Purification and Characterization of a Membrane-Associated Ganglioside Sialidase from Bovine Brain[1]

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A membrane-associated ganglioside-hydrolyzing sialidase was purified to apparent homogeneity from bovine brain. The enzyme was solubilized with Triton X-100 plus sodium cholate from the particulate fraction and purified over 100,000-fold by sequential chromatography on DEAE-cellulose, octyl-Sepharose, heparin-Sepharose, Sephacryl S-200, MonoQ, RCA-agarose, thiol-activated Sepharose, and ganglioside-affinity Sepharose. The final enzyme preparation exhibited a specific activity of 4,851.3 μmol/h/mg protein and an apparent molecular mass of 52 kDa on SDS-polyacrylamide gel electrophoresis. The enzyme preferentially hydrolyzed gangliosides other than GM1 and GM2 but demonstrated hardly any activity against glycoproteins and oligosaccharides. Gangliosides GD3, GD1a, and GT1b were much better substrates than GM3 and GD1b in the presence of Triton X-100, but the latter became more sensitive to the sialidase with addition of sodium cholate. The enzyme was activated by dithiothreitol, strongly inhibited by 4-hydroxy-mercuribenzoate, and firmly adsorbed to thiol-activated Sepharose, indicating that free sulfhydryl groups are essential for its catalytic activity. Subcellular fractionation experiments revealed that the enzyme is mainly located in the synaptosomal fraction.

Key words: bovine brain, gangliosides, sialic acid, sialidase, synaptosome.

Although the removal of sialic acid residues from glycoproteins and glycolipids has been observed to exert a great influence on the biological function of sialo-glycoconjugates (1–3), the mechanisms of physiological regulation of desialylation remain obscure, since the properties, structure, and functions of sialidases from mammalian sources are not yet fully understood. We previously demonstrated four types of sialidase in rat tissues, differing in subcellular location and in catalytic and immunological properties. They are intralysosomal (4), cytosolic (5), and two membrane-associated sialidases (6). Membrane sialidase I is mainly located in the plasma membrane, and sialidase II in lysosomal membranes. We have purified the cytosolic sialidase (5), cloned its cDNA (7), and discovered its essential role in skeletal muscle cell differentiation (8). To obtain further information on the molecular biology of mammalian sialidases, our recent studies have focused on the plasma membrane sialidase.

We previously found that a partially purified rat brain enzyme exhibits a substrate specificity distinct from that of the lysosomal membrane sialidase, *i.e.*, that it is incapable of hydrolyzing glycoproteins and oligosaccharides (6). Kopitz *et al.* (9, 10) also reported that the sialidases of human neuroblastoma cells and of human brain desialylate gangliosides selectively. Ganglioside sialidases have been suggested to be involved in the regulation of important physiological phenomena including cell differentiation and cell growth by modifying membrane gangliosides, although their identity remains to be determined. We have extensively purified and characterized the ganglioside sialidase from bovine brain particulates to analyze its properties precisely and obtain the information necessary to clone a cDNA for elucidation of its structure, function, and mechanism of expression. No membrane-associated mammalian sialidase has hitherto been purified to homogeneity, except that from rabbit erythrocyte membranes (11), whose properties are quite different from the bovine enzyme described here. The major intracellular location of the sialidase purified here was found to be the synaptosomes, and its specific activity was determined to be approximately 1,600 times higher than that of the erythrocyte enzyme.

MATERIALS AND METHODS

Materials—Bovine brain mixed gangliosides (type II) were purchased from Sigma (St. Louis, MO). GM3 and GD3 from bovine milk were products of Snow Brand (Tokyo). GM1, GM2, GD1a, GD1b, and GT1b from bovine brain were obtained from Wako (Osaka). Their sialic acid residues were all of *N*-acetylneuraminic acid type. GM3

containing *N*-glycolyl neuraminic acid was a generous gift from Dr. A. Makita, then of Hokkaido University, Sapporo. Triton X-100 was purchased from Sigma, and other detergents were from Calbiochem (La Jolla, CA). DEAE-cellulose (DE-52) was from Whatman (Kent, UK), and octyl-Sepharose, heparin-Sepharose, MonoQ, Sephacryl S-200, activated-thiol Sepharose, and ECH-Sepharose were from Pharmacia (Uppsala, Sweden). RCA lectin (RCA-120) agarose was purchased from Seikagaku Kogyo (Tokyo). Synthetic GM3 analogs GSC-211 [NeuAcα2→3Galβ1→4Glcβ1-0 (CH$_2$)$_8$NH$_2$], GSC-17 (NeuAcα2→3Galβ1→4Glcβ1→1ceramide), and GSC-61 (NeuAcα2→6Galβ1→4Glcβ1→1ceramide) were synthesized according to Hasegawa *et al.* (*12*), and GSC-211 was coupled with ECH-Sepharose by *N*-ethyl-*N'*-(3'-dimethyl-aminopropyl)carbodiimide hydrochloride according to the manufacturer's protocol. The amount of GSC-211 bound to the gel was 1.2 mg/ml of gel. The sources of the other materials are described elsewhere (*5, 6*).

