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0273-2289/97/6801-02—0041$12.00

Synthesis of *N*-Acetylneuraminyl-α2,3(6)Lactose-Malate Dehydrogenase Conjugate for Detecting Sialic Acid Terminal Groups on Glycoproteins Via Homogeneous Lectin-Based Enzyme-Linked Binding Assay

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Received January 15, 1997; Accepted April 10, 1997

ABSTRACT

An *N*-acetylneuraminyl-α2,3(6)lactose-malate dehydrogenase (MDH-Lac-Neu5Ac) conjugate is prepared via an isothiocyanate conjugation method using a *p*-aminophenethylamino derivative of sialyllactose. The newly synthesized conjugate can be utilized as a reagent in a novel homogeneous lectin-based, enzyme-linked, competitive binding assay *(1–3)* for probing the specific carbohydrate structure and content of intact glycoproteins. The enzymatic activity of the MDH-Lac-Neu5Ac conjugate is shown to be significantly inhibited (35%) by sialic acid-binding lectin, *Limax flavus* agglutinin (LFA), and this inhibition is reversed by mucin, a glycoprotein possessing sialic acid terminals. The asialo form of mucin, however, binds weakly to LFA, yielding no substantial increase in the MDH-Lac-Neu5Ac activity at comparable glycoprotein concentrations. Use of the newly synthesized conjugate in conjunction with LFA or other lectins capable of binding sialic acid may provide a rapid and convenient way to detect the presence and relative amount of sialic acid terminal groups within intact glycoprotein structures.

Index Entries: Sialic acid; malate dehydrogenase conjugate; homogeneous binding assay; glycoproteins.

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INTRODUCTION

Sialic acids are widely distributed in nature, generally at the terminal position of oligosaccharide units of glycoproteins, gangliosides, and milk oligosaccharides *(4,5)*. Among 24 different N- and O-acylated derivatives of neuraminic acid, *N*-acetylneuraminic acid (Neu5Ac) is the most commonly occurring sialic acid structure. One of the most important roles of sialic acids is their antirecognition effect. First discovered by Ashwell and Morell *(6)*, sialic acids mask the D-galactosyl residues of various serum glycoproteins, thereby protecting these molecules from being cleared from the body. Sialic acids have also been found to mask tumor antigens *(7–10)*, and can serve as the binding sites of influenza C virus, mycoplasma, hormones, and toxins *(11–14)*. The repulsive, electrostatic forces of sialic acids also appear to contribute to the rigidity of cell surfaces *(15)*, as well as influence or stabilize the conformation of proteins, including enzymes and glycolipids *(16)*.

The detection of sialic acids is challenging, since they can occur in several forms, including *N*-acetylneuraminic acid, *N*-glycolylneuraminic acid, *N,O*-diacetylneuraminic acid, and occasionally, *N*-acetyl-*O*-diacetylneuraminic acid. Sialic acids can also be present in free form or covalently bound to glycoproteins. A variety of analytical methods have been developed to quantitate sialic acids, with most being either spectrophotometric or fluorometric techniques. Warren's thiobarbituric acid (TBA) assay *(17)* is the most widely used method. Other colorimetric methods, such as acid-ninhydrin *(18)*, periodate-resorcinol *(19)*, alkali-Ehrlich *(20)*, and diphenyl-amine procedures *(21)*, are generally not as sensitive as the TBA assay. The TBA assay, however, does have its disadvantages. The assay process is long, requiring heating of the reaction mixture and subsequent extraction of the chromophore. Further, it only determines free sialic acid, necessitating an extra hydrolysis step to detect sialic acids within given glycoprotein structures. The most severe drawback of the TBA assay (as well as other colorimetric assays) is lack of specificity. Different forms of sialic acid, such as *N*-acetylneuraminic acid, *N*-glycolylneuraminic acid, *N,O*-diacetylneuraminic acid, and so on, all react with the assay reagents and produce chromophores that have very similar absorbance maxima, but different molar extinction coefficients at the detection wavelength. Therefore, in order to properly interpret colorimetric assay results, chromatographic procedures are needed to first separate and identify the forms of sialic acids present, and their relative proportions. More recently, various direct high pressure liquids chromatograph methods *(22,23)* have been developed for quantitation of sialic acids. Although these offer improved assay speed and specificity, more complex pre- or postcolumn derivatization reactions to detect the sialic acid species are usually required.

Naturally occurring lectins offer an attractive means to achieve highly specific detection of given carbohydrate structures. Numerous lectins that

bind sialic acids have been isolated from various sources (*24*). Such lectins can distinguish not only the *N*-acetyl and *N*-glycolylneuraminic acids (*25,26*), but also sialic acids with different carbohydrate moieties linked to their C-2 position (*27,28*).

