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Comparison of Analytical Methods to Detect Polysialic Acid

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Abstract

Polysialic acid (polySia) is a highly anionic polymer of sialic acid that mostly modifies the neural cell adhesion molecule (NCAM), and involved in brain functions such as learning, memory, circadian rhythm, and social behaviors, through making repulsive and attractive fields on the cell surface. Recently, polySia has been reported to have relationships with some diseases such as psychiatric disorders and cancers. To understand functions of polySia and to apply evaluation methods of polySia structures to the diagnosis of diseases, the comparison of the methods for determining the quality and quantity of polySia structures would be important and necessary. In this study, two monoclonal antibodies, 12E3 and 735, of distinct binding properties were chosen for immunochemical methods by an enzyme-linked immunosorbent assay (ELISA) and a Western-blotting. Two chemical methods, mild acid hydrolysis-fluorometric anion exchange chromatography (MH-FAEC) analysis, and fluorometric C_7/C_9 analysis were also chosen. These methods were applied to a small amount of crude adult and embryonic brain homogenates to evaluate the quantity and quality of polySia structure. The quantity of polySia can be evaluated using anti-polySia antibodies by ELISA. In addition, MH-FAEC can be also applied to the evaluation of both quantity and quality (chain length) of polySia chain. Biochemical characters of polySia-NCAM in crude homogenates can be analyzed using a mono Q-anion exchange chromatography. Combinational analyses of these methods could help our further understanding of polySia structure, and the quantity and quality of polySia will become criteria useful for diagnosis of diseases.

Keyword: Polysialic Acid; NCAM; Schizophrenia; Antibody; HPLC; DMB; Brain

Abbreviation: BDNF; Brain Derived Neurotrophic Factor; diSia: Disialic Acid; DMB: 1,2-diamino-4,5-methylenedioxybenzene; DP: Degree of Polymerization; FGF2: Fibroblast Growth Factor 2; HPLC: High Performance Liquid Chromatography; NCAM: Neural Cell Adhesion Molecule; Neu5Ac: N-acetylneuraminic acid; OligoSia: Oligosialic Acid; PolySia: Polysialic Acid; ST8SIA: alpha2,8-sialyltransferase.

Introduction

Polysialic acid (polySia, PSA) is a polymer of sialic acid with a degree of polymerization (DP) ranging from 8 to 400 and is mainly attached to neural cell adhesion molecule (NCAM) in brains of vertebrates [1]. PolySia is expressed in brains during embryonic and post-neonatal development and mostly disappears in adult brains, although expression levels of NCAM remain unchanged. In adult, polySia-NCAM expression is observed in distinct regions where neural plasticity and remodeling of neural connections or neural generation are ongoing, such as the hippocampus, and olfactory system [2]. Due to its bulky and polyanionic natures, polySia has an anti-adhesive effect on cell-cell interactions mediated by homophilic and heterophilic bindings of NCAM and other CAMs [2]. Recently, we have newly demonstrated that polySia functions as a reservoir of neurological active molecules [1] such as BDNF [3], FGF2 [4], and dopamine [5].

Two polysialyltransferases, ST8SIA2 (STX/siat8b) and ST8SIA4 (PST/siat8d), are responsible enzymes for the biosynthesis of polySia on NCAM [6]. To understand the polySia function, several types of polySia-related enzyme deficient mice were developed. ST8SIA2 and ST8SIA4 double KO (DKO) mice have no polySia structure using mAb. 735 as a polySia-detecting probe and show a severe phenotype, characterized by postnatal growth retardation, precocious death, high incidence of hydrocephalus and agenesis, and hypoplasia of major brain fiber tract [7]. Because almost all DKO mice die soon after birth (80% die before the age of 4 weeks), it appears that the presence of polySia plays a direct and important role in brain functions. Interestingly, ST8SIA2 single KO (SKO) [8] and ST8SIA4 SKO mice [9] show mild phenotypes, suggesting that remaining polySia structures compensate polySia functions. Unexpectedly, extremely large amounts of polySia are present in SKO mice (50% in ST8SIA2-SKO mice and 95% in ST8SIA4-SKO mice) however, the phenotypes are different. ST8SIA2-SKO mice exhibit misguided infrapyramidal mossy fibers and form ectopic synapses in the hippocampus. ST8SIA2 SKO mice also exhibit higher exploratory drive and reduced behavioral responses to Pavlovian fear conditioning. The phenotype of ST8SIA4 SKO mice was characterized by a marked decrease of polySia in the CA1 region of Ammon's horn, indicating that ST8SIA4 is involved in hippocampal synaptic plasticity, particularly in long-term potentiation (LTP) and long-term depression (LTD). ST8SIA4 SKO mice are predominantly characterized by decreased motivation in social interaction. These differences of phenotypes between ST8SIA2-KO mice and ST8SIA4-KO mice have been considered to come from the difference of polySia structures biosynthesized by ST8SIA2 and/or ST8SIA4.

