J. Biochem. **138**, 327–334 (2005)
DOI: 10.1093/jb/mvi126

Involvement of the C-Terminal Tail of *Arthrobacter ureafaciens* Sialidase Isoenzyme M in Cleavage of the Internal Sialic Acid of Ganglioside GM1

Masao Iwamori[1,*], Takeo Kaido[1], Yuriko Iwamori[1], Yasuhiro Ohta[2], Kentaro Tsukamoto[3] and Shunji Kozaki[3]

[1]Department of Biochemistry, Faculty of Science and Technology, Kinki University, 3-4-1 Kowakae, Higashiosaka, Osaka 577-8502; [2]Marukin Bio, Inc., 27-2 Monnomae, Todo, Uji, Kyoto 611-0013; and [3]Department of Veterinary Science, College of Agriculture, Osaka Prefecture University, 1-1 Gakuen-cho, Sakai, Osaka 599-8531

Received June 6, 2005; accepted June 20, 2005

Arthrobacter ureafaciens **sialidase comprises four isoenzymes, L, M1, M2 and S, of which L, M1, and M2, but not S, have the unique ability to cleave GM1 ganglioside, but the hydrolysis of GM3 and colominic acid by S occurs at a higher rate than that by L, M1 and M2. Since the N-terminal amino acid sequences of L, M1, M2 and S were shown to be identical on protein sequencing, they were suggested to have arisen from the same protein through truncation at different C-terminal sites. A DNA segment containing an open reading frame was cloned from a genomic library, and the structural gene was found to comprise 2,970 bp encoding a protein of 990 amino acids including a signal peptide at the N-terminus, a conserved FYRIP-region and four Asp boxes. The molecular weights of the isoenzymes determined by MALDI-TOFMS revealed that L, M1, M2 and S should comprise amino acids 39–773, 39–653, 39–655 and 39–528, respectively. In fact, recombinant enzymes M2 and S prepared in *Escherichia coli* exhibited identical substrate specificities toward gangliosides as those of the purified enzymes. Consequently, the C-terminal tail of isoenzyme M2 might be involved in the hydrolysis of the internal sialic acid of GM1.**

Key words: bacterial sialidase, ganglioside, isoenzyme, molecular cloning, sialic acid.

Abbreviations: Glycolipids are abbreviated according to the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature (*1*). The ganglioside nomenclature of Svennerholm (*2*) is employed throughout, except that GD1α denotes III^{6}NeuAc, IV^{3}NeuAc-Gg$_4$Cer. AU, *Arthrobacter ureafaciens*; CP, *Clostridium perfringens*; VC, *Vibrio cholerae*; PCR, polymerase chain reaction; MALDI-TOFMS, matrix-assisted laser desorption-time of flight mass spectrometry; TLC, thin-layer chromatography; Lac, lactose.

Sialidase [EC 3.2.1.18] is an exoglycosidase that hydrolyzes sialic acid in glycoconjugates, including gangliosides, and has been found in viruses, fungi, protozoa and animals (*3*, *4*). When sialidase activity with gangliosides as substrates was examined, enzymes of different origins were demonstrated to exhibit unique substrate specificities. The major gangliosides in mammalian brain, GM1, GD1a, GD1b and GT1b, have been cleaved into GM1 and GD1b with a sialidase from the leech, *Macrobdella decora*, due to its definite specificity to the NeuAcα2-3Gal linkage at the terminal galactose of the gangliotetraose backbone, but most enzymes available commercially of bacterial origin, such as *Vibrio cholerae* (VC), *Clostridium perfringens* (CP), *Salmonella typhimurium* and Newcastle disease virus, preferentially convert brain gangliosides to GM1, indicating that NeuAcα2-3Gal at the terminal galactose in GD1a and GT1b, and NeuAcα2-8NeuAc in GD1b and GT1b, but not NeuAcα2-3Gal at the internal galactose, are susceptible to these enzymes (*5*–*7*). Although sialic acid branching at the internal GalNAc through a NeuAcα2-6 linkage in GD1α is removed by VC and CP sialidases, such branching at the internal galactose through a NeuAcα2-3 linkage is resistant to the sialidases of both strains due to the steric hindrance of neighboring GalNAc at the internal galactose (*5*, *8*, *9*). In contrast to these sialidases, the enzyme from *Arthrobacter ureafaciens* (AU) is able to remove all sialic acid residues from brain gangliosides to yield asialo GM1 (Gg$_4$Cer) as the major product (*6*, *10*–*14*). Under the optimal conditions with suitable detergents, the rates of conversion of GM1 to asialo GM1 by VC, CP and AU sialidases are 0, 9 and 98%, respectively (*6*, *12*, *13*), and AU sialidase is the only enzyme that hydrolyzes fucosyl GM1 to fucosyl asialo GM1 (IV^{2}Fuc-Gg$_4$Cer) (*6*). Along with the unique substrate specificity toward gangliosides of AU sialidase, its enzymatic properties are different from those of other sialidases, *i.e.*, no divalent cation is required for its activity and its activity is not inhibited by EDTA and SH-inhibitors, such as *p*-chloromercuribenzoate and Hg^{2+} (*10*, *11*).

The production of sialidase from *A. ureafaciens* IFO12140 has been induced by cultivation with colominic acid or sialic acid as a sole carbon source, and mutant *A. ureafaciens* M1057, exhibiting continuous production of sialidase even in L broth containing yeast extract and peptone, has been established by mutagenesis with *N*-methyl *N*′-nitro-*N*-nitrosoguanidine (*15*). The enzymes secreted into the medium comprise four isoenzymes, L,

*To whom correspondence should be addressed. Tel: +81-6-6721-2332, Fax: +81-6-6723-2721, E-mail: iwamori@life.kindai.ac.jp

M1, M2 and S, with different molecular weights and isoelectric points (*6*, *14*). Interestingly, although isoenzymes, L, M1 and M2 effectively cleave GM1 and fucosyl GM1 at the same rates as an affinity-purified crude enzyme preparation from the culture medium of AU, isoenzyme S exhibits significantly lower activity toward GM1 than the L, M1 and M2 isoenzymes, and does not cleave fucosyl GM1 at all (*6*). In contrast, cleavage of colominic acid by isoenzyme S occurs at a higher rate than that by L, M1 and M2, indicating that the substrate specificity of isoenzyme S is similar as that of CP, and those of isoenzymes L, M1 and M2 toward gangliosides are typical of AU (*6*, *14*). To further characterize the substrate specificities of these novel isoenzymes from AU, the sialidase gene was cloned from DNA, and then the activities of the isoenzymes toward gangliosides were compared with those of the recombinant enzymes.