We have previously demonstrated (*2,3*) the feasibility of using a new lectin-based homogeneous enzyme-linked binding assay method to detect the type and relative amount of given carbohydrates appended to the surface of intact glycoprotein structures. Such a method is potentially useful in bioanalytical quality control of recombinant glycoprotein products, in which the nature of surface carbohydrate structure can vary substantially, and this could influence biological activity of the resulting products. The new enzyme-linked assay technique involves preparation of an enzyme–carbohydrate conjugate that is inhibited, homogeneously, by the binding reaction with a lectin that selectively recognizes the carbohydrate structure appended to the enzyme. In the presence of intact glycoproteins possessing the same carbohydrate groups, competitive binding yields an increase in enzyme activity proportional to the amount of the given carbohydrate structure on the surface of the glycoprotein. Although the principles of this assay scheme were demonstrated previously, using mannose-, galactose-, and *N*-acetylglucosamine-malate dehydrogenase (MDH) conjugates in conjunction with concavalin-A, jacalin, and wheat germ agglutinin (WGA) lectins as reagents (2–3) (to detect mannose, galactose, and *N*-acetylglucosamine residues within intact proteins), the preparation of enzyme–carbohydrate conjugates with covalently bound sialic acid groups, which is required to rapidly detect important terminal sialic acids within glycoprotein via this new method, remained a synthetic challenge. Here we describe a method to prepare an *N*-acetylneuraminyl-α2,3(6) lactose-malate dehydrogenase (MDH-Lac-Neu5Ac) conjugate that has the requisite properties to be useful as a sialic acid selective reagent in the new lectin-based binding assay. The catalytic activity of the new MDH-Lac-Neu5Ac conjugate is inhibited to 35% by the sialic acid binding lectin, *Limax flavus* agglutinin (LFA). This inhibition is shown to be reversed by the presence of glycoproteins with Neu5Ac terminal groups.

MATERIALS AND METHODS

Materials

MDH from pigeon breast muscle, sialyllactose, bovine submaxillary mucin and asialomucin, oxaloacetic acid, the reduced form of nicotinamide adenine dinucleotide (NADH), and Sephadex G-10 were all purchased from Sigma, (St. Louis, MO). Para-aminophenylethylamine (pAPEA), sodium cyanide borohydride, thiophosgene, and 5% palladium

charcoal were products of Aldrich (Milwaukee, WI). *Sambucus nigra* agglutinin (SNA), LFA, and *Triticum vulgare* agglutinin (WGA) were obtained from E-Y Laboratory (San Mateo, CA). Bio-Gel P-2 gel (fine) was purchased from Bio-Rad (Hercules, CA). The working assay buffer for the MDH-Lac-Neu5Ac conjugate was a 0.10 *M* sodium phosphate buffer, pH 7.5, containing 0.05 *M* NaCl, 0.01% NaN_3 (w/v) and 0.30% gelatin. Distilled-deionized water was used to prepare all buffers. All other organic solvents and reagents used were either HPLC grade or reagent grade.

Apparatus

Enzyme activities were measured with a Gilford-Stasaar-III spectrophotometer equipped with a vacuum-operated sampling system and temperature-controlled cuvette (maintained at 30°C throughout the experiments). The spectrophotometer was connected to a Syva CP-5000 EMIT Clinical Processor for automatically setting the reading intervals and recording the absorbance values. A Dynatech MR 5000 microtiter plate reader (Chantilly, VA) was used to determine the degree of saccharide conjugation. A rabbit peristaltic pump (Rainin Instrument, Woburn, MA) was used to pump solutions through the gel-filtration columns (i.e., Sephadex G-10 or P-2) and an ISCO Retriever II automatic fraction collector (ISCO, Lincoln, NE) was used to collect fractions eluted from the columns. Absorbance measurements of various solutions were performed on a Shimadzu UV160U UV-Vis spectrophotometer (Shimadzu Scientific Instruments, Columbia, MD) operated by IBM-compatible PC.

Preparation of *p*-aminophenethylamino *N*-acetylneuraminin-lactosyl pyranoside (p-APEA-Lac-Neu5Ac)

The procedure used was a modified version of the method initially reported by Zopf et al. *(29)*. In a typical preparation, 32 mg of sialyllactose (0.05 mmol) was added to 250 µL (1.75 mmol) of β-(*p*-aminophenyl) ethylamine (*p*-APEA) and stirred in a closed glass reaction vial for more than 15 h at 37°C. Two hundred and fifty µL of absolute ethanol was then added to the clear reaction mixture to reduce viscosity. The solution was adjusted to pH 4.0 by dropwise addition of glacial acetic acid. A considerable amount of white precipitate was formed, and this precipitate was dissolved by adding 1 mL of water. This was immediately followed by the addition of 0.5 mL absolute ethanol containing 10 mg sodium cyanoborohydride ($NaCNBH_3$). The ethanol solution was sonicated to an opalescent suspension. The mixture was then stirred for at least 5 h in a vented hood, with the glass reaction vial loosely capped. Following this reaction period, the mixture was diluted with 1 mL water, chilled in ice, and adjusted to pH 5.6 by glacial acetic acid as necessary. After removal of ethanol under reduced

pressure, the mixture was taken up in about 2 mL of water. The solution was then applied to a 2.5 × 110 cm Sephadex G-10 column, and eluted with 1 *M* acetic acid (adjusted to pH 5.0 with distilled pyridine) at 0.5 mL/min. The separation of product from the free amine was demonstrated by monitoring the UV absorbance of eluted fractions (vol = 4 mL) at 295 nm. The carbohydrate content of each fraction was determined by phenol sulfuric acid method *(30)*. Fractions containing the phenethylamine derivative of sialyllactose were pooled and the solvent was evaporated on a Buchi Rotavapor. The resulting solid product was characterized by FAB mass spectrometry. Electron impact mass spectro-metry was also used to detect any trace amount of *p*-APEA impurity that might be present in the purified product.

