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A NEW ENZYMATIC METHOD FOR THE DETERMINATION OF SIALIC ACID IN SERUM AND ITS APPLICATION FOR A MARKER OF ACUTE PHASE REACTANTS

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INDEXING WORDS

serum sialic acid; enzymatic substrate assay; acute phase protein; myocardial infarction

SYNOPSIS

An enzymatic method for the measurement of sialic acid in serum is described. Neuraminidase [sialidase, acylneuraminyl hydrolase, EC 3.2.1.18] from Arthrobacter ureafaciens is used for the enzymatic hydrolysis of sialyl compounds. In the presence of N-acetyl neuraminic acid (NANA) aldolase [N-acetyl neuraminate pyruvate-lyase, EC 4.1.3.3], extracted from E. coli, free sialic acid is cleaved to N-acetyl-D-mannosamine and pyruvate, then pyruvate is reduced to lactate by coexisting lactic acid dehydrogenase (LDH) and NADH. A fixed time (two point) measurement is made by measuring the delta absorbance change in 15 min.

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Sensitivity and precision were excellent and linearity extends to 200 mg/dl of sialic acid concentration. The mean value of serum sialic acid of normal adults was 37.8–64.2 mg/dl with no difference between males and females. Good correlation between sialic acid concentration and α_1-acid glycoprotein or α_1-antitrypsin was observed.

Serum sialic acid concentration of the patients with acute myocardial infarction had kept high levels after the attack with no relation to released enzymes. A positive correlation was found for acute phase proteins and sialic acid. The measurement of sialic acid in serum seems to provide a rapid and simple index as acute phase reactants.

INTRODUCTION

Sialic acid, a family of acylated derivatives of neuraminic acid, is widely distributed in mammals and usually occures as a terminal component at the non-reducing end of carbohydrate chains of glycoproteins and glycolipids.[16] In human serum a large quantity of sialic acid is involved in α_1-acid glycoproteins, α_1-antitrypsin, haptoglobin, ceruloplasmin, fibrinogen, C-reactive protein (CRP) and complement proteins. Some of these sialyl compounds are called "acute phase reactants" which rapidly increase after onset of some diseases. The useful determination of protein-bound sialic acid in serum was reported in various diseases.[20] A number of techniques were available for the determination of sialic acid.[3, 7, 13, 21, 27] For clinical purposes colorimetric methods are commonly used such as thiobarbituric acid (TBA) method[10] and periodate-resorcinol method,[17] but they are time consuming and are not specific for sialic acid. In TBA method there are interfering substances such as 2-deoxyribose and lipids bearing unsaturated fatty acids.[26] For periodate-resorcinol method, some hyperlipidemic sera may induce turbidity in color reaction giving high optical density.[17] The enzymatic method which is sensitive and specific for sialic acid was developed by Roseman and his co-workers.[5, 7] In this method, N-acetylneuraminic acid is cleaved by NANA-aldolase to N-acetyl-D-mannosamine and pyruvate. This method, however, has not yet been applied to the clinical field because of the insufficient supply of NANA-aldolase.

Recently three of the authors purified two enzymes, sialidase and NANA-aldolase, from bacterial cultures.[23, 25] By using these enzymes, we have developed an enzymatic method determining bound form sialic acid. This paper also describes the application of this assay for the measurement of sialic acid in human body fluids. It is shown that this

method provides usefulness for screening the acute phase reactant proteins in some diseases.

MATERIALS AND METHODS

Enzyme Preparation

Arthrobacter ureafaciens sialidase was prepared as previously described.[23] NANA-aldolase was obtained from E.coli grown on NANA as reported.[25]

Enzymatic Assay Procedure

The standard assay system contained the following components in a total volume of 3.0 ml, 50 μl of sample (up to 200 mg/dl sialic acid concentration) or standard solutions, 200 μl of sialidase (1 u/ml), 10 μl of LDH (1000 u/ml), 100 μl of NADH (5 mM) and 2440 μl of phosphate buffer (40 mM, pH 7.7). The preincubation was conducted at 37°C for 120 min. During this time, N-acetylneuraminc acid was liberated and the endogenous pyruvate was eliminated. The coupled enzymatic reaction was started by the addition of 200 μl of NANA-aldolase (5 u/ml). The decrease in absorbance at 366 nm due to the oxidation of NADH was followed for 15 min using the Eppendorf 1100 spectrophotometer with a 1 cm light path cuvettes. The amount of sialic acid was calculated from the decrease in optical density compared to that of standard solution. The same components except for the sample or NANA-aldolase were used as a reagent or sample blank. One unit of enzyme activity was defined as the amount that released 1 μmol of substrate per min under the reaction condition used.

