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# Identification and functional characterization of intracellular sialidase NeuA3 from *Streptomyces avermitilis*

Jia Guo [1], Yicheng Wang [1], Bo Song, Xin Wang, Ganglong Yang, Feng Guan *

*The Key Laboratory of Carbohydrate Chemistry & Biotechnology, Ministry of Education, School of Biotechnology, Jiangnan University, Wuxi 214122, PR China*

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ABSTRACT

Sialidases (EC 3.2.1.18), glycosidases that cleave the linkages whereby sialic acids are attached to glycoconjugates, are found in most bacterial species. Because sialidases can convert polysialogangliosides to monosialoganglioside GM1, they have potential clinical application for treatment of human neurological and other disorders including Alzheimer's disease, Parkinson's disease, and spinal cord injury. Sialidases with high substrate specificity are desirable for more efficient GM1 production. In this study, the sialidase *neuA3* gene from the non-pathogenic bacterium *Streptomyces avermitilis*, which is commonly used for industrial applications, was analyzed, cloned, and expressed in *E. coli* BL21 (DE3). Purified NeuA3 enzyme was characterized using 2′-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid (4-MUN) as a synthetic substrate. NeuA3 has a low molecular weight (~38 kDa), showed strong stability in the presence of various divalent metal ions and temperature and pH values, preferentially cleaved α2,3- and α2,6-linked sialic acids from gangliosides, and efficiently converted crude porcine brain gangliosides to GM1. NeuA3 treatment of malignant human bladder cancer cells YTS-1 presented enhanced cell surface expression of GM1. The novel sialidase NeuA3 will be useful for functional studies of sialylated oligosaccharides and other sialoglycoconjugates, especially for studying the functions of GM1 in cancer research.



## 1. Introduction

The terminal sialic acids of cell surface glycoconjugates have a negative charge and display specific associated physicochemical properties [1]. These glycoconjugates mediate many important cell physiological processes, including nervous system embryogenesis, inflammatory responses, and immune response pathways [2–4]. The ganglioside GM1 (monosialotetrahexosylganglioside), in which a single sialic acid is linked to galactose, has potential clinical applications to a variety of serious human disorders, including Alzheimer's disease, Parkinson's disease, spinal cord injury, and stroke [5–9]. GM1 is usually prepared from polysialogangliosides, which have two or more α2,8-linked sialic acid residues. Therefore, functional research along this line requires a high conversion ratio of polysialogangliosides to GM1 for economical production of GM1.

Sialidases (EC 3.2.1.18; also called neuraminidases) are glycosidases that cleave α2,3-, α2,6-, or α2,8-linkages binding terminal sialic acid to oligosaccharides, glycoproteins, glycolipids, or other glycoconjugates [10]. Sialidases are found in the majority of viruses, pathogenic fungi, bacteria, protozoa, and higher animals [11–13]. In certain pathogenic bacteria, some secreted sialidases were thought to function as virulence factors that recognize sialic acids exposed on the host cell surface [12], while intracellular sialidases were thought to cleave sialic acid from various sialylated oligosaccharides to use as the nutrient [14]. In various studies, sialidases from *Pseudomonas* sp. strain YF-2 and *Arthrobacter ureafaciens* M1057 were applied to conversion of crude brain gangliosides to GM1 [15,16]. However, most of the sialidases used in research are derived from pathogenic microorganisms. To elucidate the process of crude brain ganglioside conversion to GM1, it is desirable to derive a sialidase with strict substrate specificity from bacteria, particularly non-pathogenic bacteria.

Sialidase production has been reported for several species and strains of the non-pathogenic bacterial genus *Streptomyces*, including *S. griseus*, *S. purpeofuscus*, and *S. albus* [17–19], but structural identification and catalytic properties of these sialidases were not described. *S. avermitilis*, an important species used for industrial

* Corresponding author at: The Key Laboratory of Carbohydrate Chemistry & Biotechnology, Ministry of Education, School of Biotechnology, Jiangnan University, 1800 Lihu Avenue, Wuxi, Jiangsu 214122, China. Tel.: +86 051085918126; fax: +86 051085918126.
*E-mail address:* fengguan@jiangnan.edu.cn (F. Guan).
[1] These authors contributed equally to this study.

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avermectin production, has three genes (*neuA1*, *neuA2*, *neuA3*) that encode putative sialidases as evidenced by bioinformatic analysis of the complete genome [20]. Like most bacterial sialidases, the encoded proteins NeuA1 and NeuA2 are putative secreted sialidases, whereas NeuA3 is an intracellular sialidase, which is not common in bacterial sialidases. NeuA3 has the lowest molecular weight (357 amino acids) of the three. Motif information from Pfam search (http://pfam.janelia.org/search) results indicates that all three proteins have a BNR repeat-like domain (BNR_2), and NeuA1 has a concanavalin A-like lectin/glucanase superfamily (Laminin_G_3) structure that may mediate specific substrate recognition, while NeuA3 only contains the conserved catalytic domain with nonspecific substrate recognition, suggesting its potential to extend the chemoenzymatic synthesis of various sialoglycoconjugates.

In this study, the *neuA3* gene from *S. avermitilis* was cloned and expressed in *Escherichia coli* BL21 (DE3). The biochemical/enzymatic properties of purified NeuA3 and assessed its ability to convert crude brain gangliosides to GM1 were characterized.

## 2. Materials and methods

### 2.1. Bacterial strains, growth conditions, and vector

*S. avermitilis* strain ATCC31267 was kindly donated by Dr. Ying Wen (China Agricultural University; Beijing, China) and was grown on solid YMS medium at 28 °C [21]. Mycelia were grown in liquid YEME medium containing 25% sucrose [22].

