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Estimation of sialic acid in a sialoglycan and a sialoglycoprotein by capillary electrophoresis with in-capillary sialidase digestion

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Abstract

An example of application of in-capillary derivatization for CE, obtained by using the throughout-capillary format, is presented. Introduction of a sialoglycan (*N*-acetylneuraminyllactose) or a sialoglycoprotein (bovine serum fetuin) sample to a running buffer (pH 5.0) containing *N*-acetylneuraminidase followed by application of a voltage resulted in the release of *N*-acetylneuraminic acid (NANA) which could be estimated by CE with UV detection. Two-step application of voltages (5 and 20 kV) was proved to be more effective for rapid estimation of the released NANA. This format (modified throughout-capillary format) allowed differential estimation of the NANA present in the sample as an impurity and the NANA released from the substrate at the picomol level, and thereby reliable micro assay of the sialidase activity. It also allowed estimation of the rate constant of this enzymatic reaction.

Keywords: In-capillary digestion; Sialic acid; Sialoglycans; Sialoglycoproteins; Glycoproteins; Proteins; Sialidase; Acetylneuraminic acid; Acetylneuraminyllactose; Fetuin; Carbohydrates; Organic acids

1. Introduction

Capillary electrophoresis (CE) is a powerful analytical tool owing to its high capabilities in separation and detection. This method is also attractive because of other inherent advantages. We have been engaging in method development for biomedical analysis based on utilizing these inherent advantages. We would like to focus here on in-capillary derivatization based on the single phase property of CE.

The Regnier's group proposed a concept of electrophoresis-mediated microanalysis (EMMA) [1] and reported a series of works on this line. We have also independently performed a series of work on ''in-capillary derivatization''. Whichever concept is adopted, one can regard the inner space of a capillary as not only a place for separation but also a place where chemical as well as enzymatic reactions occur. Such reactions can convert a solute, not easy to separate from accompanying substances and not sensitively to detect, into a derivative that can easily be separated and sensitively detected.

We have studied the basic features of three types of in-capillary derivatization using a model reaction system composed of an amino acid mixture and *o*-phthalaldehyde. The at-inlet type derivatization [2] is based on standing the segments of a reagent solution and a sample solution adjacent to each other, at the inlet of a capillary, to react them with

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each other by molecular diffusion, followed by capillary electrophoretic analysis of the products by CE. This can most easily be automated. The zone-passing derivatization [3] is a format, in which the segments of both a sample solution and a reagent solution, adjacent to or apart from each other, migrate at different velocities in the electric field, and one of them passes the other. Reaction occurs while both segments are in contact with each other. This type of derivatization may cause different yields of derivatives for sample components, but it allows the fastest analysis and is quite useful for kinetic studies of rapid reactions. The throughout-capillary derivatization [4] is the simplest format consisting in introducing a sample solution into a running buffer containing a reagent. Reaction occurs while the sample migrates through the reagent-containing running buffer and the derivatives move immediately to the detector window. This format is liable to be interfered by high concentrations of the reagent but allows also kinetic studies. The zone-passing and throughout-capillary formats correspond to the transient EMMA and continuous EMMA by the Regnier's group, respectively. The at-inlet format is something like a hybrid of these two methods of EMMA or an advanced format of pre-capillary derivatization. All these formats can rapidly analyze minute amounts of samples.

Thus, each of these three formats of in-capillary derivatization has its own characteristic features and has been applied to suitable types of biomedical analyses. For example, the at-inlet format was applied to ultrahigh sensitive analysis of neurotransmitting amines in a single animal cell by Gilman and Ewing [5] and to automated analysis of amino acids as *o*-phthalaldehyde derivatives by our group [2]. We also reported a special technique of high-temperature at-inlet derivatization, for slow reactions such as the derivatization of reducing mono- and oligosaccharides with 1-phenyl-3-methyl-5-pyrazolone [6]. Application of the zone-passing format for micellar electrokinetic chromatography to in-capillary derivatization of D/L-amino acids was reported by Tivesten [7]. Another application of this format in conjunction with sample stacking was also attempted by Tivesten and Folestad [8]. Since the throughout-capillary format can be realized by the simplest procedure, it was successfully applied to analysis of amino acids [9] and amines [10] with fluorescence detection by Oguri et al.

