain pure grade compound **II**, the above reaction was performed for 8 h on a 12.5-fold scale, and this compound was purified. The spectra of ESI-MS of isolated compound **II** corresponded to $[M+H]^+$ and $[M+Na]^+$ species at *m*/*z* 790 and 812 (Fig. 3C-1), respectively, indicating that compound **II** is tetrasaccharide consists of 3 GlcNAc and Fru. At first, we tried decomposing compound **II** using hydrolytic action of pure grade chitinase (Thermostable Enzyme Lab., Hyogo, Japan), which releases $GlcNAc_2$ from chitin. The activity measurement and unit definition of this chitinase were performed according to the procedure described in the attached instructions. A 100 µL of 50 mM sodium acetate buffer (pH 4.5) containing 1 % (*w*/*v*) compound **II** and 10 units of this enzyme was incubated at 37 °C with gently shaking. As the result of TLC analysis of saccharides in the reaction mixture, it was confirmed that compound **II** was decomposed into 2 fragments (indicated as **IIa** and **IIb** in Fig. 3B-1) with Rf values corresponding to $GlcNAc_2$ and SucNAc, respectively. Compounds **IIa** and **IIb** were purified from the reaction mixture after 1-h incubation by charcoal column chromatography. Next, we tried decomposing compound **II** (1%, *w*/*v*) in acidic buffer solution. The experimental conditions are the same as the case for compound **I**. As the result of TLC analysis of saccharides in the reaction mixture, it was confirmed that compound **II** was decomposed into 2 fragments (indicated as **IIc** and **IId** in Fig. 3B-2) with Rf values corresponding to Fru and $GlcNAc_3$. Compounds **IId** was purified from the reaction mixture after 4-h incubation by charcoal column chromatography. The structure of the purified samples of compound **IIa**, **IIb**, and **IId** was investigated by ESI-MS and NMR. The spectra of ESI-MS of compounds **IIa**, **IIb**, and

