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Assay of sialidase activity using ion-exchange chromatography and acidic ninhydrin reaction

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ABSTRACT

A new assay method for sialidase (EC 3.2.1.18) activity using ion-exchange chromatography and acidic ninhydrin reaction has been developed. Fetuin, 4-methylumbelliferyl-N-acetylneuraminic acid (MUB-NANA), gangliosides and N-acetylneuramin-lactose were examined as substrates. Free sialic acid liberated from these substrates by sialidase reaction was isolated with a Dowex 1-X8 column (trifluoroacetate form, 1.5 cm × 0.5 cm I.D.) and determined by acidic ninhydrin reaction. Among the substrates tested, MUB-NANA was the best in the present method. N-Acetylneuramin-lactose could not be used as the substrate, because it was not separated from liberated sialic acid under the conditions used. The recovery of N-acetylneuraminic acid was above 88%, and the sensitivity of the method was 20 nmol in 300 μl of the reaction mixture. The method was applied to the sialidase assay during its purification from rat skeletal muscle, and a Michaelis constant of 1.15 m*M* was obtained with MUB-NANA as the substrate. The method using the acidic ninhydrin reaction was simple and exhibited good reproducibility.

INTRODUCTION

Many assay methods for sialidase (EC 3.2.1.18) activity have been reported. Liberated sialic acids are measured by various colorimetric methods, including the thiobarbituric acid method [1–5]. Methods using tritiated [6–8] and fluorescent [9] sialo compounds as substrates are also employed. The thiobarbituric acid method is perhaps the most used, but it requires several steps and a long period of time. In order to simplify the determination of sialic acid and shorten the time needed, we applied the acidic ninhydrin method [10–12] to the sialidase assay. In the present study, sialic acids formed by sialidase reactions were determined by the acidic ninhydrin reaction [10–12]. As the acidic ninhydrin reagent reacts with bound sialic acids as well as free sialic acids [10,11], the sialic acids liberated by the sialidase reaction were separated on an anion-exchange resin column from the unchanged substrate before the acidic ninhydrin reaction.

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EXPERIMENTAL

Materials

DEAE-cellulose (DE-52) and CM-cellulose (CM-52) were purchased from Whatman (Maidstone, UK). Dowex 1-X8 (100–200 mesh) was from Dow Chemical (Midland, MI, USA). Dimethyl sulphoxide, ninhydrin and Filter Cel were purchased from Wako (Osaka, Japan). Fetuin, N-acetylneuraminic acid (NANA), bovine serum albumin (BSA), 4-methylumbelliferyl-N-acetylneuramic acid (MUB-NANA) (sodium salt), N-

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acetylneuramin-lactose (from bovine colostrum) and gangliosides (type III) were obtained from Sigma (St. Louis, MO, USA).

To modify the acidic ninhydrin reagent 2 [13], 120 ml of dimethyl sulphoxide were used instead of 120 ml of acetic acid [10]. The modified reagent had less odour than the original.

Partial purification of sialidase

Sialidase was partially purified from the skeletal muscle of male Wistar rats according to the method, with some modifications, of Miyagi and Tsuiki [9], which was used for the rat liver enzyme. Rat skeletal muscle (315 g) was homogenized in two volumes of 0.25 *M* sucrose containing 1 m*M* EDTA. The resulting homogenate was mixed with Filter Cel (3.5%, w/v in the homogenizing medium) and centrifuged at 8000 *g* for 30 min. The supernatant was filtered through four layers of gauze and fractionated with ammonium sulphate. The fraction precipitated between 40 and 60% ammonium sulphate saturation was collected by centrifugation and dissolved in 30 ml of 5 m*M* potassium phosphate buffer (pH 6.8) containing 1 m*M* EDTA (buffer A). After dialysis against two changes (2 l each) of buffer A at 4°C for 4 h, the dialysed solution was centrifuged at 12 000 *g* for 15 min. The resulting supernatant was applied to a DEAE-cellulose column (15 cm × 4.2 cm I.D., equilibrated with buffer A) and eluted with 400 ml of the same buffer. The phosphate concentration of the combined solution of the initial effluent and eluate was adjusted to 10 m*M*, and the solution was applied to a CM-cellulose column (15 cm × 1.5 cm I.D.) equilibrated with 10 m*M* potassium phosphate buffer containing 1 m*M* EDTA (pH 6.8). After washing with 50 ml of the same buffer, elution was performed with a linear gradient of phosphate buffer from 10 to 40 m*M*. Fractions containing sialidase activity were combined. Sialidase was precipitated with ammonium sulphate at 60% saturation and stored at −20°C. One unit of sialidase was defined as the amount of enzyme that catalysed the release of 1 nmol of sialic acid per min.

