Large-scale preparation and glycan characterization of sialylglycopeptide from bovine milk glycomacropeptide and its bifidogenic properties

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ABSTRACT
Bovine glycomacropeptide (GMP) is a 7,000-Da glycopolypeptide released from κ-casein during cheese making. The O-glycan chains linked to GMP have many biological activities, but their utilization for nutraceutical products is limited due to their low content. To concentrate the functional glycan chains of GMP, we prepared sialylglycopeptide concentrate (SGC) from GMP-containing whey protein concentrate via proteolytic digestion of peptide chains and concentration of sialylglycopeptide by ultrafiltration using membranes with a molecular weight cut-off of 1,000 Da. The abundant saccharides detected in the prepared SGC were N-acetylneuraminic acid (Neu5Ac: 32.3% wt/wt), N-acetylgalactosamine (11.3%), and galactose (10.2%), which constitute O-glycans attached to GMP. The Neu5Ac content in SGC was found concentrated at approximately 4.8-fold of its content in GMP-containing whey protein concentrate (6.8%). Structural analysis of O-glycopeptides by liquid chromatography tandem mass spectrometry identified 88 O-glycopeptides. Moreover, O-acetylated or O-diacylated Neu5Ac was detected in addition to the previously characterized O-glycans of GMP. Quantitative analysis of O-glycan in SGC by fluorescence labeling of chemically released O-glycan revealed that a disialylated tetrasaccharide was the most abundant glycan (76.6% of the total O-glycan). We further examined bifidogenic properties of SGC in vitro, which revealed that SGC served as a more potent carbon source than GMP and contributes to the growth-promoting effects on certain species of bifidobacteria. Overall, our study findings indicate that SGC contains abundant O-glycans and has a bifidogenic activity. Moreover, the protocol for the preparation of SGC described herein is relatively simple, providing a high yield of glycan, and can be used for large-scale preparation.

Key words: glycomacropeptide, O-glycan, Neu5Ac, sialylglycopeptide, bifidobacteria

INTRODUCTION
Milk protein, as a rich source of EAA, has several biological functions and is vital to the healthy development of infants. Recent proteomics studies on human and bovine milk have shown that milk proteins are extensively glycosylated (O’Riordan et al., 2014). The glycan chains of these glycoproteins not only play a role in protein folding, biological recognition, and protection of proteins from digestion, but also recently have been shown to act as protection against pathogens (Wang et al., 2017b) and as substrates for bifidobacterial growth (Karav et al., 2016).

Identifying glycan structure is difficult due to the variety of their monosaccharide compositions and the nearly infinite combinations of linkage types. However, with the development of more advanced analytic techniques and instruments, such as liquid chromatography (LC)-MS, the complexity and diversity of glycans in milk glycoproteins are becoming increasingly apparent (Nwosu et al., 2012; Cao et al., 2017). The main glycoproteins found in bovine milk are lactoferrin, immunoglobulins, glycoproteins of the milk fat globule membrane, and κ-casein (O’Riordan et al., 2014). Glycopeptides released into whey from κ-casein by the action of rennet, which is used in cheese making, are termed glycomacropeptides (GMP).

The bovine GMP glycan is composed of N-acetylmuramic acid (Neu5Ac), N-acetylgalactosamine (GalNAc), and galactose (Gal). Five different types of glycan structures have been identified, namely (1) GalNAc, (2) Gal31–3GalNAc, (3) Neu5Aco2–3Gal31–3GalNAc, (4) Gal31–3(NeuAco2–6)GalNAc, and (5) Neu5Aco2–3Gal31–3( Neu5Aco2–6)GalNAc. The disialylated tetrasaccharide (type 5) is the most abundant glycan in GMP, accounting for 56% of the total glycan structures (Saito and Itoh, 1992). These glycans, especially Neu5Ac, play a vital role in the biological activities of GMP, such as inhibition of influenza vi-
Functions, such as bifidogenic activity. The properties of sialylglycopeptides and their biological food processing. Furthermore, we describe the chemi-
additives and ultrafiltration membranes available for hundred-gram scale using proteolytic enzymes as food glycan chain concentrate as sialylglycopeptides on a
preparation method for concentrating glycan chains of GMP can be expected to increase the health benefits of infant formula.

Recently, several groups reported large-scale preparation of glycopeptides from egg yolk (Liu et al., 2017), N-glycans released from glycoproteins in soybean proteins (Zhu et al., 2018), and milk proteins (Karav et al., 2016) on a milligrams to grams scale. These glyco-
peptides or N-glycans are used in the analysis of glycan structure and assessment of their biological functions (Wang et al., 2017a), as well as starting material for the synthesis of complex glycans. There remain, however, many challenges to incorporating these large-scale procedures for food ingredients. It is necessary to optimize the materials and manufacturing equipment suitable for food production with regard to food safety as well as improving yield and manufacturing costs.

The purpose of this study is to develop a large-scale preparation method for concentrating glycan chains from GMP for use as food ingredients. We prepared glycans for concentration as sialylglycopeptides on a hundred-gram scale using proteolytic enzymes as food additives and ultrafiltration membranes available for food processing. Furthermore, we describe the chemi-
properties of sialylglycopeptides and their biological functions, such as bifidogenic activity.

