学位論文

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Characterization of a recombinant *Bacteroides fragilis* sialidase expressed in *Escherichia coli*

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ABSTRACT

The human gut commensal *Bacteroides fragilis* produces sialidases that remove a terminal sialic acid from host-derived polysaccharides. Sialidase is considered to be involved in *B. fragilis* infection pathology. A native *B. fragilis* sialidase has been purified and characterized, and was shown to be post-translationally modified by glycosylation. However, the biochemical properties of recombinant *B. fragilis* sialidase expressed in a heterologous host remain uncharacterized. In this study, we examined the enzymatic properties of the 60-kDa sialidase NanH1 of *B. fragilis* YCH46, which was prepared as a recombinant protein (rNanH1) in *Escherichia coli*. In *E. coli* rNanH1 was expressed as inclusion bodies, which were separated from soluble proteins to allow solubilization of insoluble rNanH1 in a buffer containing 8 M urea and renaturation in refolding buffer containing 100 mM $CaCl_2$ and 50 mM L-arginine. The specific activity of renatured rNanH1 measured using 4-methylumberiferyl-α-D-*N*-acetyl neuraminic acid as a substrate was 6.16 μmol/min/mg. The optimal pH of rNanH1 ranged from 5.0 to 5.5. The specific activity of rNanH1 was enhanced in the presence of calcium ions. rNanH1 preferentially hydrolyzed the sialyl α2,8 linkage and cleaved sialic acids from mucin and serum proteins (e.g., fetuin and transferrin) but not from α1-acid glycoprotein, which is similar to the previously observed biochemical properties for a native sialidase purified from *B. fragilis* SBT3182. The results and methods described in this study will be useful for preparing and characterizing recombinant proteins for other *B. fragilis* sialidase isoenzymes.

1. Introduction

The human gut harbors in excess of approximately 10^{11} microorganisms per gram of wet weight [1]. The majority of these microorganisms are obligate anaerobes, and the phylum Bacteroidetes represents approximately 25% of the gut microbiota [1]. The genus *Bacteroides* is currently classified into more than 50 species. Although *B. fragilis* accounts for about 0.5% of the human colonic microbiota, this bacterium is considered to be the most virulent species in the genus *Bacteroides* due to its frequent isolation from clinical specimens [1]. In particular, *B. fragilis* is reported to account for approximately 80% of clinically isolated *Bacteroides* species [1].

For nutrients, *Bacteroides* utilize otherwise indigestible polysaccharides that derive from glycoproteins and glycolipids expressed in human intestinal mucosa [1,2]. These polysaccharides are composed of glucose, galactose, mannose and more complex sugars (e.g., *N*-acetyl-D-glucosamine and *N*-acetylneuraminic acids [sialic acids]) [2]. Sialic acid is expressed at a terminal residue of the sugar chains in three different configurations (α2,3, α2,6, and α2,8 linkages) [2] and protects sugars from sequential digestion by exoglycosidases produced by intestinal bacteria. Some human intestinal pathogens such as *Vibrio cholerae* [3], *Salmonella typhimurium* [4] and *Clostridium perfringens* [4] produce sialidases [EC 3.2.1.18] to remove sialic acids from non-reduced polysaccharides of host glycoproteins and glycolipids.

B. fragilis also produces sialidase [5], which can modulate the ability of *B. fragilis* to cause host infection [6]. However, the precise roles of sialidase in *B. fragilis* pathogenesis remain unclear.

Native sialidase purified from *B. fragilis* SBT3182 was previously shown to exist in two molecular masses, 50 kDa and 55 kDa due to differential post-translational glycosylation [7,8]. *B. fragilis* sialidase can hydrolyze three kinds of sialic acid configurations and preferentially cleaves the sialyl α2,8 linkage compared to sialyl α2,3 and

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α2,6 linkages [8,9]. On the other hand, other bacterial sialidases release only low amounts of sialic acid from the sialyl α2,8 linkage [11]. Thus, *B. fragilis* sialidase has unique substrate specificity relative to other bacterial sialidases.

Whole genome sequence analysis of *B. fragilis* YCH46 identified at least six kinds of sialidase genes on its genome (BF0734, BF1330, BF1792, BF3936, BF3937 and BF4242) [10]. Of these, the proteins encoded by BF1792, BF3936, BF3937, and BF4242 contain four or five Asp boxes [10–12], an amino acid motif (Ser/Thr-X-Asp-X-Gly-X-Thr/Ser-Trp) that is conserved among bacterial sialidases [11]. We have previously designated these sialidase genes as *nanH1*, *nanH3*, *nanH2* and *nanH4*, respectively [13]. The *nanH1* gene is located within the sgu (sialoglycoconjugate utilization) locus that was previously characterized and expressed under standard culture condition [13]. On the other hand, the mRNA expression levels of *nanH2-4* were much lower than that of *nanH1 in vitro* and *in vivo* (unpublished data). The expression levels of NanH proteins in *B. fragilis* appeared to be vary. In order to analyze their enzyme kinetics and determine whether the various *B. fragilis* sialidases have unique substrate specificity or functional roles in gut colonization and pathogenicity, recombinant versions of these proteins will be needed.

