Cat. # 4400

For Research Use

TaKaRa

Sialic Acid Fluorescence Labeling Kit

Product Manual

v201611Da

Table of content

I. Description

The fluorescence labeling of sialic acid with 1, 2-diamino-4, 5-methyleneoxybenzene (DMB) is a simple and very sensitive analytical method [1, 2] (Figure 1). In this method, free sialic acids are analyzed by means of reverse-phase HPLC after labeling with DMB. The sialic acids which are bound to sugar chain in glycoproteins or glycolipids can also be measured after pre-treatment with sialidases or acid hydrolysis. The detection limit of DMB-sialic acid is 57 fmol [1, 2]

The Sialic Acid Fluorescence Labeling Kit is designed to simplify the DMB-derivatization procedure. The reagents are provided in aqueous solutions and things to do are only mixing of reagents and incubation.

By combination of this DMB method with Pyridylamino (PA) derivatization method which is well known as a high sensitive analysis method for sugar chains, sialoglycoconjugates can be analyzed with extremely high sensitivity.

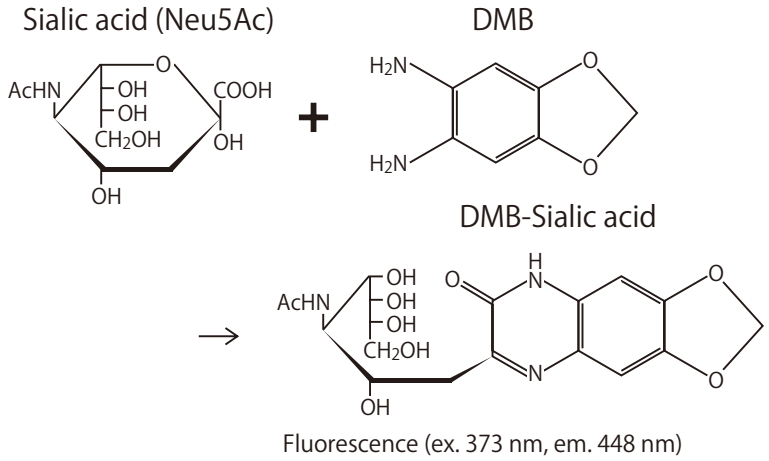

Figure 1. Fluorescence labeling of sialic acid with DMB

II. Components (100 assays)

1. DMB Solution *	1 ml x 2
2. Coupling Solution (Containing acetic acid and sodium hydrosulfite)	5 ml x 2
3. Neu5Ac (100 μM)	500 μl

* DMB Solution may be colored. It has been confirmed that this would not affect on product performance.

III. Storage

-20℃ (Store DMB solution protected from light.)

VI. Procedure

1. Prepare samples containing free sialic acids (ranged from 5 pmol to 5 nmol) in 1.5 ml tubes with screw cap.
   * Volume of samples must be kept below 50 μl. Since the labeling reaction proceeds under acidic condition, the pH of the sample solution must be kept neutral or acidic.
   * In a control reaction using Neu5Ac (100 μM), perform the reaction using 2 - 20 μl of Neu5Ac per a reaction, and then analyze 10 μl (10 - 100 pmol) of the reaction mixture.

2. Prepare the mixed solution by the ratio of Reagent 1 (DMB Solution), Reagent 2 (Coupling Solution) and H_2O=1 : 5 : 4.
   * This mixed solution is stable at least for a week at 4℃ .

3. Add 200 μl of the mixed solution to the sample and mix well.

4. Incubate at 50℃ for 2.5 hours under protection from light.

5. Cool (about 5 min) on ice to terminate the reaction.

6. Analyze 10 μl of reaction mixture by reverse-phase HPLC.
   (excitation wavelength : 373 nm, emission wavelength : 448 nm)
   The analysis should be performed at same day.
   * YMC-Pack ODS-A (YMC; Cat. #AA12S05-2546WT) etc. can be used.

