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# *Arthrobacter ureafaciens* sialidase isoenzymes, L, M1 and M2, cleave fucosyl GM1

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**Among bacterial, fungal and viral sialidases, the sialidase from *Arthrobacter ureafaciens* has the unique property of cleaving sialic acids linked to the internal galactose of gangliotetraose. In this study, we examined the ability to cleave the internal sialic acids of GM1 and fucosyl GM1 of sialidases from several bacterial and fungal origins, including *Clostridium perfringens* and *Vibrio cholerae*. We found that *A. ureafaciens* sialidase could liberate the sialic acid of GM1 at the highest rate, and was the only enzyme which could cleave fucosyl GM1 among the sialidases examined.**

**The affinity-purified sialidase derived from the culture medium of *A. ureafaciens* was comprised of four isoenzymes with different molecular weights and isoelectric points, the isoenzymes that cleaved fucosyl GM1 being L (88 kDa, pI 5.0), M1 (66 kDa, pI 6.2) and M2 (66 kDa, pI 5.5), but not S (52 kDa, pI 6.2) which showed the highest specific activity toward colominic acid among the four isoenzymes.**

***Keywords*: bacterial sialidase, fungal sialidase, FABMS, isoenzyme, internal galactose**

***Abbreviations*: SA, sialic acid; PBS, phosphate-buffered saline; PVP, polyvinylpyrrolidone; FABMS, fast atom bombardment mass spectrometry; Gal$\beta_{int}$, internal galactose of $Gg_4Cer$; Gal$\beta_{ext}$, external galactose of $Gg_4Cer$**

## Introduction

Gangliosides are defined as sialic acid (SA)-containing glycolipids, and many diverse functions have been attributed to the acidic carbohydrate chains of gangliosides [1]. Gangliosides are ubiquitous in the tissues and cells of vertebrates, and are classified into five groups according to their asialocarbohydrate chains [1], to which sialic acid is linked in the following ways: SA$\alpha$2-3Gal$\beta_{ext}$ (external galactose in $Gg_4Cer$), SA$\alpha$2-3Gal$\beta_{int}$ (internal galactose in $Gg_4Cer$), SA$\alpha$2-6Gal$\beta$, SA$\alpha$2-6GalNAc$\beta$, SA$\alpha$2-6GlcNAc$\beta$ and SA$\alpha$2-8SA$\alpha$. For structural and functional analyses of gangliosides, enzymatic removal of the SA residue is essential, and several sialidases having different substrate specificities from microorganisms and animal tissues have been used for this purpose [2]. The sialidase from the leech, *Macrobdella decora*, shows a definite specificity for SA$\alpha$2-3Gal$\beta_{ext}$, resulting in the conversion of GD1a to GM1, and GT1b to GD1b [3], and the sialidases from Newcastle disease virus and *Salmonella typhimurium* LT2 cleave SA$\alpha$2-3Gal$\beta_{ext}$ and SA$\alpha$2–8SA$\alpha$ linkages [4]. The sialidases from *Vibrio cholerae* and *Clostridium perfringens* cleave SA linkages other than SA$\alpha$2-3Gal$\beta_{int}$ in the ganglio-series gangliosides, and yield GM1 preferentially from GD1a, GD1b and GT1b, and $Gg_4Cer$ from GD1$\alpha$ [2]. The resistance to cleavage of the SA$\alpha$2-3Gal$\beta_{int}$ linkage is probably due to steric hindrance by the carbohydrate linked to the same Gal$\beta_{int}$ residue, i.e. GalNAc$\beta$ for GM2 and Gal$\beta$1-3GalNac$\beta$ for GM1, at the active site formed by the Asp-block or Arg-triad [5, 6]. However, the enzyme from *A. ureafaciens* cleaves all SA residues in gangliosides, even in GM1, to produce $Gg_4Cer$ [7, 8]. To further characterize the properties of the sialidase from *A. ureafaciens*, we examined its activity towards GM1 and fucosyl GM1 of several bacterial and fungal origins, and the activities of sialidase isoenzymes purified from the culture medium of *A. ureafaciens*.

## Materials and methods

### Materials

Gangliosides GM1 and fucosyl GM1 were purified from human brain and bovine thyroid, respectively [9]. Colominic acid (Na salt) and *N*-acetylneuraminic acid were the products of Marukin Shoyu Co., and detergents, sodium cholate, sodium deoxycholate, sodium chenodeoxycholate, sodium lithocholate, sodium ursodeoxycholate, sodium glycocholate, sodium taurocholate and Triton X-100 were

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purchased from Sigma, St Louis, Mo, USA, or Nacalai Tesque, Kyoto. The sialidases from *V. cholerae* and *Cl. perfringens* were purchased from Sigma. A colominic acid-coupled starch gel for affinity chromatography was prepared according to the method reported previously [10].

