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Structural and biochemical characterization of the broad substrate specificity of *Bacteroides thetaiotaomicron* commensal sialidase

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ABSTRACT

Sialidases release the terminal sialic acid residue from a wide range of sialic acid-containing polysaccharides. *Bacteroides thetaiotaomicron*, a symbiotic commensal microbe, resides in and dominates the human intestinal tract. We characterized the recombinant sialidase from *B. thetaiotaomicron* (BTSA) and demonstrated that it has broad substrate specificity with a relative activity of 97, 100 and 64 for 2,3-, 2,6- and 2,8-linked sialic substrates, respectively. The hydrolysis activity of BTSA was inhibited by a transition state analogue, 2-deoxy-2,3-dehydro-*N*-acetyl neuraminic acid, by competitive inhibition with a K_i value of 35 μM. The structure of BSTA was determined at a resolution of 2.3 Å. This structure exhibited a unique carbohydrate-binding domain (CBM) at its N-terminus (a.a. 23–190) that is adjacent to the catalytic domain (a.a. 191–535). The catalytic domain has a conserved arginine triad with a wide-open entrance for the substrate that exposes the catalytic residue to the surface. Unlike other pathogenic sialidases, the polysaccharide-binding site in the CBM is near the active site and possibly holds and positions the polysaccharide substrate directly at the active site. The structural feature of a wide substrate-binding groove and closer proximity of the polysaccharide-binding site to the active site could be a unique signature of the commensal sialidase BTSA and provide a molecular basis for its pharmaceutical application.

1. Introduction

Sialic acid (Neu5Ac) is found at the terminal position of the sugar chain glycans of glycoproteins, glycolipids, and polysaccharides in cells, linked through an α-(2 → 3)- or α-(2 → 6)-glycosidic linkage to galactoside, *N*-acetyl galactosaminide or *N*-acetyl glucosaminide. There are also α-(2 → 8)-glycosidic linkages found that connect polymer sialic acid residues in some tissues, such as brain or tumor cells [1,2]. Sialidases (EC 3.2.1.18), also known as neuraminidases, are retaining glycosidases that hydrolyze sialo-glycosidic bonds through a two-step, double-displcement mechanism that involves a covalent glycosyl-enzyme intermediate [3]. Sialidases are responsible for the release of sialic acids from a wide range of sialic acid-containing molecules. Sialidases contain several conserved amino acids: an arginine triad that binds to the carboxylate group of the substrate via electrostatic interactions, a distal glutamate residue that forms a salt bridge with one of the conserved arginine residues and tyrosine residues [4]. In addition to the catalytic domain, many sialidases have carbohydrate-binding domains (CBMs) at either the N- or C-terminus, or even inserted within the β-propeller domain. It has been suggested that the presence of these carbohydrate-binding domains increases the catalytic efficiency of the sialidases, particularly in the presence of polysaccharide substrates [5–7].

Sialidases in many pathogenic bacteria are considered potential virulence factors. These sialidases function to remove the terminal sialic acid. This removal exposes the binding site of the host pathogens, such as *Bacteroides fragilis*, to attachment to the human intestinal cell surface [8–10]. Therefore, many biochemical and structural analyses have been conducted to obtain data for designing selective inhibitors against pathogenic sialidases [11,12]. However, most of the sialidase-producing microorganisms are closely related to mammals, including not only the pathogens but also the commensals, such as the *Bacteroides* species. In the human colon, *Bacteroides* species are the most numerous bacterial inhabitants in the body, corresponding to 25% of intestinal bacteria [13]. Of the *Bacteroides* species, *Bacteroides thetaiotaomicron* comprises 6% of all bacteria and 12% of all *Bacteroidetes* based on the most comprehensive 16S rRNA sequence-based enumeration of the adult human colonic microbiota [14]. *B. thetaiotaomicron*, a Gram-negative anaerobe, contains a large number of glycoside hydrolases compared to other sequenced prokaryotes and appears to be able to cleave most of the glycosidic bonds found in nature [15]. *B. thetaiotaomicron* encodes 226

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glycoside hydrolases and 15 polysaccharide lyases. These enzymes allow it to break down a wide variety of dietary carbohydrates that might be available in the gut [16,17] and that humans cannot process [18]. The carbohydrate breakdown and utilization by this bacteria produces a pool of volatile fatty acids that are reabsorbed through the large intestine and utilized by the host as an energy source, providing a significant proportion of the host's daily energy requirement [19]. Carbohydrate metabolism is an important factor in nutrition. The function and population of *Bacteroides* have been linked to obesity [20–22]. To date, studies on sialidases have mainly focused on those from pathogens, and there has been little attention directed at sialidases that originate from non-pathogens or commensal microorganism.