MATERIALS AND METHODS

Reagents

Commercially available GMP-containing whey protein concentrates (G-WPC) were purchased from Arla Foods Ingredients (Lacprodan CGMP-10; Aarhus, Denmark). Lacprodan CGMP-10 is a GMP-enriched WPC containing 60 to 70% GMP (wt/wt) and 6% Neu5Ac (wt/wt). Alcalase 2.4 L FG (2.4 Anson unit/g, produced by \textit{Bacillus licheniformis}, with endopeptidase activity) and Flavorzyme 1000 L (1,000 Leucine amino-
peptidase unit/g, produced by \textit{Aspergillus oryzae}, with both endopeptidase and exopeptidase activities) were obtained from Novozymes (Copenhagen, Denmark).

Preparation of Sialylglycopeptide Concentrates from G-WPC

The preparation of sialylglycopeptide concentrates (SGC) from G-WPC is detailed in Figure 1. The G-
WPC (1.25 kg) was dissolved in 23.75 kg of water to prepare a 25-kg solution (5% [wt/wt] G-WPC solution). After the G-WPC solution was heated to 50°C and adjusted to pH 7 with KOH, Alcalase and Flavorzyme (0.15% [wt/wt], respectively) were added to the solution at the same time. The enzyme reaction was carried out at 50°C for 4 h without pH control and then stopped by cooling to 4°C. Molecular weight cut-off (MWCO) 1,000 ultrafiltration membrane (Membrelax EP19–40, Pall Corporation, New York, NY) was used to concen-
trate the glycan chains from G-WPC hydrolysate. The hydrolysate was concentrated to 20 kg (1.25-fold) by ultrafiltration. Retentate diafiltration was continuously performed in 5-fold water (vol/vol), and the retentate was then lyophilized in a freeze dryer (Nissei Limited, Tokyo, Japan). The lyophilized retentate as SGC was stored at −30°C until further use.

Size-Exclusion Chromatography

Size-exclusion chromatography (SEC) analysis was performed with an L-2000 chromatography system (GL Sciences, Tokyo, Japan) to estimate the molecular weight of G-WPC, hydrolyzed G-WPC, and SGC. Samples were applied to 2 coupled TSK gel G3000 PWXL columns (7.8 mm i.d. × 300 mm; 7 μm particle size; Tosoh Corporation, Tokyo, Japan) and eluted with 40% (vol/vol) acetonitrile containing 0.1% (vol/ vol) trifluoroacetic acid at a flow rate of 0.3 mL/min. The absorbance was monitored at 214 nm. Angiotensin II (1,046 Da) was used as a molecular weight marker (Fujifilm Wako Pure Chemical Corporation, Osaka, Japan).

Analysis of Saccharide Composition in SGC and G-WPC

For Neu5Ac and galacto-N-biose (GNB) measurements, SGC or G-WPC was treated with neuraminidase derived from \textit{Arthrobacter ureafaciens} (Nacalai Tesque Inc., Kyoto, Japan) and O-glycosidase (New England Biolabs, Ipswich, MA). A reaction mixture containing 250 μg/mL SGC or G-WPC, 0.6 U/mL neuraminidase, and 800,000 U/mL O-glycosidase in 50 mM sodium phosphate buffer solution (pH 5.0) was incubated at 37°C for 2 h. For measurement of galactose (Gal), man-
nose (Man), glucose (Glc), and fucose (Fuc), SGC or G-WPC was incubated with 1.0 M trifluoroacetic acid at 100°C for 3 h. For measurement of GalNAc and N-acetylglucosamine (GlcNAc), SGC or G-WPC was incubated with 3 M hydrochloric acid at 100°C for 2 h. Saccharides in the enzymatic or acid hydrolysates were analyzed by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD). The enzymatic or acid hydrolysates were applied to a Dionex CarboPac PA1 column (4 mm i.d. × 250 mm; 10 μm particle size; Thermo Fisher Scientific, Waltham, MA). Saccharide standards were obtained as the purest available grades (Sigma-Aldrich, St. Louis, MO). For Neu5Ac, the mobile phase consisted of water (solvent A), 200 mM NaOH (solvent B), and 600 mM sodium acetate in 100 mM NaOH (solvent C) in the following gradient elution: 0 to 7 min 45% B and 10% C; 7 to 10 min 45 to 37.5% B and 10 to 25% C; 10 to 20 min 37.5% B and 25% C; 20 to 20.1 min 37.5 to 45% B and 25 to 10% C; and 20.1 to 25 min 45% B and 10% C at a 1 mL/min flow rate. For GNB, the mobile phase was used in isocratic conditions with 100 mM NaOH for 15 min. Other monosaccharides were eluted for 15 min with an isocratic 10 mM NaOH mobile phase. The chromatograms were analyzed using Chromeleon software ver. 6.8 (Thermo Fisher Scientific).

Amino Acid Analysis

Determination of total AA content in SGC and G-WPC was performed at the Japan Food Research Laboratories (Tokyo, Japan). Samples were hydrolyzed with hydrochloric acid and then analyzed with an L-8900 automated AA analysis system (Hitachi High-Technologies Corporation, Tokyo, Japan).

