Purification and characterization of CMP-NeuAc:GM1 (Galβ1-4GalNAc) α2-3 sialyltransferase from rat brain

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A CMP-NeuAc:GM1 α2-3sialyltransferase (GD1a synthase, 2.4.99.2) has been purified from the Triton extract of rat brain. The enzyme was purified and resolved by affinity chromatography on CDP-Sepharose column by a linear NaCl gradient elution. Final purification was achieved by elution from a 'GM1-acid'-Sepharose column. SDS-PAGE of the enzyme revealed a single protein band with an apparent M_r 44 kDa. It catalyzed specifically the sialylation of GD1b, GM1 and asialo-GM1. Enzyme products were identified by TLC in three different solvent systems. The K_m value for GM1 was 7.5×10^{-2} M, and for CMP-NeuAc it was 6.5×10^{-5} M.

Glycosyltransferase; Sialyltransferase; Glycolipid; Ganglioside; GM1

1. INTRODUCTION

Gangliosides, defined as sialic acid-containing glycosphingolipids, are present at a conspicuously high concentration in the central nerve system of all chordates [1]. Structural complexity is a hallmark of gangliosides; today over 90 different species have been isolated and characterized from various tissues [2]. The sialic acid moiety in ganglioside may occur in a variety of linkages [2] such as
NeuAcα2-3Galβ1-4GalNAc-,
NeuAcα2-3/6Galβ1-4GlcNAc-,
NeuAcα2-3Galβ1-4Glc-, NeuAcα2-3Gal-,
NeuAcα2-8NeuAcα2-3Galβ1-4Glc-,
NeuAcα2-8NeuAcα2-8 NeuAcα2-3Galβ1-4Glc-,
NeuAcα2-8NeuAcα2-3Galβ1-4GalNAc-,
NeuAcα2-6GalNAcβ1-4Galβ1-4Glc-, etc. For the addition of the sialic acid to each of these structures a separate sialyltransferase may be required. To date several sialyltransferases have been purified to homogeneity, including β-galactoside α2-6sialyltransferase from bovine colostrum [3], β-galactoside α2-3sialyltransferase and *N*-acetylgalactosaminide α2-6sialyltransferase from porcine submaxillary gland [4,5], and β-galactosyl 1-3*N*-acetylgalactosaminide α2-3sialyltransferase from human placenta [6]. We recently embarked on a study to purify the various sialyltransferases in rat brain, and reported the purification of CMP-NeuAc:lactosylceramide and CMP-NeuAc:GM3 sialyltransferases (GM3 synthase and GD3 synthase) [7]. In this paper we report the purification to homogeneity and characterization of CMP-NeuAc:GM1 (Galβ1-4GalNAc) α2-3-sialyltransferase (GD1a synthase; EC 2.4.99.2) from rat brain.

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Abbreviations: The nomenclature of gangliosides is based on the system of Svennerholm [20]. The symbols and nomenclature for neutral glycolipids follow a recent recommendation (IUPAC-IUB Commission of Biochemical Nomenclature. Eur. J. Biochem. (1977) 79, 11-21) NeuAc, *N*-acetylneuraminic acid; CMP, cytidine monophosphate

2. MATERIALS AND METHODS

Ganglioside and neutral glycosphingolipids were isolated from bovine brain, or red blood cells in our laboratories [8,9]. 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 [10]. 'GM1-acid'-Sepharose was prepared by oxidation of GM1 with permanganate [11] and followed by coupling of the GM1-acid to aminohexyl-Sepharose 4B [12]. Neuraminidase (from *Vibrio cholerae)* was purchased from Sigma (St. Louis, MO). Peroxidase conjugated cholera toxin subunit B was obtained from List Biol. Lab. (Campbell, CA). Protein assay kit was obtained from Bio-Rad (Richmond, CA).

