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Purification and Characterization of a Membrane-Bound Sialidase from Pig Liver

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A membrane-bound sialidase in pig liver microsomes was solubilized with a nonionic detergent, IGEPAL CA630, and purified to homogeneity by sequential chromatographies on SP-Toyopearl, Butyl-Toyopearl (1st), SuperQ-Toyopearl, Hydroxyapatite, Butyl-Toyopearl (2nd), GM1-Cellulofine affinity, and sialic acid-Cellulofine affinity columns. The molecular weight of the purified enzyme was estimated to be 57 kDa on SDS-PAGE. The pH optimum was 4.8 for the activity measured using 4-methylumbelliferyl-α-*N*-acetylneuraminic acid (4MU-Neu5Ac) as the substrate. The enzyme activity was inhibited by 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid, iodoacetamide and *p*-chloromercuribenzoic acid. While the enzyme could effectively hydrolyze 4MU-Neu5Ac, it failed to significantly cleave a sialic acid residue(s) from sialyllactose, glycoproteins or gangliosides at pH 4.8. These results suggest that the purified enzyme is a novel sialidase with a substrate specificity distinct from those of known membrane-bound sialidases in mammalian tissues.

Key words: liver, membranes, pig, purification, sialidase.

Sialidase [EC 3.2.1.18] is an enzyme that cleaves a sialic acid residue(s) from sialoglycoconjugates and is widely distributed in the living world (*1*). In mammalian tissues, sialidase exists as isozymes with different subcellular localizations. They include two soluble enzymes localized in the cytosol and intra-lysosomal space, and two membrane-bound enzymes associated with lysosomal and plasma membranes (*1*). Additionally, a recent study provided evidence that nuclear membranes contain an intrinsic sialidase activity (*2*). The enzyme properties of these isozymes are distinct (*3–7*). In recent years, many of these isozymes have been cloned, and their amino acid sequences determined (*8–17*). Sialidase plays a central role in the catabolism of sialoglycoconjugates. Lysosomal enzyme is supposed to be critically involved in the degradation of sialoglycoproteins, as suggested by the accumulation of sialooligosaccharides in sialidosis (*18*, *19*) and galactosialidosis (*20*). Plasma membrane–bound sialidase is assumed to play an important role in ganglioside metabolism in the membrane, though concrete evidence for this hypothesis remains insufficient (*21*, *22*). An increasing body of evidence has also been accumulated suggesting that sialidase is also involved in diverse cellular functions including adhesion, proliferation, and differentiation (*23–26*).

In this study, we describe the solubilization, purification, and characterization of a membrane-bound sialidase from pig liver. The purified enzyme showed a substrate specificity different from those of known membrane-bound sialidases.

MATERIALS AND METHODS

Materials—Fresh pig liver was obtained from a local slaughterhouse. The chemicals and reagents were purchased from the following sources: SP-Toyopearl 650M, Butyl-Toyopearl 650M, and SuperQ-Toyopearl 650M (Tosoh, Tokyo), Macro-Prep Ceramic Hydroxyapatite (Hydroxyapatite) (Bio-Rad, Hercules, CA, USA), Formyl-Cellulofine (Seikagaku, Tokyo), 4-methylumbelliferyl-α-*N*-acetylneuraminic acid (4MU-Neu5Ac), sialyllactose, fetuin, bovine submaxillary mucin, transferrin, 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid (Neu5Ac2en), *N*-glycolylneuraminic acid, *p*-chloromercuribenzoic acid (PCMB), IGEPAL CA630, sodium cyanoborohydride ($NaCNBH_3$), and fetuin-agarose (Sigma, St. Louis, MO, USA), bovine serum albumin (BSA), Wakogel LP-60C18, 1,2-diamino-4,5-methylenedioxybenzene dihydrochloride (MDB), and Silver Staining Kit Wako (Wako Pure Chemicals, Osaka), BCA protein assay reagent (PIERCE, Rockford, IL, USA), and *N*-acetylneuraminic acid–containing gangliosides in pure forms (GM3, GD3, GM1, GD1a, and bovine brain ganglioside mixture) (Matreya, PA, USA). *N*-Acetylneuraminic acid was kindly donated by Snow Brand Milk Product (Sapporo).

