Photoaffinity Labeling of Sialidase with a Biotin-Conjugated Phenylaminodiazirine Derivative of 2,3-Didehydro-2-deoxy-*N*-acetylneuraminic Acid

Ramaswamy KANNAPPAN,[a] Masayuki ANDO,[a] Kimio FURUHATA,[b] and Yutaka UDA*,[a]

[a] *Department of Health Chemistry, Faculty of Pharmaceutical Sciences, Niigata University of Pharmacy and Applied Life Sciences; Niigata 956–8603, Japan: and* [b] *School of Pharmaceutical Sciences, Kitasato University; Minato-ku, Tokyo 108–8641, Japan.* Received November 14, 2007; accepted November 21, 2007; published online December 25, 2007

A biotin-conjugated photoactivatable phenylaminodiazirine derivative of 2,3-didehydro-2-deoxy-*N*-acetylneuraminic acid (DANA) was synthesized to identify sialidase. The free carboxylic group and *N*-acetyl substituent of sialic acid, which are important for recognition and enzymatic activity of sialidase, were conserved by the photolabeling compound as confirmed using analytical methods. The synthesized compound and DANA competitively inhibited starfish sialidase with a K_i value of 7.6 μM and 4.6 μM, respectively. Photo incorporation of the labeling compound to sialidase increased with irradiation time; 90% photo incorporation was achieved with more than 10-min irradiation, and labeling was completely inhibited by the addition of a competitive inhibitor. Starfish sialidase purified using high-performance gel filtration chromatography was subjected to photoaffinity labeling. A 50-kDa band was revealed to contain the sialidase active site by the photolabeling compound, and labeling was completely hindered in presence of the competitive inhibitor. Labeling specificity was ensured by the addition of the heat-deactivated standard protein chymotrypsinogen A to the reaction mixture.

Key words photoaffinity labeling; *Asterina pectinifera*; sialidase; inhibitory kinetics; protein identification

Sialidase [EC 3.2.1.18], which catalyzes the hydrolysis of terminal sialic acid residues of oligosaccharides, glycoproteins, and glycolipids, is widely distributed in species from prokaryotes to eukaryotes.[1,2] The physiological significance of sialidase was revealed from studies of the pathology of lysosomal sialidase deficiency diseases such as isolated sialidase deficiency and galactosialidosis (sialidase deficiency combined with partial β-galactosidase deficiency).[3,4] However, the isolation and characterization of mammalian sialidase have been difficult because of its extremely poor stability and membrane-bound character. So its enzymatic features of the enzyme isolated from mammalian tissues remains unclear. Recent studies have shown that lysosomal sialidase exists as a multienzyme complex with β-galactosidase, protective protein/cathepsin A, and other proteins including α-*N*-acetylgalactosaminidase.[5—9] Previously, we have highly-purified a sialidase from human placenta.[10] The final preparation of sialidase still gave five protein bands on sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with molecular masses of 78 kDa, 64 kDa, 46 kDa, 32 kDa, and 20 kDa. Of the five protein components, the 64-kDa protein was thought to be β-galactosidase and the 32-kDa and 20-kDa proteins were thought to be the components of protective protein, which has recently been identified as cathepsin A.[7,11] We have since confirmed that the 46-kDa protein is α-*N*-acetylgalactosaminidase using cDNA cloning,[12] while the 78-kDa protein was identified as the heavy chain of immunoglobulin M by van der Horst *et al.*[13] Thus the protein band on SDS-PAGE derived from human placental sialidase remains unknown.

We previously purified a sialidase from the starfish *Asterina pectinifera* and compared the enzymatic properties with those of human placental sialidase.[14] On SDS-PAGE, the purified preparation generously gave two protein bands: an upper (50-kDa) and a lower (47-kDa) band. We confirmed that the 50-kDa protein contains cathepsin D, a lysosomal protease, and its activity was also present in all the purification steps. We also demonstrated the separation of cathepsin D activity from sialidase activity.[15] The 47-kDa band was believed to be the decomposed product of the 50-kDa protein. Hence the protein band derived from sialidase could not be identified.

Photoaffinity labeling of sialidase using the radioiodinatable arylazide derivative of 2,3-didehydro-2-deoxy-*N*-acetylneuraminic acid (DANA) has been reported.[16,17] Because of its radioactive compound and low affinity toward human placental sialidase, however, it is not convenient to use it for photoaffinity labeling of sialidase. In this study, we describe the photoaffinity labeling and identification of the sialidase from starfish using a nonradioactive arylaldehyde derivative of DANA.