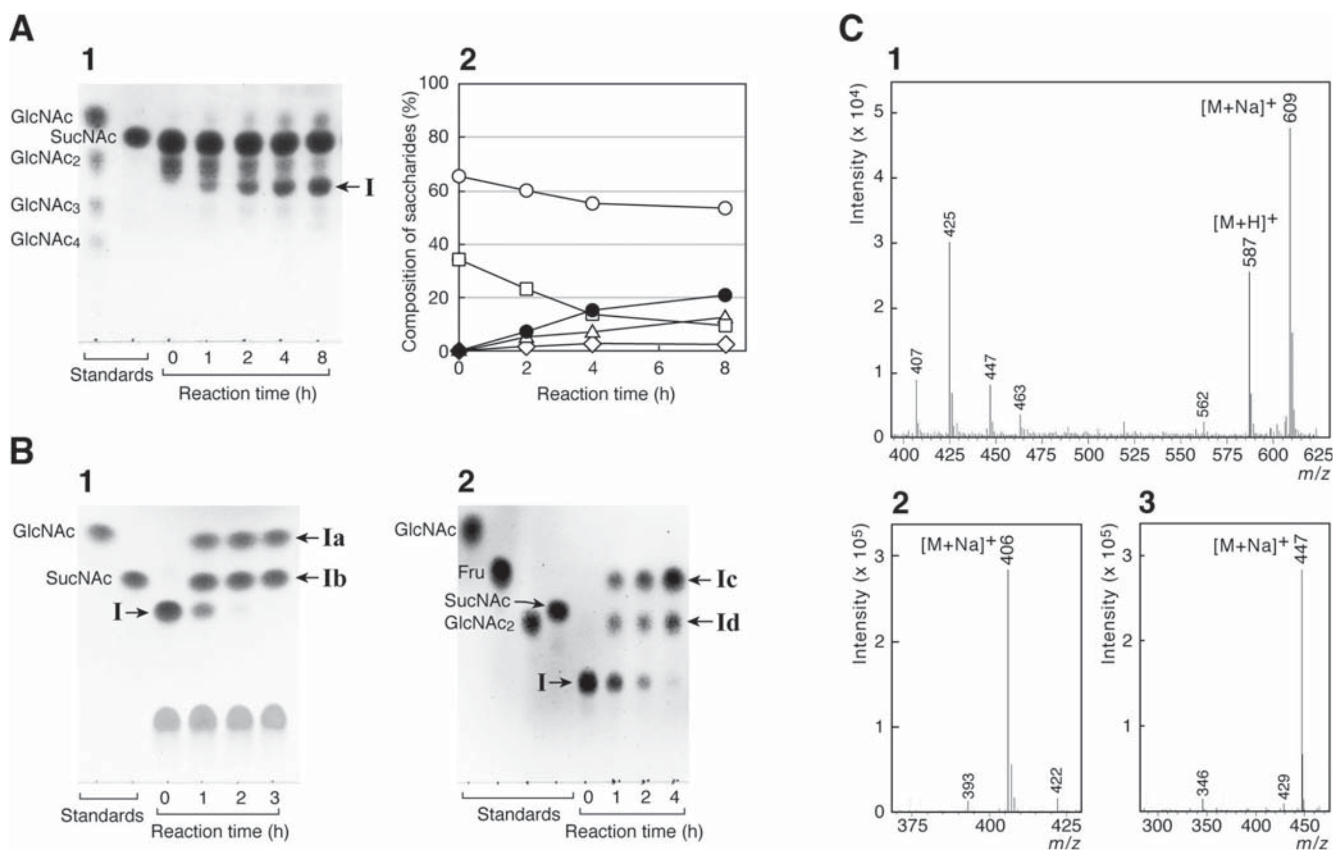

Fig. 2. Enzymatic synthesis of FCOS from $GlcNAc_2$ and SucNAc and identification of the reaction product.
A: TLC analysis result of reaction products generated by incubating $GlcNAc_2$ and SucNAc together with crude βNAHex of *S. maltophilia* (1) and HPLC analysis of composition of saccharides in the reaction solution (2). Symbols in (2): SucNAc; ○, $GlcNAc_2$; □, GlcNAc; △, $GlcNAc_3$; ◇, compound **I**; ●. B: TLC analysis results of hydrolyzates of compound **I** (**Ia** and **Ib**) produced by the action of *S. pneumonia* βNAHex (1) or those (**Ic** and **Id**) generated in sodium *p*-toluensulfonate buffer with pH 1.5 (2). C: ESI-MS analysis results of compound **I** (1) and its hydrolyzates, **Ib** (2) and **Id** (3).

IId corresponded to $[M+Na]^+$ species at *m/z* 447, 406, and 650 (Fig. 3C-2, 3, and 4), respectively. These results indicate that compound **IIa** is disaccharides consist of GlcNAc, compound **IIb** is disaccharides consist of GlcNAc and Fru, and compound **IId** is trisaccharide of GlcNAc. In ^{1}H NMR spectroscopy analysis, signals of anomeric H of reducing end αGlcNAc residue (δ 5.19, d) and βGlcNAc residue (δ 4.70, d) of $GlcNAc_2$ were observed in the spectra of compound **IIa**. Moreover, signals of anomeric