Journal of Chromatography, 523 (1990) 189–200
Elsevier Science Publishers B.V., Amsterdam

CHROM. 22 705

Analysis of N- and O-glycosidically bound sialooligosaccharides in glycoproteins by high-performance liquid chromatography with pulsed amperometric detection

SUSUMU HONDA*, SHIGEO SUZUKI, SACHIKO ZAIKI and KAZUAKI KAKEHI
Faculty of Pharmaceutical Sciences, Kinki University, 3–4–1 Kowakae, Higashi-osaka (Japan)
(Received May 8th, 1990)

ABSTRACT

N-Glycosidically bound sialooligosaccharides in a model glycoprotein of serum type (porcine thyroglobulin) were released by pronase digestion, followed by hydrazinolysis. The resulting oligosaccharides were re-N-acetylated and reduced with sodium borohydride. On the other hand, O-glycosidically bound sialooligosaccharides were released from a mucin-type glycoprotein (bovine submaxillary mucin) with alkali in the presence of sodium borohydride. The reduced oligosaccharides thus obtained from both types of glycoproteins were analysed by high-performance liquid chromatography on a column of a latex-type pellicular anion-exchange resin with strong alkali as eluent. These sequential procedures were useful for mapping of oligosaccharides in glycoproteins.

INTRODUCTION

Glycoproteins have covalently bound oligosaccharide chains in their peptide backbones, which play an important role in cell-to-cell recognition (*e.g.*, ref. 1). As the structures of the oligosaccharides in glycoproteins are dependent on variations in the enzymatic processing environment, structural variations reflect the physiological and pathological conditions to which the precursor proteins have been exposed. Therefore, oligosaccharide mapping is important for biological and clinical studies involving biosynthesis and metabolism of glycoproteins. So far, high-performance liquid chromatography (HPLC) has been most widely used for this purpose, especially in the reversed-phase partition mode as N-pyridylglycamine derivatives (*e.g.*, ref. 2). It requires, however, multi-step derivatization and clean-up processes.

An alternative method is direct separation of oligosaccharides on a latex-type anion-exchange resin with strong alkali as eluent with pulsed amperometric detection using a gold electrode [3]. This method has been applied to the analysis of oligosaccharides released from glycoproteins [4–10]. Separation is rapid and the sensitivity is down to the picomole level. Although the use of strong alkali can cause degradation or isomerization, this method seems promising for pattern analysis of oligosaccharides in glycoproteins. This paper proposes general procedures for the analysis of N- and O-glycosidically bound sialooligosaccharides in glycoproteins, including liberation processes.

0021-9673/90/$03.50

EXPERIMENTAL

Streptomyces griseus Pronase P was obtained from Boehringer (Mannheim, F.R.G.). *Clostridium perfringens* neuraminidase and coffee bean α-galactosidase were purchased from Sigma (St. Louis, MO, U.S.A.). All enzymes were used without further purification. Anhydrous hydrazine was purchased from Aldrich (Milwaukee, WI, U.S.A.). All other reagents and carbohydrate samples were of the highest grade commercially available. Water was deionized and doubly distilled before use.

Thyroglobulin was prepared from porcine thyroid glands according to the method of Ui and Tarutani [11] and was subjected to exhaustive digestion with Pronase P. The digest was applied to a column of Sephadex G-25 (30 cm × 10 mm I.D.) and the column eluted with water. The fractions corresponding to 11–15 ml of eluate were collected and evaporated to dryness. The residue was divided into three fractions (neutral, monosialo- and disialoglycopeptide fractions) on a column (80 cm × 18 mm I.D.) of DEAE-Sephadex A-25 by isocratic elution with a 2 m*M* acetic acid–1 m*M* pyridine mixture (100 ml), followed by gradient elution with the same buffer to a 100 m*M* acetic acid–50 m*M* pyridine mixture according to the procedure of Fukuda and Egami [12]. Each fraction was deionized on a column of Sephadex G-25 in a similar manner to that for Pronase P digest. Each of the mono- and disialoglycopeptide fractions were further hydrazinolysed and re-N-acetylated by the method of Takasaki *et al.* [13], then reduced with 0.1 *M* sodium borohydride in 0.05 *M* sodium hydroxide for 6 h at 27°C. The reaction mixture was decationized by passing it through a column of Amberlite CG-120 (H^+ form), and the combined eluate and water washings were evaporated to dryness. The residue was dissolved in a small volume of methanol and the solution evaporated to dryness. The treatment with methanol and evaporation was repeated twice more to remove boric acid.