The enzyme assay was performed in 1 × 7 cm glass test tubes as follows: 40 nmol of a glycolipid acceptor and 40 nmol of CMP-[^{14}C]-NeuAc (1×10^5 cpm) were pipetted first and dried with a gentle stream of nitrogen. After sonication with 50 μl of cacodylate buffer (pH 6.5, containing cacodylate 25 mM; $MnCl_2$ 10 mM and 0.15% Triton X-100), the enzyme, 50 μl (0.1–200 μg protein), was added with gentle shaking. After incubation at 37°C for 60 min, the glycolipid products were separated by gel filtration (Sephadex-G50 column 30 × 0.7 cm) [13] and their radioactivities measured. The TLC method described previously [14] was used in determining the substrate specificity of the enzyme preparations. In a separate experiment, the enzyme product, GD1a, was identified as follows: 150 nmol of GM1

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was used as substrate and the enzymatic product was extracted with 2 ml of chloroform: methanol (2:1 by volume) mixture and dried under nitrogen. The residue was dissolved in a small amount of methanol and desalted by gel filtration on a LH-20 Sepharose column [15]. The desalted glycolipid products were applied on HPTLC, and developed with three different solvent systems. They were (a) chloroform/methanol/aq. 0.2% $CaCl_2 \cdot 2H_2O$ (55:50:10) [16], (b) acetonitrile/isopropanol/aq. 0.2% $CaCl_2 \cdot H_2O$ (10:65:25) [17], and (c) chloroform/methanol/aq. 0.4% $CaCl_2 \cdot H_2O$/2.5 N NH_4OH (60:40:5:4) [15].

2.1. *Enzyme purification*

All purification steps were carried out at 4°C. Brains from 1–14-day-old rats (total 40 g) were homogenized with 200 ml of 0.32 M sucrose containing 1 mM EDTA and 10 mM 2-mercapto-ethanol in a glass homogenizer. The homogenate was centrifuged at 1000 × *g* for 10 min. The supernatant was carefully removed and centrifuged at 100000 × *g* for 90 min. The pellet thus obtained was suspended in 25 mM cacodylate buffer (pH 6.5, containing 20 mM $MnCl_2$, 25% glycerol and 1% Triton X-100). The suspension was gently stirred for 60 min and then centrifuged at 100000 × *g* for 60 min. The supernatant was collected and diluted with 9 vols of 25 mM cacodylate buffer. It was applied to a CDP-Sepharose column (20 × 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 adsorption, 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 a linear gradient of NaCl solution (from 0.1 to 1.0 M NaCl in buffer I). Aliquots of the NaCl elution were assayed for enzyme activity. Fractions with CMP-NeuAc:GM1α2-3 sialyltransferase were pooled and dialyzed against Buffer I (without NaCl) and finally concentrated with polyethylene glycol (M_r 20000). They were applied to a 'GM1-acid'-Sepharose column (10 × 0.7 cm), and the column was thoroughly washed with Buffer I and then eluted with 0.1 mM GM1 in 5 ml Buffer I, in order to remove excess GM1 in the purified enzyme preparation, the enzyme was readsorbed on a CDP-Sepharose column and eluted with Buffer I containing 1.0 M NaCl as described above. This enzyme preparation was used for the determination of enzyme specificity.

3. RESULTS

Fig. 1 shows the chromatographic profile of CMP-NeuAc:GM1α2-3sialyltransferase eluted from CDP-Sepharose. The enzyme was collected in fractions 58–72, and its specific activity arose from 0.0015 mU (one unit of activity is defined as 1 μmol/min of product formed) in the homogenate to 0.2 mU in this fraction (Table I). After the 'GM1-acid'-Sepharose affinity chromatography (Fig. 2), the enzyme was separated from other contaminants and appeared homogeneous as judged by silver staining, on SDS-PAGE electrophoresis (Fig. 3). It had an apparent molecular mass of 44000. From 40 g of rat brain, 26 μg of purified enzyme was obtained. The enzyme was specific to GD1b, GM1 and asialo-GM1. The specificity of the enzyme toward different glycolipids was examined; if the activity for GM1 was taken as 100, the activities of the enzyme toward GD1b was 120, and asialo-GM1 61. It did not catalyze the transfer of sialic acid to GalCer, LacCer, Gb3, globoside, Forssman antigen, GM2, GM3 and GD3. Product indentification was done as follows: if GM1 was used as the substrate for the enzyme reac-

