Vol. 166, No. 1, 1990
January 15, 1990
BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 387-393

PURIFICATION TO HOMOGENEITY OF GD3 SYNTHASE AND PARTIAL PURIFICATION OF GM3 SYNTHASE FROM RAT BRAIN

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Received October 20, 1989

SUMMARY: A CMP-sialic acid:GM3 sialyltransferase (GD3 synthase) and a CMP-sialic acid:LacCer sialyltransferas (GM3 synthase) have been purified 10,000- and 3,000-fold, respectively, from the Triton X-100 extract of rat brain. The two enzymes were purified and resolved by affinity chromatography on two successive CDP-Sepharose columns by NaCl gradient elution. Final purification of GD3 synthase was achieved by specific elution from a 'GM3 acid'-Sepharose column with buffer containing GM3. Sodium dodecylsulfate-gel electrophoresis of GD3 synthase revealed a single major protein band with an apparent molecular weight of 55,000.

Sialyltransferases are a group of glycosyltransferases which catalyze the transfer of a sialic acid (NeuAc) residue to the non-reducing terminal sugar of glycoproteins and glycolipids with the general reaction: CMP-NeuAc + HO-acceptor → CMP + NeuAc-O-acceptor (1). At least five sialic acid linkages, NeuAc 2-3Gal, NeuAcα2-6Gal, NeuAcα2-6GalNAc, NeuAcα2-6GlcNAc, and NeuAcα2-8NeuAc are known to occur in gangliosides (2). In accord with the one enzyme one linkage hypothesis (3), there should be at least five specific sialyltransferases to account for the synthesis of all gangliosides. However, the substrate specificities of these sialyltransferases appear to extend beyond the non-reducing terminal sugar to include the sequence of the acceptor glycosphingolipids (4, 5). Thus more than five different sialyl-transferses may be needed for the biosynthesis of gangliosides. To date, only one sialyltransferase (CMP-NeuAc:Galβ1-3GalNAc-α2-3sialyltransferase) has been purified to homogeneity (6), and

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Abbreviations: The ganglioside nomenclature is that of Svennerholm (14). LacCer, lactosylceramide; NeuAc, N-acetylneuraminic acid; CMP, cytidine monophosphate; CDP, Cytidine diphosphate; TLC, thin-layer chromatography; HPTLC, high-performance TLC.

0006-291X/90 $1.50

it prefers glycoproteins as its acceptor rather than glycolipids. One of the major difficulties in the purification of membrane-bound glycosyltransferases has been with their solubilization. Furthermore they are very labile and are present in very low amounts in tissues. Additionally, there is a lack of suitable assaying procedures that can facilitate the purification of these enzymes from the large number of sample fractions generated from column chromatography. Recently, several methods for assaying the enzyme activity have been developed, the first is a TLC method (7) which is useful for detecting the substrate specificity of the enzyme, the second a gel filtration method useful (8) in monitoring the enzyme activity in the course of enzyme purification, and the third a paper chromatographic method (9) which offers complete resolution of the glycolipid product and radiolabeled sialic acid donor but is time consuming.

In the present report, we describe the solubilization and purification of CMP-NeuAc:LacCer α2-3sialyltransferase and CMP-NeuAc:GM3 α2-8sialyltransferase from young rat brain which is relatively rich in sialyltransferases.

MATERIALS AND METHODS

Materials: Gangliosides were isolated from bovine brain or buttermilk by the method of Ledeen and Yu (10). dl-Dihydro-lactoceramide was purchased from Calbiochem (La Jolla, CA). The labeled CMP-[^{14}C]NeuAc (1.8 mCi/mmol) was purchased from New England Nuclear (Boston, MA). CDP-Sepharose was prepared as described by Wilchek and Lamed (11). 'GM3-acid'-Sepharose was prepared by oxidation of GM3 with permanganate (12) and coupling of GM3-acid to aminohexyl-Sepharose 4B (13). PD-10 Columns were purchased from Pharmacia Fine Chemicals (Piscataway, NJ). Detergents other than Myrj were supplied by Sigma (St. Louis, MO), and Myrj was purchased from ICI Inc. (Wilmington, DE). HPTLC plates were obtained from E. Merck (Darmstadt, West Germany). All other chemicals were of analytical grade. Protein assay kit was obtained from Bio-Rad Chemical Division (Richmond, CA).