It is also reported that polysialyltransferase, especially ST8SIA2 is related to schizophrenia [5,10,11] and autism [12,13]. For example, a decrease in polySia-NCAM immunoreactivity was observed in schizophrenic hippocampi [14] and patients with schizophrenia often have low olfactory volume [15]. Impairment of hippocampal functions and disturbance of its anatomical organization are also involved in the etiology of schizophrenia [16]. Recently, the chromosome where ST8SIA2 is localized, 15q26, was reported as a common susceptibility region for both schizophrenia and bipolar disorder in a genome scan of Eastern Quebec families [17]. Arai *et al* also revealed an association

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between polymorphisms in the promoter region of the ST8SIA2 gene and schizophrenia in the Japanese population [10]. Recently, we have focused on the SNP-7 found in the ORF of ST8SIA2 gene reported from a schizophrenic patient and demonstrated that polySia-NCAM biosynthesized by mutant ST8SIA2 contained less polySia with shorter chain length and exhibited impaired reservoir function for neurological active molecule such as BDNF as compared with that synthesized by wild-type ST8SIA2 [5].

Now it is demonstrated that quantity and quality of polySia which are related to some diseases are very important. Therefore, it is required to establish the quantification of the polySia structure in quantity and quality using crude samples to understand the polySia functions and diagnosis for diseases. So far there are several methods to detect polySia structure, anti-polySia antibodies-based detection methods, and chemical methods after labeling with sialic acid-specific labeling reagent; however, a comparative study among these methods especially using crude samples has not been done. In this study, we compared detection methods for polySia structure in quantity and quality using pig embryonic brain homogenate and adult brain homogenate as authentic crude samples and showed the possibility for the evaluation of polySia in quantity and quality.

Materials and Methods

Materials-α-2-3,6-neuraminidase (sialidase), bovine serum albumin, and phenylmethylsulfonyl fluoride (PMSF) were obtained from Sigma-Aldlich (St. Louis, MO). BCA assay kit and immunoplate were purchased from Thermo Fisher Scientific (Waltham, MA). Pre-stained molecular weight marker and nitrocellulose membrane were obtained from Bio-Rad (Hercules, CA). Polyvinylidene difluoride (PVDF) membrane (Immobilon P) was a product of Millipore (Bedford, MA). 1,2-diamino-3,4-methylenedioxybenzene (DMB) was purchased from Dojindo (Kumamoto, Japan). Colominic acid (polyNeu5Ac) and a Handy-ODS were from Wako (Osaka, Japan). Peroxidase-conjugated goat anti-mouse (IgG+IgM) was purchased from American Qualex (San Clemente, CA). Mono Q anion exchange chromatography and enhanced chemiluminescent detecting reagents were purchased from GE healthcare (Piscataway, NJ). *A*-2,8-Linked oligo/polyNeu5Ac mouse monoclonal antibodies 12E3 (IgM) was kindly provided by Dr. T. Seki (Juntendo University School of Medicine, Tokyo, Japan) and purified as described [18]. A mouse monoclonal antibody, mAb.735 (IgG2a) was generously provided by Dr. J. Roth (University of Zürich, Switzerland).

Pig Brain Homogenates: Embryonic and adult pig brains were purchased from Tokyo Shibaura Zouki, Co. Ltd. (Tokyo, Japan) and whole brain homogenates were prepared as described previously [19].

Enzyme-Linked ImmunoSorbent Assay (ELISA): Samples were adjusted to 100 μg/ml (as BSA) with PBS (the concentration of Triton X-100 should be below 0.03%) and 50 μl of the solutions were serially diluted and absorbed onto 96-well immunoplate. Plates were incubated at 37°C for 2 hours and washed with PBS three times. Then 100 μl of 1% BSA solution were added into the wells and incubated at 37°C for 1 hour for blocking. After washing with PBS three times, antibody solution, 12E3 (1 μg/ml) or 735 (0.8 μg/ml) were added into the wells and incubated at 4°C for overnight. Secondary antibody, peroxidase-conjugated anti-mouse IgG+IgM (2 μg/ml) were then incubated at 37°C for 1 hour after washing with PBS containing 0.05% Tween-20 (PBST) four times. Color development was performed as described [18].

SDS-PAGE and Western-blotting on PVDF membrane Samples: (10 μg protein/lane) were dissolved in Laemmli buffer and heated at 60°C for 20 min before SDS-PAGE (7.5% PAG) and blotting on the PVDF membrane. Western-blotting was performed using 12E3 (1 μg/ml) or 735 (0.8 μg/ml) as described previously. [19]

Mild acid hydrolysis-fluorometric anion-exchange chromatography analysis (MH-FAEC): Samples (1.0 mg protein as BSA) in 200 μl of 0.005 N TFA were added to 200 μl of DMB solution and incubated at 50°C for 1 hour. Then cold ethanol was added to the samples (final concentration, 95%) and stood at -80°C for 2 hours. After centrifugation, the supernatants were dried up by speed vaq. The dried samples were dissolved in water and NaOH was added to a final concentration of 0.1 N and incubated at 37°C to remove lactonization. After neutralization with 1 N HCl, the samples were diluted and subjected to an anion-exchange chromatography column (DNApac PA-100, 4×250 mm, DIONEX) and separated on a JASCO HPLC system. The sample was loaded on a column and eluted with 20 mM Tris-HCl (pH 8.0), followed by NaCl gradient (0-15 min, 0 M; 15-185 min, 0 →0.6 M; 185-190 min, 0.6 →1.0 M) in 20 mM Tris-HCl (pH 8.0) and 0.1% Triton X-100. The flow rate was 1 ml/min and fractions were monitored with a fluorescent detector (Em 373 nm, Ex 448 nm, FP-2020, JASCO, Japan) [19, 20].

