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PURIFICATION AND CHARACTERIZATION OF A SIALIDASE FROM *BACTEROIDES FRAGILIS* SBT3182

Hiroshi Tanaka, Fumio Ito and Taisuke Iwasaki

Technical Research Institute, Snow Brand Milk Products Co., Ltd., 1-1-2 Minamidai, Kawagoe, Saitama 350, Japan

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A sialidase from *Bacteroides fragilis* SBT3182 was purified 2,240-fold to apparent homogeneity by ammonium sulfate precipitation and sequential chromatographies on DEAE-Toyopearl 650M, Hydroxyapatite, MonoS and Superose6 columns. The N-terminal amino acid sequence of this sialidase, Ala-Asp- X -Ile-Phe-Val-Arg-Glu-Thr-Arg-Ile-Pro-, was determined. Substrate specificity of this enzyme using a variety of sialoglycoconjugates showed a 1.5- and 2.2-fold preference for sialyl α2-8 linkages when compared with α2-3 and α2-6 bound sialic acids, respectively. The native sialidase had a molecular weight of 165kDa, as determined by Superose6 gel filtration chromatography and consisted of three subunits each of 55kDa by SDS-polyacrylamide gel electrophoresis. This enzyme had optimal activity at pH6.1 with colominic acid as substrate.

Sialic acids are distributed in many glycoproteins, glycopeptides and glycolipids. This widespread distribution means that sugar chains containing sialic acid should associated with a variety of biological functions(1). Furthermore, sialic acids normally exist in the terminal of sugar chains on cell surface, so that these residues have an important role in the primary interaction with the other cells or materials.

Sialidases(Neuraminidases; EC 3.2.1.18), which catalyze the removal of sialic acid residues from sialoglycoconjugates, are also widespread among viruses, bacteria and animals. It has been suggested that sialidases are the key enzyme of biological functions associated with sialic acids because this enzymes regulate the number and distribution of sialic acid residues(1). Novel sialidase, which has unique substrate specificity and optimum activity in neutral pH range, is required in order to investigate the precise roles of sialic acid in glycoconjugates locating on the surface of living cells.

On the other hand, sialidases may play an important role in the pathogenicity of certain infections, because most of the bacteria producing sialidase are often pathogenic in man or other mammals(2). The *B.fragilis*, which also produces sialidase, is a dominant microflora in human intestinal tract(3) and frequently isolated from intraabdominal infections(4). These phenomena indicate that *B.fragilis* will provide a good system to investigate the relationship between bacterial sialidases and human infections.

In this study, a sialidase has been purified from *B.fragilis* SBT3182 to apparent homogeneity and its novel properties have been characterized in detail.

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MATERIALS AND METHODS

Materials

Ganglioside 9-O-acetyl-GD3(9-O-Ac-GD3) was provided by G. Hanagata(5) in our institute. N-acetylneuraminic acid(Neu5Ac), ganglioside GD3, bovine milk lactoferrin and glycomacropeptide(GMP) were prepared in our institute. Neu5Acα2-3lactose, Neu5Acα2-6lactose, gangliosides GM1, GM2 and GM3, bovine submandibular gland mucin and porcine submaxillary gland mucin were purchased from BioCarb(Lund, Sweden). 2-4(Methylumbelliferyl)-α-D-N-acetylneuraminic acid(4-MU-Neu5Ac) and fetal calf fetuin were from Sigma(St. Louis, MO, USA). Colominic acid[poly-(α2-8Neu5Ac)] was purchased from Nakarai(Kyoto, Japan). Hydroxyapatite was obtained from Seikagakukogyo(Tokyo, Japan) and polyvinylidene difluoride(PVDF) membrane was from Bio-Rad(Richmond, CA, USA).

Sialidase assay

Unless otherwise indicated, all assays were carried out using colominic acid as substrate. 50μl of enzyme solution was incubated in a reaction mixture containing 100μl of 100mM phosphate buffer(pH7.0) and 50μl of 1% colominic acid solution, for 10min at 37°C. The amount of sialic acid released was determined by the thiobarbituric acid assay(TBA-assay) of Aminoff(6). One unit of sialidase activity was defined as 1μmol sialic acid hydrolysed per min.