Enzyme assay: The complete incubation mixture contained the following components in a final volume of 100 μl: substrate (lipid acceptor) 20 nmol, CMP-NeuAc (1 x 10^5 cpm) 40 nmol, cacodylate buffer, pH 6.5 (cacodylate 25 mM, $MnCl_2$ 10 mM, 0.15% Triton X-100) 20 μl, and enzyme (0.1-200 ug of protein). The reaction was carried out at 37°C for 60 min and the glycolipid products were separated either by paper chromatography (9) or by PD-10 gel filtration and finally their radioactivities were measured. The TLC method as described previously (7) was used in determining the substrate specificity of the enzyme preparations.

Enzyme purification: All steps in the enzyme purification were carried out between 0-4°C. Brains from 12- to 14-day-old rat (total 40 to 50 g) were homogenized with 250 ml of 0.32 M sucrose containing 1 mM EDTA and 10 mM 2-mercaptoethanol in a glass

homogenizer with a Teflon pestle. The homogenate was then centrifuged at 1,000 x g for 10 min. The supernatant and the puffy layer above the pellet were carefully removed, and was centrifuged at 10,000 x g for 60 min. The second supernatant was then centrifuged at 110,000 x g for 90 min. The pellets (microsomal fraction) thus obtained were collected and suspended in 25 mM cacodylate buffer (pH 6.5), containing 20 mM $MnCl_2$, 25% glycerol, 1% Triton X-100. The suspension was gently stirred for 60 min and then centrifuged at 100,000 x g for 60 min. The supernatant was collected and diluted with 9 volumes of 25 mM cacodylate buffer. It was applied to the first CDP-Sepharose column (10 x 2.5 cm), which had been equilibrated with 25 mM cacodylate buffer containing 25% glycerol, 0.075 M NaCl and 0.1% Triton X-100 (Buffer I). After affinity absorption, the excess proteins were washed with Buffer I until no more proteins could be washed out. The enzymes adsorbed on the column were eluted with the above buffer containing 1.0 M NaCl. The eluate was dialyzed overnight against Buffer I, and then applied to the second CDP-Sepharose column (12 x 1 cm) which had been equilibrated with Buffer I. A linear gradient elution of 0.1 to 1.0 M NaCl in Buffer I was employed. Aliquots (20 ul) of the NaCl gradient elution were assayed for enzyme activity. Fractions with GD3 synthase were pooled and dialyzed against Buffer I. 'GM3-acid'-Sepharose, 1 ml suspended in Bufffer I, was poured into a small Pasteur pipette plugged with glass wool. After the dialyzed sample was added, the column was thoroughly washed with Buffer I and then eluted with 0.1 mM GM3 in Buffer I.

RESULTS AND DISCUSSION

In assaying the sialyltransferase activities, three different methods were used for the separation of radio-labeled products and substrates. The gel filtration method (PD-10 column) was time saving, but the resolution was incomplete. This problem could be alleviated by collecting the first half of the peak of the products in the eluate, e.g., when 100 μl of sample was applied to the column, we collected 2.0 to 3.2 ml of the eluate in a scintilation vial and its radioactivity was measured. The procedure was fast and was useful in monitoring the enzyme activity during affinity chromatography.

The paper chromatographic method used by many investigators gave satisfactory separation, and it was used when complete resolution and quantitative results were required. The descending chromatography eluted with a borate buffer overnight could wash away all the water soluble radioactive small molecules, and the ascending chromatography developed by the chloroform-methanol-water solvent separated the ganglioside products from the contaminants remaining at the origin. The recovery of gangliosides was about 90%, when the paper was cut between 2 to 10 cm (from the origin) and its radioactivity was determined.