H of non-reducing end βGlcNAc residue (δ 4.58–4.62, 2 d) of $GlcNAc_2$ diastereomers were also observed in ^{1}H NMR spectra of this compound. Signals of anomeric H (δ 5.39, d) and H of acetyl group (δ 2.06, s) of GlcNAc residue of SucNAc were observed in ^{1}H NMR spectra of compound **IIb**. Moreover, signals of H3 (δ 4.23, d) and H4 (δ 4.04, t) of Fru residue of SucNAc were also observed in ^{1}H NMR spectra of this compound. Signals of anomeric H of reducing end αGlcNAc residue (δ 5.20, d) and βGlcNAc residue (δ 4.70, d) of $GlcNAc_3$ were observed in ^{1}H NMR spectra of compound **IId**. Moreover, signals of anomeric H of non-reducing end βGlcNAc residues (δ 4.58–4.62, 2 d) of $GlcNAc_3$ diastereomers were also observed in ^{1}H NMR spectra of this compound. The other ^{1}H NMR signals of compounds **IIa**, **IIb**, and **IId** were corresponded to those of $GlcNAc_2$, SucNAc, and $GlcNAc_3$, respectively. From the above facts, it became clear that compound **II** produced from $GlcNAc_2$ and $GlcNAc_2$-Fru by the transglycosylation action of *S. maltophilia* βNAHex is β-D-fructofuranosyl-(2↔1)-α-*N*,*N*′,*N*′′-triacetylchitotrioside ($GlcNAc_3$-Fru). The above reaction and purification process yielded 108.4 mg of $GlcNAc_3$-Fru (molar yield from $GlcNAc_2$; 11.7 %).