MATERIALS AND METHODS

Materials DANA was purchased from Boehringer Mannheim GmbH (Germany). Affilight CHO (biotionyl-*N*-boc-phenylaminodiazirine) was from Seikagaku (Tokyo, Japan). Streptavidin-horseradishperoxidase (HRP) was purchased from Invitrogen (Carlsbad, CA, U.S.A.). Chemiluminescence Western blotting detection reagent (ECL Plus) was purchased from GE Healthcare Bio-sciences (Piscataway, NJ, U.S.A.). Bicinchoninic acid was from Sigma-Aldrich (St. Louis, MO, U.S.A.). 4-Methylumbelliferyl-α-D-*N*-acetylneuraminic acid (4MU-NeuAc) was synthesized in our laboratory. All other chemicals used in the experiments were of analytical grade.

General Analysis Melting points were measured on a Yamato melting point apparatus without correction. Elemental analyses were performed on an MT-5 elemental analyzer (Yanaco, Japan). Optical rotations were measured with a JASCO JIP-4 digital polarimeter (at 23 °C).

Chromatography Column chromatography was con-

* To whom correspondence should be addressed. e-mail: uda@nupals.ac.jp

ducted on silica gel 60 (70—230 mesh, Merck). Thin-layer chromatography (TLC) was performed on silica gel 60 F_{254} (Merck) plates, and spots were detected under ultraviolet irradiation or by spraying with 5% sulfuric acid.

Infrared Spectroscopy Infrared (IR) spectra were recorded using FT/IR-460 Plus (JASCO, U.S.A.) with KBr.

Nuclear Magnetic Resonance Spectroscopy The nuclear magnetic resonance (NMR) spectra were recorded at 300 MHz (Varian VXR-300) in solution in $CDCl_3$ (internal Me_4Si, δ 0 ppm), or D_2O (internal 3-trimethylsilyl-2,2,3,3-d_4-propionic acid sodium salt, δ 0 ppm).

Mass Spectrometry Mass spectra were measured on a JEOL JMS-AX-505HA or a JMS700MA Station mass spectrometer.

SDS-PAGE Fifty nanograms of protein were separated on 10—20% SDS-PAGE following the procedure described by Laemmli.[18] The proteins separated on the gels were visualized using Daiichi silver staining kit (Daiichi Pure Chemicals, Tokyo, Japan).

Electroblotting/Chemiluminescence Detection The photolabeled high-performance gel filtration chromatography-purified fraction (50 ng protein) or preparative PAGE-purified (100 ng protein) starfish sialidase with 50 ng of heat-deactivated chymotrypsinogen A (CTG-A) were separated on a 10—20% SDS-PAGE using the above method. Then the proteins were electroblotted on a polyvinylidene difluoride (PVDF) membrane using an electroblot apparatus (ATTO, Model AE-6677). The membrane was exposed to streptavidin–HRP (Invitrogen) (1 : 10000) in 0.1% Tween-20 Tris buffered saline (20 mM Tris–HCl, 140 mM sodium chloride, pH 7.5) after blocking with 5% nonfat milk. Chemiluminescence was performed with an ECL Plus Western blotting detection reagent (Amersham Biosciences, U.S.A.).

Starfish Sialidase Sialidase from starfish ovary was isolated and purified using the previously described procedures.[15]

Synthesis of 9-*N*-(4-Formylbenzoyl)Neu5Ac2en (6) The starting material, methyl 5-acetamido-2,6-anhydro-3,5-dideoxy-9-*O*-(*p*-toluenesulfonyl)-D-*glycero*-D-*galacto*-non-2-enonate (**2**), was prepared following the previously described procedure.[19,20] We prepared methyl 5-acetamido-4-*O*-acetyl-2,6-anhydro-9-azido-3,5,9-trideoxy-7,8-*O*-isopropylidene-D-*glycero*-D-*galacto*-non-2-enonate (**3**) utilizing the conditions reported by Warner.[17] 5-Acetamido-2,6-anhydro-3,5,9-trideoxy-9-(4-formylbenzamido)-D-*glycero*-D-*galacto*-non-2-enoic acid (**6**) was synthesized as described in Chart 1a. This structure was ascertained by analysis of ^{1}H-NMR spectra, since chemical shifts at δ 3.79 for 9-H′ are strongly indicative of the position of the 4-formylbenzamide group.

