Journal of Chromatography, 434 (1988) 51–60
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4434

ANALYSIS OF TISSUE GLYCOPROTEIN SUGAR CHAINS BY TWO-DIMENSIONAL HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC MAPPING

SUMIHIRO HASE

Department of Chemistry, Osaka University College of Science, Toyonaka, Osaka 560 (Japan)

KAZUHIRO IKENAKA* and KATSUHIKO MIKOSHIBA

Institute for Protein Research, Osaka University, Suita, Osaka 565 (Japan)

and

TOKUJI IKENAKA

Department of Chemistry, Osaka University College of Science, Toyonaka, Osaka 560 (Japan)

(First received May 16th, 1988; revised manuscript received August 16th, 1988)

SUMMARY

Excellent separation of 45 pyridylamino derivatives of oligosaccharides were achieved by the two-dimensional combination of reversed-phase and size-fractionation high-performance liquid chromatography. The sugar chains of brain glycoproteins were derivatized into a mixture of pyridylamino-oligosaccharides from lyophilized brain tissue without any purification steps, and they were well separated by the system used. The pattern obtained was reproducible, and inter-individual variation was negligible. This finding demonstrated the possibility that this method could be applied to the detection of differences in the structure of glycoprotein sugar chains in crude preparations.

INTRODUCTION

Derivatization of the reducing ends of glycoprotein sugar chains by 2-aminopyridine [1] has been found to be a useful method for their isolation and structural determination. This is because pyridylamino (PA) derivatives of sugar chains can be detected with high sensitivity, and particularly because excellent separation can be achieved by a combination of reversed-phase [2–4] and size-fractionation [2,3] high-performance liquid chromatography (HPLC). In this paper, we describe the successful separation of 45 PA-oligosaccharides, demonstrating the possibility that this method could be applied to the detection of differences in the

0378-4347/88/$03.50

structure of glycoprotein sugar chains in crude preparations. Two-dimensional gel electrophoresis of proteins has been used successfully to detect defective proteins in ill or mutant animals, although no convenient methods for analysing the structure of the sugar chains have been reported.

In this study, we showed that the sugar chains of brain glycoproteins can be derivatized to a mixture of PA-oligosaccharides from lyophilized brain tissue without any purification steps, and that they can be separated well on two-dimensional HPLC. The pattern obtained was reproducible, and inter-individual variation was negligible.

EXPERIMENTAL

Materials

A Cosmosil 5C-18P column was purchased from Nacalai Tesque (Kyoto, Japan), TSK-GEL HW-40F from Tosoh (Tokyo, Japan), MicroPak AX-5 from Varian (Walnut Creek, CA, U.S.A.) and YMC-Gel SIL S-5 from Yamamura Kagaku (Kyoto, Japan). N-Acetylneuraminidase type X (*Clostridium perfringens*) was purchased from Sigma (St. Louis, MO, U.S.A.) The PA-sugar chains and PA-oligosaccharides used are listed in Table I. Methods of preparation of F3 and F6 [2], M5A-M9A [3], M4A, M3AF3X, M2BF3X, MF3X, MF3, M3AX, M3AF3, M4X and MX [5] and M3A, MO3 and MO6 [6] have been described previously. M2A was obtained by partial acetolysis of M3A, and M2B was obtained by digestion of M2F3X with exoglycosidases. M1 was obtained from α-mannosidase digestion of M3A, and GN_2 was prepared from chitobiose. TE, TEF, TR4, TR4F and BI were prepared from corresponding glycopeptides of α_1-acid glycoprotein kindly donated by Dr. K. Schmid (Boston University), and their structures were identified by 500-MHz ^1H NMR spectroscopy. HB was prepared from hen ovalbumin and BI-G_2, TR4-G_3 and TE-G_4 were prepared by β-galactosidase digestion of the corresponding PA-sugar chains. MO3-G and MO6-G were obtained by β-galactosidase digestion of MO3 and MO6, respectively. M4B and M3B were prepared by partial acetolysis [7] of M9A and M6B, respectively. M2F3 and M2X were prepared by digestion of M2BF3X with exoglycosidases [2].

High-performance liquid chromatography

A Beckman Model 332 chromatograph, equipped with a Waters Model 710B automatic injector and a Hitachi model 650-10 fluorescence spectrophotometer, was used.

