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IMPROVED METHOD FOR LARGE-SCALE PURIFICATION OF BRAIN GANGLIOSIDES BY Q-SEPHAROSE COLUMN CHROMATOGRAPHY

IMMUNOCHEMICAL DETECTION OF C-SERIES POLYSIALOGANGLIOSIDES IN ADULT BOVINE BRAINS*

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SUMMARY

A new chromatographic method for separation of bovine brain gangliosides has been developed using Q-Sepharose. Gangliosides were separated based not only on their sialic acid numbers but also on the sialic acid molecular species and chain lengths of the skeletal oligosaccharide portions. The following results indicate that this column chromatography has practical advantages in separating mixtures of gangliosides, especially positional isomers and molecular species with N-acetyl- or N-glycolylneuraminic acid. (1) the loading capacity of Q-Sepharose for gangliosides was very high; (2) most major gangliosides such as GM1, GD1a, GD1b, GT1b and GQ1b were isolated in a single step; (3) these major gangliosides were clearly separated from gangliosides containing, N-glycolylneuraminic acid when examined using Hanganutziu–Deicher antibody; (4) polysialogangliosides that have four or more sialic acid residues were isolated efficiently. It was shown by the combination of Q-Sepharose column chromatography with thin-layer chromatography/enzyme immunostaining that adult bovine brains possess C-series polysialogangliosides as minor components which are known as embryonic molecules in avian and mammalian brains.

* The ganglioside nomenclature used is that of Svennerholm[24]. The abbreviations used for sialic acids are: Ac, N-acetylneuraminic acid; Gc, N-glycolylneuraminic acid; HD, Hanganutziu-Deicher.

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INTRODUCTION

Gangliosides are present on cell surface membranes, and especially abundant in tissues of the central nervous system. Since the first report by Momoi *et al.*[1], the two-step column chromatographic separation of gangliosides using DEAE-resins and silica gel has become one of the most popular methods for the preparation and the purification of gangliosides[2]. Quite recently, many monoclonal antibodies recognizing gangliosides on cell surface membranes have been produced[3]. The introduction of immunochemical procedures using monoclonal antibodies has revealed that mammalian cells and tissues possess many new types of gangliosides. In some cases, the quantity of gangliosides detected by monoclonal antibodies is extremely low. Therefore, good methods for purification of very minor gangliosides are required in order to characterize their chemical structures. In this report, we describe an improved method of ion-exchange column chromatography by which most ganglioside species in bovine brain can be isolated. Minor species of gangliosides, especially, C-series gangliosides which are known to be abundant in fish brains[4], were also isolated or enriched by this one-step column chromatography. The C-series gangliosides were identified by means of immunostaining on thin-layer plates. The occurrence of the C-series gangliosides in bovine brains has been demonstrated for the first time.

EXPERIMENTAL

Materials

Total gangliosides were prepared from bovine brains by the method of Ledeen *et al.*[5]. Authentic individual gangliosides were isolated from bovine brain total gangliosides as described previously[1]. Q-Sepharose was obtained from Pharmacia, Uppsala, Sweden.

Q-Sepharose column chromatography

Q-Sepharose was converted into the acetate form as follows: Q-Sepharose was suspended in 5 volumes of chloroform–methanol–4 *M* sodium acetate (30:60:8, v/v/v) for 30 min and filtered. The gel was suspended again in the same solvent mixture and left overnight at room temperature. The gel was filtered off, washed several times with 5 volumes of methanol to remove sodium acetate, resuspended in 5 volumes of chloroform–methanol–water (30:60:8, v/v/v) and then poured into a column (75 cm × 30 cm) to a height of 65 cm. The column was washed with 2 l of the same solvent.

The bovine brain total ganglioside preparation (5 g) was dissolved in 500 ml of the same solvent and applied to the column. The column was washed first with 800 ml of the solvent mixture to remove neutral lipids, and gangliosides were then eluted with a linear gradient of sodium acetate, from chloroform–methanol–water (30:60:8) to chloroform–methanol–4 *M* sodium acetate (30:60:8) as shown in Fig. 1. The flow-rate was 1.5 ml/min, and fractions of 15 ml of the effluent were collected. The gangliosides in each fraction were analyzed by thin-layer chromatography (TLC). The fractions were pooled into 23 fractions. Each fraction was concentrated to a small volume by a rotary evaporator, dialyzed against distilled water for 3 days and lyophilized.