Since a sialidase purified from *B. fragilis* SBT3182 is glycosylated [8], we aimed in this study to prepare recombinant NanH1 (rNanH1) in *E. coli* and assess whether rNanH1 without this modification shows similar properties to a native sialidase purified from *B. fragilis* SBT3182 previously reported [8].

2. Materials and methods

2.1. Bacterial strains and plasmids

B. fragilis YCH46 was isolated from the blood of a patient with septicemia at Yamaguchi Central Hospital, Yamaguchi Prefecture, Japan [10,11,14]. This strain was grown anaerobically at 37 °C in GAM broth (Nissui Pharmaceutical, Co., Tokyo, Japan). *Escherichia coli* HB101 and BL21 (DE3) [15] were used as a host for cloning experiments and for recombinant protein production, respectively. They were cultured with shaking at 37 °C in Luria-Bertani (LB) medium or HE medium (pH 7.0) [16] containing 1% bacto-yeast extract, 3.4% potassium dihydrogen phosphate, and 1% glucose.

2.2. Comparative analysis of B. fragilis YCH46 sialidases

The protein sequence similarity of the *B. fragilis* strain YCH46 sialidases (GenBank accession number D28493.1 for NanH1, YP_101212.1 for NanH2, YP_101213.1 for NanH3, and YP_101518.1 for NanH4) was assessed by Clustal W [17]. The N-terminal amino acid sequence of the SBT3182 (PC2176) sialidase was also included in the analysis. The signal sequence was identified using Signal P [18].

2.3. Construction of the overexpression vector for B. fragilis YCH46 nanH1 in E. coli

Standard DNA cloning techniques followed the protocols outlined by Maniatis et al. [19]. *B. fragilis* YCH46 *nanH1* (BF1792) was amplified by PCR using *Taq* DNA polymerase (Invitrogen, Carlsbad, CA) with 5′-GGGGTGTTGGCATATGAAAAAAGCCGTAATTCTATT TTGCG-3′ and 5′-GGGGTTTTTGCTCGAGTCATTTAATAATGTCTTTCA ACTTCACCTGG-3′ as forward and reverse primers, respectively. The underlined sequences in the forward and reverse primers indicate the recognition sites for *NdeI* and *XhoI*, respectively. We performed PCR reactions at 94 °C for 1 min, 62 °C for 1 min, and 72 °C for 2 min for 30 cycles with 2.5 U *Taq* DNA polymerase, 200 nM dNTP, and 1.0 μM of each primer. The PCR product was purified using a QIAquick-spin PCR purification kit (QIAGEN GmbH, Hilden, Germany) and digested with *NdeI* and *XhoI* at 37 °C overnight. The digested PCR product was cloned into the *NdeI* and *XhoI* sites of the pET-28b(+) vector (Novagen, Inc., Madison, WI) to yield the pFR466 construct. All restriction enzymes and T4 DNA ligase were purchased from New England Biolabs, Inc. (Beverly, MA).

2.4. Overexpression of recombinant B. fragilis YCH46 sialidase (rNanH1) in E. coli

E. coli BL21 (DE3) harboring pFR466 was cultured in 10 mL LB medium containing 30 μg/mL kanamycin with shaking overnight at 37 °C. Subsequently, 4 mL of the overnight culture was transferred to 400 mL HE medium and incubation with shaking was continued for 3 h at 37 °C. Isopropylthio-β-D-galactose (IPTG) at a final concentration of 1 mM was added to the cell culture, and the incubation was continued for another 3 h. The *E. coli* cells were harvested by centrifugation at 6000 × *g* and resuspended in 20 mL sonication buffer I (10 mM Tris-HCl [pH 7.0]). After the sonicated cell suspension was centrifuged at 9000 × *g* at 4 °C for 10 min, the supernatant was stored as a soluble fraction. The cell pellet was washed with 20 mL sonication buffer I, centrifuged at 9000 × *g* at 4 °C for 10 min, resuspended with 20 mL of sonication buffer I, and used as the insoluble fraction. These fractions were analyzed by SDS-PAGE and Coomassie blue staining to examine whether the overexpressed sialidase was present in the soluble or insoluble fraction.

