Carbohydrate Research, 112 (1983) 165–170
Elsevier Scientific Publishing Company, Amsterdam – Printed in The Netherlands

Note

Isolation and g.l.c.–m.s. analysis of the terminal, sialic acid-containing oligosaccharides obtained from intact glycoproteins by nitrous acid deamination

ILKKA MONONEN* AND TOM KRUSIUS

Department of Medical Chemistry, University of Helsinki, Siltavuorenpenger 10 A, SF-00170 Helsinki 17 (Finland)

(Received January 29th, 1982; accepted for publication in revised form, May 10th, 1982)

This paper describes a method for isolation and characterization of sialic acid-containing, terminal carbohydrates of a glycoprotein. It involves successive *N*-deacetylation and nitrous acid deamination of an intact glycoprotein, isolation of sialic acid-containing material by ion-exchange chromatography, and its analysis by g.l.c.–m.s. Two terminal trisaccharides of human α_1-acid glycoprotein, containing one sialic acid residue, D-galactose, and 2,5-anhydro-D-mannose, have been isolated and characterized. Their g.l.c. retention times differed and they appear to carry different sialic acid linkages. The position of the glycosidic linkages between sialic acid and D-galactose may be assigned directly from the mass spectra without further degradation of the molecules. The effect of different *N*-deacetylation and deamination conditions was tested, and the applicability of the method is discussed.

Deamination of *N*-deacetylated amino sugars by nitrous acid selectively cleaves their glycosidic linkages forming oligosaccharides that contain 2,5-anhydro-D-hexoses at the reducing end[1]. The procedures for *N*-deacetylation and deamination have usually been adapted to the analysis of desialylated glycopeptides, and only a few reports have been published on the deamination of glycopeptides containing *N*-acetylneuraminic acid[2–4]. In those cases, two different structures for the deaminated neuraminic acid residue have been suggested. Furthermore, little is known about the deamination of intact glycoprotein molecules with a view to isolating the terminal, sialic acid-containing oligosaccharides from them.

Recently, nitrous acid deamination of the methyl ester methyl β-glycoside of neuraminic acid was demonstrated to produce one main product, methyl 3-deoxy-D-*glycero*-β-D-*galacto*-nonulopyranosidonic acid methyl ester[5,6]. In a subsequent study, trisaccharides having a deaminated sialic acid residue were produced from glycopeptides by procedures of *N*-deacetylation and nitrous acid deamination, and their structures studied by g.l.c.–m.s.[7].

---

*To whom correspondence should be addressed.

The object of the present work was to use *N*-deacetylation and nitrous acid deamination to produce sialic acid-containing, terminal molecules from intact glycoproteins for structural analysis of the most peripheral parts of *N*-glycosylically linked carbohydrate chains. The effects of different reaction conditions were tested for the same purpose.

EXPERIMENTAL

Materials. — Human α_1-acid glycoprotein was obtained as a gift from Dr. Erkki Raaska, of the Finnish Red Cross Transfusion Service, SF-00310 Helsinki 31, Finland. All other chemicals were of analytical grade.

N-*Deacetylation and deamination procedure.* — α_1-Acid glycoprotein (100 mg) was subjected to *N*-deacetylation in alkaline dimethyl sulfoxide at 85–100° for 2–16 h according to Erbing *et al.*[8]. The samples were made neutral, purified by gel filtration on Sephadex G-10, lyophilized, and deaminated in aqueous acetic acid with sodium nitrite. Different deamination times, temperatures, and pH values were tested; the best recoveries of sialic acid-containing oligosaccharides were obtained in M sodium nitrite (pH 3.5) for 2 h at 0°. After deamination, the samples were made neutral with pyridine and purified by gel filtration on Sephadex G-10 eluted with 10mM pyridine-acetic acid buffer (pH 6.0). The samples were made alkaline and reduced by sodium borohydride, after which time borate was removed by gel filtration and repetitive evaporations to dryness with acidic methanol[9]. The sialic acid-containing oligosaccharides were isolated by anion-exchange chromatography on a column of DEAE-Sephadex A-25. A fraction of oligosaccharides containing one sialic acid residue was obtained by elution of the column with 50mM pyridine-acetic acid buffer[9]. The products were isolated by t.l.c. with plates of 0.50-mm silica gel H (Merck), and 6:2:1 (v/v) 1-propanol-water-concentrated ammonia as solvent[10]. Segments (5 mm) were scraped off, extracted with a mixture of 0.01M pyridine-acetic acid buffer (pH 5.0) and methanol (1:2, v/v), methanolyzed[5], and analyzed for monosaccharide composition as their trimethylsilyl ether derivatives by gas-liquid chromatography-mass spectrometry (g.l.c.-m.s.)[5]. Permethylation of the trisaccharides was performed by a modification[11] of the method of Hakomori[12], and symbols employed by Kochetkov[13] and Kováčik[14] are used in discussing the mass-spectral fragmentations. The positions of the glycosidic linkages were assigned by analysis of the partially methylated alditol acetates by g.l.c.-m.s.[15]. Instruments for g.l.c.-m.s. have been described[7].

