Identification and characterization of a novel, versatile sialidase from a *Sphingobacterium* that can hydrolyze the glycosides of any sialic acid species at neutral pH

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Abstract

Bacterial sialidases are widely used to remove sialic acid (Sia) residues from glycans. Most of them cleave the glycosides of *N*-acetylneuraminic acid (Neu5Ac) and *N*-glycolylneuraminic acid (Neu5Gc) under acidic pHs; however, currently available bacterial sialidases had no activity to the glycosides of deaminoneuraminic acid (Kdn). In this study, we found a novel sialidase from *Sphingobacterium* sp. strain HMA12 that could cleave any of the glycosides of Neu5Ac, Neu5Gc, and Kdn. It also had a broad linkage specificity, i.e., α2,3-, α2,6-, α2,8-, and α2,9-linkages, and the optimal pH at neutral ranges, pH 6.5-7.0. These properties are particularly important when sialidases are applied for *in vivo* digestion of the cell surface sialosides under physiological conditions. Interestingly, 2,3-didehydro-2-deoxy-*N*-acetylneuraminic acid (Neu5Ac2en), which is a transition state analog-based inhibitor, competitively inhibited the enzyme-catalyzed reaction for Kdn as well as for Neu5Ac, suggesting that the active site is common to the Neu5Ac and Kdn residues. Taken together, this sialidase is versatile and useful for the *in vivo* research on sialo-glycoconjugates.

Key words:

sialidase; Kdn-sialidase; neutral pH; Sphingobacterium; sialic acid; deaminoneuraminic acid

1. Introduction

Sialic acids (Sias[1]) are a family of nine-carbon sugars containing the 2-keto-3-deoxynononic acid skeleton. There are nearly 50 members that consist of *N*-acetylneuraminic acid (Neu5Ac), *N*-glycolylneuraminic acid (Neu5Gc), deaminoneuraminic acid (Kdn), and their derivatives. Kdn has a hydroxyl group at C5 position, instead of the acylamide group in Neu5Ac and Neu5Gc [1-4]. Sias are present at non-reducing termini of the glycan on glycoproteins and glycolipids, and play important roles on various biological phenomena, such as fertilization, immunity, neurogenesis, infection, and tumorigenesis [1,2]. The glycosides of Sia are hydrolyzed by sialidases, which widely occur in organisms from viruses, bacteria, and animals [1,2]. Apart from their biological roles, bacterial sialidases are often used in the research to elucidate the significance of Sia residues in various organisms [1,2,5]. For example, *Arthrobacter ureafaciens* sialidase (Au-sialidase), *Clostridium perfringens* sialidase (Cp-sialidase), *Vibrio cholera* sialidase (Vc-sialidase), and *Streptococcus pneumoniae* sialidase (Sp-sialidase) are commercially available and frequently used. Most bacterial sialidases are known to have the ability to hydrolyze the glycosides of Neu5Ac and Neu5Gc. However, they fail to hydrolyze the Kdn glycoside [6]. The only exception is the deaminoneuraminidase from *Sphingobacterium multivorum* (Kdnase SM), which can cleave only the KDN residues, but not Neu5Ac or Neu5Gc residues at all [7,8]. In other organisms than bacteria, so called KDN-sialidases, which can hydrolyze both Neu5Ac and KDN glycosides, have been reported, including loach liver, oyster hepatopancreas, starfish, and filamentous fungus *Aspergillus fumigatus* [9-12]. However, no bacterial sialidase that can cleave all glycosides of Neu5Ac, Neu5Gc, and KDN has been reported.

In this study, we found a novel sialidase which has the ability to hydrolyze the glycosides of Neu5Ac, Neu5Gc, and Kdn in *Sphingobacterium* sp. strain HMA12 [13]. Of 4 sialidase candidate genes in this bacterium, ORF03462, ORF03431, ORF03865, and ORF04787, that are characterized by the presence of at least one sialidase motif, Asp-box (SXDXGXTW, X=any amino acid), only ORF03865 was shown to code for the active enzyme. The recombinant enzyme was further characterized for the sialidase activity and properties.

2. Material and method

2.1. Materials

pET32a vector was purchased from Invitrogen (Carlsbad, CA). Ni-NTA agarose was purchased from Qiagen (Hilden, Germany). Colominic acid (α2,8-poly-*N*-acetylneuraminic acid), phenylmethylsulfonyl fluoride (PMSF), and Wakopak handy ODS (4.6 ×250 mm) were purchased from FUJIFILM wako (Osaka, Japan). 1,2-dimethylenedioxybenzen (DMB) was purchased from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan). *Arthrobacter ureafaciens* sialidase, 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid (Neu5Ac2en), and