Chemical analysis of α-2,8-linked oligo/polySia chains on glycoproteins blotted onto PVDF membranes: Brain glycoproteins (100 μg protein as BSA) in 14 μl were added to 4 μl of 5× Reaction buffer and 2 μl of α-2-3,6-sialidase treatment (25 μU) and incubated at 37°C for 1 hour to release monoSia residues. The sialidase treated samples were blotted onto PVDF membranes as described earlier and areas above 100 kDa were cut out. The membranes were analyzed by the fluorometric C_7/C_9 method for internal sialyl residues as previously described [1,21]

Analysis of polySia-NCAM from brain homogenates using anion-exchange chromatography: PEB and PAB samples (200 μg as BSA) were treated with α-2-3,6-sialidase as described above and the monoSia-cleaved samples were applied onto a Mono Q anion-exchange column (1 ml) and separated on a JASCO HPLC system to determine the net negative charge (NC) of polySia as described previously [3] The sample was loaded on a column and eluted with 20 mM Tris-HCl (pH 8.0), followed by a NaCl gradient (0-10 min, 0 M; 10-30 min, 0 →0.3 M; 30-105 min, 0.3 →0.5 M; 105-135 min, 0.5 →3 M) in 20 mM Tris-HCl (pH 8.0) and 0.1% triton X-100. The flow rate was 0.5 ml/min and 1 ml was collected per fraction. After the samples were dot-blotted onto a PVDF membrane, the amount of polySia in each fraction was determined by the anti-polySia staining (12E3) of the membrane. The column was calibrated with colominic acid (authentic polySia) as described [5] .

Results

ELISA and Western-blotting using anti-polySia antibodies: To characterize polySia-NCAM from pig embryonic brain (PEB) and pig adult brain (PAB), ELISA and Western-blotting were performed using two anti-polySia antibodies of different binding specificities. One was a monoclonal 12E3 antibody (IgM) that recognizes oligo/polysialic acid structures with DP 5 and greater in which the non-reducing terminal residue should be intact; the other was a monoclonal 735 antibody (IgG) that recognizes the internal sialyl residues of polysialic acid structures with DP 11 and greater [1,18]. In ELISA, both 12E3 and 735 showed strong immunoreactivities to the PEB homogenates; however, they showed far less immunoreactivities to the PAB homogenates

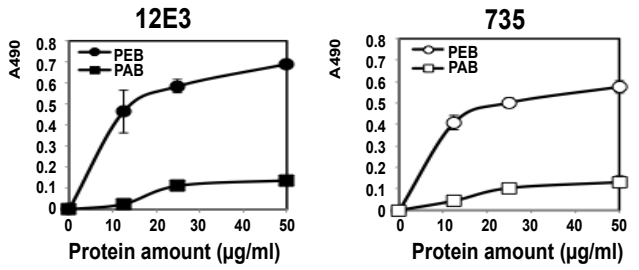

Figure 1: ELISA analysis using polySia-specific antibodies. The plastic wells were coated with various concentrations of PEB and PAB samples (0-50 μg/ml as BSA) and incubated at 37°C for 2 hours. After blocking with 1% BSA, anti-polySia antibodies, 12E3 (1 μg/ml) specific to $(Neu5Ac)_5$ or greater with non-reducing terminal end and 735 (0.8 μg/ml) specific to $(Neu5Ac)_{11}$ or greater were incubated. After washing, peroxidase conjugated anti-mouse IgG+IgM were incubated and color development was performed as described in Materials and Methods.

(Figure 1) (12E3 and 735). These results are highly reproducible, and consistent with the previous reports that polySia amounts dramatically reduced in adult brain compared with those in embryonic brains [22]. The proportion of the immunoreactivity against PAB to PEB was 21% and 22% for 12E3 and 735, respectively (Figure 1, 12E3 and 735, Table 1). These results indicate that the quantity of polySia in PAB is reduced from that in PEB by 80%.

In Western-blotting, for PEB, both 12E3 and 735 antibodies immunostained polySia-NCAM smear at greater than 250 kDa and gave the same staining patterns in intensity and molecular size range. In contrast, for PAB, 12E3 and 735 antibodies immunostained polySia-NCAM smear differently from each other: although the upper size limit of the broad smear was about 300 kDa and much the same between the two antibodies, the lower size limit was 150 kDa and 250 kDa for 12E3 and 735 antibodies, respectively. The intensity of 12E3 immunostaining toward PAB, which was densitometrically measured, was 21% of that toward PEB (Figure 2, 12E3). In the case of 735 antibody, the intensity of the immunostaining toward PAB was 10% of that toward PEB (Figure 2, 735). Based on the results of ELISA and Western-blotting, it was shown that the amount of polySia in brain homogenates could be determined by either of the two methods using 12E3 antibody. In contrast, with 735 antibody, the amount of polySia determined by Western-blotting was two times lower than that determined by ELISA (Table 1). These results indicate that polySia-NCAM species that are detectable with 735 antibody in ELISA might not be effectively immobilized on PVDF membrane probably because of longer polySia chain attached on the NCAM.