Sialidase Assay—The standard assay mixture was composed of substrates containing 50–100 nmol of bound sialic acid, 0.2 mg of bovine serum albumin, 15 μmol of sodium acetate (pH 4.6), 0.2 mg of Triton X-100, and enzyme in 0.2 ml. Bovine mixed gangliosides were routinely used as the substrate. After incubation at 37°C for 15–60 min, the reaction was terminated by immediate freezing, and the sialic acid released was determined by Warren's thiobarbituric acid method (*13*). Absorbance was measured at 532 and 549 nm. When crude enzyme preparations were assayed, for example, after step 1 or 2 described below, the reaction mixture was passed through an AG1X-2 mini column prior to the color reaction. The desialylated products from ganglioside substrates were identified by thin layer chromatography. In brief, the reaction products were lyophilized, dissolved in chloroform/methanol (C/M, 2:1 v/v), and chromatographed on a high-performance thin layer chromatographic (HPTLC) plate (Baker, NJ) in C/M/0.5% CaCl$_2$ (60:40:9 v/v/v). Glycolipids were visualized with orcinol-H$_2$SO$_4$ and resorcinol-HCl reagents. Sialidase activity towards 4-methylumbelliferyl-α-*N*-acetyl-D-neuraminic acid (4MU-Neu5Ac) was determined by spectrofluorometric measurement of 4-methyl-umbelliferone (4MU) released (*5*). One unit of sialidase was defined as the amount of enzyme which catalyzed the release of 1 nmol of sialic acid/h.

Other Assays—β-*N*-Acetylgalactosaminidase, β-galactosidase, acid phosphatase, and ouabain-sensitive (Na$^+$, K$^+$) ATPase were assayed as described previously (*6*). Protein was determined by either a dye-binding assay (Bio-Rad) or the BCA assay (Pierce Chemical).

Immunoprecipitation Study—Antiserum against rat membrane sialidase I was prepared as described previously (*6*) by immunizing rabbits with partially purified sialidase I. Immunoprecipitation was conducted as described previously (*6*). Briefly, the antiserum was immobilized to Protein A Sepharose (Pharmacia) and incubated with sialidase at 37°C for 30 min. After centrifugation, the resulting supernatant was assayed for enzyme activity. When crude particulate fractions were tested, they were solubilized prior to immunoprecipitation.

Subcellular Fractionation—Bovine brain subfractions were prepared by discontinuous sucrose density gradient centrifugation according to Whittaker *et al.* (*14*): the crude mitochondrial fraction (P$_2$) was further separated by discontinuous sucrose density gradient centrifugation into fractions P$_2$A having myelin fragments, P$_2$B synaptosomes, and P$_2$C mitochondria.

Buffers—The following buffers were used for the purification: buffer A, 20 mM potassium phosphate, pH 6.8, 0.1% (w/v) Triton X-100, 1 mM EDTA, 1 mM dithiothreitol (DTT), and 0.1 mM phenylmethylsulfonylfluoride (PMSF); buffer B, 20 mM potassium phosphate, pH 6.8, 0.04% (w/v) Triton X-100, 1 mM DTT, 1 mM EDTA, and 0.02 mM 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid (Neu5Ac2en); buffer C, 20 mM potassium phosphate, pH 6.8, 0.02% (w/v) Triton X-100, 1 mM EDTA, 1 mM DTT, and 0.02 mM Neu5Ac2en; buffer D, 20 mM potassium phosphate, pH 6.8, 1 mM EDTA, 1 mM DTT, 0.02 mM Neu5Ac2en; buffer E, 20 mM potassium phosphate, pH 6.8, 0.1% (w/v) Triton X-100, 1 mM EDTA, 1 mM DTT, 0.02 mM Neu5Ac2en, and 10% glycerol; buffer F, 10 mM potassium phosphate, pH 6.8, 0.04% Triton X-100, 1 mM EDTA, 1 mM DTT, and 20% glycerol.

Solubilization and Purification of Membrane-Associated Sialidase—All procedures were carried out at 4°C. Adult bovine brains were obtained from a slaughterhouse, put on ice immediately, and stored at −80°C until used.