The present paper gives additional examples of application of the throughout-capillary format, in a modified form, for the determination of products of in-capillary enzymatic reaction. We report herein the efficacy of in-capillary enzymatic reaction by this format by applying to the estimation of sialic acid in a sialoglycan and a sialoglycoprotein using sialidase. We also demonstrated the usefulness of this technique for simultaneous estimation of free and conjugated NANAs which can be done in a single run. The sialylation and desialylation of glycoconjugates are one of the recent topics in glycobiology as the on/off switching mechanism to control glycoprotein–protein binding [11]. Therefore, the method development presented here may contribute to stimulate related researches in this rapidly growing field.

2. Experimental

2.1. Materials

The sialidase used in this work was *N*-acetylneuraminidase from *Arthrobacter ureafaciens*. It was obtained from Nakalai Tesque (Nakakyo-ku, Kyoto, Japan) and used as obtained. The samples of *N*-acetylneuraminyllactose (NANA-Lac, a mixture of 3′- and 6′-*N*-acetylneuraminyllactoses) and bovine serum fetuin (BSF) as substrates were from Sigma (St. Louis. MO, USA) and Gibco BRL (Rockville, MD, USA), respectively, and they were used without further purification. The authentic specimen of *N*-acetylneuraminic acid (NANA) was also from Sigma. All chemicals used were of reagent grade. They were obtained from the following sources: acrylamide from Seikagaku Kogyo (Chuo-ku, Tokyo, Japan), acetic acid, sodium hydroxide and ammonium peroxodisulfate from Wako (Chuo-ku, Osaka, Japan), methacryloxypropyltrimethoxysilane (MOPTMS) from Shin-etsu Kagaku (Chiyoda-ku, Tokyo, Japan), acetonitrile and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) from Nakalai, tri-(hydroxymethyl)aminomethane (Tris) from Sigma. Deionized and glass-ware distilled water was used for preparing running buffers and rinsing capillaries.

2.2. Coating of capillaries

The inner wall of capillaries was coated basically according to the procedure of Baba et al. [12], briefly as follows. A piece of a 50-μm I. D. fused-silica capillary from Polymicro Technologies (Phoenix, AZ, USA) was rinsed with 1 *M* sodium hydroxide, water and acetonitrile in this order commonly for 15 min. An acetonitrile solution containing MOPTMS and acetic acid to a common concentration of 0.4% (v/v) was flushed through the capillary for 1 h, then the capillary was rinsed with water for 15 min followed by acetonitrile for 15 min. An acrylamide solution, prepared by adding 16 μl of 10% (v/v) TEMED and 4 μl of 10% (w/v) ammonium peroxodisulfate to 1 ml of a 3% (w/v) acrylamide solution containing Tris (50 m*M*), boric acid (50 m*M*) and EDTA (2.5 m*M*), was flushed through this silanized capillary for 10 min for polymerization, and the capillary was allowed to stand for further 30 min for completion of polymerization. All the processes of flushing the reagent solutions and rinsing the capillary were carried out by the flushing mode of the introduction system of the apparatus used.

2.3. CE

CE was performed using a model 270A capillary electrophoresis system of Applied Biosystems, equipped with a vacuum introduction device, a UV detector and a capillary oven thermostated by circulating air. A linear polyacrylamide-coated capillary (50 cm×50 μm I.D.) was installed onto the apparatus, and the capillary oven was kept at 37±0.1 °C. The capillary was rinsed with the electrophoretic solution for 5 min before each run. Detection was carried out by monitoring the absorption at 200 nm.