Mild acid hydrolysis-fluorometric anion-exchange chromatography analysis (MH-FAEC): DMB is a specific fluorescent reagent for α-keto acid and applicable for fluorescent labeling of sialic acids. DMB labeling of the reducing terminus of oligo/polySia chains obtained by mild acid hydrolysis of polySia and subsequent separation of the labeled oligo/polySia on an anion-exchange chromatography depending on the degree of polymerization (DP) (MH-FAEC) were firstly developed by us [20]. MH-FAEC has been recently improved in terms of separation on HPLC by using an high resolution column [1]. The MH-FAEC method not only enables highly sensitive detection, but also gives information on the minimum degree of polymerization (DP) of polySia chains on polySia-NCAM. Colominic acid was used as an authentic 2,8-linked polySia chain, whose DP was determined to be on average 43 by anion-exchange chromatography of the unlabeled compound [3]. MH-FAEC of colominic acid gave the comb-like peaks with various DPs, and the maximum DP detected was 65. Using this method, we analyzed the labeled supernatant obtained after ethanol precipitation of crude homogenates of PEB and PAB and the comb-like profiles could be obtained to successfully determine the maximum DP of polySia chains in PEB and PAB as 41 ± 2.1 and 31 ± 4.1, respectively (Figure 3A). The maximum DP determined for PAB was decreased to 76% of that for PEB (Figure 3B). Then we applied this method to evaluate the quantity of polySia, which can be reflected by the summation of areas of all the detected peaks with DP greater than 5. The amounts of polySia derived from PAB decreased to 18% of that from PEB (Figure 3C), consistent with those results obtained from ELISA using 12E3 and 735 and from Western-blotting using 12E3 (Table 1).

Fluorometric C_7/C_9 analysis: We previously developed a fluorometric C_7/C_9 analysis to detect α-2,8-linked oligo/polySia structures [20]. The principle of this method is as follows. A weak periodate oxidation treatment changes only a non-reducing terminal residue of α-2,8-linked oligo/polySia to C_7-Neu5Ac analogue without affecting internal sialyl (C_9-Neu5Ac) residues. After reduction, the periodate-treated oligo/polySia is subjected to a strong acid treatment to release all Neu5Ac residues as monomers. The released Neu5Ac monomers consist of C_7-Neu5Ac and C_9-Neu5Ac corresponding to the non-reducing terminal and the internal Neu5Ac residues, respectively. These monomers are then quantitated by the fluorometric HPLC analysis after DMB labeling [1,21] When the samples were crude, they were pretreated with an α-2-3,6-sialidase that can cleave α-2,3-linked and α-2,6-linked Neu5Ac residues, but not α-2,8-linked Neu5Ac. This treatment could release monoSia residues from the samples, and remaining Sia residues constituted only α-2,8-linked oligo/polySia chains. This method was applied to the PVDF membrane where α-2,8-linked oligo/polySia-glycoproteins were blotted. We could clearly detect both C_7-Neu5Ac and C_9-Neu5Ac derivatives from PEB and PAB samples (Figure 4A). The amount of C_7-Neu5Ac derived from PAB was 75% of that of PEB; however the amounts of C_9-Neu5Ac derived from

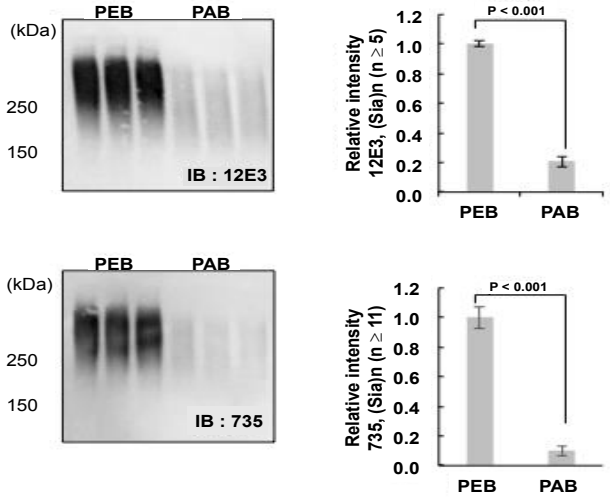

Figure 2: Western-blotting analysis using polySia-specific antibodies. The PEB and PAB crude samples (10 μg as BSA/lane) were electrophoresed and blotted onto PVDF membrane. After blocking with 1% BSA, the membrane was probed with anti-polySia antibodies, 12E3 (1 μg/ml) specific to $(Neu5Ac)_5$ or greater with non-reducing terminal end and 735 (0.8 μg/ml) specific to $(Neu5Ac)_{11}$ were incubated. After washing, peroxidase conjugated anti-mouse IgG+IgM were incubated and color development was performed as described in Materials and Methods. Left panels show the results of Western-blotting. Right panels show the relative intensity of immunostained bands with antibodies and PEB value was set to 1.0.

PAB decreased dramatically to 40% compared with PEB (Figure 4B). These values contain not only polySia but also di and oligoSia structure in protein and these structures are frequently occurred in NCAM [19]. To evaluate the quality of polySia, it is possible to compare the index of $(C^7+C_9)/C_7$ that reflects the chain length of α-2,8-linked di/oligo/polySia containing glycans. As shown in Figure 4C, the index of PEB decreased to 61% of that of PEB. This reduction is not consistent with the results obtained from MH-FAEC because MH-FAEC only evaluated the amounts of oligo/polySia with DP 5 and greater. Taken together, di/oligosialylation (DP=2~4) might occur on NCAM in PAB as suggested previously [19].