O-Glycan Analysis

Labeled SGC O-glycans were prepared using an EZ-Glyco O-Glycan Prep Kit (Sumitomo Bakelite, Tokyo, Japan). Briefly, 20 μL of SGC solution (10 mg/mL in H2O) was mixed with 15 μL of Glycan Released Reagent and incubated at 37°C for 75 min. Subsequently, released glycans were captured with Glycan Capturing Beads, after which the beads were washed with acetonitrile. Then, 4 mg of 2-aminobenzamide (2-AB) and 0.04 mg of reducing reagent in 50 μL of methanol/acetic acid/H2O (9/2/9) was added to the beads. The O-glycan-containing solution was recovered by centrifugation at 3,000 × g for 1 min. The solution was incubated at 50°C for 2.5 h and washed with acetonitrile to remove excess reagent with a Cleanup Column. 2-AB-labeled O-glycans were recovered by adding H2O and analyzed using a Q-Exactive mass spectrometer and RS Fluorescence (FL) Detector coupled with an UltiMate 3000 (Thermo Fisher Scientific). Labeled O-glycan solution (10 μL) was applied to a Glycanpac AXH-1 column (2.1 mm i.d. × 150 mm; 3 μm particle size; Thermo Fisher Scientific) at 40°C. The mobile phase consisted of 50 mM ammonium formate (pH 4.4) and acetonitrile (17/83) with a flow rate of 0.4 mL/min. The flow was split in half between MS and fluorescence detectors after the analytical column. For MS measurements, electrospray voltage and heat capillary temperature were 3.5 kV and 250°C, respectively. Nitrogen (99.5% purity) was used as sheath (set to 45) and auxiliary (set to 10) gas. Full-scan mass spectra were acquired in positive ion mode with a scan range of m/z 200 to 1,500 and resolution of 70,000. The FL detector excitation and emission wavelengths were set to 320 and 420 nm, respectively. The MS and FL chromatograms were analyzed using Xcalibur ver. 4.0 (Thermo Fisher Scientific).

Characterization of O-Glycopeptides in SGC by LC-MS

O-Glycopeptides in SGC were analyzed using a Q-Exactive mass spectrometer coupled with an UltiMate

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**Figure 1.** Schematic diagram of the large-scale preparation of sialylglycopeptide concentrate (SGC) from glycomacropeptide (GMP)-containing whey protein concentrates (G-WPC). MWCO = molecular weight cut-off.
The SGC (2 μg) was loaded onto an InertSustain AQ-C18 HP column (2.1 mm i.d. × 150 mm; 3 μm particle size; GL Sciences) at 40°C. The mobile phase consisted of 2% (vol/vol) acetonitrile with 0.1% (vol/vol) formic acid (solvent A) and 90% (vol/vol) acetonitrile with 0.1% (vol/vol) formic acid (solvent B) at a gradient elution of 0 to 5 min 0% B; 5 to 30 min 0 to 10% B; 30 to 50 min 10 to 100% B; 50 to 60 min 0% B, and a flow rate of 0.2 mL/min. Electrospray voltage and heat capillary temperature were 3.5 kV and 275°C, respectively. Nitrogen (99.5% purity) was used as sheath (set to 35), auxiliary (set to 10), and collision gas. Mass spectra were recorded in automatic data-dependent mode with one precursor scan, followed by 5 MS/MS scans. Full-scan mass spectra were acquired in positive ion mode with a scan range of m/z 100 to 1,500 and resolution of 70,000. The MS/MS spectra were acquired using higher energy collisional dissociation with a scan range of m/z 200 to 2,000, resolution of 17,500, and stepped normalized collision energies of 15, 30, and 45%.

**Data Treatment**

The MS/MS spectra were computationally analyzed to determine the glycan peptide sequence and sugar composition. Assignments were validated by manual inspection. The raw data were analyzed using Byonic (Protein Metrics, San Carlos, CA) integrated as a node into the Proteome Discoverer 2.2 software (Thermo Fisher Scientific) with the following settings: (1) no-enzyme (unspecific) digestion, (2) precursor ion mass tolerance 10 ppm, (3) fragment mass tolerance 10 ppm, (4) dynamic phosphorylation on serine and threonine, (5) protein database consisting of 2 genetic variants of bovine GMP (GMPa and GMPb), (6) dynamic glycosylation on serine and threonine, and (7) in-house O-glycan database. The glycan database contains 10 glycan masses, including 1 N-acetylhexosamine (HexNAc; +203.0894), 0–1 hexose (Hex; +162.0528), 0–2 Neu5Ac (+291.0954), and 0–2 O-acetylation per Neu5Ac (+42.0106). According to a previous report on glycan structure in GMP (Saito and Itoh, 1992), the glycans annotated as (HexNAc), (HexNAc) (Hex), or (HexNAc)(Hex)(Neu5Ac)2 were assigned to GalNAc, Galβ1–3GalNAc, or Neu5AcO2–3Galβ1–3(Neu5AcO2–6)GalNAc, respectively. The glycan annotated as (HexNAc)(Hex)(Neu5Ac) was manually assigned to Neu5AcO2–3Galβ1–3GalNAc or Galβ1–3 (Neu5AcO2–6)GalNAc considering losses of Neu5Ac and Gal from the precursor ion in the MS/MS spectra. O-Acetylation on Neu5Ac was also confirmed manually by the detection of a signal derived from O-Ac-Neu5Ac (m/z 334) or O, O-diAc-Neu5Ac (m/z 376) in the MS/MS spectra. Further, O-acetylation was determined to occur on branched Neu5Ac, manually determined by loss of Neu5Ac, Gal, and O-Ac-Neu5Ac/O, O-diAc-Neu5Ac from the precursor ion in the MS/MS spectra.