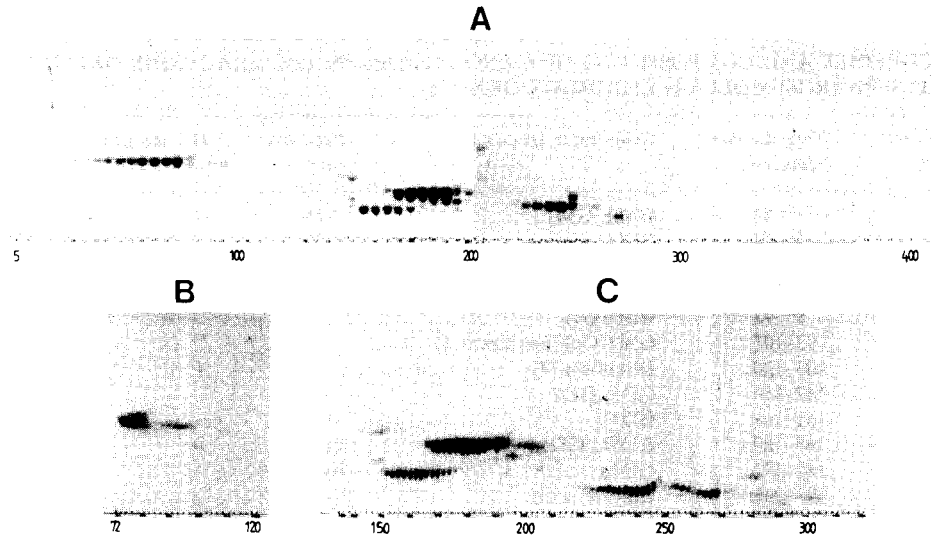

Fig. 1. Elution profile of bovine brain gangliosides by Q-Sepharose column chromatography as monitored by TLC. Aliquots (1 μl) of fractions were spotted on TLC plates, and the plates were developed with solvent system I. In plate A, every five tubes (as indicated) between tube number 5 and 410 was analyzed. Every two tubes from tube number 72 to 120 and 146 to 330 were checked again on plates B and C, respectively.

Thin-layer chromatography

TLC of gangliosides was carried out on precoated HPTLC plates (silica gel 60; E. Merck, Darmstadt, F.R.G.) using solvent systems of chloroform–methanol–15 m*M* magnesium chloride (5:4:1, v/v/v; system I) and chloroform–methanol–2.5 *M* ammonia (5:4:1, v/v/v; system II). Gangliosides were detected by spraying with the resorcinol–hydrochloric acid[6].

Enzyme immunostaining on TLC plates

Polygram Sil G plates (Macherey-Nagel, Düren, F.R.G.) were developed first with solvent system II, and then with solvent system I in the same direction. Sensitive enzyme immunostaining on TLC plates was performed by the procedure of Higashi *et al.*[7].

Polyclonal and monoclonal antibodies

Polyclonal antibodies against GM1(Ac), GD2, GA1, GA2 and GM3(Gc) were prepared as described previously[8–10]. Monoclonal anti-GD2 antibody was produced in our laboratory. Monoclonal antibody M6704 recognizing C-series gangliosides was obtained by immunizing BALB/c mice with chick embryonic brains[11].