4-methylumbelliferyl-α-D-*N*-acetylneuraminic acid (4MU-Neu5Ac) were purchased from Nacalai tesque (Kyoto, Japan). 4MU-Neu5Gc and 4MU-Kdn were kindly provided by Dr. Kimio Furuhata (Kitasato University, Tokyo) [14]. α2,9-Linked Neu5Ac polymer were isolated from capsular polysaccharide of *Neisseria meningitidis* group C and as previously described [15]. α2,9-di-*N*-acetylneuraminic acid and α2,8-di-*N*-acetylneuraminic acid were prepared as previously described [16,17]. Muromac column was purchased from Muromachi Chemicals, Inc. (Tokyo, Japan). *Clostridium perfringens* and *Vibrio cholera* sialidases were purchased from Sigma-Aldrich (St. Louis, MO). *Streptococcus pneumoniae* sialidase was purchased from New England Biolabs, Inc. (Ipswich, MA). α2,3-sialyl-*N*-acetyllactosamine, α2,6-sialyl-*N*-acetyllactosamine, recombinant enterokinase, and Silica Gel 60 TLC plate were purchased from Merck (Darmstadt, Germany). VIVASPIN20 was purchased from Funakoshi (Tokyo, Japan). The molecular weight marker was purchased from Bio-rad (Hercules, CA). Benzamidine-Sepharose was purchased from GE Healthcare Life Sciences (Amersham, England). The monoclonal antibody 12E3 was prepared as described previously [18]

2.2. Preparation of recombinant sialidases

Plasmids for *E. coli* cell expression of ORF03426, ORF03431, ORF03865, and ORF04787 genes (without predicted peptide signal sequence) with N-terminal thioredoxin (Trx)-Tag, His-Tag and S-Tag were generated using pET32a vector. *E. coli* Rosetta-gami2 (DE3) transformed with pET32a vector containing each sialidase candidate gene were cultured in 200 mL LA medium (1 % trypton, 0.5 % yeast, 1 % NaCl, 50 μg/ml ampicillin) at 15 ℃ for 20 h in the presence of 0.4 mM isopropyl-1-thio-β-D-galactopyranoside. The cells were collected by centrifugation at 6,000 × g for 10 min, washed with 10 mM sodium phosphate (pH 7.2)-0.15 M NaCl (PBS), and sonically disrupted in equilibrium buffer (20 mM Tris-HCl (pH 8.0), 5 mM imidazole, and 0.5M NaCl) containing 1 mM PMFS, 1 μg/ml leupeptin, 2 μg/ml antipain, 10 μg/ml benzamidine, 1 μg/ml pepstain, 1 μg/ml aprotinin. The suspension was centrifuged at 21,600 × *g* at 4 ℃ for 10 min to obtain the supernatant as a crude fraction. The crude fraction was rotated with the Ni-NTA agarose (1 mL) at 4℃ for 15 min, and subjected to a Ni-NTA agarose chromatography. Following collection of the flow-through fraction, the wash fraction, elute fraction, and strip fraction were obtained by eluting with wash buffer (40 mM Tris-HCl (pH 8.0), 10 mM imidazole, and 1 M NaCl), elute buffer (20 mM Tris-HCl (pH 8.0), 100 mM imidazole, and 0.5M NaCl), and strip buffer (20 mM Tris-HCl (pH 8.0), 1 M imidazole, and 0.5 M NaCl), respectively. The elute fraction was desalted and concentrated using VIVASPIN 20, followed by the treatment with recombinant enterokinase based on the manufacturer instruction to obtain the recombinant proteins without the Tags. The enterokinase digest was mixed with the benzamidine-Sepharose at 4 ℃ for 1 h, and centrifuged at 2,000 × *g* for 5 min. The supernatant was further purified by mixing with the Ni-NTA agarose at 4 ℃ for 1 h and removing the gel. The purity was determined by SDS-polyacrylamide gel electrophoresis (PAGE) followed by the Coomassie Brilliant Blue

(CBB) staining.

2.3. Assay for the sialidase activity

In total 50 µl reaction mixture including the recombinant enzyme and 4 µM of 4MU-Neu5Ac, 4MU-Neu5Gc, or 4MU-KDN in PBS was incubated at 37 °C for 30 min in the dark. The reaction was stopped by adding 50 µL of 250 mM glycine-NaOH (pH 10.4), and the fluorescence (excitation at 365 nm, emission at 437 nm) was measured for the sialidase activity with the Enspire plate reader (PerkinElmer, Waltham, MA). The pH-dependent profile of activity was measured using the following buffers instead of PBS: 0.1 M sodium acetate for pH 4.0-6.0, 0.1 M HEPES-NaOH for pH 6.0-8.0, and 0.1 M Tris-HCl for pH 8.0-10.0. For inhibition experiments using Neu5Ac2en, the reaction mixture (50 µl) was incubated in the presence of 0-40 µM Neu5Ac2en to obtain the IC_{50} value. For kinetic analyses, 0-80 µM 4MU-Neu5Ac or 4MU-KDN in the presence of 4.6 µM and 1.7 µM of Neu5Ac2en were used.