Preparation of *p*-isothiocyanate phenethylamino *N*-acetylneuraminin-lactosyl pyranoside (*p*-ITCPEA-Lac-Neu5Ac)

The procedure used to prepared the *p*-isothiocyanate derivative of APEA-Lac-Neu5Ac was adapted from ref. *31*. Briefly, 10 mg *p*-APEC-Lac-Neu5Ac (12.5 µmol) was dissolved in 1 mL of 0.1 *M* $NaHCO_3$, pH 8.5. The solution was layered over 1.25 mL of chloroform-containing thiophosgene (2.5 µL, 32.5 µmol), and stirred vigorously for 1 h. The reaction was monitored by a silica gel TLC using a mobile phase of chloroform:methanol:water = 5:5:1. After 1 h, the spot of starting material disappeared (R_f = 0.382) and the product exhibited an R_f = 0.826. The reaction mixture was then transferred to a 4-mL polypropylene centrifuge tube, and the aqueous layer was extracted twice with 1-mL vol of chloroform to remove excess thiophosgene. The aqueous layer was collected, and nitrogen was bubbled through this phase to remove the last traces of chloroform.

Coupling of *p*-ICTPEA-Lac-Neu5Ac to MDH

The *p*-ICTPEA-sialyllactose, prepared as described above, was immediately coupled to MDH, which was exhaustively dialyzed against PBS buffer, pH 7.5, for 3 d prior to the conjugation reaction. A solution of MDH (22 nmol) in PBS buffer (3 mL) was brought to pH 9.0 by the addition of 17 mg $NaHCO_3$ and 10.3 mg Na_2CO_3. Four m*M* malic acid and 4 m*M* NADH were also added to protect the active site of MDH. An appropriate amount of p-ICTPEA-sialyllactose in 0.1 *M* $NaHCO_3$ was added to 500-µL aliquots of the enzyme solution. The ligand:enzyme ratio was varied from 0:1 to 1500:1. The coupling reaction was run for 24 h at 4°C with stirring. Unreacted carbohydrate derivative was then removed from the proteins by passing the reaction mixture through a 1.5 × 32 cm Bio-Gel P-2 column (0.17 mL/min), eluting with 0.10 *M* sodium-phosphate buffer, pH 7.5, containing 0.05 *M* NaCl, 0.01% NaN_3 (w/v). One-half mL fractions were collected. The conjugate peak was identified by measuring the carbohydrate

content (by phenol– sulfuric acid method) and the enzymatic activity of the eluted fractions. Fractions containing the MDH-Lac-Neu5Ac conjugates were pooled, and the conjugates were characterized by their residual activities, degree of saccharide conjugation (i.e., number of trisaccharide attached to each MDH molecule), and percent inhibition induced by excess amount of LFA lectin. The degree of conjugation was determined by the phenol–sulfuric acid method and a modified version of Warren's TBA assay *(23)*.

Determination of Degree of Saccharide Conjugation

The degree of saccharide conjugation was defined as the number of carbohydrate molecules that were covalently attached to each enzyme macromolecule. This was first determined by a modified phenol–sulfuric acid method *(30)*. Briefly, 100 µL of conjugate or carbohydrate standard solution was pipeted into a glass tube. To this, 150 µL of 2% phenol water solution was added. Then, 750 µL of concentrated sulfuric acid was quickly added, with the stream of acid being directed against the liquid surface, rather than against the side of the test tube, in order to obtain good mixing. The tube was immediately shaken with an agitator and allowed to stand for 30 min before the absorbance of the solution was measured at 490 nm.

The number of sialic acid residues attached to each MDH enzyme molecule was then determined by a modified Warren's TBA assay *(17)*. In this assay, 200 µL of Neu5Ac standard or sialic acid-containing MDH conjugate solutions (in borosilicate glass tubes) were mixed with 20 µL 1 N H_2SO_4 and heated in a heating block at 80°C for 60 min. The solutions were cooled, and 50 µL of 0.025 *M* periodic acid in 0.125 *M* HCl was then added and vortexed. The mixtures were placed in a 37°C water bath for 30 min, with tubes uncapped. Forty µL of 2% arsenite in 0.5 N HCl were added to stop the periodic acid reaction. The solutions were then vortexed until the yellow color disappeared. Four hundred µL of TBA were then added, and the derivatization/color production reaction was allowed to proceed for 7.5 min at 100–105°C. The final reaction was stopped by placing the tubes in an ice-water bath. One mL of acidified butanol (5% HCl) was added and vortexed with the resulting solution. The absorbance of the stable pink color extracted into the butanol layer was measured at 549 nm.