In this study, we carried out biochemical and structural analyses of a sialidase from *B. thetaiotaomicron* VPI-5482 (BTSA). The characterization and three-dimensional structure, which features a unique carbohydrate binding module (CBM) at the N-terminus, provides the molecular basis for the wide substrate specificity of BTSA, which could be a useful structural signature that distinguishes the commensal sialidase from other reported pathogenic enzymes.

2. Material and methods

2.1. General experiments

Escherichia coli MC1061 [F^-, *araD*139, *recA*13, Δ(*araABC-leu*)7696, *gal*U, *gal*K, *lac*X74, *rpsL*, *thi*, *hsdR*2, *mcrB*] cells were used as the host for DNA manipulation, and *E. coli* BL21(DE3) cells were used for protein production. Genetic manipulations were performed as described by Sambrook et al. [23]. *Pwo* polymerase was purchased from Roche Molecular Biochemicals (Mannheim, Germany) and restriction enzymes were purchased from Fermentas (Germany). In addition, 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (MU-Neu5Ac) and p-nitrophenyl-α-D-*N*-acetylneuraminic acid (pNP-Neu5Ac), which were the glycoprotein substrates, were purchased from Sigma Chemical Co. Colominic acid was purchased from Nacalai Tesque Inc. (Kyoto, Japan). Sialyl-α-2,3-lactose (3′-SL) and sialyl-α-2,6-lactose (6′-SL) were kindly provided by Genchem Co. (Daejeon, Korea). In addition, 2-deoxy-2,3-dehydro-*N*-acetyl neuraminic acid (Neu5Ac2en) was synthesized as previously described [24]. Other chemicals were obtained from Sigma Chemical Co. unless otherwise specified.

2.2. Construction of sialidase-expression plasmids in E. coli

B. thetaiotaomicron VPI5482 was purchased from the American Type Culture Collection (ATCC; Manassas, VA). The genomic DNA of *B. thetaiotaomicron* was isolated using a QIAmp tissue kit (Qiagen; Hilden, Germany). The gene encoding BTSA (Genbank accession code NP_809368 and Uniprot Q8AAK9) was amplified by PCR with *Pwo* DNA polymerase using the chromosomal DNA of *B. thetaiotaomicron* as a template and the two synthetic primers [BT0455-Nd-fw (5′-CTAATACCACCA*CATATG*AAGAGAAATCAT-3') and BT0455-Xh-rv (5'-GACCTCGAGTCGAATCAAATCTTT-3')]. The synthetic primers were designed based on the genomic DNA sequence of *B. thetaiotaomicron* [16]. Twenty-five PCR cycles (45 s at 94 °C, 30 s at 55 °C and 80 s at 72 °C) were performed in a thermal cycler (Perkin Elmer; GeneAmp PCR GeneAmp PCR System 2400). The resulting PCR products were digested with NdeI and XhoI and subcloned into pTKNd119, which yielded pTKBTSA. The gene fragment encoding the truncated BTSA lacking a putative signal sequence (BTSAΔ22) was amplified by PCR using pTKBTSA as a template with two primers, BT0455-d22-fw [(5'-TGCTCTATCTTTGTGCATATGTCAGACACCGTT-3') and T7 terminator (5′-TGCTAGTTATTGCTCAGCGG-3′)]. The amplified DNA was cloned into the pET-29b vector (Novagen; Madison, WI), which generated pET29BTSAΔ22.