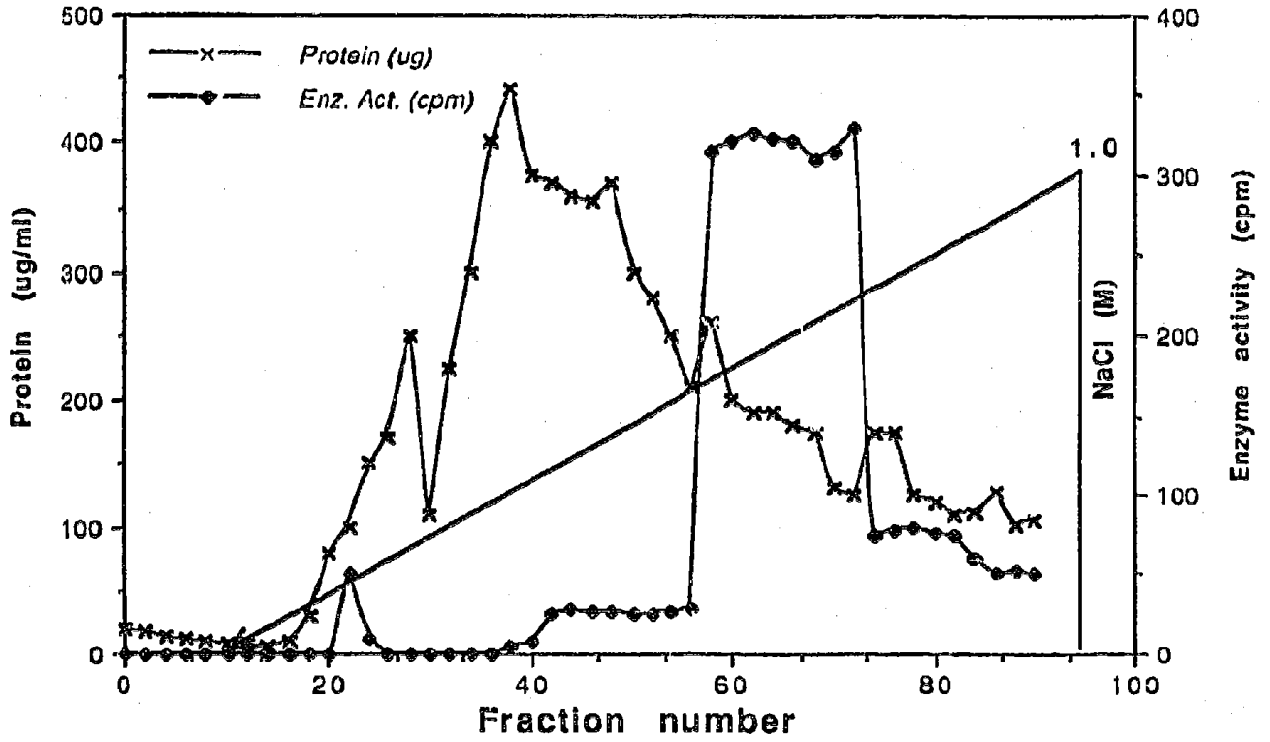

Fig. 1. The elution profile of the Triton-solubilized rat brain proteins on CDP-Sepharose column chromatography. The column (40 × 2.5 cm) was eluted with a linear gradient of NaCl. Fractions (8 ml) were collected and assayed for CMP-NeuAc:GM1 sialyltransferase activity (+) and protein (■).

Table I

Purification of CMP-NeuAc:GM1 α2-3 sialyltransferase

Step	Volume (ml)	Total protein (mg)	Specific activity (mU/mg protein)	Total activity (mU)	Yield %	Fold
Homogenate	200	3400	0.0015	5.1	100	1
Membrane preparation	45	952	0.0034	3.2	62.7	2.2
Triton extract	450	680	0.0052	3.5	68.6	3.4
CDP-Sepharose	75	13	0.2	2.6	50	133
GM1-acid-Sepharose	5.1	0.026	8.4	0.22	4.2	5610

tion, the glycolipid product was identified as GD1a by HPTLC in three different solvent systems (Fig. 4). The K_m values were determined to be 7.5×10^{-2} M for GM1, and 6.5×10^{-5} M for CMP-NeuAc.