Steps 1 and 2: Portions of 200 g of stored brains were homogenized in 9 volumes of 0.32 M sucrose containing 1 mM EDTA, 0.1 mM PMSF, and 1 mM DTT using a glass-Teflon homogenizer and centrifuged at 1,000×g for 10 min. The supernatant was centrifuged at 20,000×g. The resulting pellet (P2, step 1) was homogenized in a mixture of 180 ml of buffer A and 20 ml of 5% (w/v) sodium deoxycholate in a glass-Teflon homogenizer, and centrifuged at 100,000×g for 1 h (step 2).

Step 3. DE-52 chromatography: The supernatant obtained above was applied to a DEAE-cellulose column (4.5×20 cm) equilibrated with buffer A. After washing the column with buffer A (400 ml), the enzyme was eluted with buffer A containing 0.2 M NaCl, and the effluent was collected in fractions of 15 ml. The active fractions were pooled and dialyzed against 2 liters of buffer A for 2 h.

Step 4. Octyl-Sepharose chromatography: The dialyzed solution was applied to an octyl-Sepharose column (2.5×7 cm) equilibrated with buffer A. The column was washed with buffer A (100 ml) and developed with a linear gradient of Triton X-100, 0.1–0.4%, in 400 ml of buffer A, and the effluent was fractionated into 10-ml portions.

Step 5. Heparin-Sepharose chromatography: The active fractions from the octyl-Sepharose column were subjected to chromatography on heparin-Sepharose (1.5×1 cm) equilibrated with buffer A. After washing the column with

TABLE I. **Sialidase activity in subfractions of the crude mitochondrial pellet (P$_2$).**

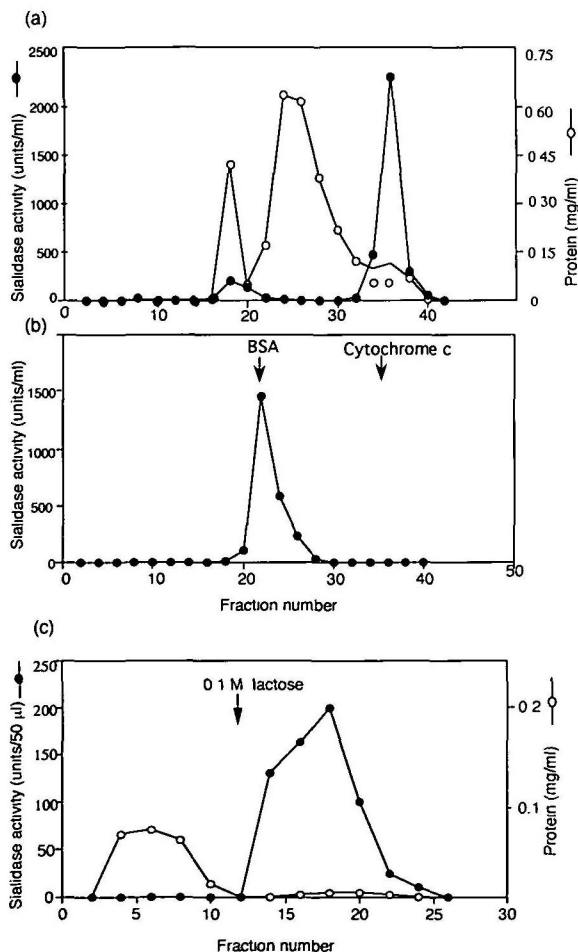|	Percent of the P$_2$ activity			Sialidase specific activity (units/mg)	Activity precipitated by anti-I sialidase[a] (%)
	Acid phosphatase	Ouabain-sensitive ATPase (%)	Ganglioside sialidase		
P$_2$A	21	23	18	19.7	40
P$_2$B	31	53	62	58.6	85
P$_2$C	48	24	20	23.4	21

[a]Solubilized particulates were assayed for ganglioside sialidase activity after immunoprecipitation with antiserum against rat membrane sialidase I.

buffer A containing 0.25 M NaCl, the enzyme was eluted with a linear gradient of 0.25–1.0 M NaCl in 200 ml of buffer A. The active fractions from three heparin-Sepharose columns (600 g of bovine brain as starting material) were pooled, diluted with three volumes of buffer A, then applied to a heparin-Sepharose column (0.8 × 1 cm) equilibrated with buffer A containing 0.2 M NaCl. The enzyme was eluted with buffer A containing 1 M NaCl and concentrated to a volume of 3–4 ml using an Amicon YM-10 membrane.
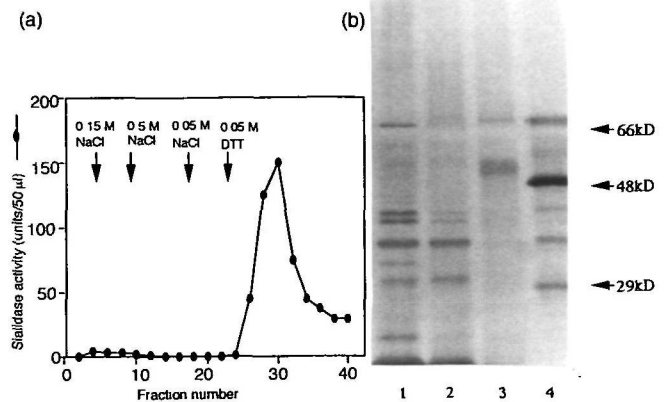
Step 6. Sephacryl S-200 chromatography: The concentrated solution was applied to a Sephacryl S-200 column (1.5 × 43 cm) equilibrated with buffer B. The enzyme was eluted with the same buffer, and 2-ml fractions were collected at a flow rate of 10 ml/h.