2.4. Substrate specificity determination by HPLC

Sialylglycans of different linkages, i.e., 100 µM Neu5Acα2,3Galβ1,4GlcNAc (α2,3Gal), Neu5Acα2,6Galβ1,4GlcNAc (α2,6Gal), Neu5Acα2,8Neu5Ac (α2,8Neu5Ac), and Neu5Acα2,9Neu5Ac (α2,9Neu5Ac), were incubated with the recombinant protein of ORF03865 (3.84 ng) in 20 µl 0.1M HEPES-NaOH (pH 7.0) at 37 ℃ for 15 min. The 5-µl aliquots were measured for released Neu5Ac by DMB-derivatization HPLC method [17].

2.5. Reactivity to polysialic acid by TLC

Five µg of α2,8-Poly-*N*-acetylneuraminic acid (α2,8polySia) or α2,9-poly-*N*-acetylneuraminic acid (α2,9polySia) were incubated with the recombinant protein of ORF03865 (5 µg) in 50 µl 0.1 M HEPES-NaOH (pH 6.5) at 30 ℃ for 15 min. The reaction mixture was spotted on a TLC plate, developed in 1-propanol, 25% aqueous ammonium, water (6:1:2.5, v/v/v) for 5 h, and visualized by spraying with the resorcinol-HCl reagent and heating [19].

2.6. *in vivo* Digestion of cultured cells

CHO cells were cultured, collected with a cell scraper and rinsed with PBS, followed by fixation in 4% paraformaldehyde at r.t. for 8 min. After washed with 0.5% BSA and 5 mM EDTA in PBS, they were incubated with 2 µg of ORF03865 in 100 µL of PBS at 37 ℃ for 30 min, and then rinsed with PBS. Cells were incubated with the 12E3 (10 µg/mL) at 4 ℃ for 30 min and then rinsed with PBS. The cells were then incubated with Alexa-labeled anti-mouse IgG and IgM (2 µg/mL) at 4 ℃ for 30 min. After washing twice with PBS, cell surface staining was measured using a flow cytometer (Gallios, BeckmanCoulter, Brea, CA) and the collected data was analyzed with the Kaluza software (Version1, BeckmanCoulter).

2.7. Molecular modeling

For molecular modeling, the Molecular Operating Environment (MOE) 2019.0101 program package (Chemical Computing Group, Inc.) was used. For multiple alignment, MUSCLE [20] was used.

3. Results

3.1. Identification of the sialidase gene encoding the active sialidase

We previously identified KDNase SM, which is capable of cleaving the Kdn glycosides in a bacterium of genus *Sphingobacterium* [7]. However, its cDNA could not be identified yet. We thus sought to find sialidase genes in the genome of *Sphingobacterium* sp. strain HMA12, which were recently isolated [13]. Four candidate genes, ORF03426, ORF03431, ORF03865, and ORF04787, that were classified under GH33 family on the carbohydrate-active enzymes database (CAZy; http://www.cazy.org), were identified. Those enzymes were expressed in *E. coli* as His-tagged enzymes without predicted signal peptide sequence, i.e., 1-21, 1-21, 1-29, and 1-20 amino acids, respectively, and assayed for the sialidase activity. Only the ORF03865 gene product showed the activity to both 4MU-Neu5Ac and 4MU-KDN (**Fig.1a**). The ORF03865 sialidase was a soluble protein with a single polypeptide chain of 59 kDa (with the tag) (**Fig. 1b**), and 42 kDa (without the tag). The deduced amino acid sequence of ORF03865 shows that it consists of 385 amino acids with signal peptide (1-19th aa), a RIP motif at 36th aa, and four Asp-boxes (SXDXGXTW; X, any amino acids) (**Fig.1c**), consistent with the presence of these two kinds of sialidase motifs in the sequence. It should be noted that the purified recombinant ORF03865 had no contamination of protease activity during at least 12 h incubation, as evaluated using denatured bovine serum albumin as test sample.

3.2. Determination of the optimal temperature and pH

The purified recombinant ORF03865 was examined for the temperature and pH dependency of the activity. For temperatures ranging from 0 to 60 ºC, the optimum one was 25 to 37 ºC for both 4MU-Neu5Ac and 4MU-Kdn (**Fig.2a**). For the pH dependency between pH 4.0 and 10.0 (**Fig.2b**), the optimal pH was pH 7.0 for both substrates. It is noted that the level of the activity for both substrates was kept high, around 50% of the maximum activity at pH 7.0, under alkaline conditions, pH 8.5-10.0. Neutral optimal pH for the activity is unique, because Au-sialidase, Cp-sialidase, Vc-sialidase, and Sp-sialidase have acidic optimal pHs [21-24].

