Purification and Characterization of Sialidase from Porcine Liver

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Sialidase [E.C.3.2.1.18] has previously been purified from porcine liver by procedures including extraction, ammonium sulfate precipitation, concanavalin A-Sepharose adsorption, activation, CM-Sepharose ion exchange chromatography, and HPLC on a Shim pack Diol 300 column. Two sialidase preparations, sialidase I and II, were obtained by CM-Sepharose column chromatography and were eluted with pH 4.5 and 5.0 buffers, respectively. The two enzyme preparations showed the same optimum pH, pH stability, and specificities for natural substrates. The two final preparations contained β -galactosidase activity and showed three protein components of 64, 30, and 21 kDa with sodium dodecyl sulfate-polyacrylamide gel electrophoresis, which are derived from the β -galactosidase multimer. The anti- β -galactosidase multimer antiserum was able to precipitate sialidase activity. It is likely that porcine liver sialidase exists as a multienzyme complex with β -galactosidase and carboxypeptidase (protective protein).

Key word sialidase; neuraminidase; β -galactosidase; protective protein; multienzyme complex

Sialidase [Neuraminidase E.C.3.2.1.18], which catalyzes the hydrolysis of terminal sialic acid residues from oligosaccharides, glycoproteins, and glycolipids, has an important function in the lysosomes of mammalian cells.²⁾ A deficiency of sialidase in man is associated with several inherited lysosomal storage disorders, which can now be classified biochemically into two types: (1) an isolated sialidase deficiency and (2) galactosialidosis being a combined deficiency of sialidase and β -galactosidase.^{3,4)} Although lysosomal sialidase in various animals and human tissues has been investigated, the enzyme has not as yet been well characterized because of the difficulty in obtaining it in a soluble and purified form.⁵⁾

Some sialidase has been copurified with β -galactosidase and carboxypeptidase (protective protein), apparently forming a stable aggregated complex.^{6–9)} In a previous study,¹⁰⁾ we demonstrated that the sialidase purified from human placenta gave five protein bands with molecular weights of 78, 64, 46, 30, and 20 kDa with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Of these, the 64 kDa protein has been identified as acid β -galactosidase, 30 and 20 kDa proteins as carboxypeptidase components,¹¹⁾ the 46 kDa protein as *N*-acetyl- α -galactosaminidase,¹²⁾ and the 78 kDa protein as immunoglobulin M.¹³⁾ Despite the characterization of these proteins, identification of the protein corresponding to sialidase and the relationship between sialidase and other protein components remains unclear.

For the purification procedure of sialidase from human placenta, the *p*-aminophenyl thio- β -D-galactoside-CH-Sepharose (PATG-Sepharose) which is a β -galactosidase-specific affinity column has been applied.¹⁰⁾ To ascertain whether mammalian sialidase is always purified as a multienzyme complex or not, we purified sialidase from porcine liver by the procedures without using PATG-Sepharose affinity chromatography. In this paper, we describe the properties of sialidase as a complex with β -galactosidase and other proteins.

MATERIALS AND METHODS

Materials Concanavalin A (Con A)-Sepharose and CM-Sepharose were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). 4-Methylumbelliferyl(4-MU)- β -galactoside from Koch-Light Laboratories (Colnbrook, U.K.), transferrin and fetuin from Sigma Chemical Co. (St. Louis, U.S.A.). Colominic acid and α -D-methylmannoside were purchased from Nacalai Tesque Co. (Kyoto, Japan), and sodium cholate from Calbiochem-Behring (La Jolla, U.S.A.). The sialyllactose isomers, α (2-3) and α (2-6) sialyllactose, were purchased from Dextra Laboratories LTD (Reading, U.K.). Bovine submaxillary glycoprotein (BSM), gangliosides GM1, GM2, GM3, GD1a, GD1b were prepared as previously described.¹⁴⁾ 4-MU- α -N-acetylneuraminic acid was synthesized by the method described previously.¹⁵⁾ All other chemicals were obtained from commercial sources and were of the highest grade available.