4. DISCUSSION

Glycosyltransferases are usually present in small amounts in tissues and are very labile [18]. Therefore, it is necessary to start with a large amount of tissues with repeated chromatographic steps. Furthermore, the similarities in enzyme activities frequently render the resolution of the various enzymes difficult to achieve. In this paper, we devised a simple procedure which included two affinity column chromatographic techniques for their purification. The use of a rapid solid-phase assay method on TLC plates further facilitated the assay of the large number of column fractions. These ensured us to start with a relatively small amount of material to obtain a relatively high yield of

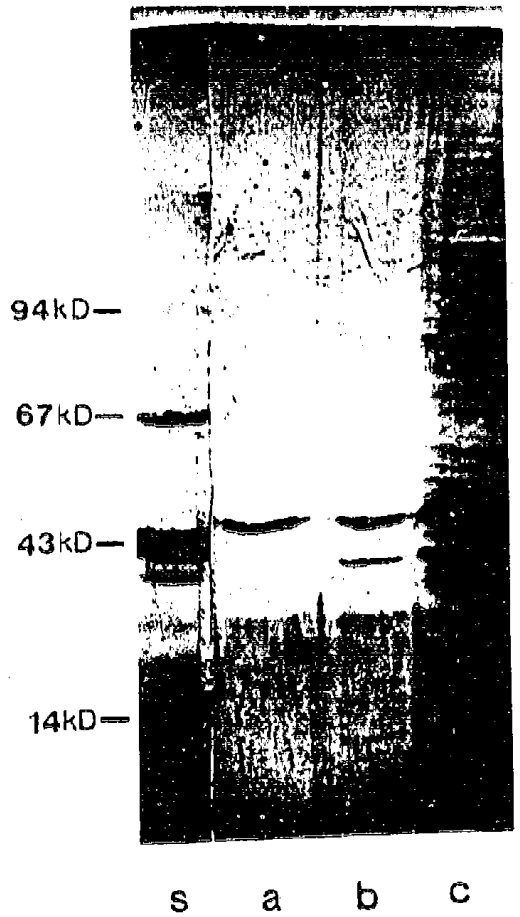

Fig. 3. SDS-PAGE patterns of the enzyme preparations obtained from the CDP-Sepharose column (lane c) and 'GM1-acid'-Sepharose column (lanes a and b); lane s contained standard protein markers. The bands were visualized by silver staining. Lane a gave a single protein band with an apparent molecular mass of 44 000. The percentage of gel was 8%.

