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Assay of sialyltransferase activity by reversed-phase ion-pair high-performance liquid chromatography

Leonard B. Spiegel

Department of Chemistry, Youngstown State University, Youngstown, OH 44555 (USA)

Jane Hadjimichael and Edward F. Rossomando*

Department of BioStructure and Function, University of Connecticut Health Center, Farmington, CT 06030 (USA)

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ABSTRACT

Sialyltransferases (CMP-N-acetylneuraminic acid:glycoprotein sialyltransferases, EC 2.4.99.1) are involved in the transfer of a sialic acid moiety from CMP-N-acetylneuraminic acid (CMP-NeuAc) to an oligosaccharide side-chain of an acceptor, asialoglycoprotein (AGP), according to the following reaction:

CMP-NeuAc + AGP → NeuAc-O-AGP + CMP

This enzyme occurs in elevated levels in the sera of patients with a wide variety of neoplastic diseases and its assay might be useful in monitoring treatment. Radioactive CMP-NeuAc has been used in assays and the radioactive sialylated product separated and counted by liquid scintillation spectrometry. This study shows that a simple, rapid, non-radiochemically based high-performance liquid chromatographic method developed for the analysis of CMP-sialic acid synthetase can be used for the quantitation of sialyltransferase activity by monitoring simultaneously the utilization of CMP-NeuAc and the release of CMP. We describe the application of this method to assay of commercially available sialyltransferase activity and to activities from synovial, ascites and gastric fluids.

INTRODUCTION

Sialyltransferases are the enzymes responsible for the transfer of sialic acid moieties (acetylneuraminic acid, for example) to an asialoglycoprotein. These enzymes add terminal residues to oligosaccharide side-chains of animal cell coat glycoproteins (see ref. 1 for review) and gangliosides.

Recent clinical interest in sialyltransferase levels has been shown by recent studies on human cells ranging from cultured melanoma cells [2], other cultured human cancer cells [3] and normal human leukocytes and granulocytes from patients with chronic myelogenous leukemia [4]. In addition, other studies have correlated the level of activity in lymphocytes with age [5,6], with the diagnosis of multiple myeloma [7,8] and colorectal cancer [9]. These findings suggest that the measurement of sialyltransferase may have clinical relevance in diagnosis and/or monitoring of treatment protocols.

Traditional sialyltransferase activity assays use a radiochemical substrate, ^{14}C-labeled cytidine 5′-monophosphate-N-acetylneuraminic acid (CMP-NeuAc), with the transfer of [^{14}C]N-acetylneuraminic acid to an unlabeled acceptor molecule with a non-reducing galactose as the terminal saccharide. After separation by precipitation or chromatographic techniques the radioactive product is quantified by liquid scintillation spectrometry.

In this report we describe a non-radiochemically based high-performance liquid chromato-

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graphic (HPLC) method similar to one previously reported for the analysis of another activity in this cascade, namely CMP-sialic acid synthetase [10]. Our method allows for the quantitation of sialyltransferase acitvity by monitoring, simultaneously, the loss of CMP-NeuAc and the formation of CMP. In this paper we describe the application of this method to a commercially available sialyltransferase activity and to activity determinations in ascites, synovial and gastric fluids.

EXPERIMENTAL

Chemicals

Neuraminidase-agarose, Type X-A, sodium cacodylate, α_1-acid glycoprotein (AGP), Triton CF-54, bovine serum albumin (BSA), CMP disodium salt (90% pure) and CMP-NeuAc sodium salt were purchased from Sigma (St. Louis, MO, USA). Rat liver sialyltransferase enzyme (EC 2.4.99.1) was purchased from Boehringer-Mannheim (Indianapolis, IN, USA). Acetonitrile, HPLC grade, was from Fisher Scientific (Fairlawn, NJ, USA) and tetrabutylammonium phosphate was purchased from Waters, Division of Millipore (Milford, MA, USA). All other chemicals were reagent grade. Biologically relevant samples were mouse ascites and synovial and gastric fluids obtained during the normal course of surgical procedures.

Asialo-α_1-acid glycoprotein preparation

AGP was prepared by using neuraminidase-agarose, Type X-A, to desialylate AGP according to a modification of the manufacturer's recommended procedure previously described [11]. In brief, 1.0 ml of the neuraminidase-agarose preparation (0.385 U) was washed three times with 50 m*M* sodium acetate buffer, pH 5.0, to remove $(NH_4)_2SO_4$, followed by centrifugation at 4°C after each wash. The enzyme-agarose preparation was resuspended in 1.3 ml of sodium acetate buffer and 0.2 ml of AGP (50 mg/ml) was added to the tube followed by incubation for 27 h at 37°C on a platform rocker. After micro-centrifugation for 4 min the supernatant was concentrated in a 10 000 nominal molecular mass exclusion filtration apparatus (Ultrafree-MC, Millipore, Bedford, MA, USA) at 4°C for 2 min. The concentrate was diluted to 2 ml with glass-distilled water and reconcentrated three times as described above. The concentrate was dried under vacuum, dissolved to 2.5 mg/ml AGP in 50 m*M* cacodylate buffer containing 0.5% (v/v) Triton CF-54 and 0.1% (w/v) BSA, pH 6.0, and stored at −20°C.