Step 7. RCA-lectin chromatography: A RCA-agarose column (1.5 × 2.5 cm) was equilibrated with buffer C. An equal volume of buffer D was added to the active fractions from step 6 (6–8 ml) to reduce the concentration of Triton X-100. The enzyme solution was then applied to the
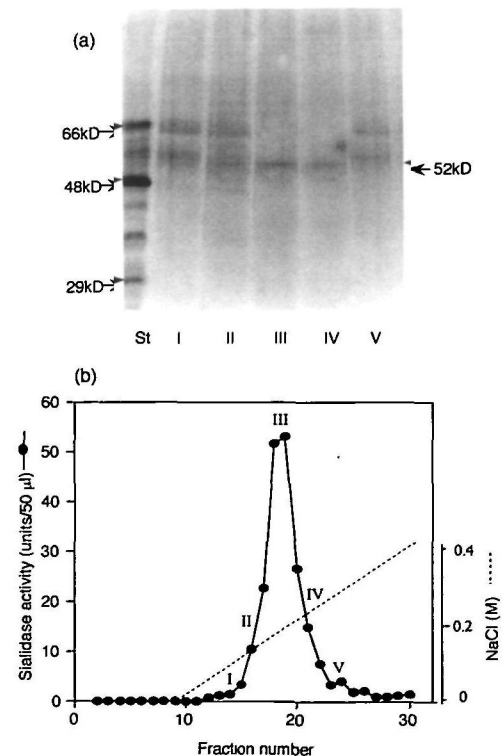
column, the column was washed with 50 ml of buffer C, and the enzyme was eluted with 100 ml of buffer C containing 0.2 M lactose.

Fig. 2. **Elution profile (a) and SDS-PAGE (b) of the ganglioside sialidase from thiol-activated Sepharose column chromatography.** The step 7 enzyme was chromatographed on thiol-activated Sepharose column and aliquots (500 μl) of the fractions eluted with 0.15 M NaCl, 0.5 M NaCl, and DTT, respectively, were dialyzed, lyophilized, and applied to lanes 1, 2, and 3 in a 10% acrylamide gel. Lane 4 is molecular mass markers. The sialidase activity was assayed with mixed gangliosides as the substrate. Protein bands were detected by silver staining.

Fig. 1. **Elution profiles of the ganglioside sialidase on Sephacryl S-200 (a, b) and RCA-lectin agarose (c) column chromatographies.** The enzyme from step 5 was chromatographed under low ionic strength (a) or in the presence of 0.15 M NaCl (b) as described in the text. BSA (bovine serum albumin) and cytochrome *c* were applied as standards. The enzyme from step 6 (fractions 34–37) was applied to a RCA-lectin column (c) and the sialidase activity was assayed with mixed gangliosides as the substrate.

Fig. 3. **SDS-PAGE (a) of the purified sialidase obtained on GM3-analogue affinity chromatography (b).** The fractions (I–V) from the affinity column were applied to the lanes indicated in the 10% acrylamide gel. The sialidase activity was assayed with mixed gangliosides as the substrate and the protein bands on the gel were detected by silver staining. St, molecular mass markers; I, fractions 14 and 15 from the affinity column; II, 16 and 17; III, 18 and 19; IV, 20 and 21; and V, 22 and 23.

TABLE II. Purification of ganglioside sialidase from brain particulate. Sialidase activity was assayed with mixed gangliosides as the substrate in the presence of Triton X-100.