Size-fractionation HPLC of PA-sugar chains was carried out using a MicroPak AX-5 column (15 cm $\times$ 0.46 cm I.D.) at a flow-rate of 1.0 ml/min at 23°C, as described previously [2]. A YMC-Gel Sil S-5 column (15 cm $\times$ 0.46 cm I.D.) was used as a precolumn to prevent damage to the MicroPak AX-5 column. Two solvents, A and B, were used. Solvent A was acetonitrile–water (80:20, v/v) containing 3% (v/v) acetic acid with the pH adjusted to pH 7.3 with triethylamine. Solvent B was 3% (v/v) aqueous acetic acid with its pH adjusted to pH 7.3 with triethylamine. The column was equilibrated with solvent A. After injection of a

TABLE I

ABBREVIATIONS AND STRUCTURES OF PYRIDYLAMINO SUGAR CHAINS

Abbreviation	Structure
GN_2	GlcNAcβ1-4GlcNAc – PA
M	Manβ1-4GlcNAcβ1-4GlcNAc – PA
M2A	Manα1-3Manβ1-4GlcNAcβ1-4GlcNAc – PA
M2B	Manα1-6Manβ1-4GlcNAcβ1-4GlcNAc – PA
M3A	Manα1-6Manβ1-4GlcNAcβ1-4GlcNAc – PA Manα1-3$\rfloor$
M3B	Manα1-2Manα1-3Manβ1-4GlcNAcβ1-4GlcNAc – PA
M4A	Manα1-3Manα1-6Manβ1-4GlcNAcβ1-4GlcNAc – PA Manα1-3$\rfloor$
M4B	Manα1-2Manα1-2Manα1-3Manβ1-4GlcNAcβ1-4GlcNAc – PA
M5A	Manα1-6$\rceil$ Manα1-3Manα1-6Manβ1-4GlcNAcβ1-4GlcNAc – PA Manα1-3$\rfloor$
M6A	*Manα1-2Manα1-6$\rceil$ Manα1-3Manα1-6Manβ1-4GlcNAcβ1-4GlcNAc – PA Manα1-3$\rfloor$
M6B	Manα1-6$\rceil$ Manα1-3Manα1-6Manβ1-4GlcNAcβ1-4GlcNAc – PA Manα1-2Manα1-3$\rfloor$
M6C	Manα1-6$\rceil$ Manα1-2Manα1-3Manα1-6Manβ1-4GlcNAcβ1-4GlcNAc – PA Manα1-3$\rfloor$
M7A	*Manα1-2Manα1-6$\rceil$ Manα1-3Manα1-6Manβ1-4GlcNAcβ1-4GlcNAc – PA Manα1-2Manα1-3$\rfloor$
M7B	Manα1-6$\rceil$ Manα1-3Manα1-6Manβ1-4GlcNAcβ1-4GlcNAc – PA Manα1-2Manα1-2Manα1-3$\rfloor$
M7C	*Manα1-2Manα1-6$\rceil$ Manα1-2Manα1-3Manα1-6Manβ1-4GlcNAcβ1-4GlcNAc – PA Manα1-3$\rfloor$
M7D	Manα1-6$\rceil$ Manα1-2Manα1-3Manα1-6Manβ1-4GlcNAcβ1-4GlcNAc – PA Manα1-2Manα1-3$\rfloor$
M8A	*Manα1-2Manα1-6$\rceil$ Manα1-3Manα1-6Manβ1-4GlcNAcβ1-4GlcNAc – PA Manα1-2Manα1-2Manα1-3$\rfloor$
M8B	*Manα1-2Manα1-6$\rceil$ Manα1-2Manα1-3Manα1-6Manβ1-4GlcNAcβ1-4GlcNAc – PA Manα1-2Manα1-3$\rfloor$
M8C	Manα1-6$\rceil$ Manα1-2Manα1-3Manα1-6Manβ1-4GlcNAcβ1-4GlcNAc – PA Manα1-2Manα1-2Manα1-3$\rfloor$
M9A	*Manα1-2Manα1-6$\rceil$ Manα1-2Manα1-3Manα1-6Manβ1-4GlcNAcβ1-4GlcNAc – PA Manα1-2Manα1-2Manα1-3$\rfloor$