Enzyme Assay—The standard reaction mixture contained 0.1 M sodium acetate buffer (pH 4.8), 0.2 mM substrate, and an enzyme preparation in a final volume of 50 μl, unless otherwise noted. The assay procedures with individual substrates were as follows. For measurement of enzyme activity toward 4MU-Neu5Ac, the reaction was terminated by the addition of 0.18 M glycine-NaOH buffer (pH 10.8),

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Abbreviations: 4MU-Neu5Ac, 4-methylumbelliferyl-α-*N*-acetylneuraminic acid; Neu5Ac2en, 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid; PCMB, *p*-chloromercuribenzoic acid; PNP-Neu5Ac, *p*-nitrophenyl-*N*-acetylneuraminic acid.

followed by spectrofluorometric measurement with excitement and emission wavelengths of 365 nm and 445 nm, respectively (*27*). To assay enzyme activity toward *p*-nitrophenyl-*N*-acetylneuraminic acid (PNP-Neu5Ac), the reaction was terminated by the addition of 0.45 M Tris-HCl buffer (pH 8.2), and the released *p*-nitrophenol was measured spectrophotometrically at 410 nm. For measurement of enzyme activity toward sialyllactose, the reaction was terminated by immediate cooling with ice water, and the released sialic acid was assayed by Warren's thiobarbituric acid method (*28*). Enzyme activities toward glycoprotein substrates (fetuin, bovine submaxillary mucin, and transferrin) were assayed using a fluorometric HPLC method (*29*). The reaction was terminated by the addition of acetone. After adding a fixed amount (50 ng) of *N*-glycolylneuraminic acid as the internal standard, the reaction mixture was centrifuged. Free sialic acids recovered in the supernatant were then labeled by treatment with the MDB reagent at 60°C for 150 min. The labeled sialic acid derivatives were analyzed by HPLC on an octadecylsilica column (Mightysil RP18 GD 250-4.6; Kanto Chemical, Tokyo) with a fluorescence detector.

The reaction mixture for sialidase activities toward ganglioside substrates (GM3, GM1, GD3, and GD1a) consisted of 0.1 M sodium acetate buffer (pH 4.8 or 6.0), 0.05% (w/v) Triton X-100, 1 mM $CaCl_2$, 1 mg/ml of BSA, 0.15 mM substrate as sialic acid, and an enzyme preparation. The reaction was terminated by the addition of chloroform/methanol (1:1). After adding a fixed amount of *N*-glycolylneuraminic acid, the reaction mixture was centrifuged, and the supernatant was dried under vacuum. The residue was dissolved in 0.5 ml of 0.1 M KCl in methanol/water (1:1), and then applied to a Wakogel LP-60C18 column. Free sialic acids were recovered in the eluate while unhydrolyzed ganglioside substrates were absorbed to the column. The sialic acids were quantitated by the fluorometric HPLC procedure described above.

Preparation of GM1-Cellulofine—Ganglioside GM1 was incubated in 1 N KOH at 102°C for 25 h, followed by reacetylation (*30*). The generated lyso-GM1 was immobilized on Formyl-Cellulofine as follows. Formyl-Cellulofine (1 g) was reacted with lyso-GM1 (1 mg) in 20 mM sodium carbonate buffer (pH 9.0, 1.5 ml) containing $NaCNBH_3$ (7 mg) and 0.2 M NaCl at room temperature for 8 h. After washing the gel with 0.2 M Tris-HCl (pH 7.0), non-reacted formyl groups of the gel were blocked by incubation with Tris-containing buffer with $NaCNBH_3$ at room temperature for 5 h.

Preparation of Sialic Acid-Cellulofine—An affinity ligand, 6-amino-1-hexanoyl 5-acetamido-3,5-dideoxy-α-glycero-D-galacto-2-nonulopyranosidonic acid, was synthesized as follows. In the presence of *N*-iodosuccinimide and trifluoromethanesulfonic acid as promoters, the coupling of methyl (phenyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-2-thio-β-D-glycero-D-galacto-2-nonulopyranosid)onate (*31*) and 6-benzyloxycarbonylamino-1-hexanol in acetonitrile gave α-glycoside in a 65% yield. The product was deacetylated with $NaOCH_3$ in methanol, followed by hydrolysis with 0.1 N KOH in methanol (64% yield in two steps). The affinity ligand was obtained by hydrogenation with $Pd(OH)_2$-C in a 70% yield; FAB-MS *m/z*: 409 $(M+H)^+$, 431 $(M+Na)^+$. Immobilization of the affinity ligand on Formyl-Cellulofine was carried out by the above-mentioned method for lyso-GM1 (Fig. 1).