Sialidase purification

Unless stated otherwise, all purification steps were performed at 4°C. A 1,000 ml culture broth of *B.fragilis* was centrifuged at 8,900xg for 10min in order to collect cells. The cells were washed with 800ml of 20mM phosphate buffer(pH7.0), and recentrifuged. The resuspended cells with 120ml of 50mM phosphate buffer(pH7.0) were subjected to ultrasonic disruption with a Tomy Seiko UR-200P sonicator in an ice bath and centrifuged at 45,000xg for 10min to obtain the crude enzyme. The crude enzyme was fractionated by ammonium sulfate precipitation. The protein precipitating between 60% and 100% saturation was collected, dissolved in a small amount of 20mM phosphate buffer(pH7.0) and dialyzed for 24h against the same buffer. Concentrated enzyme was first loaded on a DEAE-Toyopearl 650M column(1.6x17cm) equilibrated with 20mM phosphate buffer(pH7.0). The column was washed with the same buffer at a rate of 0.5ml/min. Sialidase protein was not adsorbed on this column. Active fractions were applied to a Hydroxyapatite column(2.4x10cm) equilibrated with 20mM phosphate buffer(pH7.0). The column was washed with 100ml of the same buffer followed with 100ml of 100mM phosphate buffer(pH7.0) at a flow rate of 1.5ml/min. Sialidase activity was eluted with a linear phosphate gradient from 100mM to 400mM in a total volume of 260ml of phosphate buffer(pH 7.0). The enzyme fractions were combined, concentrated with Amicon instrument using a YM-10 membrane and dialyzed against 20mM acetate buffer(pH5.0). A MonoS column(0.5x5cm) equilibrated with 20mM acetate buffer(pH5.0) was used for next chromatography. The column was washed thoroughly with the same buffer at a rate of 1ml/min after loading of the concentrated material and protein was fractionated with a linear NaCl gradient from 0M to 0.25M in a 60ml of 20mM acetate buffer(pH5.0). The enzyme fractions were finally applied to a Superose6 column(1x30cm) equilibrated with 100mM phosphate buffer(pH7.0) at a flow rate of 0.5ml/min. Every peak of the protein were fractionated by monitoring absorbance at 280nm.

Substrate specificity analysis

The following natural and synthetic substances were used for substrate specificity analysis: Neu5Acα2-3lactose, Neu5Acα2-6lactose, 4-MU-Neu5Ac, colominic acid, ganglioside GM1, GM2, GM3, GD3, ganglioside 9-O-Ac-GD3, bovine submandibular gland mucin, porcine submaxillary gland mucin, fetal calf serum fetuin, bovine lactoferrin and bovine GMP. The assay system contained the following components in a total volume of 200μl: 50μl of substance solution, 100μl of 100mM acetate buffer(pH5.5) and 50μl of purified enzyme(0.24unit). The sialic acid content of these substrates were measured by TBA-assay after mild-acid hydrolysis by 0.1N HCl at 80°C for 1h. Each substrate was prepared to contain 100nmol of bound sialic acid except for ganglioside GM2(40nmol) and bovine lactoferrin(10nmol). In the case of gangliosides using as substrate, Triton X-100 was added to the reaction mixture at a final concentration of 0.1%. After incubation at 37°C for 20min, sialic acid released was measured by TBA-assay.

Other methods

N-terminal amino acid sequence analysis was performed with a Applied Biosystems Model 477A automated sequencer. The sialidase protein was once electroblotted onto PVDF membrane and stained with Coomassie Brilliant Blue R-250 according to the method of Matsudaira(7). The

band of 55kDa sialidase protein was cut out and directly analyzed. SDS-polyacrylamide gel electrophoresis(SDS-PAGE) was carried out as described by Laemmli(8). After electrophoresis, gels were stained with silver stain kit. The native molecular weight of this enzyme was calibrated using standard proteins by gel filtration chromatography on a Superose6 column. Proteins were measured by Bio-Rad protein assay reagent using bovine serum albumin as the standard.