**In Vitro Growth of Bifidobacterium Strains in the Presence of SGC as the Sole Carbon Source**

Bifidobacterium strains used in this study were Bifidobacterium bifidum JCM1254, JCM7004, Bifidobacterium longum ssp. infantis JCM1222T, JCM1260, Bifidobacterium breve JCM1192T, JCM7016, and Bif. longum ssp. longum JCM1217T (JCM; Japan Collection of Microorganisms, Ibaraki, Japan); Bif. bifidum ATCC29521T, and Bif. breve ATCC15698 (ATCC; The American Type Culture Collection, Manassas, VA); and Bif. bifidum SB10550 and SBT2359 (SBT; Megmilk Snow Brand Co., Ltd., Tokyo, Japan). Each strain was inoculated in 8 mL of GAM broth (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) supplemented with 1% Glc and pre-cultured for 16 h at 37°C under anaerobic conditions using Anaeropack (Mitsubishi Gas Chemical Company Inc., Tokyo, Japan). In vitro growth assays were performed as previously described (Matsuki et al., 2016). Briefly, pre-cultured cells were inoculated into 200 μL of PY medium [200 mM PIPES (pH 6.7), 2 g/L peptone, 2 g/L BBL trypticase peptone, 2 g/L Bacto Yeast Extract, 8 mg/L CaCl2, 19.2 mg/L MgSO4·7H2O, 80 mg/L NaCl, 4.9 mg/L hemin, 0.5 g/L L-cysteine hydrochloride, and 100 ng/L vitamin K1] in the presence of 1.0% Glc (Wako Pure Chemical), 2.0% G-WPC, or 2.0% SGC in 96-well microplates. After 72 h at 37°C cultured under anaerobic conditions, bifidobacterial growth was evaluated for optical density at 660 nm with a Varioskan Flash spectrophotometer (Thermo Fisher Scientific). Optical density measurements were automatically corrected to a 1-cm pathlength by SkanIt Software (ver. 2.0, Thermo Fisher Scientific). Samples were grown in triplicates.

**Release of Neu5Ac and GNB from SGC by Treatment with Bif. bifidum Cell Extract**

Bacteria were inoculated in 8 mL of GAM broth (Nissui Pharmaceutical Co. Ltd.) supplemented with 1% Glc and pre-cultured for 16 h at 37°C under anaerobic conditions using Anaeropack (Mitsubishi Gas Chemical Company Inc.). The bifidobacterial cells were harvested by centrifugation (5,000 × g for 10 min, 4°C), resuspended in 2 mL of 50 mM acetate buffer (pH 5.0), and washed twice with resuspension buffer. Cells were resuspended in 1 mL of 50 mM acetate buffer (pH 5.0) and disrupted using a Multi-beads shocker (Yasui Kikai Corporation, Osaka, Japan). After removing the beads,
the obtained solution was used as the cell extract. Then, 2 mg/mL SGC (50 μL) in 50 mM acetate buffer solution (pH 5.0) and 50 μL of cell extract were mixed and incubated at 37°C for 24 h. Free Neu5Ac and GNB levels in the reaction solution were determined with HPAEC-PAD.

**Effect of SGC on In Vitro Growth of Bifidobacterium Strains in the Presence of Glucose**

*Bifidobacterium longum* ssp. *longum* JCM1217<sup>T</sup> and *Bif. longum* ssp. *infantis* JCM1222<sup>T</sup> were inoculated in 8 mL of GAM broth supplemented with 1% Glc and pre-cultured for 16 h at 37°C under anaerobic conditions. Each pre-cultured strain was inoculated into 4 mL of PY medium supplemented with 0.1% Glc in the presence or absence of 0.4% G-WPC or SGC. *Bifidobacterium* cell growth in static cultures at 37°C was automatically recorded at optical density at 660 nm every 30 min using a TVS062CA Bio-photorecorder (Advantec Toyo Kaisha Ltd., Tokyo, Japan).

**Statistical Analysis**

Statistical analyses were performed with EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan). Differences between SGC and G-WPC on the in vitro growth of *Bifidobacterium* strains as the sole carbon source were determined by 2-tailed Student’s *t*-test. Differences were considered statistically significant when the *P* value was < 0.05.