Sialyltransferase activity assay

Sialyltransferase enzyme activity was determined in a reaction mixture containing, in a reaction volume of 60 μl, 25–125 μg of AGP, 0.6 m*M* CMP-NeuAc, 50 m*M* sodium cacodylate hydrochloride containing 0.5% (v/v) Triton CF-54 and 0.1% BSA, pH 6.0, and rat liver sialyltransferase enzyme in the range 0.1–1.2 mU. One unit (U) of enzymatic activity transfers 1.0 μmol of NeuAc per minute. The reactions were initiated by the addition of CMP-NeuAc and incubated at 37°C. The reactions were terminated by precipitation of proteins with 40 μl ice cold HPLC-grade acetonitrile. The precipitate was removed by microcentrifugation (Fischer Scientific, Model 235) at 4°C, and 20 μl of the supernatant were analyzed by HPLC.

Reversed-phase ion-pair HPLC analysis

Isocratic separations of CMP-NeuAc and CMP were performed on a 250 mm × 4.6 mm I.D., 5 μm C_{18}-Ultrasphere-IP column (Altex, Division of Beckman, San Ramon, CA, USA) protected by a 30 mm × 4.6 mm I.D. guard column packed with C_{18} silica. The column was eluted at 2.0 ml/min with a mobile phase consisting of 9% acetonitrile in 5 m*M* sodium phosphate buffer (pH 7.5) and containing 5 m*M* tetrabutylammonium phosphate as an ion-pairing agent. The column eluate was monitored at 270 nm and 0.05 absorbance units full scale.

Instrumentation

The HPLC system consisted of a Model 6000 A solvent delivery system (Waters, Milford, MA, USA), a Rheodyne 7125 injector fitted with a 20-μl sample loop, a Model HM Holochrome variable-wavelength absorbance detector (Gilson, Middleton, WI, USA) and a Beckman (Palo Alto, CA, USA) dual-pen recorder. The areas under the peaks were calculated using a Model E1-A Chromatopac integrator (Shimadzu, Columbia, MD, USA).

RESULTS AND DISCUSSION

In order to observe at least two of the components of the sialyltransferase reaction a new HPLC separation system was developed consisting of a 5 μm particle size C_{18} column eluted with a mobile phase containing 9% acetonitrile in 5 m*M* sodium phosphate buffer (pH 7.5) and 5 n*M* tetrabutylammonium phosphate as an ion-pairing agent. The HPLC profile (Fig. 1) shows good separation of the components with CMP, the product, eluted at 3.4 min (Fig. 1, peak 1) and CMP-NeuAc, the substrate, eluted at 4 min (Fig. 1, peak 2). Monitoring of the CMP moieties at 270 nm prevented detection of substances with lower UV absorbance thereby allowing for a rapid recycle time for multiple analyses. The UV absorbance of the CMP moeities at 270 nm, as measured by calculating the area under the peak, was linear over the range 0–2 nmol. The amounts of CMP and CMP-NeuAc in each analytical sample were determined by calculating the area under the peak for each followed by comparison with a calibration curve.

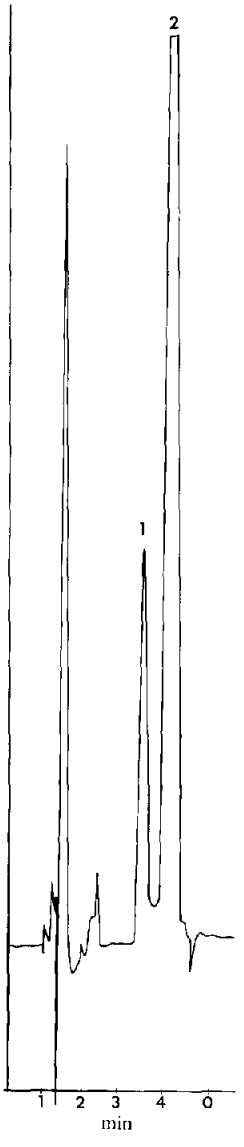

Fig. 1. Separation of CMP (peak 1) and CMP-NeuAc (peak 2) by reversed-phase ion-pair HPLC.

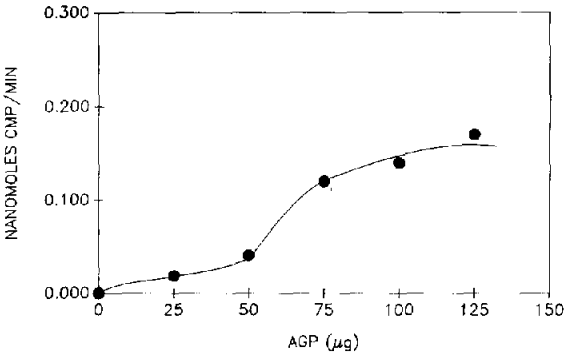

Fig. 2. Dependence of the sialyltransferase activity on acceptor protein. Reaction mixtures were prepared as described in the Experimental section with 0.6 m*M* CMP-NeuAc, 1.2 mU of sialyltransferase and the various indicated concentrations of acceptor protein (AGP). Reactions were terminated after 15 min of incubation and fractionated by HPLC. The amount of CMP was calculated from a calibration curve and plotted as a function of the amount of acceptor protein present.