Purification of Membrane-Bound Sialidase—All purification procedures were performed at 4°C. Sialidase activity was monitored using 4MU-Neu5Ac as the substrate.

Step 1. Preparation of a pig liver microsomal fraction: Fresh pig liver (1.5 kg) was perfused with PBS and then homogenized in 3 volumes of 0.25 M sucrose using a cooking blender for 2 min. After centrifugation at 10,000 ×*g* for 10 min, the supernatant was frozen at –80°C in order to disrupt vesiculated organelles. The supernatant was thawed and further centrifuged at 100,000 ×*g* for 1 h. The pellet was homogenized in 2 liters of 0.1 M sodium phosphate buffer (pH 6.0) –1 mM EDTA using a Teflon-glass homogenizer and then centrifuged at 100,000 ×*g* for 1 h. The resulting pellet was defined as the microsomal fraction.

Step 2. Solubilization: The microsomal fraction was homogenized with an equal volume (250 ml) of 10 mM sodium phosphate buffer (pH 6.0) using a Teflon-glass homogenizer. The homogenate was mixed with an equal volume (500 ml) of 0.2 M sodium acetate buffer (pH 4.5) containing 0.2 M NaCl, 1% IGEPAL CA630, 2 mM EDTA, 2 mM phenylmethylsulfonylfluoride, and 40% glycerol. The mixture was then gently agitated for 1 h, followed by centrifugation at 100,000 ×*g* for 1 h. The supernatant was dialyzed against 20 mM sodium acetate buffer (pH 4.5) containing 1 mM EDTA and 20% glycerol (buffer A) for two days.

Step 3. SP-Toyopearl chromatography: The enzyme extract was applied to an SP-Toyopearl 650M column (5 × 25 cm) equilibrated with buffer A containing 0.1% IGEPAL CA630. Enzyme activity was eluted with a linear gradient of NaCl in the buffer (from 0 to 450 mM).

Step 4. Butyl-Toyopearl chromatography: The active fraction obtained in Step 3 was supplemented with NaCl to adjust the concentration to 1 M, and then applied on a Butyl-Toyopearl 650M column (5 × 5 cm) equilibrated with 20 mM sodium acetate buffer (pH 5.5) containing 1 M NaCl, 0.1% IGEPAL CA630, 1 mM EDTA, 0.1 mM DTT, and 20% glycerol (buffer B). Enzyme activity was eluted with a linear gradient of IGEPAL CA630 in the buffer (from 0.1 to 1%).

Step 5. SuperQ-Toyopearl chromatography: The active fraction obtained in Step 4 was dialyzed against 20 mM sodium acetate buffer (pH 5.5) containing 0.1% IGEPAL

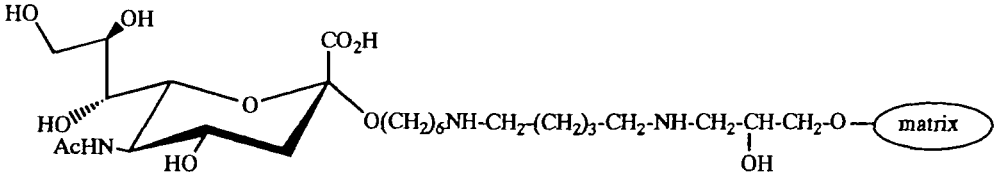

Fig. 1. **Structure of sialic acid-Cellulofine.**

CA630, 1 mM EDTA, 0.1 mM DTT, and 20% glycerol (buffer C), and then applied to a SuperQ-Toyopearl 650M column (2.5 × 20 cm) equilibrated with buffer C. Enzyme activity was eluted with a linear gradient of NaCl in the buffer (from 0 to 400 mM).

Step 6. Hydroxyapatite chromatography: The active fraction obtained in Step 5 was dialyzed against 10 mM potassium phosphate buffer (pH 5.5) containing 0.1% IGEPAL CA630, 0.1 mM DTT, and 20% glycerol (buffer D), and then applied to a hydroxyapatite column (1.5 × 16 cm) equilibrated with buffer D. Enzyme activity was eluted with a linear gradient of potassium phosphate buffer (pH 5.5) (from 0 to 500 mM).

