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Characterization of Mouse Liver Sialidase and Partial Purification of the Lysosomal Sialidase

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Sialidase activity was detected in mouse liver, and was localized predominantly in the lysosomal fraction. Weak activity was also contained in the microsomal fraction, but little was detected in the cytosolic fraction. The optimum pH values of these sialidase activities were 4.0—4.5 for 4-methylumbelliferyl-α-D-*N*-acetylneuraminate (4-MU-NeuAc) and sialyllactose.

The sialidase activity in the lysosomal fraction was very unstable, but was partially stabilized in the presence of phenylmethylsulfonyl fluoride, Ca^{2+}, Mg^{2+}, Mn^{2+} and Zn^{2+}.

The lysosomal and microsomal sialidase fractions were active preferentially toward ganglioside mixture, in the presence of sodium cholate as a detergent. These enzymes were also active toward all of the sialooligosaccharides and sialoglycoproteins tested. Those substrates possessing an α(2→3)-sialyl linkage were hydrolyzed much faster than those with an α(2→6) or α(2→8)sialyl linkage. These sialidase fractions were much more active toward glycopeptides than glycoproteins. The microsomal sialidase fraction was also active toward submaxillary mucin (bovine), suggesting that it is capable of hydrolyzing *O*-acetylated sialic acid residues.

When the lysosomal fraction was disrupted hypotonically, the activity toward 4-MU-NeuAc and sialyllactose was mainly recovered in the 15000 × *g* supernatant, and it was fractionated with ammonium sulfate. The fractionated soluble lysosomal sialidase failed to attack gangliosides, and was only weakly active toward glycoproteins but was capable of hydrolyzing glycopeptides and oligosaccharides. This enzyme was further purified by chromatography on diethylaminoethyl (DEAE)-cellulose, concanavalin A adsorption, affinity chromatography on Sephadex G-200 and high performance liquid chromatography on coupled columns of TSK-GEL G5000PW and G4000PW. When the soluble lysosomal sialidase fraction was subjected to DEAE-cellulose chromatography, most of the β-galactosidase was eluted in the unadsorbed fraction, but the sialidase was eluted in the unadsorbed and the adsorbed fractions.

Keywords——sialidase; neuraminidase; β-galactosidase; mouse liver; subcellular distribution; substrate specificity

Sialidase [EC 3.2.1.18] catalyzes the removal of sialic acid residues in sialoglycoconjugates, and this reaction is associated with the many important biological reactions, such as survival of sialoglycoconjugates,[1] antigenic expression[2] and recognition by receptors.[3] It is known that the serum sialic acid level changes in several diseases, such as inflammations, infections, cancers and rheumatism.[4] In order to elucidate the role of sialic acid residues in these diseases, it is necessary to investigate their turnover. Sialidase activity has been studied mainly in animal tissues such as rat liver,[5] pig brain,[6] rat heart,[7] rabbit spermatozoal acrosomes,[8] rabbit endometrium,[9] cultured human skin fibroblasts,[10] human liver,[11] human brain[12] and human placenta,[13] but only limited information is available on the mouse liver enzyme. Samollow *et al.* identified two kinds of sialidase activity for 4-methylumbelliferyl-α-D-*N*-acetylneuraminate (4-MU-NeuAc) in the mouse liver extract by cellulose acetate electrophoresis,[14] but little information on the general properties and substrate specificity has been reported.

This report describes preliminary investigations on the general properties and substrate

specificity of mouse liver sialidases, and a partial purification of the major sialidase activity in the lysosomal fraction.

Materials and Methods

Materials——Bovine colostrum sialyllactose (mixture of 85% α2→3 and 15% α2→6 isomers), orosomucoid, human transferrin, fetuin (type IV), bovine submaxillary mucin, bovine brain mixed gangliosides, *p*-nitrophenyl (*p*NP)-β-D-galactopyranoside and concanavalin A (Con A) were purchased from Sigma Chemical Co., Ltd. (St. Louis, U.S.A.). When specified, human milk sialyllactose (mixture of 78% α2→6 and 22% α2→3 isomers) was separated into the two isomers by using high performance liquid chromatography (HPLC) according to the procedure of Bergh *et al.*[15] Reduced trisaccharide, sialylα(2→3)galactosylβ(1→3)*N*-acetylgalactosaminitol (Siaα2→3Galβ1→3GalNAc*ol*), was prepared by β-elimination of fetuin by the procedure of Spiro and Bhoyroo.[16] 4-MU-NeuAc, colominic acid and *Arthrobacter ureafaciens* sialidase were purchased from Nakarai Chemical Co., Ltd. (Kyoto, Japan). Fetuin glycopeptides were prepared according to Spiro and Bhoyroo.[16] Glycopeptides from bovine submaxillary mucin were prepared according to Carubelli *et al.*[17] Gangliosides G_{M1} (bovine brain), G_{M2} (Tay-Sachs disease), G_{D1a} (bovine brain) and G_{D1b} (bovine brain) were kindly provided by Prof. Y. Uda (Niigata College of Pharmacy, Niigata, Japan), and G_{M3} was obtained from Iatron Laboratories, Inc. (Tokyo, Japan). Leupeptin was the product of the Protein Research Foundation (Osaka, Japan) and phenylmethylsulfonyl fluoride (PMSF) was from Sigma. All other reagents were of analytical grade. Diethylaminoethyl (DEAE)-cellulose (DE-52) and Sephadex G-200 were obtained from Whatman (Kent, U.K.) and Pharmacia Fine Chemicals (Uppsala, Sweden), respectively.