RESULTS AND DISCUSSION

Isolation and characterization of terminal oligosaccharides. After successive *N*-deacetylation, deamination, reduction, and methanolysis, the mixture of carbohydrates of human α_1-acid glycoprotein contained D-mannose, D-galactose, 2,5-anhydro-D-mannitol, some L-fucose, and a compound having retention time $T = 2.15$

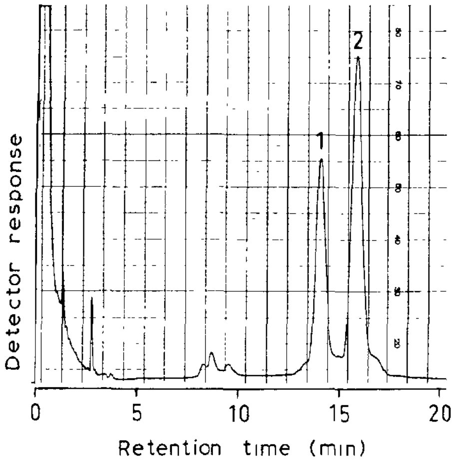


Fig. 1. G.l.c. analysis of trisaccharide fraction.

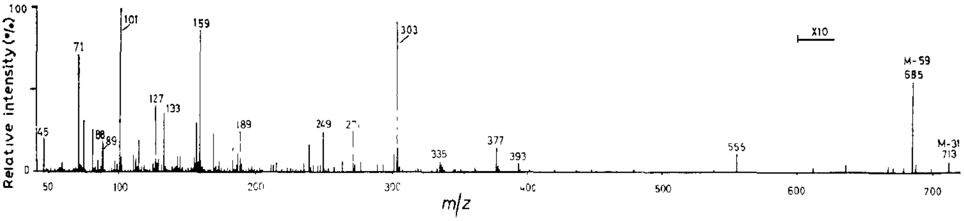
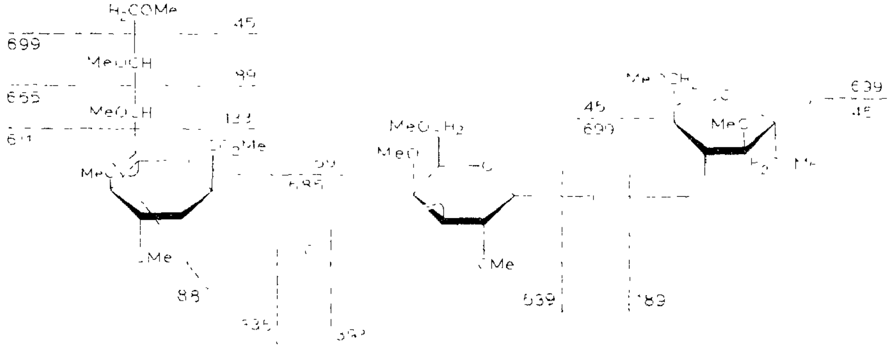


Fig. 2. Mass spectrum of trisaccharide **1**.

(190°, 2.2% SE-30) relative to *myo*-inositol. The mass spectrum of this compound, as well as the retention time, were established to be similar to those of methyl 3-deoxy-D-*glycero*-β-D-*galacto*-nonulopyranosidonic acid methyl ester, the main deamination product of the methyl ester methyl β-glycoside of neuraminic acid[5,6].

To isolate the sialic acid-containing material, the sample was fractionated on a column of DEAE-Sephadex. The fraction containing one sialic acid residue was eluted (see Experimental section), permethylated, and analyzed by g.l.c. The sample was composed of two major compounds (Fig. 1) having relative retention times of $T_1 = 2.59$ and $T_2 = 2.88$ (285°, 2.2% SE-30) relative to permethylated maltotriitol[7].