Assay of sialidase activity

The standard assay mixture, in a final volume of 300 μl, contained 10 μmol of sodium phosphate (pH 6.0), 100 μg of sodium cholate, 200 nmol (as NANA) of substrate and enzyme solution. The incubation was carried out at 37°C for 120 min and was terminated by the addition of 750 μl of 99.5% ethanol. After centrifugation at 1200 *g* for 10 min, the supernatant was applied to a Dowex 1-X8 column [1.5 cm × 0.5 cm I.D., trifluoroacetate (TFA) form]. The column was washed with 4 ml of water and eluted with 3 ml of 0.1% TFA followed by 2 ml of 2 *M* hydrochloric acid containing 50% ethanol (HCl–ethanol). When the concentration of MUB-NANA used as the substrate was more than 300 nmol per 300 μl of the reaction mixture, a Dowex 1-X8 column (2.0 cm × 0.7 cm I.D., TFA form) was used, and 5 ml of water and 4.5 ml of 0.1% TFA were used for washing and elution, respectively. Aliquots of the eluate were used for the determination of liberated sialic acid.

Routinely, 1.5 ml of the eluate were mixed with 1.5 ml of the modified acidic ninhydrin reagent in a test-tube (10 cm × 1.2 cm), and heated for 10 min in a boiling water-bath. After cooling in tap water, the absorbance at 470 nm was measured. The sensitivity of the modified reagent to NANA was not different from that of the original acidic ninhydrin reagent 2.

Liberated sialic acid was also determined by the thiobarbituric acid reactions, according to the method of Warren [3].

Determination of protein concentration

The protein concentration was determined by the biuret method [14] and by the method of Lowry *et al.* [15].

RESULTS AND DISCUSSION

Fig. 1A shows the elution profiles of free NANA normal human serum and MUB-NANA through a column of Dowex 1-X8 (1.5 cm × 0.5 cm I.D., TFA form). The human serum proteins were washed out by water, and free NANA was eluted with 0.1% TFA. MUB-NANA was not

