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Note

Rapid and sensitive sialidase assay by high-performance liquid chromatography and its application to detection of sialidase in human urine

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Sialidases are widely distributed in microorganisms and animal tissues [1]. The enzyme activity is assayed by measuring sialic acid or aglycone released from the substrate. Sialic acid is determined by the thiobarbituric acid assay [2, 3] or the more sensitive fluorometric thiobarbituric acid assay, which can detect 30 pmol of sialic acid [4]. The method used for determining aglycone depends on the substrate. 4-Methylumbelliferone N-acetylneuraminic acid ketoside has been used as a substrate for a convenient fluorometric assay of the aglycone [5]. However, the activity of sialidase may be affected by the aglycone, 4-methylumbelliferone, which is very bulky and hydrophobic. Modified natural substrates with tritium-labeled aglycones have also been used, and the aglycones released were separated from unhydrolyzed substrates by anion-exchange resin chromatography or thin-layer chromatography and then measured by scintillation spectrometry [6, 7]. Although the radiometric assay can detect amounts of product in the order of 10 pmol [7], the method for separating aglycones from unhydrolyzed substrates is time-consuming and special cautions are necessary on handling radioactive materials. Recently, Koseki et al. [8] used a fluorogenic substrate, α-D-N-acetylneuraminyl-β-D-galactopyranosyl-(1→4)-1-deoxy-1-[(2-pyridyl)-amino]-D-glucitol, which was derived from sialyllactose, for a fluorometric sialidase assay in which the fluorescent aglycone released was separated from the enzymatic reaction mixture by paper electrophoresis and extracted from the paper for quantitative measurement. The substrate is easier to handle than radioactive substrates, but the assay is not so sensitive and is time-consuming. The present report describes an improvement of the method of Koseki et al. using high-performance liquid chromatography (HPLC). Using this method, we detected sialidase for the first time in human urine.

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MATERIALS AND METHODS

Materials

Sialidase from *Clostridium perfringens* (0.5 units/mg) was purchased from P-L Biochemicals (Milwaukee, WI, U.S.A.). N-Acetylneuramin-lactose, consisting of 85% N-acetylneuraminyl-(2→3)-lactose and 15% N-acetylneuraminyl-(2→6)-lactose, was obtained from Sigma (St. Louis, MO, U.S.A.). The substrate in the sialidase assay, α-D-N-acetylneuraminyl-(2→3)-β-D-galactopyranosyl-(1→4)-1-deoxy-1-[(2-pyridyl)amino]-D-glucitol (PA-sialyllactose), was prepared by a slight modification of the method of Koseki et al. [8]. N-Acetylneuramin-lactose (17 mg) was dissolved in 0.7 ml of 2-aminopyridine reagent (prepared by mixing 830 mg of 2-aminopyridine, 320 mg of sodium cyanoborohydride, 0.37 ml of acetic acid, 3 ml of N,N′-dimethylformamide, and 0.3 ml of water), and then the mixture was heated at 80°C for 1.5 h. Water (4 ml) was then added and the mixture was applied to a Sephadex G-15 column (212 × 1.9 cm) equilibrated with 0.01 *M* ammonium bicarbonate and eluted with the same solution. The elution was monitored by the anthrone—sulfuric acid method [9] and by measuring the absorbance at 320 nm due to the pyridylamino derivative of the sugar. The 2→6 isomer of the pyridylamino derivative was eluted slightly faster than the 2→3 isomer. A chromatographically pure substrate was obtained by further purification of the substrate by HPLC in the manner described under "Sialidase assay". β-D-Galactopyranosyl-(1→4)-1-deoxy-1-[(2-pyridyl)amino]-D-glucitol (PA-lactose) and 1-deoxy-1-[(2-pyridyl)amino]-D-glucitol (PA-glucose) were prepared from lactose and glucose, respectively, by the method used for PA-sialyllactose.

Sialidase assay

A mixture of 15 μl of 0.5 m*M* PA-sialyllactose in 0.1 *M* sodium acetate buffer (pH 5.0) and 30 μl of sialidase preparation was incubated at 37°C for an appropriate period. Then 20 μl of the mixture were subjected to HPLC analysis to determine the amount of PA-lactose released by sialidase. The chromatograph used was a Gaschro-Kogyo Model 570B equipped with a stainless-steel column (300 × 4 mm) packed with TSK-Gel LS 410 (5 μm, C_{18}; Toyo-Soda Co, Tokyo, Japan). The detector was an Hitachi fluorescence spectrophotometer, Model 650-10M. The wavelengths of excitation and emission were 320 nm and 400 nm, respectively. Elution was carried out with 0.1 *M* acetic acid at a flow-rate of 1.6 ml/min at room temperature. The amount of PA-lactose released was calculated from its peak area on the chromatogram.

RESULTS AND DISCUSSION

Separation of PA-lactose and PA-sialyllactose by HPLC

PA-lactose and PA-sialyllactose were separated by HPLC under the conditions described under Materials and methods. As shown in Fig. 1, the separation of these substances was complete within 5 min. The elution position of PA-glucose completely overlapped that of PA-lactose under the conditions used. Therefore, sialidase activity can be determined even if the sample con-

tains β-galactosidase. It is possible to determine as little as 0.2 pmol of PA-lactose by this method.

Determination of sialidase

To examine the availability of the present method, we incubated PA-sialyllactose (0.167 m*M*) with various amounts of sialidase (27-133 ng/ml) from *Cl. perfringens* in 0.1 *M* sodium acetate buffer (pH 5.0) at 37°C for 15 min, and then analyzed the reaction mixtures by HPLC as described under Materials and methods. The amount of PA-lactose released was proportional to the amount of the enzyme [PA-lactose released (pmol/ml) = 0.556 × sialidase concentration (ng/ml)]. The sensitivity of the present method for sialidase assay was about two orders of magnitude greater than those of other methods so far reported.