Methyl 5-Acetamido-4-*O*-acetyl-2,6-anhydro-3,5dideoxy-7,8-*O*-isopropylidene-9-*O*-(*p*-toluenesulfonyl)-D-*glycero*-D-*galacto*-non-2-enonate (2) *p*-Toluenesulfonyl chloride (0.90 g, 4.6 mmol) was added to a solution of methyl 5-acetamido-2,6-anhydro-3,5-dideoxy-D-*glycero*-D-*galacto*-non-2-enonate[19] (**1**, Neu5Ac2en1Me) (0.91 g, 3 mmol) in pyridine (20 ml) at 0 °C. The mixture was stirred for 3 h at room temperature, poured into water (50 ml), and extracted twice with ethyl acetate (100 ml). The extract was washed with brine, dried over sodium sulfate, and evaporated to dryness. The residue was crystallized from ethyl acetate to give the 9-(*p*-toluenesulfonyl) derivative of **1** as colorless needles[20] [mp 160 °C]. The 9-(*p*-toluenesulfonyl) derivative was dissolved in acetone and 2,2-dimethoxypropane (5 ml) and anhydrous camphorsulfonic acid (20 mg) were added with stirring overnight at room temperature. The mixture was treated with Dowex-1 (OH^-) anion-exchange resin to remove the acid, and the resin was filtered off and washed with acetone. The filtrate and washings were combined and dried by evaporation to yield the 7,8-*O*-isopropylidene-9-*O*-(*p*-toluenesulfonyl) derivative of **1** as an amorphous powder, which was subjected to the next reaction step without purification. Acetic anhydride (5 ml) was added to a solution of the crude 7,8-*O*-isopropylidene-9-*O*-(*p*-toluenesulfonyl) derivative in pyridine (10 ml). The mixture was stirred for 3 h at room temperature and evaporated to give a syrup, which was purified on silica-gel column chromatography with chloroform–methanol (50 : 1) to give **2** (0.81 g, 50%) as a colorless powder. **2**: $[\alpha]_D$ +10.4 ° (c=1, MeOH). *Anal.* Calcd for $C_{24}H_{31}NO_{11}S$: C, 53.91; H, 5.77; N, 2.59. Found: C, 53.90; H, 5.81; N, 2.56. MS (EI) *m/z*: 541 (M^+). IR (KBr) cm^{-1}: 1740, 1660, 1600, 1540. ^{1}H-NMR ($CDCl_3$): δ 1.30 and 1.36 (each 3H, s, isopropylidene methyl groups), 1.97 (3H, s, NAc), 2.06 (3H, s, OAc), 2.30 (3H, s, Ph-methyl group), 3.77 (3H, s, ester methyl group), 3.95 (1H, br q, J=8.5 Hz, 5-H), 4.28—4.49 (5H, m, 6-H, 7-H, 8-H, 9-H2), 5.69 (1H, dd, J=3.0, 8.0 Hz, 4-H), 5.73 (1H, d, J=8.0 Hz, 5-NH), 5.88 (H, d, J=3.0 Hz, 3-H), 7.33, and 7.82 (each 2H, dd, J=8.0 Hz, phenyl group).

Methyl 5-Acetamido-4-*O*-acetyl-2,6-anhydro-9-azido-3,5,9-trideoxy-7,8-*O*-isopropylidene-D-*glycero*-D-*galacto*-non-2-enonate (3) Sodium azide (0.20 g, 3.1 mmol) was added to a solution of **2** (0.76 g, 1.4 mmol) in dry dimethyl sulfoxide (20 ml). The mixture was stirred for 6 h at 80 °C, poured into water (100 ml), and extracted twice with chloroform (50 ml). The extract was washed with brine, dried over sodium sulfate, and evaporated to dryness. The residue was purified on silica-gel column chromatography with chloroform–methanol (100 : 1) to give **3** (0.46 g, 80%) as a colorless powder. **3**: $[\alpha]_D$ 3.5 ° (c=1, MeOH). *Anal*. Calcd for $C_{17}H_{24}N_4O_8$: C, 49.51; H, 5.87; N, 13.59. Found: C, 49.48; H, 5.90; N, 13.55. IR (KBr) cm^{-1} : 2100, 1740, 1660, 1550. MS (EI) *m/z*: 412 (M^+). ^{1}H-NMR ($CDC1_3$): δ 1.34 and 1.48 (each 3H, s, isopropylidene methyl groups), 1.97 (3H, s, NAc), 2.08 (3H, s, OAc), 3.52 (1H, dd, J=13.0, 4.5 Hz, 9-H), 3.76 (1H, dd, J=13.0, 8.0 Hz, 9-H), 3.79 (3H, s, ester methyl group), 4.02 (1H, br q, J=7.5 Hz, 5-H), 4.27—4.38 (2H, m, 7- and 8-H), 4.50 (1H, dd, J=3.0, 8.5 Hz, 6-H), 5.63 (1H, dd, J=3.5, 7.0 Hz, 4-H), 5.98 (1H, d, J=3.5 Hz, 3-H), 6.18 (1H, d, J=8.0 Hz, 5-NH).