7. Identify and calculate the quantities of sialic acids within the sample from calibration curve based on the peak height obtained with the defined sialic acids standard.

V. Experiment Example

Example 1: Analysis of standard PA-sugar chains derived from glycoprotein

(1) Release of sialic acid (Neu5Ac) by partial acid hydrolysis
Heat each 2.5 μl (approx. 25 pmol) of PA-Sugar Chain 021, 023, 024, and 025 in 50 μl of 0.05 N HCl at 80℃ for 1 hour.

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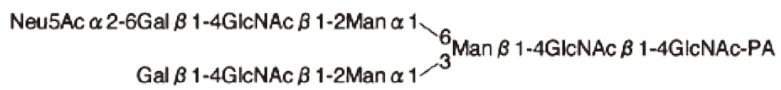

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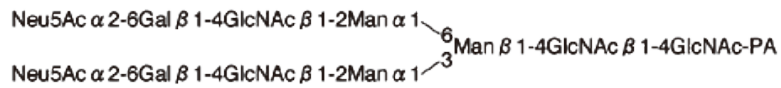

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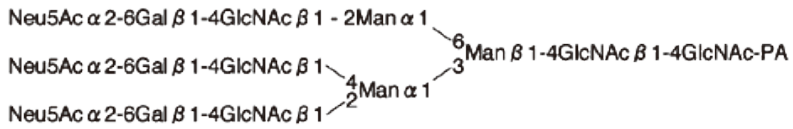

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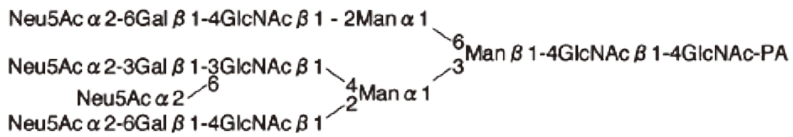
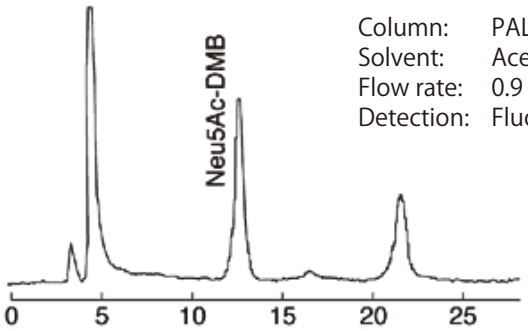


(2) Release of sialic acid (Neu5Ac) with sialidase.
Using PA-Sugar Chain 021, 023, 024, and 025 (2.5 μl each, approximately 25 pmol) as substrates, 100 mU of *Arthrobacter ureafaciens* sialidase was added to the substrates in 50 μl of a 50 mM sodium acetate buffer solution (pH 5.0), and each mixture was incubated at 37℃ for 30 minutes. Then, each mixture was heated at 100℃ for 3 minutes to terminate the enzyme reaction.

(3) Fluorescence labeling of free sialic acid with DMB and HPLC analysis.
DMB labeling was carried out using the sample obtained at the above (1) or (2), in accordance with the kit's protocol.
10 μl of each sample was analyzed by means of HPLC (e.g. Figure 2 shows the chromatogram of PA-Sugar Chain 021).
The peak height of each sample was compared with the peak height of the standard sialic acid which were simultaneously analyzed with the samples in order to quantitate the sialic acid in the samples. The results are shown in Table 1.