2.5. Measurement of protein concentration, SDS-PAGE, and Coomassie blue staining

The protein concentration was measured using a Coomassie plus protein assay reagent (Pierce Chemical Company, Rockford, IL) with bovine serum albumin as a standard according to the manufacturer's instructions. SDS-PAGE was conducted using the method of Laemmli and molecular mass markers purchased from Sigma-Aldrich (St. Louis, MO) followed by Coomassie blue staining [19,20].

2.6. Sialidase assay

To measure the activity of crude or purified rNanH1, 10 μL of a 50 μg/mL enzyme solution was added to 80 μL of 100 mM citrate buffer (pH 5.0) along with 10 μL of 0.5 mg/mL 4-methylmbelliferyl-α-D-*N*-acetylneuraminic acid (4-MU-NANA; Nacalai tesque, Kyoto, Japan), and the reaction mixture was incubated at 37 °C for 10 min. The reaction was stopped by the addition of 400 μL of 100 mM glycine-NaOH buffer (pH 10.0). Subsequently, the fluorescence of 4-methylmbelliferone (4-MU: Nacalai tesque) released from 4-MU-NANA by crude or purified sialidase was detected at excitation and emission wavelengths of 365 nm and 450 nm, respectively [21]. One unit of sialidase was defined as 1 μmol 4-MU released at 37 °C in 1 min.

2.7. Effect of ions on sialidase activity

To assess the effect of ions on rNanH1, the activity of crude rNanH1 was measured in the presence of $CaCl_2$, $CoCl_2$, $CuCl_2$, $FeCl_2$, $FeCl_3$, $FeSO_4$, $MgCl_2$, $MnCl_2$, or $ZnCl_2$ at a final concentration of 10 mM. We added 10 μL of the 50 μg/mL crude enzyme solution to 79 μL of 100 mM sodium phosphate buffer (pH 7.0) along with 10 μL of 0.5 mg/mL4-MU-NANA as a substrate and 1.0 μL of the ionic solution. The mixture was immediately incubated at 37 °C for 10 min and the reaction was stopped by the addition of 400 μL of 100 mM glycine-NaOH buffer (pH 10.0). The effect of EDTA (pH 8.0) was also tested at a final concentration of 50 mM.

2.8. Purification of recombinant sialidase

The majority of rNanH1 was present in the insoluble fraction from IPTG-induced BL21 (DE3) cells harboring pFR466. The insoluble material was resuspended in 20 mL denaturation buffer (10 mM Tris-HCl [pH 7.0], 100 mM NaCl, and 8 M Urea). After 1 h post-incubation at room temperature, the suspension was centrifuged at 9000 × g at 4 °C for 10 min, and the supernatant was collected as solubilized material. The solubilization of the recombinant sialidase from insoluble fraction was evaluated by SDS-PAGE and Coomassie blue staining. A total of 4 mL solubilized recombinant sialidase was mixed with Ni-nitrilotriacetic acid (Ni-NTA) resin (Qiagen GmbH) and applied to a 10-mL tip plugged with glass wool and connected to a silicon tube at the end of tip. Then 15 mL of wash buffer (10 mM Tris-HCl [pH 7.0], 100 mM NaCl, 20 mM imidazole [pH 7.0], and 8 M urea) was applied to the column before 15 mL of elution buffer (10 mM Tris-HCl [pH 7.0], 100 mM NaCl, 250 mM imidazole [pH 7.0], and 8 M urea) was added to the column, and the elution samples were fractionated into 40 samples that each contained approximately 1 mL of solution. The protein concentration of each fraction was measured by Coomassie plus protein assay reagent. The elution sample containing the purified recombinant sialidase was dialyzed with 500 mL refolding buffer (10 mM Tris-HCl [pH 7.0], 100 mM NaCl, 100 mM $CaCl_2$, 1 mM DTT, and L-arginine at various concentrations between 0 and 500 mM) at 4 °C for 2 h. Dialysis was continued for additional 14 h in 500 mL of fresh refolding buffer.

2.9. Biochemical characterization of rNanH1

The optimal pH for rNanH1 activity was determined with 80 mM sodium acetate, 80 mM sodium citrate, and 80 mM Tris-malate at pH values ranging between 4.5 and 7.0 at 0.5 pH intervals. Additionally, V_{max} and K_m values with 4-MU-NANA at concentrations between 0.00 and 0.50 mM were measured. Moreover, the K_i value with 4-MU-NANA and 2,3-dehydro-2-deoxy-N-acetylneuraminic acid (Neu5Ac2en) (Boehringer, Mannheim, Germany) ranging between 0.02 and 0.10 mM and 0.00 and 0.10 mM, respectively, was calculated.

