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Chemoenzymatic synthesis and application of a sialoglycopolymer with a chitosan backbone as a potent inhibitor of human influenza virus hemagglutination

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Abstract—Sialoglycopeptide (SGP) is referred as the glycopeptide in hen's egg yolk, which has an N-linked, complex-type, disialyl biantennary oligosaccharide with an α-(2→6)-sialyl *N*-acetyllactosamine residue. The residue is known as a binding ligand of type-A human influenza virus hemagglutinin. We describe herein a simple synthesis of a sialoglycopolymer with a chitosan backbone as a potent inhibitor of human influenza virus hemagglutination that makes use of the natural source ingredient, SGP, and the transglycosylation activity of *endo*-β-*N*-acetylglucosaminidase from *Mucor hiemalis* (Endo-M). Its inhibitiory activity for influenza virus hemagglutination is 40 times higher than that of SGP, and its competitive inhibition is determined to be over 300 times higher than that of fetuin. These results indicate that a sialoglycopolymer having a multivalent sialooligosaccharide could potentially be used for the prevention of influenza virus infection.

Keywords: Chitosan; Complex-type glycopeptide; Sialo-oligosaccharides; *Mucor hiemalis*; Transglycosylation; Endo-M; Influenza virus; Hemagglutination

1. Introduction

Influenza viral diseases, which have been known since ancient times, are highly contagious viral infections and are among the most severe respiratory illness known. Influenza viruses frequently mutate and rapidly become resistant to vaccines. The sialidase inhibitor, oseltamivir phosphate, has been developed as an antiviral drug and is widely utilized in the treatment of influenza.[1] However, this drug is effective only after the onset of infection in the host and is not suitable for prophylactic purposes. Moreover, a recent report has shown evidence of a virus resistant to this drug.[2] Thus, the prevention of influenza viral infection is still difficult, and the development of an effective therapy is required. Influenza viruses are known to infect host tissues by binding to sialoglycoconjugates on the cell surface through their hemagglutinins (HAs). Type-A human influenza virus, which has a strong pathogenicity, is known to infect host tissues by first binding to the α-(2→6)-sialyl *N*-acetyllactosamine [α-(2→6)-Neu5Ac-β-(1→4)-Gal-β-GlcNAc-] residue of a sugar chain on the cell surface through their HAs.[3] Recent studies showed that α-sialoside-containing polymers, which were prepared by chemical methods, were potent inhibitors of

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the human erythrocyte hemagglutination by influenza viruses on the basis of multivalent and cluster effects.[4–7] Thus an α-(2→6)-sialyl-*N*-acetyllactosamine-containing polymer is expected to have higher inhibition activity. But chemical synthesis of a sialyl *N*-acetyllactosamine residue is difficult, and the preparation of its polymer requires highly developed techniques.