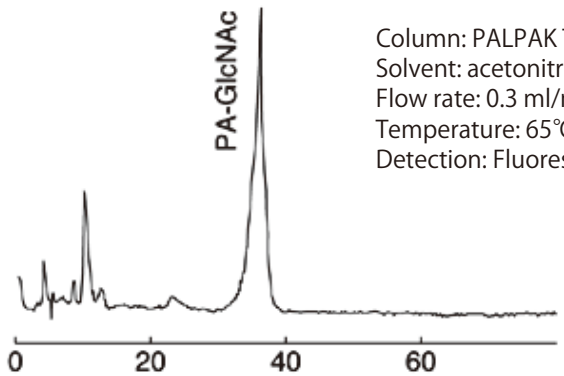
Column: PALPAK Type R (4.6 mm φ x 250 mm)*
Solvent: Acetonitrile/methanol/H_2O=9/7/84 (v/v/v)
Flow rate: 0.9 ml/min
Detection: Fluorescence (ex. 373 nm, em.; 448 nm)
* discontinued

Figure 2. HPLC analysis of DMB-labeled release sialic acid from PA-Sugar Chain 021

(4) Quantification of PA-GlcNAc at the reducing end of PA-sugar chains.
PA-Sugar Chain 021, 023, 024, and 025 (5.0 μl each, approximately 50 pmol) were poured into individual glass tubes and dehydrated. 50 μl of 5.7 N hydrochloric acid (HCl) that has a constant boiling point was added to each tube. The tubes were vacuum-sealed. Next, the tubes were heated at 100℃ for 16 hours to effect acid hydrolysis. The tubes were opened and the mixture in each tube was dehydrated. Small volume of water was added and each mixture was boiled in order to completely remove HCl. Freshly prepared saturated $NaHCO_3$ was added in 48 μl to each tube to dissolve the residue. After the pH of each mixture was confirmed to be slightly alkaline, 2 μl of acetic anhydride was added and mixed well. After left at room temperature for 15 minutes, a further 48 μl of saturated $NaHCO_3$ and 2 μl of acetic anhydride were added. Each mixture was mixed well and left at room temperature for 30 minutes to effect *N*-acetylation. 20 μl of each acetylated mixture (the sample) was analyzed (Figure 3 shows the chromatogram of PA-Sugar Chain 021).
The peak height of each sample was compared with the peak height of the standard PA-GlcNAc (that was analyzed at the same time as the samples) in order to quantify PA-GlcNAc at the reducing terminal. The results are shown in Table 1. The number of moles of the conjugated sialic acid per mole of sugar chains is obtained by dividing the amount of the sialic acid (obtained in (3)) by the amount of PA-GlcNAc (Table 1).

Table 1. Number of the conjugated sialic acid per sugar chain

PA-Sugar Chain	Neu5Ac(pmol/μl)		PA-GlcNAc (pmol/μl)	The numbers of the conjugated sialic acid per sugar chain	
	Acid hydrolysis	Enzyme hydration		Acid hydrolysis	Enzyme hydration
021	13.0	12.9	11.6	1.1	1.1
023	25.0	26.1	11.2	2.2	2.3
024	59.7	60.8	18.6	3.2	3.3
025	58.9	65.0	14.7	4.0	4.4



Column: PALPAK Type A (4.6 mmφ x 250 mm)＊
Solvent: acetonitrile/0.7 M H_3BO_3-KOH (pH 9.0)=1/9 (v/v)
Flow rate: 0.3 ml/min
Temperature: 65℃
Detection: Fluorescence (ex. 310 nm, em. 380 nm)
＊ discontinued

Figure 3. HPLC Analysis of the reducing terminal from PA-Sugar Chain 021

Example 2: Analysis of *N*-linked sugar chains derived from bovine fetuin

(1) Analysis of *N*-linked sugar chains
N-linked sugar chains were obtained from bovine fetuin by means of hydrazinolysis and *N*-acetylation. The sugar chains were fluorescence labeled with 2-aminopyridine using *GlycoTAG*, and were analyzed by HPLC (ion-exchange mode) using PALPAK Type N.* Figure 4 shows the chromatogram of *N*-linked sugar chains. As seen in the figure, asialo, monosialo, disialo, trisialo, and tetrasialo fractions were obtained.