Sialidase Assay——(a) Thiobarbituric Acid (TBA) Method: The standard assay mixture contained 100 nmol of substrate as bound sialic acid and enzyme in 0.4 ml of 10 mM citrate–phosphate buffer (pH 4.0) containing 20 mM sodium chloride. After incubation at 37 °C for 3 h, the reaction was stopped by rapid cooling, and when insoluble material was present in the reaction mixture, it was removed by centrifugation. The resulting supernatant was applied to a column of Dowex AG1-X8 (AcO^-), then the column was washed with distilled water and the absorbed sialic acid was eluted with 1 ml of 1 M sodium acetate buffer (pH 4.6). The sialic acid released was determined by the TBA method as modified by Horgan.[18] The values of blanks for enzyme (without substrate) and substrate (with heat-inactivated enzyme) were subtracted from the experimental value. One unit of enzyme was defined as the amount of enzyme which catalyzed the release of 1 nmol of sialic acid per hour.

(b) Fluorometric Method: The assay mixture contained 25 nmol of 4-MU-NeuAc and enzyme in 0.4 ml of 10 mM citrate–phosphate buffer (pH 4.5) containing 20 mM sodium chloride. After incubation at 37 °C for 30 min, the reaction was terminated by the addition of 1.6 ml of ethanol, and the mixture was allowed to stand at −20 °C for 30 min. Then protein was removed by centrifugation, and the supernatant (1.5 ml) was added to 2 ml of 0.25 M glycine–NaOH buffer (pH 10.4). 4-Methylumbelliferone released was determined spectrofluorometrically (excitation at 360 nm and emission at 440 nm).

(c) Thin-Layer Chromatographic (TLC) Method: Sialidase activity toward gangliosides was determined by the TLC method. The reaction mixture contained 10 μg of ganglioside as the substrate, 400 μg of sodium cholate and 500 μg of enzyme protein in 0.1 ml of 10 mM citrate–phosphate buffer (pH 4.0). After incubation at 37 °C for 3 h, the reaction mixture was extracted with 500 μl of chloroform–methanol (2 : 1, v/v), then the organic layer was separated from the aqueous layer by centrifugation. The organic layer was removed, then the aqueous layer was reextracted with 500 μl of chloroform–methanol (2 : 1, v/v) saturated with water. Both organic layers were combined and evaporated to dryness under a stream of nitrogen. These samples were spotted on a silica gel plate (Merck No. 5721) which had been activated at 121 °C for 4 h, and developed with chloroform–methanol–water (60 : 35 : 8, v/v/v). The spots on the chromatogram were visualized with orcinol-sulfuric acid reagent[19] or resorcinol reagent.[20] Gangliosides in the reaction mixture were identified by comparison with authentic standards.

Fractionation of Mouse Liver——ICR mice (6—7 weeks old, 19—33 g) starved for 20 h were anesthetized with sodium pentobarbital and the livers were flushed with 0.01 M phosphate-buffered saline (pH 7.2) to remove blood, and then excised. Subsequent preparative procedures described below were conducted at 0—4 °C. Fractionation of mouse liver was carried out by a modification of the procedures described by Miyagi and Tsuiki.[5f] Livers (23.0 g) were homogenized in 4 volumes of 0.25 M sucrose/1 mM ethylenediaminetetraacetic acid (EDTA) with 4 strokes of a glass/Teflon homogenizer, and the homogenate was centrifuged at 1000 × *g* for 10 min. The supernatant was then centrifuged at 11000 × *g* for 20 min; the pellet was washed twice with 0.25 M sucrose/1 mM EDTA by centrifugation and suspended in this solution (the lysosomal fraction), while the supernatant was further centrifuged at 105000 × *g* for 1 h. The supernatant was saved (the cytosolic fraction), and the pellet was suspended in 0.25 M sucrose/1 mM EDTA (the microsomal fraction). The 1000 × *g* pellet was solubilized using a Polytron homogenizer (Kinematica GmbH, Lucerne). After centrifugation at 15000 × *g* for 15 min, the supernatant was saved (Polytron-solubilized fraction).

Partial Purification of the Lysosomal Sialidase——All isolation and purification procedures were carried out at 0—4 C.

Step 1. Solubilization of the Lysosomal Fraction and Ammonium Sulfate Precipitation: The lysosomal fraction was suspended in 10 mM sodium acetate buffer (pH 5.5) containing 10 mM $MnCl_2$ and 1 mM PMSF, and homogenized with 6 strokes of a glass/Teflon homogenizer. The homogenate was stirred gently at 0 °C for 20 min, and then centrifuged at 15000 × *g* for 15 min. Solid ammonium sulfate was added to the supernatant to obtain 60% saturation and then the mixture was stirred for 30 min. The resultant precipitate was collected by centrifugation at 15000 × *g* for 15 min, dissolved in a minimum amount of 10 mM sodium acetate buffer (pH 5.5) containing 10 mM $MnCl_2$ and 1 mM PMSF, and then dialyzed overnight against the same buffer. The dialysate was centrifuged at 15000 × *g* for 15 min to remove insoluble materials.