EXPERIMENTAL PROCEDURES

Materials—Glycolipids were purified from various sources in our laboratory: LacCer and GM3 from human erythrocytes, GM1 from bovine brain, and fucosyl GM1 from bovine thyroid. Asialo GM1 and fucosyl asialo GM1 were prepared from GM1 and fucosyl GM1 by treatment with AU sialidase, respectively (*6*). Colominic acid (Na salt) and *N*-acetylneuraminic acid were the products of Marukin-Chuyu Co., Kyoto. The following reagents were from commercial sources: restriction enzymes, *i.e.*, *Eco*RI, *Kpn*I, *Bam*HI, *Pst*I, *Xba*I, *Nru*I, *Nhe*I, *Mun*I, *Sma*I, *Dra*I, *Sca*I, *Ssp*I, *Sac*I, *Sal*I, *Pvu*I, *Sac*II, *Xho*I and *Nde*I, EX-Taq and a deletion kit from Takara, Kyoto; *Hin*dIII, *Nco*I, alkaline phosphatase and a Ligation Pack from Nippon Gene, Tokyo; a pET Directional TOPO Expression Kit from Invitrogen, Tokyo; a DNA and gel band purification kit, and an Alkphos Direct Labelling and Detection System from Amersham, Tokyo; and NeuAcα2-3Lac, Neuα2-6Lac, and CP and VC sialidases from Sigma, St. Louis, MO, USA.

Cultivation of Bacteria—*A. ureafaciens* IFO 12140 was cultured in medium comprising 0.5% colominic acid, 0.2% $(NH_4)_2HPO_4$, 0.2% NaCl, 0.1% KH_2PO_4, 0.02% $MgSO_4 7H_2O$, 0.005% $FeSO_4 7H_2O$ and 0.05% yeast extract (pH 7.0), and the mutant cells, M1057, in L broth comprising 1% peptone, 0.5% NaCl and 0.5% yeast extract (pH 7.0) at 30°C, with shaking (*10*, *11*, *14*).

Sialidase Assay—Sialidase activity was determined using colominic acid and gangliosides as substrates. The standard assay mixtures comprised 200 μg colominic acid in 50 mM acetate buffer, pH 4.5, and 100 μmol ganglioside in 50 mM acetate buffer, pH 5.0, containing 1 mg/ml of sodium cholate, respectively, with a suitable amount of enzyme in a total volume of 200 μl. After incubation at 37°C, the amount of sialic acid liberated was determined by the thiobarbituric acid procedure (*11*). In addition, for characterization of the products by TLC, 5 μg of gangliosides was hydrolyzed with 50 milliunits of sialidase in 30 μl of 20 mM acetate buffer, pH 5.0, containing 30 μg of sodium cholate. After reaction at 37°C for 1 h, glycolipids in the reaction mixture were isolated with a Sep-Pak C18 cartridge (Waters, Milford, MA, USA) and then applied to a thin-layer plate (Silica gel 60; Merck, Darmstadt, Germany), which was developed with chloroform/ methanol/ 0.5% $CaCl_2$ in water (55:45:10, v/v). The spots were visualized with orcinol-H_2SO_4 reagent and by TLC-immunostaining with monoclonal anti-fucosyl asialo GM1 antibodies (*6*), and the density of spots was determined at an analytical wavelength of 420 nm and a control wavelength of 710 nm using a dual-wavelength TLC densitometer (CS-9000; Shimadzu, Kyoto).

Purification of Sialidase Isoenzymes—The procedure for purification of the sialidase isoenzymes from the culture medium of AU or its mutant, M1057, was essentially the same as reported previously (*11*, *14*). In brief, cells were cultivated until the tubidity at 660 nm reached 0.8–2.6, and then the culture medium was collected by centrifugation at 10,000 × *g* for 20 min. Purification of isoenzymes L, M and S from the medium was performed by DEAE-cellulose, colominic acid-coupled affinity and gel exclusion column chromatographies, and isoenzyme M was further fractionated into M1 and M2 by chromatofocusing as described previously (*14*).

Analysis of Enzyme Proteins—Protein concentrations were determined by the dye-binding method with bovine serum albumin as the standard (*16*). SDS-PAGE was carried out according to the method of Laemmli (*17*), and the molecular weights of proteins were determined by matrix-assisted laser desorption-time of flight mass spectrometry (MALDI-TOFMS, REFREX™, Bruker; Tsukuba) with sinapinic acid as a matrix (*18*). The enzymatic cleavage of isoenzyme L was performed with lysyl endopeptidase (0.01 mg; Wako Chemicals, Tokyo) in carbonate buffer, pH 8.9, containing 0.02% Tween 20 at 37°C overnight, and the resultant peptides were purified by reverse phase-high performance liquid chromatography (PU-1580; Jasco, Tokyo) on a C18-column (RP-18GP250-2.0; Kanto Chemicals, Tokyo). The N-terminal amino acid sequences of the isoenzymes and peptides obtained on digestion with lysyl endopeptidase were determined by Edman degradation according to the manufacturer's instructions (Protein Sequencer 241; Hewlett Packard, Foster City, CA, USA).

Polymerase Chain Reactions—The general conditions for PCR amplification of DNA were 20–30 cycles of denaturation at 95°C for 1 min, annealing at 62°C for 30 s and extension at 72°C for 1 min with 50–100 pmol of primers, 10 ng of template DNA and 0.5 unit of EX-Taq (Takara, Kyoto). Five-minute predenaturation at 95°C and additional 5-min extension at 72°C were performed before and after the cycles, respectively.

The Primers Used—Unless otherwise indicated, the nucleotide sequences of the primers used for cloning, sequencing and expression of cDNA are listed in Table 1. The sequence of Primer F was determined by PCR with primers, 20F and 20R, constructed from the 1st to 5th amino acids for the forward primer and the 15th to 20th amino acids for the reverse primer of isoenzyme L, and AU-DNA as the template. Also, primer LR was prepared on the basis of the peptide sequence by cleavage of isoenzyme L with lysyl endopeptidase.

Blotting and Hybridization—DNA was isolated from AU according to the method of Marmur (*19*) as modified by Gebers *et al.* (*20*). The basic procedures for DNA including preparation of plasmid DNA, agarose gel electrophoresis, and elution of DNA fragments from the gel were as described by Sambrook and Russell (*21*).

Table 1. **Primers used for cloning, sequencing and expression of sialidase DNA.**

Primer	Oligonucleotide sequence (5′ to 3′)	Used for
20F	GCT(C/A/G)CCT(C/A/G)ACT(C/A/G)CCT(C/A/G)CCT(C/A/G)A	Coding region PCR
20R	AA(G)A(G)TTA(G/T/C)GTT(C)TCA(G/T/C)GAA(G)AA	Coding region PCR
F	GCCCCCACTCCGCCCAATTC	Coding region PCR
LR	CT(G/C)GCT(G/C/A)GCT)G/C)GGGTTT(G/C)GT(T/C)TT	Probe
500R	TTGGTGTCGGAGGTGATGAG	Probe
Ex-F	CACCATGGCCCCCACTCCGCCCAATT	Expression
Ex-SR	TTGCGGAGCCACGGGCGTGGT	Expression
Ex-MR	GTCCGCCGAAATCAGGCCATCCC	Expression

DNA sequencing was carried out with a DNA-Analysis System (CEQ 2000 xL, Beckman) according to the manufacturer's instructions. DNA fragments derived from AU-DNA through cleavage with restriction enzymes, *i.e.*, *Kpn*I, *Hin*dIII, *Bam*HI, *Pst*I, N*co*I, *Nhe*I and *Nru*I, were electrophoresed on agarose gels, transferred to nylon sheets (Hybond-N™, Amersham), denatured and then immobilized, and then hybridization was performed according to Wallace *et al.* (*22*) using an alkaline phosphatase-labeled [^{32}P]-probe corresponding to DNA between primers F and 500R, according to the instructions for the Alkphos Direct Labelling and Detection System (Amersham).