* discontinued

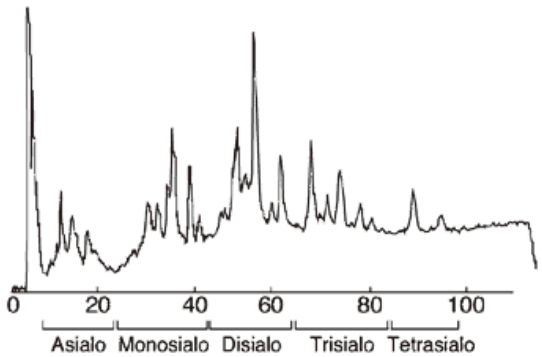


Figure 4. HPLC analysis of *N*-linked sugar chains from bovine fetuin

(2) Quantifying the sialic acid in each fraction
Approximately 100 pmol of each fraction (obtained in (1)) was heated in 50 μl of 0.05 N HCl at 80℃ for 1 hour in order to release the sialic acid by means of partial acid hydrolysis. The sialic acid was DMB-labeled in accordance with the kit's protocol and then analyzed with HPLC.
The results are shown in Table 2.

(3) Quantification of PA-GlcNAc at the reducing terminal in each fraction
In accordance with the method described above ((4) in Example 1: Analysis of standard PA-sugar chains derived from glycoprotein), approximately 100 pmol of each fraction was acid-hydrolyzed and *N*-acetylated. The amount of PA-GlcNAc at the reducing terminal was quantified by means of HPLC analysis. The numbers of the conjugated sialic acid per sugar chain in each fraction were obtained by dividing the amount of sialic acid by the amount of PA-GlcNAc; they were 0.16, 0.98,1.86, 2.86, and 4.26 (Table 2).

Table 2. Numbers of the conjugated sialic acid per *N*-linked sugar chain derived from bovine fetuin

	Neu5Ac (pmol/μl)	PA-GlcNAc (pmol/μl)	The numbers of the conjugated sialic acid per sugar chain
Asialo fraction	1.01	6.41	0.16
Monosialo fraction	8.23	8.45	0.98
Disialo fraction	26.36	14.20	1.86
Trisialo fraction	18.15	6.34	2.86
Tetrasialo fraction	7.10	1.67	4.26

Example 3: Analysis of standard PA-sugar chains derived glycolipid

Sialic acids are normally linked to complex carbohydrates, through α-linkage which is unstable in acid. If they are linked to the terminal of sugar chain, they can be easily separated by heating the complex carbohydrate in 50 mM HCl at 80℃ for 1 hour.[8] However, it is well known that the sialic acid that is linked through the structure {-GalNAc β 1 - 4 (Neu5Ac α 2-3) Gal-} is difficult to hydrolyze due to the steric hindrance with *N*-acetylgalactosamin. This linkage structure is seen in glycolipid GM1. Such a sialic acid is not released by the normal enzyme amount of *Clostridium perfringens* or *Vibrio cholerae* sialidase (specific to α-2,3 and α-2,6 linkages). Even in the case of partial acid hydrolysis of such a sialic acid in PA-sugar chains (GM1-PA), only 80% of the sialic acid is released after 1 hour of heat treatment, as shown in Figure 5. The figure also shows that almost 100% of the sialic acid is released after 3 hours of heat treatment.

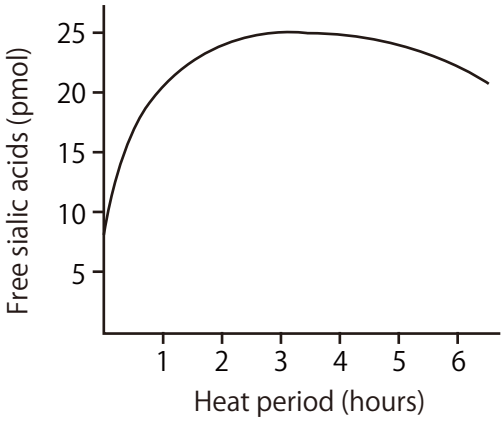


Figure 5. The amount of free sialic acid after heating of 50 mM HCl (50 μl) containing 25 pmol GM1-PA at 80℃
(measured by means of DMB labeling)

(1) Desialylation condition

[I. Partial acid hydrolysis condition]

PA-Sugar Chain 029, 032, 033 (discontinued), 035, 036, and 037 (2.5 μl each, approximately 25 pmol) were incubated under conditions A, B, and C, described below.