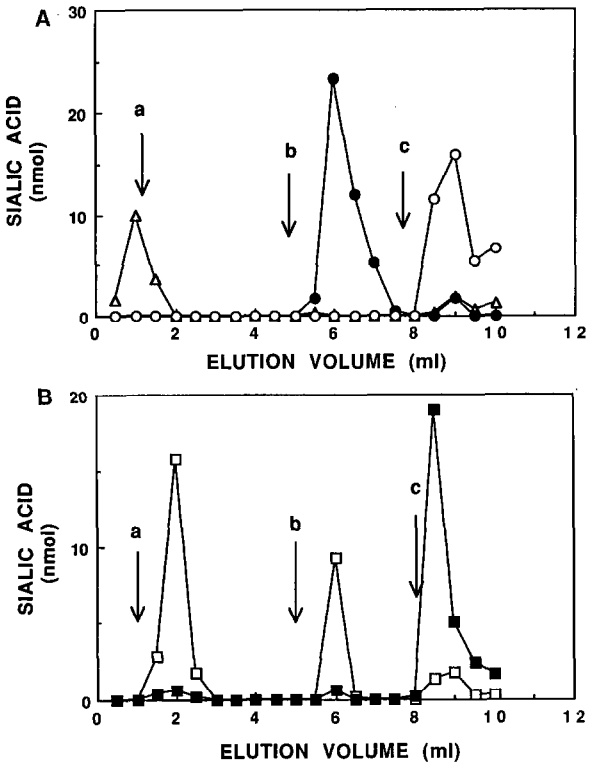

Fig. 1. Elution profiles of (A) N-acetylneuraminic acid (NANA), 4-methylumbelliferyl-N-acetylneuraminic acid (MUB-NANA) and human serum, and (B) fetuin and gangliosides, from a Dowex 1-X8 column (1.5 cm × 0.5 cm I.D., TFA form). NANA (●, 50 nmol), MUB-NANA (○, 50 nmol), human serum (△, 3.0 mg of protein), fetuin (□, 125 μg of protein) or gangliosides (■, 50 μg), dissolved in 1.0 ml of 10 m*M* potassium phosphate buffer (pH 6.0) containing 100 μg of sodium cholate, was applied to the column. Elution was performed successively with 4 ml of water, 3 ml of 0.1% TFA and 2 ml of 2 *M* HCl–ethanol. Fractions of 0.5 ml were collected. Each fraction was treated with 500 μl of acidic ninhydrin reagent as described under Experimental. Arrows indicate the positions of the start of elution with (a) water, (b) 0.1% TFA and (c) 2 *M* HCl–ethanol.

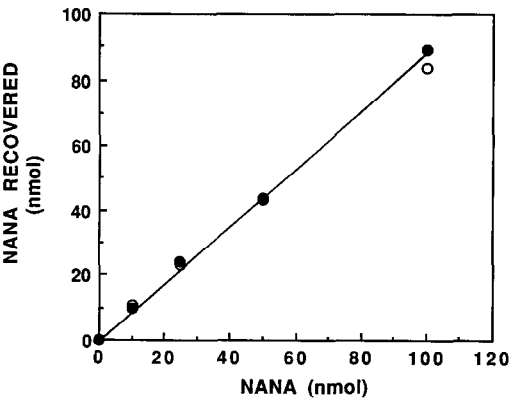

Fig. 2. Standard curve of NANA, added to the standard assay mixture instead of the substrate and processed according to the standard assay procedures. After incubation at 37°C for 120 min, ethanol (99.5%, 750 μl) was added to the incubation mixture. Then it was centrifuged at 1200 *g* for 15 min. The resulting supernatant was applied to Dowex 1-X8 (1.5 cm × 0.5 cm I.D., TFA form). NANA was determined in the eluate by the acidic ninhydrin reaction (○), and in the supernatant (before application to the Dowex 1-X8 column) by the thiobarbituric acid method (●).

eluted with 0.1% TFA, but it was eluted with HCl–ethanol. Thus, free NANA was separated from MUB-NANA and human serum proteins.

Fig. 1B shows the elution profiles of gangliosides and fetuin from the same Dowex 1-X8 column. Fetuin was washed out with water. However, as shown in Fig. 1B, an appreciable amount of NANA was liberated from fetuin during the chromatographic procedures and eluted with 0.1% TFA. Free sialic acids are also detected in

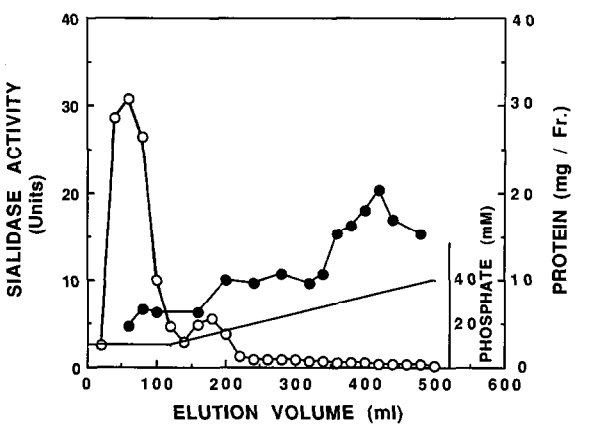

Fig. 3. Elution profile of sialidase from a CM-cellulose column. A sialidase fraction obtained by DEAE-cellulose column chromatography was applied to a CM-cellulose column (15 cm × 1.5 cm I.D.) equilibrated with 10 m*M* potassium phosphate buffer. (pH 6.8) containing 1 m*M* EDTA. Fractions of 20 ml were collected, and the sialidase activity was assayed by the acidic ninhydrin reaction using MUB-NANA as the substrate. Protein contents were measured according to Lowry *et al.* [15]. (○) Protein concentration; (●) sialidase activity.