The residues finally obtained from the neutral glycopeptide and reduced sialooligosaccharide fractions were dissolved in water and 20-μl portions were analysed by HPLC with isocratic elution. For HPLC with gradient elution, a sample (50 μg) of thyroglobulin was directly hydrazinolysed (without being digested with Pronase P), re-N acetylated and reduced with sodium borohydride in a similar manner to that for the sialoglycopeptide fractions. The residue was dissolved in water and the whole solution injected into the HPLC system.

Bovine submaxillary mucin was isolated by the method of Tettamanti and Pigman [14]. It was treated with 1 *M* sodium borohydride in 0.05 *M* sodium hydroxide for 24 h at 45°C to liberate oligosaccharides as reduced forms, according to the procedure of Iyer and Carlson [15]. The reduced oligosaccharides from this mucin-type glycoprotein were analysed by HPLC with isocratic elution in a similar manner to that for thyroglobulin, after removal of boric acid by decationization and treatment with methanol. Erythrocyte ghost was prepared from porcine blood by the method of Dodge et al. [16] and treated with sodium borohydride in alkali in a similar manner to that for bovine submaxillary mucin. HPLC was performed by isocratic elution.

In all HPLC experiments with isocratic elution the HPLC system consisted of a Hitachi 655 dual plunger pump, a Rheodyne 7125 sample injector with a 20-μl loop, a Dionex HPIC-AG 6 guard column (5 cm × 4 mm I.D.), a Dionex HPIC-AS 6 analytical column (25 cm × 4 mm I.D.) and a Dionex triple pulse amperometric

detector equipped with a gold electrode (PAD II). In analytical HPLC (0.5–2.5 μg as saccharide for each run), all portions of the eluates were introduced to the detector, whereas in preparative HPLC (5–25 μg as saccharide for each run), the eluate for each sample was continuously divided into two portions (volume ratio 1:9) by use of a splitter, and the smaller portion was led into the detector for monitoring. The larger portion was fractionated, and the combined fractions for every peak were neutralized and deionized on a column of Sephadex G-25. In analytical HPLC with gradient elution, a Dionex pump capable of programmed gradient elution (GPM) was used, but other parts were the same as those for isocratic elution.

^{1}H NMR spectra of the major fractions from preparative HPLC were recorded in deuterium oxide at room temperature with a JEOL JNM GSX-500 spectrometer. The proton signals were referenced to the methyl proton signal of acetone (2.225 ppm) in δ (ppm). The water signal was eliminated by measuring the spectra in the homogate decoupling mode. Fast atom bombardment mass spectra were obtained in a glycerol matrix using a JEOL HX-100 spectrometer. Each sample was dissolved in aqueous 10% glycerol and a 2-μl portion of each sample solution was loaded on a stainless-steel sample plate. The energy of the primary xenon beam was 6 kV. Scanning was carried out every 10 s up to m/z 2000. The ion source had an accelerating potential of 5 kV. Calibration of mass number was carried out by using Ultra Mark (PCR Research Chemicals, FL, U.S.A.) as the mass reference.

RESULTS AND DISCUSSION

Problem of interference by amino acids and proteins

As the present system employed pulse amperometry on a gold electrode for detection, which is also sensitive to amino group-containing substances, interference by amino acids and proteins was examined. Fig. 1a and b shows the chromatograms obtained with 0.5 *M* sodium hydroxide after injection of a mixture of 1 μmol each of eighteen kinds of amino acids and 5 μg of bovine serum albumin, respectively.

The amino acid mixture gave intense peaks (not assigned) at *ca.* 5 and 13 min together with a few peaks near the void volume, but the protein sample gave no peaks within 30 min except for a few small ones near the void volume. These results indicate that in the analysis of oligosaccharides from glycoproteins amino acids must be removed before HPLC analysis. However, this problem was solved simply by passing sample solutions through a Sephadex G-25 column (30 cm × 1.0 cm I.D.) and collecting the 11–15-ml fraction of the aqueous eluate.

Separation of oligosaccharides derived from glycoproteins

Oligosaccharide chains in glycoproteins can be either N- and O-glycosidically bound to the polypeptide cores. In this work procine thyroglobulin and bovine submaxillary mucin were used, respectively, as the models of these two types.

Oligosaccharide in porcine thyroglobulin. Three fractions corresponding to neutral, monosialo- and disialoglycopeptides were obtained by clean-up of the Pronase P digest of porcine thyroglobulin on a column of Sephadex GA-25, followed by fractionation on a column of DEAE-Sephadex A-25.