A: 50 μl of 50 mM HCl, 80℃, 3 hours
B: 50 μl of 2 M acetic acid, 80℃, 3 hours
C: 50 μl of 1 M formic acid, 100℃, 1 hour

DMB labeling was carried out in accordance with the kit's protocol, and the sialic acid was quantified by means of HPLC. The results are shown in Table 3.

[II. Enzymatic hydrolysis]

Each 2.5 μl of PA-Sugar Chain 029, 032, 033, 035, 036, and 037 (approximately 25 pmol each) were incubated in 25 μl of a 50 mM sodium acetate buffer solution (pH 6.0) containing 500 mU of *Arthrobacter ureafaciens* sialidase at 37℃ for 30 minutes. Next, each mixture was heated at 100℃ for 3 minutes to terminate the reaction.

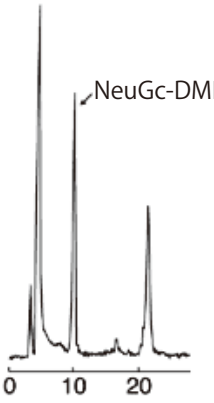
DMB labeling was carried out in accordance with the kit's protocol, and the sialic acid was quantified by means of HPLC. The results are shown in Table 3.

(2) Fluorescence labeling of free sialic acid by DMB and HPLC analysis
DMB labeling was carried out using samples prepared in (1)-I or II in accordance with the kit's protocol. Each sample volume equivalent to 1 pmol of sugar chains was analyzed by means of HPLC (Figure 6 shows the chromatogram of PA-Sugar Chain 029).
The peak height of each sample was compared with the peak height of the standard sialic acids in order to quantify the sialic acid in the samples.

Column: PALPAK Type R (4.6 mmφ x 250 mm)
Solvent: acetonitrile/methanol/H_2O=9/7/84 (v/v/v)
Flow rate: 0.9 ml/min
Detection: Fluorescence (ex. 373 nm, em. 448 nm)

Figure 6. HPLC analysis of free sialic acid from PA-Sugar Chain 029

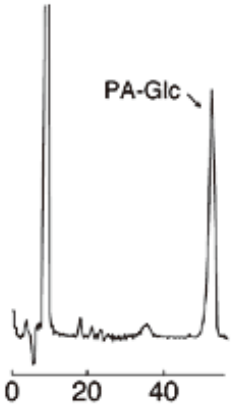
(3) Analysis of NeuGc
2.5 μl of PA-Sugar Chain 030 (approximately 25 pmol) was dehydrated and 50 μl of 50 mM HCl was added. The mixture was heated at 80℃ for 3 hours to release NeuGc. DMB labeling was carried out in accordance with the kit's protocol, and then HPLC analysis was carried out. The results are shown in Figure 7. DMB labeling is useful for analysis of NeuGc, as well as analysis of Neu5Ac.