Step 2. DEAE-Cellulose Column Chromatography: The supernatant (5.4 mg protein) obtained in Step 1 was applied to a DEAE-cellulose column (3.0 × 7.5 cm) previously equilibrated with 50 mM sodium acetate buffer (pH 5.5) containing 10 mM $MnCl_2$ and 1 mM PMSF. The column was eluted with the starting buffer and then with the same buffer containing 0.5 M sodium chloride, collecting 5 ml fractions. Two sialidase fractions were obtained from the eluates with the starting buffer and 0.5 M NaCl in the same buffer. Both fractions were pooled and then concentrated by ultrafiltration with a Toyo Soda Ultracent-30.

Step 3. Con A Adsorption and Sephadex G-200 Affinity Chromatography: Con A (1 mg) was dissolved in 50 mM sodium acetate buffer (pH 6.0) containing 1 M NaCl, 10 mM $MnCl_2$ and 1 mM PMSF, and added to the enzyme solution obtained by DEAE-cellulose chromatography. After standing at 0 °C for 1 h, this mixture was centrifuged at 1200 × *g* for 20 min. The resulting supernatant was applied to a Sephadex G-200 column (2.0 × 5.0 cm) previously equilibrated with the above buffer. The column was eluted with the same buffer and then with 0.5 M methyl α-D-mannopyranoside in the same buffer, collecting 2 ml fractions. The fractions containing sialidase activity were pooled and concentrated by ultrafiltration with a Toyo Soda Ultracent-30.

Step 4. HPLC: The enzyme solution (0.7 ml) containing 470 μg of protein was applied to a Shimadzu LC-6A HPLC instrument equipped with coupled TSK-GEL G5000PW and G4000PW columns (0.75 × 60 cm, each). The columns were eluted with 50 mM sodium acetate buffer (pH 5.5) containing 10 mM $MnCl_2$ and 1 mM PMSF at a flow rate of 1 ml per min. Fractions were collected at 1 ml per tube. The fraction containing sialidase activity was rechromatographed in the same way.

Other Methods——Protein was determined by the method of Lowry *et al.*[21] with bovine serum albumin as the standard. The bound sialic acid of sialoglycoconjugates was determined by the TBA method after digestion with *A. ureafaciens* sialidase or hydrolysis with 0.1 M hydrochloric acid at 80 °C for 50 or 60 min. β-Galactosidase activity was determined by modifications of the procedures described by Li *et al.*[22] with *p*NP-β-galactoside as the substrate. The enzyme solution (10 μl) was added to 100 μl of 1 mM *p*NP-β-galactoside dissolved in 25 mM citrate–phosphate buffer (pH 3.2) containing 50 mM sodium chloride in a microtiter plate. After incubation of the mixture at 37 °C for 30 min, 200 μl of 0.2 mM sodium borate buffer (pH 9.8) was added and then the *p*-nitrophenol liberated was determined from the absorption at 405 nm. The absorbance was measured with a Titertek Multiskan. One unit of enzyme was defined as the amount of enzyme which hydrolyzed 1 μmol of *p*NP-β-galactoside per min under the conditions described above.

Results

Sialidase Activity in Mouse Liver

Sialidase activity in mouse liver was assayed with 4-MU-NeuAc and sialyllactose as substrates in citrate–phosphate buffer. The lysosomal, microsomal and the 1000 × *g* pellets exhibited maximal sialidase activity at pH 4.0—4.5 (Table I). Among these fractions, the lysosomal fraction exhibited the highest sialidase activity, and more than 60% of the sialidase activity toward sialyllactose and more than 80% of the activity toward 4-MU-NeuAc were found in this fraction (Table I). Weak activity was also detected in the microsomal and 1000 × *g* pellets but little sialidase activity could be detected in the soluble cytosolic fraction. Sialidase specific activity was also greatest in the lysosomal fraction (Table I).

When the lysosomal and the microsomal fractions were treated by freeze-thawing twice, and then centrifuged at 105000 × *g* for 60 min, about 35% of sialidase activity toward sialyllactose was recovered in the soluble part from the lysosomal fraction, and 16% of the activity was recovered in the supernatant from the microsomal fraction.