Methyl 5-Acetamido-4-*O*-acetyl-2,6-anhydro-3,5,9-trideoxy-9-(4-formylbenzamido)-7,8-*O*-isopropylidene-D-*glycero*-D-*galacto*-non-2-enonate (5) A solution of **3** (0.42 g, 1 mmol) in methanol (30 ml) was hydrogenated with hydrogen over Lindlar catalyst (0.1 g) for 3 h at room temperature. The solution was filtered through celite and evaporated to dryness at 20 °C to the yield the 9-amino derivative (**4**) as a brownish residue, which was subjected to the next reaction step without purification. **4**: ^{1}H-NMR ($CDCl_3$): δ 1.34 and 1.48 (each 3H, s, isopropylidene methyl groups), 1.99 (3H, s, NAc), 2.08 (3H, s, OAc), 2.93 (1H, ddd, J=13.0, 4.5 Hz, 9-H), 3.09 (1H, ddd, J=13.0, 8.0 Hz, 9-H), 3.40 (2H, br s, 9-NH_2), 3.80 (3H, s, ester methyl group), 4.12 (1H, dt, J=6.0,

7.0 Hz, 5-H), 4.27—4.38 (1H, ddd, J=5.0, 4.5, 8.0 Hz, 8-H), 4.37 (1H, br t, J=6.0 Hz, 6-H), 4.53 (1H, dd, J=5.0, 6.0 Hz, 7-H), 5.53 (1H, dd, J=3.5, 6.5 Hz, 4-H), 6.02 (1H, d, J=3.5 Hz, 3-H), 6.18 (1H, d, J=7.0 Hz, 5-NH).

4-Formylbenzoyl chloride (0.25 g, 1.5 mmol) was added to a solution of **4** (0.40 g, 1.04 mmol) in pyridine (5 ml) at 0 °C. The mixture was stirred for 3 h at room temperature, poured into ice-water, and extracted twice with chloroform (20 ml). The extract was washed with brine, dried over sodium sulfate, and evaporated to dryness. The residue was purified on silica-gel column chromatography with chloroform–methanol (100 : 1) to give **5** (0.38 g, 73%) as a colorless powder. **5**: $[\alpha]_D$ +23.0 ° (c=0.27, MeOH). *Anal*. Calcd for $C_{25}H_{30}N_2O_{10}S$: C, 57.91; H, 5.83; N, 5.40. Found: C, 57.95; H, 5.83; N, 5.42. MS (FAB) m/z: 519 (M^++1). IR (KBr) cm^{-1}: 1740, 1700, 1660, 1605, 1540. ^{1}H-NMR ($CDCl_3$): δ 1.33 and 1.46 (each 3H, s, isopropylidene methyl groups), 1.99 (3H, s, NAc), 2.08 (3H, s, OAc), 3.61 (1H, ddd, J=14.0, 5.0, 6.0 Hz, 9-H), 3.89 (3H, s, ester methyl group), 3.99 (1H, ddd, J= 14.0, 4.5, 7.0 Hz, 9-H), 4.11 (1H, br q, J=9.0 Hz, 5-H), 4.37 (1H, dd, J=2.5, 6.0 Hz, 7-H), 4.41 (1H, br q, J=6.0 Hz, 8-H), 4.63 (1H, dd, J=2.5, 9.0 Hz, 6-H), 5.74 (1H, dd, J=3.5, 7.5 Hz, 4-H), 5.89 (1H, d, J=8.5 Hz, 5-NH), 5.98 (H, d, J= 3.5 Hz, 3-H), 7.53 (1H, dd, J=4.5, 6.0 Hz, 9-NH), 7.92, and 7.95 (each 2H, d, J=8.0 Hz, phenyl group), 10.05 (1H, s, formyl group).