Step 7. Butyl-Toyopearl chromatography: The active fraction obtained in Step 6 was supplemented with NaCl to adjust the concentration to 1 M, and then chromatographed on a Butyl-Toyopearl 650M column (1.5 × 12 cm) in a similar manner to as described for Step 4.

Step 8. GM1-Cellulofine affinity chromatography: The active fraction obtained in Step 7 was dialyzed against buffer C and then applied to a GM1-Cellulofine affinity column (1 × 2 cm) equilibrated with buffer C. Enzyme activity was eluted with a linear gradient of NaCl in the buffer (from 0 to 500 mM).

Step 9. Sialic acid-Cellulofine affinity chromatography: The active fraction obtained in Step 8 was dialyzed against buffer C and then applied to a sialic acid-Cellulofine affinity column (1 × 2 cm) equilibrated with buffer C. Enzyme activity was eluted with a linear gradient of NaCl in the buffer (from 0 to 1 M). The active fractions were pooled and then dialyzed against buffer C.

SDS-PAGE—SDS-PAGE was performed on a 10% polyacrylamide gel by the method of Laemmli (*32*). Proteins were detected by Coomassie blue staining or silver staining.

Binding to Ganglioside GM1—An enzyme preparation was applied to a small GM1-Cellulofine affinity column equilibrated with buffer C. The column was washed consecutively with buffer C, 20 mM sodium acetate buffer (pH 5.5) containing 2% IGEPAL CA630, 1 mM EDTA, 0.1 mM DTT, and 20% glycerol, buffer C, and finally buffer C containing 5 mM *N*-acetylneuraminic acid, and then the enzyme activity in each fraction was measured.

Protein Determination—Protein concentrations were determined by the Lowry method (*33*) or BCA method (*34*) with BSA as a standard.

RESULTS

Purification of Sialidase—To purify a membrane-bound

TABLE I. **Purification of membrane-bound sialidase from pig liver.** Enzyme activity was measured using 4MU-Neu5Ac as the substrate.

Step	Total protein (mg)	Total activity (pmol/min)	Specific activity (pmol/min/mg)	Purification (fold)	Yield (%)
Microsomal fraction (10,000–100,000 ×*g* ppt)	9,193	1,499,400	163	1	100
Solubilization	2,830	266,000	94	0.58	17.7
SP-Toyopearl	474	105,360	222	1.36	7.0
Butyl-Toyopearl (1st)	80.7	59,360	736	4.51	4.0
SuperQ-Toyopearl	10.0	22,399	2,247	13.8	1.5
Hydroxyapatite	0.23	6,112	26,474	162	0.41
Butyl-Toyopearl (2nd)	0.090	4,757	53,012	325	0.32
GM1-Cellulofine	0.035	3,786	107,005	656	0.25
Sialic acid-Cellulofine	0.017	2,000	115,760	710	0.13

