A Microplate Assay for Sialidase Activity Using Plant Lectin Binding to *N*-Acetylactosamine

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This paper presents a sensitive assay for sialidase activity based on the specific binding of lectin to *N*-acetylactosamine. The substrate used for sialidase assay is fetuin (30–100 ng/50 μ l) with sialylated oligosaccharides, which was then coated on a 96-well microtiterplate. After removing sialic acids from the terminal positions of the glycoconjugate glycans by sialidase, it was subjected to biotin-labeled lectin (*Ricinus communis* agglutinin 120), which binds specifically to *N*-acetylactosamine. This was followed by the addition of a peroxidase conjugated avidin–biotin complex. The amount of bound peroxidase was determined by a colorimetric assay. The sensitivity was enhanced 1000- to 10000-fold compared to the colorimetric assay using a synthetic substrate such as 2-*O*-(*p*-nitrophenyl)-*N*-acetyl- α -D-neuraminic acid (PNPN). In the established method, only very small amounts of substrate and sialidase were required; therefore, it can be applied to the quantitative assay of some sialidases from *Vibrio cholerae*, streptococcus, the influenza virus and rat liver.

Keywords sialidase; lectin; avidin–biotin; neuraminidase

Sialidase (EC 3.2.1.18) is one of the important exoglycosidases, which are involved in the metabolism of glycoproteins and glycolipid sugar chains. Sialic acid at the terminal, nonreducing end of many oligosaccharide chains of glycoconjugates have significant biological functions such as the clearance of circulating sialoglycoproteins, acting as a receptor for various peptide hormones and viruses, and also as a regulator of cell–cell interactions.^{1,2)} Quantitative variations in binding sialic acid to an oligosaccharide may be regulated by the balance between sialidase and sialyltransferase activity in tissues. In connection with this function, there has been recent interest in these enzymes in terms of infection by bacteria and the influenza virus, cell growth and differentiation, and tumor cells.

Up to this time, the main assay of sialidase activity has been done using the following methods: 1) colorimetric assay of sialic acids released from sialyl compounds such as glycoproteins and gangliosides^{3,4)}; 2) measurement of the chromogen released from a synthetic chromogenic substrate⁵⁾; 3) measurement of the fluorescence of methylumbelliferone released from a synthetic fluorescent substrate⁶⁾; 4) radioisotopic assay using tritium-labeled sialyl oligosaccharide substrates⁷⁾; and 5) peroxidase or lectin amplified assay.^{8–11)} The above assays have been routinely utilized. However, methods 1) and 2) cannot measure very low sialidase activity and often do not give reliable results with crude enzyme preparations. Systems 3–5 are extremely sensitive. However, in most areas of study it is generally inconvenient to provide a special place for using a radioisotope, they involve time-consuming systems which utilize chromatographic methods prior to measurement, and the cost of using these is prohibitive.

The procedure using a microplate, in this paper, is based on the specific binding of lectin to desialylated *N*-acetylactosamine, and was effective for measuring many samples, such as multiple samples from body fluids and tissues. It can also be used to monitor sialidase activity

during chromatography for sialidase purification.

MATERIALS AND METHODS

Materials Bovine fetuin was purchased from Canadian Bioclinical, Ltd., Scarborough, Canada. Asialofetuin (type I from fetal calf serum) and taurodeoxycholic acid (sodium salt) were purchased from Sigma Chemical Co., St. Louis, U.S.A. Sialidase from *Streptococcus S.P.*, β -galactosidase (Jack bean), Biotin-RCA₁₂₀ (*Ricinus communis* agglutinin 120) and PNPN [2-*O*-(*p*-nitrophenyl)-*N*-acetyl- α -D-neuraminic acid (Na salt)] were obtained from Seikagaku Kogyo Co., Ltd., Tokyo, Japan. Sialidase from *Vibrio cholerae* was supplied by Boehringer Mannheim, Germany. The vectastain ABC kit (Avidin DH and biotinylated horseradish peroxidase H) was obtained from Vector Laboratories, Inc., Burlingame, U.S.A. 4-Methylumbelliferyl-*N*-acetyl- α -D-neuraminic acid ammonium salt was purchased from Nacalai Tesque, Inc., Kyoto, Japan. Bovine albumin (Chon fraction V powder) was from Amour Pharmaceutical Co., Tokyo, Japan. Gelatin was supplied by Funakoshi Co., Ltd., Tokyo, Japan. The 96-well polystyrene microtiter plate (Sumilon) was purchased from Sumitomo Bakelite, Ltd., Tokyo, Japan. *o*-Phenyldiamine was obtained from Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan. The sialic acid test reagents were purchased from Kyokuto Pharmaceutical Industrial Co., Ltd., Tokyo, Japan. The Bio-Rad Protein Assay Dye Reagent Concentrate was obtained from Bio-Rad Laboratories, Richmond, U.S.A. D(+)-Mannose, lactose monohydrate, galactose, dextrose anhydrous (glucose), *N*-acetyl-D(+)-glucosamine, polyoxyethylene(20) sorbitan monolaurate (Tween 20) and polyoxyethylene(10) octylphenyl ether (Triton X-100) were purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan. *N*-Acetyl-D-galactosamine was obtained from E·Y LABS, Inc, San Mateo, U.S.A. The purified influenza virus (A/Aichi/2/68, H3N3) was a generous gift from Prof. Y. Suzuki (University of Shizuoka, School of Pharmaceuti-

