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CHEMOENZYMIC PREPARATION OF A GLYCOCONJUGATE POLYMER HAVING A SIALYLOLIGOSACCHARIDE: Neu5Acα(2→3)Galβ(1→4)GlcNAc

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SUMMARY: Water-soluble polyacrylamide having 3'-sialyl *N*-acetyl-lactosamine [Neu5Acα(2→3)Galβ(1→4)GlcNAc] was enzymatically prepared by stepwise sugar-elongation on a water-soluble GlcNAc-bearing polyacrylamide. It was demonstrated that the flexible GlcNAc branches of the polymer chains allow quantitative galactosylation with bovine galactosyl transferase and partial sialylation by *Trypanosoma cruzi trans*-sialidase. Unsialylated *N*-acetyl-lactosamine side chains can be removed with β-D-galactosidase and *N*-acetyl-β-D-glucosaminidase to afford the targeted polymer containing 3'-sialyl *N*-acetyl-lactosamine.

Synthetic glycopolymers are actively investigated as biomimetic, diagnostic, and therapeutic ligands that can manifest amplified specific carbohydrate-protein interactions based on the glycoside clustering effects (1). One of the most effective methods to increase the valency of the target sugars is to polymerize simple glycosides by facile chemical reactions (2). Water-soluble polyacrylamide derivatives and stable polymer gels or solid matrices bearing pendant glycoside chains are excellent polyvalent ligands which interact specifically with proteins (3-7), intact hepatocytes (8-10), and influenza virus (11-13). Successful chemical synthesis of the polymer carrying a complex oligosaccharide sequence such as tumour-associated Lewis x (Le^x) trisaccharide has been recently reported (14). Although enzymatic oligosaccharide-syntheses on polymer supports have been previously reported (15-17), there is no practical and efficient method for the enzyme-assisted preparation of biologically significant glycopolymers having finely defined chemical structures.

Enzymatic syntheses of carbohydrates are gaining importance as an excellent alternative method for preparation of a variety of oligosaccharides because of their stereo- and regioselectivity (18-21). Some biologically significant sialo-oligosaccharides have been

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successfully synthesized with glycosyltransferases. When appropriate, combinations of chemical and enzymatic methods have been used also (22-26). We reasoned that enzyme-mediated reactions can also be applied to the syntheses of specific glycopolymers, if the polymers possess suitable acceptor sugars on the flexible spacer-arms (10), rendering them accessible to the enzymes.

We demonstrate here the feasibility of the enzymatic modifications of synthetic glycopolymers by synthesis of a novel polymer having pendant 3'-sialyl *N*-acetyl-lactosamine [Neu5Acα(2→3)Galβ(1→4)GlcNAc] by a combination of a readily available bovine milk β-1,4-galactosyltransferase (27) and a unique *trans*-sialidase from *Trypanosoma cruzi* (28-32).

MATERIALS AND METHODS

Acceptor GlcNAc-polymer. A polyacrylamide containing GlcNAc residues (**1**), was prepared from *n*-pentenyl 2-acetamido-2-deoxy-β-D-glucopyranoside (10) and acrylamide by radical copolymerization promoted with ammonium persulfate and *N,N,N',N'*-tetramethylethylenediamine according to the previous report (33). Average molecular weight was 190,000 and the GlcNAc content of the polymer was 34.6 wt% (Figure 1).

Enzymes and substrates. Bovine milk galactosyl transferase (GalT) (EC 2.4.1.90), uridine 5'-diphosphate galactose (UDP-Gal), and *Aspergillus niger* β-galactosisdase (EC 3.2.1.23) were obtained from Sigma Chem. Co. Beef kidney *N*-acetyl-β-D-glucosaminidase (EC 3.2.1.30) was purchased from Beohringer Mannheim GmbH. *Trypanosoma cruzi trans*-sialidase (TcTs) is a gift from Dr. V. Nussenzweig of New York University Medical Center. One TcTs unit defined as the amount of enzyme that yield 1 nmol of sialylated radiolabeled lactose during 30 min incubation in 50 μL of 50 mM HEPES, pH 7.0, containing 50 nmol of unlabeled sialyllactose and 0.2% BSA at room temperature. *p*-Nitrophenyl 5-acetamido-3,5-dideoxy-α-D-glycero-D-galacto-2-nonulopyranosidonic acid (PNP-Neu5Ac) was prepared by the method of Eschenfelder et al. (34).