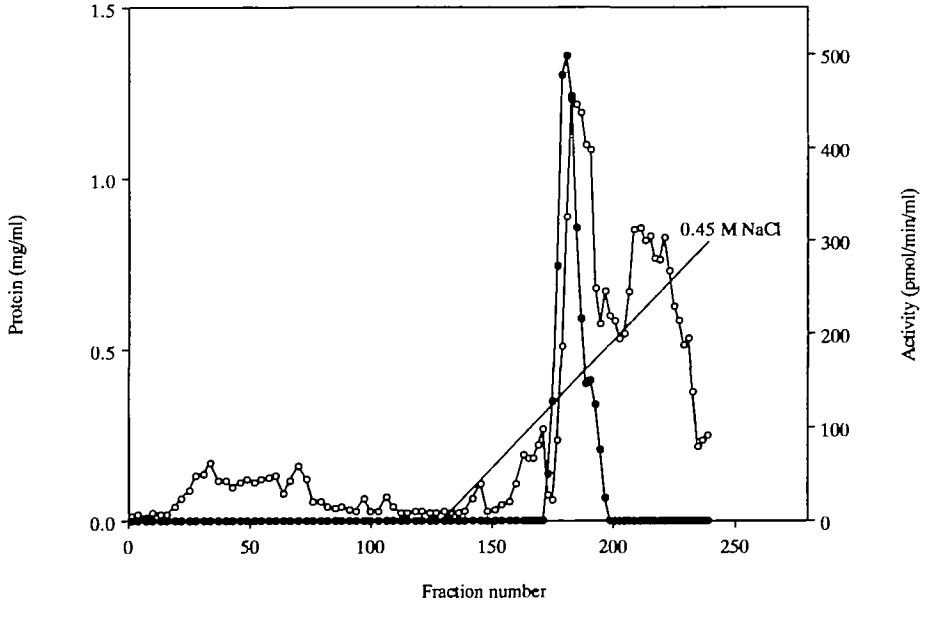

Fig. 2. **SP-Toyopearl column chromatography.** (●) Sialidase activity; (○) protein; (—) NaCl concentration. The enzyme activity was measured using 4MU-Neu5Ac as the substrate. Protein was determined by the Lowry method.

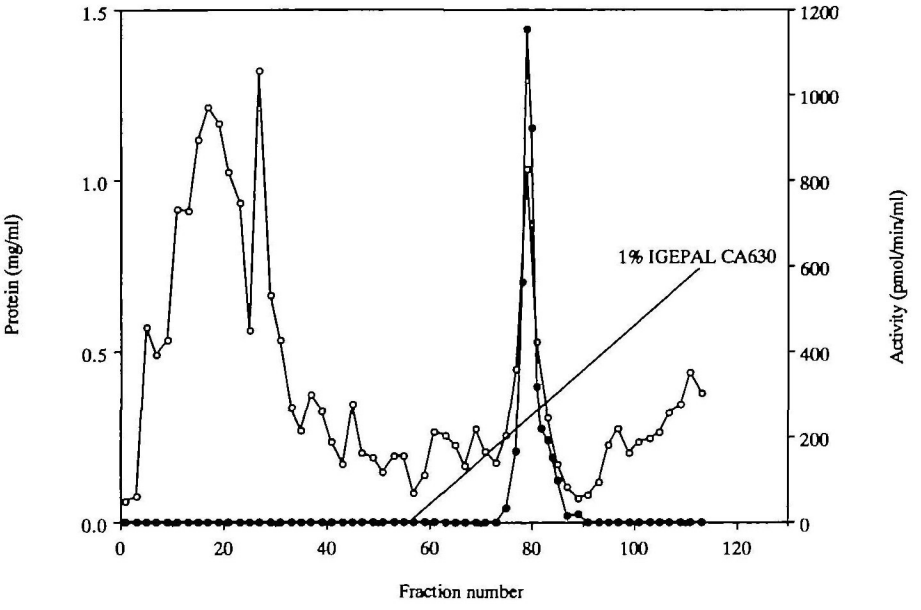

Fig. 3. **1st Butyl-Toyopearl column chromatography.** (●) Sialidase activity; (○) protein; (—) IGEPAL CA630 concentration. The enzyme activity was measured using 4MU-Neu5Ac as the substrate. Protein was determined by the Lowry method.

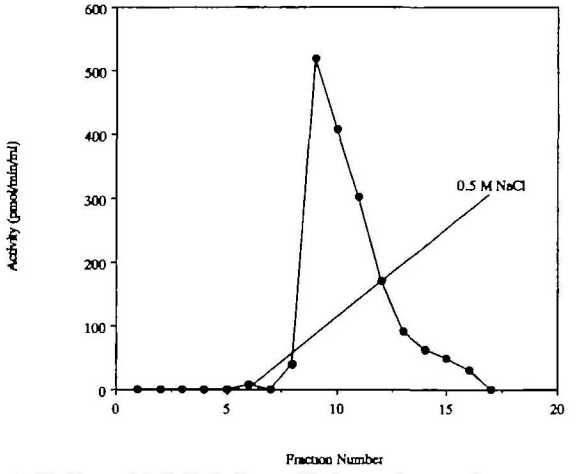

Fig. 4. **Sialic acid-Cellulofine affinity column chromatography.** (●) Sialidase activity; (—) NaCl concentration. The enzyme activity was measured using 4MU-Neu5Ac as the substrate.