5-Acetamido-2,6-anhydro-3,5,9-trideoxy-9-(4-formylbenzamido)-D-*glycero*-D-*galacto*-non-2-enoic Acid (6) A solution of the fully protected 9-(4-formylbenzoylamido) derivative (**5**) (0.3 g, 0.57 mmol) in 90% acetic acid (50 ml) was stirred for 1.5 h at 60 °C. The mixture was evaporated to dryness. The residue was dissolved in 4% aqueous sodium hydroxide (5 ml), kept at room temperature for 3 h, diluted with water (20 ml), and acidified to pH 3.0 with Dowex-50 (H^+) resin. The resin was filtered off and washed with water. The filtrate and washings were combined and lyophilized. The residue was purified by recrystallization with water–methanol (1 : 2) to give **6** (0.14 g, 58%) as a colorless needles. **6**: mp (dec.) 155 °C. $[\alpha]_D$ +20.3 ° (c=0.36, MeOH). *Anal*. Calcd for $C_{19}H_{22}N_2O_9$: C, 54.03; H, 5.25; N, 6.63. Found: C, 54.09; H, 5.27; N, 6.55. IR (KBr) cm^{-1}: 3300, 1720, 1700, 1640, 1600, 1550, 1520. MS (FAB) m/z: 423 (M^++1). ^{1}H-NMR (D_2O): δ 2.00 (3H, s, NAc), 3.57 (1H, dd, J=14.0, 7.5 Hz, 9-H), 3.60 (1H, dd, J=1.0, 9.5 Hz, 6-H), 3.79 (1H, dd, J=14.0, 3.5 Hz, 9-H), 4.07 (1H, ddd, J=10.5, 7.5, 3.5 Hz, 8-H), 4.08 (1H, t, J=9.5 Hz, 5-H), 4.28 (1H, dd, J=1.0, 10.5 Hz, 7-H), 4.48 (1H, dd, J=2.5, 9.0 Hz, 4-H), 5.94 (H, d, J=2.5 Hz, 3-H), 7.90, and 8.02 (each 2H, d, J=8.5 Hz, phenyl group), 9.98 (1H, s, formyl group).

Synthesis of Photoreactive Neu5Ac2en Derivative The photoreactive Neu5Ac2en derivative was synthesized as shown in Chart 1b. Deprotection of compound **a** (1.6 mg, 2.3 μmol), dissolved in dichloromethane (20 μl), was performed with trifluroacetic acid (20 μl) at 0 °C for 60 min and after evaporation the residue was dissolved in 50 μl of 80% acetonitrile containing compound **6** (0.5 mg). The pH was adjusted to 5—6 with diisopropylethylamine and the mixture was incubated at 37 °C for 40 h in the dark. After evaporation to dryness and dissolving in 80% acetonitrile (10 μl), it was purified on a silica gel 60 column by gradient elution with chloroform : methanol : water (10 : 2 : 0.1 to 8 : 5 : 1) to give **7**. The yield (0.9 mg, 1.2 μmol) was determined from the UV spectra of methanol solution using the ε value of compound **a** (ε^{360}=400) as a standard. TLC was carried out following the above procedure using chloroform : methanol : water (8 : 5 : 1) as the mobile phase to confirm the purity of **7** (Rf=0.37).

Photoaffinity Labeling Photolabeling was carried out in 100 mM of acetate buffer, pH 4.3, in the presence of 15 μM of probe **7**, 200 ng of protein, and with or without 1 mM of DANA. The mixture was allowed to equilibrate by incubating at 4 °C on ice in the dark for 30 min. The mixture was kept on ice, 7 cm away from the light source in a UV light box (Model LS-D, Super Light Hayashi Rikagaku, Japan). Irradiation was carried out for 5 min at 365 nm.

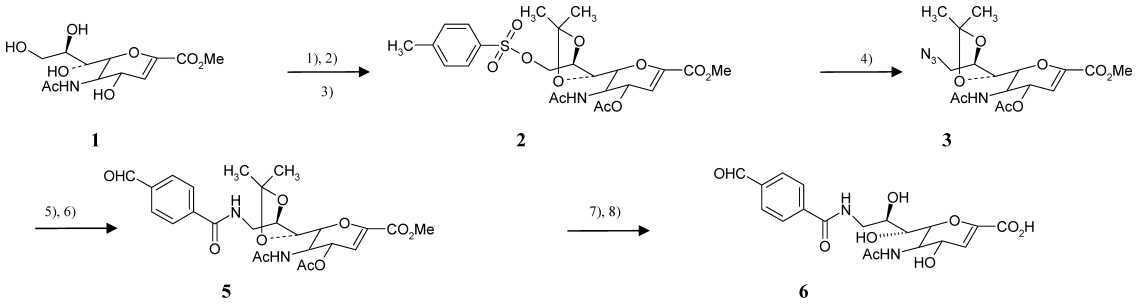
Enzyme Assay and Inhibitory Kinetics Sialidase activity toward the synthetic substrate 4MU-NeuAc was determined according to the method reported in the literature.[21] One unit of enzyme is defined as the amount of enzyme which catalyzed the release of 1 nmol of sialic acid per minute. For inhibitory studies, stock solutions of DANA and **7** were added to the enzyme preparation to yield final concentrations of 20 μM and 10 μM, respectively. The inhibitors were allowed to bind to the enzyme for 5 min at 4 °C prior to adding the substrates. The residual sialidase activity was determined using the above procedure.