Enzyme Assay Sialidase activities toward 4-MU- α -N-acetylneuraminic acid, sialyllactose, colominic acid, glycoproteins, and gangliosides were determined as previously described.¹⁴⁾ When gangliosides were used as substrates, sodium cholate (200 μ g) was added to the reaction mixture. After incubation at 37 °C for an appropriate time, the sialic acid released was determined by the thiobarbituric acid method.¹⁴⁾ One unit of enzyme was defined as the amount which catalyzed the release of 1 nmol of sialic acid per min. β -Galactosidase activity was determined with 4-MU- β -D-galactoside as a substrate.¹⁶⁾

Production of Antisera and Immunoprecipitation Two male white rabbits were immunized by subcutaneous multiple injections of the β -galactosidase multimer and monomer purified from bovine brain,¹⁷⁾ respectively. Immune sera were collected at 10 d after the final booster injection. Immunotitration using the anti-bovine β -galactosidase multimer and monomer antisera was performed by the procedure described previously.¹⁰⁾

Analytical Methods Protein concentrations were determined by spectrophotometry at 280 nm or by a bicinchoninic

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acid assay¹⁸⁾ using bovine serum albumin as the standard. SDS-PAGE was carried out according to the procedure described by Laemmli.¹⁹⁾ Gels were stained with silver nitrate according to the method described by Merrill *et al.*²⁰⁾

Purification of Sialidase All isolation and purification procedures were carried out 0–4 °C unless otherwise indicated.

Step 1. Extraction and Ammonium Sulfate Precipitation: Fresh porcine liver (160 g) was homogenized in 6 volumes (v/w) of 0.25 M sucrose containing 0.5 mM EDTA with a Polytron homogenizer for 2 min. The homogenate was centrifuged at 10000×*g* for 10 min and the pellet was discarded. The supernatant was centrifuged at 105000×*g* for 1 h and the residue was discarded. To the supernatant, 1.0 M potassium phosphate buffer (pH 7.0) was added to obtain a 10 mM solution, and then solid ammonium sulfate was added to 35% saturation. After standing overnight, the precipitated protein which contained sialidase was collected by centrifugation (25000×*g*, 20 min) and dissolved in 40 ml of 10% glycerin. The solution was centrifuged at 25000×*g* for 20 min to remove insoluble materials.

Step 2. Con A-Sepharose Affinity Chromatography and Activation: To the supernatant, 1.0 M potassium phosphate buffer (pH 6.5) and 5.0 M NaCl were added to obtain a 30 mM phosphate buffer solution containing 0.5 M NaCl and 10% glycerin (Buffer A). The enzyme solution was applied to a Con A-Sepharose column (1×15 cm) which had been equilibrated with Buffer A. After loading, the column was washed with 1 l of equilibrated Buffer A. Then, the column was brought to 37 °C and washed with 50 ml of 20 mM potassium phosphate buffer (pH 6.0) containing 0.5 M NaCl and 10% glycerin (Buffer B) and 200 ml of Buffer B containing 10 mM methyl α -D-mannopyranoside. Sialidase was eluted with Buffer B containing 0.4 M methyl α -D-mannopyranoside. The fractions containing sialidase activity were pooled and then concentrated by ultrafiltration with a Diaflo YM-100 membrane. To the enzyme solution, 1.0 M acetate buffer (pH 4.8) was added to obtain a 40 mM solution, the solution was incubated at 37 °C for 120 min to activate the enzyme and then centrifuged at 15000×*g* for 20 min to remove insoluble matter.