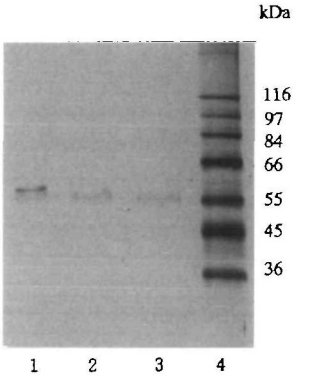

Fig. 5. **SDS-PAGE of membrane-bound sialidase preparations obtained in the last three steps during purification.** The pooled samples recovered during purification of the pig liver membrane-bound sialidase were analyzed by SDS-PAGE. Lanes 1, 2, and 3: pooled fractions obtained on 2nd Butyl-Toyopearl chromatography (Step 7), GM1-Cellulofine affinity chromatography (Step 8), and sialic acid-Cellulofine affinity chromatography (Step 9), respectively. Lane 4, molecular weight markers. The gel was silver-stained.

sialidase from the microsomal fraction of pig liver, different solubilization conditions were first examined. While detergents such as sodium deoxycholate, octylglucoside, and Triton X-100 could solubilize less than 50% of the enzyme activity, IGEPAL CA630 gave a higher yield (60–70% of total). The addition of glycerol and salt further improved the yield of enzyme activity (90% of total). After solubilization, the enzyme activity became unstable especially outside the pH range of 4.5 to 6.0. The optimal pH for the stability of the enzyme activity was around 5.5. Therefore, this pH was maintained throughout the purification procedures, except for the step on the SP-Toyopearl column, to which the enzyme binds at pH 4.5 but not at pH 5.5. The sialidase was purified to homogeneity with a purification magnitude of 710-fold and an overall yield of 0.13% (Table I). Figures 2–4 show the elution profiles on SP-Toyopearl, Butyl-Toyopearl, and sialic acid-Cellulofine affinity chromatography, respectively. Figure 5 shows SDS-PAGE of the enzyme preparations in the last three steps (*i.e.*, steps 7–9). The pooled fraction obtained on 2nd Butyl-Toyopearl chromatography (Step 7) gave multiple protein bands. The final preparation of the enzyme (Step 9) gave a single band. Figure 6 shows SDS-PAGE of the purified enzyme after staining with Coomassie brilliant blue R250. The molecular weight of the enzyme was estimated to be 57 kDa.

pH Optimum and Kinetic Parameters—The purified sialidase showed the maximal enzyme activity at pH 4.8 when 4MU-Neu5Ac was used as the substrate (Fig. 7). The V_{max} and K_m values were 150 nmol/min/mg protein and 73 μM, respectively.

Effects of Various Compounds on the Enzyme Activity—Table II shows the effects of various compounds on the sialidase activity of the purified enzyme. Neu5Ac2en, a well-known inhibitor of sialidases from various sources (*35*), strongly inhibited the enzyme activity. Among divalent cat-

ions, Ca^{2+} and Mg^{2+} had no effect on the activity up to 10 mM, whereas Cu^{2+} was inhibitory at 10 mM. The enzyme activity was also inhibited by an oxidizing agent, potassium ferricyanide ($K_3[Fe(CN)_6]$), and thiol-modifying agents, PCMB and iodoacetamide.

Substrate Specificity—Table III summarizes the relative sialidase activities of the purified enzyme toward various substrates at two different pHs. At pH 4.8, the enzyme hydrolyzed PNP-Neu5Ac at the rate of 8% when the activity toward 4MU-Neu5Ac was assumed to be 100%. The enzyme hardly hydrolyzed sialyllactose (below 5%), or ganglioside and glycoprotein substrates (below 0.1%). Interestingly, a small but definite amount of ganglioside GD3 was hydrolyzed by the enzyme at pH 6.0; the activity was 0.3 ± 0.1% (mean of three determinations ± SD) of that with 4MU-Neu5Ac. The enzyme activity at a higher pH could not be examined because of the instability of the enzyme activity. These results suggested that the enzyme might be more active on ganglioside substrates at physiological pH. The activity toward glycoproteins or sialyllactose was below the detection limits at pH 6.0. In order to compare the substrate specificity of the purified enzyme with that of pig liver lysosomal sialidase, the activities of the purified enzyme toward GD3 and GM3 were assayed in the presence of sodium cholate at pH 4.5, as described by Nagaoka *et al.*

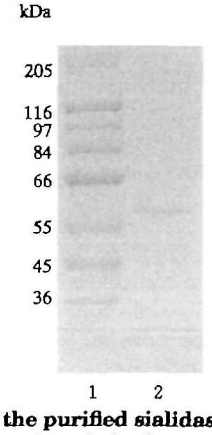

Fig. 6. **SDS-PAGE of the purified sialidase.** The purified enzyme was subjected to electrophoresis in the presence of SDS and then stained with Coomassie brilliant blue R250. Lane 1: molecular weight markers; lane 2: purified enzyme (0.43 μg).