3.3. Substrate specificity

The ORF03865 sialidase, together with other bacterial sialidases (Au-sialidase, Cp-sialidase, Vc-sialidase, and Sp-sialidase), were examined for their enzyme activity using 4MU glycosides of Neu5Ac, Neu5Gc, and Kdn as substrates (**Fig. 3a**). All the bacterial

sialidases examined in this study had the activity to 4MU-Neu5Ac and 4MU-Neu5Gc, although the levels of activity were different between them. Notably, only ORF03865 sialidase, but not other enzymes, could cleave 4MU-Kdn, thus indicating that the new sialidase shows broad specificity in Sia species recognition. The kinetic analysis of the enzyme (see **Fig. 4b, 4d** for the Lineweaver-Burk plot) showed that the k_{cat}/K_m values ($s^{-1}M^{-1}$) were (1.24 ± 0.09) × 10^4 for 4MU-Kdn and (1.74 ± 0.21) × 10^5 for 4MU-Neu5Ac, suggesting that 4MU-Neu5Ac is 14 times more efficient substrate than 4MU-Kdn.

For the linkage specificity, the ORF03865 sialidase could act on all of four different linkages of Neu5Ac with a preference of α2,3Gal > α2,9Neu5Ac > α2,6Gal = α2,8Neu5Ac in this order (**Fig.3b**). The TLC analysis showed that the products from α2,8polySia and α2,9polySia by the ORF03865 treatment were only Neu5Ac, but not di/oligoNeu5Ac (**Fig.3c**). These results indicate that the ORF03865 sialidase is an exo-sialidase.

3.4. Application to *in vivo* digestion

Since the ORF03865 sialidase works in PBS at neutral pH, whether it can be applied to digestion of the surface sialosides in live cells was investigated. FACS analysis of the cells before and after digestion with the ORF03865 sialidase was performed (**Fig.3d**). The surface polySia amount was decreased after digested for 30 min. It is suggested that the enzyme is applicable for *in vivo* analysis of the surface sialylation state.

3.5. Inhibition by Neu5Ac2en

Effects of a transition state analog of sialidase-catalyzed reactions, Neu5Ac2en, on the ORF03865 sialidase activity were tested with 4MU-Neu5Ac and 4MU-Kdn as substrates (**Fig. 4a, 4c**). Neu5Ac2en was shown to inhibit the hydrolysis of both 4MU-Neu5Ac and 4MU-Kdn by the enzyme with the IC_{50} of 4.6 μM and 1.7 μM, respectively. Kinetic analysis was also performed, and the Lineweaver-Burk plots were drawn to determine the inhibition mode of Neu5Ac2en (**Fig.4b, 4d**). These results showed that Neu5Ac2en competitively inhibited the hydrolysis of the glycosides of both Neu5Ac and Kdn by the enzyme. It is thus suggested that the same active site is shared by the Neu5Ac and Kdn glycosides.

4. Discussion

In this study, we first identified Kdn-sialidase, which can hydrolyze both Kdn and Neu5Ac/Neu5Gc glycosides, as a bacteria-derived one from a Gram-negative *Sphingobacterium* sp. strain HMA12 [13]. Actually, no bacterial sialidases examined in this study acted on 4MU-Kdn at all (**Fig.3a**). We previously reported that a Kdn-specific sialidase (Kdnase) existed in *Sphingobacterium multivorum* [7]. This Kdnase can only act on Kdn residues, but not on Neu5Ac/Neu5Gc residues [7]. Thus, the genus *Sphingobactrium* appears to be unique in utilizing Kdn glycosides, different than *Arthrobacter*, *Clostridium*, *Vibrio*, and

Streptococcus genera. In other organisms than bacteria, Kdn-sialidases are known to occur, including filamentous fungus *Aspergillus fumigatus*, oyster, starfish, and loach [9-12], while no Kdn-sialidase has been known in mammals [6,7,25]. Although further studies on the occurrence of Kdn-sialidase and Kdnase are necessary, it would be interesting to pursue how Kdn and Neu5Ac/Neu5Gc are selected in evolution in relation to adaptation and survival strategies.

Bacterial sialidases have been used not only as a tool for structural and functional analyses of sialo-glycoconjugates [1,2,5], but also as a Sia-targeted medicinal probe for tumor therapy [26]. For the application purposes, merits of the ORF03865 sialidase are two-fold. The first one is its broader specificity in the Sia species and linkages. As already discussed above, it can act on any glycosides of Neu5Ac, Neu5Gc, and Kdn, and can hydrolyze the known major linkages of α2,3Gal, α2,6Gal, α2,8Neu5Ac, and α2,9Neu5Ac. Most prominent is the susceptibility to Kdn residues which are resistant to the action of most bacterial sialidases. The second one is its neutral pH optimum for the activity. This property is advantageous, when it is applied to enzymatic treatments of acid labile glycans, such as polysialic acid and Kdn residues, under neutral pHs [14,16]. In addition, its maximal activity obtained under physiological pH allows us to perform *in vivo* digestion of the surface of live cells and *in situ* application to animals through its injection.