The substrate specificity of rNanH1 was examined by incubating sialo-glycoconjugates (0.6 mM total bound sialic acid content) in a total volume of 50 μL 0.1 M citrate buffer (pH 5.0) at 37 °C. To determine the substrate specificity of rNanH1, sialyl-α2,3 lactose, sialyl-α2,6 lactose, colominic acid (a sialyl-poly-α2,8 *N*-acetylneuraminic acid [Funakoshi, Tokyo Japan]), α1-acid glycoprotein (also called orosomucoid) from human plasma, human fetuin, type III gangliosides from bovine brain, type 1-S mucin obtained from bovine submaxillary glands, and human transferrin (Sigma-Aldrich) were used. The substrates were reacted with rNanH1 in a total volume of 50 μL 100 mM citrate buffer (pH 5.0) at 37 °C. Sialic acids bound to substrates were experimentally premeasured with a thiobarbituric acid (TBA) assay [22]. *N*-acetylneuraminic acid (NANA) was used to generate a standard curve.

3. Results

3.1. Relationship among B. fragilis YCH46 sialidase genes

The proteins encoded by six different genes (BF0734, BF1330, BF1792, BF3936, BF3937 and BF4242) in *B. fragilis* YCH46 have been assigned as sialidases (Supplemental Fig. S1 and Table S1). Of these, four gene products encoded by BF1792, BF3936, BF3937 and BF4242 contained four or five "Asp boxes", which is a conserved motif of bacterial sialidases. The gene products for BF1792, BF3937 and BF4242 have five Asp boxes, whereas BF3936 has four Asp boxes (Supplemental Fig. S2). In this study, these proteins were sequentially designated as NanH1, NanH3, NanH2 and NanH4, with molecular masses of 59.6, 41.0, 61.5 and 60.4 kDa, respectively. N-terminal signal sequences were found in four kinds of the NanH proteins, suggesting that these localize extracellularly or in the periplasmic space. Amino acid sequence comparisons showed high sequence identity between NanH1 and NanH4 (78.4% identity), but NanH1 was more distant from NanH2 and NanH3 (23.5%–40.4% identity, respectively) (Supplemental Table S2). NanH1 contains at least one amino acid mismatch along with one unidentified amino acid in the N-terminal amino acid sequence relative to the SBT3182 sialidase, but overall these two sialidases are predicted to be essentially equivalent.

Coyne et al. reported that a large number of *B. fragilis* proteins were glycosylated by the Bacteroidetes phylum-wide protein glycosylation system, which O-glycosylates a D(S, T)(A,L,V,I,M,T) motif [23]. NanH1 and NanH4 have two glycosylation motifs predicted from the glycosylation system (Supplemental Fig. S2). Since a native sialidase purified from SBT3182 was glycosylated *in vivo* [8], we aimed to prepare recombinant NanH1 (rNanH1) in *E. coli* and characterize the properties without this modification.

3.2. Preparation of B. fragilis YCH46 sialidase in E. coli

The *B. fragilis* YCH46 *nanH1* gene was cloned into pET-28b(+) to yield pFR466 (Supplemental Fig. S3). rNanH1 expression was induced by adding IPTG at a final concentration of 1 mM to *E. coli* BL21 (DE3) harboring pFR466. A protein of approximately 60 kDa was overproduced in *E. coli* BL21 (DE3) harboring pFR466, whereas the corresponding band was not observed in uninduced cells (Fig. 1, lanes 1 vs. 3). This 60 kDa protein was localized in the insoluble fraction and no visible band was observed in the soluble fraction (Fig. 1, lanes 3 and 4). The sialidase activities in the soluble and insoluble fractions were 1.50 and 2.13 μmol/min/mg total protein,