(Continued on p. 54)

TABLE I (continued)

Abbreviation	Structure
HB	Manα1-6⎫ Manα1-3Manβ1-6⎬ GlcNAcβ1-4Manβ1-4GlcNAcβ1-4GlcNAc–PA GlcNAcβ1-2Manα1-3⎭
F3	GlcNAcβ1-4GlcNAc–PA Fucα1-3⎭
F6	Fucα1-6⎫ GlcNAcβ1-4GlcNAc–PA
MX	Xylβ1-2Manβ1-4GlcNAcβ1-4GlcNAc–PA
MF3	Manβ1-4GlcNAcβ1-4GlcNAc–PA Fucα1-3⎭
MF3X	Xylβ1-2Manβ1-4GlcNAcβ1-4GlcNAc–PA Fucα1-3⎭
M2BX	Manα1-6Manβ1-4GlcNAcβ1-4GlcNAc–PA Xylβ1-2⎭
M2BF3	Manα1-6Manβ1-4GlcNAcβ1-4GlcNAc–PA Fucα1-3⎭
M2BF3X	Manα1-6Manβ1-4GlcNAcβ1-4GlcNAc–PA Xylβ1-2⎭ Fucα1-3⎭
M3AX	Manα1-6⎫ Manα1-3Manβ1-4GlcNAcβ1-4GlcNAc–PA Xylβ1-2⎭
M3AF3	Manα1-6⎫ Manα1-3Manβ1-4GlcNAcβ1-4GlcNAc–PA Fucα1-3⎭
M3AF3X	Manα1-6⎫ Manα1-3Manβ1-4GlcNAcβ1-4GlcNAc–PA Xylβ1-2⎭ Fucα1-3⎭
M4AX	Manα1-3Manα1-6⎫ Manα1-3Manβ1-4GlcNAcβ1-4GlcNAc–PA Xylβ1-2⎭
MO3	Manα1-6⎫ Galβ1-4GlcNAcβ1-2Manα1-3Manβ1-4GlcNAcβ1-4GlcNAc–PA
MO6	Galβ1-4GlcNAcβ1-2Manα1-6⎫ Manα1-3Manβ1-4GlcNAcβ1-4GlcNAc–PA
MO3-G	Manα1-6⎫ GlcNAcβ1-2Manα1-3Manβ1-4GlcNAcβ1-4GlcNAc–PA
MO6-G	GlcNAcβ1-2Manα1-6⎫ Manα1-3Manβ1-4GlcNAcβ1-4GlcNAc–PA
BI	Galβ1-4GlcNAcβ1-2Manα1-6⎫ Galβ1-4GlcNAcβ1-2Manα1-3Manβ1-4GlcNAcβ1-4GlcNAc–PA
BI-G_2	GlcNAcβ1-2Manα1-6⎫ GlcNAcβ1-2Manα1-3Manβ1-4GlcNAcβ1-4GlcNAc–PA
TR4	Galβ1-4GlcNAcβ1-2Manα1-6⎫ Galβ1-4GlcNAcβ1-4Manα1-3Manβ1-4GlcNAcβ1-4GlcNAc–PA Galβ1-4GlcNAcβ1-2⎭

TABLE I (continued)

Abbreviation	Structure
TR4F	Galβ1-4GlcNAcβ1-2Manα1-6⎫ Galβ1-4GlcNAcβ1-4Manα1-3Manβ1-4GlcNAcβ1-4GlcNAc–PA Fucα1-3⎭ Galβ1-4GlcNAcβ1-2⎭
TR4-G_3	GlcNAcβ1-2Manα1-6⎫ GlcNAcβ1-4Manα1-3Manβ1-4GlcNAcβ1-4GlcNAc–PA GlcNAcβ1-2⎭
TE	Galβ1-4GlcNAcβ1-6⎫ Galβ1-4GlcNAcβ1-2Manα1-6⎫ Galβ1-4GlcNAcβ1-4Manα1-3Manβ1-4GlcNAcβ1-4GlcNAc–PA Galβ1-4GlcNAcβ1-2⎭
TEF	Galβ1-4GlcNAcβ1-6⎫ Galβ1-4GlcNAcβ1-2Manα1-6⎫ Galβ1-4GlcNAcβ1-4Manα1-3Manβ1-4GlcNAcβ1-4GlcNAc–PA Fucα1-3⎭ Galβ1-4GlcNAcβ1-2⎭
TE-G_4	GlcNAcβ1-6⎫ GlcNAcβ1-2Manα1-6⎫ GlcNAcβ1-4Manα1-3Manβ1-4GlcNAcβ1-4GlcNAc–PA GlcNAcβ1-2⎭

sample, the proportion of solvent B was increased linearly to 20% in 2 min, and then to 45% in 18 min.