Stability of Sialidase Activity in the Lysosomal Fraction

When the lysosomal fraction was preincubated in citrate–phosphate buffer ranging in pH from 2.5 to 7.0 at 4 °C for 48 h prior to assay at pH 4.5, the sialidase activity towards 4-MU-NeuAc was relatively stable in the pH range of 4.0 to 5.0 compared with more acid-

TABLE I. Subcellular Distribution of Sialidase Activity in Mouse Liver

Fraction	Protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Optimum pH	Yield (%)
Sialyllactose					
1000 × *g* pellet	739	916	1.3	4.0	20
Lysosomal	879	2902	3.3	4.5	63
Microsomal	698	711	1.0	4.0	15
Cytosolic	958	109	0.1	—	2
4-MU-NeuAc					
1000 × *g* pellet	739	517	0.7	4.5	6
Lysosomal	879	7477	8.5	4.5	83
Microsomal	698	847	1.2	4.0	9
Cytosolic	958	143	0.1	—	2

TABLE II. Effect of EDTA, Carbohydrates, Dithiothreitol, Protease Inhibitors, Bovine Serum Albumin and Cations on the Lysosomal Sialidase Activity

Compounds added	Concentration (mM)	Remaining activity[a] (%)
No addition	—	48 (100)[b]
+ Lactose	50	53 (99)
+ NeuAc	50	5 (4)
+ EDTA	50	40 (52)
+ Dithiothreitol	50	27 (86)
+ PMSF	1	68 (98)
+ Leupeptin	2	49 (95)
+ Bovine serum albumin	0.015	54 (100)
+ $CaCl_2$	10	80 (83)
+ $MgCl_2$	10	77 (76)
+ $ZnCl_2$	10	72 (45)
+ $HgCl_2$	10	5 (45)
+ $MnCl_2$	10	83 (74)
+ $FeCl_3$	10	0 (6)

a) The lysosomal fraction was preincubated at 4 °C for 48 h (pH 5.5) in the presence of these compounds at the indicated concentration, followed by sialidase assay using 4-MU-NeuAc as described in Materials and Methods. *b*) The sialidase activity was determined using 4-MU-NeuAc in the presence of these compounds. The results were expressed as a percentage of the initial activity.

ic or alkaline pH. When the enzyme was preincubated at various temperatures for 30 min in the above buffer (pH 4.5), the activity toward 4-MU-NeuAc decreased with increase of temperature, and was completely lost at over 60 °C.

Storage of the lysosomal fraction in citrate–phosphate buffer (pH 5.5) at 4 °C for 48 h resulted in a 52% loss of the activity toward 4-MU-NeuAc. Therefore, the effects of various substances on the activity were investigated. As shown in Table II, the enzyme activity was stabilized to some extent by addition of PMSF or divalent cations such as Mn^{2+}, Ca^{2+}, Mg^{2+} and Zn^{2+} during storage at 4 °C, pH 5.5 for 48 h. Of these divalent cations, Mn^{2+} was most effective. The enzyme activity was slightly inhibited in the presence of these divalent cations but not PMSF. NeuAc, Hg^{2+} and Fe^{3+} did not stabilize the activity but inhibited it significantly. Lactose, leupeptin, EDTA and dithiothreitol were all without stabilizing effect; the latter two were rather inhibitory to the enzyme activity.

Fractionation of the Lysosomal Sialidase

When the lysosomal fraction was subjected to hypotonic disruption followed by

TABLE III. Fractionation of Sialidase from Mouse Liver Lysosomal Fraction

Fraction	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Recovery (%)	Purification (-fold)
Sialyllactose					
Lysosomal fraction	1161	722	0.6	100	1.0
Lysosomal supernatant	325	518	1.6	72	2.6
Ammonium sulfate precipitate[a]					
Soluble	48	288	6.0	40 (68)[b]	9.7
Insoluble	36	201	5.6	28	9.0
4-MU-NeuAc					
Lysosomal fraction	1161	2069	1.8	100	1.0
Lysosomal supernatant	325	1273	3.9	62	2.2
Ammonium sulfate precipitate[a]					
Soluble	48	469	9.8	23 (27)[b]	5.5
Insoluble	36	83	2.3	4	1.3

a) The 60% ammonium sulfate precipitate was soluble in the buffer, but a part of the activity was insolubilized during dialysis. *b*) Total recovery in 60% ammonium sulfate precipitate.

TABLE IV. Partial Purification of Sialidase from Mouse Liver Lysosomal Soluble Fraction

Fraction	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Recovery (%)
Sialyllactose				
Ammonium sulfate precipitation soluble part	5.4	214	39.9	100
DEAE-cellulose				
Unadsorbed fraction	0.14	2.4	17.7	1.1
Adsorbed fraction	0.06	1.6	27.0	0.7
4-MU-NeuAc				
Ammonium sulfate precipitation soluble part	5.4	859	160	100
DEAE-cellulose				
Unadsorbed fraction	0.14	15	112	1.8
Adsorbed fraction	0.06	19	318	2.2

centrifugation at $15000 \times g$, approximately 72% of the original activity toward sialyllactose and 62% of that toward 4-MU-NeuAc were solubilized in the resultant supernatant (Table III) while 20% of the activity toward sialyllactose and 4% of that toward 4-MU-NeuAc remained in the pellet. The supernatant was further fractionated with ammonium sulfate, and the precipitate formed at 60% saturation exhibited 68% of the original activity toward sialyllactose and 27% toward 4-MU-NeuAc, while the supernatant showed little activity toward either substrate. This precipitate was soluble in the buffer but a part of the activity was insolubilized during the dialysis. The soluble enzyme recovered from the dialysate was purified 9.7-fold (toward sialyllactose) and 5.5-fold (toward 4-MU-NeuAc) from the lysosomal pellet (Table III).