Analysis of the neutral glycopeptide fraction by isocratic elution gave three major peaks and one minor peak, as shown in Fig. 2a.

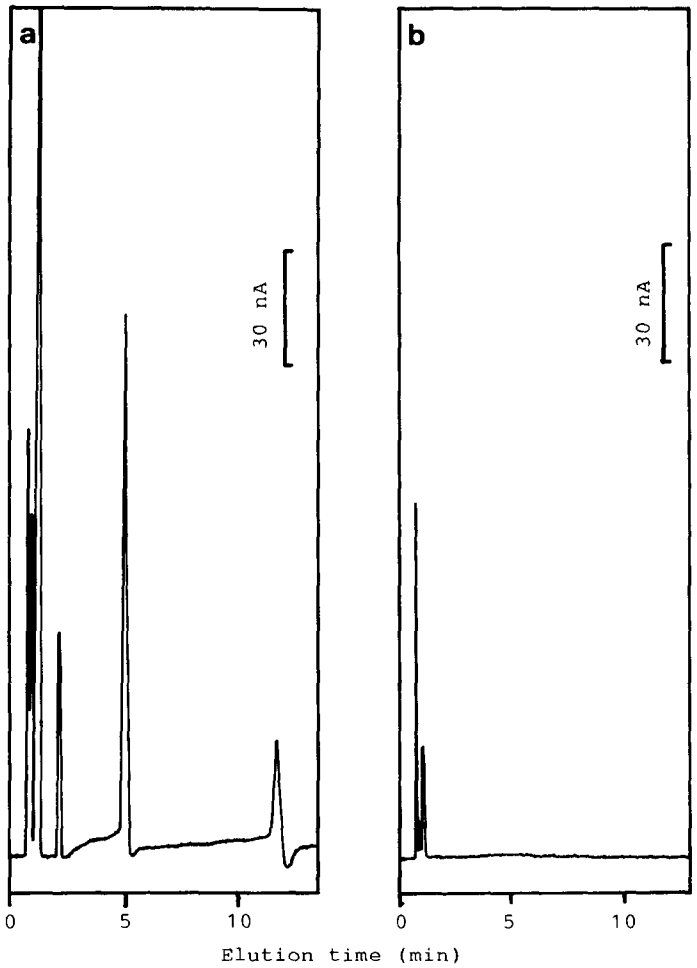


Fig. 1. Analysis of (a) a mixture of amino acids (1 μmol each) and (b) bovine serum albumin (5 μg). Column, Dionex HPIC AS-6 (25 cm × 4 mm I.D.); flow-rate, 1.0 ml/min; detection, pulsed amperometry on a gold electrode; E_1 = + 0.05 V (0.6 s), E_2 = + 0.60 V (0.12 s), E_3 = −0.80 V (0.42 s).

The compounds corresponding to the major peaks were isolated by preparative HPLC, followed by deionization. The ^{1}H NMR spectra of the compounds giving peaks 1 (**1**) and 2 (**2**) indicated the presence of seven and eight anomeric protons (H-1s), respectively, whose chemical shifts (Table I) are consistent with those of the hepta- and octasaccharides of high-mannose type shown above the elution profile. Each of them also showed the presence of two N-acetyl groups giving the signals of the methyl protons at 2.04 and 2.06 ppm together with two β-methylene protons (ca. 2.8 ppm) in the asparagine residue. The chemical shifts of the anomeric protons and the protons at C-2 (H-2s) of individual monosaccharide residues were in good agreement with those in the literature [17]. The spectrum of the compound corresponding to peak 3 (**3**) showed the presence of two N-acetyl and two methylene groups. It also

gave complex signals of H-1 and H-2 protons which could be assigned as shown in Table I.

The multiplicity of the signals in the 5.0–4.9 ppm region indicated the presence of three isomers of high mannose-type nonasaccharides linked to asparagine, differing in the position of attachment of the D-mannose residues. Substitution of the A-, B- and C-mannose residues by the D-mannose residue resulted in a downfield shift of the anomeric protons as given in statics and from the intensities of these signals the molar ratio of the A-, B- and C-bound positional isomers were estimated to be 23:13:64. Peak 4 is probably a $Man_8GlcNAc_2Asn$ analogue, judging from the k' value, although the ^1H NMR data were not conclusive owing to limited availability of the sample.