Although underlying mechanisms how the ORF03865 sialidase exhibits those unique properties remain to be elucidated, its molecular modeling by the MOE interestingly shows the highest similarity with *Aspergillus fumigatus* KDNase (D84A mutant, PDB: 4M4U.A) in the protein folding, irrespective of the low identities (29%) of amino acid sequence with each other. AfKDNase is a Kdn-sialidase that can cleave the Kdn and Neu5Ac glycosides [12]. The k_{cat}/K_m values ($s^{-1}M^{-1}$) of AfKDNase are 1.82×10^5 and 53.3, for the 4MU-glycosides of Kdn and Neu5Ac, respectively [12, 27], showing that 4MU-Kdn is 3,400 fold more efficient substrate than 4MU-Neu5Ac. Considering that the ORF03865 sialidase prefers Neu5Ac to Kdn with 14-fold higher efficiency, it is concluded that the ORF03865 sialidase has much broader Sia-species specificity than AfKDNase. Notably, the AfKDNase R171L mutant, in which the size of binding pocket around C-5 substituent of Sia is enlarged, increases the k_{cat}/K_m value for the Kdn glycoside from 53.3 to 74.2 [27]. In the ORF03865 sialidase, T146 is corresponding to R171 of AfKDNase, and the T146R mutant would have narrower binding pocket around C-5 substituent. However, the mutation did not result in any changes on Sia species preference (data not shown). Based on MOE modeling, T177 and S204, which are also located near the C5-position of Sia, might be important in recognition of Kdn by the ORF03865 sialidase. In addition, Neu5Ac2en, a transition state analog, competitively inhibits the ORF03865 sialidase for the hydrolysis of both 4MU-Neu5Ac and 4MU-KDN (**Fig.4b, 4d**). The ORF03865 sialidase is suggested to have a common active site to Neu5Ac and KDN glycosides. Previously, we isolated KDNase SM from the same genus *Sphingobacterium*, and it is shown to cleave only KDN, but not Neu5Ac/Neu5Gc glycosides. It should be noted that KDNase

SM is not inhibited by Neu5Ac2en, but by Kdn2en [28]. These results indicate the importance of the structure of a binding pocket to accommodate the C5-substituents of Kdn and Neu5Ac. Thus, the X-ray crystallographic analysis of the mutated ORF03865 enzyme around the binding pocket would be required to understand how the ORF03865 recognizes Sia species. Furthermore, it is also interesting to point out that the pI of the ORF03865 sialidase is 9.0, while those of other bacterial sialidases that have acidic optimal pHs are 5-6. Since the pKa of Neu5Ac is 2.5 [29], the ionization state of Neu5Ac remains unchanged under both optimal pHs. Therefore, the pIs of the sialidase may be related with optimal pHs for the sialidase reaction.

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Legends for Figures

Fig.1 Identification of a Kdn-sialidase from *Sphingobacterium* sp. strain HMA12. (a) Relative sialidase activities for four candidate genes of the GH33 family from the bacterium. The recombinant proteins were expressed in *E. coli*, and measured for the sialidase activity using 4MU-Neu5Ac and 4MU-Kdn as substrates. (b) SDS-PAGE-CBB staining of the purified ORF03865 sialidase. The enzyme was affinity-purified as described under Materials and Methods. (c) The deduced amino acid sequence of the ORF03865 sialidase. Predicted signal peptide sequence (bold letters), RIP motif (shaded), and Asp-boxes (square). The numbers represent the amino acid number.

Fig.2 Temperature and pH dependency of the ORF03865 sialidase activity. (a) Temperature-dependent profiles. The enzyme reaction was performed at pH 7.0 for 30 min at 0-60 ℃, using 4MU-Neu5Ac and 4MU-KDN. Released 4MU was fluorometrically quantified (excitation at 365 nm, emission at 437 nm). The experiments were performed in triplicate, and the standard deviations are shown by the bar. (b) pH-dependent profiles. The enzyme reaction was performed at 37 ℃ for 30 min at pH 4.0-10.0: 0.1 M sodium acetate for pH 4.0-6.0, 0.1 M HEPES-NaOH for pH 6.0-8.0, and 0.1 M Tris-HCl for pH 8.0-10.0. The experiments were performed in triplicate, and the standard deviations are shown by the bar.