The TLC method was developed recently by us (7). In this method various glycolipid substrates acting as sialic acid

TABLE 1. Activation of Enzyme Activity by Various Detergents

DETERGENT	ENZYME ACTIVITY
Triton X-100	100
Myrj 59	375
Sodium deoxycholate	206
Triton CF-54	205
Tween 20	175
Tween 80 : Triton CF 54 (1:2)	156
Tween 80	88

The concentration of detergents was 0.15%.
The microsomal enzyme activity in Triton X-100 (0.008 units/mg protein) is arbitrarily designated as 100. One unit of activity is defined as 1 nmol/min of product formed.

acceptors were spotted and developed on a HPTLC plate. The enzyme reaction was carried out on the plate; therefore, the specificity of the enzyme preparation toward various glycolipid acceptors could be determined simultaneously.

As is true for many other membrane-bound enzymes, the catalytic activity of the sialyltransferases was strongly influenced by the presence of detergents. As shown in Table 1, among the various detergents tested, Myrj was the most potent activator. However, its ability to solubilize the membrane-bound sialyltransferases was much less than Triton X-100. For this reason, Triton X-100 was used for the initial solubilization of the enzymes.

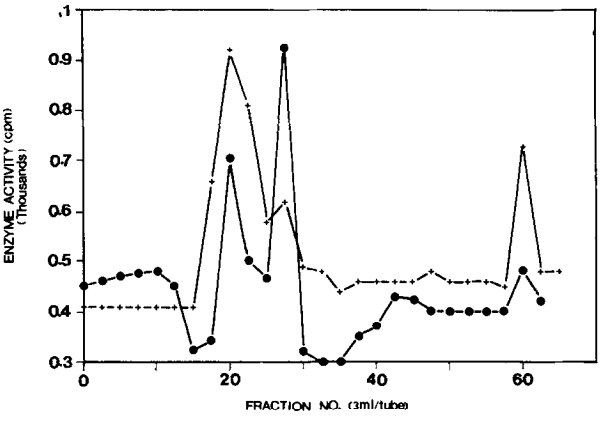

Fig. 1. Chromatography of the detergent-solubilized proteins adsorbed on CDP-Sepharose I. The Triton X-100 extractable proteins of the microsomal fraction were loaded onto a CDP-Sepharose column (I) (10 x 2.5 cm). The column was eluted with 1.0 M NaCl (in Buffer I); proteins (+) and the activity of GD3 synthase (●) in the eluate are shown.

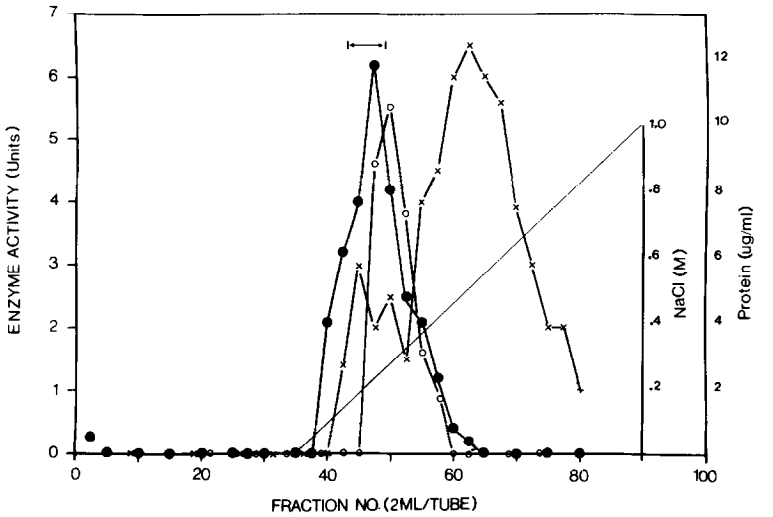

Fig. 2. Elution profile of GM3 and GD3 synthases. The dialyzed enzyme from the CDP-Sepharose column (I) was adsorbed on the CDP-Sepharose column (II) (12 x 1 cm) and eluted at the arrow with a linear NaCl gradient. Fractions (2 ml) were assayed for GM3 (o) and GD3 (●) synthases, and protein (x).