*E. coli* strains JM109 and BL21 (DE3) (Novagen; Darmstadt, Germany), used respectively as cloning host and expression host, were grown on LB medium at 37 °C [23]. Vector pET-28a (+) (Novagen) containing a $His_6$ tag was used for gene expression.

### 2.2. Cell culture

Malignant bladder cancer cell line YTS-1 was kindly donated by Dr. S. Hakomori (The Biomembrane Institute; Seattle, WA, USA). Cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum (HyClone; Logan, UT, USA) and 1× penicillin/streptomycin (Gibco; Carlsbad, CA, USA) at 37 °C in 5% $CO_2$ atmosphere.

### 2.3. Cloning and expression of NeuA3 in E. coli

The nucleotide sequence of *neuA3* gene is obtained from "Genome project of *Streptomyces avermitilis*" (http://avermitilis.ls.kitasato-u.ac.jp). The *neuA3* gene (Gene ID: SAV_5934), encodes a putative sialidase of 357 amino acids (GenBank Accession No. NP_827111.1). Genomic DNA was obtained from *S. avermitilis* by the phenol/chloroform method as described previously [23]. An 1138-kb DNA fragment of the *neuA3* open reading frame was amplified by polymerase chain reaction (PCR) from the genomic DNA by primers ETneuA3-S, containing an *Eco*RI site (5′-GGAATTCCATATGGCGATGACAGAAGCCAGTACAC), and ETneuA3-AS, containing a *Hin*dIII site (5′-CCCAAGCTTCAGTAGAGGTCATGGGGTGA). The PCR fragment was digested with *Eco*RI/*Hin*dIII and inserted into expression vector pET-28a(+) to generate pET28-NeuA3. pET28-NeuA3 was confirmed by DNA sequencing and then introduced into *E. coli* BL21 for protein expression. The recombinant NeuA3 protein tagged with $His_6$ at the N-terminus was induced by isopropyl-β-D-thiogalactopyranoside (IPTG). Next, cells were harvested and resuspended in 10 mM imidazole lysis buffer, and disrupted with sonicator for 20 min. After centrifugation at 12,000 rpm for 20 min at 4 °C, the supernatant was filtrated using 0.4 μm filter membrane, loaded onto a Ni-NTA column (Roche; Basel, Switzerland) which had been equilibrate with three bed volumes of 20 mM imidazole, washed with five bed volumes of 20 mM imidazole and eluted with 500 mM imidazole. The recombinant proteins in the elution buffer were further purified by dialysis and freeze drying, and were dissolved in 50 mM potassium phosphate (pH 6.8).

### 2.4. Gel separation and staining

Enriched proteins were loaded onto 10% SDS-PAGE [24] and separated using a Mini-PROTEAN Tetra System (Bio-Rad; Berkeley, CA, USA). Proteins were visualized by 2 h treatment with Coomassie Brilliant Blue R250 and then partially destained with a solution containing 10% acetic acid and 5% ethyl alcohol. Individual protein bands were excised from the gel.

### 2.5. In-gel digestion

In-gel digestion was performed as described previously [25]. In brief, protein bands obtained as above were cut into pieces, destained with 100 μL of 40 mM ammonium bicarbonate/acetonitrile (1:1 v/v) (Sigma; St. Louis, MO, USA), added with 50 μL acetonitrile, and vortexed. Proteins were reduced and alkylated by dithiothreitol and iodoacetamide (Sigma), respectively, and then trypsinized (Promega; Madison, WI, USA) at 37 °C for 12 h. Peptide samples were desalted using ZipTip μ-C18 pipette tips (Millipore; Billerica, MA, USA), spotted onto an MTP 384 AnchorChip sample target, and air-dried.

### 2.6. Mass spectrometry

Peptides were characterized by MALDI-TOF/TOF-MS (Matrix assisted laser desorption/ionization time-of-flight/time-of-flight mass spectrometry; UltrafleXtreme, Bruker Daltonics; Bremen, Germany). Peptides from mass spectra of in-gel digested samples were compared with those in National Center for Biotechnology Information (NCBI) databases.

### 2.7. Sialidase activity assay

Sialidase enzymatic activity was assayed using 2′-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid (4-MUN) (Sigma) as a substrate [26]. The incubation reaction was performed for 10 min in a 100 μL volume containing 0.1 mM 4-MUN in 100 mM sodium acetate buffer (pH 5.1), and then quenched by adding 100 μL of 1 M glycine–NaOH buffer (pH 10.4). Free 4-methylumbelliferone (4-MU) was determined by spectrofluorometry (Synergy H4 Hybrid Multi-Mode Microplate Reader, BioTek; Winooski, VT, USA) with excitation wavelength 365 nm and emission wavelength 450 nm. One unit (U) of sialidase was defined as the amount of enzyme required to liberate 1 nmol 4-MU per minute in this system [26,27].

### 2.8. NeuA3 substrate specificity

NeuA3 substrate specificity was determined using α2,3- and α2,6-sialyllactose (Tokyo Chemical Industry Co.; Tokyo, Japan) as substrates. The reaction was performed in a total volume of 50 μL 1 mM sialyllactose containing 300 mM acetate buffer (pH 5.0) and 0.1 mg/mL NeuA3 at 37 °C for 1 hr, and quenched at 100 °C for 3 min. The released terminal sialic acids were detected by periodic acid/thiobarbituric acid-based (TBA) assay [28]. The mixture was oxidized by 125 μL of 25 mM potassium periodate in 125 mM $H_2SO_4$ at 37 °C for 20 min, added with 250 μL of 2% (w/v) sodium arsenite in 500 mM HCl, vortexed, added with 1 mL of 0.3% thiobarbituric acid (pH 9.0), boiled at 100 °C for 15 min, and cooled to