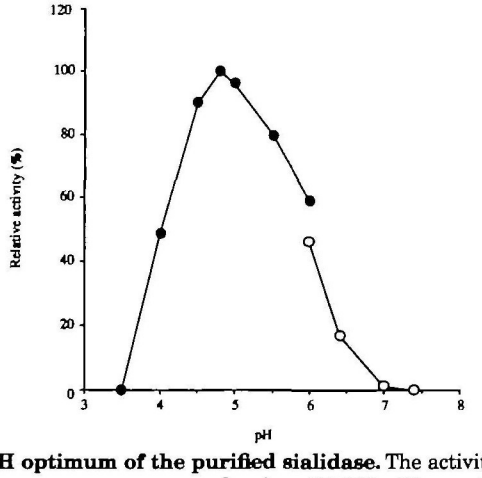

Fig. 7. **pH optimum of the purified sialidase.** The activity of the purified enzyme was measured using 4MU-Neu5Ac as the substrate. (●) 0.1 M sodium acetate buffer; (○) 0.1 M sodium phosphate buffer.

TABLE II. **Effects of various compounds on sialidase activity of the purified enzyme.** Enzyme activity was measured using 200 μM 4MU-Neu5Ac as the substrate.

Inhibitor	Concentration	Relative activity (%)
(–)	(–)	100
Neu5Ac2en	100 μM	67
$CaCl_2$	10 mM	105
$MgCl_2$	10 mM	105
$CuCl_2$	1 mM	91
	10 mM	50
$K_3[Fe(CN)_6]$	100 μM	63
Iodoacetamide	100 μM	75
PCMB	100 μM	69
DTT	10 mM	108

TABLE III. **Substrate specificity of the purified enzyme.** Enzyme activity toward 4MU-Neu5Ac at pH 4.8 is expressed as 100%. The final concentration of each substrate was 150 μM as sialic acid.

Substrate	Relative activity (%)
pH 4.8	
4MU-Neu5Ac	100
PNP-Neu5Ac	8
Gangliosides[a]	ND
Glycoproteins[b]	ND
Sialyllactose[c]	ND
pH 6.0	
4MU-Neu5Ac	50
GD3	0.3
GM3	ND
Glycoproteins[b]	ND
Sialyllactose[c]	ND

[a]GM3, GD1a, GM1, and bovine brain ganglioside mixture. [b]Fetuin, bovine submaxillary mucin and transferrin. [c]Sialyl-(α2→3)-lactose and sialyl-(α2→6)-lactose. ND, not detectable (<0.1%, for gangliosides and glycoproteins, <5% for sialyllactose).

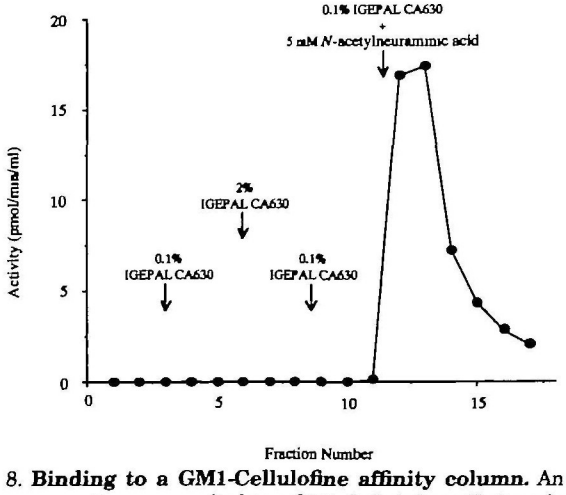

Fig. 8. **Binding to a GM1-Cellulofine affinity column.** An enzyme preparation was applied to a GM1-Cellulofine affinity column. The column was successively washed with different buffers with or without 5 mM *N*-acetylneuraminic acid as described under "MATERIALS AND METHODS."

(*36*). No measurable hydrolysis of GD3 or GM3 was detected.

Binding to Ganglioside GM1—The binding of sialidase to gangliosides was examined using a GM1-Cellulofine affinity column. The enzyme activity was bound to the column and was only eluted with the buffer containing 5 mM *N*-acetylneuraminic acid (Fig. 8). The sialidase did not bind to a fetuin-agarose column under the same conditions (data not shown).