Analysis of polySia-NCAM with an anion-exchange chromatography: We previously analyzed the net negative charge (NC) of soluble polySia-NCAM-Fc without membrane-spanning region on an anion-exchange chromatography [5,23]. Here we applied this method to membrane-bound polySia-NCAM derived from crude brain homogenates. After treated with α-2-3,6-sialidase to remove monoSia residues on glycoproteins, the homogenates were subjected to a mono Q anion exchange chromatography with detergent. The polySia-NCAM thus obtained is expected to contain one or at most two polySia-containing *N*-glycans on its Ig domain V. The eluted fractions were analyzed by dot blotting with anti-polySia antibody, 12E3. After the column was calibrated for the elution position of polySia, or NC, with colominic acid, PEB and PAB samples were analyzed on the same column under the same conditions (Figure 5A). For PEB, polySia-containing glycoproteins were eluted at 0.66-1.07 M NaCl, corresponding to NC=24-114. In contrast, for PAB, polySia-containing glycoproteins unexpectedly gave two peaks eluted at higher salt concentrations, 1.48-1.69 M (NC=870-2100) and 1.80-2.10 M NaCl (NC=3300 and greater). Then the peak fraction 22 at 0.87 M NaCl from PEB and fractions 40 and 48 at 1.6 M and 2.0 M NaCl, respectively, from PAB were analyzed by MH-FAEC and C_7/C_9 analyses. Based on the MH-FAEC profiles, the maximum chain length detected in fraction 22 from PEB was DP 31, while those in fractions 40 and 48 from PAB were DP 27 and DP 34, respectively (Figure 5B). The fraction 22 from PEB and 40 and 48 from PAB were also analyzed by the C_7/C_9 analysis (Figure 5C *upper*). The $(C_7+C_9)/C_7$ value, an index for the quality of polySia-NCAM, was also calculated (Figure 5C *lower*). The index value for fraction 22 from PEB was estimated to be 25, while those for fractions 40 and 48 from PAB were 7 and 5, respectively. These results suggest that, although both PEB and PAB contain the polySia-NCAM with longer polySia chains, i.e., DP=around 30, PAB contain NCAM with shorter polySia chain (or di and oligoSia) much more frequently than PEB. However, it remains unknown why the polySia-NCAMs from PAB were eluted at unexpectedly higher NaCl concentration on the anion-exchange chromatography (Figure 5A), while it had far less negative charges than that from PEB (Figure 5C). This may come from unknown structural factor of polySia-NCAM other than sum of net negative charge of polySia.