Reversed-phase HPLC was carried out using a Cosmosil 5C18-P column (15 cm × 0.46 cm I.D.) as described previously [2], at a flow-rate of 1.5 ml/min. Two solvents, C and D, were used. Solvent C was 0.1 *M* ammonium acetate buffer (pH 4.0) and solvent D was 0.1 *M* ammonium acetate buffer (pH 4.0) containing 0.5% 1-butanol. Two elution systems were used. In elution system I, the column was equilibrated with a mixture of solvents C and D (ratio 95:5), and after injection of a sample the ratio of solvent D was increased linearly to 100% in 55 min. In elution system II, the column was equilibrated with a mixture of solvents C and D (70:30), and after injection of a sample, the ratio of solvent D was increased linearly to 100% in 14 min. Chromatography was performed at 24°C. For detection of PA-sugar chains, an excitation wavelength of 320 nm and emission wavelength of 400 nm were used.

Preparation of N-linked sugar chains from mouse brains

Mice were killed by dislocation. The brains were removed and placed on ice, and each portion of the brain, still on ice, was prepared. The portions were quickly crushed between blocks of dry ice and lyophilized. Hydrazinolysis and N-acetylation were carried out as described previously [8]. Lyophilized mouse cerebellum or cerebrum (1.5 mg) was hydrazinolysed with 0.2 ml of anhydrous hydrazine in an evacuated sealed tube at 100°C for 10 h. At this point no insoluble materials were found in the tube, indicating that all glycoproteins in mouse brain, including insoluble glycoproteins, were hydrazinolysed well. Excess hydrazine was removed by repeated evaporation with toluene under reduced pressure. The residue was N-acetylated with 400 μl of sodium bicarbonate solution (9.8%, w/w) and 16 μl

of acetic anhydride for 30 min at room temperature. Dowex 50-X2 (H^+) was added to the solution to lower the pH to 3, and then the resin and the solution were transferred to a small chromatography column and washed with five bed-volumes of water. The effluent and the washings were combined and concentrated to dryness.

Fluorescence labelling of sugar chains

The residue was mixed with 40 μl of the 2-aminopyridine solution as described previously [8]. The reaction mixture was heated at 100°C for 13 min in a sealed tube. Then the tube was opened and 4 μl of a reducing reagent (sodium cyanoborohydride solution [8]) was added for reductive amination. After resealing, the tube was heated at 90°C for 15 h. The excess reagents were removed by gel chromatography using a TSK-GEL HW-40F column (34 cm × 1.5 cm I.D.), which had been equilibrated with 0.01 *M* ammonium acetate buffer (pH 6.0). The column was washed with the same buffer at a flow-rate of 10 ml/min, and 2.2-ml fractions were collected. Fractions between the void volume and the M3A fraction were collected and lyophilized.

Preparation of asialo PA-sugar chains

To the residue were added 20 μl of 0.02 *M* citrate buffer, (pH 5.0), 0.2 unit of N-acetylneuraminidase and a small amount of toluene. The mixture was incubated overnight at 37°C. After heating at 100°C for 2 min, the solution was lyophilized.