## Cultivation of bacteria and fungi

As reported previously [10–12], colominic acid was added to the media as the sole source of carbon to induce sialidase production. The medium for bacteria consisted of 0.5% colominic acid, 0.2% $(NH_4)_2HPO_4$, 0.2% NaCl, 0.1% $KH_2PO_4$, 0.02% $MgSO_4 \cdot 7H_2O$, 0.005% $FeSO_4 \cdot 7H_2O$ and 0.05% yeast extract (pH 7.0), and that for fungi of 0.5% colominic acid, 0.2% $NaNO_3$, 0.1% $KH_2PO_4$, 0.05% KCl, 0.02% $MgSO_4 \cdot 7H_2O$ and 0.05% yeast extract (pH 6.2).

## Purification of sialidase from the culture medium of *Arthrobacter ureafaciens*

The procedure for purification of the sialidase from the culture medium of *A. ureafaciens* was essentially the same as reported previously [10, 12]. In brief, cells were cultivated until the turbidity at 660 nm reached 0.8–2.6, and then the culture medium obtained on centrifugation at 10 000 × **g** for 20 min was concentrated by ultrafiltration through a Diaflo UM-10 membrane (Amicon Co., Beverly, MA, USA), followed by salting-out at 40–60% saturation with ammonium sulfate. The precipitate was dissolved in 10 mM acetate buffer (pH 5.8), dialysed against the same buffer at 4 °C overnight, and then applied to an affinity column packed with the colominic acid-coupled starch gel. The column was washed with 10 mM acetate buffer (pH 4.5) until the absorbance of the eluate at 280 nm became negligible, and then the bound sialidase was eluted with 100 mM carbonate buffer (pH 9.0). The sialidase-containing fractions were concentrated by ultrafiltration through a Diaflo UM-10 membrane and salting-out at 70% saturation with ammonium sulfate, and the resulting pellet was dissolved in 100 mM phosphate buffer (pH 6.8) and then fractionated by Ultrogel AcA 44-column chromatography. Isoenzyme M was further fractionated into M1 and M2 by isoelectric focusing as described previously [12]. The elution was monitored at 280 nm and as the sialidase activity measured with colominic acid as the substrate.

## Determination of sialidase activity

The reaction mixture for the colorimetric assay of sialidase activity comprised 200 μg of colominic acid and 50 mM acetate buffer, pH 4.5, in a final volume of 200 μl. After incubation at 37 °C for 10 min, the reaction was terminated by the addition of 100 μl of 25 mM periodic acid in 0.125 N $H_2SO_4$, and then the solution was incubated at 37 °C for 30 min. Excess periodate was removed until the yellow colour disappeared by the addition of 100 μl of 1.6% sodium arsenite in 0.4 N HCl, and then to this solution 1.0 ml of 0.1 M thiobarbituric acid-reagent, pH 9.0, was added. After heating in boiling water for 7.5 min, the tube was chilled in ice and the solution was diluted with 2.0 ml of acetone containing 2.5% (by vol.) HCl. The absorbance of the diluted solution was measured at 551 nm [10]. *N*-Acetylneuraminic acid was used as a standard and the standard curve was linear over the range of 1–15 μg. One unit of enzyme activity was defined as 1 μmol of sialic acid released per minute under the reaction conditions used. When gangliosides were used as substrates, the reaction mixture comprised 100 μmol gangliosides, 20 mM acetate buffer, pH 5.0, and 1.5 mM sodium cholate, in a final volume of 200 μl. After incubation at 37 °C for 1 h, the amount of sialic acid liberated was determined as above. In addition, for characterization of the products, the solution after the reaction was diluted with 2 ml of 0.1% KCl in water and then applied to a Sep-Pak™ cartridge (Waters, Milford, MA, USA), which had previously been washed successively with 20 ml each of water, methanol, chloroform : methanol (1 : 1, by vol.), methanol, and 0.1% KCl in water. The cartridge was washed with 20 ml of water, and then glycolipids bound on the cartridge were eluted with 10 ml of methanol and 15 ml of chloroform : methanol (1 : 1, by vol.). The products were examined by thin-layer chromatography (TLC) with a solvent system of chloroform : methanol : 0.5% $CaCl_2$ in water (55 : 45 : 10, by vol.), and with orcinol-$H_2SO_4$ and resorcinol-HCl as detection reagents for carbohydrates and sialic acids, respectively. Furthermore, the products were isolated from the substrates by DEAE-Sephadex A-25 (acetate form; Pharmacia, Uppsala, Sweden) column chromatography as described previously [13], and identified by negative ion FABMS with triethanolamine as the matrix solution [14].