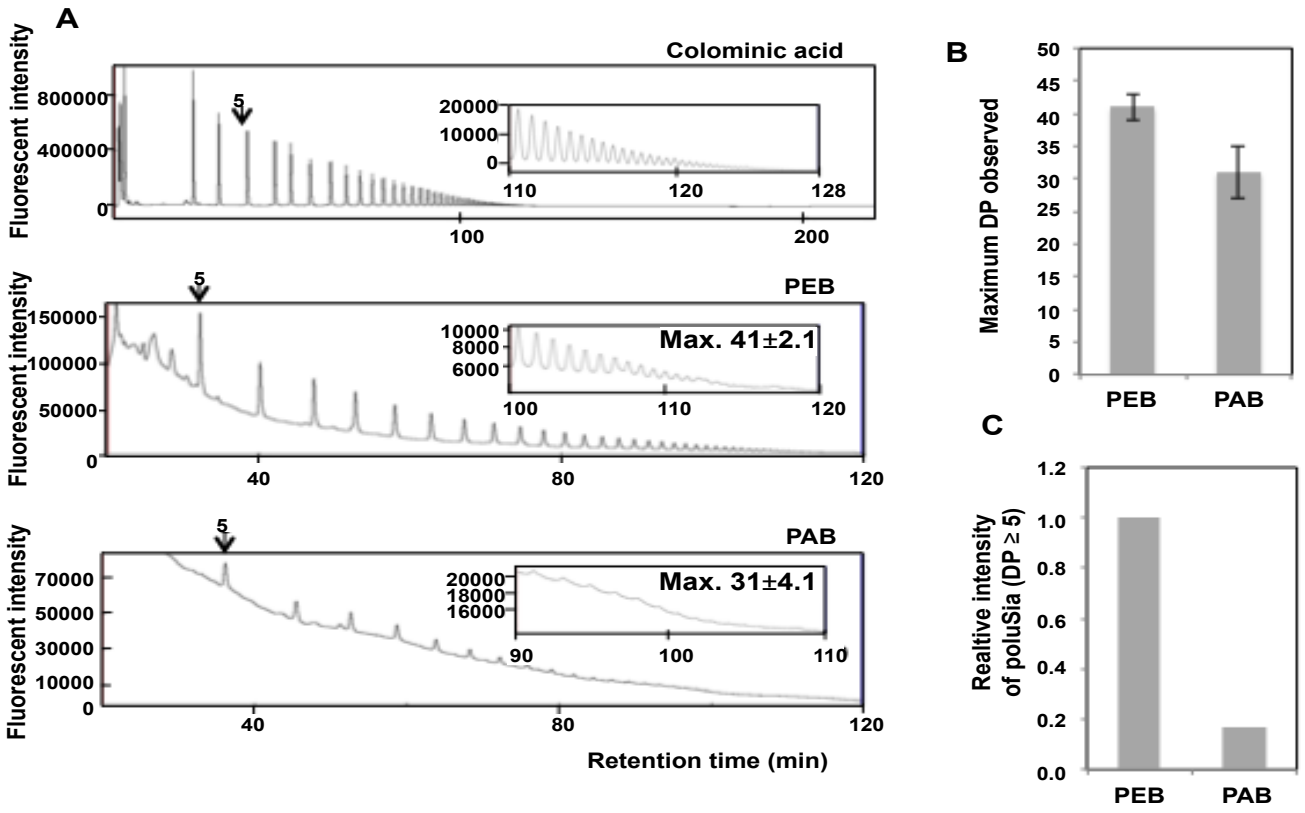

Figure 3: Mild acid hydrolysis-fluorometric anion-exchange chromatography analysis (MH-FAEC). The PEB and PAB crude samples (1 mg as BSA) were mild acid hydrolyzed and simultaneously labeled with DMB. Released and labeled oligo/polySias were separated with DNA-Pac PA-100 anion exchange chromatography and eluted with a gradient of NaCl (0-15 min, 0 M; 15-185 min, 0 →0.6 M; 185-190 min, 0.6 →1.0 M) in 20 mM Tris-HCl (pH 8.0). The flow rate was 1 ml/min. Eluted oligo/polySia-DMBs were monitored with fluorescent detector (Ex 373 nm, Em 448 nm). (A) Chromatograms of MH-FAEC. Colominic acid, PEB and PAB were analyzed. Three independent experiments were performed and typical chromatograms were shown. The average of Maximum DP (B) and the relative amounts of oligo/polySia ((Neu5Ac) n-DMB, n ≥ 6) obtained by three independent experiments. The sum of intensity of oligo/polySia (Neu5Ac) n-DMB, n ≥ 6) was set to 1.0.

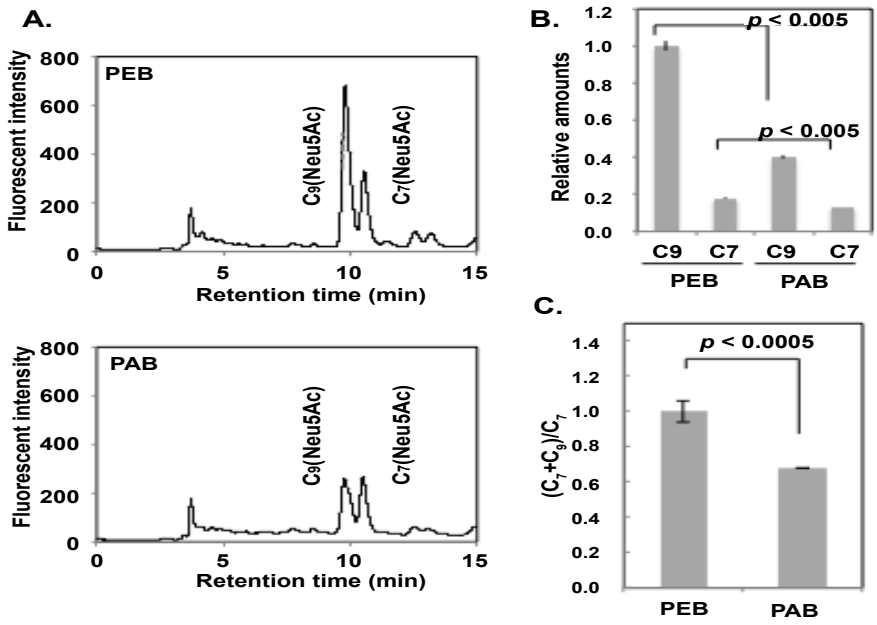

Figure 4: Fluorometric C_7/C_9 analysis. The PEB and PAB crude samples (100 µg as BSA) were treated with monoSia-specific sialidase, electrophoresed and blotted onto PVDF membrane. PolySia-NCAM at greater than 100 kDa area were cut out and subjected to fluorometric C_7/C_9 analysis. The samples were treated with mild periodate and non-reducing terminal end of α-2,8-linked oligo/polySia was changed to C_7-analogues while internal sialic acid (C_9-sialic acid) remain unchanged. After acid treatment, C_7- and C_9-Sias were released and labeled with DMB. The DMB-labeled sialic acids were applied to the HPLC equipped with a Wako Handy ODS column (250×4.6 mm, Wako), and a fluorescence detector (FP-2025, JASCO). The column is equilibrated using methanol/acetonitrile/water (7:9:84, v/v/v) at 26˚C. Samples were applied to HPLC analysis isocratically flow at 1.0 ml/min. (A) Typical chromatograms of C_7/C_9 analysis of PEB and PAB samples. (B) Relative amounts of C_9-Neu5Ac and C_7-Neu5Ac derived from PEB and PAB samples. The amount of C_9-Neu5Ac derived from PEB was set equal to 1.0. (C) The $(C_9+C_7)/C_7$ index. The index of PEB was set to 1.0.