According to m.s. analysis, both of the compounds were trisaccharides (**1** and **2**) containing a deaminated sialic acid residue attached to a hexopyranosyl-2,5-anhydro-D-mannitol moiety; the mass spectrum of the (2→3)-linked compound **1** is shown in Fig. 2 and the major fragments in Scheme 1. Characteristic ions of the deaminated sialic acid residue are observed at *m/z* 335 (aA_1), 303 (aA_2), and 271 (aA_3). The 2,5-anhydro-D-mannitol residue (cA_1) gives rise to the ion at *m/z* 189, and the galactosyl-2,5-anhydro-D-mannitol residue (bcA_1) to that at *m/z* 393. The molecular weight of trisaccharide **1** was readily deduced from the ions at *m/z* 713



Scheme 1. Main m.s. fragmentation of trisaccharide 1

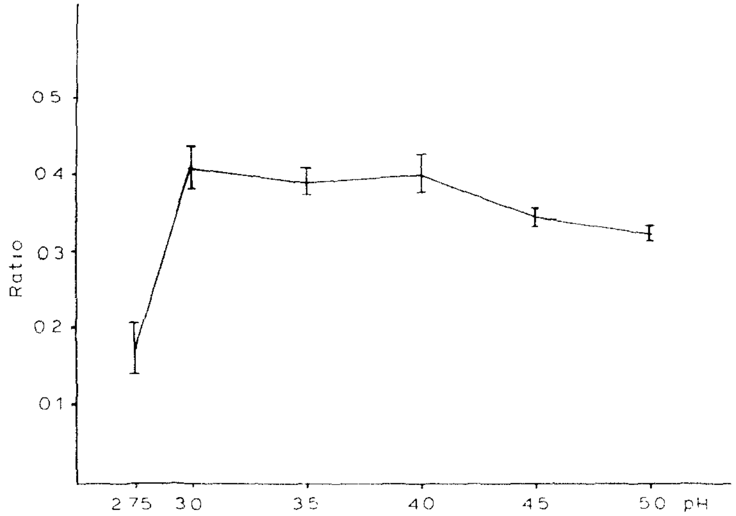


Fig. 3. Effect of pH on recovery of main trisaccharide products (**1** and **2**) relative to other, unidentified products

(M − 15) and 685 (M − 59). The position of the glycosidic linkage between sialic acid and D-galactose was confirmed by the analysis of the partially methylated alditol acetates[15] obtained from the isolated (see Experimental section) oligosaccharide: an intense g.l.c. peak for 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylgalactitol indicated a (2→3) linkage. In the mass spectra of trisaccharides composed of simple hexoses, this linkage could be assigned on the basis of intense ions at *m/z* 159 and the base peak at *m/z* 101 (H_1)[16]. Similarly, the (2→6)-linked compound (**2**) was shown to have a structure identical to that of compound **1** except for a (2→6) linkage between the sialic acid and D-galactose components. Details of the mass spectra of different deaminated, neuraminic acid-containing trisaccharides have been published[7].

Effect of deamination conditions on recovery of the neuraminic acid residue. — These experiments were performed with *N*-deacetylated α_1-acid glycoprotein as the test substance. The recovery was estimated by comparing the peak area of deaminated neuraminic acid to that of the main peak of D-galactose in g.l.c., after methanolysis and trimethylsilylation. The most suitable conditions, with good reproducibility, were found to be between pH 3 and 4. Under conditions more or less acidic, the recovery of the main product was lower and the amount of other, unidentified products increased (Fig. 3). The effects of temperature and the concentration of sodium nitrite were also tested. The best recoveries were obtained in reactions performed in an ice-bath in the presence of high concentrations of sodium nitrite. In an ice-bath, in the presence of M sodium nitrite at pH 3.5, the g.l.c. detector-response for deaminated neuraminic acid was ~40%, as compared with that of *N*-acetylneuraminic acid obtained from intact α_1-acid glycoprotein by methanolysis.