Colorimetric Assay Method for Sialic Acid

Free sialic acid was determined by modified Aminoff's method.[23]

Estimation of Other Serum Proteins

α_1-acid glycoprotein, haptoglobin, α_2-macroglobulin, transferrin, α_1-antitrypsin and CRP were estimated by the laser nephelometry method (Boehring Laser Nephelometer, Boehring Institute).

Reagents

LDH from pig heart and β-NADH was obtained from Oriental Yeast Company (Tokyo). Sialyllactose (N-acetyl-neuraminosyl-D-lactose) was obtained from Boehringer Mannheim Yamanouchi Company (Tokyo). N-acetylneuraminic acid (NANA, sialic acid) was obtained from Nakarai Chemicals Company (Kyoto).

RESULTS

Effects of pH and Buffer Concentration

The optimal pH of sialidase measured by Aminoff's method was 5.5, whereas that of NANA-aldolase was 7.7 as shown in Fig. 1(a). Since there was no marked depression of sialidase activity at around pH 7.7, we carried out the coupled enzyme reaction at this pH. The relationship between phosphate buffer concentration and reaction rate is shown in Fig. 1(b). This result demonstrates that reaction rate is maximum around 40 mM, showing gradual decrease with increasing buffer concentration.

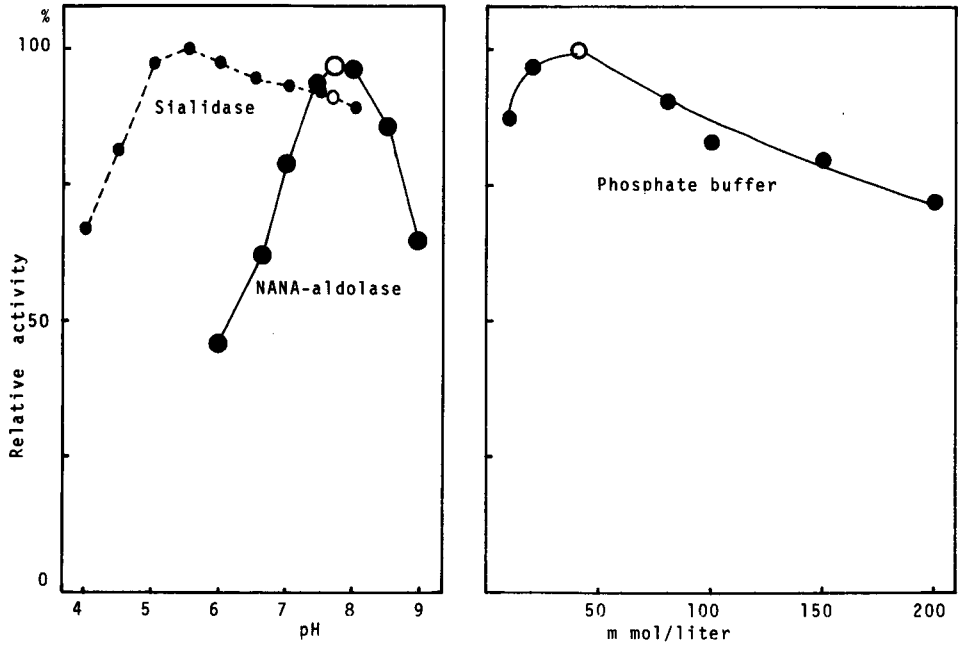

Fig. 1 Effect of pH and buffer concentration.

Effect of Sialidase Concentration on Liberation of Sialic Acid

The time course of the liberation of sialic acid by sialidase were followed with human serum and sialyl-lactose as a substrate (Fig. 2). The release of sialic acid increased with increasing addition of sialidase. When 0.06 u/cuvette sialidase was used, as much as 95% of sialic acid of human serum was liberated within 120 min incubation. If 0.2 u of sialidase was used, sialic acid was nearly completely liberated within 60 min incubation time.