RESULTS

A two-dimensional map of PA-sugar chains

The separation of 45 PA-oligosaccharides by HPLC using two columns is illustrated in Fig. 1. The elution positions of PA-sugar chains on size-fractionation HPLC were plotted on the ordinate and those obtained on reversed-phase HPLC on the abscissa. Molecular sizes were expressed as M_n ($Man_nGlcNAc_2$-PA), where n represents the total number of mannose residues, and their equivalent (e.g. galactose), and thus the n value increased by 1.0 when one sugar residue as mentioned above was added. However, the n value only increases by 0.6–0.7 for an N-acetylhexosamine residue, by 0.5 for a fucose residue linked to a reducing-end GlcNAc residue by an α1–6 linkage, and by 0.3–0.8 for a xylose residue. Under the present HPLC conditions, PA-sugar chains were fractionated according to their molecular sizes and not according to the linkage positions of sugar residues or anomeric configurations. There also seemed to be some rules governing the elution positions of PA-sugar chains on reversed-phase HPLC, and this aspect will be discussed later. This separation method, which uses two different HPLC separation principles, may be an excellent tool for the isolation and identification of PA-sugar chains derived from either purified glycoproteins or a crude mixture of tissue glycoproteins.

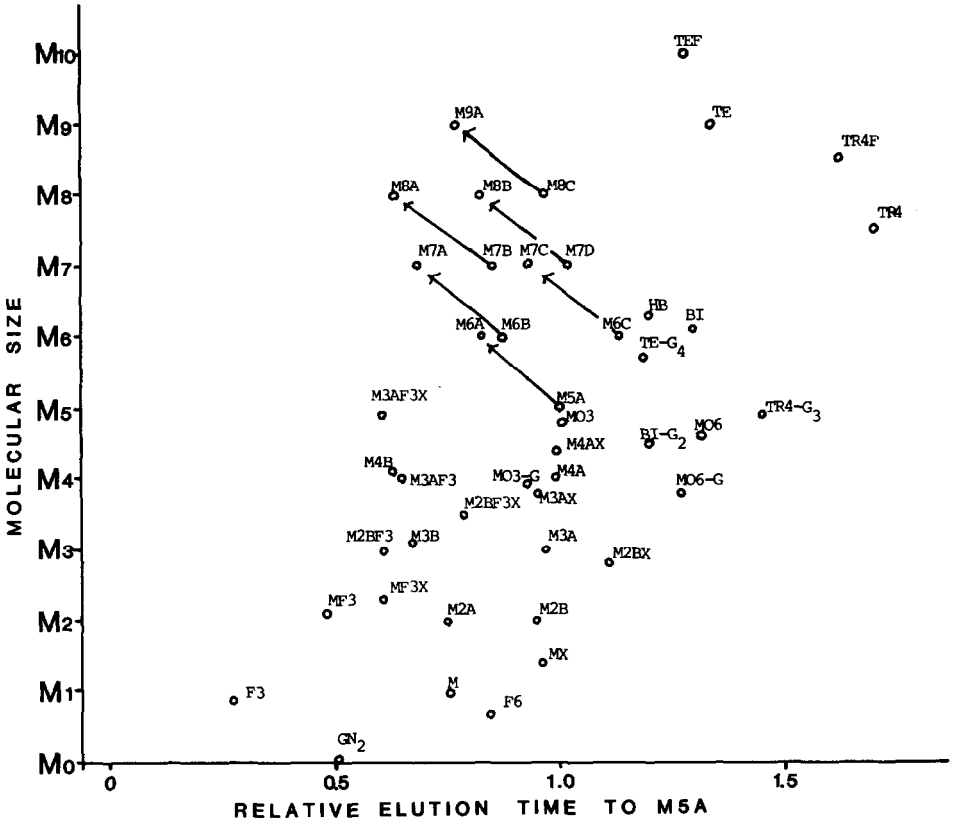

Fig. 1. Two-dimensional map of PA-sugar chains. The elution positions of PA-sugar chains by size-fractionation HPLC (MicroPak AX-5) were expressed in terms of molecular size using M_n ($Man_nGlcNAc_2$-PA) on the ordinate and that obtained by reversed-phase HPLC (Cosmosil 5C18-P) as elution time relative to M5A on the abscissa. Elution system I was used, and the elution time of M5A was 24 min. For abbreviations of PA derivatives, see Table I.

Preparation and fractionation of asialo PA-sugar chains from mouse brains

Cerebra and cerebella of mice brains were lyophilized, and sugar chains were isolated from the glycoproteins in the tissue samples by reductive pyridylamination, according to the standard method used for preparation of PA-oligosaccharides from a pure glycoprotein preparation.

