SUSCEPTIBILITY OF GANGLIOSIDE GM_1 TO A NEW BACTERIAL NEURAMINIDASE

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1. Introduction

Recently, a new bacterial neuraminidase (*N*-acetylneuraminate glycohydrolase (EC 3.2.1.18)) was isolated and purified from the culture filtrate of a non-pathogenic bacterium, *Arthrobacter ureafaciens* [1]. This preparation was reported to liberate *N*-acetylneuraminic acid (NeuNAc) from substrates containing the terminal α,2→3, α,2→6 and α,2→8 linkages of the NeuNAc residue, and to show no activities of contaminating enzymes, such as protease, NeuNAc-aldolase and other glycosidases [1]. However, the action of this neuraminidase on gangliosides has not yet been studied in detail. This report described the specific action of this new neuraminidase on ganglioside GM_1, which was believed to be resistant to various neuraminidases of viral, bacterial and mammalian origin [2–5].

The new bacterial neuraminidase hydrolyzed not only ganglioside GM_1 but an oligosaccharide having the same saccharide structure as GM_1. Addition of detergents, especially bile salts, resulted in a marked increase in the enzymatic hydrolysis of GM_1, but, in sharp contrast, did not influence the hydrolysis of this oligosaccharide. In addition, the activities of various neuraminidases toward GM_1 correlated well with their activities on this oligosaccharide. It is suggested that recognition of the specific saccharide structure of GM_1 by various neuraminidases primarily determines the release of NeuNAc from GM_1 and that the hydrophobic ceramide moiety of GM_1, which detergents are supposed to attack chiefly, also participates in the hydrolysis of ganglioside GM_1.

Abbreviations: GM_1, Galβ1→3GalNAcβ1→4(NeuNAcα2→3)-Galβ1→4Glc–Cer; GM_2, GalNAcβ1→4(NeuNAcα2→3)-Galβ1→4Glc–Cer; GM_3, NeuNAcα2→3Galβ1→4Glc–Cer; Gal, galactose; Glc, glucose; GalNAc, *N*-acetylgalactosamine; NeuNAc, *N*-acetylneuraminic acid; Cer, ceramide (2-*N*-acylsphingosine); CMC, critical micelle concentration; GLC, gas–liquid chromatography; TLC, thin-layer chromatography

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2. Materials and methods

2.1. *Enzyme source*

Neuraminidase from *A. ureafaciens* was kindly given by Y. Uchida (Marukin-shoyu Co., Kyoto) or commercially obtained from Nakarai Chemicals (Kyoto). Neuraminidase from *Clostridium perfringens* (type VI) was purchased from Sigma Chemicals (St Louis, MO) and neuraminidase from *Vibrio cholerae* was from Calbiochem (San Diego).

2.2. *Preparation of substrates*

Bovine brain gangliosides were prepared as in [6]. They were extensively hydrolyzed with clostridial neuraminidase in the absence of detergents, and then, monosialoganglioside GM_1 was purified from the mixture by combined column chromatography with DEAE–Sephadex and Iatrobeads [6]. The purified

GM_1 was subjected to ozonolysis as in [7], and the oligosaccharide liberated was purified by Sephadex G-25 (superfine) column chromatography (1.8 × 150 cm, equilibrated and eluted with 0.1 M acetic acid–pyridine buffer, pH 5.0).