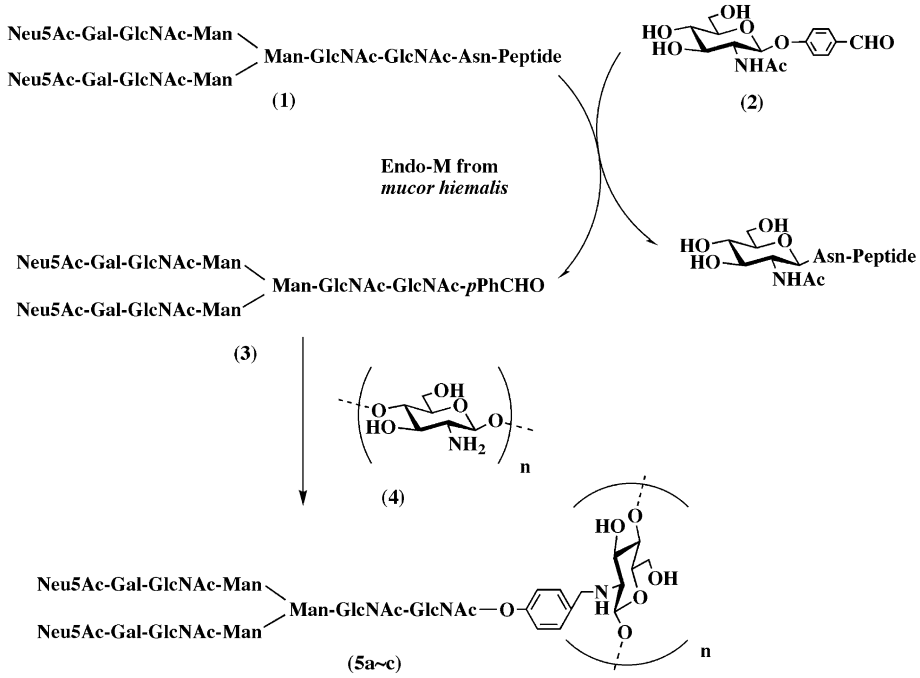
Recently, we developed a chemoenzymatic method for the synthesis of sialoglycoconjugates via the transglycosylation activity of *endo*-β-*N*-acetylglucosaminidase (EC 3.2.1.96). *endo*-β-*N*-Acetylglucosaminidase hydrolyzes an *N*,*N*-diacetylchitobiosyl residue in the N-linked oligosaccharides of glycoproteins and glycopeptides.[8] This enzyme from *Mucor hiemalis* (Endo-M) has an especially high transglycosylation activity and can transfer the oligosaccharides of glycopeptides to the GlcNAc moiety of the compound.[9] The sialoglycopeptide of hen's egg yolk (SGP) has an N-linked, complex-type disialyl biantennary glycan with an α-(2→6)-sialyl *N*-acetyllactosamine residue[10] that amounts to ~10 mg per one hen's egg, and its isolation is comparatively easy. We used SGP as an oligosaccharide donor for the transglycosylation reaction of *endo*-β-*N*-acetylglucosaminidase when we synthesized the sialoglycoconjugates by a chemoenzymatic method.[11] Recently, Bovin et al. reported the synthesis of a polyacrylamide-based sialoglycopolymer using SGP,[12] and this polymer effectively inhibited influenza virus infection.[13] However, we wonder about safety for the host cells using such a polyacrylamide. Safe biocompatible materials must be used to develop such a potent inhibitor of the influenza virus infection. Thus the use of chitosan as polymer base came to our attention. Chitosan is a polysaccharide formed from a repeating β-(1→4)-D-glucosamine residue, which has an amino moiety that can be used for selective condensation with an aldehyde group. It is known as a relatively nontoxic and biodegradable material. For these reasons we opted to use chitosan as the backbone of a sialoglycopolymer.

We report herein the novel and simplified synthesis of α-(2→6)-sialyl-*N*-acetyllactosamine-containing glycopolymers with a chitosan backbone as a potent influenza virus hemagglutination inhibitor using the transglycosylation reaction of endoglycosidase, followed by chemical modification.

2. Results and discussion

2.1. Enzymatic transfer of N-linked sialo-oligosaccharides from the sialoglycopeptide (SGP)

We attempted to transfer sialo-oligosaccharide from SGP of hen's egg yolk **1**[10] to *p*-formylphenyl 2-acetamido-2-deoxy-β-D-glucopyranoside (*p*-CHOPh-GlcNAc, **2**), which was synthesized according to the procedure reported by Roy et al.,[14] using the transglycosylation activity of Endo-M. The reaction mixture was composed of SGP as an oligosaccharide donor, *p*-CHOPh-GlcNAc as an oligosaccharide acceptor, and Endo-M in a potassium phosphate buffer (pH 6.5) (see Scheme 1). After incubation for 12 h, the reactant was analyzed by HPLC using



Scheme 1. (1) SGP, (2) *p*-formylphenyl-*N*-acetylglucosaminide, (3) transglycosylation product of Endo-M (*p*-formylphenyl sialo-oligosaccharide), (4) sialoglycopolymer conjugated with chitosan (reacted with different quantity of donor, (a) 0.3, (b) 0.4, (c) 0.5).

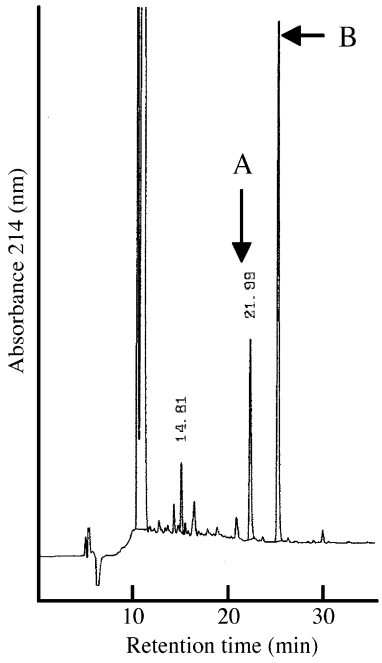


Figure 1. HPLC profile of the transglycosylation reaction mixture of Endo-M; HPLC elution conditions; column: Cosmosil AR-II (10 × 250 mm) linear increase of 0.05% trifluoroacetic acid–acetonitrile concentration from 0% to 15% in 0.05% trifluoroacetic acid–water until 30 min at a flow rate of 2.5 mL/min. (A) Transglycosylation product (*p*-formylphenyl sialooligosaccharide). (B) Oligosaccharide acceptor (*p*-formylphenyl *N*-acetylglucosaminide).

an ODS column and detected by UV absorbance (214 and 280 nm). As shown in Figure 1, a new single peak was observed at 22 min. The new peak fraction was collected, desalted over a Sephadex G-25 column and lyophilized. The product was determined to be *p*-formylphenyl sialo-oligosaccharide **3** by ^{1}H NMR spectroscopy; yield ~50%.