RESULTS AND DISCUSSION

In the present study a Q-Sepharose column was used for the fractionation of bovine brain gangliosides with a linear gradient of sodium acetate in chloroform–methanol–water. The elution order of the bovine brain gangliosides was examined by HPTLC as shown in Fig. 1. According to the elution profile, the fractions were

TABLE I

RECOVERIES AND COMPOSITIONS OF GANGLIOSIDES IN THE FRACTIONS OBTAINED BY Q-SEPHAROSE COLUMN CHROMATOGRAPHY

Fraction number	*Tube number collected*	*Ganglioside species*	*Recovery (mg)*	*HD-antigen**
1	1–55	GM2, GM1	215	
2	56–74	GM1	459	
3	75–82	GM1	110	
4	83–85	GM1(Gc), unknown (2)**	7	
5	86–92	GM1(Gc)	19	
6	93–102	GM1(Gc), unknown (1)	22	
7	103–120	Unknown (4)	4	+
8	142–150	GD3, GD2	48	
9	151–164	GD1b	348	
10	165–180	GD1b, GD1a	1487	
11	181–192	GD1a	1022	
12	193–200	Unknown (3)	177	
13	201–212	GalNAc-GD1a(?)	91	+
14	213–218	Unknown (2–3)	2	
15	219–228	GT1b	68	
16	229–242	GT1b	539	
17	243–250	GT1b, unknown (1)	218	+
18	251–260	Unknown (1)	0.2	+
19	261–270	GQ1b	92	
20	271–277	Unknown (3)	1.7	+
21	278–284	Unknown (2)	10	
22	285–294	Unknown (3)	6	
23	295–310	Unknown (4)	14	+
Total			4969.9	

* The HD antigenic gangliosides were examined by TLC/enzyme immunostaining as shown in Fig. 3.

** The number of unknown gangliosides revealed by TLC analysis.

pooled to give 23 fractions, and the recoveries and the ganglioside species in these fractions are shown in Table I. Fig. 2 shows thin-layer chromatograms of these 23 fractions. Fractions 1–6 contained different species of monosialogangliosides. GM2 travelled a little faster that GM1, and it was obtained in fraction 1. GM1(Ac) recovered in fraction 2 was revealed to be homogeneous by TLC using two different solvent systems. Following GM1(Ac), GM1 containing N-glycolylneuraminic acid (Gc) appeared.

GD3 and GD2 gangliosides were eluted simultaneously from the column, and then GD1b, followed by GD1a (Fig. 1). As both GD3 and GD2 are minor components that comprise only 1.5% of the total disialogangliosides (Table I), it may be easy to isolate them from large amounts of disialogangliosides by conventional methods. GD3 and GD2 recovered in fraction 8 can be separated by Iatrobeads column chromatography[1]. Major gangliosides, GD1a, GD1b, GT1b and GQ1b, were obtained almost homogeneous in fractions 11, 9, 15 and 16 and 19, respectively, by this single-step Q-Sepharose column chromatography.