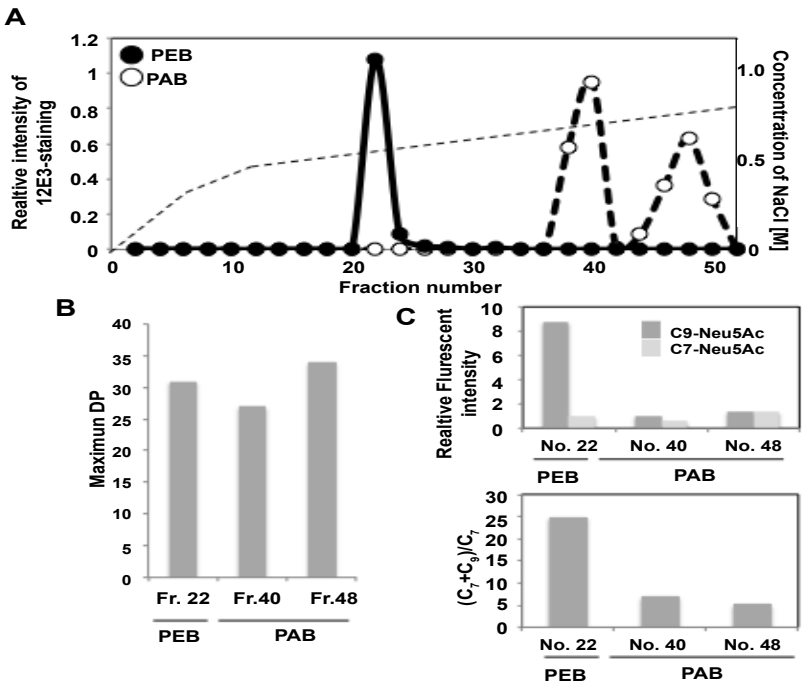

Figure 5: Mono Q anion exchange chromatography of PEB and PAB. PEB and PAB were treated with monoSia-specific sialidase and subjected to the mono Q anion exchange chromatography. The samples were eluted with a NaCl gradient (0-10 min, 0 M; 10-30 min, 0 →0.3 M; 30-105 min, 0.3 →0.5 M; 105-135 min, 0.5 →3 M) in 20 mM Tris-HCl (pH 8.0). The flow rate was 0.5 ml/min and 1 ml was collected per fraction. After the samples were dot-blotted onto a PVDF membrane, the amount of polySia in each fraction was determined by the anti-polySia staining (12E3) of the membrane. (A) Chromatogram of mono Q anion exchange chromatography. (B) MH-FAEC analysis of peaks detected with 12E3 staining, fraction (fr.) 22 from PEB and fr. 40 and 48 from PAB. Maximum DP of the samples were shown. (C) Fluorometric C_7/C_9 analysis. Upper panel shows relative amounts of C_7-Neu5Ac and C_9-Neu5Ac. Samples derived from peaks detected with 12E3 staining, fraction 22 from PEB and fraction 40 and 48 from PAB were analyzed. Lower panel shows the $(C_9+C_7)/C_7$ value, index as quality of di/oligo/polySia.

Discussion

PolySia is an essential glycan for normal brain functions and its structural alterations caused by impairments of the polysialyltransferase genes often lead to several diseases such as psychiatric disorders and cancers [1,11] Recently, an increasing number of studies have reported that the immunostaining of polySia in brains is greatly deviated depending on diseases, such as schizophrenia [14] Alzheimer's disease [24], Parkinson's disease [25], and drug abuse [26]. Therefore, the immunostaining profiles of polySia could be a good diagnostic marker for those diseases. The differences in the immunostaining profiles of polySia are often discussed for the quantity difference of polySia. However, this is not always the case, because the immunostaining profiles are greatly affected by the immunospecificity of anti-polySia antibodies used in the experiments. We previously pointed out that there are large repertoires of anti-polySia antibodies whose immunospecificities are different in terms of DP and sialic acid species [19,27]. In addition to the DP, the number of polySia chains per NCAM glycan also affects the intensity of immunostaining with these antibodies. Accordingly, we should understand that the intensity of the immunostaining does not precisely tell the quantity or the quality of polySia structure, i.e., the DP and the number of polySia chains per NCAM glycan. Therefore, to understand the precise structural features of polySia, we need methods other than immunostaining. In this study, we sought to understand the quantity and quality of polySia of PEB and PAB with μg-order amount of crude samples using several methods, including highly sensitive immunochemical and chemical methods. Here we compare the results obtained by those methods using the same crude samples and summarized them in Table 1.