Determination of Residual Enzyme Activity and Maximum Percent Inhibition

The activity of the MDH-Lac-Neu5Ac conjugates was determined by measuring the rate of decrease of NADH concentration at 340 nm, after addition of 100 µL of 5.25 m*M* NADH, 100 µL of 4.25 m*M* oxaloacetic acid (OAA), and 100 µL of an appropriately diluted MDH-saccharide conjugate (all in assay buffer) to a disposable polypropylene centrifuge tube containing

700 µL of assay buffer. All solutions were kept at 0°C until the mixing of the reagents. For each assay, after mixing the reagents and subsequent agitation on a vibrator for a couple of seconds, the reaction mixture was aspirated into the thermostated flow cell of the spectrophotometer and the absorbance at 340 nm was measured over 1 min period, after an initial 20 s delay.

In order to determine the maximum percent inhibition of conjugate by LFA or other lectins, 100 µL of the assay buffer (of 700 µL) was replaced with 100 µL of lectin solution prepared in assay buffer. In addition, the conjugates were incubated first with the lectin for 30 min at room temperature before addition of the substrate solution. Unless otherwise indicated, all incubations for the assays were carried out at room temperature.

Association Kinetics Between MDH-Lac-Neu5Ac Conjugates and Various Lectins

The rate of binding between the MDH-Lac-Neu5Ac conjugate and various lectins was measured as follows: 100 µL of an appropriately diluted MDH-Lac-Neu5Ac conjugate in assay buffer, plus 100 µL of lectin solution (for concentrations, *see* the legend of Fig. 4), were mixed and incubated for varying time periods. After each incubation period, enzymatic activity of the conjugates was determined as described in the Determination of Enzyme Activity Section, above.

Dose–Response Curves Toward Saccharides and Glycoproteins

Standard solutions of different glycoproteins were prepared in the assay buffer. In the assay protocol, 100 µL of standard or glycoprotein solution was first incubated with 100 µL of lectin solution for 15 min. One hundred µL of appropriately diluted conjugate solution was then added, and the mixture was incubated for 30 min. The resulting enzyme activity was measured as described in the Determination of Enzyme Activity Section, above. Dose-response curves were prepared by plotting percent inhibition vs the logarithm of the concentration of carbohydrate species in the 100 µL of solutions added to the assay mixture.

RESULTS AND DISCUSSION

Preparation of an enzyme–saccharide conjugate with terminal sialic acid residue is the essential step in the development of a lectin-based binding assay for detecting the sialyl terminal groups on intact glycoproteins. Although isothiocyanatephenyl derivatives of α(β)-galactose, α-mannose, and β-*N*-acetylglucosamine are readily available and have been used in previous work *(1–3)* for covalent attachment of these mono-

saccharides to MDH (or other enzymes), there is no analogous isothiocyanatephenyl sialic acid derivative commercially available. Although *p*-nitrophenyl neuraminic acid is available (Sigma), it is too unstable in acidic solution to be converted to its isothiocyanatephenyl form. Thus, in this work, to prepare an appropriate enzyme conjugate with pendent sialic acid groups, the trisaccharide sialyllactose was derivatized and conjugated to MDH enzyme in accordance with the reaction sequence illustrated in Fig. 1.

The starting material, *N*-acetylneuraminin-lactose (or sialyllactose) trisaccharide, is a mixture of α2-6 and α2-3 isomers. The solid material obtained from Sigma contains 64% Neu5Acα2-3lactose and 16% Neu5Acα2-6lactose; the rest is mainly sodium chloride salt. Sialyllactose was first reacted with p-APEA, and the resulting Schiff base was then reduced by sodium cyanide borohydride. The reaction product was subsequently purified using a Sephadex G-10 column, and the eluted fractions were monitored by measuring the UV absorbance peak of the aromatic ring (λ = 295 nm), and carbohydrate content. Figure 2 illustrates a typical elution profile. The first UV absorbing peak overlapped with the carbohydrate band, indicating a carbohydrate derivative possessing an aromatic ring, was formed after the reaction. Moreover, the product band was well-separated from the second UV absorbing band, in which excess *p*-APEA was eluted. FAB mass spectrometry yielded a major peak at a m:z ratio corresponding to the *p*APEA-Lac-Neu5Ac derivative, with no starting sialyllactose detected.

Purified *p*-APEA-Lac-Neu5Ac was further converted to *p*-ICTPEA-Lac-Neu5Ac according to the procedures described in Materials and Methods. The reaction was carried in aqueous–organic two-phase solution system to avoid the hydrolysis of sialic acid glycosyl bond in acidic solution (1 mol of thiophosgene generates 2 mol HCl after reaction with *p*-APEA-Lac-Neu5Ac). Para-APEA-Lac-Neu5Ac was dissolved in $NaHCO_3$ buffer, pH 8.5; thiophosgene was kept in the chloroform organic phase. Nonetheless, the isothiocyanate functional group in the product can hydrolyze readily at pH 8.5; therefore, the isothiocyanate-derivative product was not isolated, but directly coupled to MDH immediately after the reaction. The resulting MDH-Lac-Neu5Ac conjugate was purified using a Bio-Gel P-2 column. The eluted fractions were detected by measuring the enzymatic activity and carbohydrate content. As expected, the conjugate eluted at the void volume of the column, and it was well separated from the excess amount of unreacted p-ICTPEA-Lac-Neu5Ac.