The mono- and disialoglycopeptides gave multiple peaks due to heterogeneity of the peptide moity, because the peptide linkages were not hydrolysed completely. Therefore, each of the sialoglycopeptide fractions was submitted to sequential hydrazinolysis, re-N-acetylation and borohydride reduction, then fractionated on a column of DEAE-Sephadex. The amount of desialylated products appearing at the void volume was roughly calculated to be less than 10% of the starting fraction on a weight basis for both sialoglycopeptide fractions. This result confirms that the partial degradation was almost negligible and this series of derivatizations gave reliable oligosaccharide mapping. Fig. 2b and c show the chromatograms for reduced mono- and disialooligosaccharide fractions, respectively. They also include the proposed structures of the compounds giving the major peaks. The compound corresponding to peak 5 (**5**) from the reduced monosialooligosaccharide, as its ^1H NMR spectrum (Table II) was in good agreement with that in the literature [17].

The compound giving peak 6 (**6**) was thought to have an additional α-Gal residue linked to the peripheral Gal residue in **5**, because digestion of **6** with coffee bean α-galactosidase removed peak 6 from the elution profie and yielded a peak identical with peak 5. Further, **6** gave a ^1H NMR spectrum identical with that of **5**, except for the presence of additional H-1 signal at 5.135 ppm with a coupling constant of 3.5 Hz and a slightly upfield shift of the H-1 signal of the 6′-GlcNAc residue from 4.471 to 4.446 ppm. This situation was the same as that for the tetra-antennary oligosaccharide reported by Dorland *et al.* [18], which has also a peripheral α-Gal residue linked to the Gal residue. The minor peaks in Fig. 2b are considered to arise from isomerically monosialylated bi- and triantennary oligosaccharides, although the positions of attachment of the NeuNAc residue are not known.

Peak 7 from the reduced disialooligosaccharide fraction was assignable to the biantennary oligosaccharide in which both peripheral Gal residues are sialylated. Peak 8 from the same fraction is presumably due to a mixture of triantennary oligosaccharides having two NeuNAc residues at different Gal residues, because partial desialylation with *Clostridium perfringens* neuraminidase gave peaks identical with one of the minor peaks from the reduced monosialooligosaccharide fraction.

Hence the present system allowed excellent separations of neutral glycopeptides and also reduced sialooligosaccharides, possibly owing to slight difference in hydrophilicity. The reproducibility of the retention times was fairly high, the relative standard deviation being less than 0.1% for these peaks.

With thyroglobulin oligosaccharides, prior fractionation on an anion-exchange column was essential, as isocratic elution could not separate, in one run, all types of