Effect of N-*deacetylation on the recovery of terminal trisaccharides.* — In these experiments, the glycoproteins were successively *N*-deacetylated under different conditions. deaminated under the optimal conditions just described, reduced, and the products isolated by DEAE-Sephadex. The effect of different reaction conditions was estimated on the basis of the recoveries of sialic acid bound to D-galactose obtained from the anion-exchange columns. Up to 40% of the total amount of D-galactose was obtained in the sialic acid fraction, and the recovery showed a correlation to the incubation temperature (85–100°) and time (2–16 h), so that, under the mildest possible conditions (85°, 2 h), it was highest. In the samples treated under the mildest conditions, however, the *N*-deacetylation was unsatisfactory and trisaccharides incorporating *N*-acetylneuraminic acid, D-galactose, and 2,5-anhydro-D-mannitol could be detected. In all experiments, special attention was paid to the proportion of (2→6)- and (2→3)-linked trisaccharides. This appeared to remain the same no matter what was the recovery of trisaccharides. These results demonstrate that the conditions for *N*-deacetylation of sialic acid residues are quite close to those causing cleavage of its glycosidic linkages, which makes the *N*-deacetylation step the most critical one in the production of deaminated sialyloligosaccharides.

Recently, Strecker *et al.*[4] published results on g.l.c.–m.s. analysis of a number of oligosaccharides produced by hydrazinolysis and subsequent deamination of glycopeptides. On the basis of mass spectra, they concluded that deamination causes changes in the ring structure of the sialic acid residues. This conclusion is in disagreement with the results presented here and earlier[7] concerning trisaccharides, and with those concerning deamination of the methyl ester methyl β-glycoside of neuraminic acid[6]. Several parameters, such as temperature, ionic strength, pH, and solvents, are known to affect the deamination reaction, and more than one product is usually obtained[1]. Under our conditions, where the procedure is performed in an ice-bath, only small amounts of other, unidentified products have been detected. On the other hand, very small changes in the structure of a trisaccharide may be difficult to identify by m.s. alone, so that other means of analysis, such as n.m.r. spectroscopy, must be applied to solve this problem.

Our results indicate that *N*-deacetylation, combined with deamination, may be used for production of sialic acid-containing oligosaccharides from the terminal parts of the carbohydrate chains of intact glycoprotein molecules. By using mass-fragmentographic detection, the oligosaccharides, produced by the present method from 5 mg of human α_1-acid glycoprotein, were analyzed without prior isolation of the glycopeptides. Furthermore, the use of similar, deuterium-labelled oligosaccharides as internal standards makes quantitation of the oligosaccharides possible. The present method thus affords a useful procedure for analysis of the terminal structures of glycoproteins.

ACKNOWLEDGMENTS

The authors are indebted to Professor J. Järnefelt, M. D., Docent J. Finne, M. D., and Docent J. Kärkkäinen for their keen interest in this work. This work was supported by the Sigrid Jusèlius Foundation, Finland.

REFERENCES

1 J. M. Williams, *Adv. Carbohydr. Chem. Biochem.*, 31 (1975) 9–79.
2 M. Isemura and K. Schmid, *Biochem. J.*, 124 (1971) 591–604.
3 B. Bayard and B. Fournet, *Carbohydr. Res.*, 46 (1976) 75–86.
4 G. Strecker, A. Pierce-Cretel, B. Fournet, G. Spik, and J. Montreuil, *Anal. Biochem.*, 111 (1981) 17–26.
5 I. Mononen, *Carbohydr. Res.*, 88 (1981) 39–50.
6 I. Mononen, B. Lengstad, and J. Lonngren, *Acta Chem. Scand.*, B34 (1980) 775–776.
7 I. Mononen, *Carbohydr. Res.*, 104 (1982) 1–9.
8 C. Erbing, K. Granath, L. Kenne, and B. Lindberg, *Carbohydr. Res.*, 47 (1976) c5–c7.
9 J. Finne and T. Krusius, *FEBS Lett.*, 66 (1976) 94–97.
10 R. J. Penic, M. H. Meisler, and R. H. McCluer, *Biochim. Biophys. Acta*, 116 (1966) 279–287.
11 J. Finne, T. Krusius, and H. Rauvala, *Carbohydr. Res.*, 80 (1980) 336–339.
12 S.-I. Hakomori, *J. Biochem. (Tokyo)*, 55 (1964) 205–208.
13 N. K. Kochetkov and O. S. Chizhov, *Adv. Carbohydr. Chem.*, 21 (1966) 39–93.
14 V. Kováčik, Š. Bauer, J. Rosík, and P. Kováč, *Carbohydr. Res.*, 8 (1968) 282–290.
15 P.-E. Jansson, L. Kenne, H. Linegren, B. Lindberg, and J. Lonngren, *Chem. Commun. Stockholm Univ.*, (1976) (8) 1–75.
16 J. Kärkkäinen, *Carbohydr. Res.*, 17 (1971) 11–18.