Cloning of the Sialidase Gene—Since a DNA fragment of about 7,500 bp in length obtained with *Nru*I was found to contain the sialidase gene in the hybridization experiment, this fragment was isolated from the gel with a Gel Band Purification kit (Amersham) and then ligated into the *Sma*I site of the pUC19 vector by means of the Ligation Pack (Nippon Gene, Tokyo). Then, transformation with the resultant plasmid into *E. coli* strain DH5α was accomplished by the calcium phosphate-procedure (*21*), and plasmid-bearing cells were selected on ampicillin-containing agar plates, and by direct colony PCR with primers F and R500. The clone that contained the DNA insert was characterized by sequence analysis, the obtained plasmid, E54, being found to contain the full length of the enzyme gene. Then, subcloning of the DNA fragment obtained on cleavage of plasmid E54 with *Xba*I and *Pst*I into the pUC19 vector was performed as above. After transformation of the resultant vector into *E. coli* JM109, plasmid AU-D3 containing the sialidase gene was selected by direct colony PCR with primers F and R500.

Sequencing of the Sialidase Gene—For sequencing of the DNA insert in plasmid AU-D3, deletion mutants with several lengths of AU-DNA were prepared with a Deletion kit for Kilo-Sequences (Takara, Kyoto) as follows. The DNA insert was cut out from the plasmid with XbaI and SacI, and then cleaved with exonuclease III at 37°C for periods from 1 to 15 min. Then, after treatment of the products with nuclease (mung bean), followed by with Klenow fragment, they were ligated into the *Xba*l site of plasmid pUC19, which was transformed into *E. coli* JM109 to produce deletion mutants. The lengths of the deleted DNA inserts were determined by electrophoresis after cleavage with *Eco*RI and *Pst*I, and their sequences were determined with a DNA analysis system (CEQ2000 xL, Beckman) according to the manufacturer's instructions.

Expression of Isoenzymes M2 and S—The DNAs corresponding to the molecular weights of isoenzymes M2 and S determined by MALDI-TOFMS were multiplied by PCR with plasmid AU-D3, as the template, and primers Ex-F and Ex-MR, and primers Ex-F and Ex-SR, respectively, and then ligated into the pET vector (Invitrogen, Tokyo). The selection of plasmids with the DNA insert in *E. coli* TOPO and the expression of proteins in *E. coli* BL21Star (DE3) on stimulation with IPTG were carried out according to the manufacturer's instructions. The cells producing the recombinant enzymes and those with the empty vector were homogenized in 4 volumes of 0.25 M sucrose with a Polytron homogenizer for determination of sialidase activities.

RESULTS

Sialidase Isoenzymes from A. ureafaciens—The sialidases found in the culture medium of *A. ureafaciens* IFO12140 in the presence of colominic acid comprised four isoenzymes, *i.e.*, L, M1, M2 and S (Fig. 1), the recoveries from 2 g of the proteins in the culture medium through the purification procedure being 5.9, 2.4, 3.4 and 1.6 mg, respectively, and the relative ratio was identical to that obtained for the medium of mutant M1057 cells. The molecular weights of L, M1, M2 and S determined by MALDI-TOFMS were 76,796.2, 64,120.5, 64,326.2 and 51,364.1, respectively (Fig. 2), and their N-terminal amino acid sequences were found to be identical (Table 2), indicating that the four isoenzymes produced by AU

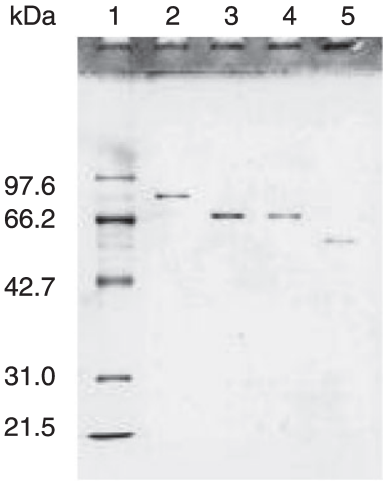

Fig. 1. **SDS-PAGE of AU sialidase isoenzymes L, M1, M2 and S.** The proteins were electrophoresed on a 12% gel under reducing conditions and then stained with Coomassie Brilliant Blue. Lane 1, molecular markers; lane 2, L; lane 3, M1; lane 4, M2; lane 5, S.

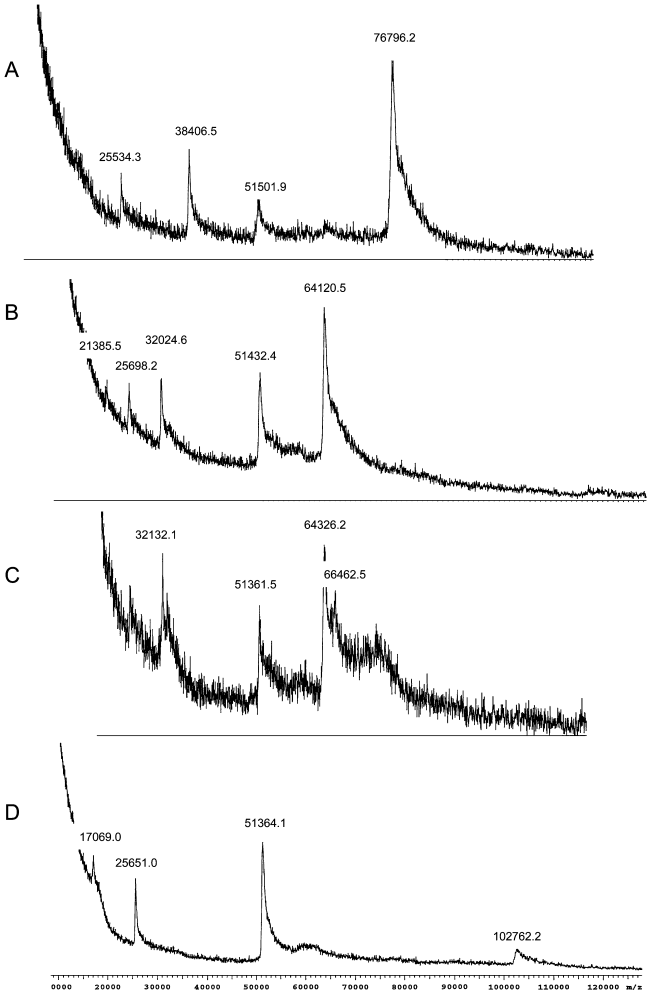

Fig. 2. **MALDI-TOFMS spectra of AU sialidase isoenzymes L (A), M1 (B), M2 (C), and S (D).** The proteins were analyzed by MALDI-TOFMS with sinapinic acid as a matrix.