Bovine brain gangliosides contain very minor gangliosides together with N-

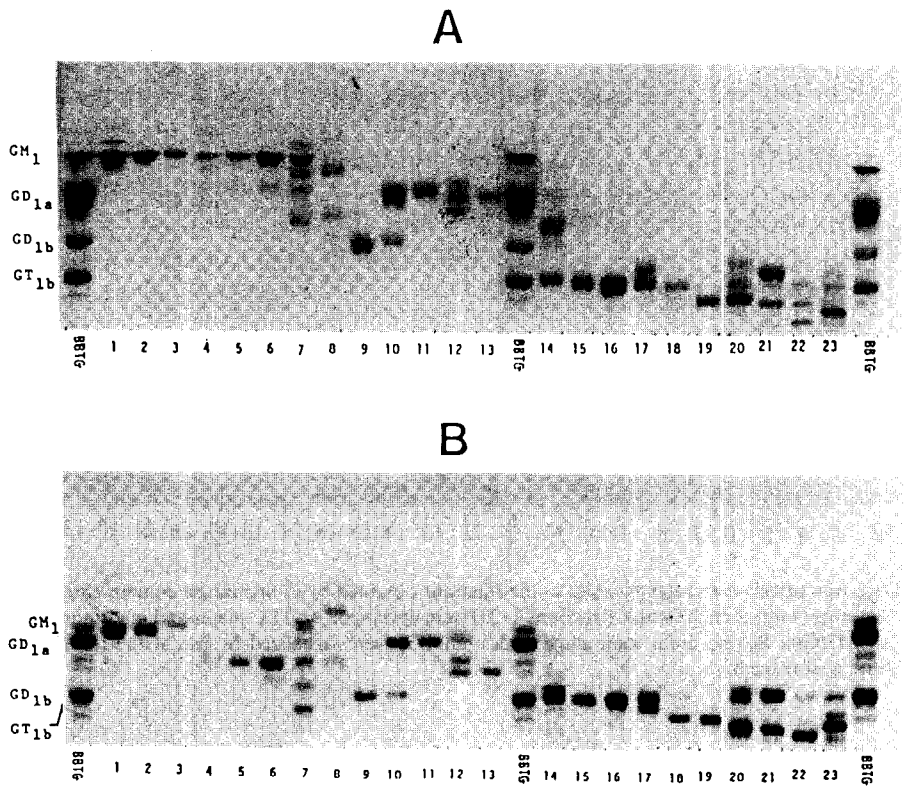

Fig. 2. Thin-layer chromatograms of fractioned (1–23) gangliosides by Q-Sepharose column chromatography. Plates A and B were developed with solvent systems I and II, respectively. BBTG = Bovine brain total gangliosides.

glycolyleuraminic acid[12], some of which are recognized by Hanganutziu–Deicher (HD) antibody. Employing enzyme immunostaining on a TLC plate using anti GM3(Gc) which recognizes the outer portion of N-glycolylneuraminic acid in gangliosides[13], we examined whether the purified ganglioside fractions were contaminated with HD antigenic gangliosides. As shown in Fig. 3, the major gangliosides including GM1 (fraction 2), GD2 and GD3 (fraction 8), GD1a (fraction 11), GD1b (fraction 9), GT1b (fraction 15 and 16) and GQ1b (fraction 19) did not contain any HD-antigenic gangliosides. Instead, the HD-active gangliosides were detected in fractions 7, 13, 17, 18, 20 and 23. This result indicates that Q-Sepharose is a very powerful chromatographic system for the separation of gangliosides having different molecular species of sialic acid.

Another minor ganglioside group found in bovine brain was the C-series gangliosides. This type of gangliosides was found in fish brains[4,14] and chick embryonic brains[15]. Recently, we have produced a monoclonal antibody (M6704) by immunizing BALB/c mice with chick embryonic brain tissues, and found that the antibody recognizes C-series gangliosides including GT3, GT2, GT1c and GQ1c (data not shown). By using this antibody, bovine brain gangliosides were found to contain the C-series gangliosides as shown in Fig. 4. Among the C-series gangliosides detected,

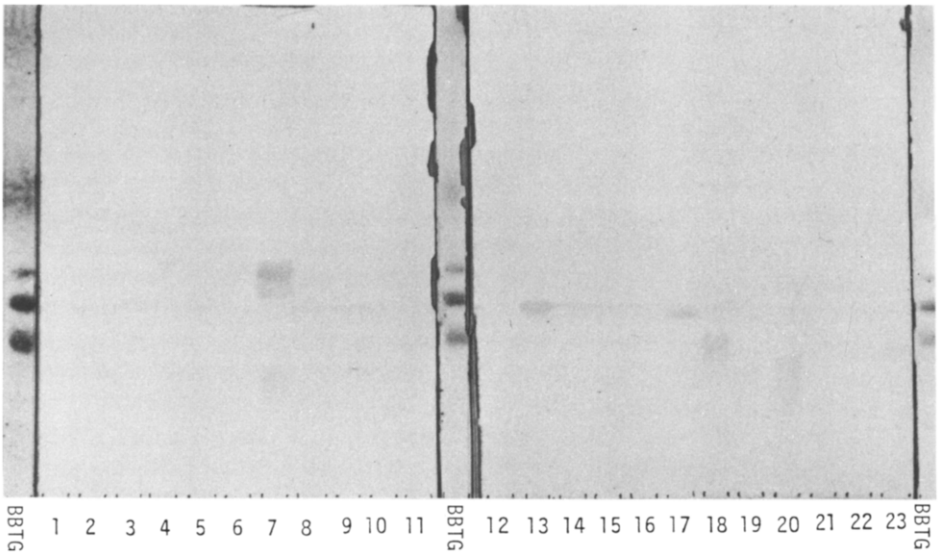

Fig. 3. Detection of HD-active gangliosides in the fractions obtained by Q-Sepharose column chromatography. Aliquots (5 μg) of each sample (fractions 1–23) were developed on TLC plates and immunostained with anti-GM3(Gc). Bovine brain total gangliosides (BBTG) were visualized by use of the resorcinol–hydrochloric acid reagent.