2.3. *Enzyme assay*

The typical reaction mixture contained 500 μg GM_1, or oligosaccharide, 5–50 mU neuraminidase, 10 mM buffer solution (acetate buffer, pH 4.8, Tris–acetate buffer, pH 6.8 and pH 7.1 for neuraminidases from *A. ureafaciens*, *V. cholerae* and *C. perfringens*, respectively) with or without an appropriate amount of detergent in final vol. 200 μl. In the assay for *V. cholerae* neuraminidase, calcium chloride was also added at final conc. 1 mM. One unit of the enzyme was defined as the amount that liberated 1 μmol of NeuNAc from *N*-acetylneuraminyllactose/min at pH 5.0 and 37°C. After incubation for 60 min at 37°C, released NeuNAc was measured by the thiobarbituric acid (TBA) procedure [8], using NeuNAc type IV (Sigma) as a standard. In the study of the enzyme activity on GM_1 in the absence of detergent, the more sensitive fluorometric TBA method [9] was employed. The relative fluorometric intensity was measured in a Hitachi model MPF-4 at 20°C with excitation and emission wavelengths of 550 nm and 570 nm, respectively. By this method, 50–500 ng free NeuNAc could be reliably determined. All other chemicals used were of reagent grade.

2.4. *Identification of the reaction product*

Enzyme reaction using 20 mU each enzyme was carried out essentially as described above except that the incubation time was longer (20 h). The enzyme reaction was terminated by adding a small amount of chloroform. Then the mixture was evaporated under a stream of N_2, the residue was dissolved in a small amount of chloroform–methanol (2:1, v/v), and the solution was directly applied to a precoated silica gel 60 plate (Merck, Darmstadt). The plate was developed with a solvent system of either chloroform/methanol/water (60:35:8, v/v/v), or chloroform/methanol/3 N NH_4OH (60:40:9, v/v/v). Spots were detected by spraying the plate with either anthrone or resorcinol reagent (fig.1). The product recovered from the TLC plate was analyzed by GLC, as in [6].

3. Results

When ganglioside GM_1 was incubated with *A. ureafaciens* neuraminidase in the presence of various detergents, the end-product of the reaction was asialo-GM_1, as determined by analysis on TLC (fig.1). The product recovered from the TLC plate was confirmed to be Gal–GalNAc–Gal–Glc–Cer (asialo-GM_1) by GLC analysis (data not shown). As shown in fig.1, the kind of enzyme as well as the detergent used greatly influenced the amount of asialo-GM_1 produced; the neuraminidase from *A. ureafaciens* showed greater activity than that from *C. perfringens* toward GM_1, and bile salts, such as sodium cholate and sodium deoxycholate, had strong activating effects. These findings are shown quantitatively in table 1. The degrees of activation by detergents depended upon both their species and concentrations, and sodium cholate was the strongest activator of both neur-

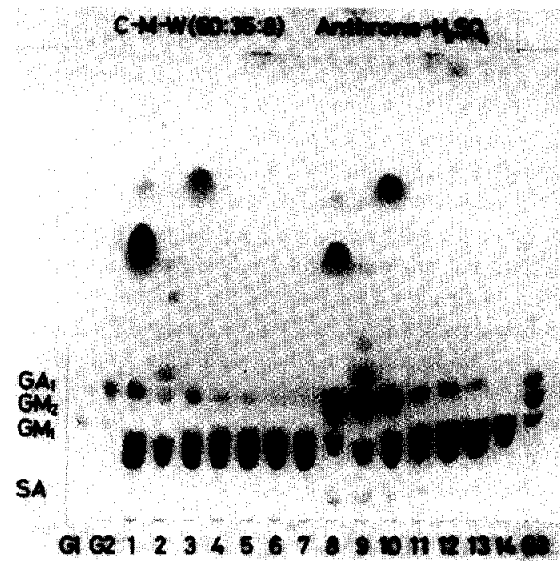

Fig.1. Thin-layer chromatography of the reaction products. Experimental details are described in the text. The enzymes used were *C. perfringens* neuraminidase (lanes 1–7) and *A. ureafaciens* neuraminidase (lanes 8–14). The detergents used were sodium cholate (lanes 1, 8), sodium deoxycholate (lanes 2, 9), taurocholic acid (lanes 3, 10), Triton X-100 (lanes 4, 11), Tween 80 (lanes 5, 12) and Lubrol WX (lanes 6, 13). No detergent was added for lanes 7, 14. Ganglioside standards used: G_1, ganglioside GM_1; G_2, asialo-GM_1 (GA_1); G_3, a mixture of GM_1, GM_2 and asialo-GM_1; SA, *N*-acetylneuraminic acid.

