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

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Abstract: Utilizing transglycosylation reaction catalyzed by β-N-acetylhexosaminidase of Stenotrophomonas maltophilia, β-D-fructofuranosyl-(2→1)-α-N, N′-diacetylchitobioside (GlcNAc-Fru) was synthesized from N-acetylsucrosamine and N, N′-diacetylchitobiose (GlcNAc2), and β-D-fructofuranosyl-(2→1)-α-N, N′, N″′-triacetylchitotrioside (GlcNAc3-Fru) was synthesized from GlcNAc2-Fru and GlcNAc3. Through purification by charcoal column chromatography, pure GlcNAc2-Fru and GlcNAc3-Fru were obtained in molar yields of 33.0 % and 11.7 % from GlcNAc2, 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.10)11)12)13)14) We are particularly interested in lacto-N-biose, a disaccharide derived from human milk 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 hetero-oligosaccharides containing GlcNAc, utilizing regio- and anomere-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 (GlcNAc2) 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 GlcNAc2 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-D-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

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Abbreviations: COS, chitin oligosaccharide; ESI, electrospray ionization; FCOS, D-fructofuranose-linked chitin oligosaccharide; Fru, D-fructofuranose; βFFase, β-fructofuranosidase; GlcNAc, N-acetyl-D-glucosamine; GlcNAc2, N, N′-diacetylchitobiose; GlcNAc3, N, N′, N″′-triacetylchitotrioside; GlcNAc2-Fru, β-D-fructofuranosyl-(2→1)-α-N, N′-diacetylchitobioside (GlcNAc2-Fru); GlcNAc3-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.
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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*,2223 because the raw material cost is low in this methodology. 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 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. 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 previous 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 GlcNAc₂, and 72.3 mg SucNAc (molar ratio of GlcNAc₂ 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 GlcNAc₂ 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 H₂O, 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 obtained by NMR of those oligosaccharides. In the previous studies (Fig. 1A,B, and C),[7][8][9] 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 GlcNAc2 and Fru, we decided to confirm the structure of obtained disaccharides by comparing their 1H 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-toluenesulfonate 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 GlcNAc2, 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+H]+ and [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 1H NMR signals that can be indicators of these compounds. In 1H 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 1H 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 GlcNAc2 were observed in 1H NMR spectra of compound Id. Moreover, signals of anomeric H of non-reducing end βGlcNAc residues (δ 4.58-4.62, 2 d) of GlcNAc2 diastereomers were also observed in 1H NMR spectra of this compound. The other 1H NMR signals of compounds Ib and Id were corresponding to those of SucNAc and GlcNAc2, respectively. From the above facts, it became clear that compound I produced from SucNAc and GlcNAc2 by the transglycosylation action of S. maltophilia βNAHex is β-D-fructofuranosyl-(2→1)-α-N′-diacetylmuramiduronic acid (GlcNAc2-Fru). The above reaction and purification process yielded 228 mg of GlcNAc2-Fru (molar yield from GlcNAc2; 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 GlcNAc2 and GlcNAc2-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 GlcNAc2-Fru. A 0.4 mL of 20 mM sodium phosphate buffer (pH 7.0) containing 0.1 unit of crude βNAHex of S. maltophilia, 40 mg of GlcNAc2, and 111 mg GlcNAc2-Fru (molar ratio of GlcNAc2 to GlcNAc2-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 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 GlcNAc2 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 GlcNAc2 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 GlcNAc2, respectively. Compounds IIa and IIb were purified from the reaction mixture after 4-h incubation by charcoal column chromatography. Next, 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 IId were similar to those of GlcNAc2 and Fru.
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 1H 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 1H NMR spectra of this compound. Signals of anomeric H of reducing end αGlcNAc residue (δ 5.39, d) and βGlcNAc residue (δ 4.70, d) of GlcNAc 3 were observed in 1H 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 1H NMR spectra of this compound. The other 1H 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 3-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 1H 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.
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, Darmstadt, 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 developed twice using 2-PrOH/AcOH/H₂O 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 developed three times using 1-BuOH/MeOH/16 % aqueous solution of NH₃ 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 % H₃(PO₄)₂•6H₂O, 5 % H₂SO₄, and 1.5 % H₃PO₄, followed by heating.

Quantitative analysis of saccharides was carried out by 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/H₂O 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: H₂O) after heated for 5 min in boiling water to stop the enzymatic reaction. To separate GlcNAc₂-Fru (I) from other saccharides, GlcNAc was at first eluted with H₂O, and then each oligosaccharides were eluted stepwise with different concentration of aqueous solution of ethanol (EtOH): SucNAc, 5 % (v/v) EtOH; GlcNAc₂-Fru and GlcNAc₂, 9 % (v/v) EtOH. Since the elution of GlcNAc₂-Fru and GlcNAc₂ partially overlapped, we recovered these compounds almost completely by repeating charcoal column chromatography. To separate GlcNAc₂-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$_2$-Fru and GlcNAc$_3$, 14 % (v/v) EtOH. Since the elution of GlcNAc$_2$-Fru and GlcNAc$_3$ was 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-toluenesulfonate buffer (pH 1.5) were loaded onto a charcoal column (size: φ1.0 × 5 cm; solvent: H$_2$O). To separate SucNAc (IIb) and GlcNAc$_3$ (IIc) from GlcNAc$_2$ (Ia) and Fru (Ic), respectively, each monosaccharide was at first eluted with H$_2$O, and then SucNAc or GlcNAc$_2$ was eluted with 7 % (v/v) EtOH. To separate GlcNAc$_3$-Fru and GlcNAc$_3$, 14 % (v/v) EtOH. 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).

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