2.2. Synthesis of sialoglycopolymer with a chitosan backbone

This synthetic reaction of the sialoglycopolymer was carried out as per Shigemasa's chitosan N-alkylation,[15] which is a very convenient method for the modification of chitosan. The reaction mixture was composed of chitosan **4**, *p*-formylphenyl sialo-oligosaccharide **3** (0.3, 0.4, and 0.5 equiv), and the reducing agent, $NaBH_3CN$. The mixture was stirred for 2 days at room temperature and then filtered by centrifuge using a membrane. The degree of substitution (DS) with the sialooligosaccharide derivatives was calculated by the increase of the weight from that of the chitosan. DS and yields of each reaction were 0.21 (DS), 70% yield as a **5a** (0.3 equiv of **3**), 0.28, 70% as a **5b** (0.4 equiv of **3**), and 0.33, 67% as a **5c** (0.5 equiv of **3**) (see Table 1). Compound **5c** was also determined by ^{1}H NMR spectroscopy. Because of the poor solubility of chitosan in water, the water solubilities of these polymers were examined, and it was found that **5c** was soluble. Therefore, **5c** was used for the hemagglutination inhibition (HI) assay.

Table 1. Degree of substitution and yield of N-alkylation of chitosan **4** with *p*-formylphenyl sialooligosaccharide **3**

3 (equiv)	DS[a]	Yield[a] (%)
0.3	0.21	70
0.4	0.28	70
0.5	0.33	67

[a] DS and yield were determined by weight recovery.

2.3. Hemagglutination inhibition (HI) assay of the sialoglycopolymer using A-type human influenza viruses

We estimated the HI activity of sialoglycopolymer **5c**, using two A-type human influenza viruses (A/Memphis/1/71 and A/new Caredonia/20/99). The results are shown in Table 2. Contrary to anticipation, SGP scarcely inhibited the hemagglutination by influenza virus. But sialoglycopolymer **5c** showed quite a high HI activity that is four times higher than that of fetuin at 10-fold higher concentration. When we estimated the inhibition activity after removal of sialic acid from sialoglycopolymer using sialidase, HI activity was completely lost.

2.4. Competitive inhibition assay by sialoglycopolymer

We also carried out a competitive inhibition assay using A/new Caredonia/20/99 influenza virus. The results are shown in Table 3 and Figure 2. According to these results, the IC_{50} value of sialoglycopolymer **5c** is 12.8 ng/mL. It was found to be much lower than those of fetuin (4000 ng/mL) and SGP (52,000 ng/mL).

Table 2. Hemagglutination inhibition of human influenza virus by sialoglycopolymer

Compounds (1 mg/mL)	Hemagglutinating inhibition (HI)	
	A/New Caledonia/20/99 (H3N2)	A/Memphis/1/71 (H3N2)
SGP	<2	<2
Sialoglycopolymer	2048	512
Sialoglycopolymer (sialidase treated)		<2
Fetuin (10 mg/mL)	512	128

Table 3. IC_{50} values of sialoglycopolymer

Compounds	IC_{50} (ng/ML) Influenza virus A/New Caledonia/20/99 (H3N2)
SGP	52,000
Sialoglycopolymer	12.8
Fetuin	4000

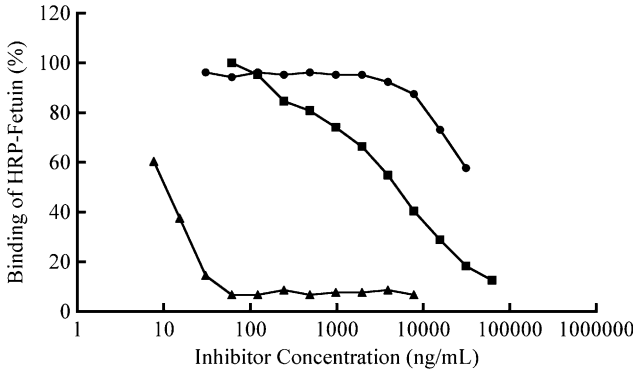


Figure 2. Competitive inhibition of sialoglycopolymer for binding to HRP-fetuin by A/New Caredonia/20/99 influenza virus (●) SGP, (■) fetuin, (▲) sialoglycopolymer.