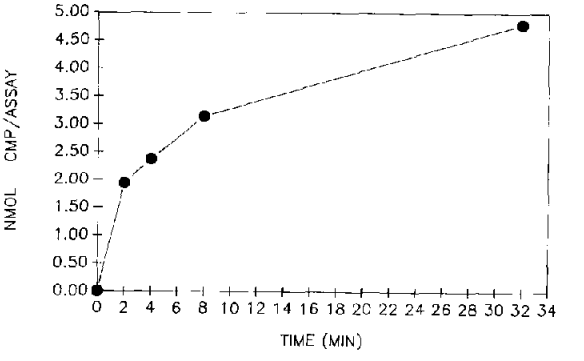

Fig. 3. Time course of the sialyltransferase reaction. Several identical (60 μl) reaction mixtures were prepared as described in the Experimental section with 1.2 m*M* CMP-NeuAc, 50 μg of AGP and 0.1 mU of sialyltransferase. At the times indicated, a reaction was terminated and the amount of CMP determined. The amount of CMP was calculated from a calibration curve and plotted as a function of time.

In order to study the dependence of product formation on acceptor protein, a series of experiments was performed with varying amounts of the acceptor protein, AGP. As shown in Fig. 2, when the amount of acceptor present was increased, the amount of CMP increased. When the acceptor protein was omitted some CMP was released due to hydrolysis at pH 6. Despite this slow hydrolysis of the substrate, CMP-NeuAc, Fig. 2 supports the conclusion that the CMP recovered in the presence of AGP was not the result of acid hydrolysis but was due to the presence of sialyltransferase activity.

The time course of the formation of CMP during the sialyltransferase reaction is shown in Fig. 3. The reactions were carried out as described in the Experimental section and the amount of CMP recovered was determined from the area under the curve with the aid of the calibration curve and plotted as a function of time. As shown in Fig. 3, the amount of CMP increased over the course of the 30-min incubation.

The effect of enzyme concentration on CMP recovered was determined and is shown in Fig. 4. Reaction mixtures were prepared containing 0.4, 0.8 or 1.2 mU of sialyltransferase, 125 μg of AGP, and 36 nmol of CMP-NeuAc. The reaction mixtures were incubated for 15 min prior to analysis by HPLC. The amount of CMP recovered was normalized and plotted as a function of enzyme concentration. As shown in Fig. 4, the recovery of CMP increased in a linear manner, three-fold for a three-fold increase in the amount of enzyme present, over the range of enzyme concentrations tested.

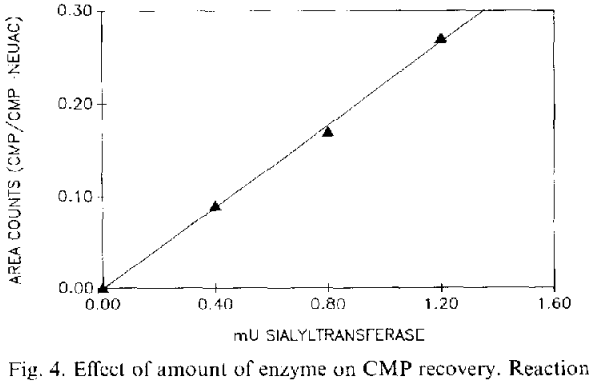

Fig. 4. Effect of amount of enzyme on CMP recovery. Reaction mixtures (60 μl) were prepared as described in the Experimental section containing 0.4, 0.8 or 1.2 mU of sialyltransferase, 0.6 m*M* CMP-NeuAc and 125 μg of AGP, incubated for 15 min, terminated and analyzed by HPLC.

TABLE I

SIALYLTRANSFERASE ACTIVITIES IN SEVERAL BIOLOGICAL FLUIDS

Fluid	Activity[a] (mU/ml)
Ascites	5.4
Synovium	9.7
Gastric	6.1

[a] Values represent amount found in 1 ml of fluid, where 1 mU is defined as 1 nmol of CMP released per minute.

The sialyltransferase activity present in several biological fluids was assayed and the results are presented in Table I. Detectable levels of sialyltransferase activity were found in mouse ascites, human synovial fluid from a traumatized knee and gastric secretions obtained by lavage.

In conclusion, this new HPLC assay for sialyltransferase activity is rapid, simple and avoids the hazard and expense of use of radiochemicals used in methods for the assay of this activity. It is also easily applied to biological samples from various sources. The assay is sensitive in the biologically relevant range and therefore provides a good alternative method for analysis of sialyltransferase activity.

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