**RESULTS**

**Preparation of SGC from G-WPC and SGC**

To concentrate the GMP glycan chains, we removed the GMP peptide chains by proteolytic digestion and ultrafiltration (Figure 1). The G-WPC, which contains 60 to 70% GMP, was digested with protease. The G-WPC digestion products were confirmed by SEC as the peaks shifted to lower molecular regions (Figure 2a, b). By ultrafiltration through MWCO 1,000 membranes followed by diafiltration, peptides and AA pass through the ultrafiltration membrane while the glycopeptides are concentrated in the retentate. The concentrated glycopeptides were lyophilized to obtain SGC powder. The solid yield of SGC from G-WPC was approximately 15%. The SEC analysis showed that the molecular weights of most SGC glycopeptides were more than 1,000 Da (Figure 2c). The Neu5Ac content was 32.3% in SGC, which was an approximate 4.8-fold increase compared with its content in G-WPC (6.8%; Table 1), indicating that Neu5Ac became concentrated. Similarly, Gal and GalNAc, which are component neutral monosaccharides of O-glycan attached to GMP, also became concentrated in SGC. Small amounts of Fuc, Man, and GlcNAc, which are suspected to be component N-glycan monosaccharides attached to whey proteins, were detected in SGC; these monosaccharides were also more concentrated in SGC compared with G-WPC. However, total AA content decreased in SGC by about 50%. These results indicate that the glycan chains in G-WPC were concentrated in SGC and that more than half of the peptide chains were removed. The GNB (Galβ1–3GalNAc), which is the core O-glycan structure in GMP, was released from G-WPC or SGC by neuraminidase and O-glycosidase. The GNB content in G-WPC and SGC was 4.9% or 21.0% (wt/wt), respectively. The molar ratio of Neu5Ac to GNB was 1.75:1 in G-WPC and 1.78:1 in SGC, suggesting that 2 Neu5Ac molecules are bound to most GNB. We further performed quantitative analysis of O-glycans in G-WPC and SGC using fluorescently labeled, chemically released O-glycan and found that disialyl-GNB is a major component of both G-WPC and SGC O-glycans, accounting for 76.8% and 76.6%, respectively. Moreover, the SGC O-glycan proportion was identical to that of G-WPC (Table 2).

**LC-MS/MS Structural Analysis of the Glycopeptides in SGC**

The SGC O-glycopeptide structures were identified by LC-MS/MS. Mass spectra were analyzed using Proteome Discoverer and Byonic software, and the assignments were validated by manual inspection. Seven types of glycans attached to peptides were identified in SGC (Table 3). The glycan types 1 to 5 in Table 3 were previously reported as O-glycans in GMP (Saito and Itoh, 1992). The Neu5Ac of type 5 was modified by acetylation, specifically the Neu5Ac bound to GalNAc (glycan type 6 and 7 in Table 3). Figure 3 shows a representative MS/MS spectrum of a glycopeptide possessing O-Ac-Neu5Ac with m/z 798.8. The peptide sequence and glycan composition were computationally identified as ESTVAT + (GalNAc)(Gal)(Neu5Ac)(O-Ac-Neu5Ac). Product ions resulting from Gal and Neu5Ac loss (m/z 1,143.48) and O-Ac-Neu5Ac (m/z 1,263.53) loss were detected, indicating that Neu5Ac and O-Ac-Neu5Ac must be bound to Gal and GalNAc, respectively. Therefore, we determined that the glycan structure was Neu5Aco2-3Galβ1–3(O-Ac-Neu5Aco2-6)GalNAc. The glycan structure Neu5Aco2-3Galβ1–3(O, O-diAc-Neu5Aco2-6)GalNAc was also determined.

Supplemental Table S1 (https://osf.io/u3rv5/) summarizes the 88 O-glycopeptides detected in SGC. Most
O-glycopeptides contained 6 well-defined glycosylation sites at T121, T131, T133, T136, T142, and T165 (residue numbering is based on the Swiss-Prot entry for the mature form of bovine κ-casein; accession number P02668; Hernandez-Hernandez et al., 2016). Glycan type 5 [Neu5Acα2–3Galβ1–3(Neu5Acα2–6)GalNAc] was the most frequently detected among the 7 glycan types occurring in 55 of 88 glycopeptides. Furthermore, the peptide chain was composed of 11 AA residues or less.

**Table 1.** Saccharides and AA in glycomacropeptide-containing whey protein concentrates (G-WPC) and sialylglycopeptide concentrate (SGC)

<table>
<thead>
<tr>
<th>Saccharides¹ and AA²</th>
<th>% (wt/wt)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>G-WPC</td>
</tr>
<tr>
<td>Neu5Ac</td>
<td>6.8</td>
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<tr>
<td>Gal</td>
<td>2.2</td>
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<tr>
<td>GalNAc</td>
<td>3.6</td>
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<tr>
<td>Man</td>
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<tr>
<td>GlcNAc</td>
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</tr>
<tr>
<td>Glc</td>
<td>0.5</td>
</tr>
<tr>
<td>Fuc</td>
<td>0.1</td>
</tr>
<tr>
<td>AA</td>
<td>86.8</td>
</tr>
</tbody>
</table>

¹Neu5Ac = N-acetylneuraminic acid; Gal = galactose; GalNAc = N-acetylgalactosamine; Man = mannose; GlcNAc = N-acetylglucosamine; Glc = glucose; Fuc = fucose.
²Sum of individual AA.
³ND = not detected.

**Table 2.** Molar ratio of 2-aminobenzamide (2-AB) labeled O-glycan derived from glycomacropeptide-containing whey protein concentrates (G-WPC) and sialylglycopeptide concentrate (SGC)

<table>
<thead>
<tr>
<th>2-AB labeled O-glycan</th>
<th>G-WPC</th>
<th>SGC</th>
</tr>
</thead>
<tbody>
<tr>
<td>GNB²-AB</td>
<td>4.1</td>
<td>4.2</td>
</tr>
<tr>
<td>Monosialyl-GNB-AB (linear)</td>
<td>12.0</td>
<td>11.9</td>
</tr>
<tr>
<td>Monosialyl-GNB-AB (branched)</td>
<td>7.1</td>
<td>7.2</td>
</tr>
<tr>
<td>Disialyl-GNB-AB</td>
<td>76.8</td>
<td>76.6</td>
</tr>
</tbody>
</table>

²GNB = galacto-N-biose.