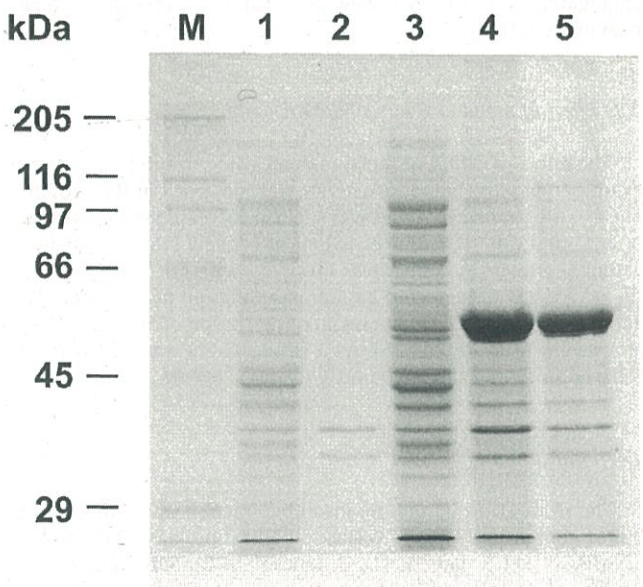

Fig. 1. Localization in *E. coli* and solubilization of rNanH1 derived from *B. fragilis* YCH46. We analyzed the localization of hexahistidine-tagged sialidase in BL21 (DE3) cells carrying pFR466 by SDS-PAGE followed by Coomassie blue staining. Lane M, molecular mass markers in kilodaltons (15 μg). Lane 1: soluble materials from uninduced cells (56.2 μg), Lane 2: insoluble materials from uninduced cells (7.3 μg), Lane 3: soluble materials from induced cells (128.9 μg), Lane 4: insoluble materials from induced cells (72.4 μg), Lane 5: solubilized materials using 8 M urea (68.7 μg).

Table 1
Overexpression of rNanH1 in *E. coli*.

IPTG	Fraction	Total volume (ml)	Total protein (mg)	Total activity (μmol/min)	Specific activity (μmol/min/mg)
–	Soluble material	20.0	112.4	<0.28	$<2.58 \times 10^{-3}$
–	Insoluble material	20.0	14.4	<0.04	$<2.54 \times 10^{-3}$
+	Soluble material	20.0	257.8	386.7	1.50
+	Insoluble material	20.0	144.8	244.5	2.13

These samples were obtained from 400-mL cultures of *E. coli* carrying pFR466.

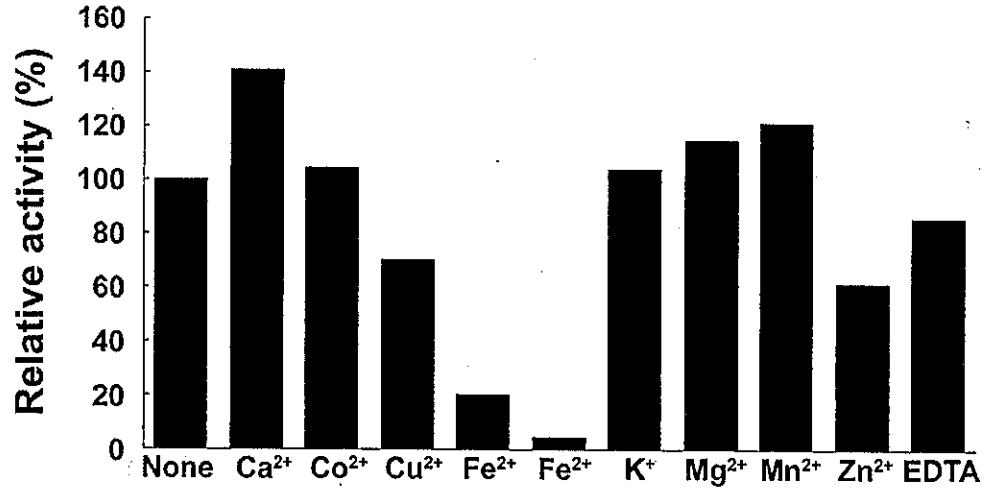

Fig. 2. Effect of ions and EDTA on sialidase activity. We measured sialidase activity with ions or EDTA at a final concentration of 10 mM or 50 mM, respectively (pH 8.0), using 4-MU-NANA as a substrate. Sialidase activity is relative to the activity in the absence of ions.

respectively (Table 1). These specific activities were approximately 600- and 850-fold higher, respectively, than those detected in uninduced cells (Table 1). This result suggested that rNanH1 aggregated as inclusion bodies in *E. coli* cells, but still retained enzymatic activity.

3.3. *Effect of metal ions on sialidase activity*

The effect of different metal ions on crude rNanH1 activity was examined *in vitro*. Sialidase activity was increased in the presence of Ca^{2+}, Mg^{2+} and Mn^{2+}, but was slightly decreased in the presence of Zn^{2+} and Cu^{2+} (Fig. 2). Ferric ions effectively inhibited sialidase activity, whereas no effect was observed with Co^{2+} and K^{+} treatment (Fig. 2). We detected a minimal inhibition of neuraminidase activity by adding EDTA at a final concentration of 50 mM (pH 8.0) as previously reported [26]. As a result, $CaCl_2$ was added to the renaturation buffer used to purify rNanH1.