Steps	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification (-fold)	Yield (%)
Particulate	36,622	1,666,327	45.5	1	100
Solubilized particulate	23,057	1,616,337	70.1	1.5	97
DE-52	13,440	1,051,740	78.3	1.7	63
Octyl-Sepharose	1,581	486,000	307	6.7	29
Heparin-Sepharose	112	245,520	2,188	48	15
Sephacryl S-200	5.37	53,322	9,930	218	3.2
RCA-agarose	1.38	43,325	31,394	690	2.6
MonoQ	0.385	19,996	51,996	1,143	1.2
Thiol-Sepharose	0.0103	18,660	1,811,650	39,816	1.1
Ganglioside-affinity	0.0012	5,773	4,851,260	106,621	0.34

TABLE III. Substrate specificity of bovine brain membrane sialidase. Sialidase from step 10 was assayed using substrates at the concentrations of 30-50 nmol of bound sialic acid per assay mixture in the presence of 0.05% of Triton X-100 or sodium cholate. The activities were expressed as the percentage of sialic acid released relative to the desialylation of GD3. Sialic acids released from GD3 were 25.0 and 29.5 nmol for Triton X-100 and sodium cholate, respectively.

Substrate	Relative hydrolysis rate (%)	
	Triton X-100	Sodium cholate
Gangliosides:		
GD3	100	100
GD1a	57	59
GD1b	16	53
GT1b	67	50
GM3	25	92
GM3 (*N*-glycolyl)	11	43
GM2	3	4
GM1	0	1
Mixed gangliosides	70	68
Synthetic GM3:		
GSC-17(α2-3)	ND	99
GSC-61(α2-6)	ND	39
Sialyllactose	9	7
4-MUNeuAc	15	13
Orosomucoid	2	1
Fetuin	2	2
Glycophorin	3	2
Ovine submaxillary mucin	3	0
Bovine submaxillary mucin	0	0

ND, not determined.

TABLE IV. Kinetic constants of membrane sialidase. Step 9 enzyme was used under the standard conditions with 0.05% of Triton X-100 or sodium cholate.

Substrates	Triton X-100		Sodium cholate	
	K_m (μM)	V_{max} (nmol/h)	K_m (μM)	V_{max} (nmol/h)
GD3	219	49.7	131	59.6
GD1a	554	24.2	277	34.5
GD1b	985	6.2	326	28.9
GM3	457	9.7	142	54.2
Sialyllactose	6,250	45.2	6,250	47.0
4MU-Neu5Ac	265	10.4	282	8.9

same buffer containing 0.5 M NaCl.

Step 10. GM3 analog (GSC-211)-affinity chromatography: The concentrated enzyme was dialyzed against buffer F, then applied to a column (0.7 × 3 cm) equilibrated with the same buffer. The column was allowed to stand for 2 h and the flow-through fractions were applied again to the column. After washing the column with 20 ml of buffer F, the enzyme was eluted with a 0-0.5 M NaCl gradient in 40 ml of buffer F, and 1.5-ml fractions were collected. Active fractions were stored at −20°C until use.

Molecular Weight Determination—A Sephacryl S-200 column (1.5 × 43 cm) was operated as described for step 6 except for use of buffer A containing 0.15 M NaCl. Catalase (M_r 240,000), aldolase (150,000), bovine serum albumin (67,000), and cytochrome c (12,400) were used for calibration. The molecular weight was also determined by sucrose density gradient centrifugation as described previously (5) and by SDS-polyacrylamide gel electrophoresis according to Laemmli (15).

RESULTS

Purification of Ganglioside Sialidase—To search for an appropriate tissue for extensive purification of membrane ganglioside sialidase, particulate fractions of several animal tissues were assayed for sialidase activity. Ganglioside sialidase activities in bovine, porcine, rabbit, rat, and mouse brains were 580, 42, 144, 57, and 67 units/g tissue, respectively. When the cross-reactivity with rat membrane sialidase I, whose major localization was previously demonstrated to be synaptosomes, was examined by immunoprecipitation, 79% of the ganglioside sialidase in bovine brain particulates was found to be immunoprecipitated with the antiboby, suggesting that the major ganglioside sialidase of this tissue is similar or identical to rat membrane sialidase

Step 8. MonoQ chromatography: Step 7 enzyme was subjected to MonoQ (HR5/5) anion exchange FPLC. The column was equilibrated with buffer E and developed with a linear 0-0.5 M NaCl gradient in 20 ml buffer E, and 0.5-ml fractions were collected. The active fractions were stored at −20°C until the enzymes from the three MonoQ columns were pooled.