Column: PALPAK Type R (4.6 mmφ x 250 mm)
Solvent: acetonitrile/methanol/H_2O=9/7/84 (v/v/v)
Flow rate: 0.9 ml/min
Detection: Fluorescence (ex. 373 nm, em. 448 nm)

Figure 7. HPLC analysis of free NeuGC from PA-Sugar Chain 030

(4) Quantification of PA-Glc at the reducing terminal of PA-sugar chains
PA-Sugar Chain 029, 032, 033, 035, 036, and 037 (5.0 μl each, approximately 50 pmol) were poured in individual glass tubes and dehydrated.
50 μl of a mixture of 2 N HCl/2 N TFA was added to each tube. The tubes were vacuum-sealed. Next, the tubes were heated at 100℃ for 6 hours to effect acid hydrolysis. The tubes were opened and the mixture in each tube was dehydrated. A little water was added and each mixture was boiled. 25 μl of water was added to each tube to dissolve the residue. 5 μl of each mixture (sample) was analyzed by means of HPLC under the conditions shown in Figure 3.
Figure 8 shows the chromatogram of PA-Sugar Chain 032. The peak height of each sample was compared with the peak height of the standard PA-Glc (which was analyzed at the same time as the samples) in order to quantify PA-Glc at the reducing terminal. The results are shown in Table 3.



Column: PALPAK Type A (4.6 mmφ x 250 mm)
Solvent: acetonitrile/0.7M H_3BO_3-KOH (pH 9.0)=1/9 (v/v)
Flow rate: 0.3 ml/min
Temperature: 65℃
Detection: Fluorescence (ex. 310 nm, em. 380 nm)

Figure 8. HPLC analysis of PA-Glc from PA-Sugar Chain 032

(5) The amount of the conjugated sialic acid per sugar chain
The number of moles of the conjugated sialic acid per mole of sugar chain can be obtained by dividing the amount of the sialic acid (obtained in (2)) by the amount of PA-Glc at the reducing terminal (Table 3). Table 3 shows that PA-Sugar Chain 032, 033, 036, and 037, which have the structure {-GalNac β1 - 4 (-Neu5Ac α2 - 3) Gal-}, tended to have less moles of the conjugated sialic acid than PA-Sugar Chain 029 and 035, which do not have such a structure. The results of partial acid hydrolysis (A: 50 mM HCl, 80℃, 3 hours) were good.

Table 3. Number of the conjugate sialic acid per sugar chain

PA-Sugar Chain	I. Sialic acid amount released by partial acid hydrolysis (pmol/μl)			II. Sialic acid amount released by digestion with sialidase (pmol/μl)	(4) Amount of reducing end PA-Glc. (pmol/μl)	Numbers of the conjugated sialic acid per sugar chain.			
	A	B	C			A	B	C	Digestion with sialydase.
029	13.88	13.05	12.02	13.24	13.71	1.01	0.95	0.88	0.97
032	9.11	6.01	6.20	6.16	8.62	1.06	0.70	0.72	0.71
033	26.38	22.12	24.76	20.36	13.08	2.02	1.69	1.89	1.56
035	31.83	30.75	30.24	30.19	5.77	2.02	1.95	1.92	1.91
036	37.00	35.37	36.35	33.05	13.93	2.66	2.54	2.61	2.37
037	50.19	46.99	48.86	45.02	13.76	3.65	3.41	3.55	3.27

VI. Application Example

Study of protocol optimized for accurate and quick quantification of many samples.

In manufacturing and analysis sites of pharmaceuticals or foods that contain sialic acids (e.g. erythropoietin in pharmaceuticals, milk oligosaccharides in food industry), it is necessary to quantify sialic acid in many samples, precisely and rapidly. A protocol suitable for this purpose is shown.

[HPLC conditions]
Based on a pilot study, the column temperature was set at 40℃, and a gradient elution method was adopted using Solvent A (methanol/water=7/93, v/v), and Solvent B (acetonitrile/methanol=93/7 v/v). In analyzing a large number of samples, a reagent-derived peak is eluted very late. So a broad peak sometimes appeared in a chromatogram may obstruct the analysis. Therefore, the column was washed using the elution of Solvent B in each analysis.