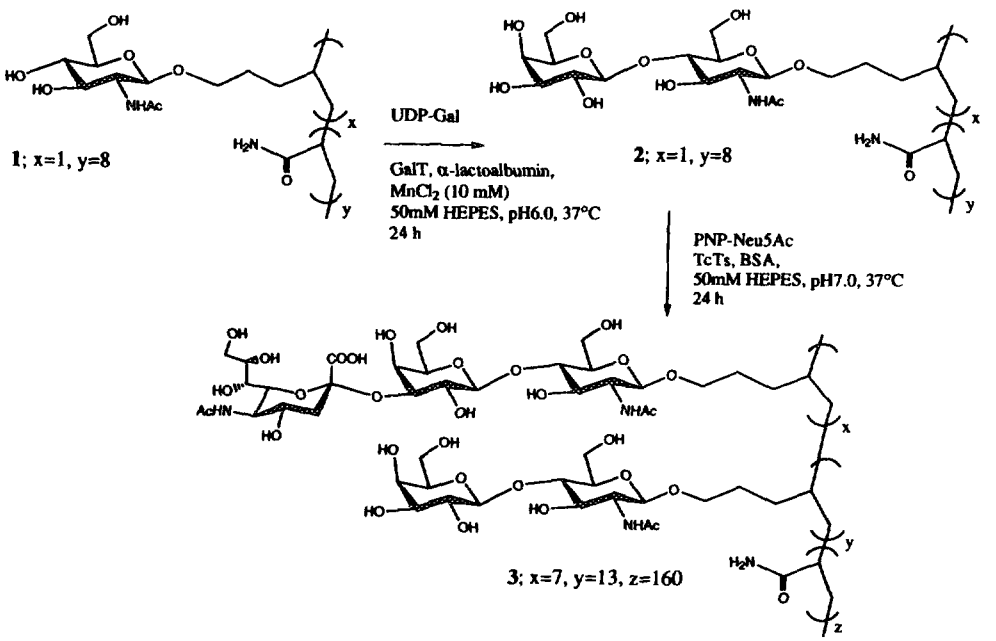

Figure 1. Enzymatic Sugar-Elongation of A Synthetic Glycopolymer.

Preparation of polyacrylamide having N-acetyl-lactosamine (2). Acceptor glycopolymer **1**, (10.0 mg, 12 μmol GlcNAc), UDP-Gal (9.63 mg, 17 μmol), α-lactalbumin (100 μg), and GalT (1.0 unit) were incubated in 50 mM HEPES (pH 6.0) for 24 h at 37℃. The mixture was directly purified by chromatography on a Sephadex G-50 column (2.5 x 100 cm) eluted with 50 mM CH_3COONH_4. The polymer fractions were collected and lyophilized to give a glycopolymer having *N*-acetyllactosamine, **2** (11.8 mg) in quantitative yield.

Partial 3'-sialylation and the subsequent trimming of 2. TcTs (48 unit) was added to a mixture of **2** (10.0 mg, 8 μmol of *N*-acetyl-lactosamine unit), PNP-Neu5Ac (3.6-18 mg, 8.0-40 μmol), BSA (4 μg) in 50 mM HEPES buffer (pH 7.0, 300 μL). After incubation at 37℃ for 24-72 h, the reaction mixture was chromatographed on a Sephadex G-50 column (2.5 x 100 cm) eluted with 50 mM CH_3COONH_4, and the fractions at the void volume containing glycopolymer were collected and lyophilized to yield the sialylated product, **3**. The content of the sialic acid was determined by ^{1}H-NMR and by an enzyme-assisted method based on the reactions of sialidase and aldolase (35). Since the trans-sialylation of the N-acetyl-lactosamine groups was no complete, the unsialylated *N*-acetyl-lactosamine residues on the polymer was trimmed as follows. A mixture of the intermediate **3** (10.3 mg) and β-galactosidase (0.5 unit) in 50 mM citrate-phosphate buffer (pH 4.4, 500 μL) was incubated at 25℃ for 24 h. The mixture was chromatographed on a Sephadex G-50 column as described above. The polymer-containing fractions were collected, lyophilized, and dissolved in 50 mM citrate buffer (pH 4.4, 500 μL). *N*-Acetyl-β-D-glucosaminidase (2.5 unit) was added to the solution and a mixture was incubated at 37℃ for 24 h. Finally, the mixture was subjected to the Sephadex G-50 column chromatography, eluted with 50 mM CH_3COONH_4. The polymer fractions were pooled and lyophilized to give the derivative **4** (9.0 mg) (Figure 2).

NMR spectroscopy. ^{1}H-NMR spectra of polymers were recorded on Bruker AMX-300 spectrometer. Prior to the NMR measurement, the samples were dissolved in D_2O and lyophilized to remove the labile protons. This step was repeated twice before finally dissolving in 500 μL of 99.998% D_2O. The sample concentration was about 20 mg/mL.