RESULTS AND DISCUSSION

Synthesis and Characterization of Photoaffinity Labeling Compound Previously, we demonstrated the inhibitory effects of sialic acid derivatives against starfish sialidase and the importance of the free carboxylic group and *N*-acetyl substituent of sialic acid for recognition and enzymatic activity of sialidase.[14] Therefore throughout the synthesis of the photoaffinity labeling compound, we preserved these two functional groups of the inhibitor DANA and introduced the photoreactive group at the C-9 position which is a primary hydroxyl group and open for selective derivatization. The starting material, compound **2**, was prepared following the previously described procedure.[19,20] Compound **6**, was synthesized as shown in Chart 1a. Each compound was confirmed by mass, IR, and NMR spectroscopy. Compound **a** was deprotected by TFA to give **b** and coupled to **6**. The outline of the coupling reaction is shown in Chart 1b. After coupling, the mixture was purified on silica gel 60 to give the pure glycoconjugates **7**. The completion of the reaction, product formation, and purity of the synthesized compound were confirmed on TLC.

Inhibitory Kinetics We first investigated whether **7** has the same high affinity for sialidases as DANA by comparing the inhibitory effects of these compounds on the activity of CM-Sephadex purified *A. pectinifera* sialidase. Figure 1 shows the double-reciprocal plot of the enzyme activity as a function of the substrate concentration. The Lineweaver–Burk analysis data obtained at four concentrations of 4MU-NeuAc (K_m=58.7 μM) indicated that **7** and DANA were competitive inhibitors with K_i values of 7.5 μM and 4.6 μM, respectively. This shows that the incorporation of the photoreactive carbene-generating group, biotinyl-phenylaminodiazirine, in to DANA did not influence the affinity of this compound for the enzyme.

Next we investigated the time-dependent photo incorpora-



1) *p*-TsCl, py., 0 ºC. 2) $(MeO)_2CMe_2$, camphorsulfonic acid, acetone. 3) Ac_2O-py.. 4) NaN_3, DMSO, 6 h, 80 ºC.
5) H_2, Lindlar catalyst. 6) 4-formylbenzoyl chloride, py.. 7) 90 % AcOH, 1.5 h, 60 ºC. 8) 4% NaOH, 3 h, RT, Dowex-50 (H^+)

Chart 1a

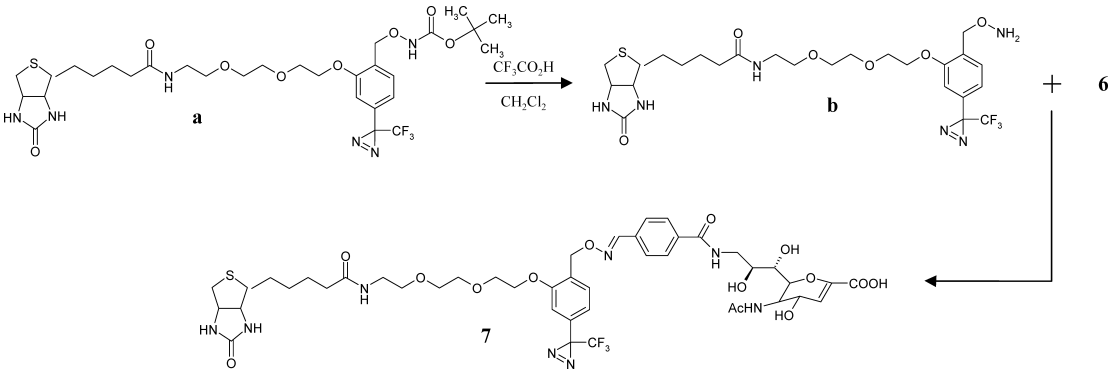


Chart 1b

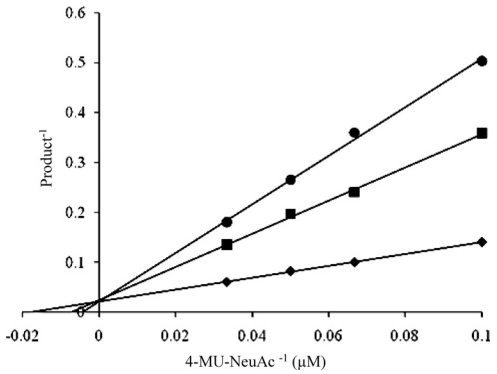


Fig. 1. Effects of the Synthesized Photolabeling Compound on the Kinetic Parameters of the Hydrolysis of 4MU-NeuAc by Starfish Sialidase

CM-Sephadex-purified starfish sialidase was assayed in the absence (◆) or presence of **7** (■) or DANA (●). The results were calculated with the Lineweaver–Burk reciprocal plot.