The soluble enzyme was further purified by DEAE-cellulose column chromatography. The sialidase activity emerged from the column as unadsorbed and adsorbed fractions. Recently, the human placental sialidase was partially purified by Verheijen *et al.*,[13b] who showed that it exists as a complex with β-galactosidase. In the present case, most of the β-galactosidase activity emerged as the unadsorbed fraction from the DEAE-cellulose column,

TABLE V. Substrate Specificity of Mouse Liver Sialidases

Substrates[a]	Lysosomal fraction		Microsomal fraction		Polytron fraction		Lysosomal soluble fraction	
	Released NeuAc (nmol)	Relative activity (%)	Released NeuAc (nmol)	Relative activity (%)	Released NeuAc (nmol)	Relative activity (%)	Released NeuAc (nmol)	Relative activity (%)
Oligosaccharides								
Sialyllactose					2.0	100		
α(2→3)	12.7	100	7.0	100	n.d.[b]	—	9.2	100
α(2→6)	8.0	63	4.6	66	n.d.[b]	—	4.7	51
Siaα(2→3)Galβ(1→3)-GalNAc*ol*	7.7	61	4.3	61	1.3	66	9.9	108
Glycopeptides								
Fetuin	9.3	73	4.9	70	1.2	61	5.9	64
Bovine submaxillary mucin	2.9	23	2.8	40	n.d.[b]	—	1.8	20
Glycoproteins								
Fetuin	3.3	26	1.4	20	0.2	12	0.6	7
Orosomucoid	3.8	30	1.4	20	0.5	26	0.9	10
Transferrin	3.2	25	1.2	17	0.3	16	0.3	3
Bovine submaxillary mucin	0	0	1.1	16	0.5	23	0.5	5
Gangliosides								
Bovine brain mixed[c]	2.2	17	3.1	44	1.3	63	0	0
Bovine brain mixed[d]	13.3	105	7.7	110	n.d.[b]	—	0	0
Colominic acid	0.1	1	2.8	40	0.5	26	1.0	11

a) The substrates (100 nmol as bound NeuAc) were incubated with the lysosomal fraction (1 mg as protein), the microsomal fraction (2 mg as protein), the Polytron soluble fraction (4 mg as protein) or the lysosomal soluble fraction (150 μg as protein). *b*) Not determined. *c*) In the absence of sodium cholate. *d*) In the presence of sodium cholate.

though a little activity could be detected in the adsorbed fraction. The sialidase activity of the adsorbed fraction toward 4-MU-NeuAc was further purified 2-fold from the soluble part of the ammonium sulfate precipitate by DEAE-cellulose column chromatography, while the sialidase specific activity of the adsorbed fraction toward sialyllactose was reduced in comparison with that of the same soluble part (Table IV). The unadsorbed fraction from the DEAE-cellulose column also contained significant sialidase activity toward 4-MU-NeuAc and sialyllactose, but the specific activity of this fraction was lower than that of the soluble part. Only 0.7—2.2% of the sialidase activities toward these substrates were recovered in the unadsorbed and adsorbed fractions on DEAE-cellulose column chromatography.

Substrate Specificity

Table V summarizes the sialidase activities in mouse liver toward various substrates (100 nmol as bound sialic acid) in the assay mixture. Although the substrate specificity of rat liver sialidase has been reported in detail,[5f,g] little information on substrate specificity is available for the mouse liver enzyme. Therefore, the substrate specificity of mouse liver sialidase was investigated and compared with that of the rat liver enzyme. All fractions of sialidases were able to hydrolyze a wide variety of sialooligosaccharides, sialoglycoproteins and gangliosides. The lysosomal and microsomal sialidases were preferentially able to hydrolyze bovine brain ganglioside mixture (in the presence of sodium cholate) in all substrates, while the lysosomal sialidase hydrolyzed glycoproteins (orosomucoid and transferrin) better than the microsomal enzyme. The sialidase activities of the lysosomal fraction toward glycoproteins were little influenced by the presence of a mixture of leupeptin (40 μg) and PMSF (1 mM) (data

TABLE VI. Mouse Liver Sialidase Activity toward Gangliosides

Gangliosides[a]	Lysosomal fraction		Microsomal fraction	
	Released NeuAc (nmol)	Relative activity (%)	Released NeuAc (nmol)	Relative activity (%)
Bovine brain mixed	4.8	100	8.3	100
G_{M3}	5.5	115	9.6	115
G_{D1a}	5.2	109	9.5	114
G_{D1b}	4.6	96	8.4	101

a) Gangliosides (100 nmol as bound NeuAc) were incubated with the lysosomal fraction (1 mg as protein) or the microsomal fraction (2 mg as protein) in the presence of sodium cholate (0.4 mg).