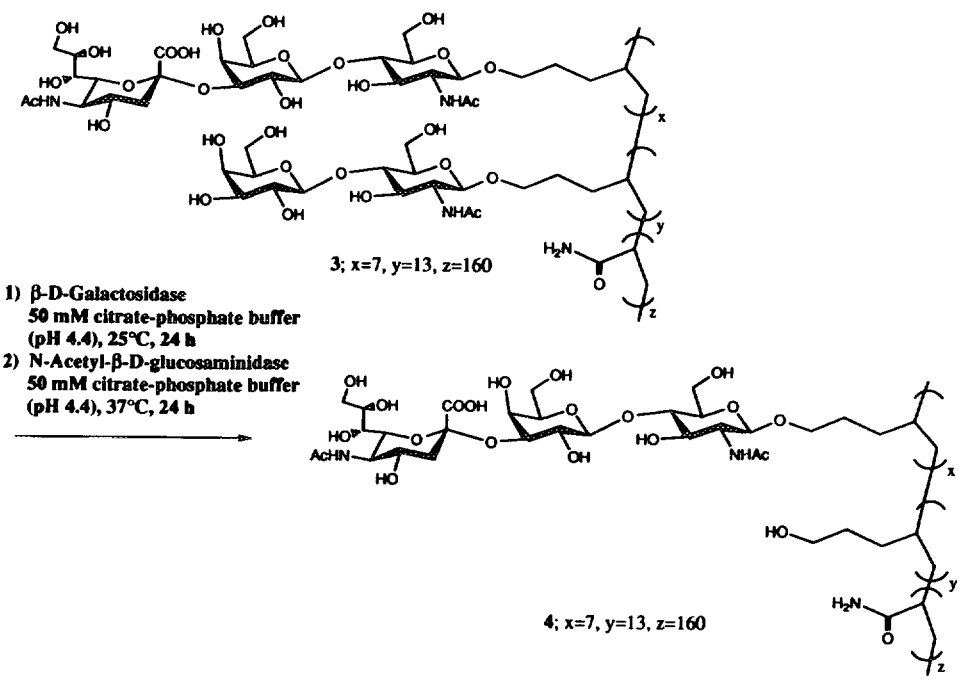

Figure 2. Trimming by Glycosidases.

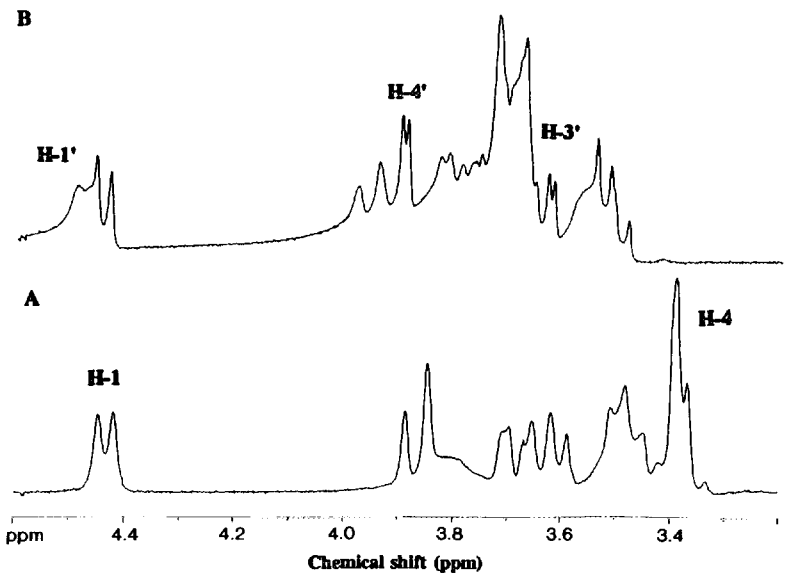

Figure 3. ^{1}H-NMR spectra of (A) acceptor polymer **1** and (B) *N*-acetyllactosamine polymer **2** in D_2O.

RESULTS AND DISCUSSION

Galactosylation. β-1,4-Galactosyltransferase transfers the D-galactosyl group from UDP-Gal to the 4-OH of the terminal β-D-GlcNAc unit resulting in the synthesis of *N*-acetyl-lactosamine terminal (36). The same reaction was also used in formation of human type 2 determinant trisaccharide (37), and chemically modified GlcNAc derivatives can be used as acceptors (38).