**Growth of Bifidobacterium Strains in the Presence of SGC as the Sole Carbon Source**

Eleven infant gut-associated *Bifidobacterium* strains were cultivated in medium supplemented with glucose, SGC, or G-WPC as the sole carbon source. *Bifidobacterium* growth was evaluated by cell density (optical density at 660 nm), and all strains grew well in glucose medium. Four strains (*Bif. bifidum* JCM1254, JCM7004, SBT10550, and SBT2359) grew in both SGC- and G-WPC-medium, whereas the remaining 7 strains did not grow in either SGC or G-WPC media (Figure 4). Four strains grew significantly better in SGC medium than in G-WPC medium, whereas *Bif. breve* JCM1192T and *Bif. longum* ssp. *longum* JCM1217T grew slightly in none, SGC medium, and G-WPC medium (optical density <0.1). This minimal growth was considered to be independent of the carbon source.
Release of Neu5Ac and GNB from SGC by Bif. bifidum Cell Extract

The SGC was incubated with the cell extracts of 5 Bif. bifidum strains. The released Neu5Ac and GNB were analyzed by HPAEC-PAD. The proportion of Neu5Ac and GNB released from SGC was higher in the cell extracts of 4 strains (JCM1254, JCM7004, SBT10550, and SBT2359) that grew in SGC medium compared with that of ATCC29521T cell extracts (Table 4). These results indicate that these 4 strains have high neuraminidase and endo-α-N-acetylgalactosaminidase activity.

Effect of SGC on In Vitro Growth of Bifidobacterium Strains in the Presence of Glucose

Bifidobacterium longum ssp. longum JCM1217T and Bif. longum ssp. infantis JCM1222T showed no growth in media with SGC as the sole carbon source (Figure 4). We thus investigated whether SGC can promote their growth in the presence of glucose (Figure 5). Addition of G-WPC or SGC into glucose-containing PY medium had no effect on growth of either strain. However, SGC addition shortened the time needed to reach optical density >0.2 by 3 h for Bif. longum ssp. longum JCM1217T and 8.5 h for Bif. longum ssp. infantis JCM1222T. The addition of G-WPC shortened Bif. longum ssp. longum JCM1217T growth time by 1 h and Bif. longum ssp. infantis JCM1222T by 1.5 h. These results indicate that SGC promotes Bif. longum ssp. longum JCM1217T and Bif. longum ssp. infantis JCM1222T growth more potently than can G-WPC.

DISCUSSION

In this study, we developed a simple protocol to concentrate GMP glycan chains using a 2-step process consisting of (1) proteolytic digestion of peptide chains and (2) glycopeptide concentration by ultrafiltration.

Table 3. Proposed glycan structure of sialylglycopeptide concentrate (SGC) based on liquid chromatography MS/MS results

<table>
<thead>
<tr>
<th>Glycan type</th>
<th>Glycan chain</th>
<th>Mass (Da)</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>GalNAc</td>
<td>203</td>
</tr>
<tr>
<td>2</td>
<td>Galβ1–3GalNAc</td>
<td>365</td>
</tr>
<tr>
<td>3</td>
<td>Neu5Acox2–3Galβ1–3GalNAc</td>
<td>656</td>
</tr>
<tr>
<td>4</td>
<td>Galβ1–3(Neu5Acox2–6)GalNAc</td>
<td>656</td>
</tr>
<tr>
<td>5</td>
<td>Neu5Acox2–3Galβ1–3(Neu5Acox2–6)GalNAc</td>
<td>947</td>
</tr>
<tr>
<td>6</td>
<td>Neu5Acox2–3Galβ1–3(O-Ac-Neu5Acox2–6)GalNAc</td>
<td>989</td>
</tr>
<tr>
<td>7</td>
<td>Neu5Acox2–3Galβ1–3(O, O’-diAc-Neu5Acox2–6)GalNAc</td>
<td>1,031</td>
</tr>
</tbody>
</table>

1Neu5Ac = N-acetylneuraminic acid; O-Ac-Neu5Ac = O-acetylated N-acetylneuraminic acid; O, O’-diAc-Neu5Ac = O, O’-diacetylated N-acetylneuraminic acid; GalNAc = N-acetylgalactosamine; Gal = galactose.
We predicted that glycopeptides, which are O-glycans bearing peptide fragments consisting of several AA residues, remain undigested after proteolytic digestion of GMP due to steric hindrance by glycans, and that glycopeptide molecular weights are more than 1,000 Da. Therefore, ultrafiltration membranes with MWCO 1,000 were used to enrich glycopeptides. The lyophilized powder of retentate obtained by ultra- and diafiltration, which we named SGC, contained 32.3% Neu5Ac, 11.3% GalNAc, and 10.2% Gal. These saccharides were present in O-glycans attached to GMP, indicating that GMP glycan chains became approxi-
mately 4- to 5-fold concentrated. The SGC prepared in this study is an O-glycan-rich ingredient derived from bovine milk. We used G-WPC as the starting material because of its high GMP content (80% of the protein). In contrast, cheese whey-derived WPC or whey protein isolates contain 20 to 25% GMP (Wang and Lucey, 2003). In preliminary experiments using cheese whey-derived WPC or whey protein isolates, Neu5Ac was successfully concentrated by more than 5-fold, similar to when G-WPC was used (data not shown). We examined the enzymatic hydrolysis of G-WPC for some proteases used as food additives. The G-WPC hydrolysates were evaluated by SEC as the peaks shifted to low molecular weight regions. As a result, the combination of Alcalase and Flavorzyme was most effective for the hydrolysis of G-WPC (data not shown). Therefore, we used a combination of Alcalase and Flavorzyme. It has been reported that these 2 enzymes can efficiently hydrolyze milk proteins (Jaiswal et al., 2015; Ghosh et al., 2017). Our results also revealed that these enzymes completely hydrolyzed α-LA and β-LG as well as the GMP contained in G-WPC. Alcalase has endoprotease activity (Lapeña et al., 2018) and Flavorzyme has both endoprotease and exopeptidase activities (Ghosh et al., 2017). The combination of these 2 proteases appears to be suitable for the hydrolysis of GMP and whey proteins.