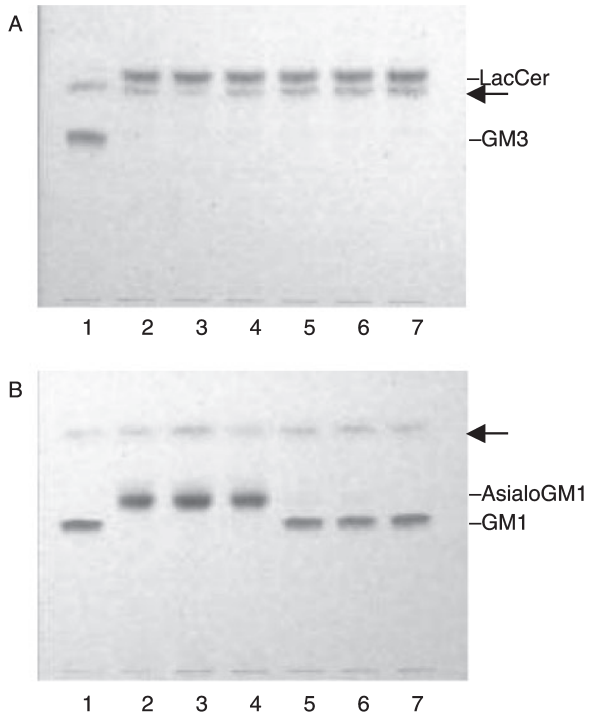

Fig. 3. **TLC of the products derived on hydrolysis of gangliosides GM3 (A) and GM1 (B) by sialidases.** Gangliosides GM3 and GM1 (5 μg) were hydrolyzed without an enzyme (1), and with 50 milliunits of AU sialidases L (2), M1 (3), M2 (4), and S (5), and CP (6) and VC sialidases (7). The buffers used were 20 mM acetate (pH 5.0) for AU, 20 mM tris-acetate (pH 7.1) for CP sialidase, and 20 mM tris-acetate (pH 6.8) with 1 mM $CaCl_2$ for VC sialidase, respectively, 30 μg of sodium cholate being added, in the total volume of 30 μl. TLC was performed with chloroform/methanol/0.5% $CaCl_2$ (55:45:10, v/v), and the spots were visualized with orcinol-sulfuric acid reagent. The arrows indicate sodium cholate.

are proteins modified post-translationally at different C-terminal sites of the same pre-protein.

The enzymatic activities of the four isoenzymes toward various substrates are shown in Table 3. Isoenzyme S hydrolyzed NeuAcα2-6Lac, NeuAcα2-3Lac, colominic acid and GM3, showing higher specific activity than those of isoenzymes L, M1 and M2, but the susceptibility of sialic acid at the internal galactose residue of GM1 to isoenzymes L, M1 and M2 was much higher than that to isoenzyme S. In particular, although fucosylGM1 was readily de-sialylated by isoenzymes L, M1 and M2, it was entirely resistant to the action of isoenzyme S. On incubation of gangliosides GM3 and GM1 with 50 milliunits of AU sialidases L, M1, M2 and S, and CP and VC sialidases for 1 h, conversion of GM3 to the product LacCer was completely achieved with all enzymes. However, while VC sialidase did not remove sialic acid from GM1 at all, AU sialidases L, M1 and M2 were able to convert most of the GM1 to Gg_4Cer, the rates of conversion of GM1 to Gg_4Cer by isoenzyme S and CP sialidase being less than 5% (Fig. 3). The specific activities of isoenzyme

Table 2. **N-Terminal amino acid sequences of isoenzymes L, M1, M2 and S, and the peptides obtained on cleavage of isoenzyme L with lysylendopeptidase.**

Isoenzyme	Amino acid sequence from N-terminal
L	APTPPNSPTLPPGSFSETNL
M1	APTPPNSPTL
M2	APTPPNSPTLPPGSF
S	APTPPNSPTLPPG
Peptide 1 from L	KTNPAA

Table 3. **Specific activities of sialidase isoenzymes.**

Substrate	NeuAc released (μmol/mg protein/min)			
	L	M1	M2	S
NeuAcα2-3Lac	85.1	89.5	93.7	145.2
NeuAcα2-6Lac	89.0	108.6	112.6	170.8
Colominic acid	15.6	18.4	20.9	83.3
GM3	92.8	93.5	98.4	145.7
GM1	57.7	68.0	75.5	8.0
Fucosyl GM1	12.7	14.2	18.3	0.0

Substrates containing 1 mmol of NeuAc were hydrolyzed with 25 milliunits of enzymes in 50 mM acetate buffer, pH 4.5, without a detergent for NeuAcα2-3Lac and colominic acid, and in the same buffer with sodium cholate (1 mg/ml) for GM3, GM1 and fucosyl GM1, respectively, at 37°C for 10 min. The sialic acid released was determined by the thiobarbituric acid procedure as described in the text.