Step 3. CM-Sepharose CL-6B Column Chromatography:

The supernatant obtained in Step 2 was dialyzed against 20 mM acetate buffer (pH 4.0) containing 0.15 M NaCl and 10% glycerin for 6 h and applied to a CM-Sepharose CL-6B column (1.5×3 cm) previously equilibrated with the same buffer. The column was eluted with 20 mM acetate buffer (pH 4.0) containing 0.15 M NaCl and 10% glycerin to wash out the protein not adsorbed to the column, and then with 20 mM acetate buffers (pH 4.5 and 5.0) containing 0.15 M NaCl and 10% glycerin. Sialidase was eluted with both 20 mM acetate buffers (pH 4.5 and pH 5.0) containing 0.15 M NaCl and 10% glycerin. The two sialidase fractions were respectively pooled and concentrated by ultrafiltration of a Diaflo membrane YM-100.

Step 4. HPLC: The enzyme solution was applied to a Shim-pack Diol-300 column (0.7×25 cm). The column was eluted with 20 mM acetate buffer (pH 4.6) containing 0.15 M NaCl and 10% glycerin. Fractions were collected at 100 μ l per tube.

RESULTS

Purification of Sialidase In this experiment sialidase was purified in a different manner from that described previously.¹⁰⁾ Sialidase was purified from porcine liver by successive procedures including extraction, ammonium sulfate precipitation, Con A-Sepharose chromatography, activation, CM-Sepharose ion exchange chromatography, and HPLC on a Shim-pack Diol-300 column. Of these preparative processes, the Con A-Sepharose affinity chromatography was very useful for purification of the enzyme because many protein contaminants including some glycoproteins were removed from the enzyme (Fig. 1) and the specific activity of sialidase was increased 30 fold. When the enzyme preparation obtained by Con A-Sepharose column chromatography was incubated at pH 4.8 and at 37 °C, a 2.5 fold increase in sialidase activity was observed. The activation of sialidase was affected by the pH of the incubation medium and the most effective activation was observed in the pH range between 4.5 and 5.0 (data not shown). Sialidase activity was also increased by placing the preparation at 4 °C over a period of a few weeks. When the enzyme preparation obtained in Step 2 was applied to a CM-Sepharose column, sialidase

Fig. 1. Con A-Sepharose Column Chromatography of the Sialidase Preparation Obtained by Ammonium Sulfate Precipitation

The sample (ca. 4 g protein) was applied to a Con A-Sepharose column (1×15 cm) which had been equilibrated with Buffer A. The column was eluted with Buffer A followed by Buffer B and with the same buffers containing 0.01 M and 0.4 M methyl α -D-mannoside, and 10 ml fractions were collected. -----, Absorption at 280 nm; ●, sialidase activity; ○, β -galactosidase activity.

Fig. 2. CM-Sepharose CL-6B Column Chromatography of the Sialidase Preparation Obtained by Con A-Sepharose Chromatography

The sample (ca. 12 mg protein) was applied to a CM-Sepharose CL-6B column (1.5×3 cm) which had been equilibrated with 0.02 M acetate buffer (pH 4.0) containing 0.15 M NaCl and 10% glycerin. The column was eluted with the same buffer, followed by 0.02 M acetate buffers of pH 4.5 and pH 5.0 containing 0.15 M NaCl and 10% glycerin, and 3.5 ml fractions were collected. -----, Absorption at 280 nm; ●, sialidase activity; ○, β -galactosidase activity.

activity was separated into two fractions. One was eluted at pH 4.5 (sialidase I) and the other at pH 5.0 (sialidase II) as shown in Fig. 2. By this column chromatography, β -galactosidase was also eluted in the pH 4.5 and the 5.0 fractions together with sialidases, although most of the β -galactosidase was eluted at pH 4.5. The two fractions containing sialidase activity were pooled separately and used at the next purification step, HPLC on a Shim pack Diol 300 column; the results are shown in Fig. 3. Sialidase and β -galactosidase were partially separated by gel filtration. This filtration yielded three peaks with different molecular weights: >700 kDa (a), 600–700 kDa (b), and 120 kDa (c); peak b and peak c did not contain sialidase activity. Table 1 summarizes the specific activities during the purification procedure for the sialidase from porcine liver. The two finally purified preparations showed a purification factor of 4700 for sialidase I and 2300 for sialidase II; even in the final preparation, they showed 6.8 and 4.6 times higher specific activities of the β -galactosidase than that of the sialidase, respectively.