Seko et al. (1997) reported that sialylglycopeptide, a complex bi-antennary N-glycan bearing a hexa-peptide fragment, is present in hen egg yolk. Liu et al. (2017) further developed an improved method for isolating sialylglycopeptide from commercially available egg yolk powder by means of ethanol extraction followed by active carbon/celite column chromatography and SEC, which yielded 1.82 g of sialylglycopeptide from 2.27 kg of egg yolk powder. This method was developed for the purpose of synthesizing N-glycans needed in the field of glycobiology and analytical chemistry but not for the preparation of food ingredients. Sialylglycopeptide prepared in this study was obtained without the use of organic solvents or chromatography techniques. To our

Table 4. Release of N-acetylneuraminic acid (Neu5Ac) and galacto-N-biose (GNB) from sialylglycopeptide concentrate (SGC) by treatment with Bifidobacterium bifidum cell extract

<table>
<thead>
<tr>
<th>Strain</th>
<th>Released saccharides (%)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Bif. bifidum JCM1254</td>
<td>Neu5Ac: 78.8</td>
<td>GNB: 39.7</td>
</tr>
<tr>
<td>Bif. bifidum JCM7004</td>
<td>Neu5Ac: 83.0</td>
<td>GNB: 21.0</td>
</tr>
<tr>
<td>Bif. bifidum SBT10550</td>
<td>Neu5Ac: 75.3</td>
<td>GNB: 52.3</td>
</tr>
<tr>
<td>Bif. bifidum SBT2359</td>
<td>Neu5Ac: 88.6</td>
<td>GNB: 40.0</td>
</tr>
<tr>
<td>Bif. bifidum ATCC29521T</td>
<td>Neu5Ac: 7.8</td>
<td>GNB: 0.0</td>
</tr>
</tbody>
</table>

1Proportion of Neu5Ac or GNB released from SGC.

Figure 5. Effect of sialylglycopeptide concentrate (SGC) and glycomacropeptide-containing whey protein concentrates (G-WPC) on the in vitro growth of Bifidobacterium longum ssp. longum and Bifidobacterium longum ssp. infantis in the presence of glucose (Glc). Bifidobacterium longum ssp. longum JCM1217T (a) and Bif. longum ssp. infantis JCM1222T (b) cultured in broth containing 0.1% Glc supplemented with 0.4% G-WPC or 0.4% SGC. Growth was monitored by measuring the optical density (OD) at 660 nm.
knowledge, the protocol described here is the first to report large-scale preparation of SGC from milk materials on the scale of hundreds of grams.

Human milk is a rich source of Neu5Ac (0.3–0.7 g/L in mature human milk) as oligosaccharides, glycoproteins, and glycolipids (Idota et al., 1994b; Wang et al., 2001), whereas mature bovine milk-based infant formula contains low Neu5Ac content (0–0.2 g/L; Wang et al., 2001; Spichtig et al., 2010). Therefore, increasing Neu5Ac content is important for making infant formula as similar as possible to human milk. To this end, GMP-enriched ingredients, such as G-WPC in this study, are a valuable Neu5Ac source for infant formula (Brück et al., 2006). However, the main component of GMP-enriched ingredients is protein (>80% wt/wt), not Neu5Ac (about 6% wt/wt), and thus supplemental GMP dosing of infant formula should be limited to avoid protein content excess and AA imbalance. Recently, protein content in infant formula was reduced to that in human milk (European Union, 2016). Although it is possible to add Neu5Ac but not increase protein content in infant formula by replacing GMP with casein or whey protein, there is concern regarding AA imbalances, as GMP has no aromatic AA. The SGC has higher Neu5Ac (4.8-fold) and lower protein (2-fold) levels than does G-WPC. Therefore, SGC is a more valuable Neu5Ac source for infant formula.