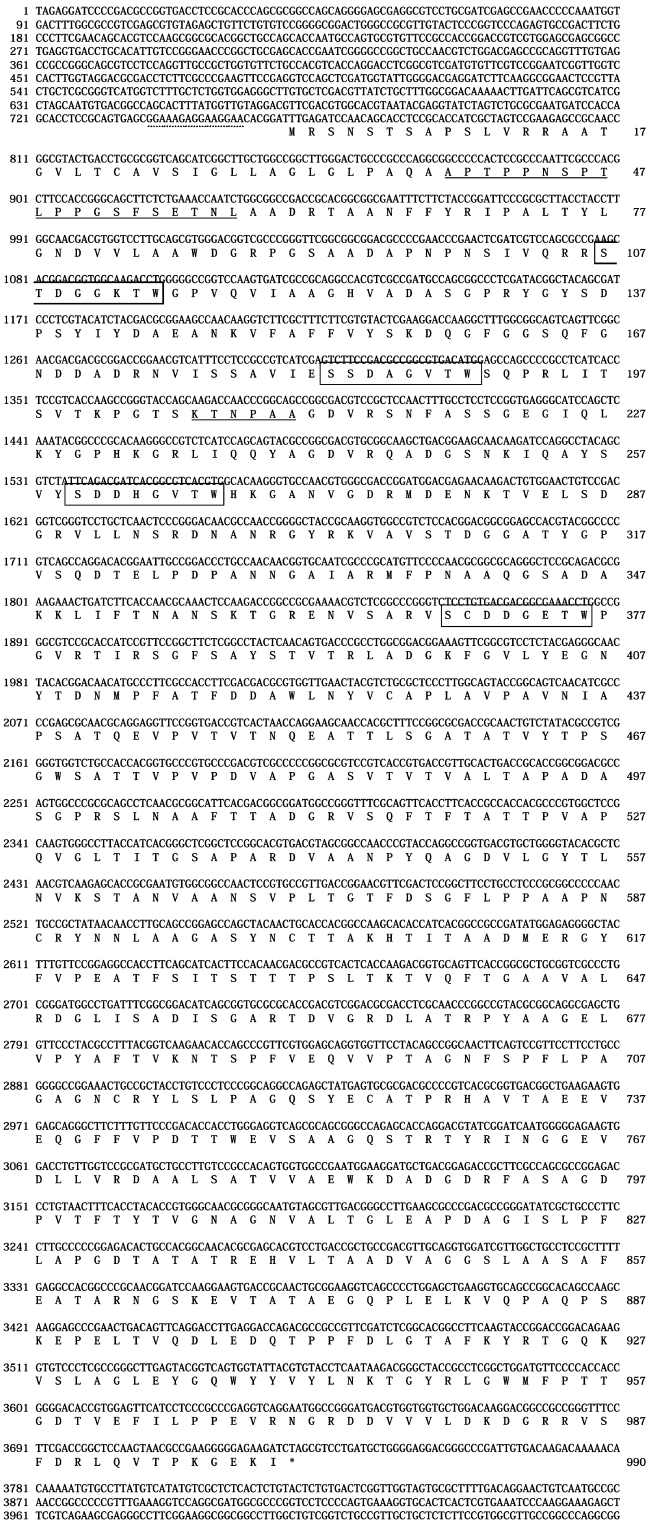

Fig. 4. **Sequence of the DNA insert in plasmid AU-D3 and the deduced amino acid sequence of AU sialidase.** The N-terminal regions of the enzymes purified and of the peptides obtained on digestion with lysyl endopeptidase are underlined with a straight line, and the Shine-Dalgarno sequence with a dotted line. The boxed amino acids are the Asp boxes conserved in bacterial sialidases. Nucleotides 1 to 11 are derived from the pUC19 vector on subcloning of the gene.

M2 toward GM1 and fucosyl GM1 were highest among those of the isoenzymes of AU sialidase.

Cloning of the Sialidase Gene—PCR with AU-DNA as the template, and primers F and LR allowed amplification of nucleotides with a length of 505 bp, which included the sequences for the N-terminal amino acids in Table 1 and two sialidase consensus Asp-boxes, RRST-DGGKTWGP and IESSDAGUTWSQ, and the resultant nucleotides were used as templates for preparation of a radioactive [^{32}P]-probe (488 bp) by PCR with primers F and 500R, according to the instructions for the Alkphos Direct Labelling Kit (Amersham). On Southern blotting with the probe, the following fragments were found to contain the sialidase gene, 3 kbp with *Kpn*I, 6 kbp with *Bam*HI, 7 kbp with *Nco*I, 7.5 kbp with *Nru*I, 8 kbp with *Nhe*I, 10 kbp with *Hin*d III and 10 kbp with *Pst*I. As to the plasmids containing the above fragments, the directions and lengths of DNA inserts were examined by PCR with primers M13 reverse and 500R, and a full length open reading frame was found in plasmid E54, which was obtained on ligation of the *Nru*I fragment into the *Sma*I site in the pUC19 vector. Digestion of plasmid E54 at the *Xba*I site in the vector and the *Pst*I site in AU-DNA yielded a fragment with a length of 4.3 kbp, this length being estimated to include the whole of the sialidase gene (Sia-AU), and the resultant fragment was ligated into the pUC19 vector to generate plasmid AU-D3, whose sequence was determined by preparation of a deletion mutant as described under "EXPERIMENTAL PROCEDURES."

Primary Structure of the Sialidase Gene—Figure 4 shows the nucleotide sequence of the DNA insert in plasmid AU-D3 and the deduced amino acid sequence of AU sialidase. In contrast to the bacterial sialidases reported previously, an initiation codon, ATG, was not observed in the upstream region, and TTG in frame at nucleotide position 760 was thought to be a putative initiation codon, since the proximal stop codon, TGA, in the upstream region was at 733. Also, a twin arginine translational signal, RRAA, was present at nucleotide positions 796–807. In addition, a Shine-Dalgarno sequence (dotted underline in Fig. 4) with ribosomal binding activity and a promoter were located at –8 to –22 and –46 to –100 from the initiation codon, respectively (*24–26*). The stop codon, TAG, in frame was located at nucleotide 3730, and thus the entire gene was postulated to encompass 2,970 bp, corresponding to 990 amino acids and a calculated molecular mass of 103.8 kDa for the unprocessed protein. In the downstream region from the termination codon at 3730–3733, inverted repeat sequences were present at positions 3820–3822 and 3839–3841, followed by four successive Ts, which was a preferable site for the termination of mRNA synthesis (*24*, *25*). The N-terminal peptide sequences of the native protein and those obtained on cleavage with lysyl endopeptidase shown in Table 2 were found in the sequence underlined in Fig. 4. The Asp box, S-X-D-X-G-X-T-W, which is conserved in bacterial and mammalian sialidases, was repeated at four positions, that is, amino acid residues 107–114, 183–190, 260–267 and 369–376 from the N-terminus, and another conserved sequence, FYRIP, was located at amino acid residues 68–72 in the upstream region from the first Asp-box.

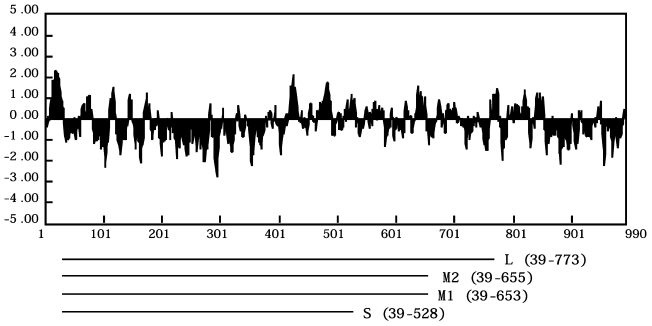

Fig. 5. **Hydropathy profile of the deduced amino acid sequence of AU sialidase.** A Kyte-Doolittle hydropathy profile of sialidase is plotted with an 11-residue window (*28*). The regions of amino acids for isoenzymes L, M2, M1 and S are shown under the profile.