From the above results, it was found that FCOSs can be synthesized by the transglycosylation action of *S. maltophilia* βNAHex, by using SucNAc and GlcNAc as basic raw materials. Moreover, it was found that the structure of FCOSs can be easily confirmed by instrumental analyses (MS and ^{1}H NMR) of fragments obtained by enzymatic hydrolysis using βNAHex or chitinase and acid hydrolysis. As mentioned at the beginning of this report, we have a plan to use the obtained FCOSs to the application fields of functional oligosaccharides. However, since synthetic yield of FCOSs is not so high, it is currently difficult to carry out research to achieve this plan. Although the synthetic methodology of FCOSs has been established by this study, we think it is necessary to find out βNAHex with higher transglycosylation activity to produce FCOSs in higher yield. We will search for βNAHex showing higher transglycosylation activity between SucNAc and $GlcNAc_2$.

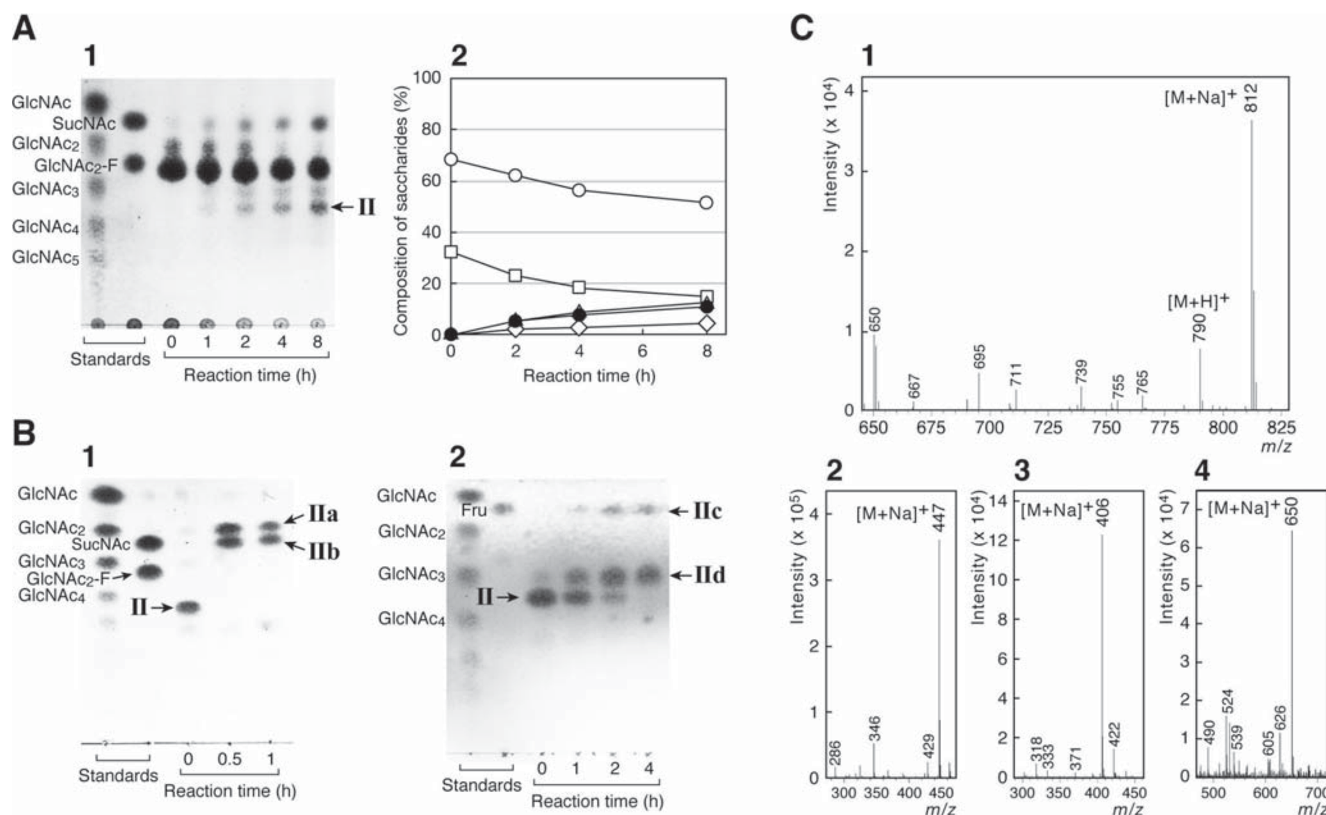

Fig. 3. Enzymatic synthesis of FCOS from $GlcNAc_2$ and $GlcNAc_2$-Fru and identification of the reaction product.

A: TLC analysis result of reaction products generated by incubating $GlcNAc_2$ and $GlcNAc_2$-Fru together with crude βNAHex of *S. maltophilia* (1) and HPLC analysis of composition of saccharides in the reaction solution (2). Symbols in (2): $GlcNAc_2$-Fru; ○, $GlcNAc_2$; □, SucNAc; △, $GlcNAc_3$; ◇, compound **II**; ●. B: TLC analysis results of hydrolyzates of compound **II** (**IIa** and **IIb**) produced by the action of chitinase (1) or those (**IIc** and **IId**) generated in sodium *p*-toluensulfonate buffer with pH 1.5 (2). C: ESI-MS analysis results of compound **II** (1) and its hydrolyzates, **IIa** (2), **IIb** (3), and **IId** (4).

EXPERIMENTAL

Analysis of reaction products by TLC and HPLC.

Qualitative analysis of saccharides was carried out by TLC using silica gel-coated plate (Silica gel 60, 0.25 mm, Merck KGaA, Darmstads, Germany). A solution containing FCOS synthesized by βNAHex was diluted 10-fold with water, 2 μL of the diluted solution was spotted on TLC plate, and then the plates were developped twice using 2-PrOH/AcOH/H_2O 4:4:1 (*v*/*v*/*v*) as a mobile phase solvent. In addition, 1 μL of a solution containing FCOS decomposition products generated by enzymatic hydrolysis using βNAHex or chitinase and acid hydrolysis using 100 mM sodium *p*-toluensulfonate buffer (pH 1.5) were spotted on TLC plates, and then each plate was developped three times using 1-BuOH/MeOH/16 % aqueous solution of NH_3 5:4:3 (*v*/*v*/*v*) as a mobile phase solvent. After development, saccharides on the plates were visualized by spray-coating them with an aqueous solution containing 2.4 % $H_3(PMo_{12}O_{40})\bullet nH_2O$, 5 % H_2SO_4, and 1.5 % H_3PO_4, followed by heating.