TABLE I

PURIFICATION OF SIALIDASE FROM RAT SKELETAL MUSCLE

Purification of sialidase from 315 g rat skeletal muscle is summarized in Experimental. Sialidase activity was assayed using the acidic ninhydrin method.

Step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)
1. Crude extract	5273	7919	0.67	100
2. Ammonium sulphate (40–60%)	703	949	0.74	13.3
3. DEAE-cellulose column chromatography	521	614	0.85	9.9
4. CM-cellulose column chromatography	503	15	33.56	9.5

aged solutions of sialo compounds, such as serum, fetuin and gangliosides, even when stored at −20°C. Gangliosides were not eluted with 0.1% TFA, but were eluted with HCl–ethanol. The elution pattern of N-acetylneuramin-lactose through the Dowex 1-X8 column was the same as that of free NANA under the present conditions (data not shown). These findings show that free sialic acids can be separated from these sialic acid-containing substrates, except for N-acetylneuramin-lactose. Results also show that MUB-NANA is a better substrate than the others used in this investigation, because it is more stable during storage and chromatography.

Fig. 2 shows the standard curves of NANA measured by the acidic ninhydrin and thiobarbituric acid reactions. The recovery of NANA applied to the Dowex 1-X8 column was 88.2 ± 5.8% in eight separate experiments determined by the acidic ninhydrin reaction, and this value was comparable with that determined by the thiobarbituric acid reaction.

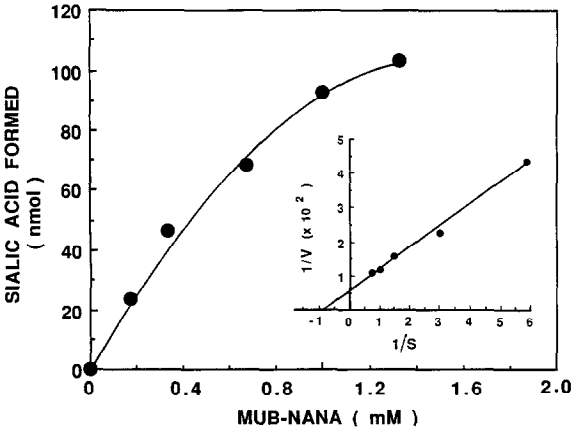

Fig. 4. Effect of MUB-NANA on sialidase activity from rat skeletal muscle. Step 3 of the purification in Table I was used as the enzyme solution, and MUB-NANA as the substrate. The inset shows the Lineweaver–Burk plot.

The present method was applied to the assay of sialidase during its purification from rat skeletal muscle. Fig. 3 shows an elution profile of sialidase from a CM-cellulose column (15 cm × 1.5 cm I.D.). The sialidase activity was assayed by the acidic ninhydrin method using MUB-NANA as the substrate. Sialidase was eluted at phosphate concentrations between 30 and 40 m*M*. Table I summarizes the results of the purification of sialidase from rat skeletal muscle.

The effect of the MUB-NANA concentration on the activity of sialidase from rat skeletal muscle was studied using the present method and the enzyme preparation of step 3 in Table I. As shown in Fig. 4, Michaelis–Menten kinetics were observed. The Michaelis constant (K_m) obtained from the Lineweaver–Burk plot was 1.15 m*M*. This value was close to 0.67 m*M* obtained for sialidase from rat liver [9]. The thiobarbituric acid method for the determination of sialic acid is able to determine only free sialic acid in the presence of both free and bound sialic acids. However, some bound sialic acids are liberated in the heating step, mostly sialic acid from MUB-NANA. Therefore, the separation of liberated sialic acid from the remaining substrate is necessary, as performed in the present method. As de-

scribed above, the procedure for the separation of free sialic acid is simple, and the acidic ninhydrin reaction is very simple and time-saving compared with the thiobarbituric acid reaction, and thus the present sialidase assay method gives reproducible results.

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