Step 9. Activated thiol-Sepharose chromatography: The pooled step 8 enzyme was dialyzed against buffer E containing 0.15 M NaCl for 2.5 h, then loaded at a flow rate of 8 ml/h on an activated thiol-Sepharose column (1.5 × 2 cm) that had been equilibrated with buffer E containing 0.15 M NaCl. The column was washed sequentially with 50 ml each of the same buffer, buffer E containing 0.5 M NaCl, and buffer E containig 0.05 M NaCl. The enzyme was then eluted with 100 ml of buffer E containing 0.05 M NaCl and 50 mM DTT. The active fractions were concentrated immediately by adsorption onto a MonoQ column equilibrated with buffer E containing 0.05 M NaCl, then eluted with the

I. Bovine brain was thus used as the enzyme source for the purification.

To confirm that the bovine sialidase is located in the synaptosomes, subcellular fractions of bovine brain homogenate were prepared and assayed for sialidase activity as shown in Table I. The homogenates were fractionated by differential centrifugation into the crude nuclear pellet (P_1), crude mitochondrial pellet (P_2), and microsomes (P_3), then P_2 was subfractionated into P_2A, P_2B, and P_2C by sucrose density gradient centrifugation. With mixed gangliosides as the substrate, more than 70% of the initial sialidase activity was recovered in P_2, and the highest activity both in distribution and specific activity was associated with P_2B, which was judged to be synaptosome fraction on the basis of enrichment of the ouabain-sensitive (Na^+, K^+) ATPase. In addition, 85% of the activity in the P_2B fraction was immunoprecipitated with anti-membrane sialidase I. The P_2B fraction was then isolated from 200 g of bovine brain and used as starting material for the sialidase purification. Ganglioside sialidase in the fraction was solubilized and purified by the procedures of steps 1-8 as outlined in the "MATERIALS AND METHODS." However, much larger amount of starting material was found to be needed for the complete purification. The P_2 fraction showed essentially identical activity profiles on column chromatographies to those of the P_2B fraction. We therefore decided to use P_2 instead of the P_2B fraction to improve purification efficiency.

The procedures for steps 1-4 were similar to those employed for the rat enzyme, and the following steps 5-9 were added for further purification. To maintain the enzyme in a soluble state and to minimize non-specific adsorption, a low concentration of Triton X-100 (0.02-0.1%) was included in all solutions used for column chromatography. NeuAc2en (0.02 mM), a known sialidase inhibitor, DTT (1 mM), and glycerol were also added at later chromatography steps, since they were found to stabilize the enzyme. When the sialidase was applied to a Sephacryl S-200 column in the absence of NaCl, the enzyme unexpectedly emerged from the column mainly at about a position corresponding to that of cytochrome *c*, a reference protein of 12.4 kDa, with a small amount of the activity in the void volume and over 95% of the protein contaminants eluted prior to the sialidase (Fig. 1a). In the presence of NaCl, however, the sialidase was eluted much earlier at the position for molecular mass 65 kDa (Fig. 1b). The change of elution positions was reversible by adding or removing NaCl, and the condition without NaCl was employed for the purification. Almost all the sialidase activity in the fractions obtained by the gel filtration chromatography bound to a RCA$_{180}$ lectin column (Fig. 1c), suggesting that the enzyme is a glycoprotein having nonreducing galactose residues on its sugar chains. The enzyme concentrated by a MonoQ column was further purified by activated thiol-Sepharose chromatography. As shown in Fig. 2, low molecular weight contaminants were removed by the column, and the SDS-PAGE pattern of the eluate with DTT showed two diffuse, main bands of 50-52 kDa and a faint band of 66 kDa. We then used GM3 analog-immobilized affinity chromatography with a gradient elution of increasing NaCl concentrations, resulting in removal of the 66 kDa band (Fig. 3). The enzyme thus obtained gave a band corresponding to a molecular mass of 52 kDa together with other faint bands on SDS-PAGE. The elution profile of the 52-kDa band coincided with the distribution of sialidase activity, which was confirmed by densitometric measurement of the intensity of the bands. Furthermore, the band was concentrated by this affinity chromatography, whereas the intensity of other bands was decreased. We therefore conclude that the 52 kDa band is the sialidase. The enzyme did not enter polyacrylamide gels under non-denaturing conditions even in the presence of Triton X-100, and neither was it able to be reconstituted from SDS-polyacrylamide gels even under mild conditions. The purification was repeated three times and the results were reproducible. The purification procedure with 3.5 kg of bovine brain as starting material is summarized in Table II. Overall, the sialidase was purified over 100,000-fold from bovine brain particulates with a yield of 0.34%. β-Galactosidase and β-hexosaminidase activities were not detectable in enzyme fractions after step 7. The enzyme was stable at $-20°C$ in the presence of 10% glycerol, 1 mM DTT, and 0.2 mM Neu5Ac2en for at least 2 months without a significant loss of activity.