Sialidases Encoded by the Gene—As shown in Fig. 5, a Kite-Doolittle hydropathy plot of the entire protein suggested that the N-terminal sequence of this enzyme could be a signal sequence that directs the secretion of proteins. The removal of the N-terminal 38 amino acids including the hydrophobic signal sequence provided a common N-terminal structure for isoenzymes L, M1, M2 and S, and the resultant peptide comprising 386 amino acids from Ala at the 39th position was included in the catalytic domain of sialidase (*7*, *27*). Consequently, additional C-terminal peptides with the catalytic domain were found to form isoenzymes L, M1, M2 and S, which were produced by truncation at different C-termini in the entire protein. On the bases of the molecular weights determined by MALDI-TOFMS, sialidase isoenzymes L, M1, M2 and S were supposed to comprise amino acids 39 to 773 with a molecular weight of 76,837, amino acids 39 to 653 with a molecular weight of 64,157, amino acids 39 to 655 with a molecular weight of 64,343, and amino acids 39 to 528 with a molecular weight of 51,382, respectively. To confirm the enzyme activities of their peptides, the nucleotides corresponding to the regions for isoenzymes M2 and S were ligated into the expression pET vector, by which were then transformed into *E. coli* BL21Star (DE3) and then expressed to generate the recombinant enzymes. As shown in Fig. 6, recombinant enzyme M2 completely converted GM3 and GM1 to the products LacCer and asialo GM1, and about 60% of fucosyl GM1 to fucosyl asialo GM1. However, although the conversion of GM3 to LacCer by recombinant enzyme S was complete, that of GM1 to asialo GM1 was only 3% and fucosyl GM1 was entirely resistant to its action. Also, the specific activities toward colominic acid, NeuAcα2-6Lac and NeuAcα2-3Lac of recombinant enzyme S (88.9, 124.5 and 105.1 μmol/mg protein/min, respectively) were significantly higher than those of recombinant enzyme M2 (20.2, 75.1 and 66.6 μmol/ mg protein/min, respectively), indicating that recombinant enzymes M2 and S retain the substrate specificities of native enzymes M2 and S. Thus, the C-terminal region comprising amino acids 529 to 655 in M2 was indicated to be essential for formation of the active site interacting with the sialic acid residue at the internal galactose of GM1, a unique feature of AU sialidase.

DISCUSSION

Among bacterial sialidases reported so far, AU sialidase is the sole enzyme that hydrolyzes all linkages of sialic acid found in gangliosides, *i.e.*, NeuAcα2-3Galβ, NeuAcα2-6Gal, NeuAcα2-6GalNAcβ, NeuAcα2-6GlcNAcβ and NeuAcα2-8NeuAcα, particularly being unique in the hydrolysis of the NeuAcα2-3Galβ linkage at the internal galactose residue of gangliotetraose in GM1 and fucosyl GM1 (*6*), whose cleavage was found to be achieved by isoenzymes L, M1 and M2, but not by isoenzyme S of AU sialidase. To determine the molecular bases of the substrate specificities toward gangliosides of the four isoenzymes, determination of the molecular weights, by MALDI-TOFMS, and the N-terminal amino acid sequences, by protein sequencing, of the purified isoenzymes, molecular cloning and expression of the gene, and analysis of the substrate specificities of the recombinant enzymes were performed in this study. The AU sialidase gene (SiaAU) we cloned was identical to that reported previously (*29*), and the open reading frame comprised 2,970 bp, cor-

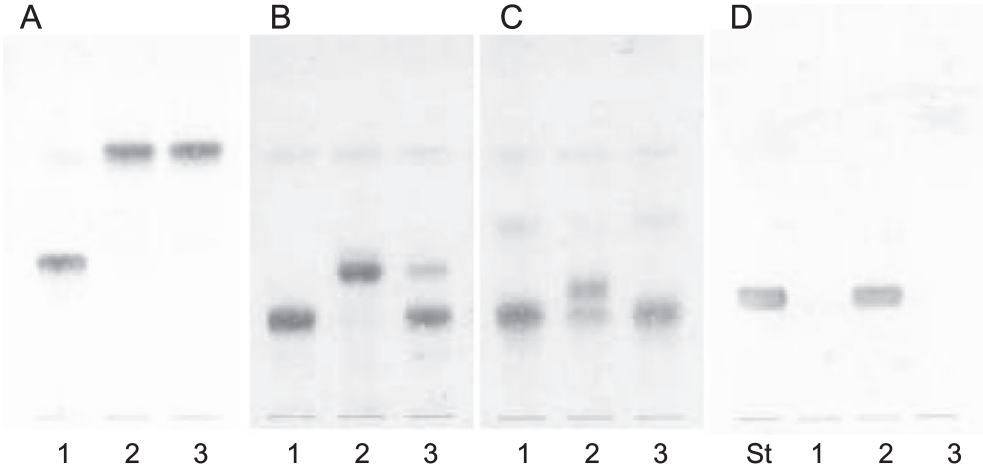

Fig. 6. **TLC of the products derived on hydrolysis of gangliosides GM3 (A), GM1 (B), and fucosyl GM1 (C, D) by recombinant AU sialidases M2 and S.** Gangliosides GM3, GM1 and fucosyl GM1 (5 μg) were hydrolyzed without an enzyme (1), and with 50 milliunits of recombinant AU sialidases M2 (2) and S (3). TLC was performed with chloroform/methanol/0.5% $CaCl_2$ (55:45:10, v/v), and the spots were visualized with orcinol-sulfuric acid reagent (A, B, C), and by immunostaining with monoclonal anti-fucosyl asialo GM1 antibodies (D). St, fucosyl asialo GM1. The products derived on hydrolysis of GM3 (A) and GM1 (B) were LacCer and asialo GM1, respectively.