cal Science, Japan). The rat liver sialidase was prepared as described previously.¹²⁾

Coating of 96-Well Microtiter Plate with Fetuin The wells were coated with 50 μ l of the 0.1 M carbonate buffer solution, pH 9.7, containing 30–100 ng/50 μ l of fetuin for 4 h at room temperature, or overnight at 4 °C. The coating solution was removed by aspiration and the wells were rinsed three times with 100 μ l of 1/15 M phosphate buffered saline–0.05% Tween 20, pH 7.4 (PBS–Tween 20), then 100 μ l of PBS–Tween 20 containing 5% bovine serum albumin (BSA) was added for blocking. After being left for 1 h at room temperature, the blocking solution was aspirated and the wells were rinsed again three times with 100 μ l of PBS–Tween 20. The coated plates were stored for up to two weeks at 4 °C in a solution of PBS containing 0.03% NaN₃.

Assays of Sialidase Activity The enzymes used were from *Vibrio cholerae*, streptococcus, rat liver and the influenza A virus. The wells coated with fetuin were incubated for 1 h at 37 °C after adding 50 μ l of 0.05 M sodium acetate buffer adjusted to the optimum pH for each of the sialidases. The reaction was stopped with 100 μ l of 250 mM glycine–NaOH buffer, pH 10.4. The reaction mixture was removed by aspiration and the wells were washed three times with 100 μ l of PBS–Tween 20. Next, 50 μ l of biotinylated RCA₁₂₀ (50 μ g/ml) in PBS–Tween 20 was added to the wells. After standing for 1 h at room temperature, the wells were washed three times with 100 μ l of PBS–Tween 20, and 100 μ l of the ABC kit (a solution mixture of avidin DH and biotinylated horse-radish peroxidase H in PBS–Tween 20) was then added. After being left to stand for 1 h at room temperature, the wells were washed three times with 100 μ l of PBS–Tween 20. Following this, 100 μ l of a chromogenic solution consisting of 12 mg of solubilized *o*-phenyldiamine and 25 μ l of 5% H₂O₂ in 30 ml of 0.1 M citrate buffer, pH 4.5, was added to the wells. The reaction was stopped with 50 μ l of 3 M H₂SO₄ and measured at 490 nm on the microplate reader. Fetuin and the enzyme added to the mixture solution were used as a background for the assay. Another

assay using PNP as a substrate was performed by incubating the solution, including the sialidase and substrate, for 1 h at 37 °C, followed by measurement at 415 nm.

Inhibition of Lectin Binding to Asialo Fetuin by Some Sugars The microplate was coated with 30 ng/50 μ l of fetuin. Mixtures of 50 μ l of biotinylated RCA₁₂₀ (50 μ g/ml) and individual sugars (1, 10 and 100 mM) such as lactose, galactose, glucose, mannose, *N*-acetyl-galactosamine and *N*-acetyl-glucosamine, were incubated for 60 min at 37 °C and placed in the asialo fetuin-coated well. The continuous methods were performed as described above. The percentage of inhibition was determined by the following formula:

inhibition (%) =

$$\frac{\text{absorbance (without sugar)} - \text{absorbance (with sugar)}}{\text{absorbance (without sugar)}} \times 100$$

Hemagglutination Assay (HA) of Influenza Virus¹³⁾ HA was titrated by combining 25 μ l of serial two-fold dilutions of the virus in PBS containing 0.01% gelatin with 25 μ l of 0.5% chicken erythrocytes. After incubation for 1 h at 4 °C, the end point was estimated visually, and the reciprocal of the end point dilution was defined as the titer.