## Preparation of anti-IV $^2$Fucα-Gg$_4$Cer antiserum and its application for detection of the products after sialidase treatment of fucosyl GM1

One mg of IV$^2$Fucα-Gg$_4$Cer purified as above and 0.5 mg of bovine serum albumin were dissolved in 1 ml of PBS, and then mixed well with 1 ml of Freund's complete adjuvant to form a stable water-in-oil emulsion. The emulsion was injected intradermally into the footpads of a rabbit and the antibody titre of the serum was monitored by means of an ELISA. When the titre reached the maximum level (about 1 : 200 000), the animal was bled and the serum was collected. The antiserum reacted with IV$^2$Fucα-Gg$_4$Cer, but not with Fucosyl GM1, GM1 or Gg$_4$Cer. For TLC-immunostaining with the anti-IV$^2$Fucα-Gg$_4$Cer antiserum, fucosyl GM1 (0.05–2.0 μg) was reacted with 0.1 U of sialidase in 30 μl of the following buffers: 25 mM acetate buffer, pH 5.0, containing 1.5 mM sodium cholate for *A. ureafaciens* sialidase isoenzymes L, M1 and M2, and *Cl. perfringens* sialidase, 25 mM acetate buffer, pH 4.0, containing 1.5 mM sodium cholate for *A. ureafaciens* sialidase isoenzyme S, and

25 mM acetate buffer, pH 5.5, containing 1.5 mM sodium cholate, 2.2 mM $CaCl_2$ and 37 mM NaCl for *V. cholerae* sialidase. After incubation at 37 °C for 1 h, followed by dilution with 15 µl of ethanol, 5 µl of the solution was chromatographed on a TLC plate (Art. 805013; Sigma) with a solvent system of chloroform : methanol : 0.5% $CaCl_2$ in water (55 : 45 : 10, by vol.), and then the plate was blocked with a blocking buffer (1% polyvinylpyrrolidone, 1% ovalbumin and 0.02% $NaN_3$ in PBS) at 37 °C for 2 h. The plate was then incubated with the anti-IV$^2$Fucα-Gg$_4$Cer antiserum diluted 1 : 1000 with a dilution buffer (3% polyvinylpyrrolidone in PBS) at 4 °C overnight, and then the antibody bound to the plate was detected by reaction at 37 °C for 1 h with horseradish peroxidase-conjugated anti-rabbit Ig(G + M + A) antibodies diluted 1 : 500 with the dilution buffer, followed by reaction with 4-chloro-1-naphthol and $H_2O_2$ in 50 mM Tris-HCl (pH 7.4) containing 200 mM NaCl at 37 °C for 10 min, and then the density of the spots was quantitated at 500 nm with a TLC-ensitometer.

## Results and discussion

### The activities of sialidases from several microorganisms towards GM1 and fucosyl GM1

The sialidases in the culture media of several microorganisms were determined with colominic acid, GM1 and fucosyl GM1 as substrates. As shown in Table 1, the sialidase of *Sporothrix schenckii* exhibited relatively high activity towards colominic acid, but the enzyme was unable to cleave GM1. The culture media of strains other than *S. schenckii* contained GM1-sialidases, the highest activity, determined in the presence of sodium cholate, being in the culture medium of *A. ureafaciens*. The activity of GM1-sialidase was approximately 10 times higher than those in the culture media of *A. oxydans* and *B. protophormiae*. Furthermore, the sialidase from *A. ureafaciens* was found to