In addition, in order to correct the injection volume of the samples by an auto-injector, one of sialic acids, 3-deoxy-D-glycero-D-galacto-2-nonulosonic acid (keto-deoxy-nonulosonic acid, KDN[9]; Toronto Reseach Chemicals Inc.), was used as the internal standard.
KDN has a very similar structure to *N*-acetylneuraminic acid (NANA) and *N*-glycolylneuraminic acid (NGNA) (Figure 9), thus its reactivity against DMB is assumed to be almost the same.

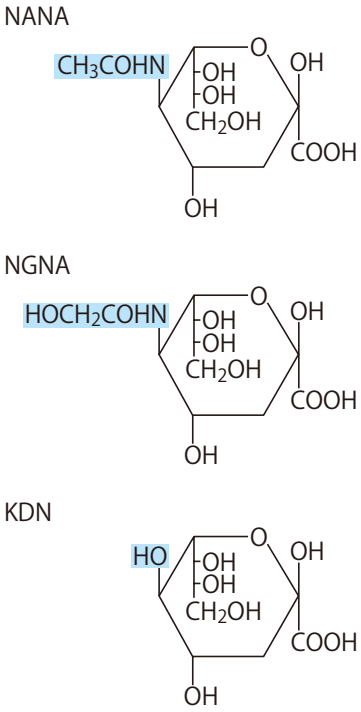
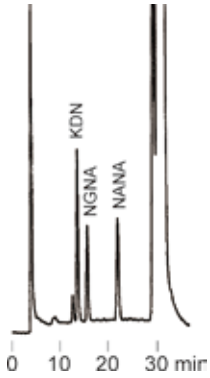


Figure 9. Structure of sialic acid

[Experiment 1: Time courses of the DMB labeling of various sialic acids]
200 μl of the labeling reagent mixture of this kit was added to 10 μl of an aqueous solution containing each 10 pmol of NANA, NGNA, and KDN. The mixture was incubated at 50℃ in a dark place. The mixture was sampled in 0.5, 1.25, 2.5, and 5.0 hours after the incubation (reaction), and 10 μl of each sample (476 fmol of each type of sialic acid) was analyzed by means of HPLC (Figure 10).



Column:	PALPAK Type R (4.6 mmφ x 250 mm)
SolventA:	methanol/H_2O=7/93 (v/v)
SolventB:	acetonitrile/methanol=93/7 (v/v)
Elution:	0 - 25 min, 5% Solvent B
	25 - 35 min, 100% Solvent B
	35 - 50 min, 5% Solvent B (re-equilibration)
Flow rate:	1 ml/min
Temperature:	40℃
Detection:	Fluorescence (ex. 373 nm, em. 448 nm)

Figure 10. Isolation of DMB labeled sialic acid

Results
Each sialic acid (NANA, NGNA, and KDN) showed a clear peak at a different time under the conditions described in Figure 10. After column wash using Solvent B in 10 minutes, contaminants which were observed as a large peak near 30 minutes were removed. The time course of the DMB reaction in each type of sialic acid is shown in Figure 11 (The peak heights at specified times were plotted assuming that the peak height of each DMB-labeled sialic acid at 2.5 hours after the reaction was 1.) The time courses of NANA, NGNA, and KDN were all similar one another. This suggests that KDN has the same reactivity to DMB as NANA and NGNA do, and that KDN is a valid internal standard for sialic acid analysis using the DMB reaction method.

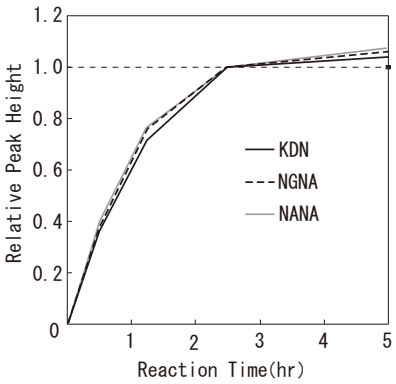
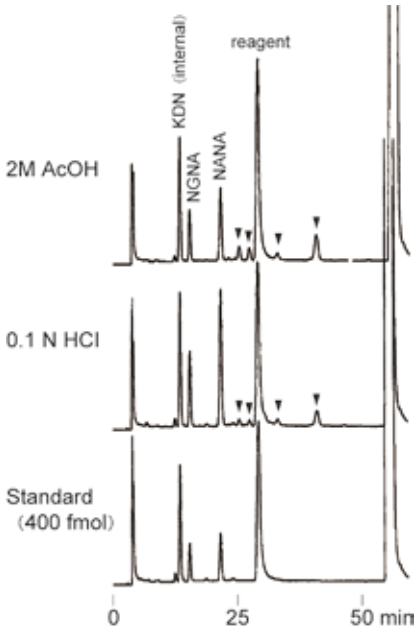