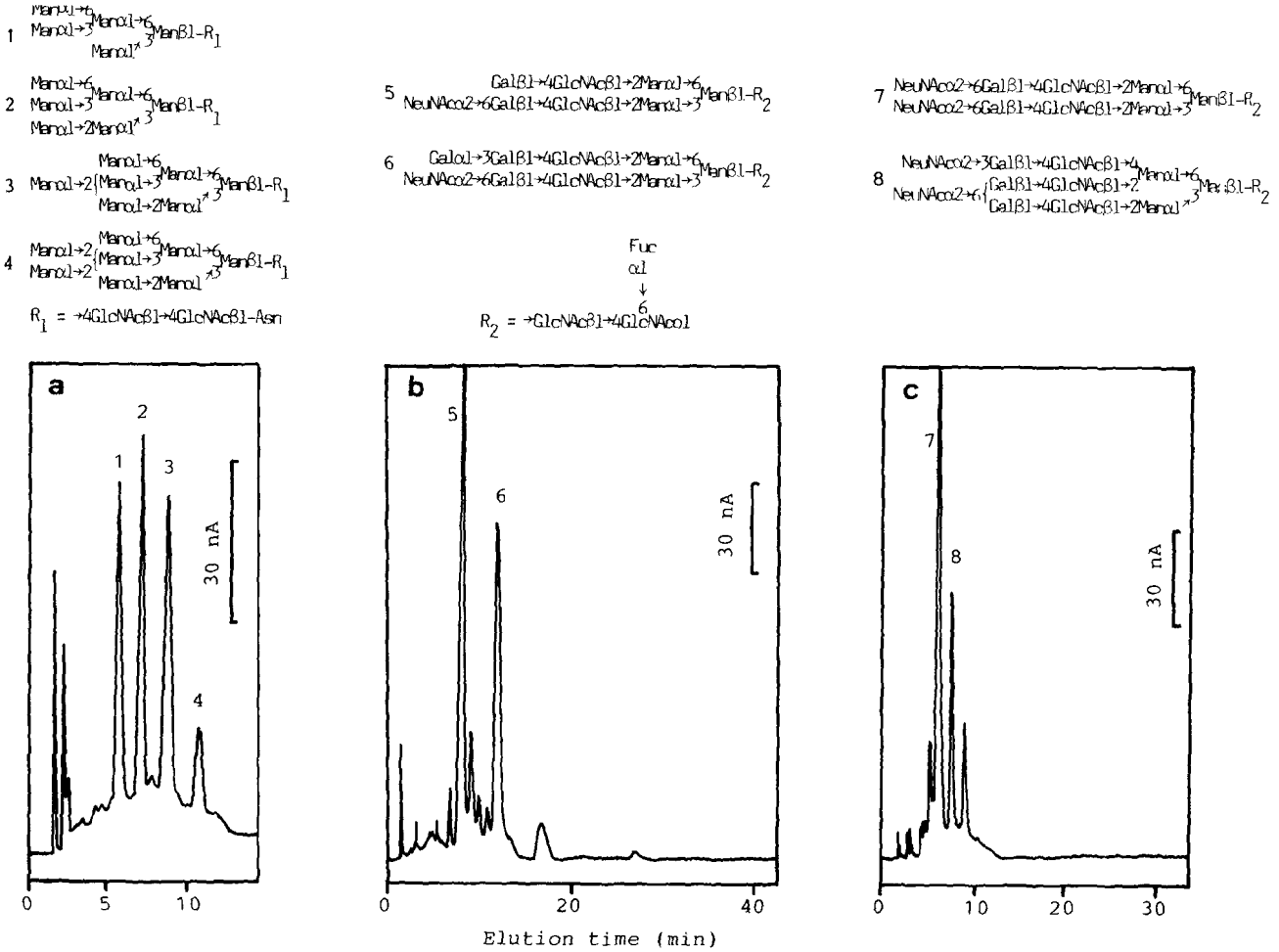


Fig. 2. Analysis of the (a) neutral glycopeptides, (b) reduced monosialo- and (c) reduced disialooligosaccharides, derived from porcine thyroglobulin. Eluent: (a) 0.5, (b) 0.5 and (c) 0.8 *M* sodium hydroxide solutions. Other conditions as in Fig. 1. Sample scale: 11–30 μg as glycoprotein.

TABLE I

ASSIGNMENT OF THE CHEMICAL SHIFTS OF REPRESENTATIVE PROTON SIGNALS OF MAJOR NEUTRAL GLYCOPEPTIDES DERIVED FROM PORCINE THYROGLOBULIN

Numbering of monosaccharide residue:

M = mannose; N = N-acetylglucosamine.

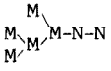
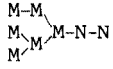
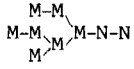
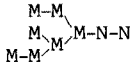
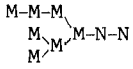
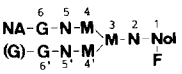
Signal	Monosaccharide residue	**1**	**2**	**3**		
						
H-1	1	5.072	5.072	5.072	5.072	5.072
NAc	1	2.037	2.038	2.037	2.037	2.037
	2	2.063	2.063	2.064	2.064	2.064
H-1	4	5.094	5.348	5.340	5.340	5.340
	4′	4.870	4.874	4.871	4.871	4.871
	A	5.094	5.094	*5.404*	5.093	5.093
	B	4.907	4.909	4.908	*5.149*	4.908
	C		5.054	5.056	5.056	*5.304*
	D			5.043	5.043	5.043
H-2	3	4.25	4.23	4.23	4.23	4.23
	4	4.07	4.11	4.09	4.09	4.09
	4′	4.15	4.15	4.15	4.15	4.15
	B	3.99	3.99	3.99	3.99	3.99
	C		4.07	4.07	4.07	4.07
	D			4.07	4.07	4.07

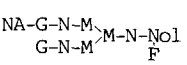
TABLE II

ASSIGNMENT OF THE CHEMICAL SHIFTS OF REPRESENTATIVE PROTON SIGNALS OF MAJOR SIALOOLIGOSACCHARIDES DERIVED FROM PORCINE THYROGLOBULIN

Numbering of monosaccharide residue:



M = mannose; N = N-acetylglucosamine; Nol = N-acetylgucosaminitol; F = fucose; G = galactose.