Characterization of Ganglioside Sialidase—The molecular weight of purified sialidase was determined by three different methods. As described above, gel filtration on Sephacryl S-200 and SDS-PAGE gave values of 65,000 and 52,000, respectively. On sucrose density gradient centrifugation, the sialidase sedimented with an $s_{20,w}$ value of 4.5S, corresponding to an apparent molecular weight of 63,000. The pH optimum of the purified sialidase activity was 4.6-4.8 in either sodium acetate buffer or phosphate-citrate buffer. The enzyme activity after step 4 was reduced by 80% when bovine serum albumin was removed from the assay mixture. Triton X-100, sodium cholate, and sodium deoxycholate maximally activated the sialidase at the concentration of 0.05% (w/v), while sodium deoxytaurocholate and sodium taurocholate showed less effects even at higher concentrations. The divalent cations Ca^{2+}, Mn^{2+}, and Mg^{2+} had no effect on the activity at a 1 mM concentration, but Cu^{2+} and Hg^{2+} demonstrated complete inhibition. K^+ and Na^+ did not affect the activity even at 0.5 M in the assay system. A thiol-modifying reagent, 4-hydroxymercuribenzoate, caused a total loss of activity at a 5 μM concentration, while DTT activated the sialidase to the maximum (140%) at 2 mM, indicating that sulfhydryl groups are essential for the catalytic activity.

Table III summarizes the activities of the purified sialidase toward various substrates relative to that toward GD3 in the presence of Triton X-100 or sodium cholate. The sialidase preferentially hydrolyzed gangliosides other than GM1 and GM2 but acted poorly on sialyllactose and 4MU-Neu5Ac. This tendency was particularly clear in the presence of sodium cholate. None of the glycoproteins tested could serve as substrate for the enzyme. GM3 containing *N*-glycolyl-neuraminic acid was hydrolyzed at a lower rate than that containing Neu5Ac. The rate of hydrolysis of a synthetic GM3 analog (GSC-61) having $\alpha 2\rightarrow6$ sialyllinkage was less than half that for GM3 itself. The internal sialic acids of GM1 and GM2 were resistant to the action of this sialidase, and GD1b was also a poor substrate as compared to GD1a and GT1b, probably because both the sialic acids are present at internal sites. The end products from GD1a, GD1b, and GT1b were demonstrated to be GM1 by HPTLC (data not shown).

When the kinetic properties of the sialidase were compared using GM3, sialyllactose, and 4MU-Neu5Ac as substrates (see Table IV), the K_m for sialyllactose (6.25 mM) was found to be 13 and 44 times higher than those for GM3 with Triton X-100 and with sodium cholate, respectively, although the V_{max} values for sialyllactose and GM3 with sodium cholate were almost same, indicating that GM3 was hydrolyzed much more preferentially than sialyllactose. The V_{max} for 4MU-Neu5Ac was only one-fifth to one-seventh of that for GM3, while the K_m values were similar for both. Altogether these data indicate that the purified sialidase desialylates the terminal sialic acids of gangliosides in a specific manner.

The hydrolysis rates for ganglioside substrates with Triton X-100 differed from those with sodium cholate: GD3, GD1a, and GT1b were much better substrates than GM3 and GD1b in the presence of Triton X-100, while the latter were hydrolyzed almost as well as GD3 and GD1a after addition of sodium cholate. Sodium cholate generally reduced the K_m values and increased the V_{max} (Table IV). When the step 6 enzyme was used to examine the substrate specificity, it hydrolyzed GM3 equally effectively with either Triton X-100 or sodium cholate. Addition of step 6 enzyme fraction to step 9 enzyme did not enhance the hydrolysis rate of these gangliosides over the sum of the two enzyme fractions (data not shown). After prolonged storage (more than 3 weeks) at 4°C, the step 6 enzyme was found to become less active against GM3 and GD1b (71 and 80% decrease, respectively, from the initial hydrolysis rates after 2 months) on being assayed with Triton X-100. The activity was restored by addition of sodium cholate, whereas the hydrolysis rate for GD3 and GD1a hardly changed.

DISCUSSION

Through our experience of partial purification of rat membrane sialidase I, we found that large amounts of the enzyme source are needed for extensive purification. Assays of ganglioside-hydrolyzing sialidases of various mammalian tissues and their immunoprecipitation revealed bovine brain to be an appropriate source for membrane sialidase I. Although bovine brain had been utilized for the studies of ganglioside sialidase (*16-19*), the properties of its major sialidase are not fully understood. Therefore, we purified and characterized a ganglioside sialidase from the particulate fraction of bovine brain.

The low apparent molecular weight of this sialidase on gel filtration at low ionic strength on Sephacryl S-200 columns allowed removal of a large proportion of contaminating proteins. Although the underlying molecular mechanism is unclear, this phenomenon appears to be due to a few negatively charged groups on the crosslinked dextrans. The discrepancy among molecular weight values demonstrated by gel filtration, sucrose density gradient, and SDS-polyacrylamide gel electrophoresis might be due to the hydrophobic and glycoprotein nature of the enzyme, which has been shown to possess galactose residues at non-reducing ends by its binding to RCA-lectin (*20*). Our studies on the subcellular fractionation, shown in Table I, indicate that the majority of this sialidase is present in the synaptosomal fraction. This is consistent with the observations of Schengrund and Rosenberg (*17*) and is supported by our previous

finding (*6*) that rat membrane sialidase I cross-reactive with this bovine sialidase is located in plasma membranes. The fact that the sialidase is sensitive to sulfhydryl reagents and is activated by DTT indicates a requirement for cysteine residues for its enzymatic action.