3.4. *Purification of rNanH1*

The rNanH1 protein in the insoluble fraction was solubilized in buffer containing 8 M urea. The solubilization was confirmed by SDS-PAGE (Fig. 1, lane 5, Fig. 3, lanes 1 to 3). Subsequently, the denatured rNanH1 was fractionated and purified using Ni-NTA column affinity chromatography. We identified fractions containing purified rNanH1 by measuring protein concentrations (data not shown). SDS-PAGE analysis demonstrated that a single band of approximately 60 kDa was detected in fractions 24–27 (Fig. 3, lanes 4 to 7). However, the purified enzyme had no sialidase activity due to protein denaturation by urea (Table 2).

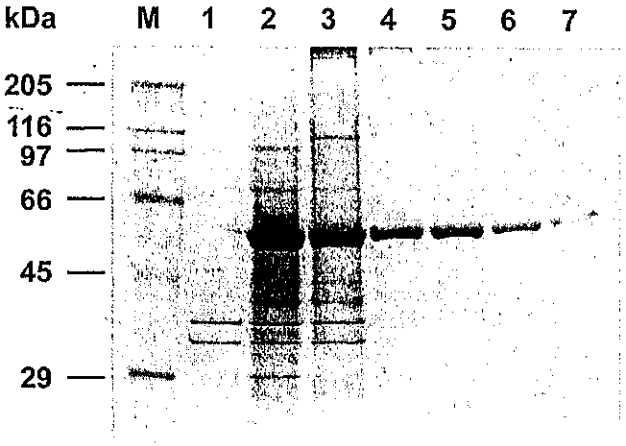

Fig. 3. Purification of rNanH1 derived from *B. fragilis* YCH46. SDS-PAGE analysis of purified recombinant sialidase is shown. Lane M, molecular mass markers in kilodaltons (15 μg), Lanes 1 to 7, 10 μL of each sample was applied to SDS-PAGE. Lane 1: insoluble materials obtained from uninduced cells (7.3 μg), Lane 2: insoluble materials obtained from IPTG-induced cells (72.4 μg), Lane 3: materials solubilized by 8 M urea (68.7 μg), Lanes 4 to 7, purified hexahistidine-tagged sialidase contained in fraction numbers 24 to 27 (1.1, 1.0, 0.6, and 0.2 μg, respectively).

3.5. *Protein refolding of rNanH1*

L-arginine is reported to suppress protein aggregation [24]. Here, addition of L-arginine at final concentrations between 25 and 75 mM in the dialysis buffer dose-dependently increased rNanH1 sialidase activity (Fig. 4). However, L-arginine at concentrations

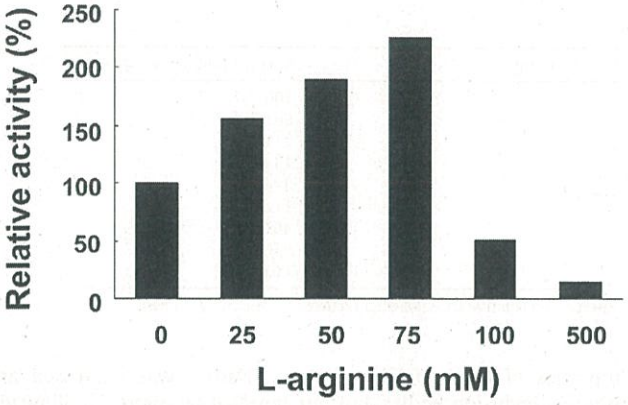

Fig. 4. Effect of L-arginine on sialidase activity during dialysis. An equivalent of 4 μg of purified protein was dialyzed with 500 mL of dialysis buffer containing L-arginine at various concentrations for 2 h and continued dialysis with 500 mL of fresh buffer for another 14 h. Sialidase activity was subsequently measured using 4-MU-NANA as a substrate. Sialidase activity is relative to that obtained using dialysis buffer lacking L-arginine.

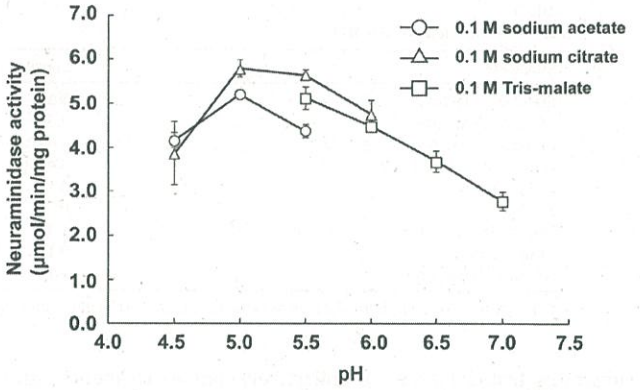

Fig. 5. Effect of pH on enzymatic activity of purified sialidase. Enzyme activity was assayed with 4-MU-NANA as a substrate in 0.1 M sodium acetate buffer (pH 4.5–5.0; circle); 0.1 M sodium citrate buffer (pH 4.5–6.0; triangle); 0.1 M Tris-malate buffer (pH 5.5–7.0; square).