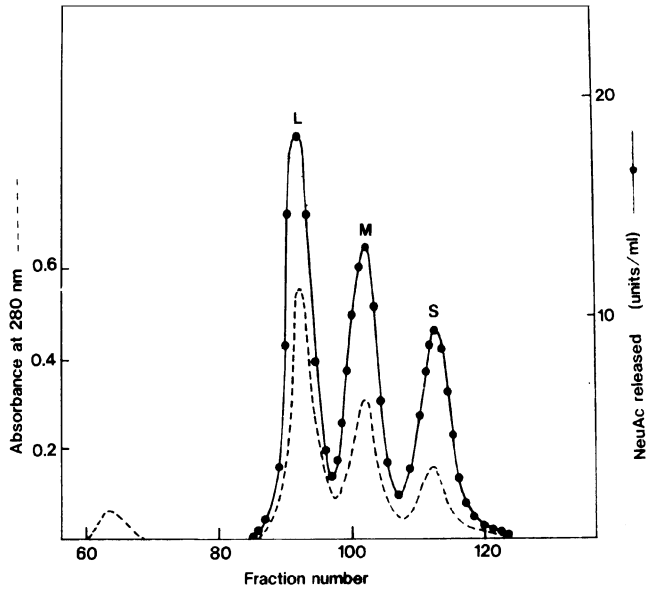


**Figure 1.** Elution profile of sialidases from *A. ureafaciens* on Ultrogel AcA 44. Sialidases purified by affinity chromatography were applied to a column of Ultrogel AcA 44 (2.6 cm × 100 cm) and then eluted with 100 mM phosphate buffer (pH 6.8). The absorbance at 280 nm (·····), and the sialidase activity measured with colominic acid as the substrate (——) were monitored. Three peaks representing sialidases are designated as isoenzymes L, M and S.

**Table 1.** Sialidase activities towards colominic acid, GM1 and fucosyl GM1 in the culture media of several microorganisms.

| *Strains* | *Sialidase activity (mU ml$^{-1}$)* | | | |
|---|---|---|---|---|
| | *Colominic acid* | *GM1* | | *Fucosyl GM1 with sodium cholate* |
| | | *Without sodium* | *With cholate* | |
| *Arthrobacter ureafaciens* IFO 12140 | 279 | 4 | 114 | 56 |
| *Arthrobacter aurescens* IFO 12136 | 44 | 0 | 5 | 0 |
| *Arthrobacter oxydans* IFO 12138 | 31 | 1 | 11 | 0 |
| *Corynebacterium aquaticum* IFO 12154 | 50 | 4 | 6 | 0 |
| *Corynebacterium sialophilum* KMS 3608 | 52 | 4 | 9 | 0 |
| *Brevibacterium protophormiae* IFO 12128 | 100 | 5 | 14 | 0 |
| *Streptomyces* sp. M 39-1 | 136 | 0 | 2 | 0 |
| *Streptomyces* sp. MB 53-2 | 119 | 1 | 3 | 0 |
| *Streptomyces* sp. M 148-2 | 198 | 2 | 6 | 0 |
| *Sporothrix schenckii* IFO 5983 | 862 | 0 | 3 | 0 |
| *Sporothrix schenckii* IFO 5984 | 694 | 0 | 0 | 0 |
| *Sporothrix schenckii* IFO 6073 | 470 | 0 | 0 | 0 |
| *Penicillium urticae* IFO 7011 | 25 | 0 | 3 | 0 |

be the only enzyme capable of liberating the sialic acid from fucosyl GM1, and the GM1-sialidases produced by the other microorganisms were unable to cleave fucosyl GM1 even with ten times higher activity than that of *A. ureafaciens.*

**Table 2.** Effects of various detergents on the cleavage of the sialic acid of fucosyl GM1 by *A. ureafaciens* sialidase isoenzyme L. The activity was determined in 25 mM acetate buffer (pH 5.0) as described in the text.

| *Detergent* | *Concentration (mM)* | *Fucosyl GM1-sialidase activity (mU mg$^{-1}$ protein)* |
|---|---|---|
| None | | 2 |
| Sodium cholate | 0.38 | 165 |
| | 0.75 | 345 |
| | 1.50 | 366 |
| Sodium deoxycholate | 0.75 | 311 |
| | 1.50 | 50 |
| Sodium chenodeoxycholate | 0.75 | 354 |
| | 1.50 | 105 |
| Sodium lithocholate | 0.75 | 12 |
| | 1.50 | 9 |
| Sodium ursodeoxycholate | 0.75 | 151 |
| | 1.50 | 103 |
| Sodium glycocholate | 0.75 | 43 |
| | 1.50 | 48 |
| Sodium taurocholate | 0.75 | 100 |
| | 1.50 | 16 |
| Triton X-100 | 0.75 | 30 |
| | 1.50 | 18 |

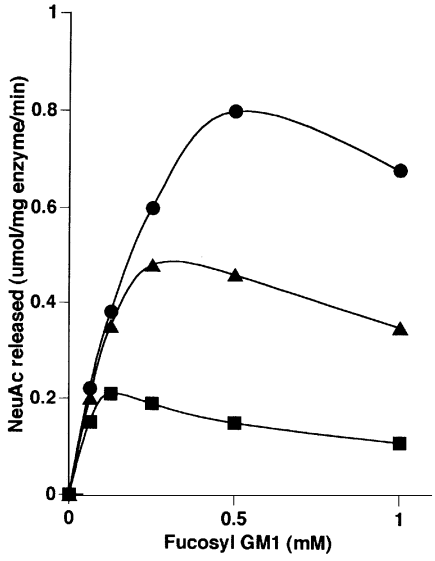


**Figure 2.** Effect of sodium cholate on the initial reaction velocity of isoenzyme L with fucosyl GM1. The activity was determined in 25 mM acetate buffer, pH 5.0, with various concentrations of sodium cholate and fucosyl GM1. ●, 1.5 mM; ▲, 0.75 mM, and ■, 0.375 mM sodium cholate.