In conclusion, we have succeeded in the development of a simple synthesis of a complex-type sialo-oligosaccharide epitope-conjugating polymer as an inhibitor of human influenza virus hemagglutination. The hemagglutination inhibition of human influenza virus is quite strong, and this compound has the potential for development into a drug for the prophylaxis of human influenza viral infections.

3. Experimental

3.1. Materials

SGP was prepared from hen's egg yolk as described previously.[10] Chitosan (Cp = 100) was purchased from Wako Pure Chemicals Industry. *endo*-β-*N*-Acetylglucosaminidase from *M. hiemalis* (Endo-M) expressed in *Candida boidinii* was prepared according to the previous paper[16] [1 unit (U) of the enzyme was defined as the amount of protein that yields 1 μmol of dansyl (DNS)-Asn-GlcNAc from DNS-Asn-$(GlcNAc)_2$-$(Mannose)_6$ per min at 37 °C at pH 6.0]. Type-A human influenza viruses tested were propagated in the allantoic sac of 10-day-old embryonated eggs and were purified as described previously.[17]

3.2. Analytical methods

High-performance liquid chromatography (HPLC) was performed using a Hitachi L-7100 chromatograph with an L-7420 UV-detector. ^{1}H NMR spectra were recorded on a Bruker PMX-500 (500 MHz) spectrometer using D_2O with TSP (sodium 3-(trimethylsilyl)propionate-d_4) as the internal standard. Matrix-assisted laser-desorption ionization time-of-flight mass spectra (MALDI-TOF-MS) were recorded on a Shimadzu/Kratos Kompact MALDI I mass spectrometer equipped with a SPARC workstation. Each sample was mixed with triethylamine (TEA) matrix on a target. The ion accelerating voltage was 8.0 kV, and the primary beam for the bombardment was 6.0 keV of xenon. Thin-layer chromatography (TLC) of the reaction mixtures was carried out using E. Merck silica gel aluminum plates no. 60F-254.

3.3. Transfer of oligosaccharide from SGP to *p*-formylphenyl 2-acetamido-2-deoxy-β-D-glucopyranoside by *M. hiemalis endo*-β-*N*-acetylglucosaminidase (Endo-M) (3)

SGP **1** (28.6 mg, final 25 mM) and *p*-formylphenyl 2-acetamido-2-deoxy-β-D-glucopyranoside **2** (13 mg, final 100 mM), which was prepared according to the method of Roy et al.,[14] were dissolved in 100 mM potassium phosphate buffer (380 μL, pH 6.0). To the mixture was added the Endo-M preparation (60 mU, 150 mU/mL), and the mixture was incubated at 37 °C for 12 h. The reaction was stopped after boiling for 3 min, and the transglycosylation product was separated and isolated by HPLC (10 × 250 mm, Cosmosil AR-II Nacalai Tesque: eluted by linear gradient of acetonitrile 0 to 25% containing 0.05% TFA solution), followed by Sephadex LH-20 gel filtration (10 × 500 mm: 5 g, water), and finally lyophilization gave **3** as an amorphous solid (11.6 mg, yield 50%). ^{1}H NMR (D_2O) of compound **3**: δ 9.71 (s, 1H, CHO), 7.83 (d, 2H, $J_{2,3} = J_{6,5} = 8.77$ Hz, H-2 and H-6 of Ph), 7.09 (d, 2H, $J_{3,2} = J_{5,6} = 8.78$ Hz, H-3 and H-5 of Ph), 5.20 (d, 1H, $J_{1,2}$ 8.45 Hz H-1 of GlcNAc-1), 5.02 (s, 1H, H-1 of Man-4), 4.84 (s, 1H, H-1 of Man-4′), 4.53 (d, 1H, $J_{1,2}$ 7.96 Hz, H-1 of GlcNAc-2), 4.49 (2d, 2H, $J_{1,2}$ 7.76 Hz, H-1 of GlcNAc-5,5′), 4.15 (s, 1H, H-2 of Man-3′), 4.09 (d, 1H, $J_{2,3}$ 2.29 Hz, H-2 of Man-4), 4.00 (d, 1H, $J_{2,3}$ 2.02 Hz, H-2 of Man-4′), 2.55 (2dd, 2H, H-3eq of Neu5Ac-7,7′), 1.89, 1.91 × 2, 1.95 × 2, 1.99 (6s, 18H, AcN), and 1.60 (2t, 2H, $J_{gem} = J_{3ax,4} = 12.19$ Hz H-3ax of Neu5Ac-7,7′). MALDI-TOF-MS of compound **3**: calcd for $C_{91}H_{142}N_6O_{63}$ (2328.10); found a quadruply charged ion as a broad peak enveloping the corresponding isotopic distribution centered at *m*/z 2205 and 2361.