Characterization of MDH-Lac-Neu5Ac Conjugates

Conjugates obtained using various enzyme:ligand ratios were characterized by determining their residual activity, degree of saccharide con-

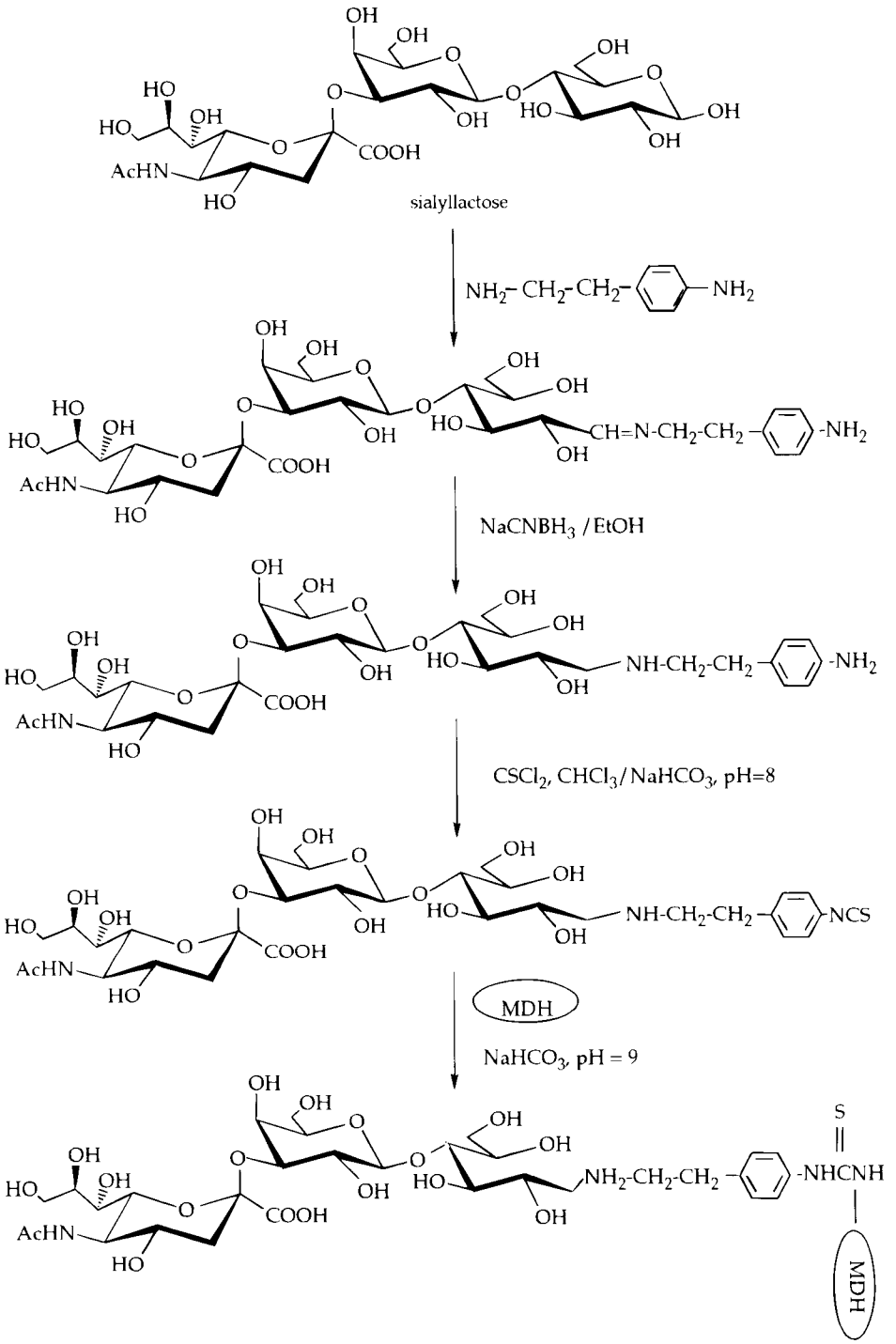

Fig. 1. Synthetic reaction route used to prepare MDH-Lac-Neu5Ac conjugate.