Antibodies are most useful, essential probes to determine polySia structures. Although not so many studies have taken care of the specificities of antibodies used, the immunospecificity of the antibodies is very important. Previous reports determined the binding specificities of anti-polySia antibodies in detail [18,27]. However, there are still some antibodies whose specificities are unknown, such as 12F8 that are often used as the anti-polySia antibody. We used two typical anti-polySia antibodies, 12E3 and 735, whose specificities are determined precisely to be greater than 5 and 11, respectively [1]. Interesting binding properties of these two antibodies are that 12E3 recognizes polySia-chain that has an intact non-reducing terminal end, while 735 requires only internal sialyl residues of polySia for binding [18] Using these antibodies, the quantity of polySia in pig brain homogenates can be evaluated by ELISA and Western-blotting using 12E3 (Figure 1 and 2, Table 1). In case of 735, the amount of polySia determined by Western-blotting is lower than that determined by ELISA. This is probably because the blotting efficiency of components with longer polySia chain, or with high reactivity to 735, on PVDF membrane is very low. The difference of the immunostaining patterns of polySia-NCAM from PAB between 12E3 and 735 supports the low efficiency of blotting of components with longer polySia chain (Figure 2, 735). Notably, the quantity of polySia can be also evaluated by MH-FAEC that has been developed for detection of the maximum DP of polySia chain (Figure 3C). We sum up the amount of (Neu5Ac)n-DMB peaks with DP greater than 6, which are corresponding to the same epitopes that 12E3 recognizes. The quantities of polySia can be evaluated using crude homogenates that contain large amounts of contaminated proteins other than polySia-NCAM. The quantity value obtained by MH-FAEC shows a good correlation with those obtained by ELISA and Western-blotting. Oligo/polySia chains are characterized by the presence of internal sialyl residues, whose quantity can be evaluated by the amount of C_9-Neu5Ac (C_9) obtained by the C_7/C_9 analysis [21]. The amount of C_9 in PAB was reduced from that in PEB by 40%. This reduction was much lower than expected for that of the polySia quantity between PEB and PAB, i.e., 80% reduction in ELISA (Table 1). The higher C_9 value for PAB suggests that not only polySia-NCAM with longer polySia chain is decreased from PEB to PAB, but also that NCAM and other proteins in PAB still contain di/oligoSia chains [1,19].

Evaluation of	Methods	Estimation for	PEB	PAB
Quantity	ELISA	Amount of the 12E3 epitope	100 (%)	21 (%)
		Amount of the 735 epitope	100 (%)	22 (%)
	Western blotting	Intensity of the 12E3 immunostaining	100 (%)	21 (%)
		Intensity of the 735 immunostaining	100 (%)	10 (%)
	MH-FAEC	Total area of the peaks with DP ± 6	100 (%)	18 (%)
	C_7/C_9 analysis	Amount of internal sialyl residues (C_9)	100 (%)	40 (%)
Quality	MH-FAEC	Maximum DP	100 (%)	76 (%)
	C_7/C_9 analysis	Average DP index (C_7+C_9)/C_7	100 (%)	73 (%)

Table 1: Comparison of quantity and quality of polySia in PEB and PAB as estimated by various methods.

There are several points for the quality of polySia-NCAM. One is the chain length of polySia on NCAM. In this point, the maximum DP obtained by the MH-FAEC analysis can be used as a quality index. The maximum DP for PEB and PAB were obviously different and the maximum DP of polySia-NCAM from PAB was shorter than that from PEB by around 10 residues. Therefore, the quality of polySia chain decreases 76% in terms of maximum DP of polySia. The average DP, which is obtained as the (C_7+C_9)/C_7 value by the C_7/C_9 analysis [21], is also used as a quality index. The average DP of polySia directly coincides with the DP of the single di/oligo/polySia chain. However, the *N*-glycans usually have two to four antennas and we do not know how many polySia chains are attached on two *N*-glycan chains of the Ig5 domain of NCAM [1,2]. Therefore, the average DP in crude homogenates does not always mean the DP of polySia. This problem may be improved, albeit not completely, if the samples are treated with α2-3,6-sialidase to remove the monoSia residues, remaining only di/oligo/polySia chain, before the C_7/C_9 analysis. The average DP for PAB was decreased to 61% compared with that for PEB.

It is interesting that polySia-NCAM in adult brain (PAB) homogenates was eluted at higher salt concentration (1.4-2.1 M or NC= 870 or greater) using mono Q anion-exchange chromatography with Triton X-100 (Figure 5A). However, the maximum DP by MH-FAEC and the average DP index by C_7/C_9 analysis indicate that polySia chains from PAB are shorter than that from PEB (Figure 3B). This is the first demonstration of the big difference between polySia-NCAM derived from PEB and PAB. The reason why polySia-NCAM from PAB is retarded on the anion-exchange chromatography compared with the polySia-NCAM from PEB is still unknown. NCAM is known to bind homophilically to each other and heterophilically to other proteins [2]. If the polySia-NCAM from PAB might have the similar property, the polySia-NCAM from PAB would easily make a large complex in the homogenate. This property is unique to the polySia-NCAM from PAB, but not to that from PEB. In this regard, we have recently found that a NCAM has a sialic acid-binding property (Hane *et al.* unpublished results). This property may help the formation of large complex in PAB, but not in PEB.

Based on the immunochemical and chemical results obtained in this study, we can conclude as follows. In embryonic brain, NCAM is

modified exclusively with longer polySia chains. In adult brain, NCAM is mostly modified with di/oligoSia chains, while a small population (20%) of NCAM is modified with long polySia chains. It is interesting to point out that adult brain polySia has much the same chain length (quality) with the embryonic brain polySia. This aspect is first clarified in this study. There are many reports discussing that the quantity of polySia in brain decreases during development from embryo to adult based on the intensity of immunostaining [22] however, our study indicates that the quantity estimation is greatly different depending on the antibody used, i.e., 12E3 and 735. In addition, our study can first describe the abundance of di/oligoSia (quality of polySia) in adult brain in more detail using combinational analyses by sensitive immunochemical and chemical methods. In conclusion, combinational analyses of these methods are useful for the evaluation of the quantity and quality of polySia chain, and might be applicable for the diagnosis of pathological states of diseases using a small amount of crude samples.

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