Quantitative analysis of saccharides was carried out by a HPLC. A solution containing FCOS synthesized by βNAHex was diluted 10-fold with water, and then 1 μL of the diluted solution was loaded onto the HPLC column. The HPLC analysis was performed using an LC-10AS pump (Shimadzu, Kyoto, Japan) equipped with a Shodex RI-101 differential refractometer (Showa Denko, Tokyo, Japan) and a COSMOSIL Sugar-D column (φ4.6 × 250 mm, Nacalai Tesque, Kyoto, Japan). The system was operated under isocratic conditions using a solvent of acetonitrile/H_2O 77:23 (*v*/*v*) as the mobile phase (flow rate, 0.8 mL min^{-1}). The amounts of various saccharides in the reaction mixture were estimated using a calibration curve created based on the peak area of each pure sample.

Purification of reaction products by charcoal column chromatography.

Charcoal (particle size: 63–300 μm) used for a column chromatography was purchased from FUJIFILM Wako Pure Chemical Ind. Osaka, Japan. The solution containing FCOS synthesized by βNAHex was loaded onto a charcoal column (size: φ3.0 × 25 cm; solvent: H_2O) after heated for 5 min in boiling water to stop the enzymatic reaction. To separate $GlcNAc_2$-Fru (**I**) from other saccharides, GlcNAc was at first eluted with H_2O, and then each oligosaccharides were eluted stepwise with different concentration of aqueous solution of ethanol (EtOH): SucNAc, 5 % (*v*/*v*) EtOH; $GlcNAc_2$-Fru and $GlcNAc_2$, 9 % (*v*/*v*) EtOH. Since the elution of $GlcNAc_2$-Fru and $GlcNAc_2$ partially overlapped, we recovered these compounds almost completely by repeating charcoal column chromatography. To separate $GlcNAc_3$-Fru (**II**) from other saccharides, each oligosac-

charides were eluted stepwise with following concentration of aqueous solution of EtOH: SucNAc, 5 % (*v*/*v*) EtOH; $GlcNAc_2$, 7 % (*v*/*v*) EtOH; $GlcNAc_2$-Fru, 9 % (*v*/*v*) EtOH; $GlcNAc_3$-Fru and $GlcNAc_3$, 14 % (*v*/*v*) EtOH. Since the elution of $GlcNAc_3$-Fru and $GlcNAc_3$ were partially overlapped, we recovered these compounds almost completely by repeating charcoal column chromatography. A solution containing FCOS decomposition products generated by enzymatic hydrolysis using pure grade βNAHex or chitinase and acid hydrolysis using 100 mM sodium *p*-toluensulfonate buffer (pH 1.5) were loaded onto a charcoal column (size: φ1.0 × 5 cm; solvent: H_2O). To separate SucNAc (**Ib**) and $GlcNAc_2$ (**Id**) from GlcNAc (**Ia**) and Fru (**Ic**), respectively, each monosaccharide was at first eluted with H_2O, and then SucNAc or $GlcNAc_2$ was eluted with 7 % (*v*/*v*) EtOH. To separate SucNAc (**IIb**) and $GlcNAc_2$ (**IIa**), these compounds were eluted stepwise with 5 % (*v*/*v*) EtOH and 7 % (*v*/*v*) EtOH, respectively. To separate $GlcNAc_3$ (**IId**) from Fru (**IIc**), this monosaccharide was at first eluted with H_2O, and then $GlcNAc_3$ was eluted with 14 % (*v*/*v*) EtOH.

Identification of reaction products.

Identification of reaction products was carried out by ESI-MS using a LCMS-2020 instrument (Shimadzu) and ^1H- and ^{13}C-NMR spectroscopy using a Varian NMR system 600 spectrometer (Varian, Palo Alto, CA, USA). $[M-H]^+$ and $[M+Na]^+$ shown in each mass spectrum indicate a protonated molecular ion peak and a sodium adduct molecular ion peak, respectively. The NMR spectra were recorded in D_2O at 25 °C using 2,2-dimethyl-2-silapentane-5-sulfonate-d_6 that was used an external standard for setting up 0 ppm.