DISCUSSION

In this study, we purified a sialidase in pig liver microsomes to homogeneity. One of the critical steps in the purification was solubilization from microsomal membranes. Among the detergents examined, non-ionic detergent IGEPAL CA630 gave the best result in the presence of 20% glycerol and 0.1 M NaCl. Another crucial tactic for the purification was related with the pH-dependent stability of the enzyme activity. Since the enzyme activity became unstable outside a narrow pH range, it was essential to maintain a proper pH (*i.e.*, pH 5.5) to minimize the loss of enzyme activity during the purification. Successful purification of the solubilized enzyme was achieved by sequential column chromatographies. Throughout the procedures, the enzyme activity was eluted as a single peak, and not multiple peaks, suggesting that the purified enzyme may represent a major sialidase species associated with microsomal membranes from pig liver.

The purified sialidase had an estimated molecular weight of 57 kDa, which falls within the range reported for membrane-bound sialidases from bovine brain (52 kDa) (*37*), rabbit erythrocytes (54 kDa) (*38*), and human brain (60 kDa) (*39*). The optimal pH value also resembled those reported for other membrane-bound sialidases (*37–39*). The activity of the purified sialidase was inhibited by Neu5Ac2en, as observed for other sialidases (*7*, *39*). Mg^{2+} and Ca^{2+} did not inhibit the sialidase activity of the enzyme. A similar result was obtained for a membrane-bound enzyme from bovine brain (*37*). The activity of this enzyme was also inhibited by $K_3[Fe(CN)_6]$, iodoacetamide, and PCMB, but not by DTT (Table II). These results suggest that a free thiol group(s) of cysteine may be necessary for sialidase activity. The requirement of thiol group(s) for full activity was also reported for a membrane-bound sialidase from bovine brain (*37*). Structural or catalytic similarity may exist between these two membrane-bound sialidases.

The purified enzyme had a distinct substrate specificity. Among the substrates tested, 4MU-Neu5Ac was the best substrate for the enzyme, whereas gangliosides (GM3, GD3, GD1a, GM1, and bovine brain gangliosides mixture), glycoproteins (fetuin, bovine submaxillary mucin, and transferrin), and sialyllactose were hardly hydrolyzed by the enzyme at pH 4.8. It has been reported that a group of sialidases hydrolyzes gangliosides at comparable or higher rates as compared with 4MU-Neu5Ac or sialyllactose (*3*, *5*, *36–42*). Another group exhibits low activities toward gangliosides, but effectively hydrolyzes sialyllactose and 4MU-Neu5Ac (*4*, *41*, *43*, *44*). To our knowledge, there is no known sialidase showing a substrate specificity similar to that observed for the enzyme in this study. Thus, the purified enzyme is probably a novel sialidase species with a distinct substrate specificity. Because of its membrane-bound nature and different substrate specificity from that of the lysosomal enzyme in pig liver (*36*), it was assumed that the enzyme is of neither cytosolic nor lysosomal origin. The present study showed that the enzyme, although at a slow rate, hydrolyzed GD3, but neither GM3, GD1a, nor GM1, at pH 6.0. This observation suggests the possibility that the enzyme is more active on $\alpha2 \rightarrow 8$ linked sialic acids than $\alpha2 \rightarrow 3$ linked residues.

While the biological function of the purified enzyme from pig liver microsomes is not known, several possibilities can be raised. First, the enzyme may be involved in the catabolism of glycoconjugates, even though it hardly hydrolyzed "natural" substrates at pH 4.8. This hypothesis may be supported by the observation that the enzyme, although at a slow rate, hydrolyzed GD3 at a higher pH. Second, the sialidase may play an important role in adhesion through binding with "poor substrates" such as GM1, as suggested for myelin-associated sialidase, which is possibly involved in the formation of the multilamellar structure of myelin (*45*). GM3, GD1a, and some sialoglycoproteins may also serve as ligands for the enzyme. Since GM3 is the major ganglioside species in liver tissues (*46*), it would be important to characterize the possible interaction between GM3 and the enzyme. To obtain further insights into its biological roles, studies on the subcellular localization, catalytic properties, and binding to sialoglycoconjugates of the enzyme are being planned.

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