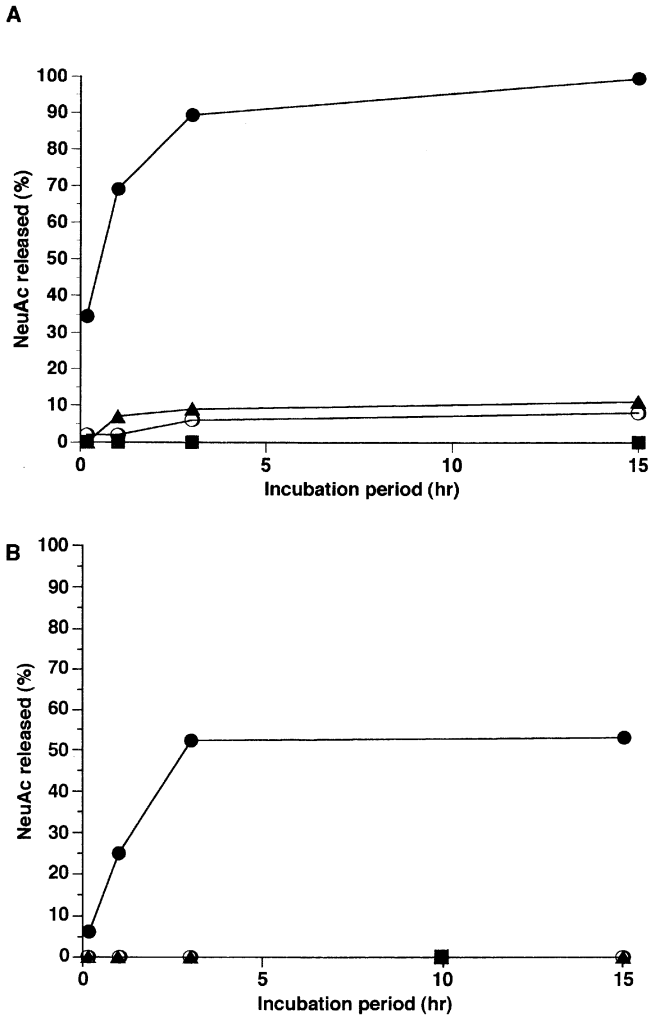


**Figure 3.** Time courses of several sialidases with GM1 (A) and fucosyl GM1 (B). ●, *A. ureafaciens* sialidase isoenzyme L; ○, *Cl. perfringens* sialidase (Sigma); ▲, *A. ureafaciens* sialidase isoenzyme S; and ■, *V. cholerae* sialidase (Sigma). Fucosyl GM1 (60 μg) or GM1 (80 μg) was used as the substrate, and the reaction conditions for *A. ureafaciens* sialidase isoenzyme L and the *Cl. perfringens* sialidase were as described in the text. *A. ureafaciens* sialidase isoenzyme S was incubated in 25 mM acetate buffer (pH 4.0) containing 1.5 mM sodium cholate, and *V. cholerae* sialidase was incubated in 25 mM acetate buffer (pH 5.5) containing 1.5 mM sodium cholate, 2.2 mM $CaCl_2$ and 37 mM NaCl. The enzyme activities used were 0.5 U ml$^{-1}$, as determined with colominic acid as the substrate.

## Isoenzymes exhibiting GM1- and fucosyl GM1-sialidase activities from *A. ureafaciens*

Gel filtration of the affinity purified sialidase from *A. ureafaciens* on Ultrogel AcA 44 resulted in the separation of three isoenzymes with molecular weights of 88, 66 and 52 kDa, which were designated as isoenzymes L, M and S, respectively (Fig. 1). Isoenzyme M was further separated by isoelectric focusing into isoenzymes M1 (pI 6.2) and M2 (pI 5.5) [12]. The specific activities towards colominic acid of isoenzymes L, M1, M2 and S were 1.64, 1.88, 2.13 and 2.94