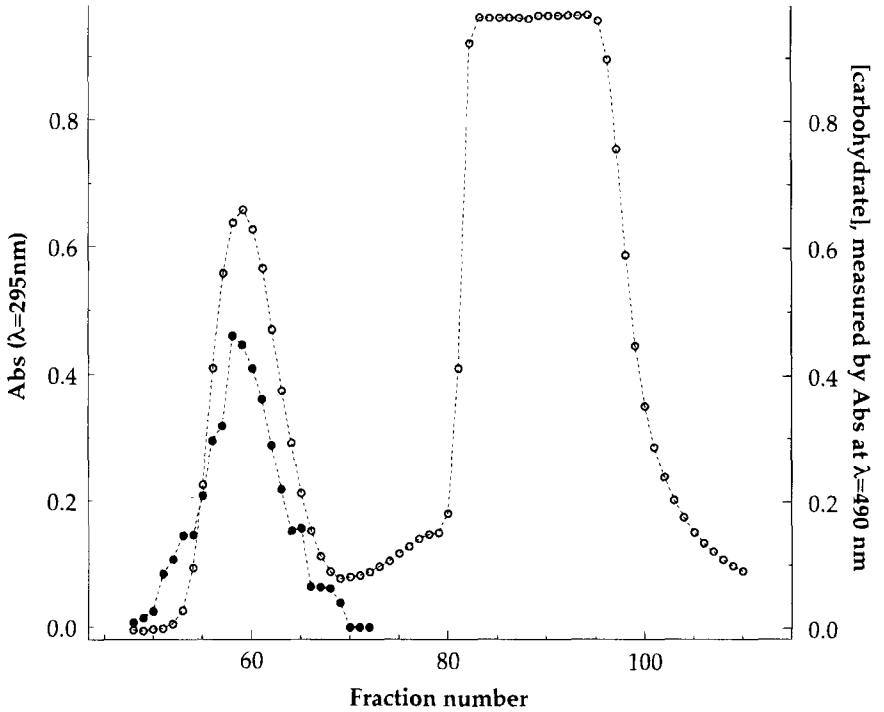

Fig. 2. Elution profile of APEA-Lac-Neu5Ac using a 2.5 × 110 cm Sephadex G-10 column. The compound was eluted with 1 *M* acetic acid adjusted to pH 5.0 with pyridine at 0.5 mL/min. The fractions (4 mL) were monitored by measuring the UV absorbance peak of the aromatic ring at 295 nm (○), and their carbohydrate content (●). The latter assay employed the colorimetric phenol–sulfuric acid method.

jugation, and maximum percent inhibition induced by the presence of excess amounts of lectins. As summarized in Table 1, the various conjugates synthesized retained 48–72% of their native enzyme activity, depending on the degree of conjugation. All conjugates prepared possessed relatively high degrees of conjugation (number of carbohydrate moeities per enzyme molecule). This was confirmed by the phenol—sulfuric acid method, which quantitates the amount of lactose, and by a specific TBA assay that quantitates sialic acid content. Both methods gave almost the same degree of conjugation (*see* Table 1). This agreement of assay results not only confirmed the reliability of both carbohydrate assay methods, but also indicated that the glycosyl bond of sialic acid was preserved during the sequence of synthetic steps indicated in Fig. 1.

Conjugate 3 (*see* Table 1) was chosen for further study with lectins, because of its high degree of saccharide conjugation. A given amount of the conjugate 3 was incubated with various concentrations of four different sialic acid-binding lectins. As shown in Fig. 3, among all sialic acid-binding lectins examined, only LFA and WGA significantly inhibit the enzymatic

Table 1
Characterization of MDH-Lac-Neu5Ac Conjugates

	Conjugate 1	Conjugate 2	Conjugate 3
Enzyme:ligand ratio	1:500	1:1000	1:1500
Residual activity	72%	60%	48%
Degree of conjugation			
by phenyl sulfuric method	16.4	52.8	65.1
by TBA assay	16.0	52.3	60.4
Maximum % inhibition			
by WGA	23%	51%	54%
by LFA	>8%[a]	>30%[a]	>35%[a]

[a] The binding curve for LFA does not completely plateau at highest lectin concentration tested.

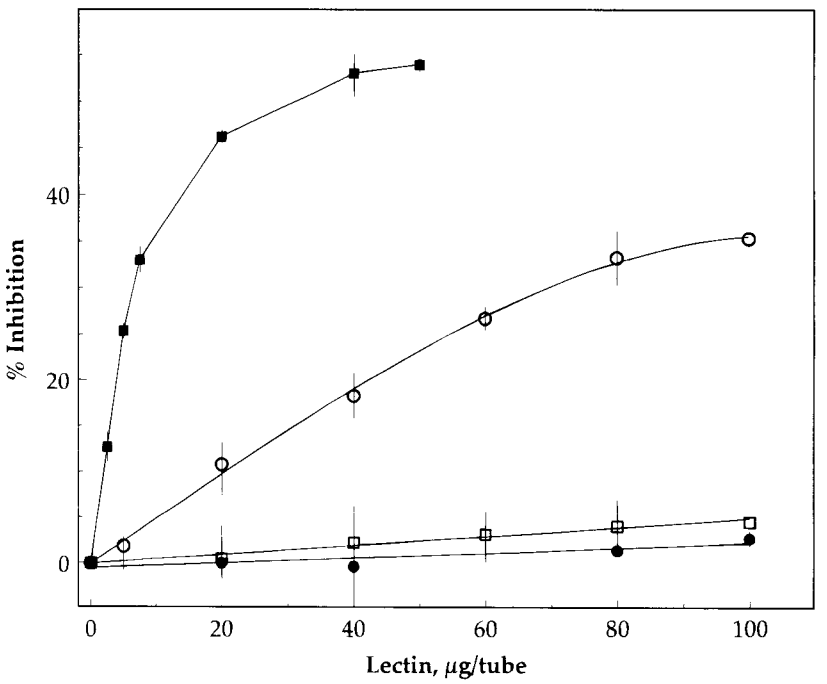

Fig. 3. Effect of concentration of various lectins on the catalytic activity of MDH-Lac-Neu5Ac conjugate: WGA (■), LFA (○), SNA (□), and MAA (●). The conjugate concentration used was 1.48×10^{-9} *M* in a 100-μL aliquot. Data points are average of duplicate measurements ± range.