TABLE VII. Substrate Specificity of DEAE-Cellulose Unadsorbed and Adsorbed Sialidases from Mouse Liver Lysosomal Soluble Fraction

Substrates[a]	Unadsorbed fraction		Adsorbed fraction	
	Released NeuAc (nmol)	Relative activity (%)	Released NeuAc (nmol)	Relative activity (%)
Sialyllactose[b]	0.4	100	0.2	100
Fetuin glycopeptides	0.1	16	0.4	170
Fetuin	0.2	54	0	0
Ganglioside mixture	0	0	0	0
Colominic acid	0.1	30	0.2	71

a) The substrates (100 nmol as bound NeuAc) were incubated with DEAE-cellulose unadsorbed fraction (7 μg as protein) or adsorbed fraction (3 μg as protein). *b*) An α(2→3)- and α(2→6)sialyllactose mixture.

not shown). The lysosomal sialidase was incapable of hydrolyzing bovine submaxillary mucin, but the microsomal and the Polytron-solubilized fractions hydrolyzed this substrate significantly. The lysosomal and microsomal sialidases attacked oligosaccharides possessing an α(2→3)sialyl linkage more efficiently than those containing an α(2→6)sialyl linkage. The microsomal sialidase hydrolyzed colominic acid possessing an α(2→8)sialyl linkage efficiently, but the lysosomal sialidase fraction showed negligible activity toward this substrate. As for gangliosides, it should be noted that the lysosomal and microsomal sialidases cleaved G_{M3}, G_{D1a} and G_{D1b} (Table VI). When G_{D1a} and G_{D1b} were treated with the lysosomal or the microsomal sialidases, G_{M1} was detected by TLC. Asialo G_{M3} was also detected as a reaction product of G_{M3} by TLC. When G_{M1} and G_{M2} were treated with these sialidases, decreases of G_{M1} and G_{M2} were detected in the reaction mixture by TLC but no reaction products could be detected by TLC. Weak lysosomal and microsomal sialidase activities toward G_{M1} were detected by TBA assay, but the activities toward G_{M2} could not be assayed by the TBA method because only a small amount of G_{M2} was available.

The activities of the fractionated lysosomal sialidase (soluble enzyme from 60% ammonium sulfate precipitate) towards various substrates relative to that towards α(2→3)-sialyllactose are also shown in Table V. Although fetuin was not significantly hydrolyzed by this enzyme, sialoglycopeptides prepared by pronase digestion of fetuin were hydrolyzed rather efficiently. Although the lysosomal fraction hydrolyzed gangliosides rather efficiently, the fractionated lysosomal sialidase failed to exhibit this activity even in the presence of sodium cholate. It should be noted that colominic acid bearing an α(2→8)sialyl linkage was

hydrolyzed without any previous treatment. These results indicate that the enzyme which can hydrolyze only low-molecular-weight substrates such as sialooligosaccharides and sialoglycopeptides (except colominic acid) was fractionated as the soluble sialidase from mouse liver lysosomal fraction.

The DEAE-cellulose-unadsorbed fraction liberated sialyl residues from sialyllactose, fetuin, colominic acid and fetuin glycopeptide, while the adsorbed fraction liberated them from sialyllactose, fetuin glycopeptides and colominic acid. The relative activities of these enzymes towards various substrates are summarized in Table VII. The adsorbed fraction exhibited almost the same substrate specificity as the soluble lysosomal sialidase, but the unadsorbed fraction hydrolyzed fetuin more efficiently than fetuin glycopeptide.

Partial Purification of the Lysosomal Sialidase

The soluble lysosomal fraction was reacted with Con A solution, then the reaction mixture was centrifuged. The supernatant was applied to a Sephadex G-200 column. The column was washed with 50 mM sodium acetate buffer (pH 6.0) containing 1 M NaCl, 10 mM $MnCl_2$ and 1 mM PMSF, then the Con A-binding substance was eluted with methyl α-D-mannoside. The sialidase activity toward 4-MU-NeuAc was detected in the precipitate after Con A addition, and Sephadex G-200 unadsorbed and adsorbed fractions (Fig. 1). β-Galactosidase activity also detected in all these fractions.

When the soluble lysosomal fraction was subjected to HPLC on coupled TSK-GEL G5000PW and G4000PW columns, the sialidase activity toward 4-MU-NeuAc was eluted as two peaks, one major and the other minor, leaving most of the protein contaminant behind (Fig. 2). The sialidase activity toward sialyllactose and the β-galactosidase activity were also eluted as distinct single peaks, but the retention time of β-galactosidase was close to that of the minor 4-MU-NeuAc sialidase. The relative molecular weight (M_r) of the major 4-MU-NeuAc sialidase was estimated to be 680000 by HPLC with immunoglobulin M (M_r 970000), thyroglobulin (M_r 669000), ferritin (M_r 450000) and catalase (M_r 240000) as standards. The HPLC was also performed with the buffer containing 0.1 M NaCl, but the retention times of 4-MU-NeuAc sialidase and β-galactosidase were not changed. When the same fraction was