Several studies have demonstrated that GMP contains 5 different O-glycan chains (glycan types 1–5; Table 3; Saito and Itoh, 1992; Hernandez-Hernandez et al., 2010). We demonstrated that SGC contains these 5 glycan chains as well as additional glycan chains containing O-acetylated or O-diacetylated Neu5Ac (glycan types 6 and 7). The Neu5Ac is often modified with O-acetylation at the C-7 or C-9 positions, or both (Varki, 1992). O-Acetylated Neu5Ac is predominant on cell surface glycoconjugates (Krishna and Varki, 1997) and salivary mucin (Kawabata et al., 2000). In bovine milk, Neu5Ac linked to ganglioside GD3 is partly O-acetylated (Ren et al., 1992), but there have been no reports on GMP. The O-acetylated Neu5Ac is involved in specific cell recognition by influenza C (Herrler et al., 1985) and coronaviruses (Kreml et al., 1995). Thus, O-acetylated Neu5Ac in SGC may act as decoy receptors and protect against viral infection.

The molar ratio of Neu5Ac to GNB was approximately 1.7 for both G-WPC and SGC, suggesting that mono- and disialylglycan composition was almost identical between them. Indeed, relative quantification of O-glycan conjugated with 2-AB revealed that the molar ratios of monosialyl-GNB (linear and branched) and disialyl-GNB to total O-glycans derived from G-WPC or SGC were similar. These results suggest that glycans in G-WPC were recovered in SGC without altering glycan composition.

Glycomacropeptide has growth-promoting effects on Bifidobacterium species via O-linked glycosylation (O’Riordan et al., 2018). Therefore, we hypothesized that SGC would have stronger growth-promoting effects than those of GMP on Bifidobacterium species, as SGC glycan content was higher than that of G-WPC. Four Bif. bifidum strains (Bif. bifidum JCM1254, JCM7004, SBT10550, and SBT2359) grew better in SGC-supplemented medium than G-WPC-supplemented medium, whereas growth of Bif. bifidum ATCC29521T and the other Bifidobacterium species was not affected. Bifidobacterium bifidum is among the first colonizers of the infant gut, along with Bif. breve and Bif. infantis (Turroni et al., 2009, 2012). A peculiar property of Bif. bifidum is its ability to use mucin as a sole carbon source by hydrolyzing the glycosidic bonds of Gal, GalNAc, Fuc, and Neu5Ac in mucins (Turroni et al., 2010, 2011). In this study, cell extracts derived from strains capable of growth in SGC-medium released Neu5Ac and GNB from SGC, indicating that these strains possess neuraminidase and endo-α-N-acetylgalactosaminidase activity. Indeed, Bif. bifidum JCM1254 and JCM7004 both have neuraminidase and endo-α-N-acetylgalactosaminidase, whereas Bif. bifidum ATCC29521T does not have neuraminidase (Fujita et al., 2005; Kiyohara et al., 2011). Conversely, Bif. bifidum lacks the nan gene cluster involved in Neu5Ac metabolism (Turroni et al., 2010). Thus, we speculated that Bif. bifidum does not use the Neu5Ac released from SGC, but rather the released GNB. A recent study reported that Bif. breve harbors the nan gene cluster but lacks extracellular sialidase and that cross-feeding occurs between Neu5Ac-releasing Bif. bifidum and Neu5Ac-utilizing Bif. breve (Egan et al., 2014; Nishiyama et al., 2018). These findings suggest that SGC glycans may be used via cross-feeding between Neu5Ac-releasing Bif. bifidum and nan gene cluster-harboring bacteria, such as Bif. breve. Further studies are needed to fully elucidate this issue.

O’Riordan et al. (2018) demonstrated that GMP supplementation of glucose-containing medium promoted the growth of Bif. longum ssp. infantis. We also confirmed the growth-promoting effect of G-WPC or SGC supplementation of glucose-containing medium on Bif. longum ssp. infantis JCM1222T and Bif. longum ssp. longum JCM1217T. Furthermore, these effects were more potent in medium supplemented with SGC than with G-WPC, suggesting that GMP glycan chains play a critical role in growth. Moreover, as Bif. longum ssp. infantis JCM1222T and Bif. longum ssp. longum JCM1217T were unable to grow in SGC-medium without
glucose, it appears that SGC works as a growth factor but not as a carbon source. O’Riordan et al. (2018) also showed that exposure of *Bif. longum* ssp. *infantis* to GMP upregulates 2 genes encoding glycoside hydrolase from family 25 (GH25), which is involved in cell wall and peptidoglycan catabolism. We are interested in whether SGC, compared with GMP, more potently upregulates the expression of these GH25 genes. In addition, it is not clear in our study whether the growth-promoting effect of SGC can be attributed to glycans, because SGC also contains peptide chains to which glycans are bound. Further studies are needed to elucidate the molecular mechanisms underlying SGC activity.

The in vitro bifidogenic effect of intact GMP has been reported using pure cultures (Azuma et al., 1984; Janer et al., 2004); however, this effect was not supported by fermentation experiments with infant fecal slurry (Brück et al., 2002) or with administration tests of GMP-supplemented infant formulas for initially breast-fed infants (Brück et al., 2006). Whether GMP has bifidogenic activity in vivo remains controversial, and thus further in vivo studies are warranted to clarify the bifidogenic effects of SGC.

Glycans contained in GMP are functional materials involved in maintaining and improving health. In this study, we demonstrated that SGC prepared from GMP contains abundant *O*-glycans and has a bifidogenic activity. Moreover, the protocol for the preparation of SGC described herein is relatively simple and provides a high yield of glycan and can be used for large-scale preparation; nevertheless, several challenges remain for production on an industrial-plant scale.

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**REFERENCES**