Figure 11. Time courses of the DMB reaction of various sialic acids
(Relative peak height was calculated assuming the peak height of each DMB-labeled sialic acid at 2.5 hours was 1.)

[Experiment 2: Analysis of sialic acids in glycoprotein]

It has been reported that bovine submandibular gland mucin contains more than 10 kinds of *O*-acetylated sialic acid, as well as NANA and NGNA.[10] Some *O*-acetyl groups are detached in desialylation using a strong acid such as HCl or sulfuric acid. Therefore, desialylation was performed under a condition in which *O*-acetyl groups are not easily detached (2 M AcOH, 80℃, 3 hours)[11], as well as under a condition in which sialic acids can completely separated (0.1 N HCl, 80℃, 1 hours).

20 pmol of KDN was added to 1 μg of bovine submandibular gland mucin as internal standard. Sialic acids were separated in 50 μl of 0.1 N HCl or 2 M acetic acid. DMB labeling of free sialic acids was carried out in accordance with the kit's protocol. 10 μl of the reaction mixture (equivalent to 40 ng of protein) was analyzed by means of HPLC (Figure 12). Table 4 shows the amount of free sialic acids that was quantified, based on the standard sialic acids.



Column:	PALPAK Type R (4.6 mm φ x 250 mm)
SolventA:	methanol/H_2O=7/93 (v/v)
SolventB:	acetonitrile/methanol=93/7 (v/v)
Elution:	0 - 50 min, 5% Solvent B 50 - 60 min, 100% Solvent B 60 - 75 min, 5% Solvent B (re-equilibration)
Flow rate:	1 ml/min
Temperature:	40℃
Detection:	Fluorescence (ex. 373 nm, em. 448 nm)

Figure 12. Sialic acid analysis using bovine submandibular gland mucin.

Table 4. Comparison of acid hydrolysis conditions in quantifying sialic acids

	NGNA (pmol/μg)	NANA (pmol/μg)
0.1 N HCl	17.1	24.7
2 M AcOH	13.6	15.8

Results

The amount of free sialic acids (NGNA and NANA) was larger when they were treated with HCl in acid hydrolysis. Given that sialic acids are released totally in the hydrolysis using acetic acid, the difference in the amount is thought to be due to *O*-acetylated sialic acids. As there is no difference between them about the KDN peaks (internal standard), it is not likely that DMB labeling reaction itself was influenced by the difference in the conditions of acid hydrolysis. In fact, some peaks that were suspected to be *O*-acetylated sialic acids were observed (worked with in figure 12. ▼). These peaks obtained in acetic acid were almost double of those treated with HCl.
At present, there is no commercially available standard *O*-acetylated sialic acid. Therefore, it is impossible to quantify *O*-acetylated sialic acids. Only for identification purpose, Oxford GlycoSystems Plc can be used. For quantifying the total amount of sialic acids including *O*-acetylated sialic acids, it is necessary to de-*O*-acetylate samples under an alkaline condition (0.1 N NaOH, 37℃, 30 min)[10] before hydrolysis and DMB labeling.

VII. Related Products

Various kinds of PA-Sugar Chain (PA-Sugar Chain 023 (Cat. #4123) etc.)

VIII. References

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