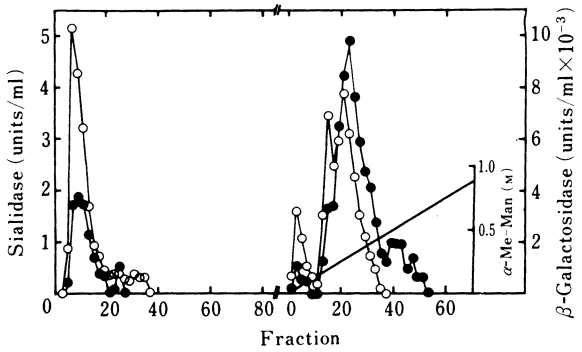

Fig. 1. Sephadex G-200 Column Chromatography of the Lysosomal Soluble Sialidase Mixed with Con A

The lysosomal soluble sialidase (1.4 mg protein) was mixed with Con A, followed by centrifugation. The supernatant was applied to a Sephadex G-200 column (2.0 × 5.0 cm). The column was eluted with 50 mM sodium acetate buffer (pH 6.0) containing 1 M NaCl, 10 mM $MnCl_2$ and 1 mM PMSF, followed by a linear gradient of methyl α-D-mannoside in the same buffer, and 2 ml fractions were collected. ○, 4-MU-NeuAc sialidase; ●, β-galactosidase; ——, methyl α-D-mannoside concentration.

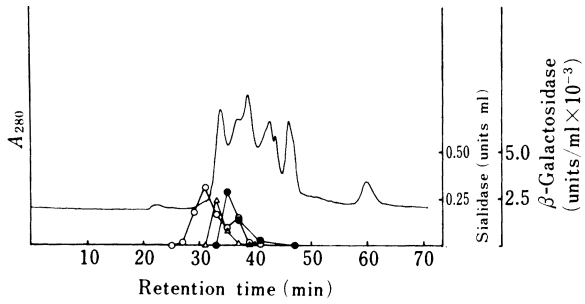

Fig. 2. HPLC of the Lysosomal Soluble Sialidase

The sample (470 μg protein) was applied to an HPLC instrument equipped with coupled TSK-GEL G5000PW and G4000PW columns (0.75 × 60 cm, each). The column was eluted with 50 mM sodium acetate buffer (pH 5.5) containing 10 mM $MnCl_2$ and 1 mM PMSF, and 1 ml fractions were collected. ○, 4-MU-NeuAc sialidase; △, sialyllactose sialidase; ●, β-galactosidase; ——, absorbance at 280 nm.

applied to a Sephadex G-200 column, the sialidase activity was eluted as a single peak near the void volume (data not shown).

When the DEAE-cellulose adsorbed fraction was reacted with Con A and then subjected to Sephadex G-200 affinity chromatography, the sialidase activity was mainly detected in the Sephadex G-200 unadsorbed fraction. This Sephadex G-200 unadsorbed fraction was further purified by HPLC, and sialidase activity toward 4-MU-NeuAc was purified as a single protein peak. This purified enzyme showed the same retention time as the major 4-MU-NeuAc sialidase, as shown in Fig. 2, but the specific activity was decreased because of the lability of this sialidase.

Discussion

The subcellular distribution of mammalian sialidase has been studied in human skin fibroblasts,[10c] rat liver[5a,b] and human liver.[11a] In rat liver, sialidase activity is localized in cytosol,[5e] plasma membrane,[5d] Golgi apparatus[5c] and lysosomes.[5a,b,e] Miyagi and Tsuiki also detected sialidase activity in the lysosomal, microsomal and cytosolic fractions from rat liver.[5f,g]

In the present study, the subcellular distribution of sialidase activity in mouse liver was investigated as a preliminary experiment. We found sialidase activity in the lysosomal, microsomal and $1000 \times g$ pellets of mouse liver homogenate when the fractionation was carried out by the same method used in the case of rat liver.[5f] In rat liver, the major sialidase was localized in lysosomes, and was most active at pH 4.5, but the cytosolic enzyme was most active at pH 6.5, and the microsomal enzyme showed a double peak at pH 4.5 and 6.5.[23] The mouse enzyme activity was predominantly localized in the lysosomal fraction. However, the sialidase activity which is most active at neutral pH was not detected in mouse liver, and little sialidase activity was detected in the cytosolic fraction. The subcellular localization of sialidase in mouse liver is similar to that of human liver enzyme because sialidase activity in human liver was also predominantly detected in the lysosomal fraction,[11a] and the microsomal and nuclear ($1500 \times g$ pellet) fractions contained some activity, but no cytosolic activity could be detected.[11a] Schengrund *et al.* reported that the nuclear fraction of rat liver cells contained the plasma membrane fraction, and the plasma membrane fraction exhibited potent sialidase activity towards ganglioside mixture and sialyllactose.[5d] Therefore, the activity of the nuclear fraction of human liver and $1000 \times g$ pellet fraction of mouse liver may correspond to the plasma membrane enzyme of non-disrupted cells.

Mammalian sialidases are very unstable and this lability prevents their purification. Mouse liver lysosomal sialidase activity was also very unstable, but this enzyme was stabilized partially in the presence of a protease inhibitior, PMSF. This result suggested that the enzyme is inactivated by the action of some protease(s).