Signal	Monosaccharide residue	**5**	**6**
			
H-1	4	5.138	5.139
	4′	4.927	4.929
	5	4.609	4.607
	5′	4.581	4.583
	6	4.446	4.446
	6′	4.471	4.446
	Fuc	4.897	4.898
	α-Gal		5.135
H-2	3	4.26	4.26
	4	4.19	4.20
	4′	4.11	4.11
H-3ax	NeuNAc	1.777	1.777
H-3eq	NeuNAc	2.668	2.669
H-5	Fuc	4.11	4.11
H-6	Fuc	1.212	1.212
NAc	1	2.018	2.017
	2	2.076	2.074
	5	2.058	2.058
	5′	2.044	2.043
	NeuNAc	2.035	2.035

saccharides differing in the number of sialic acid residues. Gradient elution alleviated the problem of prior fractionation, and simultaneous gradient separation of neutral, mono-and disialooligosaccharides was accomplished, although with lower reproducibility of retention times (*e.g.*, the relative standard deviation for peak 1 was 5.3%, $n = 5$). Fig. 3 shows an example of the separation of all the oligosaccharides with gradient elution.

The sample was prepared by direct hydrazinolysis, re-N-acetylation and borohydride reduction, and gradient elution was performed by adding 0.1 *M* sodium hydroxide containing 0.5 *M* sodium acetate to 0.1 *M* sodium hydroxide in 200 min. Peaks were tentatively assigned by comparing the elution pattern with those obtained by isocratic elution of neutral glycopeptide and reduced mono- and disialooligosaccharide fractions. Neutral, high-mannose-type oligosaccharides were eluted first at *ca.* 20 min, reduced monosialooligosaccharides followed in the range of 35–40 min and reduced disialooligosaccharides appeared after 45 min. The unassigned peaks

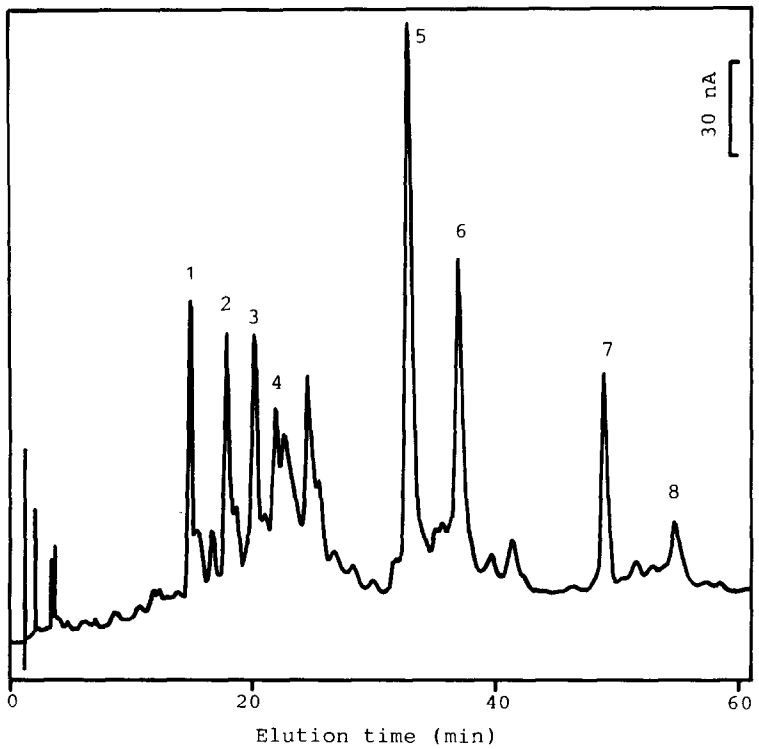


Fig. 3. Analysis of the reduced neutral and sialooligosaccharides derived from porcine thyroglobulin. Gradient elution from 0.1 *M* sodium hydroxide to 0.1 *M* sodium hydroxide containing 0.5 *M* sodium acetate in 200 min. Other conditions as in Fig. 1. Sample scale: 50 μg as glycoprotein. Numbering of peaks as in Fig. 2.

between 22 and 23 min were presumably due to oligopeptides formed by partial degradation of the polypeptide core, which escaped clean-up with Sephadex G-25 and Amberlite CG-120 columns. Such compounds would be difficult to remove by this clean-up procedure, and digestion of glycoprotein samples with Pronase P prior to hydrazinolysis is desirable to eliminate the problem of non-carbohydrate contaminants.

Oligosaccharides from bovine submaxillary mucin. Bovine submaxillary mucin contains large amounts of NeuNAc and NeuNGc-containing di- and trisaccharides, which can be easily released as reduced forms with dilute alkali containing sodium borohydride. Fig. 4 shows the separation of the reduced oligosaccharides obtained after clean-up on a column of Sephadex G-25, using 0.3 *M* sodium hydroxide.