An aliquot (1/20) of PA-sugar chains thus prepared from 1.5 mg of lyophilized sample was first separated on a MicroPak AX-5 column according to molecular size. An HPLC profile of PA-sugar chains obtained from the cerebrum of a wild-type mouse is shown in Fig. 2. Fractions corresponding to M_5–M_{13} (fractions A–I) were collected (indicated by bars in Fig. 2). An aliquot (1/8) of each fraction (M_5–M_{13}) thus obtained was analysed by reversed-phase HPLC. Fig. 3 shows the elution profiles for the cerebrum of a wild-type mouse brain, and the elution positions of some oligomannose-type PA-sugar chains, N-acetyllactosamine-type sugar chains and a hybrid (HYB)-type PA-sugar chain are marked. The presence

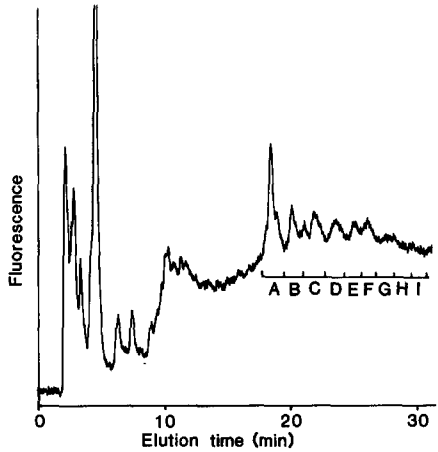

Fig. 2. Size-fractionation HPLC of asialo PA-sugar chains obtained from the cerebrum of a wild-type mouse. The column used was a MicroPak AX-5. PA-sugars obtained from the cerebrum (corresponding to 75 μg dry mass) were injected. Fractions A to I, where M_5 to M_{13} were eluted, respectively, were collected. Peaks eluting between 0 and 10 min are due to contaminating materials.

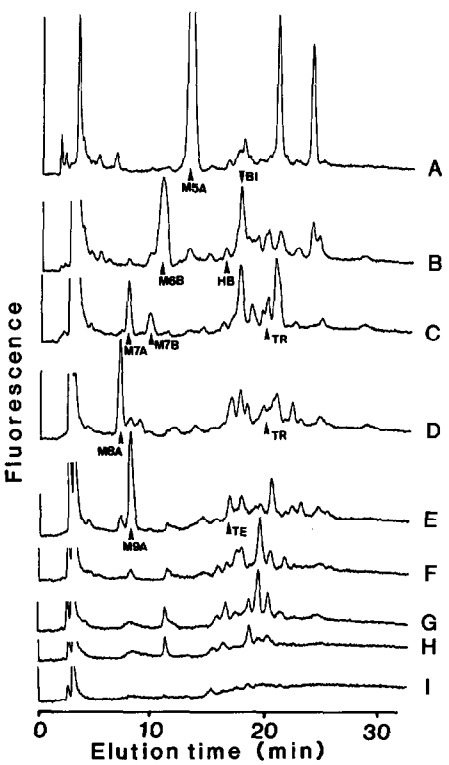

Fig. 3. Reversed-phase HPLC patterns of PA-sugar chains in fractions A to I (Fig. 2) obtained from the cerebrum of a wild-type mouse. A Cosmosil 5C18-P column and elution system II were used. A portion (corresponding to 0.27 mg of cerebrum) of each fraction was injected. Peaks eluting between 2 and 4 min are due to contaminating materials. Arrows indicate the elution positions of PA-sugar chains, whose abbreviations are listed in Table I.

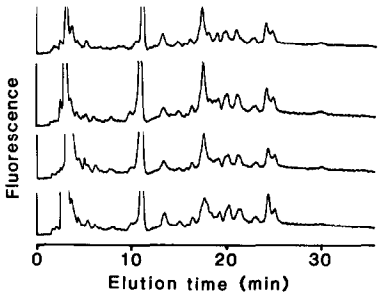

Fig. 4. Comparison of M6 fractions obtained from four different cerebra. Preparation and conditions for HPLC were the same as for Fig. 3.

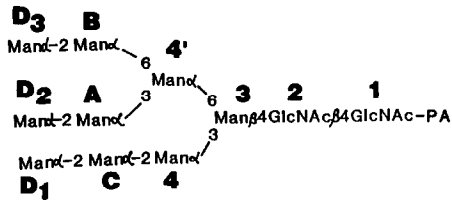

Fig. 5. Coding of sugar residues in M9A.

of considerable amounts of six major PA-sugar chains of the oligomannose type, several of the N-acetyllactosamine type and the hybrid type was observed.