CONFLICTS OF INTEREST

The authors declare no conflict of interests.

ACKNOWLEDGMENTS

We thank Asaki Uehara and Takuto Omatsu for their cooperation in instrumental analyses. We thank Dr. Koki Fujita and Ensuiko Sugar Refining Co., LTD to which he belongs for donating pure grade β-fructofuranosidase of *M. saccharophilum*.

This work was supported by a Grant-in-Aid for Scientific Research (C) (No. 18K05540) from the Ministry of Education, Culture, Sports, Science, and Technology (Japan); and a Grant-in-Aid from the Mishima Kaiun Memorial Foundation.

REFERENCES

1) Y. Tamai, K. Miyatake, T. Okamoto, Y. Takamori, K. Sakamoto, and S. Minami: Enhanced healing of cartilaginous injuries by *N*-acetyl-D-glucosamine and glucuronic acid. *Carbohydr. Polym*., **54**, 251–262 (2003).

2) C.X. Tu, R.X. Zhang, X.J. Zhang, and T. Huang: Exogenous *N*-acetylglucosamine increases hyaluronan production in cultured human dermal fibroblasts. *Arch Dermatol Res.,* **301**, 549–551 (2009).

3) T. Mammone, D. Gen, C. Fthenakis, and K. Marenus: The effect of *N*-acetyl-glucosamine on stratum corneum desquamation and water content in human skin. *J. Soc. Cosmet. Chem*., **60(4)**, 423–428 (2009).

4) J.K. Chen, C.R. Shen, and C.L. Liu: *N*-Acetylglucosamine: Production and applications. *Mar. Drugs.,* **8**, 2493–2516 (2010).

5) B.B. Asm, E.B. Heggset, A.L. Norberg, M. Sørlie, K.M. Vårum, and V.G.H. Eijsink: Production of chitooligosaccharides and their potential applications in medicine. *Mar. Drugs*., **8**, 1482–1517 (2010).

6) F. Khoushab and M. Yamabhai: Chitin research revisited. *Mar. Drugs*., **8**, 1988–2012 (2010).

7) T. Yamaguchi, Y. Ito, and N. Shibuya: Oligosaccharide elicitors and their receptors for plant defense responses. *Trends Glycosci. Glycotechnol*., **12(64)**, 113–120 (2000)

8) W.M. Khan, B. Prithiviraj, and D.L. Smith: Effect of foliar application of chitin and chitosan oligosaccharides on photosynthesis of maize and soybean. *Phptosynthetica*, **40(4)**, 621–624 (2002).

9) H. Yin, Y. Du, and Z. Dong: Chitin oligosaccharide and chitosan oligosaccharide: Two similar but different plant elicitors. *Front. Plant Sci*., **7**, 522 (2016).

10) M. Slevin, S. Kumar, and J. Gaffney: Angiogenic oligosaccharides of hyaluronan induce multiple signaling pathways affecting vascular endothelial cell mitogenic and wound healing responses. *J. Biol. Chem*., **277**, 41046–41059 (2002).

11) P.W. Noble, C.M. Mckee, M. Cowman, and H.S. Shin: Hyaluronan Fragments Activate an NF-$_K$B/I-$_K$Bα Autoregulatory Loop in Murine Macrophages. *J. Exp. Med.,* **183**, 2373–2378 (1996).

12) R. Thomas,and T. Brooks: Common oligosaccharide moieties inhibit the adherence of typical respiratory pathogens. *J. Med. Microbiol.*, **53**, 833–840 (2004).

13) R. Thomas and T. Brooks: Attachment of *Yersinia pestis* to human respiratory cell lines is inhibited by certain oligosaccharides. *J. Med. Microbiol.*, **55**, 309–315 (2006)

14) M. Nishimoto and M. Kitaoka: Practical preparation of lacto-*N*-biose I, a candidate for the bifidus factor in human milk. *Biosci. Biotechnol. Biochem.,* **71(8)**, 2101–2104 (2007).