The experiments on substrate specificity of the purified enzyme revealed a unique character. Unlike cytosolic and lysosomal sialidases, this enzyme acts preferentially on gangliosides other than GM1 and GM2 but hardly on glycoproteins and oligosaccharides. In this it is similar to sialidases partially purified from rat brain (*6*) and human brain (*10*), although the human enzyme seems to differ from the bovine and rat enzymes in its apparent molecular weight and the low involvement of its sulfhydryl groups in the catalytic activity. The bovine enzyme is quite different from the rabbit erythrocyte sialidase reported by Chen *et al.* (*11*) in terms of substrate specificity and specific activity. The rabbit enzyme hydrolyzes GM1 and GM2 as well as glycoprotein and oligosaccharides and has much lower specific activity than the bovine enzyme. The bovine sialidase was found to be stimulated by both Triton X-100 and sodium cholate. Interestingly, the preference of the purified bovine enzyme for ganglioside substrates differs from that of the partially purified enzyme in the presence of Triton X-100. The former hydrolyzes GD3 and GD1a much faster than GM3, probably due to the better solubility of GD3 and GD1a compared to GM3. However, the partially purified enzyme cleaves GM3 as fast as GD3. The reasons for this discrepancy are not known, but two possibilities are (a) that factors necessary for GM3 hydrolysis such as the sulfatide (*21, 22*) and GM2 (*23*) activators, which were demonstrated to stimulate sialidase reaction, are removed during the purification; and (b) that a structural conformation of the sialidase needed for GM3 hydrolysis is lost during purification, probably at the thiol-activated Sepharose step. The latter possibility seems reasonable, since accessibility of the partially purified enzyme to GM3 was changed by long-term storage, and addition of step 6 enzyme fraction did not enhance the activity. Since anionic cholate-type detergents have been suggested to mimic the activator action in making gangliosides more sensitive to sialidase, the quick hydrolysis of GM3 and GD1b in the presence of sodium cholate could be a result of an increase in solubility of these gangliosides caused by the detergent. Triton X-100, on the other hand, seems to act on the enzyme rather than the substrate and to keep enzyme soluble. Cantz and his coworkers (*9, 24*) have presented evidence that Triton X-100-stimulated activity represents plasma membrane sialidase activity with cell homogenates as the enzyme source. Their proposal does not conflict with our present data, although cholate may also stimulate the plasma membrane sialidase, as shown here and earlier (*21, 25*). With regard to the sialyllinkage preference, the sialidase was found capable of cleaving gangliosides having $\alpha2{\rightarrow}8$, $\alpha2{\rightarrow}3$, and $\alpha2{\rightarrow}6$ in this order. This is in contrast with results for lysosomal sialidase published by Fingerhut *et al.* (*21*), who demonstrated that the sialidase purified from human placenta does not act on GD3 or GD1b, even in the presence of sulfatide activator protein or taurodeoxycholate. It is therefore likely that the longer-chain gangliosides including GD3, GD1a, and GT1b are degraded mainly in plasma membranes, while GM3 is also hydrolyzed in lysosomes.

Cell surface gangliosides have been implicated in cell–cell interactions and transmembrane signaling (*26*). Therefore, the sialidase resident in plasma membranes might be involved in some of these important functions by modulating the gangliosides content. Ganglioside sialidases, in fact, have been suggested to play a role in cell differentiation of cerebellar granule (*27*) and human neuroblastoma cells (*28*), and the observations by Usuki *et al.* (*29, 30*) led them to propose the participation of ganglioside sialidase in cell growth regulation. Alterations of the levels of ganglioside sialidase expression associated with malignant transformation have been described: loss of cell density–dependent suppression in 3T3-transformed cells (*31*) and appearance of ganglioside sialidase activity in BHK-transformed cells (*32*), although no information is available as to what type of sialidase is involved. We previously demonstrated an increase of membrane sialidase I during induction of anchorage-independent growth and tumorigenicity in mouse epidermal JB6 cells exposed to phorbol ester, along with a decrease of lysosomal-type sialidase (*33*). To obtain further insights into the biological function, the significance of pathological alterations, and the expression mechanism of this sialidase, we have undertaken cDNA cloning. Based on the partial amino acid sequence of the enzyme purified in the present work, we have just succeeded in cloning a cDNA (*34*).

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