SDS-PAGE The final preparations of sialidase I and II were subjected to SDS-PAGE to investigate the subunit composition of the two enzymes. Sialidase I exhibited two strong protein bands with molecular weights of 64 and 54 kDa and five weak bands with molecular weights of 40, 35, 30, 26 and 21 kDa (Fig. 4). Sialidase II gave a strong band with molecular weight of 54 kDa, and several additional bands with higher than sialidase I.

Optimum pH and pH Stability The effect of pH on the activities of the two sialidases was determined in citrate, acetate and phosphate buffers using 4-MU- α -N-acetylneuraminic acid as a substrate. As shown in Fig. 5(a), sialidase I and II exhibited the same optimum pH and were most active at pH 4.2. The optimum pHs for the hydrolysis of natural substrates, sialyllactose, fetuin and ganglioside GM3, lay between 4.0 and 4.5, and no significant differences were observed between sialidase I and II (Fig. 5(b)). The stability of the two enzymes at various pHs was determined by keeping the enzymes in 0.05 M citrate, acetate and phosphate buffers ranging in pH from 3.0 to 7.0 at 37°C prior to standard assaying at pH 4.2. As shown in Fig. 6, both sialidases were quite stable at pH 4.0 or 5.2, but rapidly lost their activity at

Fig. 3. HPLC of the Sialidase Preparation Obtained by CM-Sepharose CL-6B Column Chromatography

The sample (ca. 100 μ g protein) was applied to an HPLC instrument equipped with a Shim pack Diol 300 column (0.75×25 cm). The column was eluted with 0.02 M acetate buffer (pH 4.6) containing 0.15 M NaCl and 10% glycerin, and 100 μ l fractions were collected. -----, Absorption at 280 nm; ●, sialidase activity; ○, β -galactosidase activity.

pH 3.4 or pH 7.0. At 4°C, the two sialidases were stable in the pH range of 4.0 to 6.0, but had moderately decreased activities under pH 3.4 and at pH 7.0 (data not shown). The two enzymes showed the same profile of pH stability.

Substrate Specificity The substrate specificities of sialidase I and II toward sialyloligosaccharides, glycoproteins, and gangliosides were examined, and their activities toward various substrates are summarized in Table 2. As to the relative hydrolysis rates, sialyllactose, colominic acid, fetuin, GM3, GD1a, and GD1b were hydrolyzed at higher rates than the others. Transferin, bovine submaxillary mucin, GM1, and GM2 were hydrolyzed moderately by the two sialidases from porcine liver.

Table 1. Purification of Sialidase from Porcine Liver

	Total protein (mg)	Sialidase				β -Galactosidase
		Total activity (U)	Specific activity (U/mg)	Recovery (%)	Purification (fold)	Specific activity (U/mg)
105000 $\times$ g	110880	17280	0.2	100.0	1.0	9.7
(NH ₄) ₂ SO ₄ precipitation	8107	9800	1.2	56.7	7.8	9.4
Con A Sepharose	25.5	938	36.8	5.4	236	1010
Post activation	16.2	1583	97.7	9.2	627	1590
CM Sepharose pH 4.5 (I)	1.53	149	97.4	0.86	625	7495
pH 5.0 (II)	0.87	214	246.0	1.24	1578	2220
Gel filtration (HPLC) (I)	0.06	45.7	761.7	0.26	4887	5080
(II)	0.22	80.0	363.6	0.46	2333	1715

Fig. 4. SDS-Polyacrylamide Gel Electrophoresis of Purified Sialidase I and II

Each enzyme (2.5 μ g protein) was subjected to electrophoresis in a 10% gel and the gel was stained with silver nitrate. The scale of molecular weight was determined using the following standards (Bio Rad Lab.); phosphorylase B (111 kDa), bovine serum albumin (77 kDa), ovalbumin (48.2 kDa), carbonic anhydrase (33.8 kDa), soybean trypsin inhibitor (28.6 kDa), and lysozyme (20.5 kDa).