15) M. Kiyohara, A.Tachizawa, M. Nishimoto, M. Kitaoka, H. Ashida, and K. Yamamoto: Prebiotic effect of lacto-*N*-biose I on Bifidobacterial growth. *Biosci. Biotechnol. Biochem.,* **73(5)**, 1175–1179 (2009).

16) K. Kadokura, Y. Sakamoto, K. Saito, T. Ikegami, T. Hirano, W. Hakamata, T. Oku, and T. Nishio: Production and secretion of a recombinant *Vibrio parahaemolyticus* chitinase by *Escherichia coli* and its purification from the culture medium. *Biosci. Biotechnol. Biochem.,* **71(11)**, 2484–2851 (2007).

17) T. Minamoto, N. Takahashi, S. Kitahara, Y. Shinozaki, T.Hirano, W. Hakamata, and T. Nishio: Saccharification of β-Chitin from squid pen by a fermentation method using recombinant chitinase-secreting *Escherichia coli*. *Appl. Biochem. Biotechnol.*, **175**, 3788–3799 (2015).

18) A. Uehara, N. Takahashi, M. Moriyama, T. Hirano, W. Hakamata, and T. Nishio: Synthesis of chitin oligosaccharides using dried *Stenotrophomonas maltophilia* cells containing

a transglycosylation reaction-catalyzing β-*N*-acetylhexosaminidase as a whole-cell catalyst. *Appl. Biochem. Biotechnol.*, **184**, 673–684 (2018).

19) T. Hirano, T. Wada, S. Iwai, H. Sato, M. Noda, M. Juami, M. Nakamura, Y. Kumaki, W. Hakamata, and T. Nishio: Synthesis of β-D-fructofuranosyl-(2→1)-2-acetamido-2-deoxy-α-D-glucopyranoside (*N*-acetylsucrosamine) using β-fructofuranosidase-containing *Aspergillus oryzae* mycelia as a whole-cell catalyst. *Carbohydr. Res.*, **353**, 27–32 (2012).

20) H. Sato, S. Yokochi, T. Kasama, T. Hirano, W. Hakamata, and T. Nishio: Continuous production of β-D-fructofuranosyl-(2→1)-2-acetamido-2-deoxy-α-D-glucopyranoside (*N*-Acetylsucrosamine) Using a Column reactor packed with β-fructofuranosidase-containing mycelia of *Aspergillus oryzae* immobilized on a porous carrier. *J. Appl. Glycosci.,* **59**, 153–160 (2012).

21) H. Hosaka, M. Kawamura, T. Hirano, W. Hakamata, and T. Nishio: Utilization of sucrose and analog disaccharides by human intestinel bifidobacteria and lactobacilli: Search of the bifidobacteria enzymes involved in the degradeation of these disaccharides. *Microbiol. Res.*, **240**, 126558 (2020).

22) K. Fujita, K. Hara, H. Hashimoto, and S. Kitahata: Purification and some properties of β-fructofuranosidase I from *Arthrobacter* sp. K-1. *Agric. Biol. Chem.,* **54**, 913–919 (1990).

23) K. Fujita, K. Hara, H. Hashimoto, and S. Kitahata: Transfructosylation catalyzed by β-fructofuranosidase I from *Arthrobacter* sp. K-1. *Agric. Biol. Chem.,* **54**, 2655–2661 (1990).

24) M. Ogawa, M. Kitagawa, H. Tanaka, K. Ueda, T. Watsuji, T. Beppu, A. Kondo, R. Kawachi, T. Oku, and T. Nishio: A β-*N*-acetylhexosaminidase from *Symbiobacterium thermophilum*; gene cloning, overexpression, purification and characterization. *Enzyme Microb. Technol.*, **38**, 457–464 (2006).