Effect of NANA-aldolase Concentration

The rate of reaction increased with the increasing addition of NANA-aldolase. Fig. 3 shows that the reaction did not reach to the end points within 30 min unless such a large amounts of aldolase as 4 units were used. However it is also important to reduce the assay time and the amount of enzyme since the enzyme is rather expensive. In the enzymatic assay procedure the fixed time assay is often employed instead of the end point assay to save the enzymes and the analysis time.[22]

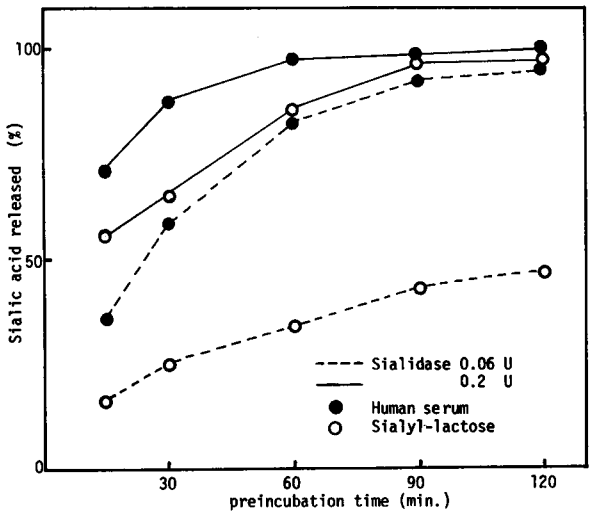

Fig. 2 Effect of sialidase concentration on liberation of sialic acid.

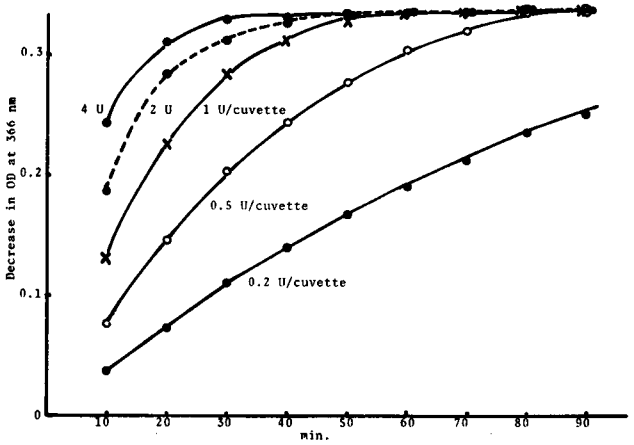

Fig. 3 Effect of NANA-aldolase concentration.

Calibration Curve

Known amounts of sialic acid were allowed to react under the standard assay condition and the decrease in optical density at a fixed time (5,10,15 min) were plotted against the sialic acid concentration (Fig. 4). A linear relationship existed up to the sialic acid concentration of 200 mg/dl at 5, 10, and 15 min incubation time. Delta optical density at 366 nm in such a short time as 5 min was good enough for calculating the corresponding concentration of sialic acid.

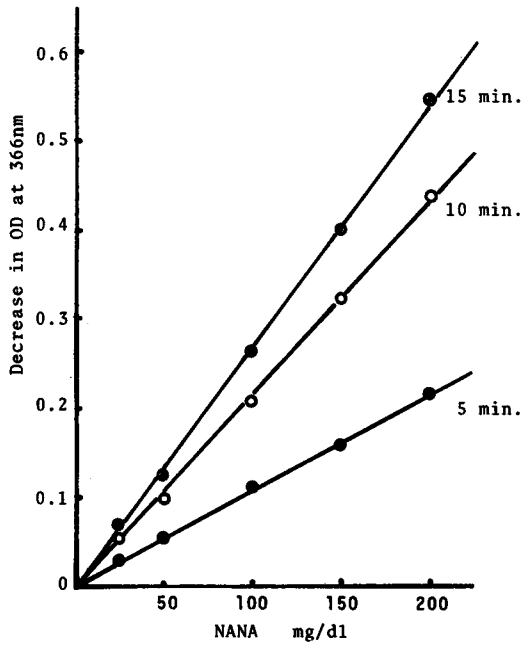

Fig. 4 Standard curve for determination of sialic acid by the enzymatic method.