Fig. 5. Effect of pH on the Activities of the Porcine Liver Sialidases

(a) 4-MU-Sialidase: ●, sialidase I; ○, sialidase II. (b) Hydrolysis of natural substrates by sialidase I. △, Sialyllactose; ▲, fetuin; □, G_{M3}.

Immunological Analysis To investigate whether the final sialidase preparations occur as the enzyme complex with β -galactosidase, immunoprecipitation experiments were performed using anti- β -galactosidase multimer and anti- β -

Fig. 6. Effect of pH on the Stability of Sialidase Activity

The enzyme was placed in 0.05 M citrate, acetate and phosphate buffers ranging in pH from 3.0 to 7.0 at 37 °C and then the sialidase activity was measured with 4MU-*N*-acetylneuraminic acid as a substrate at pH 4.2. (a) Sialidase I, (b) sialidase II, △, pH 3.0; ▲, pH 3.4; ○, pH 4.0; ●, pH 5.2; □, pH 6.0; ■, pH 7.0.

galactosidase monomer antisera. As shown in Fig. 7, the anti- β -galactosidase multimer antibody precipitated the activities of sialidase I and II as well as human placental sialidase in a dose dependent manner, while the anti- β -galactosidase monomer antibody did not precipitate these activities.

DISCUSSION

Lysosomal sialidases from mammalian tissue are often copurified with β -galactosidase.⁶⁻⁹ In our previous study, we have highly purified sialidase from human placenta using PATG-Sepharose affinity chromatography which is a β -galactosidase-specific column.¹⁰ Although most of the β -galactosidase was removed by HPLC on a Shim-pack Diol 300 column, the final preparation gave three bands with molecular weights of 64, 30, and 20 kDa which were derived from the β -galactosidase multimer, in addition to the bands with molecular weights of 78 and 46 kDa on SDS-PAGE. In the present study, we attempted to purify the enzyme without using PATG-Sepharose affinity chromatography. The two sialidase preparations, sialidase I and II, were obtained by CM-Sepharose column chromatography and were eluted with pH 4.5 and pH 5.0 buffers, respectively. Sialidase I was eluted with β -galactosidase at pH 4.5, while sialidase II was accompanied by only a small amount of β -galactosidase at pH 5.0. Although the two preparations were eluted at different pHs from the CM-Sepharose column, they showed almost

Table 2. Substrate Specificity of Sialidase from Porcine Liver

Substrate	Bound NeuAc (nmol)	Sialidase I		Sialidase II	
		Release NeuAc (nmol)	Relative activity (%)	Released NeuAc (nmol)	Relative activity (%)
Oligosaccharides					
Sialyllactose					
α (2-3)	100	30.1	100.0	26.8	100.0
α (2-6)	100	13.9	46.3	11.8	43.9
colominic acid	100	20.4	67.9	15.0	56.0
Glycoprotein					
Fetuin	100	20.7	68.7	13.5	50.3
Trans ferrin	100	1.8	6.1	0.5	1.8
BSM	100	3.1	10.3	1.7	6.4
Gangliosides					
GM1	100	4.7	15.7	3.0	11.0
GM2	100	6.6	21.9	5.3	19.8
GM3	100	21.0	69.7	19.3	72.0
GD1a	100	17.6	58.7	15.1	56.3
GD1b	100	17.5	58.3	14.8	55.2

Fig. 7. Immunoprecipitation of Sialidase Activity by Anti- β -galactosidase Multimer and Monomer Antisera

Sialidase I and II, and human placental sialidase were immunotitrated with anti- β -galactosidase multimer and monomer antisera as described in Materials and Methods. (a) Sialidase I, (b) sialidase II, (c) human placental sialidase, ●, anti- β -galactosidase multimer antiserum; ○, anti- β -galactosidase monomer antiserum, △, non-immune serum.

the same optimum pH, pH stability, and substrate specificity profiles as described above. These findings suggest that the sialidase I and II activities are elicited by the same enzyme protein which associates with different proteins, like β -galactosidase related proteins, to form complexes having different electric charges.