When the lysosomal fraction was solubilized by hypotonic disruption followed by ammonium sulfate precipitation, the resulting soluble enzyme was much more active against oligosaccharides and glycopeptides but inactive against glycoproteins and glycolipids in comparison with the lysosomal fraction, which is active against glycoproteins and glycolipids in addition to oligosaccharides and glycopeptides. Miyagi and Tsuiki reported that rat liver lysosomes contain at least two kinds of sialidase, one located in the intralysosomal space and the other in the lysosomal membrane.[5f] In mouse liver, it was observed that the undisrupted pellet in the lysosomal fraction was active toward gangliosides (T. Nagai and H. Yamada, unpublished observation). They also reported that protease inhibitors markedly reduced the rat liver intralysosomal sialidase activity toward glycoproteins, and suggested that fragmentation of the protein core by protease may be prerequisite to glycoproteins becoming substrates of this enzyme.[5f] In the case of mouse liver lysosomal sialidase, it was expected that

the fractionated lysosomal fraction would be less active toward glycoproteins on removal of the protease. However, when the sialidase activities of the original lysosomal fraction of mouse liver towards fetuin, orosomucoid and transferrin were assayed in the absence and presence of the mixture of leupeptin and PMSF, the activities of this fraction toward all substrates tested were little influenced by the protease inhibitors. These results suggested that the lysosomal fraction of mouse liver contained at least two kinds of sialidases. One is active toward low-molecular substrates, and the other is active towards glycolipids and glycoproteins. One of the lysosomal sialidases in mouse liver may be related to the metabolic turnover of serum glycoproteins such as orosomucoid and transferrin.

The microsomal sialidase of mouse liver was most active toward gangliosides among the tested substrates in the presence of sodium cholate as a detergent. The presence of membrane sialidase has been reported in rat liver,[5d] calf brain[24] and cultured human skin fibroblasts.[10c] These sialidases hydrolyze gangliosides preferentially. In mouse liver, the microsomal sialidase may also hydrolyze the membrane-bound gangliosides and regulate the biological functions of gangliosides.

The mouse liver microsomal fraction preferentially hydrolyzed bovine submaxillary mucin, which is known to have 9-*O*-acetylated sialic acid residues.[25] Rat liver partially purified intralysosomal and purified cytosolic sialidases and human placental purified sialidase were incapable of hydrolyzing bovine submaxillary mucin.[5f,g,13d] Since sialate-*O*-acetylesterase activity has been detected in mammalian and human livers when determined with 4-*O*-acetyl- and 9-*O*-acetylsialic acid as substrates,[11a,26] it was expected that the microsomal sialidase would hydrolyze bovine submaxillary mucin after the removal of *O*-acetyl groups by sialate-*O*-acetylesterase. However, the present result indicated that the microsomal sialidases in mouse liver are able to hydrolyze *O*-acetylated sialic acid residues, because when the reaction product formed from bovine submaxillary mucin by the mouse microsomal sialidase was saponified before the TBA reaction, the absorbance in the TBA reaction was greatly increased (T. Nagai and H. Yamada, unpublished observation).

Miyagi and Tsuiki reported that lysosomal soluble sialidase of rat liver emerged from Sephadex G-200 at the void volume in the absence of KCl, but when the enzyme recovered from the carboxymethyl (CM)-cellulose column was rechromatographed on Sephadex G-200 in the presence of 0.3 M KCl, four peaks of sialidase activity were found, and the M_r of the slowest (the major) peak was estimated to be 60000.[5f] On the other hand, Hiraiwa *et al.* reported that the human placental soluble sialidase was eluted near the void volume on HPLC in the presence of 0.1 M NaCl.[13c,d] The molecular weight of the lysosomal soluble sialidase from mouse liver was estimated to be 680000 by HPLC, and this molecular weight did not change on HPLC even in the presence of 0.1 M NaCl. These results indicated that the molecular weight of mouse liver lysosomal soluble sialidase was higher than that of the rat liver lysosomal sialidase, but the possibility that this enzyme forms an aggregate could not be ruled out.

Verheijen *et al.* reported that sialidase and β-galactosidase are not separable and that the two enzymes are present as a complex in human placenta[13b] and bovine testis.[27] However, Hiraiwa *et al.* recently showed that these two enzymes in human placenta were separable by HPLC.[13c,d] The present study confirmed that β-galactosidase and sialidase in mouse liver are separable by HPLC. When this soluble lysosomal fraction was subjected to DEAE-cellulose column chromatography, most of the β-galactosidase was separated from DEAE-cellulose-adsorbed sialidase. Verheijen *et al.* also demonstrated that the activated and stabilized human lysosomal sialidase exists as a complex with β-galactosidase.[13b] The present results showed that lysosomal soluble sialidase activity in mouse liver became very unstable after purification by DEAE-cellulose column chromatography and HPLC, even when protease inhibitor was present during the purification step. It was expected that this lability of the partially purified

sialidase may be caused by the removal of β-galactosidase. However, we could not separate the sialidase completely from β-galactosidase activity in this study.

So far, sialidases have only been purified from rat heart,[7] rat liver,[5g] rabbit spermatozoal acrosomes[8] and human placenta.[13d,28] Purification of the lysosomal sialidase in mouse liver, now in progress in this laboratory, should provide further insights into the biological function of mammalian sialidase.

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