3.4. Condensation of transglycosylation product (*p*-formylphenyl sialo-oligosaccharide) with chitosan (5c)

Compound **3** (4.2 mg, 1.8 μmol) and chitosan **4** (0.42 mg, 2.6 μmol, 2 equiv/hexosamine residue) were dissolved in water (4.5 mL) containing HOAc (5 mg) and MeOH (0.5 mL). To the mixture was added $NaBH_3CN$ (0.19 mg, 3 μmol), and the mixture was stirred at room temperature for 24 h. The product mixture was filtered by centrifuge using a membrane cutoff of MW 30,000 at 8000 rpm, and the product was then lyophilized to give **5** as an amorphous mass (3.3 mg, 67% yield, DS 0.33). ^{1}H NMR (D_2O) of compound **5**: δ 7.33 (d, 2H, $J_{2,3} = J_{6,5} = 6.22$ Hz, H-2 and H-6 of

Ph), 7.09 (d, 2H, $J_{3,2} = J_{5,6} = 8.31$ Hz, H-3 and H-5 of Ph), 5.14 (d, 1H, H-1 of GlcNAc-1), 4.96 (s, 1H, H-1 of Man-4), 4.60 (d, 1H, H-1 of GlcNAc-2), 4.46 (2d, 2H, H-1 of GlcNAc-5,5′), 4.27 (s, 1H, H-2 of Man-3′), 4.13 (d, 1H, $J_{2,3}$ 2.29 Hz, H-2 of Man-4), 4.02 (d, 1H, $J_{2,3}$ 2.02 Hz, H-2 of Man-4′), 2.68 (2dd, 2H, H-3eq of Neu5Ac-7,7′), 2.03, 2.04, 2.05, 2.06, 2.07, 2.08, 2.10 (7s, 21H, AcN) and 1.73 (2t, 2H, $J_{gem} = J_{3ax,4} =$ 12.17 Hz, H-3ax of Neu5Ac-7,7′).

3.5. Hemagglutination inhibition (HI) assay

Sialoglycopolymers (1 mg/mL) were diluted serially with 25 μL of phosphate-buffered saline (PBS) on a microtiter plate (U-bottom 353911, Becton–Dickinson, NJ, USA) as previously described.[18] A suspension (25 μL) of influenza virus containing 4 U of hemagglutinin was added to each well. After incubation at 4 °C for 1 h, 50 μL of a 0.5% (v/v) guinea pig erythrocyte suspension was added to each well. The hemagglutination inhibition (HI) titer was defined as the maximum dilution of each sialoglycopolymer that caused complete inhibition of hemagglutination. As a control, bovine fetuin (1.0 mg/mL; Sigma–Aldrich Corporation, St. Louis, USA) was examined in the same manner.

3.6. Preparation of horseradish peroxidase (HRP)-labeled bovine fetuin

Periodate oxidation of HRP (Sigma–Aldrich Corporation, St. Louis, MO USA) was performed as described previously.[19] The activated HRP (10 mg), lactose (7.2 mg), and bovine fetuin (8 mg) were dissolved in 2 mL of 0.2 M carbonate buffer (pH 9.5) and incubated at room temperature for 2 h. The Schiff's bases were formed by adding 0.1 mL of 100 mM $NaBH_4$, with further incubation at 4 °C for 1 h. The HRP-labeled bovine fetuin was then purified by Superose-6 column chromatography (Amersham Biosciences, NJ, USA).

3.7. Competitive inhibition assay of sialoglycopolymers

Sialoglycopolymers carrying complex-type disialo-oligosaccharides were diluted serially with phosphate-buffered saline (PBS) containing 0.01% polyoxyethylene (20) sorbitan monolaurate (Wako Pure Chemical Industries Ltd, Japan). Each sialoglycopolymer serial dilution (25 μL) and 50 μL of HRP-labeled bovine fetuin (47 nM) solution were added to the wells coated with influenza virus A/New Caledonia/20/99 (H1N1) (300 ng as protein) as previously described.[20] After incubation for 3 h at 4 °C, the plate was washed, and the amount of bound HRP-labeled bovine fetuin was determined by measuring the absorbance at 492 nm with *O*-phenylenediamine as the substrate.[18] The concentration causing 50% inhibition of the binding of HRP-labeled bovine fetuin was determined by plotting the percentage inhibition against the concentration of each sialoglycopolymer.

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