RESULTS

A sialidase from *B.fragilis* SBT3182 was purified from the crude extract by ammonium sulfate precipitation and sequential four chromatographies. Table1 shows the individual purification step and the yield of sialidase from a 4,000ml of culture medium of *B.fragilis*. The 2,240-fold purification of sialidase in final step was attained with a yield of 2.7%. In the DEAE-Toyopearl 650M chromatography, the sialidase was not retained. The increased activity of 5.3-fold purification in this step was due to the removal of other proteineous compounds from the clarified lysate. The second chromatography, using a Hydroxyapatite column, was a most effective procedure to remove the inert proteins. The sialidase activity was completely adsorbed and eluted with a linear phosphate gradient when its concentration became 0.2M ahead of the uninteresting proteins. Fig.1 demonstrates the result of the MonoS chromatography, used for the third chromatography, was most successful for the separation of three sialidase-active fractions: fraction S1, S2 and S3. These activities were once adsorbed and eluted sequentially by NaCl gradient. The first S1 fraction with less contaminant proteins in these three sialidase fractions was used for further purification. At the last chromatography using a Superose6 column, sialidase was purified to electrophoretic homogeneity of a 55kDa protein by SDS-PAGE analysis as shown in Fig.2 and this final preparation is free from protease or activities of the tested glycosidases.

In order to determine substrate specificity, the purified enzyme was incubated with various compounds. As shown in Table2, the relative hydrolysis rates of various substrates compared to that for Neu5Acα2-3lactose clearly show that this sialidase prefer to hydrolyze colominic acid which is a homopolymer of Neu5Ac linked by α2-8 linkage. This is the first sialidase found to be most active for α2-8 linkage among three major sialyl linkage styles. No cleavage were observed against gangliosides GM1 and GM2. In the use of ganglioside 9-O-Ac-GD3 as a substrate, 9-O-Ac-Neu5Ac was released by this enzyme but the hydrolysis rate was 0.59-times slower than that of original ganglioside GD3.

Table 1. Purification of a sialidase from *B.fragilis* SBT3182

Step	Total activity (units)	Recovery (%)	Total protein (mg)	Specific activity (units/mg)	Purification (fold)
Crude enzyme	117.4	100.0	4,133.7	0.028	1.0
Ammonium sulfate precipitation	52.6	44.8	441.9	0.12	4.3
DEAE-Toyopearl 650M	52.6	44.8	82.2	0.64	22.9
Hydroxyapatite	25.7	21.9	4.52	5.69	203.2
Mono S (S1)	5.12	4.4	0.10	51.2	1,828.6
Superose 6 (purified)	3.20	2.7	0.051	62.7	2,239.3

The values are from a 4,000ml culture.

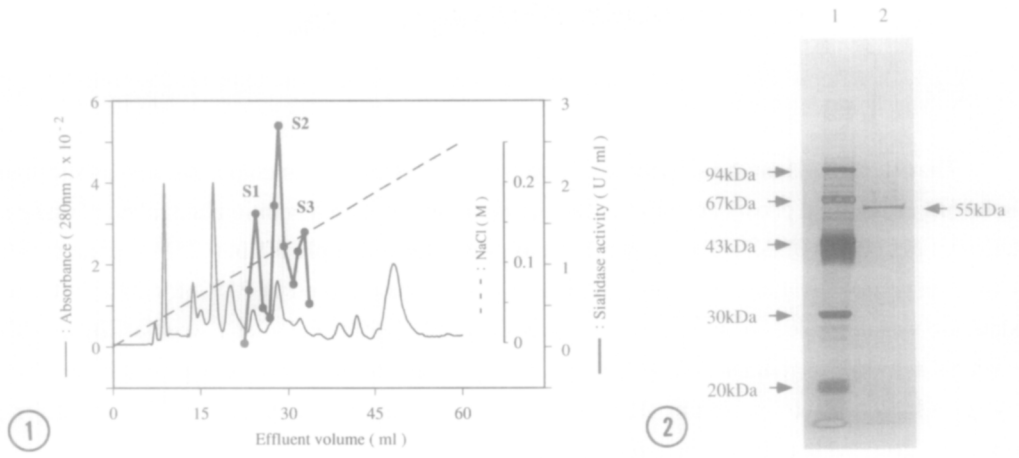

Fig.1. Elution profile of a MonoS column chromatography. This chromatogram shows the protein elution by NaCl after washing the column. Three sialidase active fractions(S1,S2,S3) were eluted sequentially.