Fast atom bombardment mass spectrometry (FAB-MS) of the substance corresponding to peak 1 gave an intense signal at *m/z* 513 together with a weak signal at 716. They can be assigned to the $[M-H]^-$ ions resulting from NeuNAcα2→6GalNAc–OH (disaccharide a) and GlcNAcβ1→3(NeuNAcα2→6)GalNAc–OH (trisaccharide a) reported by Tsuji and Osawa [19]. The compound giving peak 2 gave a

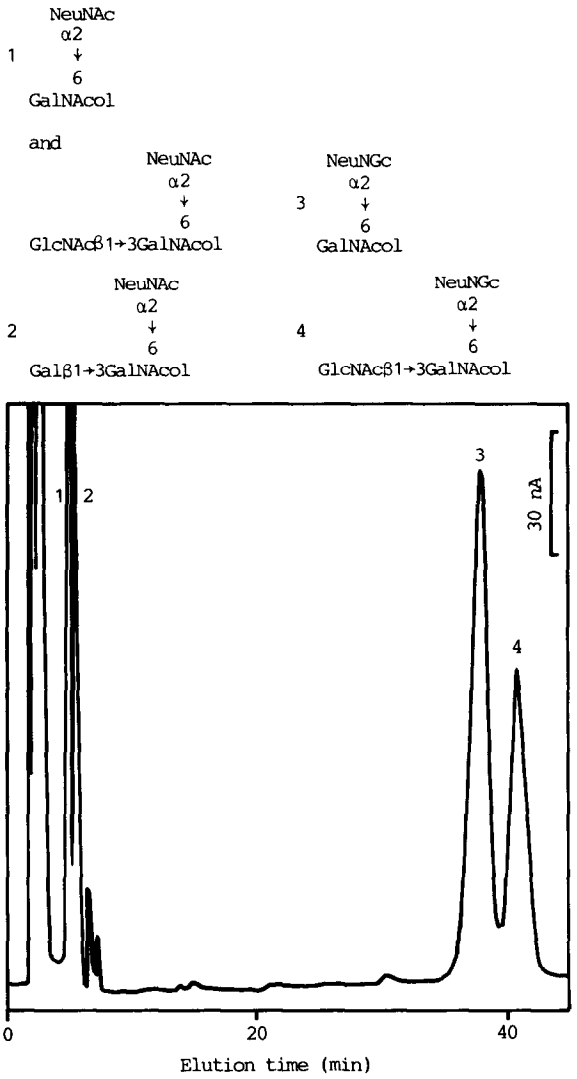


Fig. 4. Analysis of the reduced oligosaccharides derived from bovine submaxillary mucin. Eluent: 0.3 *M* sodium hydroxide solution. Other conditions as in Fig. 1. Sample scale: 11 μg as glycoprotein.

signal at *m/z* 674, which similarly resulted from Galβ1→3(NeuNAcα2→6)GalNAc–OH (trisaccharide b). Slowly eluting peaks 3 and 4 are considered to be due to the NeuNGc analogues of disaccharide a and trisaccharide a, respectively, because the isolated compounds corresponding to these peaks gave signals at *m/z* 529 and 732 respectively, in FAB-MS. Although the separation of faster eluting oligosaccharide was not efficient under these conditions, use of lower concentrations of sodium hydroxide gave better resolution.

The O-glycosidically bound oligosaccharides are widely distributed, ranging from soluble glycoproteins inside and outside cells to insoluble ones on cell surface.

As a representative of the latter type of glycoproteins, porcine erythrocyte membrane was treated with sodium borohydride in dilute alkali in the same manner as described for bovine submaxillary mucin, although in suspension. Analysis of the released oligosaccharides by the present system with 0.5 *M* sodium hydroxide as eluent (Fig. 5) yielded two later eluting peaks (peaks 1 and 2), which are presumably due to sialooligosaccharides.

The amounts of these oligosaccharides were too low to allow definitive assignment by ^{1}H NMR spectrometry. However, as the main oligosaccharides in glycophorin, the major erythrocyte membrane glycoprotein, has been reported to have O-glycosidically bound oligosaccharides, Galβ1→3(NeuNGcα2→6)GalNAc and Galβ1→3GlcNAcβ1→4GlcNAcβ1→3Galβ1→3(NeuNGcα2→6)GalNAc [20], peaks 1 and 2 might be assigned to these NeuNGc-containing oligosaccharides. Although further studies are required for unequivocal assignment, it is clear that the present method is also useful for pattern analysis of oligosaccharides on cell surfaces.