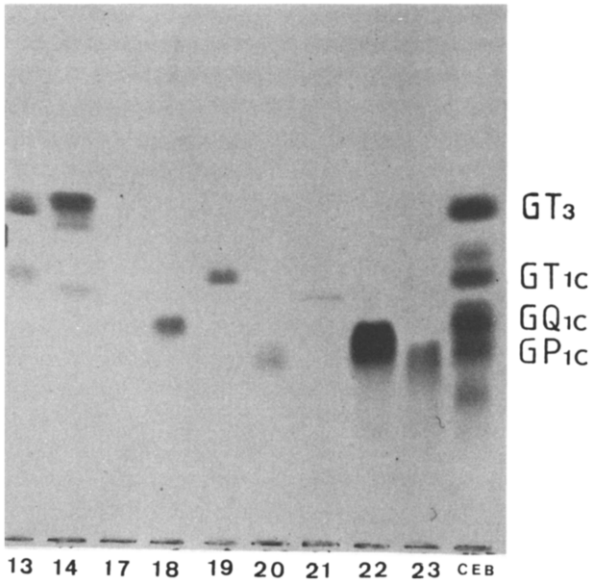

Fig. 4. Detection of C-series gangliosides in the fractions of bovine brain gangliosides by TLC/enzyme immunostaining. Samples from fractions 13–23 (5 μg) were spotted on TLC plates and developed successively with the solvent systems II and I. The plates were treated with M6704 monoclonal antibody which recognizes the C-series structure. CEB = Gangliosides isolated from 10-day chick embryonic brains.

a ganglioside in fraction 22 was most strongly stained with this monoclonal antibody. Further work on the chemical characterization of these minor gangliosides is now in progress in our laboratories.

The use of a DEAE-cellulose column for the separation of gangliosides was first reported by Winterbourn[16]. Since then, many different weak anion-exchange resins have been used, *i.e.*, DEAE-Sephadex A-25[1], DEAE-Sepharose[17], DEAE-Sephacel[18], DEAE-dextran[19] and DEAE-silica gel[20,21]. Recently a new type of strong anion-exchange resin, Mono Q has become available for the preparation of gangliosides[22,23]. In this paper, we report that a new type of resin, Q-Sepharose, has high resolution power for the separation of brain gangliosides. Q-Sepharose is a strong anion-exchange resin similar to Mono Q, but it seems to be better than Mono Q since GQ1b is coeluted with monosialogangliosides from the latter, as reported by Månsson *et al.*[23]. This kind of phenomenon had never been observed with Q-Sepharose.

Several practical advantages in using Q-Sepharose are as follows: (1) it has high sample loading capacity (about 20 μmol of ganglioside-sialic acid per ml gel); (2) gangliosides are quantitatively recovered from the column in 98–99% recovery; (3) gangliosides are separated according not only to their sialic acid contents but also to their small structural differences. The third point is very important in the chromatography of gangliosides. It is remarkable that bovine brain total gangliosides are fractionated into 23 groups by one-step column chromatography. Most major gangliosides were isolated almost pure, and many unidentified species which showed resorcinol-positive reactions were separated. N-Glycolylneuraminic acid-containing gangliosides that were detected with HD antibody appeared to be separated from N-acetylneuraminic acid-containing gangliosides. Thus, Q-Sepharose column chromatography seems to be a good method for purification of gangliosides from bovine brain as a starting material, because the coexistence of N-acetyl- and N-glycolylneuraminic acid-containing gangliosides in bovine brain results in a complex mixture and makes it difficult to purify them by conventional column chromatographies. To our surprise, two structural isomers of GM1 which differ only in N-substitution of sialic acid, acetyl and glycolyl, were clearly separated by an ion-exchange chromatography (Fig. 1, Table I). The possibility is not excluded that hydroxyl groups have some interaction with Q-Sepharose gel and one additional hydroxyl group in GM1(Gc) makes the interaction stronger than in GM1(Ac), but it is more likely that the hydroxy group of the glycolyl residue behaves as a weak acid towards a strong cation. If this is the case, the elution of GM1(Gc) from the column will be retarded. Thus, Q-Sepharose is a very useful anion-exchange resin for large-scale and fine separation of brain gangliosides.