2.3. Purification of recombinant sialidases

The recombinant *E. coli* BL21(DE3) carrying pET29BTSAΔ22 was cultured overnight in 1 L of Luria–Bertani broth [1% (w/v) Bacto-tryptone, 0.5% (w/v) yeast extract, and 0.5% (w/v) NaCl] supplemented with kanamycin (20 μg/mL) at 37 °C until the optical density at 600 nm reached 0.5, and 0.2 mM isopropylthiogalactoside (IPTG) was added to the culture broth to induce gene expression. After induction for 20 h at 20 °C with shaking, the cells were harvested, resuspended in 50 mM Tris–HCl (pH 7.5) and disrupted by sonication. The crude extracts were clarified by centrifugation. The cell extract was subjected to Ni-affinity chromatography using an AKTAprime™ plus (GE Healthcare; Uppsala, Sweden) equipped with a His-Trap® column (GE Healthcare). The eluted target proteins were dialyzed against 50 mM Tris–HCl buffer (pH 7.0).

2.4. Sialidase activity assay

The sialidase activity of recombinant BTSA was determined with MU-Neu5Ac as follows: The reaction was performed in a total volume of 250 μL consisting of 100 μL of 50 mM Tris–HCl buffer (pH 7.0), 125 μL of 0.2 mM MU-Neu5Ac, and 25 μL of a diluted enzyme solution. The reaction mixture was incubated at 37 °C for 10 min. After the enzyme reaction, 250 μL of 1 M $NaHCO_3$ was added to stop the reaction. Two hundred microliters of the reaction mixtures were transferred to 96-well plates, and the fluorescence of 4-methylumbelliferone (MU) released from MU-Neu5Ac was measured with a spectrofluorometer (VICTOR^3 1420 Multi-label plate readers; Perkin-Elmer, USA) with excitation and emission wavelengths of 365 nm and 450 nm, respectively. One unit was defined as the amount of enzyme required to release 1 μmol of MU from MU-Neu5Ac per min. The effects of pH on the hydrolysis activity were determined at 37 °C in 50 mM sodium acetate buffer (pH 4.0 to 6.0), 50 mM sodium phosphate buffer (pH 6.0 to 8.0), 50 mM Tris–HCl buffer (pH 7.0 to 9.0), and 50 mM glycine buffer (pH 9.0 to 10.0) with 0.1 mM MU-Neu5Ac using the same methods described above. To determine the optimal temperatures of the enzymes, different temperatures ranging from 20 to 50 °C were tested. The effects of divalent ions and EDTA on sialidase activity were determined by measuring the enzymatic activity at 37 °C in 50 mM Tris–HCl (pH 7.0) after pre-incubation in 50 mM Tris–HCl (pH 7.5) at 4 °C for 1 h in the presence of divalent ions or EDTA (0.1 mM, 1 mM, and 5 mM).

2.5. Sialidase activity assay for sialo-substrates

To assay sialidase activity using sialooligosaccharides and sialoglycoproteins as sialidase substrates, the EnzyChrom™ sialic acid assay kit (BioAssay Systems; Hayward, CA, USA), which is based on the periodic acid/thiobarbituric acid method [25], was used to determine the amount of free sialic acid released by the sialidase reaction. The substrate concentration of the sialolactoses was 1 mM and colominic acid and sialoglycoproteins were at a concentration of 0.5% (w/v) in 50 mM Tris–HCl (pH 7.0). The reaction was performed in a total volume of 250 μL, which consisted of 100 μL of 50 mM Tris–HCl buffer (pH 7.0), 125 μL of substrate solution, and 25 μL of a diluted enzyme solution. The reaction mixture was incubated at 37 °C for 10 min. The change in absorbance was measured at 540 nm with a micro-plate reader (VERSAmax, Molecular device; Sunnyvale, USA). The final absorbance was converted into the amount of Neu5Ac using a standard curve prepared using Neu5Ac.

2.6. Hydrolysis kinetics of BTSA

The kinetic parameters of BTSA were determined using MU-Neu5Ac, pNP-Neu5Ac, 3′-SL, 6′-SL, and colominic acid as the substrates. The initial reaction velocity of either MU release from MU-Neu5Ac or pNP