Precision

In order to study the reproducibility of this method, experiments were repeated 15 times with two kinds of sera containing high and low amount of sialic acid. The good reproducibility was obtained as shown in Table 1. Coefficient of variation were 1.3 to 1.6%. Day to day precision with standard pool serum was below 5%.

Sialic Acid Concentration of Normal Human Serum

The normal level of sialic acid content in human serum was determined in samples of venous blood obtained from normal adults subjects.

Table 1. Reproducibility.

(1)	38 mg/dl	(2)	69 mg/dl
	38		70
	37		73
	37		73
	38		72
	37		71
	37		71
	38		70
	37		70
	37		70
	37		71
	37		71
	37		71
	37		70
	37		71
$\bar{x}$	37.3		70.9
SD	0.48		1.13
CV	1.3%		1.6%

The average sialic acid content of serum, from 9 control male individuals (of 22 to 52 years of age) was ranged from 42.1 to 59.2 with a mean of 49.6 ± 6.5 (SD) mg/dl. The average sialic acid contents obtained from 12 females individuals (of 23 to 46 years of age) was 52.4 ± 6.7 with a range from 45.7 to 66.0.

Correlation between Serum Glycoproteins and Serum Sialic Acid Concentration

The correlation between sialic acid and glycoproteins in serum such as α_1-acid glycoprotein, haptoglobin showed a positive correlation but α_2-macroglobulin, transferrin showed a negative correlation (Fig. 5). Especially α_1-acid glycoprotein and sialic acid showed the best coefficient correlation of 0.9175.

Glycoproteins and Sialic Acid Concentration Patterns in Acute Myocardial Infarction

It is well known that the acute myocardial infarction elevates the

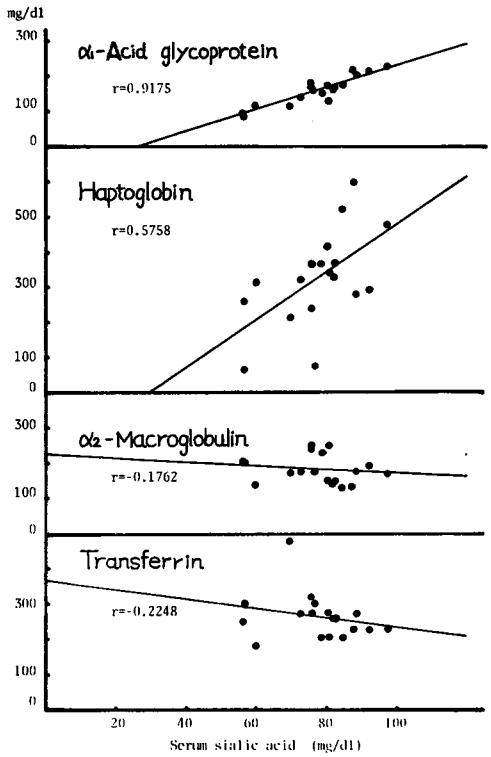

Fig. 5 Correlation between serum glycoprotein level and serum sialic acid concentration.

levels of serum enzymes and acute phase reactant proteins. Fig. 6 shows the changes of sialo-glycoproteins and sialic acid after acute myocardial infarction. Among the enzymatic activities, CPK and GOT activities reached maxima at second day after the episode of infarction and restored to normal levels at the 5th day (not shown). In contrast to these observations, acute phase reactant proteins changed with no relation to the released enzymes. CRP rapidly increased and then gradually decreased to normal levels. α_1-acid glycoprotein and α_1-antitrypsin reached maxima at 14th day after the episode and gradually returned to normal levels. **Sialic acid changed in parallel with these glycoproteins.**

DISCUSSION

A new fully enzymatic method for the determination of sialic acid in the body fluid is described using recently prepared enzymes, sialidase [23, 24] and NANA-aldolase. [25] Roseman and his co-workers [5, 7] had