2.4. In-capillary digestion with sialidase

The electrophoretic solution was prepared by adding sialidase to 50 m*M* acetate buffer (pH 5.0) to a concentration of 250 mU/ml and degassed before use. A capillary was filled with this sialidase-containing electrophoretic solution, and a sample solution of NANA-Lac or BSF in 50 m*M* acetate buffer (pH 5.0) was introduced from the cathodic end of the capillary. In the established procedure two voltages (5 kV followed by 20 kV) were applied stepwise at various intervals between both ends of the capillary.

2.5. Pre-capillary digestion (reference experiment)

A sialidase solution (500 U/ml) in 50 m*M* acetate buffer (pH 5.0) was added to an equivalent volume (10 μl) of NANA-Lac (5 mg/ml) or BSF (10 mg/ml) solution in the same buffer. The mixture was incubated at 37 °C for 20 h for digestion, then subjected to NANA analysis by CE.

3. Results and discussion

3.1. Matching of conditions between enzymatic reaction and electrophoretic separation

Basically the throughout-capillary format was adopted in the present work, because the enzymatic reaction to be employed is not so fast and needs rather long reaction time for completion. The zone-passing format is outside consideration for this reason. The at-inlet format may also be applicable, but total analysis time is generally longer than in the throughout-capillary format. In the throughout-capillary format the conditions for the enzymatic reaction should be identical with those for the separation of the products by CE in order to obtain reproducible results.

In the present work, the hydrolytic release of NANA from a sialoglycan (NANA-Lac) with sialidase (*Arthrobacter N*-acetylneuraminidase) was adopted as a model system. The optimum pH of this sialidase was reported to be 5~5.5 [13], but it varies with substrates (for example, 4.5~7.0 for bovine submaxillary mucin; 4.3~4.8 for colominic acid). From this reason pH 5.0, which may cover most siloglycans and sialoglycoproteins, was adopted in the present work.

3.2. Electropherogram of the in-capillary released NANA

Based on the pH optimization strategy mentioned above a model experiment of the in-capillary digestion of NANA-Lac with sialidase was performed by

the throughout-capillary format at pH 5.0. A sufficiently high concentration of sialidase should be used for such in-capillary technique to ensure rapid release of NANA. Therefore, a 250-mU/ml concentration was employed, which permits the release of $2.5 \cdot 10^{-7}$ mol of NANA per ml from a substrate in 1 min (for the definition of activity unit please see Section 3.6). Since the minimum detectable concentration of NANA under the conditions employed was ca. 25 μg/ml ($8.1 \cdot 10^{-8}$ mol/ml), this sialidase concentration was enough for rapid release of NANA. A few kinds of buffers can be chosen as media for the enzymatic reaction and separation by CE. In the present case 50 m*M* acetate buffer was selected, because it generated relatively low electric current (~10 μA), causing no enzyme inactivation by Joule heating. Fig. 1 compares the electropherograms of NANA-Lac in the absence and presence of sialidase.

Fig. 1a shows an electropherogram of NANA-Lac obtained in 50 m*M* phosphate buffer (pH 5.0) not containing sialidase. The large broad peak between 40 and 50 min is of NANA-Lac. The broadness of the peak is probably because the NANA-Lac sample used was a mixture of positional isomers having the NANA residue at the 3′- and 6′-positions of lactose.

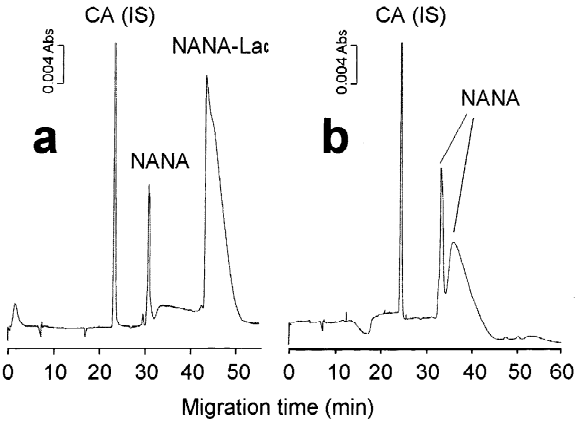

Fig. 1. Analysis of NANA-Lac in the absence (a) and presence (b) of sialidase. Capillary, polyacrylamide-coated fused-silica (50cm ×50 μm I.D.); electrophoretic solutions, 50 m*M* acetate buffer (pH 5.0) (a) or the same buffer containing sialidase (250 mU/ml) (b); applied voltage, 5 kV; detection, UV absorption at 200 nm; temperature, 37 °C; sample, NANA-Lac (8 mg/ml) in 50 m*M* acetate buffer (pH 5.0). CA=Cinnamic acid.