Table 1
Hydrolysis of ganglioside GM_1 in the presence of various detergents

Detergent	(mg/ml)	Neuraminidase activity[b] (ng NeuNAc released/h/mU)	
		A. ureafaciens	*C. perfringens*
None		n.d.[c]	n.d.
Sodium cholate	0.5	441.7	n.d.
	1.0	716.7	n.d.
	2.5	475.0	19.2
Sodium deoxycholate	0.5	566.7	n.d.
	1.0	83.3	16.6
	2.5	133.3	–[a]
Sodium taurocholate	0.5	n.d.	n.d.
	1.0	n.d.	–[a]
	2.5	50.0	–[a]
Triton X-100	1.0	33.3	n.d.
	2.5	29.2	n.d.
Lubrol WX	1.0	20.8	n.d.
Tween 80	1.0	25.0	n.d.

[a] not determined
[b] Enzyme assays were performed as described in the text except that the amount of enzyme used were 10 mU and 50 mU for *A. ureafaciens* and for *C. perfringens* neuraminidase, respectively, and the amount of ganglioside GM_1 used was 250 μg

n.d., not detectable

aminidases. Neuraminidase from *A. ureafaciens* in the presence of 1.0 mg/ml sodium cholate showed about 40-fold greater activity toward GM_1 than neuraminidase from *C. perfringens* with 2.5 mg/ml sodium cholate. The maximum activation of *A. ureafaciens* neuraminidase with sodium cholate at a given concentration of GM_1 was obtained when the molar ratio of sodium cholate to GM_1 was about 3.

Furthermore, by a more sensitive method, the neuraminidase from *A. ureafaciens*, but not from *C. perfringens*, was shown to cause slight, but significant, hydrolysis of GM_1, even in the absence of any

Table 2
Effect of sodium cholate on the activity of *A. ureafaciens* neuraminidase toward ganglioside GM_1 at concentrations above and below the critical micelle concentration (CMC)

Substrate concentration	Neuraminidase activity (ng NeuNAc released/h/mU)	
Ganglioside GM_1 ($\times 10^{-4}$ M)	Sodium cholate (–)[a]	Sodium cholate (+)[b]
0.32 (below CMC)	0.26	224
3.16 (above CMC)	0.76	743

[a] The activity of neuraminidase in the absence of detergent was assayed as in [9], as described in the text
[b] In this assay, sodium cholate was added to the incubation mixture at 3-times the concentration of ganglioside GM_1 on a molar basis. The activity of neuraminidase was assayed essentially as in [8], as described in the text

detergents. Neither the enzyme activity nor the activity effect of sodium cholate was influenced appreciably by the physical state of GM_1, which changes from monomeric to micellar form at a concentration (CMC) of about 0.85×10^{-5} M [10] (table 2).

Figure 2 showed that in the absence of detergent, the oligosaccharide derived from the sugar moiety of GM_1 was more susceptible than GM_1 to neuraminidase, and in sharp contrast with the results on GM_1, the addition of detergent had no appreciable effect on the enzymatic activity toward the oligosaccharide. The neuraminidase from *A. ureafaciens* showed about 20-fold more activity than clostridial neuraminidase on the oligosaccharide, while *V. cholerae* neuraminidase did not hydrolyze either the oligosaccharide or GM_1, irrespective of the presence of detergents. Thus, the activities of different neuraminidases toward the oligosaccharide correlated well with their activities toward GM_1.