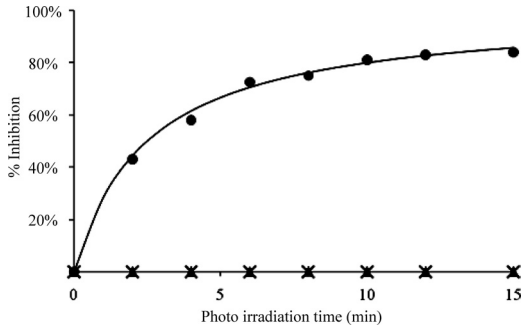


Fig. 2. Time Course of Label Incorporation

Residual starfish sialidase activity against 4MU-NeuAc was measured after photolabeling with **7** (●) in the presence of DANA (▲) or nonirradiated dark control (×).

tion of **7** in to the CM-Sephadex-purified starfish sialidase fraction. For this study, photolabeling of sialidase with **7** was performed as described in Materials and Methods at various time intervals in the presence or absence of DANA. Then the reaction mixture was dialyzed to wash out the unbound photolabeling compound, and the residual enzyme activity was measured to plot the graph of photoirradiation time *vs*. percentage of inhibition (Fig. 2). The enzyme inhibition by photolabeling reached the maximum after 10 min and did not increase further with time. This photolabeling was completely inhibited by the addition of 1 mM DANA. In the dark, **7** is sufficiently stable under our experimental conditions, and no detectable photolabeling (inhibition of sialidase activity) was observed. From the graph, it is clearly seen that photolabeling is effective when exposed to UV light for 10 min and it is completely inhibited by the excess addition of DANA. However, 10-min exposure also increased nonspecific photolabeling. When the 10-min photolabeled protein sample was examined for biotin on a PVDF membrane, even the DANA-inhibited sample showed biotin incorporation (data not shown). This means that nonspecific labeling also occurred with 10-min exposure. For this reason, 5-min irradiation, where more than 60% of sialidase activity was inhibited, was set as the standard procedure.

Photoaffinity Labeling High-performance gel filtration chromatography-purified sialidase (Fig. 3a) was photoaffinity labeled with **7** in presence or absence of the competitive in-

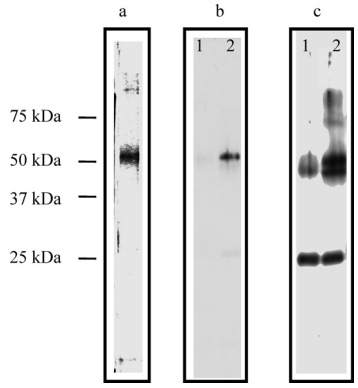


Fig. 3. Photoaffinity Labeling of Starfish Sialidase

(a) Silver-stained high-performance gel filtration chromatography (HPGFC) fraction of starfish sialidase. (b) Photoaffinity labeling of HPGFC-purified starfish sialidase with **7** in the presence (lane 1) or absence (lane 2) of DANA. (c) CTG-A (25-kDa band) was added to the preparative PAGE-purified starfish sialidase as a standard to confirm equimolar loading, and photoaffinity labeling was performed with **7** in the presence (lane 1) or absence (lane 2) of DANA.

hibitor DANA (Fig. 3b). The 50-kDa protein (lane 2) was labeled effectively by **7** and its labeling was inhibited in presence of DANA (lane 1). This reveals that **7** has high affinity toward the catalytic site of starfish sialidase, and this affinity is competitively inhibited by DANA. The preparative gel electrophoresis-purified starfish sialidase after removing cathepsin D activity using high-performance gel filtration chromatography was subjected to photolabeling and the specificity of photoaffinity labeling was confirmed by the addition of a standard protein CTG-A (25-kDa) (Fig. 3c). The photolabeling compound, which has no affinity for CTG-A, bound to CTG-A nonspecifically and this binding was not influenced by DANA. Whereas, the 50-kDa protein, which has the sialidase catalytic site influenced the photolabeling process of **7** and was subsequently inhibited by DANA.

In our earlier studies, we showed the purification of a sialidase from the starfish *A. pectinifera*,[14] and the copurification of cathepsin D. On SDS-PAGE, the purified preparation generously gave two protein bands: an upper (50-kDa) and a lower (47-kDa) band. We confirmed that the 50-kDa band has cathepsin D domains and also demonstrated the separation of cathepsin D activity from sialidase activity.[15] The current study clearly showed that after removal of cathepsin D activity, the 50-kDa protein band remained in the SDS-PAGE and contained the sialidase catalytic site, which concludes that starfish cathepsin D and sialidase have similar molecular weights.