Four samples obtained from four other mice were also analysed in the same way, and Fig. 4 shows the elution patterns of PA-oligosaccharides in the M_6 fraction on reversed-phase HPLC. There appeared to be little difference among individuals. Similarities in the elution pattern were observed in all the fractions analysed (data not shown).

DISCUSSION

At present, there is no easy and convenient method for detecting abnormalities of sugar-chain structure using whole-tissue preparations. The method presented in this paper allowed us to analyse the pattern of the N-linked sugar chains from as little as 1 mg of lyophilized tissue. If abnormal peaks do appear in the elution profile of a sample, it is possible to deduce its structure by the following strategy. First, we compare the elution position with those of standard samples, since we mapped 45 PA-oligosaccharides using our present HPLC conditions. Second, we isolate the peak of interest and digest it with various enzymes (e.g. hexosaminidase, mannosidase and so on), followed by HPLC analysis. The number of residues released from its non-reducing ends can be estimated by size-fractionation HPLC analysis. Moreover, information on the structure of the newly generated non-reducing end can be obtained as described below.

When a Manα1–2 residue was attached to Man B of M5A (thus forming M6A, see Fig. 5), the relative elution time decreased by 0.17 on reversed-phase HPLC

(see Fig. 1). This shift in relative elution time was constant (0.17–0.20), regardless of the structure of the other part of the sugar chain, and seemed to be governed solely by addition of a *Manα1–2 residue (see Table I) to Man B; i.e. M6b to M7A, M6C to M7C, M7B to M8A, M7D to M8B and M8C to M9A (indicated by the arrows in Fig. 1). We have previously shown that similar phenomena are observed following addition of a Manα1–2 residue to any mannose residue at the non-reducing ends of M5A or M6B [3]. In the present study, we were able to extend this rule to other sugar residues, such as those in N-acetyllactosamine-type sugar chains and xylose-containing sugar chains. Therefore, it is highly likely that the shift in elution time, observed on reversed-phase HPLC after addition of a sugar residue to a PA-sugar chain, is mainly influenced by the change in microenvironment and is independent of the structure of the other part of the sugar chain. This rule was applicable to all the sugar chains listed in Table I and Fig. 1, although further more investigation may be required in order to generalize this rule. Thus, analysis of the peak of interest after enzyme digestion on reversed-phase HPLC and measurement of the difference in elution time would seem to provide much information about the structure to which a released sugar residue was attached.

When we analysed the structural patterns of sugar chains present in normal mouse brain, they were strikingly similar from one individual to another. This result indicates that the present method is useful for the detection of sugar chains with abnormal structures in ill or mutant animals.

ACKNOWLEDGEMENTS

The authors are grateful to Dr. K. Schmidt for his gift of the glycopeptide sample. This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan (No. 62580120).

REFERENCES

1 S. Hase, T. Ikenaka and Y. Matsushima, Biochem. Biophys. Res. Commun., 85 (1978) 257.
2 S. Hase, S. Koyama, H. Daiyasu, H. Takemoto, S. Hara, Y. Kobayashi, Y. Kyogoku and T. Ikenaka, J. Biochem., 100 (1986) 1.
3 S. Hase, S. Natsuka, H. Oku and T. Ikenaka, Anal. Biochem., 167 (1987) 321.
4 N. Tomiya, M. Kurono, H. Ishihara, S. Tejima, S. Endo, Y. Arata and N. Takahashi, Anal. Biochem., 163 (1987) 489.
5 Y. Kimura, S. Hase, Y. Kobayashi, Y. Kyogoku, G. Funatsu and T. Ikenaka, J. Biochem., 101 (1987) 1051.
6 S. Hase, T. Sugimoto, H. Takemoto, T. Ikenaka and K. Schmidt, J. Biochem. 99 (1986) 1725.
7 S. Natsuka, S. Hase and T. Ikenaka, Anal. Biochem., 167 (1987) 154.
8 S. Hase, T. Ibuki and T. Ikenaka, J. Biochem., 95 (1984) 197.