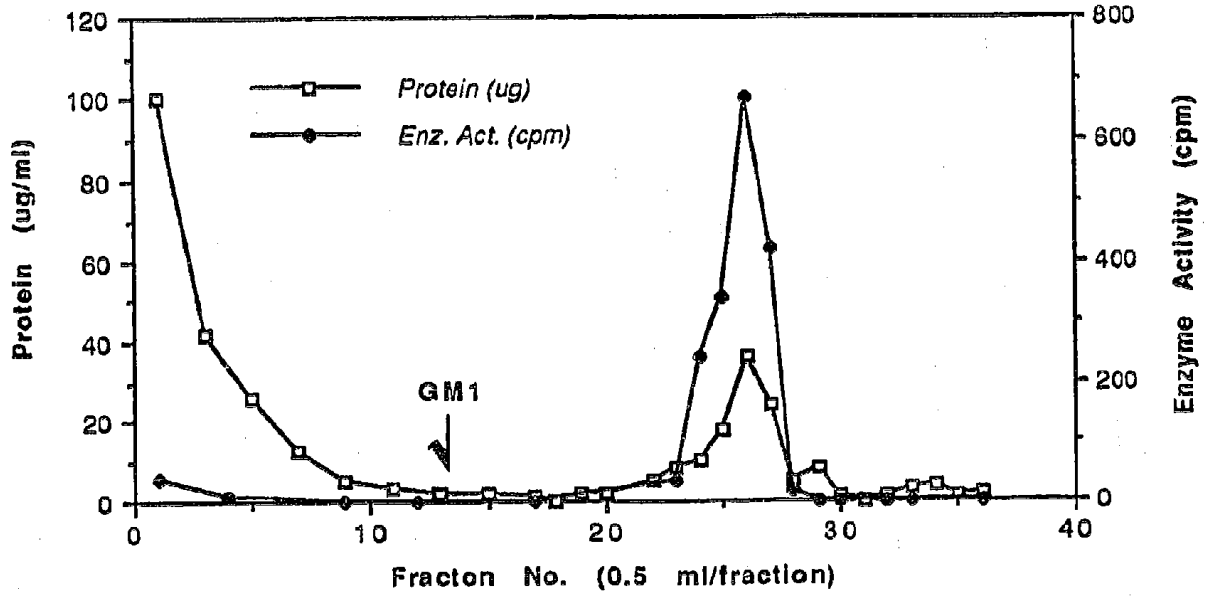

Fig. 2. The elution profile of the sialyltransferase on GM1-acid-Sepharose affinity chromatography.

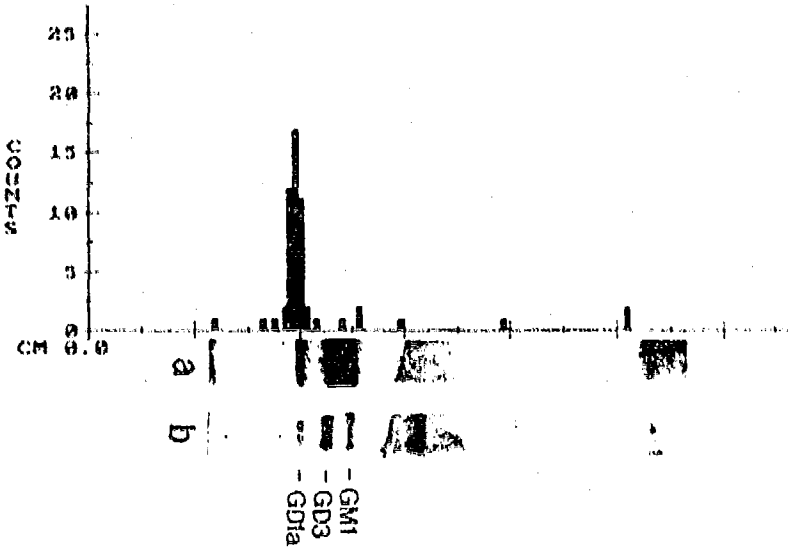

Fig. 4. Thin layer chromatography of the enzymatic product, GD1a. Isolation of the ganglioside product was described in section 2. Cold GD1a, 10 μg, was added before isolation. The HPTLC plate was developed in chloroform/methanol/aq. 0.2% $CaCl_2 \cdot 2H_2O$ (55:50:10 v/v). The positions of ganglioside were revealed by staining with iodine, and the radioactivity was measured by scanning on System 20i Imaging Scanner (Bioscan Inc., Washington, DC). Lane a contained the sample, and lane b authentic standards.

the pure enzyme (Table I). The enzyme shares a number of properties of other β-galactoside α2-3sialyltransferases in that it is a membrane-bound glycoprotein (judged from its binding with Con A), and it has a relatively high K_m value for the glycolipid acceptors (10^{-2} M) [18]. There are several papers reporting [4-6] that the β-galactoside α2-3sialyltransferases from human placenta, porcine submaxillary gland and liver occur in several molecular weight forms. Our data indicate, however, that the enzyme isolated from rat brain appears to exist as single protein entity. It was proved by kinetic and competition experiments [19] that GM1b, GD1a and GT1b synthase are identical. This was confirmed in this study. The enzyme catalyzes the sialylation of β-galactosides which possess a terminal Galβ1-4GalNAc-structure. Further studies are in progress to determine the molecular properties of the various sialyltransferases.

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