The sharp peak at 23 min is from cinnamic acid as internal standard. A relatively small sharp peak was also observed at ca. 31 min, which was due to NANA present in the sample as an impurity. This is obvious because the intensity of this peak increased by co-migration with the authentic specimen of NANA (figure not shown). Analysis of a pre-capillary digestion mixture of NANA-Lac also gave a sharp peak of NANA, similarly to the pre-capillary incubation mixture of BSF shown later in Fig. 3c. This is another evidence that the sharp peak was due to NANA present in the sample solution. When sialidase was added to the running buffer, the NANA-Lac peak disappeared and instead a different broad peak appeared between 30 and 40 min (Fig. 1b). This was due to the NANA formed by in-capillary digestion during application of voltage. Since the mobility of NANA was higher than that of NANA-Lac under the conditions employed, the peak of the NANA released by the digestion with sialidase was detected at a shorter migration time than that of NANA-Lac. The peak of NANA as an impurity was not substantially affected by the addition of sialidase, giving its sharp peak only slightly retarded at ca. 33 min. Thus, two kinds of NANA peaks were observed in this throughout-capillary format. The broadening of the peak of the newly formed NANA reflects the time course of the release of NANA from NANA-Lac. The sialidase *from Arthrobacter ureafaciens* employed in this work is known to catalyze hydrolytic release of NANA from both positional isomers of NANA-Lac, but the activities to these isomers are known to be different between the isomers. This might be another reason of the broadness of the peak of the newly formed NANA.

3.3. Effect of applied voltage

Although the NANA-Lac peak was no longer detected in Fig. 1b under the conditions employed (5 kV), we could observe the strong influence of applied voltage on the enzymatic reaction. Fig. 2 compares the in-capillary digestions at 10 and 15 kV.

The lower voltage (10 kV) (Fig. 2a) allowed longer reaction time, thus giving a large NANA peak (peak top at ca.17 min) and a small NANA-Lac peak (at ca. 23 min). The higher voltage (15 kV) (Fig. 2b)

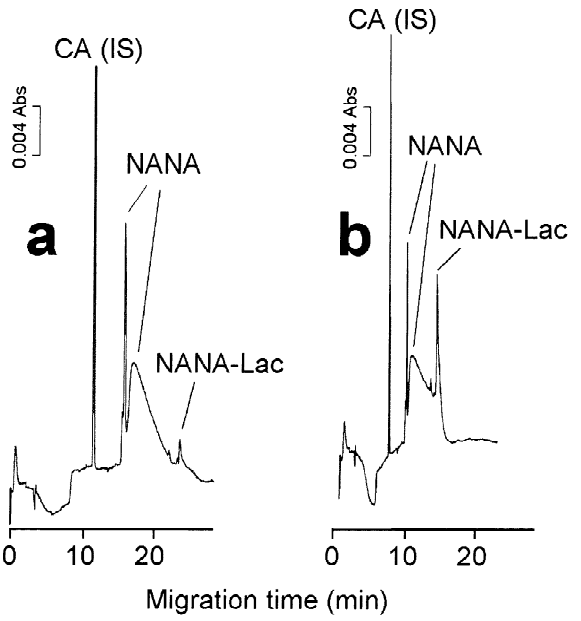

Fig. 2. Effect of applied voltage. Electrophoretic solutions, 50 m*M* acetate buffer (pH 5.0) containing sialidase (250 mU/ml); applied voltage, 10 kV (a) or 15 kV (b). The other conditions as in Fig. 1.

caused less complete digestion, the NANA and NANA-Lac peaks being smaller and larger, respectively, than in Fig. 2a. Their migration times were naturally shorter than those in Fig. 2a because their velocities were faster.