Fig.2. SDS-PAGE analysis for purified sialidase. Lane1 shows standard proteins and lane2 indicates the purified sialidase as estimated to be 55kDa.

We also clarified the N-terminal amino acid sequence of this sialidase as shown in Fig.3. Only the third residue from the N-terminal was not determined. The molecular weight of the native sialidase was estimated to be 165kDa by gel filtration on a Superose6 column, while SDS-PAGE analysis showed an average molecular weight of 55kDa. These results indicated that the *B.fragilis* SBT3182 sialidase was composed of three homologous 55kDa subunits.

Table 2. Substrate specificity of a sialidase purified from *B. fragilis* SBT3182

Substrate	Relative rate (%)
Neu5Acα2-3lactose	100.0
Neu5Acα2-6lactose	70.3
Colominic acid [poly-(α2-8Neu5Ac)]	151.9
4-MU-Neu5Ac	86.9
Ganglioside GM1	0
GM2	0
GM3	5.1
GD3	13.1
9-O-Ac-GD3	7.7
Mucin	
Submandibular gland	10.0
Submaxillary gland	2.4
GMP	114.8
Fetuin	75.1
Lactoferrin	0.3

The hydrolysis rate of various substrates were compared to that for Neu5Acα2-3lactose.

5 10
Ala - Asp - X - Ile - Phe - Val - Arg - Glu - Thr - Arg - Ile - Pro -

Fig.3. N-terminal amino acid sequence of the 55kDa sialidase. The third residue from the N-terminal was not determined.

The effect of pH on the activity of this enzyme was assayed with colominic acid as substrate in 0.25M acetate and phosphate buffer. The optimum pH for these condition laid in the pH ranges between pH6.0 and pH6.5. The highest activity was achieved at pH 6.1 in 0.25M acetate buffer. Crude enzyme was stable between pH5.0 and pH9.0 when stored at 37°C for 24h, but pure sialidase was unstable. After storage for 3 weeks at -80°C, 60% and after 1 week at -20°C, 20% of initial activity was remained. The stability of the purified enzyme could be appreciably increased by the addition of bovine serum albumin at 5% concentration. Enzyme could be stored for 3 weeks at -20°C without loss of activity.

DISCUSSION

In this study, sialidase activity from *B.fragilis* SBT3182 was separated into three fractions(fraction S1, S2, S3). SDS-PAGE of the individual fractions with sialidase activity from a Superose6 chromatography demonstrated that the isolated sialidase molecules were separated into two proteins in their electrophoretic mobilities. The fraction S1, S2 and S3 at the step of MonoS chromatography contained 55kDa protein, both of 55kDa and 50kDa and 50kDa, respectively. The 55kDa protein in the fraction S1 was purified as a sialidase protein(Fig.2), but the 50kDa protein which may be the other sialidase protein eluted in the fraction S2 and S3 was not purified completely in this study.

Sialidase from *B.fragilis* SBT3182 showed a novel characteristics in substrate specificity. This sialidase prefers to hydrolyze α2-8 linkage rather than α2-3 and α2-6 linkages(Table1), while sialidases from other bacteria and organisms can cleave α2-3 or α2-6 linkages much faster than α2-8 linkage. Only the human placental sialidase was reported to hydrolyze a2-8 linkage as well as α2-3 linkage(9). The enzyme from *B.fragilis* SBT3182 is the first described sialidase with a preference for sialyl α2-8 linkage. When gangliosides were used for substrate, ganglioside GD3 was hydrolyzed faster than ganglioside GM3(Table2). This observation supports the substrate specificity of this enzyme described above. On the other hand, O-acetylations of sialic acid residue are reported to reduce the hydrolysis rate by sialidases or block enzyme activity completely(10). The investigation of the effect of 9-O-acetylation itself on the sialidase activity was carried out using pure ganglioside 9-O-Ac-GD3. Table1 shows that the presence of 9-O-acetylation reduced 41.2% of its activity. This inhibitory rate almost agrees with the rate reported by Corfield and Schauer(10).