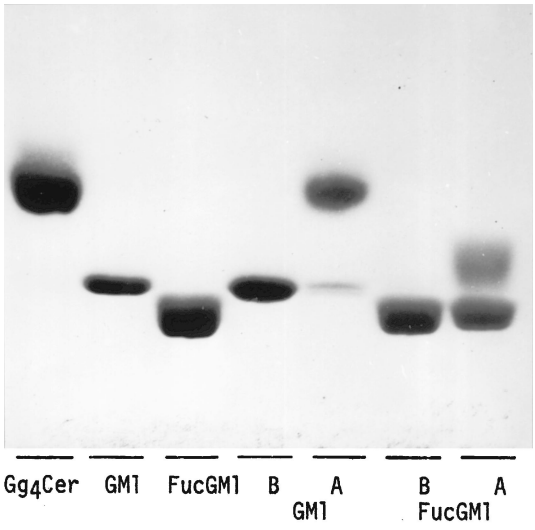


**Figure 4.** TLC of GM1 and fucosyl GM1 before (B) and after (A) treatment with *A. ureafaciens* sialidase isoenzyme L. The TLC plate was developed with chloroform : methanol : 0.5% $CaCl_2$ in $H_2O$ (55 : 45 : 10, by vol.), and the spots were visualized with orcinol-$H_2SO_4$ reagent.

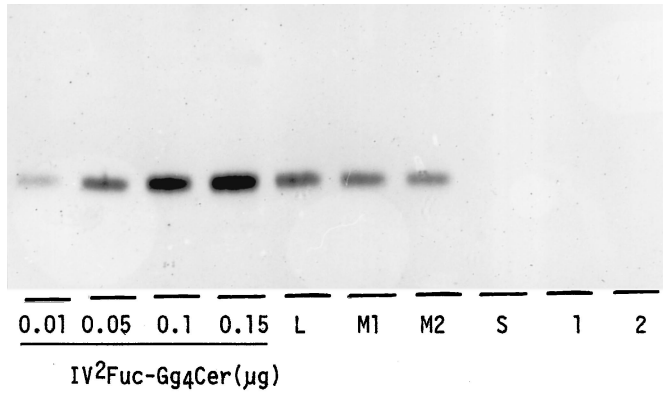


**Figure 6.** TLC-immunostaining, with the anti-IV$^2$Fuc$\alpha$-Gg$_4$Cer antiserum, of the products after treatment of fucosyl GM1 with various sialidases. Fucosyl GM1 (0.5 μg) was hydrolysed with 0.1 U of *A. ureafaciens* sialidase isoenzymes L, M1, M2 and S, and the *Cl. perfringens* (1) and *V. cholerae* sialidases (2) under the conditions described in the text.

units per mg of protein, respectively. Isoenzymes L, M1, M2 and S exhibited GM1-sialidase activity, and the specific activity towards GM1 of isoenzyme L was slightly higher than those of isoenzymes M1 and M2, and much higher than those of isoenzyme S. The optimum conditions for liberation of sialic acids from fucosyl GM1 were determined using isoenzyme L. Among the detergents tested, sodium cholate, sodium deoxycholate and sodium chenodeoxycholate in the reaction mixture provided conditions in which the sialidase activity was sufficiently high enough to cleave fucosyl GM1, but we selected sodium cholate for further analysis because it was most effective even at 1.5 mM (Table 2). The rate of cleavage and the range of linearity of fucosyl GM1-sialidase in the optimum pH range of 4.8–5.0 were affected by the concentration of sodium cholate in the reaction mixture (Fig. 2), the optimum concentration of which was 1.5–5 mM. With 1.5 mM sodium cholate, the enzyme activity was linear up to 0.5 mM fucosyl GM1. Isoenzymes M1 and M2 cleaved fucosyl GM1 at their optimum pHs of 4.8–5.0 to extents similar to that in the case of isoenzyme L, and the $K_m$ and $V_{max}$ for fucosyl GM1 were $4.0 \times 10^{-4}$ M and 2.5 U per mg protein for isoenzyme L,