The results shown in Figs. 1 and 2 indicate the preponderant effect of applied voltage in this throughout-capillary format of in-capillary reaction. Lower voltages were obviously more favorable for digestion, but required longer analysis times. The lower limit that allowed complete release of NANA was 5 kV, as seen from Fig. 1b. However, the total analysis time at 5 kV was as long as 45 min.

In order to reduce analysis time we applied voltages at two-steps. For example, we analyzed NANA-Lac for 20 min at 5 kV, then the voltage was switched to 20 kV to accelerate the appearance of the NANA peak at the detector window. The total analysis time was reduced to ca. 25 min. Under these conditions the NANA in NANA-Lac was completely released and its peak was separated from the peak of the NANA as an impurity.

3.4. Estimation of NANA in NANA-Lac and BSF

Under the optimized conditions mentioned above (5 kV/20 kV program) the content of NANA was estimated by the throughout-capillary format of in-capillary digestion with sialidase. Good linearity ($y=3.55\cdot10^{5}x+6.17\cdot10^{3}$, $R=1.000$) was observed for 0.025~10.0 mg/ml of the authentic specimen of NANA. Although the peak of the authentic specimen of NANA was sharp like that of NANA as an impurity (Figs. 1 and 2) different from the released NANA, we dared to estimate the content of the released NANA by using this calibration curve.

Since the authentic specimen of NANA-Lac was extremely hygroscopic, it was difficult to weigh exact amounts of NANA-Lac, the obtained value (30.1%, w/w%) of the NANA content in NANA-Lac was considerably smaller than expected. However, it was in good agreement with the value obtained after pre-capillary digestion (30.4%). The NANA content of BSF was also estimated by the same method. Fig. 3a shows the electropherogram of BSF in the absence of sialidase.

A huge peak of BSF was observed after 26 min, but the peak of the NANA as an impurity (at ca. 22 min) was small. In the presence of sialidase (Fig. 3b), however, the peak of released NANA was observed at ca. 24 min just after the voltage change. The peak of desialylated BSF was not visible within 30 min, because it reduced negative charge due to the removal of the NANA residue. The NANA content estimated by using the calibration curve mentioned above was 6.9%. The NANA content was also estimated after pre-capillary digestion (Fig. 3c). It should be noted that in Fig. 3c the initial BSF concentration was one fourth of the concentration of BSF in Fig. 3a and b. The obtained value was 6.6%. The consistence of the values of NANA obtained by the throughout-capillary format of in-capillary derivatization with those obtained by pre-capillary digestion, in both NANA-Lac and BSF, means that the calibration curve can be applied to both types of NANA, almost indifferent to peak shape.

Thus, it was demonstrated that the throughout-capillary format of in-capillary reaction could easily estimate the NANA content in a sialoglycan and a sialoglycoprotein. It should be noticed that this method allowed the estimation of the released