Figs. 1 and 2 show the chromatographic profiles of GD3 and GM3 synthases eluated from the CDP-Sepharose. The first chromatographic step was used to concentrate the enzymes from a large volume of the Triton extract. It also served to remove most of the extraneous proteins. The specific activity of GD3 synthase was increased 50-fold after this step (Table 2). The second chromatographic step employed a NaCl gradient for elution. It did not resolve GD3 from GM3 synthase but other sialyltransferases involved in ganglioside biosynthesis were separated from them. The final 'GM3-acid'-Sepharose column specifically adsorbed GD3 synthase,

TABLE 2. Purification of GD3 Synthase From Rat Brain

Step	Volume (ml)	Total protein (mg)	Total activity (unit*)	Specific activity (unit/mg Protein)	Yield (100%)	Fold
Homogenate	200	3400	6.80	0.002	100	1
Microsome	18	183.6	1.47	0.008	21.6	4
Triton extract	102	108.1	1.73	0.016	25.4	8
CDP-Sepharose I	10	1.6	1.27	0.79	18.74	395
CDP-Sepharose II	42	0.126	0.782	6.21	11.51	3105
GM3-Sepharose	2	0.0016	0.015	18.9	0.22	10000

*One unit of activity is defined as 1 nmol/min of product formed.

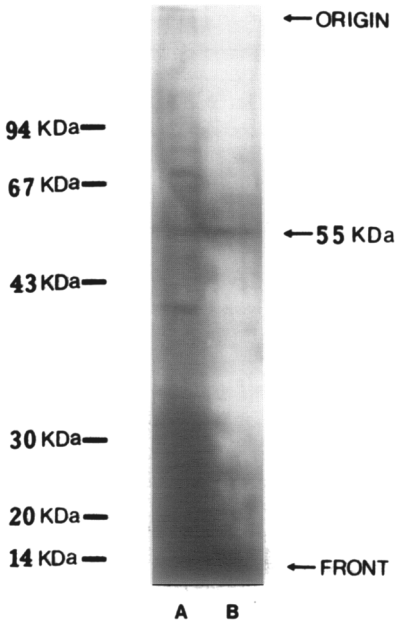

Fig. 3. SDS-PAGE patterns of the enzyme preparations obtained from the last two steps of purification. The fraction eluted from the second CDP-Sepharose column gave four protein bands on silver staining (column A). The final preparation from 'GM3-acid'-Sepharose column gave a single protein band (column B). The percentage of SDS was 10%.

which was then separated from the GM3 synthase by elution with GM3 in buffer.

The purification scheme for GD3 synthase from 50 rat brains (about 50 g) is shown in Table 2. Representative elution profiles for steps 4 and 5 are shown in Figs. 1 and 2. The overall yield of the GD3 synthase from the starting homogenate was 0.22% with a purification factor of 10,000.

Assessment of the purified enzyme was carried out by SDS-gel electrophoresis. As shown in Fig. 3 (column A), the fraction containing GM3 and GD3 synthase activities from the second CDP-

TABLE 3. Activity of GD3 Synthase in Various Rat Organs

ORGAN	ENZYME ACTIVITY
Brain	100
Kidney	175
Heart	48
Lung	39
Pancreas	38
Liver	21

The enzyme activity in brain is arbitrarily designated as 100.

Sepharose chromatography exhibited four protein bands. After the final 'GM3-acid'-Sepharose column chromatography, only GD3 synthase activity was detected. The preparation gave a single band on silver staining, with an apparent molecular weight of 55,000 (Fig. 3, column B). The GM3 synthase obtained from the final column had a purification factor of over 3,000-fold from the homogenate of rat brain.

Finally, the distribution of GD3 synthase in various rat organs was compared (Table 3). Kidney was abundant in GD3, and it also had the highest GD3 synthase activity. Studies are in progress to characterize the various specific sialyltransferases in various tissues.

Acknowledgment: This work was supported by USPHS grant NS-11853.

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