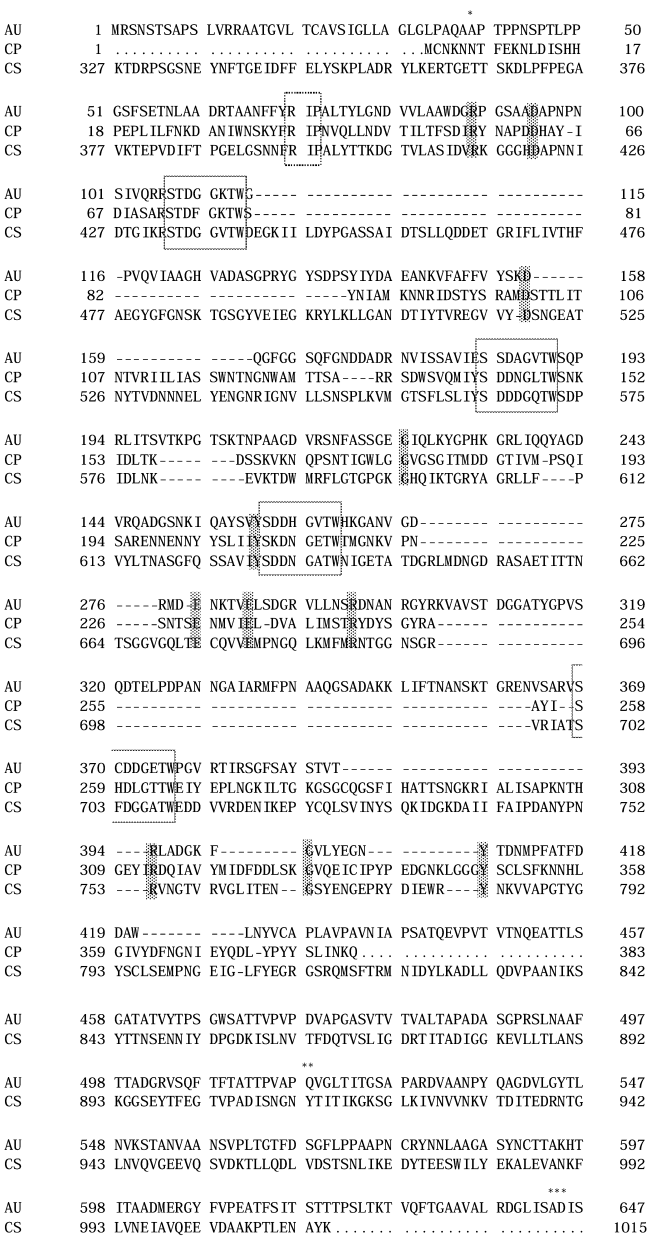

Fig. 7. **Alignment of the amino acid sequences of AU (AU), CP (CP), and *C. septicum* (CS) sialidases.** The FRIP region and the Asp box are boxed with dotted and straight lines, respectively. The shaded amino acids are the conserved ones in bacterial sialidases. *Common N-terminal of the purified AU sialidase isoenzymes; **C-terminal of isoenzyme S; ***C-terminal of isoenzyme M2.

responding to 990 amino acids. Differing from those of other bacterial sialidases reported, the initiation codon was TTG, instead of ATG, and one of the conserved sequences, FRIP, of bacterial sialidases was FYRIP for AU sialidase (*30*). There were four copies of the Asp-box typical of all microbial sialidases in the protein, although the distance between the 3rd and 4th Asp boxes of AU sialidase was greater than that in Clostridium species sialidases (Fig. 7) (*30*). The arginine triad that was demonstrated to form a part of the active site through interaction with the carboxylic acid group of sialic acid was also conserved at Arg^{70} in FYRIP, Arg^{89} between FYRIP and Asp-box 1, and Arg^{361} between Asp-boxes 3 and 4 (*31*). The other conserved amino acids in the sequences of bacterial and protozoan sialidases, which were aligned by Roggentin *et al.*, were observed in the deduced amino acid sequence of AU sialidase, Arg^{89}, Asp^{95}, Val^{154}, Asp^{158}, Gly^{224}, Tyr^{259}, Glu^{279}, Glu^{284}, Arg^{295}, Arg^{394}, Gly^{401} and Tyr^{408} (Fig. 7) (*32*). The enzymes secreted into the culture medium were found to be post-translationally modified at both the N- and C-terminals. Namely, elimination of the N-terminal 38 amino acids including the signal anchor domain was necessary for secretion of the enzymes into the medium, and all isoenzymes, *i.e.*, L, M1, M2 and S, in the medium had an identical N-terminal structure. Therefore, the differences in molecular weight and isoelectric point among the four isoenzymes were found to be due to cleavage at different C-terminal positions. Since the catalytic domain was located on the N-terminal side, the unique substrate specificity toward GM1 of isoenzymes L, M1 and M2, distinct from that of S, was presumed to be due to the additional C-terminal amino acids linked to isoenzyme S.

As reported previously (*6*), the molecular weights (MW) and isolectric points of the isoenzymes were determined, on SDS-PAGE and isoelectric gel electophoresis, to be as follows, 88 kDa and pI 5.0 for L, 66 kDa and pI 6.2 for M1, 66 kDa and pI 5.5 for M2, and 52 kDa and pI 6.2 for S, respectively, but the precise molecular weights, *i.e.*, MW 76,796 for L, MW 64,121 for M1, MW 64,326 for M2 and MW 51,364 for S, determined by MALDI-TOFMS were used for calculation of the numbers of amino acids, that is, amino acids 39 to 773, calculated MW 76,837 and pI 4.97 for L; amino acids 39 to 653, MW 64,157 and pI 5.18 for M1; amino acids 39 to 655, MW 64,343 and pI 5.08 for M2; and amino acids 39 to 528, MW 51,382 and pI 5.02 for S, respectively. The differences in molecular weight and isoelectric point between the calculated and measured ones were thought to be due to the three-dimensional structures in solution. The activities due to amino acids 39–655 for M2 and 39–528 for S were confirmed by preparation of the recombinant enzymes with an expression vector ligated with their DNA segments, since M2 exhibited the highest specific activity toward GM1 among the isoenzymes, and S had lost this activity. As shown in Fig. 6, recombinant enzymes M2 and S exhibited identical substrate specificities toward gangliosides to those of the purified enzymes. Recombinant enzyme M2 cleaved GM1 and fucosyl GM1 at significantly higher rates than S, but the cleavage of GM3 and sialyl lactose, as well as the trans-sialylation of lactose by S readily proceeded compared to in the case of M2. Corfield *et al.* reported that the K_ms of GM1 for CP and AU sialidases were 0.46 mM and 0.08 mM, respectively, when they determined the activity with GM1 under conditions without a detergent, and GM1-oligosaccharide was cleaved by AU sialidase, but not by CP sialidase, indicating a significantly high affinity of AU sialidase toward sialic acid branching at the internal galactose of GM1 (*34*). The enzymatic activity of isoenzyme S resembled that of CP sialidase, and consequently amino acids 259–655 at the C-terminal of M2 were suggested to be involved in the affinity with GM1. On the contrary, the high activity toward GM3, sialyl lactose and colominic acid seen for isoenzyme S was significantly reduced for

isoenzyme M2, due to the addition of the C-terminal peptide.

NMR studies on gangliosides revealed interaction of the amide carbonyl group of GalNAc and the carboxylic acid of the sialic acid in GM1, resulting in line-broadening of the H-3ax signal of the terminal sialic acid (δ 1.913), in comparison to that of the internal sialic acid (δ 1.798), and in the steric hindrance of its rotation (*33*). One can suggest that the C-terminal peptide of M2 prevents the effect of the neighboring GalNAc to permit cleavage of the sialic acid at the internal galactose residue. Since the numbers of amino acids from the 4th Asp box to the C-terminal were 118 amino acids for CP sialidase, 142 amino acids for isoenzyme S, and 279 amino acids for isoenzyme M2, amino acids 529–655 of the C-terminal peptide of M2, which is longer than that of S, are responsible for the high affinity with GM1. Replacement of the C-terminal peptide of CP sialidase with that of isoenzyme M2 should provide a clue as to the unique substrate specificity toward GM1 and fucosyl GM1 of AU sialidase, and work along these lines is in progress in our laboratory.

The nucleotide sequences reported in this paper have been submitted to GenBank/NCBI under accession number AB193297.