above 100 mM decreased the activity. Dialysis in renaturation buffer containing 50 mM L-arginine, 100 mM $CaCl_2$ and 1 mM DTT restored the specific activity to 6.16 μmol/min/mg, which was a 2.9-fold increase over that of the starting material (Table 2).

3.6. Enzymatic characterization of recombinant B. fragilis sialidase

The optimum pH of rNanH1 enzyme activity was determined with 4-MU-NANA as a substrate in three kinds of buffers with pH between 4.5 and 7.0 increasing by 0.5 pH intervals. The pH for optimal activity ranged from 5.0 to 5.5 (Fig. 5). The K_m and V_{max} values with 4-MU-NANA were 0.055 mM and 1.05 nmol/min, respectively (Supplemental Fig. S5A). Subsequently, an inhibition assay was performed using Neu5Ac2en, a common sialidase inhibitor. Neu5Ac2en inhibited rNanH1 with the K_i value at a concentration of 0.032 mM (Supplemental Fig. S5B).

3.7. rNanH1 preferentially cleaves sialic acids with an α2,8 linkage

Substrate specificity of rNanH1 was determined using chemical compounds and sialo-glycoproteins. The data indicated that rNanH1 could hydrolyze sialic acids with three different configurations of sialyl α2,3, α2,6 and α2,8 linkages (Table 3). Particularly, it preferentially hydrolyzed colominic acid, which contains a polysialyl α2,8 linkage, relative to sialyl α2,3 and sialyl α2,6 linkages (Table 3). rNanH1 released sialic acid residues from bovine submandibular mucin more efficiently than the other sialoglycoproteins and gangliosides tested. However, rNanH1 could not remove sialic acid residues from α1-acid glycoproteins (Table 3). Consequently, our data revealed that rNanH1 preferentially hydrolyzes substrates containing polysialyl α2,8 linkages under acidic conditions.

4. Discussion

A sialidase purified from *B. fragilis* SBT3182 has been previously characterized, but it is an unavailable material. Additionally, the approaches to produce recombinant NanHs in *E. coli* are not established. In this study, to determine whether an unmodified sialidase had biochemically similar properties to a native enzyme purified from SBT2182, we purified and biochemically characterized rNanH1 of *B. fragilis* YCH46 expressed in *E. coli*.

Sialidases from *C. chauvoei*, influenza virus and *V. cholerae* are all known as metalloenzymes [25–27]. *B. fragilis* sialidase also seemed to be a metalloenzyme since its activity was increased in the presence of Ca^{2+} (Fig. 2). Interestingly, the enzymatic activity of rNanH1 was inhibited by copper, iron, and zinc ions, as was previously reported for the influenza virus [28,29], *C. perfringens* [30] and *Streptococcus pneumoniae* [31]. Refolding buffers containing L-arginine increased the rNanH1 activity relative to buffer lacking L-arginine (Fig. 4). As reported, L-arginine could be a useful reagent to prevent protein aggregation [24].

The optimal pH of the rNanH1 was between 5.0 and 5.5. (Fig. 5), which was within the range of many other viral and bacterial sialidases (pH 5.0–6.0) [26,27,32–35], although its value was slightly lower than the pH 6.1 seen for the 55 kDa sialidase purified from SBT3182 [7]. Recombinant NanH1 hydrolyzed three sialic acid configurations with sialyl α2,3, α2,6, and sialyl α2,8 linkages (Table 3). Of these linkages, rNanH1 as well as an SBT3182 sialidase [7,8] preferentially hydrolyzed the sialyl α2,8 linkage (Table 3), whereas other bacterial and viral sialidases hydrolyze sialyl α2,3 and α2,6 linkages with preference [36]. Moreover, rNanH1 effectively released sialic acids from mucin relative to the other natural

Table 2
Purification of hexahistidine-tagged neuraminidase using Ni-NTA column.