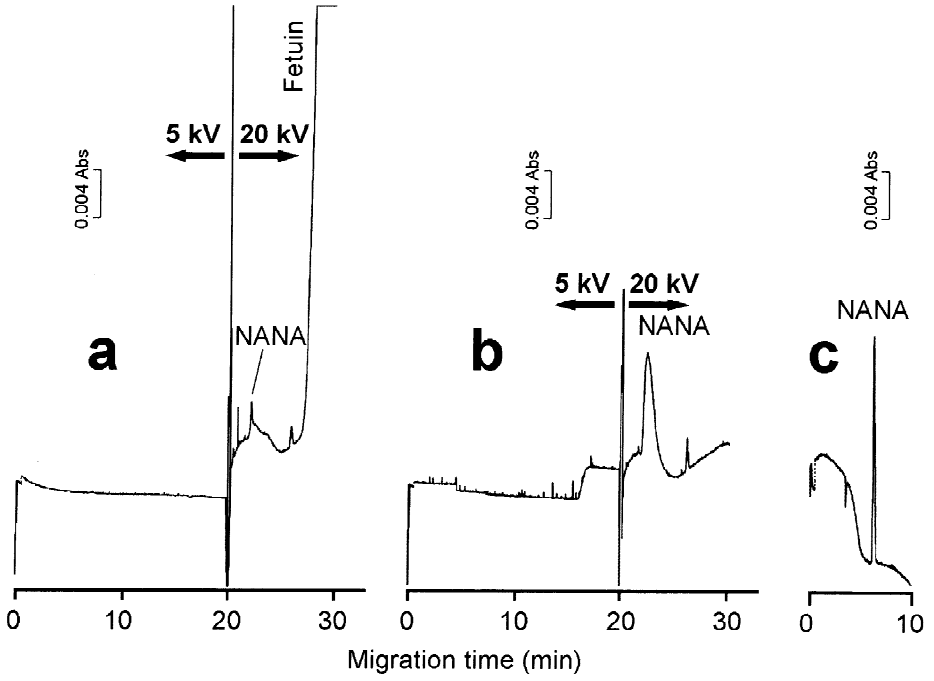

Fig. 3. Analysis of NANA in BSF by the modified throughout-capillary format of in-capillary reaction in the absence (a) and presence (b) of sialidase. Electrophoretic solutions, 50 m*M* acetate buffer (pH 5.0) containing sialidase (250 mU/ml); applied voltage, 5 kV (20 min) followed by 20 kV (10 min); sample, BSF (20 mg/ml) in 50 m*M* acetate buffer (pH 5.0). Analysis of NANA in BSF after pre-capillary digestion with sialidase (c). Sample, BSF (initial concentration, 5 mg/ml) digested with sialidase (250 mU/ml) for 20 h at 37 °C in 50 m*M* acetate buffer (pH 5.0); applied voltage, 20 kV. The other conditions as in Fig. 1.

NANA from a minute amount of sample at the picomol level. It is also important that it was free from the interference from the NANA present in sample as an impurity, since both types of NANA were clearly separated from each other. This characteristic feature of the present method will also be useful for the studies of the function of NANA, since it allows simultaneous estimation of free and conjugated NANAs in biological samples. Successive analyses of NANAs in intact and sialidase-digested samples might also estimate the amounts of the free and total NANAs, respectively. The conjugated NANA can be quantified by subtracting the amount of the free NANA from that of the total NANA. This has been a much more popular strategy for such studies but requires two operations that are tedious, time-consuming, and may cause additional errors. The present method does not need such dual operations.

3.5. Kinetic study

It is an interesting feature of this format that reaction time can be controlled by changing applied voltage, and kinetic study is possible by utilizing this characteristic. Fig. 4 shows an electropherogram of NANA-Lac obtained by the stepwise application of 5 kV/20 kV.

When the lower voltage was applied for 10 min, the enzymatic reaction did not complete as observed from the peak of the remaining NANA-Lac (at ca. 17 min). The magnified figure in the inset clearly shows the two peaks of NANA, the first (peak 1) and the second (peak 2) peaks being of the contaminant and newly formed NANA, respectively. The shape of peak 2 changed as the duration of lower voltage application increased. At first it formed a heavy tailing peak, but it became symmetric after 20 min, the duration for complete release of NANA. The