The K_m value of the sialidase for PA-sialyllactose at pH 5.0 and 37°C was calculated to be 50 μ*M* from plots of $\frac{s}{v} - s$ [10]. The enzyme concentration was 0.96 μg/ml, and the substrate concentration was varied from 15 to 74 μ*M*. It is noteworthy that the K_m value was much lower than that for sialyllactose, which was reported to be 2.4 m*M* [11].

Sialidase activity in human urine

This sensitive method for sialidase assay was applied to the detection of sialidase activity in human urine. Urine from an adult person was dialyzed

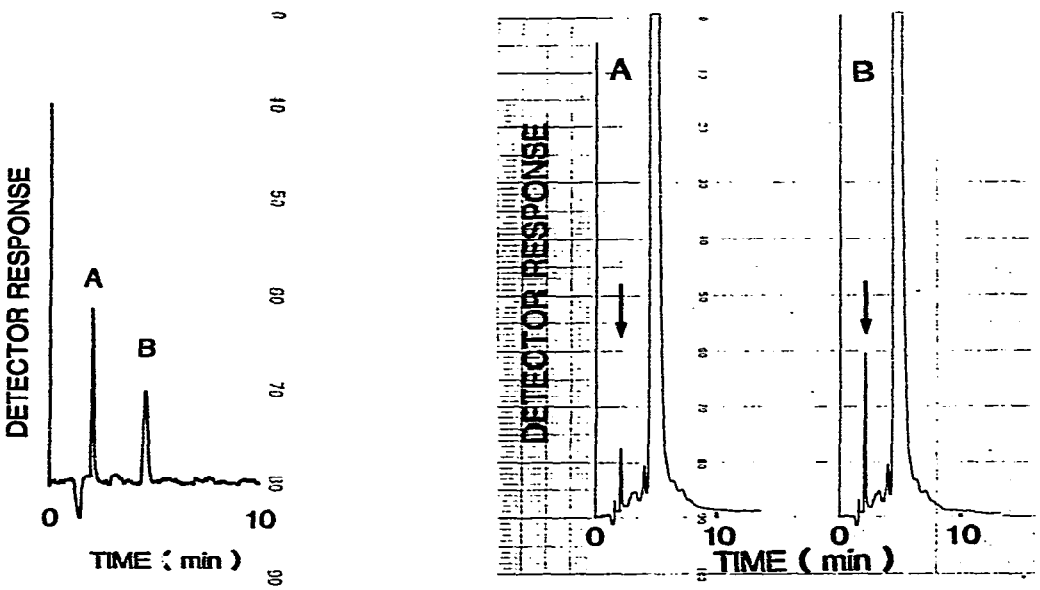

Fig. 1. Separation of PA-lactose and PA-sialyllactose by HPLC. HPLC was carried out as described under Materials and methods. Peaks: (A) PA-lactose (0.6 pmol); (B) PA-sialyllactose (0.6 pmol).

Fig. 2. Chromatogram of the digest of PA-sialyllactose by urine. PA-sialyllactose (0.167 m*M*) was incubated with dialyzed urine in 0.1 *M* sodium acetate buffer (pH 5.0) at 37°C for 2 h. The reaction mixture was analyzed as described under Materials and methods. (A) Substrate + urine heated at 100°C for 10 min (control). (B) Substrate + urine. The arrows show the elution position of PA-lactose.

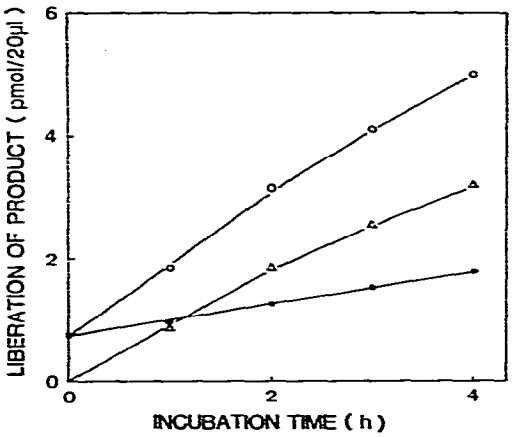

Fig. 3. Time course of hydrolysis of PA-sialyllactose by sialidase in urine. (•; *A*) PA-sialyllactose + urine heated at 100°C for 10 min. (○; *B*) PA-sialyllactose + urine. (△), *B* — *A*.

against 0.1 *M* sodium acetate buffer (pH 5.0) containing 0.0001% sodium ampicillin. The precipitate produced during dialysis was removed by centrifugation and the supernatant was used as enzyme solution. PA-sialyllactose was incubated with the dialyzed urine and the reaction mixture was analyzed as described under Materials and methods. The chromatogram of the reaction mixture after incubation for 2 h is shown in Fig. 2. The time course of the enzymatic reaction is shown in Fig. 3. Control experiments should be carried out because PA-lactose was produced during incubation of the substrate alone for many hours, as shown in Figs. 2A and 3. The decrease in activity during incubation for a long time is probably due to inactivation of the enzyme. The above result indicates the existence of sialidase in human urine, and illustrates the availability of the assay system.

The present assay using HPLC is more rapid and convenient and also more sensitive (1000-fold) than the assay method reported by Koseki et al. [8]. The sialidase assay for urine may be useful for diagnosis and investigation of the diseases associated with the enzyme, for example, sialidosis.

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