activity of MDH-Lac-Neu5Ac. WGA, a less selective lectin that binds terminal and internal GlcNAc and GalNAc structures, as well as terminal neuraminic acid residues *(32,33)*, yields the steepest inhibition curve and greatest degree of inhibition. More specific LFA inhibited the activity of the

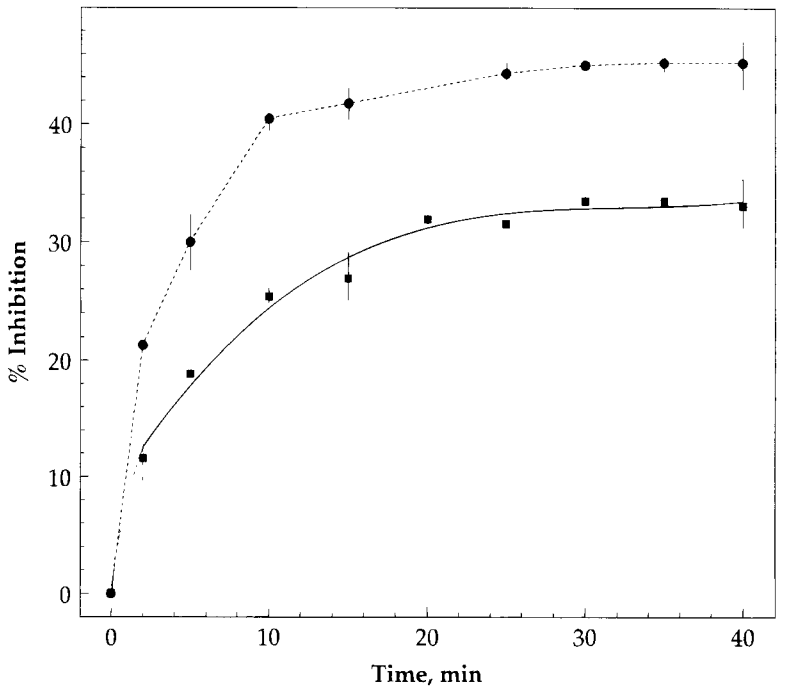

Fig. 4. Kinetics of association between MDH-Lac-Neu5Ac conjugate (1.48×10^{-9} *M* in a 100-μL aliquot) and lectins: LFA (■), and WGA (●). The concentrations of LFA and WAG in 100-μL aliquot are 800 μg/mL and 400 μg/mL, respectively. Data points are average of duplicate measurements ± range.

new MDH-Lac-Neu5Ac up to about 35%. As shown in Fig. 3, SNA lectin (*Sambucus nigra* agglutinin) does not inhibit the activity of the MDH-Lac-Neu5Ac conjugate. This is not surprising, given the fact that SNA only recognizes the Neu5Acα2-6Gal/GalNAc sequence *(34)*. The sialyllactose trisaccharide used in preparation of the conjugate, however, contained only 20% of the Neu5Acα2-6lactose isomer. The remaining 80% was Neu5Acα2-3lactose, the sequence that is not bound by SNA. The conjugate was also not inhibited by *Maackia amurensis* agglutinin (MAA), a sialic acid-binding lectin that is known to bind the Neu5Ac-α2-3Gal structure. It has been shown in the literature that this lectin's specificity is complex. Indeed, MAA only binds to asparagine-linked oligosaccharides with an Neu5Acα2-3Gal terminal group, but not to O-linked oligosaccharide with the same terminal group *(35)*. The glycans of the neoglycoprotein conjugate (MDH-Lac-Neu5Ac) synthesized here are neither N-linked nor O-linked type; thus, it is not surprising that MAA does not interact with this conjugate to any measurable degree.

The kinetics of the association between the MDH-Lac-Neu5Ac and WGA and LFA were studied in detail. As shown in Fig. 4, WGA exhibits somewhat faster binding kinetics with the MDH-saccharide conjugate than