Indeed, galactosylation of the polymer substrate **1** proceeded smoothly in the presence of the small excess of UDP-Gal (1.42 molar equivalent to the acceptor GlcNAc residues) and gave a quantitative yield of *N*-acetyllactosamine polymer **2**. ^{1}H-NMR spectrum of the product (Figure 3B) clearly shows the complete substitution of the GlcNAc residues on the polymeric acceptor **1**. Two anomeric protons at 4.43 ppm (H-1) and 4.46 ppm (H-1') were observed concomitant with disappearance of a signal at 3.40 ppm attributable to the H-4 of unsubstituted GlcNAc residue. these data are in good agreement with the chemically synthesized LacNAc-polymer (10). Zehavi et al. (17) had previously showed only 27% of galactose-transfer reaction using a polyvinyl alcohol derivative having 2-nitrobenzyl glycoside of glucose as an acceptor substrate. Our data suggest that the longer and the more flexible spacer-arm structure derived from *n*-pentenyl glycosides described here may be the key to the excellent glycosylation yield.

Trans-sialylation and trimming. Some glycosidases can catalyze transglycosylation effectively. The *trans*-sialylation by TcTs has been recently demonstrated and the reaction results in the formation of a unique α2→3 linkage between the terminal Neu5Ac and Gal (28-32). Since TcTs can use as donor substrates simple sialosides such as p-nitrophenyl and 4-methylumbelliferyl derivatives which can be easily prepared, this enzyme offers a powerful

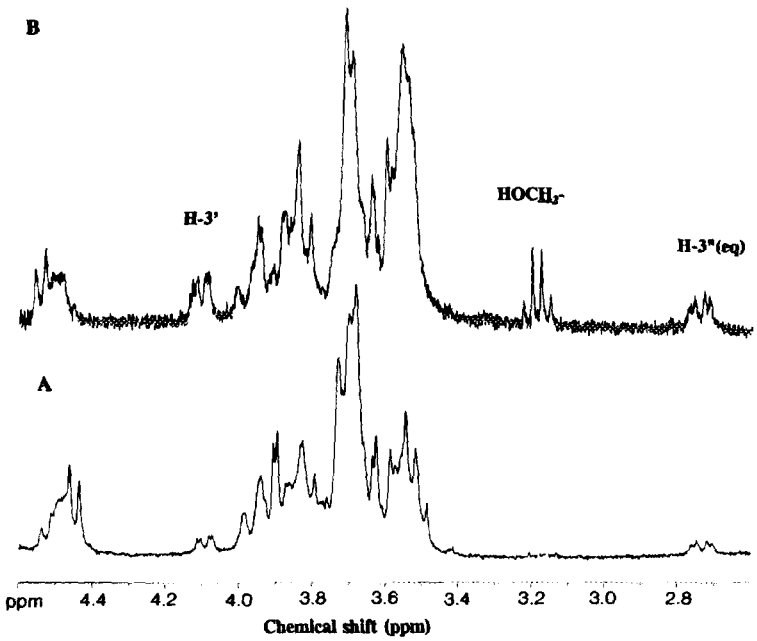

Figure 4. ^{1}H-NMR spectra of (A) partially sialylated polymer **3** and (B) sialyl *N*-acetyllactosamine polymer **4** in D_2O.

alternative for the syntheses of the 3'-sialyloligosaccharides to the reaction with CMP-Neu5Ac and sialyltransferases. When an equi-molar ratio of PNP-Neu5Ac (8 μmol) with the acceptor **2** (8 μmol of GlcNAc residues) was used for the reaction of TcTs, sialic acid content of the product **3** showed approximately 35% of the theoretical maximum and no significant increase was observed by increasing the concentration of the donor substrate. As shown in the ^{1}H-NMR spectrum of the compound **3** (Figure 4A), characteristic signals of α2→3 sialosides are observed in the galactosyl protons at 4.07 ppm (dd, H-3') in addition to the signal due to α-sialosides at 2.72 ppm (dd, H-3"eq). However, the unsialylated H-3' signal at 3.63 ppm was also detected.

Using the selective hydrolytic activities of *exo*-type β-D-galactosidase and *N*-acetyl-β-D-glucosaminidase, we were able to prepare a defined glycopolymer having only α2,3-sialyl-LacNAc residues. The complete removal of the unsialylated *N*-acetyl-lactosamine was confirmed from the integration ratios of H-3' at 4.07 ppm and anomeric protons at 4.48 and 4.52 ppm, respectively. Moreover, ^{1}H-NMR spectrum of compound **4** exhibited a new signal at 3.17 ppm due to the methylene protons of the terminal methylol groups (Figure 4B).

The present results demonstrate that chemically synthesized soluble glycopolymers can be modified with galactosyltransferase and *trans*-sialydase. The water-soluble polymers with larger molecular weights are advantageous in one regard that product purification is simple gel filtration and thus eliminate or reduce laborious workup and chromatography required for the chemical syntheses of polymerizable glycosides. Although applicability of this procedure to sugar transferring activity of other enzymes have not been examined yet, the enzyme-assisted strategy offers a valuable alternative approach to the syntheses of glycoconjugate polymers as potential ligands or inhibitors in biochemical and medical fields.

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