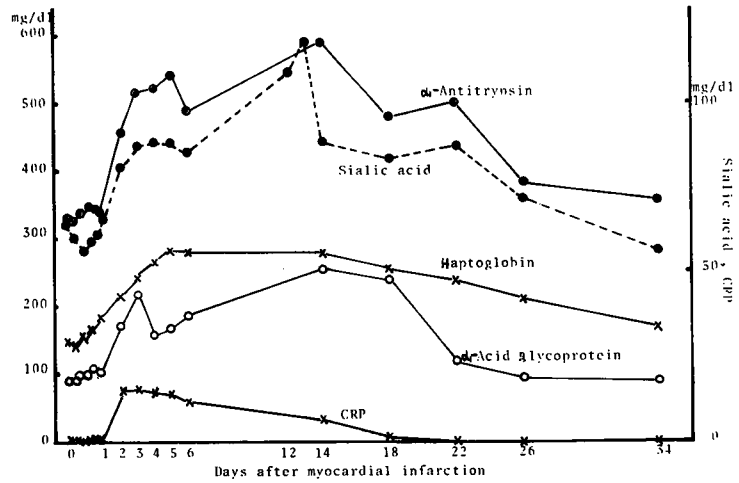

Fig. 6 **Glycoprotein and sialic acid patterns of a 61-year-old man with acute myocardial infarction.**

already described an enzymatic method for quantification of free sialic acid based on the cleaving of N-acetylneuraminic acid by NANA-aldolase to pyruvate and the corresponding N-acetyl-mannosamine. But the assay of this procedure must be preceded by acid or enzyme treatment to hydrolyze the ketosidic bond to get a free sialic acid.

The optimal pH of A. ureafaciens sialidase was 5.6 and those of the other bacterial sialidase lie generally in the range of pH 4.5 to 6.5. [9] However pH optima and the pH activity profiles vary with the substrate. [24] The pH activity profile of A. ureafaciens enzyme with serum as a substrate gave broad optimum range from 4.5 to 8.0 (Fig. 1). The same result was reported by Uchida et al. with bovine submaxillary mucin as a substrate. [24] This makes the coupling reaction proceed in the single buffer system of pH 7.7, optimum pH of NANA-aldolase.

E.coli NANA-aldolase, purified by ion-exchange column chromatography followed by gel filtration, contained no NADH oxidase. [25] The pH profile of this enzyme was similar to those previously reported with the enzymes from Cl. perfringens [8] and other sources. [4, 7] The reaction rate of NANA-aldolase was inhibited by high concentration of phosphate buffer (Fig. 1) and also of Tris buffer (not shown). The alteration of enzymatic activities caused by strong electrolyte in buffer were reported by Barton et al. with Cl. perfringens sialidase [2] but hitherto not described with NANA-aldolase. [5, 7, 8, 15]

The fixed time assay (two point measurement) is often used in enzy-

matic analysis.[22] Advantages of this analysis method are in shortening the analysis time and in reducing the amount of enzymes to be used. Calibration curves made by this fixed time absorbance changes as a function of sialic acid concentration passed through the zero point and is linear up to the sialic acid concentration of 200 mg/dl (Fig. 4).

Changes in the levels of plasma sialoglycoproteins have been reported in a variety of diseases.[16, 20] Sialic acid levels in human serum in a normal and pathological condition have been studied extensively. Rey[17] reported a fully automated micromethod for determining sialic acid in biological fluids and also described the simultaneous determination of sialic acid and protein in erythrocyte membrane from diabetic patients.[15]

Silver et al.[19] reported that serum sialic acid concentration apparently bears a relationship to tumor burden and can provide a method for monitoring the therapy in individual patients similar to the carcinoembryonic antigen (CEA) assay. Plasma proteins whose concentration alter following trauma (surgery, myocardial infarction) or tissue necrosis of inflammation are known as acute phase reactant proteins. The production of these proteins increases in individuals subjected to these stresses. The sialic acid contents is proportional to that of normal glycoproteins. Good correlation was found between sialic acid concentration and α_1-acid glycoprotein or α_1-antitrypsin (Fig. 5). The measurement of sialic acid seems to provide a more rapid and simple index as acute phase reactants. It is proposed, however, that the patients with chronic diseases such as Hodgkin's disease, diabetes and psychiaric arthritis have elevated α_1-acid glycoprotein in serum with reduced sialic acid contents.[18] Similar cases were reported in urine of a Hodgkin's disease[1] and in serum of a patient with hepatobiliary dysfunction.[14] The measurement of sialic acid might not be a marker for these desialylated glycoproteins of these patients.

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