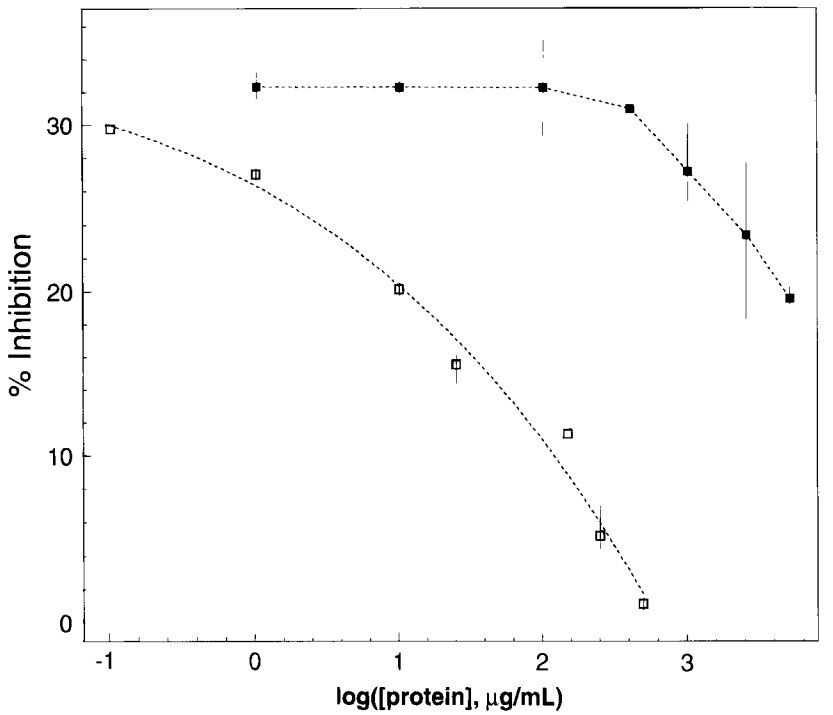

Fig. 5. Dose-response curves of mucin (□) and asialomucin (■). MDH-Lac-Neu5Ac conjugate and LFA lectin concentrations are 1.48×10^{-9} *M* and 800 μg/mL, respectively, in 100-μL aliquot. Data points are average of duplicate measurements ± range.

LFA. Although less than 20 min was needed for WGA to reach its maximum binding with the conjugate, a 30-min incubation time was required for LFA to achieve the full binding. It should be noted that maximum/full binding in the present assay scheme means the time required to achieve the highest degree of homogeneous inhibition of MDH activity. Since homogeneous inhibition is highly dependent on surrounding the enzyme–saccharide conjugate with a suitable number of lectin molecules that causes either steric hindrance (for substrate access to the active site) and/or a conformation change in the enzyme structure, the time required for substantive inhibition is likely to be somewhat longer than that required for a single lectin-appended saccharide interaction to achieve full equilibrium binding in solution.

Competitive Binding Assays Using MDH-Lac-Neu5Ac Conjugate and LFA

As previously suggested *(1–3)*, appropriate MDH–saccharide conjugates are useful reagents for devising a homogeneous assay method to detect the presence of given carbohydrate structures on intact glycoproteins. To demonstrate that the MDH-Lac-Neu5Ac conjugate can be used for

similar purposes, the new conjugate was used in conjunction with LFA to detect the presence of sialic acid terminal groups on bovine submaxillary mucin. It should be noted that LFA, rather than WGA, was used for these studies, since WGA is not specific for sialic acid groups (i.e., most glycoproteins have GlcNAc and GalNAc within their glycan chains that are also recognized by WGA). Figure 5 illustrates the results of a competitive binding assay in which both mucin and asialomucin are probed as intact glycoproteins. Mucin exhibits a more sensitive dose response, with an ED_{50} value of 48 μg/mL, which is more than 3 orders of magnitude lower than that observed for asialomucin. The result clearly indicates that MDH-Lac-Neu5Ac, together with LFA, can easily distinguish glycoproteins with sialic acid terminal groups from their respective asialo-forms. No hydrolysis of the glycoprotein, or discrete separation steps are required to assess the presence of sialic acid terminal groups, making this homogeneous enzyme-linked assay faster than any existing sialic acid measurement method. However, the proposed assay is only a semiquantitative method most suitable for quality control situations or research purposes, when knowledge regarding the absence or presence (or changes in the extent) of sialation on the surface of intact glycoproteins may be desired.

In summary, MDH-Lac-Neu5Ac conjugates with varying degrees of carbohydrate substitution were successfully synthesized using a pure organic synthetic route. The MDH-Lac-Neu5Ac, when used in conjunction with LFA, can be employed to conveniently distinguish glycoproteins with sialic acid terminal residues from their asialo-form. It should be noted, however, that at present, the high cost of LFA lectin ($125/mg) would make such assays rather expensive to carry out on a routine basis. This issue could be overcome with the availability of a recombinant form of this protein in the future. Further, the organic synthesis method employed here can also be applied to prepare other MDH-oligosaccharide conjugates with various sialic acid terminal structures/linkages. For example, it is expected that if a purified trisaccharide with an Neu5Acα2-6Gal/GalNAc terminal is attached to MDH enzyme, SNA should inhibit the conjugate to a greater degree than LFA. Such a system could then be used to specifically detect glycoproteins with Neu5Acα2-6Gal/GalNAc terminal groups, hence achieving some level of isomer selectivity. By employing the present MDH-Lac-Neu5Ac conjugate in conjunction with the array of MDH–saccharide reagents already reported *(1–3)* and a suitable array of lectins, a more complete and rapid finger-print type assessment of the relative carbohydrate content/structure of intact glycoproteins should be possible. Use of these new homogeneous assay reagents in 96-well microtiter plate format (as described in Ref. *3*) will greatly aid in such bioanalytical applications.

ACKNOWLEDGMENT

We gratefully acknowledge the National Science Foundation for supporting this work (NSF # CHE 9423063)

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