In this study, we established a reliable procedure for photoaffinity labeling of sialidase using a nonradioactive probe. The active site of sialidase from starfish ovary was labeled effectively and we clarified that the 50-kDa band in SDS-PAGE has the sialidase catalytic site. The arylaldehyde derivative of 2,3-didehydro-2-deoxy-*N*-acetylneuraminic acid conjugated with biotinyl phenylaminodiazirine will be a valuable tool to identify the human protein that contain sialidase active site.

REFERENCES

1) Rosenberg A., "Biology of the Sialic Acids," Chap. 2, ed. by Schauer R., Kelm S., Reuter G., Roggentin P., Shaw L., Plenum Press, New York, 1995, p. 31.
2) Rosenberg A., "Biology of the Sialic Acids," Chap. 8, ed. by Saito M., Yu R. K., Plenum Press, New York, 1995, pp. 261—277.
3) Bonten E. J., Arts W. F., Beck M., Covanis A., Donati M. A., Parini R., Zammarchi E., d'Azzo A., *Hum. Mol. Genet*., **9**, 2715—2725 (2000).
4) Zhou X. Y., van der Spoel A., Rottier R., Hale G., Willemsen R., Berry G. T., Strisciuglio P., Morrone A., Zammarchi E., Andria G., d'Azzo A., *Hum. Mol. Genet.*, **5**, 1977—1987 (1996).
5) Hoogeveen A. T., Verheijen F. W., Galjaard H., *J. Biol. Chem.*, **258**, 12143—12146 (1983).
6) Hiraiwa M., Saitoh M., Arai N., Shiraishi T., Odani S., Uda Y., Ono T., O'Brien J. S., *Biochim. Biophys. Acta*, **1341**, 189—199 (1997).
7) Verheijen F. W., Palmeri S., Hoogeveen A. T., Galjaard H., *Eur. J. Biochem.*, **149**, 315—321 (1985).
8) Pshezhetsky A. V., Potier M., *J. Biol. Chem.*, **271**, 28359—28365 (1996).
9) Hiraiwa M., Saitoh M., Uda Y., Norihiro A., Martin B. M., Kishimoto Y., O'Brien J. S., *Comp. Biochem. Physiol. B, Biochem. Mol. Biol.,* **115**, 541—546 (1996).
10) Hiraiwa M., Nishizawa M., Uda Y., Nakajima T., Miyatake T., *J. Biochem.* (Tokyo), **103**, 86—90 (1988).
11) Galjart N. J., Gillemans N., Harris A., van der Horst G. T., Verheijen F. W., Galjaard H., d'Azzo A., *Cell*, **54**, 755—764 (1988).
12) Tsuji S., Yamauchi T., Hiraiwa M., Isobe T., Okuyama T., Sakimura K., Takahashi Y., Nishizawa M., Uda Y., Miyatake T., *Biochem. Biophys. Res. Commun.*, **163**, 1498—1504 (1989).
13) van der Horst G. T., Galjart N. J., d'Azzo A., Galjaard H., Verheijen F. W., *J. Biol. Chem.*, **264**, 1317—1322 (1989).
14) Iriyama N., Takeuchi N., Shiraishi T., Izumi K., Sawada M. T., Takahashi N., Furuhata K., Ogura H., Uda Y., *Comp. Biochem. Physiol. B, Biochem. Mol. Biol.*, **126**, 561—569 (2000).
15) Kannappan R., Satoh Y., Iriyama N., Ando M., Sawada M. T., Takahashi N., Furuhata K., Uda Y., *J. Biochem.* (Tokyo), **143**, 117—122 (2008).
16) van der Horst G. T., Mancini G. M., Brossmer R., Rose U., Verheijen F. W., *J. Biol. Chem.*, **265**, 10801—10804 (1990).
17) Warner T. G., *Biochem. J.*, **285**, 957—964 (1992).
18) Laemmli U. K., *Nature* (London), **227**, 680—685 (1970).
19) Furuhata K., Sato S., Goto M., Takayanagi H., Ogura H., *Chem. Pharm. Bull.*, **36**, 1872—1876 (1988).
20) Warner T. G., *Biochem. Biophys. Res. Commun.*, **148**, 1323—1329 (1987).
21) Potier M., Mameli L., Belisle M., Dallaire L., Melancon S. B., *Anal. Biochem.*, **94**, 287—296 (1979).