Fraction	Volume (ml)	Protein concentration (mg/ml)	Total activity (μmol/min)	Specific activity (μmol/min/mg)	Purification factor
Insoluble material[a]	4.0	7.24	61.68	2.13	1
Solubilized insoluble material	4.0	6.87	<0.07	$<2.5 \times 10^{-3}$	$<8.52 \times 10^{-4}$
Ni-NTA column eluted material	3.0	0.90	NT[b]	NT[b]	NT[b]
Dialysis sample[c]	52.9	0.05	16.29	6.16	2.9

[a] Insoluble material was isolated from 80-mL culture of *E. coli* cells containing plasmid pFR466.
[b] NT: not tested.
[c] Dialysis sample was represented by values calculated from results obtained in the renaturation experiment.

Table 3
Substrate specificity of rNanH1.

Substrate	Specific activity (μmol/min/mg)	Relative hydrolysis rate (%)[a]
Sialyl-α2,3 lactose	3.05	100
Sialyl-α2,6 lactose	2.65	86.9
Colominic acid (Polysialyl-α2,8)	6.43	211
Gangliosides+1% Triton X-100	0.47	15.4
Gangliosides	0.11	3.61
Fetuin	0.35	11.5
Mucin (bovine submaxillary gland)	1.43	46.9
Transferrin	0.17	5.57
α1-acid glycoprotein	<0.01	<0.01

[a] The rate of relative hydrolysis of sialic acid released from each substrate was represented relative to sialyl-α2,3 lactose.

substrates tested (Table 3). Collectively, our experiments using synthetic and natural substrates demonstrated that rNanH had similar substrate specificities to SBT3182 sialidase [7]. Recombinant NanH1 was unlikely to be modified by glycosylation in *E. coli*, and thus was detected as a single band of ~60 kDa molecular mass that was consistent with the mass estimated from the amino acid sequence (Fig. 3). These results indicated that carbohydrate modification is likely not involved in determining substrate specificity, but instead could play a role in the catalytic activity or localization of the enzyme.

Salmonella typhimurium LT2 expresses a 42 kDa sialidase, which hydrolyzes sialic acid residues with sialyl α2,3 linkages as compared to sialyl α2,6 linkages [33]. It also poorly cleaves sialyl α2,8 linkages in colominic acids due to very slow reaction rates [33]. Recent analysis showed that *B. thetaiotaomicron* promotes the growth of an enteric pathogen *S. typhimurium* lacking the sialidase gene [37]. Since *S. typhimurium* encodes the *nan* operon gene to metabolize sialic acids, their liberation by *B. thetaiotaomicron* sialidase is predicted to accelerate the emergence of *S. typhimurium* [37]. These findings imply that sialidases derived from polysaccharide-degrading commensal *Bacteroides* species facilitate growth of enteric pathogens in human intestinal mucins. Since *B. fragilis* has both the *nanH* genes and the *nan* operon [10], those gene products will promote not only emergence of enteric pathogens but also cell growth *per se*. The preference of *B. fragilis* sialidase for sialyl α2,8 linkages likely contributes to the ability of this bacterium to establish a particular niche in the lower gut. Although a previous report found that sialic acids with α2,6 linkages are dominant in human colonic mucins [38], glycoproteins and glycolipids displaying sialic acids with sialyl α2,8 linkages will nonetheless be present in the human intestinal mucosa since human α2,8-sialyltransferases I and VI are expressed by gastrointestinal tract cells [39].

NanH1 and NanH4 contain two motifs that would be predicted to have the modification by Bacteroidetes phylum-wide general O-glycosylation system [23] (Supplemental Fig. S2). On the other hand, NanH2 and NanH3 have no such modification site. Thus, *in silico* analysis suggested that each *B. fragilis* sialidase isoenzyme may have a different physiological role and comparisons of the biochemical properties of *B. fragilis* sialidase isoenzymes may provide novel insights into the role of sialidases in colonic adaptation or pathogenicity of human gut symbionts.

Collectively, whole genomic analysis demonstrated that the *B. fragilis* genome encodes not only the *nanH1* gene but at least five other paralogous genes. To analyze functional differences, the other *B. fragilis* sialidase isoenzymes will need to be purified. However, purifying native forms could be challenging since quantitative real-time PCR analysis showed undetectable mRNA levels for the other sialidase genes (*nanH2* and *nanH4*) in both the *in vitro* and *in vivo* (mouse intestine) models (unpublished data). As such, recombinant proteins will be helpful for characterizing the biochemical properties of these sialidases. Here, rNanH1 was expressed as insoluble inclusion bodies, but our purification approach allowed the separation of rNanH1 from *E. coli*-derived soluble proteins and we were able to successfully solubilize and renature rNanH1 while retaining enzymatic activity. This purification method may be useful for purifying other *B. fragilis* proteins, including other sialidase isoenzymes.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.anaerobe.2018.02.003.

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