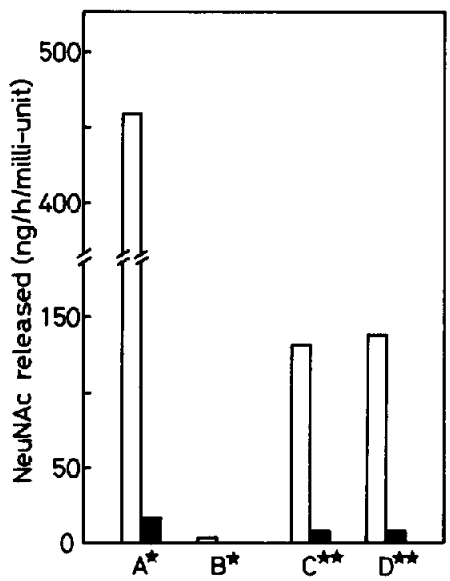

Fig. 2. Comparison of neuraminidase activities of *A. ureafaciens* (▭) and *C. perfringens* (▬) toward ganglioside GM_1 in the presence or absence of detergent. * When ganglioside GM_1 was used as substrate in the presence (A) or absence (B) of sodium cholate, the enzyme reaction was started by addition of 20 mU of either bacterial neuraminidase in terms of the activity toward N-acetylneuraminyllactose. ** When the oligosaccharide prepared from GM_1 was used as substrate in the presence (C) or absence (D) of sodium cholate, the enzyme reaction was started by addition of 50 mU of either bacterial neuraminidase in terms of the activity toward *N*-acetylneuraminyllactose.

4. Discussion

Monosialoganglioside GM_1 has long been believed to be resistant to neuraminidase, due to the steric hindrance exerted by the neighboring Gal–GalNAc residue [3]. In the presence of bile salts this resistant NeuNAc could be hydrolyzed by *C. perfringens* neuraminidase [11]. However, their experiments did not show whether the hydrolysis of GM_1 was due to specific activation of clostridial neuraminidase by bile salts or to their modification of the micellar substrate to forms that were susceptible to the action of neuraminidase. The present work demonstrated that the neuraminidase from *A. ureafaciens* had much greater activity toward GM_1 than that from *C. perfringens*, the only bacterial neuraminidase so far reported to be able to cleave GM_1 in the presence of bile salts. Moreover, contrary to the results [11], this new enzyme could hydrolyze GM_1 in the absence of any detergents, and the activating effect was not confined to bile salts. These results indicate that a detergent is not essential, but that it plays an auxiliary role, in the enzymatic hydrolysis of GM_1.

GM_1 below the CMC became susceptible to the *C. perfringens* enzyme even in the absence of bile salts [12]. In our experiments, neither the susceptibility of GM_1 to the action of *A. ureafaciens* neuraminidase nor the activation effect of sodium cholate was affected appreciably by the physical state of GM_1. Consequently, the proposed hypothesis that the monomeric form of GM_1 is more susceptible to neuraminidase does not seem to be correct. The discrepancies between our results and those of [11,12] may be due to a considerably lower activity of *C. perfringens* neuraminidase toward GM_1, which makes it difficult to estimate exactly minute differences in GM_1 hydrolysis without detergent, or they may be due to the less sensitive assay procedure employed in previous experiments.

Our results on hydrolysis of the oligosaccharide suggest that there is a great difference between different neuraminidases in their recognition of the specific saccharide structure of GM_1, which primarily

determines the release of NeuNAc from GM_1. The differences in the susceptibilities of the oligosaccharide and GM_1 to neuraminidases and in the effect of detergent on their hydrolyses also suggest that the hydrophobic ceramide moiety of GM_1 restricts the enzymatic hydrolysis, and that detergents may release this restriction, possibly by modification of the interaction between the hydrophobic portion of GM_1 and the enzyme molecule. More detailed investigations on the enzymatic mechanism of ganglioside hydrolysis by *A. ureafaciens* neuraminidase are now in progress.

The high specific activity of this new bacterial enzyme toward GM_1 will enable us not only to prepare asialo-GM_1 enzymatically, but also to analyze the chemical structure of other complex sialo–sugar compounds.

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