The results obtained here by using model proteins of serum type and mucin type demonstrate the usefulness of these procedures for the analysis of both N- and O-glycosidically bound sialooligosaccharides derived from these glycoproteins. Conversion to borohydride-reduced derivatives is quantitative, as is well established, and eliminates the problem of the presence of anomers and avoids on-column epimerization and degradation. In addition, the sensitivity was not lowered by this treatment, permitting analysis at the several tens of micrograms level as glycoprotein.

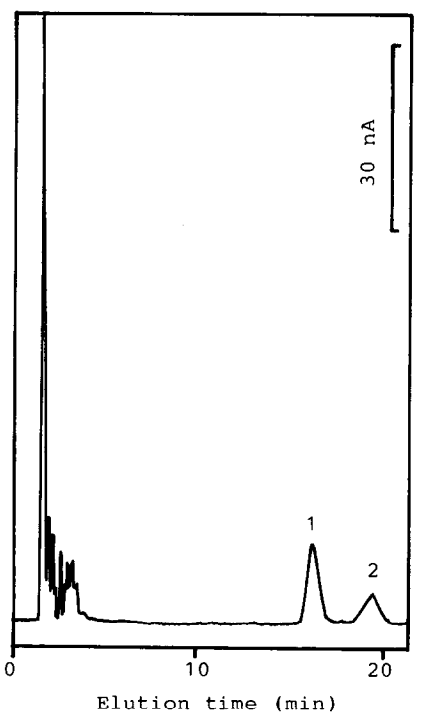


Fig. 5. Analysis of the reduced oligosaccharides derived from porcine erythrocyte ghost. Eluent: 0.5 *M* sodium hydroxide solution. Other conditions as in Fig. 1. Sample scale: 73 μl as blood.

ACKNOWLEDGEMENT

The authors wish to express their gratitude to Professor Y. C. Lee from The Johns Hopkins University, for useful discussion throughout the work.

REFERENCES

1 T. W. Rademachen, R. B. Parekh and R. A. Dwek, *Annu. Rev. Biochem.*, 57 (1988) 785.
2 N. Tomiya, J. Awaya, M. Kurono, S. Endo, Y. Arata and N. Takahashi, *Anal. Biochem.*, 171 (1988) 73.
3 R. D. Rocklin and C. A. Pohl, *J. Liq. Chromatogr.*, 6 (1983) 1577.
4 M. R. Hardy and R. R. Townsend, *Proc. Natl. Acad. Sci. U.S.A.*, 85 (1988) 3289.
5 R. R. Townsend, M. R. Hardy, O. Hindsgaul and Y. C. Lee, *Anal. Biochem.*, 174 (1988) 459.
6 R. R. Townsend, M. R. Hardy, D. A. Cummings, J. P. Carver and B. Bendiak, *Anal. Biochem.*, 182 (1989) 1.
7 R. R. Townsend, M. R. Hardy and Y. C. Lee, *Methods Enzymol.*, 179 (1989) 65.
8 M. R. Hardy and R. R. Townsend, *Carbohydr. Res.*, 188 (1989) 1.
9 W. T. Wang and D. Zopf, *Carbohydr. Res.*, 189 (1989) 1.
10 L. J. Basa and M. W. Spellman, *J. Chromatogr.*, 499 (1990) 205.
11 N. Ui and O. Tarutani, *J. Biochem. (Tokyo)*, 50 (1961) 508.
12 M. Fukuda and F. Egami, *Biochem. J.*, 123 (1971) 407.
13 S. Takasaki, T. Mizuochi and A. Kobata, *Methods Enzymol.*, 83 (1982) 263.
14 G. Tettamanti and W. Pigman, *Arch. Biochem. Biophys.*, 124 (1968) 41.
15 R. N. Iyer and D. M. Carlson, *Arch. Biochem. Biophys.*, 142 (1971) 101.
16 J. T. Dodge, C. Mitchell and D. J. Hanahan, *Arch. Biochem. Biophys.*, 100 (1963) 119.
17 J. F. G. Vliegenthart, L. Dorland and H. van Halbeek, *Adv. Carbohydr. Chem., Biochem.*, 41 (1983) 209.
18 L.Dorland, H. van Halbeek and J. F. G. Vliegenthart, *Biochem. Biophys. Res. Commun.*, 122 (1984) 859.
19 T. Tsuji and T. Osawa, *Carbohydr. Res.*, 151 (1986) 391.
20 I. Kawashima, K. Fukuda, M. Tomita and A. Hamada, *J. Biochem. (Tokyo)* 91 (1982) 865.