Analytical and Chemical Analysis The protein content was determined using the Bio-Rad Protein Assay procedure based on dye-binding assay on the 96-well microplate.¹⁴⁾ The sialic acid content was determined by colorimetric assay as previously described.¹⁵⁾

RESULTS AND DISCUSSION

The small scale multiscreening solid phase method for the sialidase activity is schematically outlined in Fig. 1. The fetuin had trisialyl triantennary sugar chains (74%)¹⁶⁾ and was a good substrate for this assay system, compared to transferrin and the α 1-acid glycoprotein-bearing exposed galactose residue, resulting in low background levels. In

Fig. 1. Schematic Representation of Sialidase Assay in a Solid Phase Using Fetuin

SA, sialic acid; Gal, galactose; RCA₁₂₀, *Ricinus communis* agglutinin 120; OPD, *o*-phenyldiamine.

Fig. 2. Inhibition of Lectin Binding to Asialo Fetuin by Some Sugars

A preincubated suspension of lectin and each of the sugars was added to asialo fetuin-coated wells. The continuous procedure was performed as described in Materials and Methods. —●—, lactose; —○—, galactose; —■—, *N*-acetyl-galactosamine; —△—, glucose; —▲—, mannose; —□—, *N*-acetyl-glucosamine.

Fig. 3. Sialidase Activity Using Sialidase from *Vibrio cholerae* in Various Concentrations of Fetuin

The assay was done with (●) and without (○) sialidase. The absorbance at 490 nm was monitored. The results represent the means $\pm$ S.D. of triplicate determinations.

this assay, the key point for the high sensitivity is the utilization of the avidin-biotin peroxidase complex. It was reported that the lectin, RCA₁₂₀, bound with a high affinity to the structure of the Gal β 1-4 GlcNAc disaccharides, but significantly reduced the association with sialylated Gal β 1-4 GlcNAc.¹⁷⁾ As shown in Fig. 2, the binding of RCA₁₂₀ to Gal β 1-4 GlcNAc disaccharides was inhibited by mono- or disaccharides; especially, by lactose, rather than galactose, which displayed strong inhibition. Inhibition assay using other sugars was low. However, inhibition for increasing sugars (100 mM) may be caused by steric hindrance. In addition, when asialofetuin was treated with β -galactosidase, the binding to RCA₁₂₀ significantly decreased (data not shown). These results are consistent with the specificity of RCA₁₂₀ described previously.¹⁷⁾

Figure 3 shows sialidase activity in various concentrations of fetuin to determine the optimum concentration

Fig. 4. Sialidase Activity in Various Dilutions of Sialidase from *Vibrio cholerae* Using Either Fetuin (●) or PNP (○) as a Substrate

Microplates were coated with 30 ng/50 μ l of fetuin. Sialidase was diluted in 10^{-2} – 10^{-10} U/50 μ l with 0.1 M sodium acetate buffer, pH 6.5. The absorbances at 490 nm for fetuin and at 415 nm for PNP were monitored. The results represent the means $\pm$ S.D. of triplicate determinations.

Fig. 5. The Time Course in Various Dilutions of Sialidase from *Vibrio cholerae* Using Fetuin (A) or PNP (B) as Substrates

Microplates were coated with 30 ng/50 μ l of fetuin. Sialidase for PNP was diluted in 10^{-2} – 10^{-8} U/50 μ l with a 0.1 M sodium acetate buffer, pH 6.5, and sialidase for fetuin was diluted in 10^{-4} – 10^{-8} U/50 μ l with the same buffer. —●—, 10^{-2} U/50 μ l; —○—, 10^{-5} U/50 μ l; —▲—, 10^{-6} U/50 μ l; —△—, 10^{-8} U/50 μ l; —■—, 10^{-2} U/50 μ l; —□—, 10^{-3} U/50 μ l. The reaction time was 0–120 min. The absorbances at 490 nm for fetuin and at 415 nm for PNP were monitored.