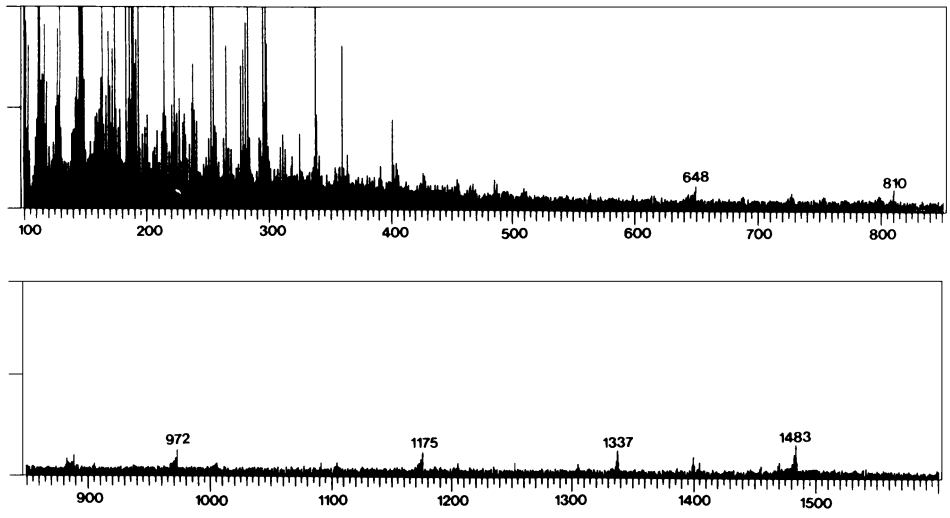


**Figure 5.** Negative ion FABMS spectrum of the product after treatment of fucosyl GM1 with *A. ureafaciens* sialidase isoenzyme L. Fucosyl GM1 (10 μg) was hydrolysed with 0.1 U of *A. ureafaciens* sialidase isoenzyme L under the conditions described in the text, and the products were separated from the unreacted substrate by DEAE-Sephadex column chromatography.

$2.2 \times 10^{-4}$ M and 1.3 U per mg protein for isoenzyme M1, and $2.0 \times 10^{-4}$ M and 1.3 U per mg protein for isoenzyme M2, respectively. Under the same conditions, the reaction velocity of the GM1-sialidase of isoenzyme L was significantly higher than that of fucosyl GM1-sialidase, as shown in Fig. 3. When 10 μg each of GM1 and fucosyl GM1 were treated with 0.1 U of *A. ureafaciens* sialidase isoenzyme L at 37 °C for 1 h, 98% of GM1 and 43% of fucosyl GM1 were converted to their asialo derivatives (Fig. 4). The structures of the products were confirmed by negative ion FABMS after removal of the substrate by DEAE-Sephadex column chromatography. As shown in Fig. 5, the molecular ion of $IV^2Fuc\alpha$-$Gg_4Cer$ with lignoceroyl 4-sphingosine as the ceramide appeared at $m/z$ 1483, and the ions originating from the following sequential cleavage of the carbohydrate chain detected were as follows: $m/z$ 1337 of $(Gg_4Cer\text{-}H)^-$, $m/z$ 1175 of $(Gg_3Cer\text{-}H)^-$, $m/z$ 972 of $(LacCer\text{-}H)^-$, $m/z$ 810 of $(GlcCer\text{-}H)^-$, and $m/z$ 648 of $(ceramide\text{-}H)^-$, indicating that $IV^2Fuc\alpha$-$Gg_4Cer$ is produced without any structural modification. Under the conditions used, isoenzyme S of *A. ureafaciens* sialidase and the *Cl. perfringens* sialidase cleaved the sialic acid of GM1 at rates significantly lower than that in the case of isoenzyme L, but did not cleave fucosyl GM1. The *V. cholerae* sialidase was unable to cleave either GM1 or fucosyl GM1 (Fig. 6). As shown in Table 3, isoenzyme L readily hydrolysed NeuAcα2-6GalNAcβ in GD1α, and NeuAcα2-8NeuAcα in GD1b and GT1b, as well as NeuAcα2-3Gal$\beta_{ext}$, at higher rates than NeuAcα2-3Gal$\beta_{int}$, and the same substrate specificities were observed for isoenzymes M1 and M2. When we compared the rates of cleavage of the SA residue with different linkages by isoenzyme L at 25 °C for 10 min, we found that $II^6$NeuAcα-LacCer was converted to LacCer at a higher rate than $II^3$NeuAcα-LacCer, indicating that the SAα2-6Galβ linkage is more susceptible to cleavage by isoenzyme L than the SAα2-3Galβ one (data not shown). However, the susceptibilities of the SAα2-6GAlNAcβ and SAα2-3Galβ linkages in GD1α, and of the SAα2-8SAα and SAα2-3Galβ linkages in GT1b could not be clearly established from the results obtained on reaction at 25 °C, or comparison of the products, GM1b and GM1α from GD1α, and GD1a and GD1b from GT1b, by TLC-densitometry, suggesting similar rates of cleavage of these linkages by isoenzyme L. On the other hand, isoenzyme S cleaved $II^6$NeuAα-LacCer at a much higher rate than $II^3$NeuAcα-LacCer, and the initial reaction velocity with $II^6$NeuAcα-LacCer was higher than that in the case of isoenzyme L. These properties might be related to the exclusive production of the NeuAcα2-6-structure through the reverse reaction with the *A. ureafaciens* sialidase [19, 20].