Fig.3 Substrate specificity of the ORF03865 sialidase. (a) Comparison of the Sia species specificity with known bacterial sialidases. 4MU-Neu5Ac, 4MU-Neu5Gc, and 4MU-KDN was digested with sialidases derived from *Sphingobacterium* sp. (ORF03865), *Arthrobacter ureafaciens* (Au), *Clostridium perfringens* (Cp), *Vibrio cholera* (Vc), and *Streptococcus pneumoniae* (Sp). Values represent relative activities to the 4MU-Neu5Ac hydrolyzing one set to 1.0. The experiments were performed in triplicate, and the standard deviations are shown by the bars. (b) Linkage specificity. The sialylglycans, i.e., α2,3Gal, α2,6Gal, α2,8Neu5Ac, and α2,9Neu5Ac, were digested with the ORF03865. The released Neu5Ac was quantitated by the DMB-derivatization HPLC method. The experiments were performed in triplicate, and the standard deviations are shown by the bars. (c) TLC analysis of products from polySia. α2,8polyNeu5Ac and α2,9polyNeu5Ac were digested at pH 7.0 at 37 ℃ for 15 min. The digests were analyzed by TLC. +, with enzyme :-, without enzyme. Arrow head, Neu5Ac. (d) FACS analysis with anti-polySia antibody. CHO cells were treated with and without the ORF03865 sialidase and applied to FACS analysis.

Fig.4 Inhibition and kinetic analysis of the ORF03865 sialidase. (a) Effect of Neu5Ac2en on the 4MU-Neu5Ac-hydrolyzing reaction. The reaction was performed in the presence of 0-10 μM Neu5Ac2en at pH 7.0 at 37 ℃ for 30 min. The released 4MU was fluorometrically

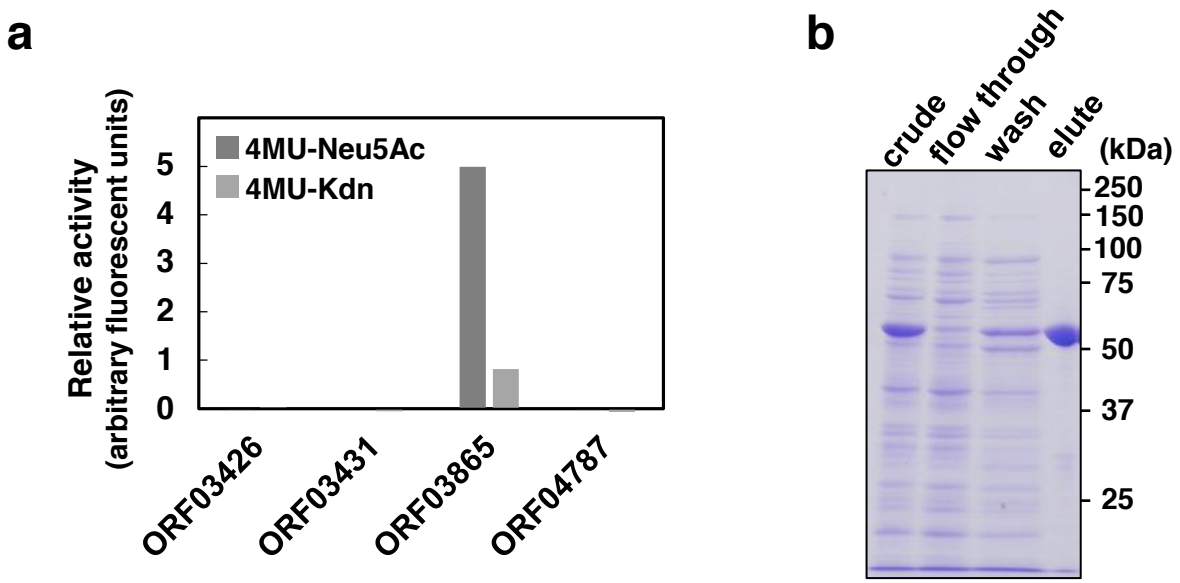
quantitated (excitation at 365 nm, emission at 437 nm). (b) The Lineweaver-Burk plots for 4MU-Neu5Ac. The substrate (0-80 μM) was incubated with the ORF03865 sialidase in the presence and absence of 4.6 μM Neu5Ac2en at pH 7.0, at 37 ℃ for 30 min. (c) Effect of Neu5Ac2en on the 4MU-Kdn-hydrolyzing reaction. See (a) for the detail. (d) The Lineweaver-Burk plots for 4MU-Kdn. The substrate (0-80 μM) was incubated with the ORF03865 sialidase in the presence and absence of 1.7 μM Neu5Ac2en at pH 7.0, at 37 ℃ for 30 min. All the experiments were triplicated, and the standard deviations are shown by the bar.

Footnote

[1]The abbreviations used are: Kdn, deminoneuraminic acid; Neu5Ac, *N*-acetylneuraminic acid; Neu5Ac2en, 2,3-didehydro-2-deoxy-*N*-acetylneuraminic acid; Neu5Gc, *N*-glycolylneuraminic acid; Sia, Sialic acid;

Figure1

Fig. 1 (Iwaki et al.)