REFERENCES

1 T. Momoi, S. Ando and Y. Nagai, *Biochim. Biophys. Acta*, 441 (1976) 488–497.
2 S. Ando, H. Waki, K. Kon and Y. Kishimoto, in *NATO ASI Series*, Vol. H7, Springer, Heidelberg 1987, pp. 167–177.
3 S. Hakomori, *Annu. Rev. Immunol.*, 2 (1984) 103–126.
4 S. Ando and R. K. Yu, *J. Biol. Chem.*, 254 (1979) 12224–12229.
5 R. W. Ledeen, R. K. Yu, L. F. Eng, *J. Neurochem.*, 21 (1973) 829–839.
6 L. Svennerholm, *Biochim. Biophys. Acta*, 24 (1957) 604–611.

7 H. Higashi, Y. Fukui, S. Ueda, S. Kato, Y. Hirabayashi, M. Matsumoto and M. Naiki, *J. Biochem (Tokyo)*, 95 (1984) 1517–1520.
8 Y. Hirabayashi, K. Koketsu, H. Higashi, Y. Suzuki, M. Matsumoto, M. Sugimoto and T. Ogawa, *Biochim. Biophys. Acta*, 876 (1986) 178–182.
9 T. Yamanaka, Y. Hirabayashi, M. Hirota, M. Kaneko, M. Matsumoto and N. Kobayashi, *Biochim. Biophys. Acta*, 920 (1987) 181–184.
10 Y. Hirabayashi, T. Suzuki, Y. Suzuki, T. Taki, M. Matsumoto, H. Higashi and S. Kato, *J. Biochem. (Tokyo)*, 94 (1983) 327–330.
11 K. Obata, Y. Hirabayashi, M. Hirota, M. Matsumoto, K. Obata and S. Ando, unpublished results.
12 S. Ando, *Neurochem. Int.*, 5 (1983) 507–537.
13 Y. Hirabayashi, H. Higashi, S. Kato, M. Taniguchi and M. Matsumoto, *Jpn. J. Cancer Res.*, 78 (1987) 614–620.
14 R. K. Yu and S. Ando, *Adv. Exp. Med. Biol.*, 125 (1980) 33–45.
15 H. Rösner, H. Rahmann, G. Reuter, R. Schauer, J. P. Katalinic and H. Egge, *Biol. Chem. Hoppe-Seyler*, 366 (1985) 1177–1181.
16 C. C. Winterbourn, *J. Neurochem.*, 18 (1971) 1153–1155.
17 M. Iwamori and Y. Nagai, *Biochim. Biophys. Acta*, 528 (1978) 257–267.
18 T. Itoh, Y. T. Li, S. C. Li and R. K. Yu, *J. Biol. Chem.*, 256 (1981) 165–169.
19 P. Fredman, O. Nilsson, J. L. Tayot and L. Svennerholm, *Biochim. Biophys. Acta*, 618 (1980) 42–52.
20 K. Kundu, S. K. Chakravarty, S. K. Roy and A. K. Roy, *J. Chromatogr.*, 170 (1979) 65–72.
21 S. Ando, H. Waki and K. Kon, *J. Chromatogr.*, 408 (1987) 285–290.
22 M. Blaszczyk, A. H. Ross, C. S. Ernst, M. Marchisio, B. F. Atkinson, K. Y. Pak, Z. Steplewski and H. Koprowski, *Int. J. Cancer*, 33 (1984) 313–318.
23 J. E. Månsson, B. Rosengren and L. Svennerholm, *J. Chromatogr.*, 322 (1985) 465–472.
24 L. Svennerholm, *J. Neurochem.*, 10 (1963) 613–623.