**Table 3.** Cleavage of various gangliosides by isoenzymes L, M1, M2 and S of the *A. ureafaciens* sialidase, and the *Cl. perfringens* and *V. cholerae* sialidases. The gangliosides (2 μg) were incubated at 37 °C for 1 h with 0.1 U of sialidase in 30 μl of the same buffer as described in the legend to Fig. 3, and then after dilution with 15 μl of ethanol, 5 μl of the solution was applied on a TLC plate. The densities of glycolipids were quantitatively determined by TLC-densitometry after visualization with orcinol-$H_2SO_4$ reagent [15].

| *Substrate* | *Substrate and products after reaction* | *Molar percentage (%)* | | | | | |
|---|---|---|---|---|---|---|---|
| | | *L* | *M1* | *M2* | *S* | Cl. perfringens | V. cholerae |
| GM3 | GM3 | 0 | 0 | 0 | 0 | 0 | 0 |
| | LacCer | 100 | 100 | 100 | 100 | 100 | 100 |
| GM2 | GM2 | 0 | 0 | 0 | 65 | 70 | 100 |
| | $Gg_3Cer$ | 100 | 100 | 100 | 35 | 30 | 0 |
| GM1 | GM1 | 2 | 4 | 4 | 93 | 91 | 100 |
| | $Gg_4Cer$ | 98 | 96 | 96 | 7 | 9 | 0 |
| Fucosyl GM1 | Fucosyl GM1 | 57 | 62 | 64 | 100 | 100 | 100 |
| | $IV^2Fuc\alpha$-$Gg_4Cer$ | 43 | 38 | 36 | 0 | 0 | 0 |
| GD1a | GD1a | 0 | 0 | 0 | 0 | 0 | 0 |
| | GM1 | 1 | 10 | 8 | 95 | 94 | 100 |
| | $Gg_4Cer$ | 99 | 90 | 92 | 5 | 6 | 0 |
| GD1α | GD1α | 0 | 0 | 0 | 0 | 0 | 0 |
| | $Gg_4Cer$ | 100 | 100 | 100 | 100 | 100 | 100 |
| GD1b | GD1b | 0 | 0 | 0 | 0 | 0 | 0 |
| | GM1 | 2 | 4 | 5 | 90 | 95 | 100 |
| | $Gg_4Cer$ | 98 | 96 | 95 | 10 | 5 | 0 |
| GT1b | GT1b | 0 | 0 | 0 | 0 | 0 | 0 |
| | GM1 | 2 | 8 | 7 | 95 | 98 | 100 |
| | $Gg_4Cer$ | 98 | 92 | 93 | 5 | 2 | 0 |

Thus, among the four isoenzymes of *A. ureafaciens* sialidase having different molecular weights and pIs, L (88 kDa, pI 5.0), M1 (66 kDa, pI 6.2), M2 (66 kDa, pI 5.5), and S (52 kDa, pI 6.2), only L, M1 and M2 cleave the SA in fucosyl GM1. This was in constrast to isoenzyme S and the *Cl. perfringens* sialidase, the abilities of which to cleave the SA at Gal$\beta_{int}$ of GM1 were lost on substitution of GM1 with fucose, indicating that the potential of *A. ureafaciens* isoenzymes L, M1 and M2 to recognize the SA residue together with the neighbouring structure is highest among the bacterial sialidases reported to date. On the other hand, the substrate specificity of isoenzyme S was similar to that of the *Cl. perfringens* sialidase, which was able to cleave the SA at Gal$\beta_{int}$ of GM1, indicating that *A. ureafaciens* cultured in the presence of colominic acid produces two sialidases with different substrate specificities with regard to the reactivity to the SA in fucosyl GM1. The involvement of a Trp or Arg residue in the active sites of the *Cl. perfringens* and *S. griseus* sialidases has been deduced from the results of an inhibition experiment [16, 17], and the replacement of Arg 37 with Lys in a short conserved region upstream of the four repeated sequences of the *Cl. perfringens* sialidase gene has been shown to cause changes in the $K_m$, $V_{max}$ and $K_i$ of the sialidase inhibitor [18], indicating the involvement of Arg 37 in the active site. Also, a hydrophobic pocket formed by an Arg-triad has been shown to be essential in the active site of bacterial sialidases [6]. However, since the actual function of the Asp-box and the structural background of the active site remains to be clarified, a comparison of sequences among the isoenzymes of *A. ureafaciens* sialidase should provide new insights not only into the structural differences of the active site, but also into the use of the enzyme for the synthesis of sialocompounds by the reverse reaction [19, 20], and is now in progress in our laboratory.

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