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101 DLLDPRYPQGRLLLFYNTGNNHEGEVRKGNGLRECWSISSTDAGKTWSHP 150
151 ENITLETHRPNQPLVNTQYNFQEDWRTYANTPGHALQFDSGKYKGRIYIP 200
201 ANHSEGNPKANGKDYFAHSYYSDDHGKTFKIGASVKFEGSNETMAAQISN 250
251 TGLYMNSRNQQGNVKSRIVSYSNDGGVTWDTTYYDKNLPDPVNQGSVLSW 300
301 RRKGRYLLAVCNAATANRRDNLTLRISRDQGKTWFFNQVVSKAPEGVKGD 350
351 YAAYSDLVLLDKNRIGVLFEKENYSKIVFVPVNLK                385
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Figure2

Fig. 2 (Iwaki et al.)

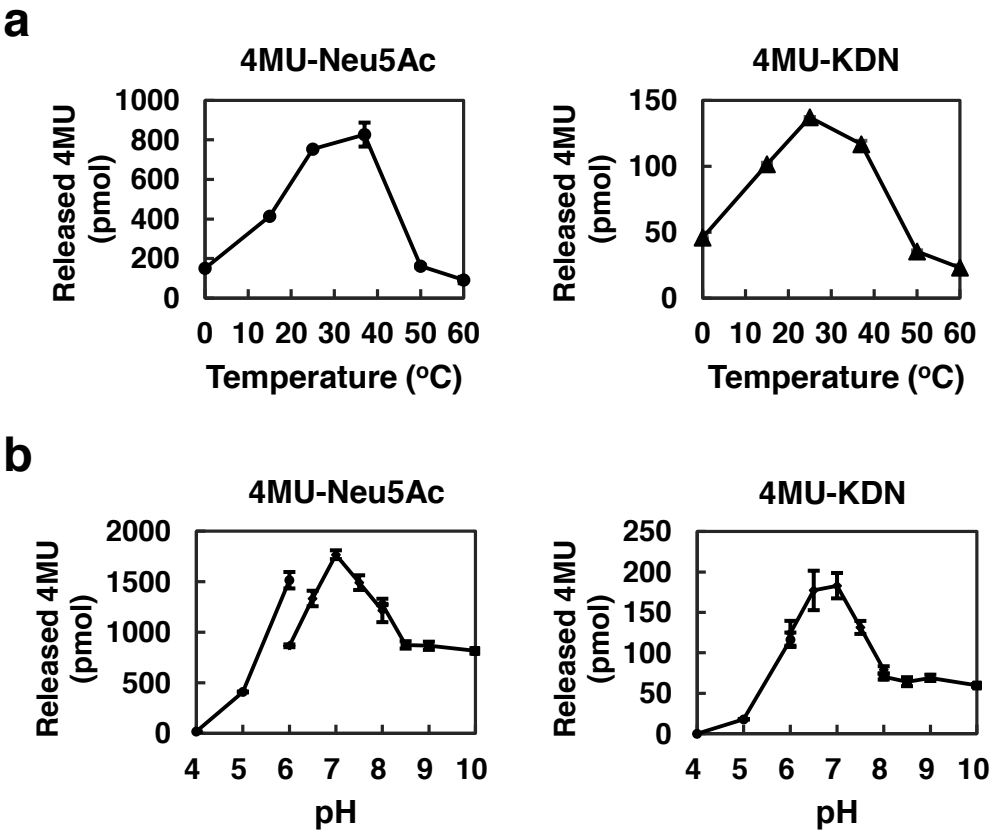

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Figure3

Fig. 3 (Iwaki et al.)