Most of the β -galactosidase was removed by the present procedures without using a PATG affinity column which is specific for β -galactosidase, but we were unable to obtain a sialidase preparation from porcine liver which was sufficiently free from β -galactosidase. The two final preparations, sialidase I and II, both contained β -galactosidase activity and rechromatography of these preparations obtained on HPLC did not completely remove this activity. The purified enzyme, sialidase I, gave seven bands with molecular weights of 64, 54, 40, 35, 30, 26, and 21 kDa on SDS-PAGE. In a previous report,¹⁷⁾ we described that the multimeric form of GM1 β -galactosidase purified from bovine brain gave three bands with molecular weights of 62, 32, and 20 kDa on SDS-PAGE. The anti- β -galactosidase monomer (62 kDa) and anti-

32 kDa protein antisera were specifically reactive with the 62 kDa protein and 32 kDa protein on immunoblotting analysis, respectively (unpublished data). Therefore, the three sub-components with molecular weights of 64, 30, and 21 kDa in the two final preparations of sialidase from porcine liver appear to be derived from the β -galactosidase multimer. However, SDS-PAGE of sialidase I and II both showed a strong band with 54 kDa and weak bands with 40, 35 and 26 kDa, besides the bands of 64, 30, and 21 kDa. A certain protein component in these bands, possibly at 54 kDa, may be produced from sialidase as the molecular mass of the sialidase from bovine testis is known to be 55 kDa using a photoaffinity labeling technique.²¹⁾

The immunoprecipitation experiments showed that the activities of sialidase I and II were precipitated by an anti- β -galactosidase multimer antibody in a dose dependent manner but not by an anti- β -galactosidase monomer antibody, similarly to the case of human placental sialidase which is considered to be a complex with β -galactosidase multimer. This observation indicated that porcine liver sialidase exists as an enzyme complex with the β -galactosidase multimer. Prior to the complex formation of sialidase with the β -galactosidase multimer, it seems necessary to form a β -galactosidase multimer with β -galactosidase and a protective protein as described previously.^{22,23)}

In the present study, activation and stabilization of the enzyme were observed on incubation of the concentrated enzyme preparation at 37 °C for 60 min or storage at 4 °C for a few weeks. The activation of the enzyme was accelerated by chloride ion. We previously showed that the activation of a sialidase from human placenta was significantly inhibited by amastatin which is an inhibitor of aminopeptidase A.²⁴⁾ This suggests the possible participation of an aminopeptidase function in the activation process of sialidase. However, the activation of the sialidase from porcine liver was not affected by amastatin (data not shown), so that activation may involve different mechanisms from that of human placental sialidase. Sialidase copurified with lipid transfer protein from human plasma has been shown to activate by placing it at 4 °C for a few weeks.²⁵⁾

The sialidase from porcine liver hydrolyzed all of the sialooligosaccharides, glycoproteins, and gangliosides tested. The enzyme was particularly active toward sialooligosaccharides, fetuin, and gangliosides GM3, GD1a, and GD1b, and showed pH optima between 4.0 and 4.5 for 4MU-NeuAc, sialyllactose, fetuin, and GM3 as substrates. In addition, the substrate specificity of porcine liver sialidase was similar to that of human placental sialidase.¹⁰⁾ This may mean that the enzyme in porcine liver originates from lysosomes.

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