Fig. 6. The pH Range of Four Different Sialidases Using Fetuin (●) or PNP (○) as a Substrate

The sialidase was from *Vibrio cholerae* (A), streptococcus (B), influenza A virus particle (C), and the homogenized supernatant of the lysosomal fraction from rat liver (D). The pH range was adjusted to 4–8 with 0.1 M sodium acetate buffer. The absorbances at 490 nm for fetuin and at 415 nm for PNP were monitored.

of fetuin. The reaction reached a plateau at the concentration of 100 ng/50 μ l of fetuin. The background levels (no sialidase) were relatively low, indicating that the fetuin had very little exposed galactose residue to react with RCA₁₂₀. This is the reason fetuin was used as a substrate.

The dilution of sialidase was compared as to the sensitivity of the enzymatic assay using the avidin-biotin-peroxidase complex with that of the colorimetric assay using PNP as a substrate. As shown in Fig. 4, the reaction was linear, ranging from 10^{-4} to 10^{-9} U/50 μ l; therefore, the sensitivity obtained by utilizing fetuin (30 ng/50 μ l) was 1000- to 10000-fold higher in comparison with the colorimetric assay. Additional results were obvious in the time course of the diluted sialidase (Fig. 5). The reaction mixture using PNP as a substrate was incubated for 120 min but had a very low activity in 10^{-4} or 10^{-5} U/50 μ l of the diluted sialidase. However, the reaction using fetuin reached a plateau after 15 min under the condition of 10^{-4} U/50 μ l, as above. These results indicate that the assay developed in this paper has a much higher sensitivity than the colorimetric assay. Furthermore, low activity such as 10^{-6} or 10^{-8} U/50 μ l can be monitored by employing a long incubation time. For example, it may be possible to measure low sialidase activity in body fluids, cells and tissues. The above results were the same in terms of sensitivity as the method using a synthetic fluorescent substrate⁶⁾ or the radiolabelled substrate.⁷⁾ Furthermore, this assay system was ascertained to have high reproducibility according to the levels of standard deviation shown in Figs. 3 and 4. The diluted sialidase was stable for at least two weeks at 4 °C in the presence of 0.1% BSA.

Figure 6 shows the pH range of the four types of

Fig. 7. The Effects of Three Detergents on Sialidase Activity

Sialidase from *Vibrio cholerae* was used at 10^{-4} U/50 μ l. Detergents used were taurodeoxycholic acid (●), Tween 20 (△) and Triton X-100 (○). The absorbance at 490 nm was monitored.

sialidases. Except for the sialidase derived from rat liver, sialidase activity was effective at pH 5–7. The optimal pH of lysosomal sialidase from rat liver was 4. A system using a microplate is most useful in cases which investigate the pH range at once by adding an adjusted pH buffer to the wells coated with fetuin. The selection of a detergent is very important in the extraction of sialidase from tissues and cells without incurring a significant loss of activity. Figure 7 shows the effects of detergents that were used on the sialidase activity. The sialidase activity was inhibited by more than 50% using 0.01% Tween 20 and Triton X-100; whereas, in the case of 0.1% taurodeoxycholic acid, sialidase activity was maintained at more than 90%. Therefore, it appears that taurodeoxycholic acid is appropriate for extracting sialidase from tissues while

Fig. 8. The Relationship between Sialidase Activity and the Hemagglutinin Titer of the Influenza Virus (A/Aichi/2/68, H3N3)

The plates were coated with 50 ng/50 μ l of fetuin. Serial two-fold dilutions of the influenza A virus were combined with 0.1 M sodium acetate buffer, pH 5.5. The sialidase activity and hemagglutinin titer were determined as described in Materials and Methods.

maintaining its activity.

Figure 8 shows the relationship between sialidase activity and the hemagglutinin titer of the influenza A virus. The culture of the influenza virus required more time and cost; hence, Fig. 8 indicates that the assay system described in this paper is useful to measure the sialidase activity of only a small amount of the influenza virus for the preparation of sialidase.

This method is currently used in our laboratories, both

for monitoring column fractions for sialidase activity in the course of purification of the enzyme, and in screening clinical body fluid samples for alterations in sialidase activity. Sialidase activity can be specifically detected by this assay in crude biological samples, and the procedure of this assay is simpler and safer with higher reproducibility than that of conventional radiochemical methods.

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