REFERENCES

1. IUPAC-IUB Commission on Biochemical Nomenclature (1977) The nomenclature of lipids. Recommendations (1976) *Lipids* **12**, 455–468
2. Svennerholm, L. (1963) Chromatographic separation of human brain gangliosides. *J. Neurochem.* **10**, 613–623
3. Schauer, R. and Vliegenthart, J.G. (1982) in *Sialic Acids: Chemistry*, *Metabolism and Functions* (Schauer, R., ed.) pp. 1–3, Springer-Verlag, Vienna
4. Saito, M. and Yu, R.K. (1995) in *Biology of Sialic Acids: Biochemistry and Function of Sialidases* (Rosenberg, A., ed.) pp. 261–313, Plenum Press, New York
5. Corfield, T. (1992) Bacterial sialidases—roles in pathogenicity and nutrition. *Glycobiology* **2**, 509–521
6. Iwamori, M., Ohta, Y., Uchida, Y., and Tsukada, Y. (1997) *Arthrobacter ureafaciens* sialidase isoenzymes, L, M1 and M2, cleave fucosyl GM1. *Glycoconj. J.* **14**, 67–73
7. Chou, M.Y., Li, S.C., Kiso, M., Hasegawa, A., and Li, Y.T. (1994) Purification and characterization of sialidase L, a NeuAc α 2→3Gal-specific sialidase. *J. Biol. Chem.* **269**, 18821–18826
8. Iwamori, M., Sunada, S., Ishihara, E., Moki, M., Fujimoto, S., and Nagai, Y. (1986) Differential expression of fucosyl GM1 and a disialoganglioside with a NeuAc α 2-6GalNAc linkage (GD1ε) in various rat ascites hepatoma cells. *FEBS Lett.* **198**, 66–70
9. Sillerud, L.O., Yu, R.K., and Schafer, D.E. (1982) Assignment of the carbon-13 nuclear magnetic resonance spectra of gangliosides GM4, GM3, GM2, GM1, GD1α, GD1β, and GT1β. *Biochemistry* **21**, 1260–1271
10. Uchida, Y., Tsukada, Y., and Sugimori, T. (1974) Production of microbial neuraminidases induced by colominic acid. *Biochim. Biophys. Acta* **350**, 425–431
11. Uchida, Y., Tsukada, Y., and Sugimori, T. (1977) Distribution of neuraminidase in *Arthrobacter* and its purification by affinity chromatography. *J. Biochem.* **82**, 1425–1433
12. Sugano, K., Saito, M., and Nagai, Y. (1978) Susceptibility of ganglioside GM1 to a new bacterial neuraminidase. *FEBS Lett.* **89**, 321–325
13. Saito, M., Sugano, K., and Nagai, Y. (1979) Action of *Arthrobacter ureafaciens* sialidase on sialoglycolipid substrates. Mode of action and highly specific recognition of the oligosaccharide moiety of ganglioside GM1. *J. Biol. Chem.* **254**, 7845–7854
14. Ohta, Y., Tsukada, Y., and Sugimori, T. (1989) Purification and properties of neuraminidase isozymes in *Arthrobacter ureafaciens* mutant. *J. Biochem.* **106**, 1086–1089
15. Uchida, Y., Tsukada, Y., and Sugimori, T. (1985) Distribution of N-acylneuramininate lyase in bacteria and its production by *Escherichia coli*. *Agric. Biol. Chem.* **49**, 181–187
16. Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254
17. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685
18. Wilm, M. (2000) Mass spectrometric analysis of proteins. *Adv. Protein Chem.* **54**, 1–30
19. Marmur, J. (1961) A procedure for the isolation of desoxyribonucleic acid from microorganisms. *J. Mol. Biol.* **3**, 208–218
20. Gebers, R., Wehmeyer, U., Roggentin, T., Schlesner, H., Kölbel-Boelke, J., and Hirsch, P. (1985) Desoxyribonucleic acid base compositions and nucleotide distributions of 65 strains of budding bacteria. *Int. J. Syst. Bact.* **35**, 260–269
21. Sambrook, J. and Russell, D.W. (2001) *Molecular cloning. A Laboratory Manual*, 3rd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York
22. Wallace, R B., Johnson, M.J., Hirose, T., Miyake, T., Kawashima, E.H., and Itakura, K. (1981) The use of synthetic oligonucleotides as hybridization probes. II. Hybridization of oligonucleotides of mixed sequence to rabbit beta-globin DNA. *Nucleic Acids Res.* **9**, 879–894
23. Roggentin, P., Schauer, R., Hoyer, L.L., and Vimr, E.R. (1993) The sialidase superfamily and its spread by horizontal gene transfer. *Mol. Microbiol.* **9**, 915–921
24. Hawley, D.K. and McClure, W.R. (1983) Compilation and analysis of *Escherichia coli* promoter DNA sequences. *Nucleic Acids Res.* **11**, 2237–2255
25. Siebenlist, U., Simpson, R.B., and Gilbert, W. (1980) *E. coli* RNA polymerase interacts homologously with two different promoters. *Cell* **20**, 269–281
26. Reese, M.G. (2001) Application of a time-delay neural network to promoter annotation in the *Drosophila melanogaster* genome. *Comput. Chem.* **26**, 51–56
27. Henningsen, M., Roggentin, P., and Schauer, R. (1991) Cloning, sequencing and expression of the sialidase gene from *Actinomyces viscosus* DSM 43798. *Biol. Chem. Hoppe Seyler*. **372**, 1065–1072
28. Kyte, J. and Doolittle, R.F. (1982) A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* **157**, 105–132
29. Christensen, S. and Egebjerg, J. (2004) Cloning, expression and characterization of a sialidase gene from *Arthrobacter ureafaciens*. *Biotechnol. Appl. Biochem.* BA20010144
30. Grobe, K., Sartori, B., Traving, C., Schauer, R., and Roggentin, (1998) Enzymatic and molecular properties of the *Clostridium tertium* sialidase. *J. Biochem.* **124**, 1101–1110
31. Crennell, S.J., Garman, E.F., Laver, W.G., Vimr, E.R., and Taylor, G.L. (1993) Crystal structure of a bacterial sialidase (from *Salmonella typhimurium* LT2) shows the same fold as an influenza virus neuraminidase. *Proc. Natl Acad. Sci. USA* **90**, 9852–9856
32. Roggentin, P., Schauer, R., Hoyer, L.L., and Vimr, E.R. (1993) The sialidase superfamily and its spread by horizontal gene transfer. *Mol. Microbiol.* **9**, 915–921
33. Sillerud, L.O., Yu, R.K., and Schafer, D.E. (1982) Assignment of the carbon-13 nuclear magnetic resonance spectra of gangliosides GM4, GM3, GM2, GM1, GD1a, GD1b, and GT1b. *Biochemistry* **21**, 1260–1271
34. Corfield, A.P., Schauer, R., Dorland, L., Vliegenthart, J.F., and Wiegandt, H. (1985) Studies on the interaction of *Clostridium perfringens* sialidase with sialic acid linked to the internal galactose in monosialogangliotetraosyl ceramide. *J. Biochem.* **97**, 449–461