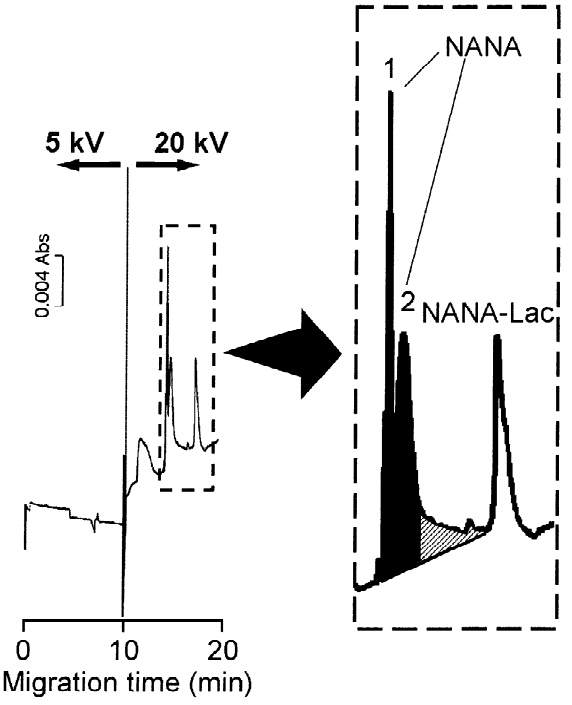

Fig. 4. Estimation of the NANA released from NANA-Lac by the modified throughout-capillary format of in-capillary digestion with sialidase with two-step application of voltages. Electrophoretic solutions, 50 m*M* acetate buffer (pH 5.0) containing sialidase (250 mU/ml); applied voltage, 5 kV (10 min) followed by 20 kV; sample: NANA-Lac (5 mg/ml) in 50 m*M* acetate buffer (pH 5.0). The other conditions as in Fig. 1. The inset shows the magnification of the framed portion. The front area in peak 2 (indicated in black) corresponds to the amount of NANA formed at 5 kV, and the rest of the area (indicated in slanted lines) that formed at 20 kV.

heavy tailing was due to the additional formation of NANA at 20 kV. Therefore, the area of the front part of peak 2 cut out by the tangential line of the rear downhill of peak 2 (indicated in black) corresponded to the NANA released during the application of 5 kV, while the rest of the area in the rear part of peak 2 (indicated by slanted lines) was due to the additionally released NANA upon application of the higher voltage of 20 kV. Since the duration of lower voltage application is freely changeable, the black peak area, accordingly the estimated concentration of NANA released at 5 kV (*C*, mg/ml) could be correlated to the duration of this lower voltage application (*t*, in min). The $C-t$ plot showed the change of the concentration of NANA released at 5 kV as time elapsed. We could observe that this plot gave an almost straight line ($C=0.063\ t-0.10$, $R=0.986$), and zero order rate constant could be estimated as 63 $\mu g\ ml^{-1}\ min^{-1}$ from the slope of this straight line.

3.6. Sialidase activity

The sialidase activity is defined as the amount of sialidase that releases 1-μmol amount of NANA from NANA-Lac in 1 min. We could estimate this value based on the experimental data described in Section 3.5. Since 1.49 μmol amount of NANA was released in 1 min, the activity of the *N*-acetylneuraminidase employed in the present work was estimated to be 65 U/mg. This was considerably lower than the value (85 U/mg) reported by the supplier. This difference was presumably due to partial deactivation during storage.

Since the proposed method can be done in micro scale, it will be useful for sialidase activity assay for small amounts of biological samples.

4. Concluding remarks

We presented herein examples of application of the two-step voltage throughout-capillary format of in-capillary derivatization for enzyme (sialidase) activity assay, based on the CE estimation of NANA formed by in-capillary digestion. We optimized analytical conditions and demonstrated that this method allowed the estimation of NANA formed by sialidase digestion of a sialoglycan and a sialoglycoprotein without interference from NANA present in samples in a minimum analysis time among the complete digestion-type methods (excluding the initial rate methods). It does not require blank experiments by which the amount of the NANA present as an impurity can be subtracted. Since the sample scale was quite low (at the picomol level), this method will be widely applied to sialidase assay for glycobiology research. It may also be useful for simultaneous estimation of free and conjugated NANAs in biological samples. The obtained data will provide important information on the function of NANA in many biological phenomena. We are extending application studies of the proposed method

and the results will appear elsewhere. The usefulness of this method for kinetic study of this enzymatic reaction was also demonstrated. This advantage will benefit studies on the mechanism of enzymatic reactions for not only sialidase but also many other enzymes.

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