Primary structures have been reported in some sialidases(11,12,13,14,15). In the case of *B.fragilis* TAL2480, sialidase gene, nanH, have been cloned, expressed in *Escherichia coli* and partial amino acid sequence except for the N-terminal region have already determined(13). In this study, we reported the N-terminal sequence of the *B.fragilis* sialidase that can use to elucidate the complete sialidase gene and primary structure. The *B.fragilis* SBT3182 sialidase had a molecular weight of 165kDa consisted of three subunits of each 55kDa, while Berg et al.(16) reported that

the molecular weight of sialidase from *B.fragilis* B70 was 92kDa as determined by Sephadex G-200 gel filtration chromatography. This oligomer structure of sialidase estimated from our experiments is unusual among bacterial sialidases only except for *Clostridium chauvoei*(17), which sialidase had a homodimer structure consisted of each 150kDa subunits.

In general, the optimum pH of bacterial sialidases lie in the pH range of pH5-6(10), but the optimum value of *B.fragilis* SBT3182 was pH6.1. This activity was remained up to pH6.5. In addition, crude enzyme was stable between pH5.0 and pH9.0. From these observations, this enzyme could be used for the treatment on living cells with minimum artificial effects and it may display the function of sialic acid residue in living condition directly.

The unique properties of *B.fragilis* SBT3182 sialidase such as substrate specificity, pH activity and stability will give us to investigate what sialyl and polysialylmaterials play in vivo. With regard to the relationship between sialidases and human infections, a sialidase from *B.fragilis* SBT3182 purified in this paper should provide a trigger to understand the interactions which occur in human intestine.

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REFERENCES

1. Reutter, W., Kottgen, E., Bauer, C. and Gerok, W. (1982) In Sialic Acids (R. Schauer, Ed.), pp. 263-305. Springer-Verlag, Wien.
2. Schauer, R. (1983) In Structual Carbohydrates in the Liver (H. Popper, W. Reutter, E. Kottgen, F. Gudat, Ed.), pp. 83-97. MTP Press Ltd, Boston.
3. Heimdahl, A., Kager, L., Malmborg, Q. S. and Nord, C. E. (1982) Infection 10, 120-124.
4. Pipper, R., Kager, L., Weintraub, A. and Nord, C. E. (1982) Acta. Chir. Scand. 148, 39-44.
5. Hanagata, G., Kobayashi, T., Yanahira, S., Deya, E. and Gasa, S. (1991) Glycoconjugate J. 8, 254.
6. Aminoff, D. (1961) Biochem. J. 81, 384-392.
7. Matsudaira, P. (1987) J. Biol. Chem. 262, 10035-10038.
8. Laemmli, U. K. (1970) Nature 227, 680-685.
9. Hirakawa, M., Uda, Y., Nishizawa, M. and Miyatake, T. (1987) J. Biochem. 101, 1273-1279.
10. Corfield, A. P. and Schauer, R. (1983) In Sialic Acids (R. Schauer, Ed.), pp. 225-234. Springer-Verlag, Wien.
11. Vimr, E. R., Lawrisuk, L., Galen, J. and Kaper, J. (1988) J. Bacteriol. 170, 1495-1504.
12. Roggentin, P., Rothe, B., Lottspeich, F. and Schauer, R. (1988) FEBS Lett. 238, 225-234.
13. Russo, T. A., Thompson, J. S. Godoy, V. G. and Malamy, M. H. (1990) J. Bacteriol. 172, 2594-2600.
14. Rothe, B., Rothe, B., Roggentin, P. and Schauer, R. (1991) Mol. Gen. Genet. 226, 190-197.
15. Pereira, M. E. A., Mejia, J. S., Ortega-Barrira, E., Matzilevich, D. and Prioli, R. P. (1991) J. Exp. Med. 174, 179-191.
16. Berg, J. O., Lindqvist, L., Andersson, G. and Nord, C. E. (1983) Applied Environ. Microbiol. 46, 75-80.
17. Heuermann, D., Roggentin, P., Kleineidam, R. and Schauer, R. (1991) Glycoconjugate J. 8, 95-101.