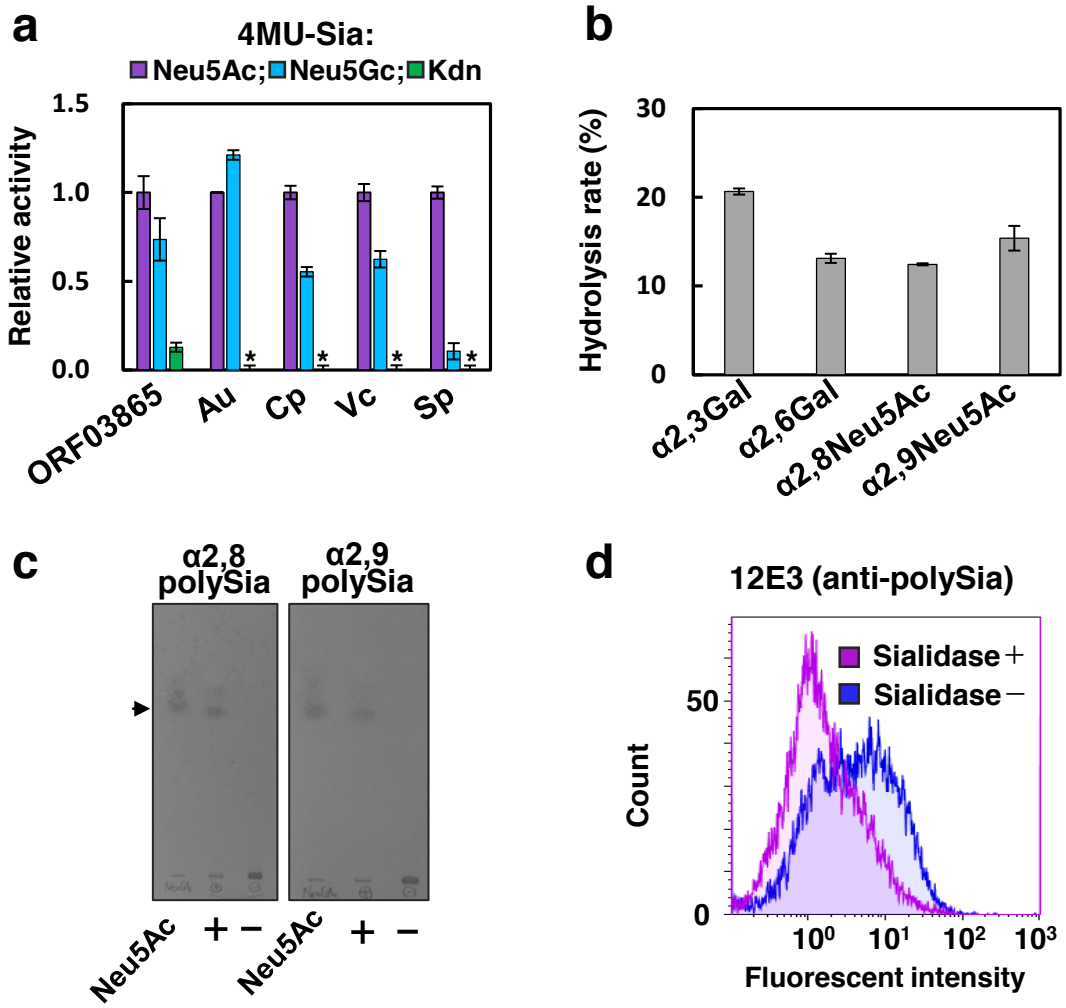

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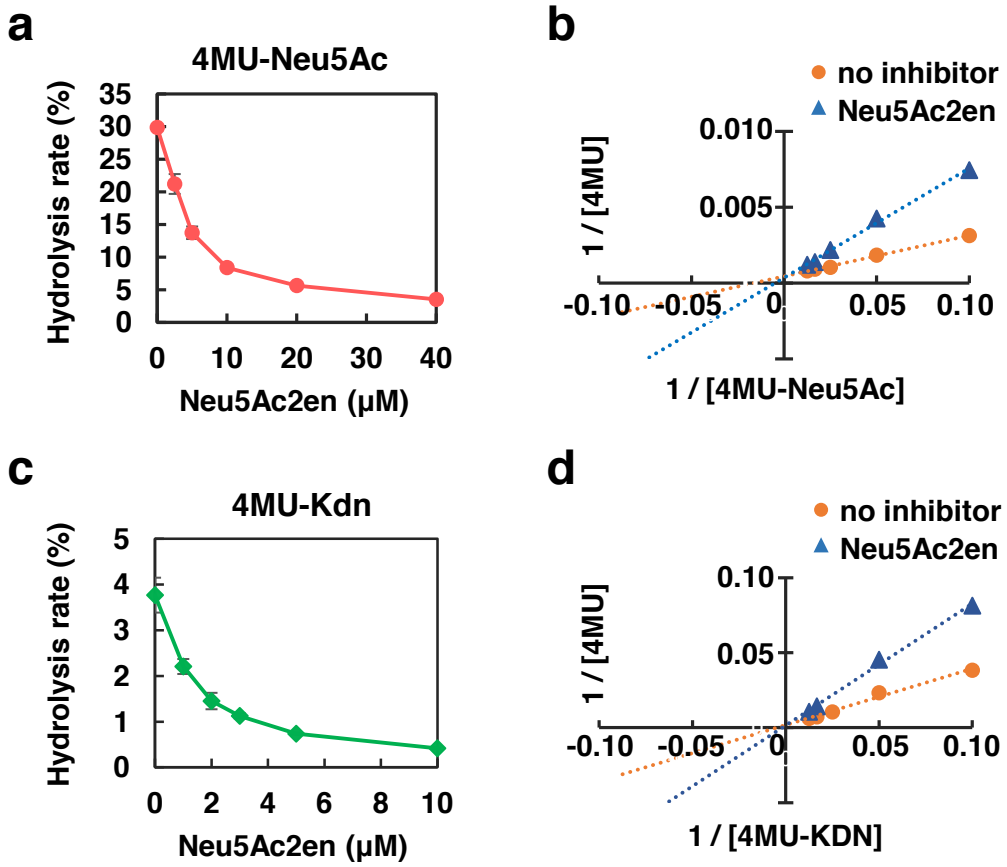
Figure4

Fig. 4 (Iwaki et al.)



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Conflict of Interest

On behalf of all the authors for this manuscript, the corresponding author declare:
None declared.