Acta Biochimica Polonica

Vol. 44 No. 2/1997

285–292

QUARTERLY

Determination of single monosugars bound to a peptide

Hubert Krotkiewski[1] and Bożena Krotkiewska[2]

[1]*Department of Immunochemistry, Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, R. Weigla 12, 53-114 Wrocław, Poland*
[2]*Department of Biochemistry, Medical Academy, T. Chałubińskiego 10, 50-368 Wrocław, Poland*

Received: 28 October, 1997; revised: 8 January, 1997; accepted: 14 April, 1997

Key words: glycoprotein glycosylation, alditols, β-elimination, g.l.c.-m.s. analysis

A method is described which allows detection and quantitative determination of single monosugar units bound O-glycosidically to a peptide. A glycoprotein or a glycopeptide is chemically degraded under the modified conditions of Carlson degradation (β-elimination performed in weakly alkaline conditions in the presence of sodium borohydride). An aliquot of the neutralized reaction mixture, supplemented with an internal standard, is peracetylated, extracted and directly analyzed by g.l.c.-m.s. All the O-linked oligosaccharides split off from the peptide are derivatized, but under gas-liquid chromatography at 150–230°C only monosugar peracetylated alditols reach the detector. By comparing the retention times of appropriate peaks with standards and by checking their mass spectra the monosugar alditols are unequivocally identified. The detectable amount of a reduced monosugar in the analyzed sample is about 0.3 μg. Several glycoproteins were analyzed using this method. Free *N*-acetylgalactosaminitol was detected in the degradation products of human glycophorin A and ovine submaxillary mucin, additionally free galactitol was detected in the degradation products of glycophorin. This result suggests that some single galactose units, O-glycosidically linked to the peptide are present in human glycophorin A.

Glycosylation of a polypeptide chain is a very common post-translational modification of proteins. It is assumed that more than 90% of proteins known so far contain carbohydrates, and the sugar content in glycoproteins varies from about 1% to as much as 80% of the total mass [1]. The carbohydrate moiety of glycoproteins consists of sugar chains of different length and different structure, bound to the polypeptide by N- and/or O-linkages. The linkage between *N*-acetylglucosamine and asparagine is relatively resistant to alkali, whereas the O-glycosidic linkage is susceptible to degradation even under weak alkaline conditions.

The N-linked oligosaccharides of glycoproteins may consist of as many as about 70 monosugar units and these poly(glycosyl)chains were found in the glycoproteins of human erythrocyte membranes [2]. The short N-linked oligosaccharides, consisting of four or even three monosugar units, were

Abbreviations: g.l.c.-m.s., gas-liquid chromatography with mass-spectrometric detection; GlcNAc, *N*-acetylglucosamine; GalNAc, *N*-acetylgalactosamine; Gal-ol, GalNAc-ol, reduced forms (alditols) of the respective sugars; OSM, ovine submaxillary mucin; Tn antigen, non-substituted GalNAc residue, bound to a peptide.

found in insect [3, 4] and plant [5] glycoproteins. These chains represent always a part of a branched core pentasaccharide $Man_3GlcNAc_2$, present in all N-linked oligosaccharides [6].

The O-linked sugar chains in glycoproteins are usually not so long as the N-linked ones; they have been reported to contain up to about 20 monosugar units, as in soluble blood-group substances [7]. The short O-glycosidic oligosaccharides in glycoproteins may consist of two or even only one monosugar residue. They include such structures as Galβ1-3GalNAc (T antigen), Neu5Acα2-6GalNAc (sialyl Tn antigen) and GalNAc (Tn antigen). These structures are frequently found in cancer cell mucins and in erythrocytes of persons with the rare Tn syndrome [8]. These structures were also found, among others, in Antarctic fish anti-freeze glycoproteins [9] and some animal mucins [10]. Since the late eighties a new type of monoglycosylation in glycoproteins has been known, i.e. the single GlcNAc units are linked O-glycosidically to the side chain hydroxyls of serine or threonine. The first described single GlcNAc residue-bearing proteins were the nuclear pore proteins; later these mono GlcNAc units were found in glycoproteins from lymphocytes and erythrocytes, and in chromatin proteins [11].

A few years ago immunochemical studies on the *Moluccella laevis* lectin and its receptor residing on the human glycophorin A molecule, showed that specificity of this lectin can be defined as anti-Tn, which means that this lectin recognizes single GalNAc residues bound to a peptide [12]. The major O-glycan of human glycophorin is a disialylated Galβ1-3GalNAc disaccharide [13, 14]; more recent results suggested that due to microheterogeneity of glycosylation a minor number of incomplete structures, including single GalNAc residues, could be present in this glycoprotein. These observations were fully confirmed by identification of a small amount of GalNAc-ol in a salt fraction obtained by gel filtration of the products of mild alkaline degradation of glycophorin A [15].

The results obtained in the latter paper prompted us to elaborate a general method to determine the monosugar residues bound to a polypeptide in any type of glycoprotein. The method, based on a direct chemical analysis of the β-elimination products of a glycoprotein/glycopeptide, does not involve any fractionation and, by applying an internal standard, allows also quantitative determinations.

MATERIALS AND METHODS

Glycoproteins. Human, mouse and chicken glycophorins were obtained by phenol-water extraction of erythrocyte membranes, according to Lisowska *et al.* [16]. Mouse monoclonal antibodies: IgM (B006) and IgA (A008) were isolated from hybridoma culture fluids as described previously [17, 18]. Ovalbumin and fetuin were purchased from Sigma (U.S.A.) and ovine submaxillary mucin (OSM) was purchased from BioCarb AB (Lund, Sweden). Haptoglobin was a gift from Prof. I. Kątnik of the Department of General Chemistry, Medical Academy (Wrocław, Poland).

Carlson degradation (β-elimination). A sample of glycoprotein (50–400 μg) was treated with 0.1 M NaOH/ 1 M $NaBH_4$ (2 ml) at 50°C for 18 h, in the presence of Cd^{2+} ions as described by Likhosherstov *et al.* [19] then the solution was cooled and neutralized with 50% acetic acid.

Acetylation. A sample of the neutralized reaction mixture (100 μl) was supplemented with a known amount of internal standard (xylitol or perseitol), evaporated to dryness, then evaporated three times with methanol and peracetylated with 1:1 (0.15 ml, v/v) mixture of pyridine/acetic anhydride at 100°C for 35 min. The solvents were then removed by co-evaporation with ethanol (3 times) and the sample was extracted with chloroform.

g.l.c.-m.s. Analysis. The chloroform phase was analyzed using gas-liquid chromatography. A Hewlett-Packard 5890 gas chromatograph was used, equipped with a mass selective detector 5971A and a capillary column HP-1 (0.2 mm × 12 m); the temperature program 150°–230°C (8°C/min) was applied. The content of detected monosugars was calculated by comparing their g.l.c. peaks with

the peak of the internal standard (xylitol or perseitol).

Desialylation of glycoproteins. Human glycophorin and OSM were desialylated by mild acid hydrolysis in 0.025 M sulfuric acid at 60°C for 4 h. The solution was cooled, neutralized with NaOH and the glycoproteins were desalted on a BioGel P-4 column (2.5 cm × 44 cm), run in water.

RESULTS AND DISCUSSION

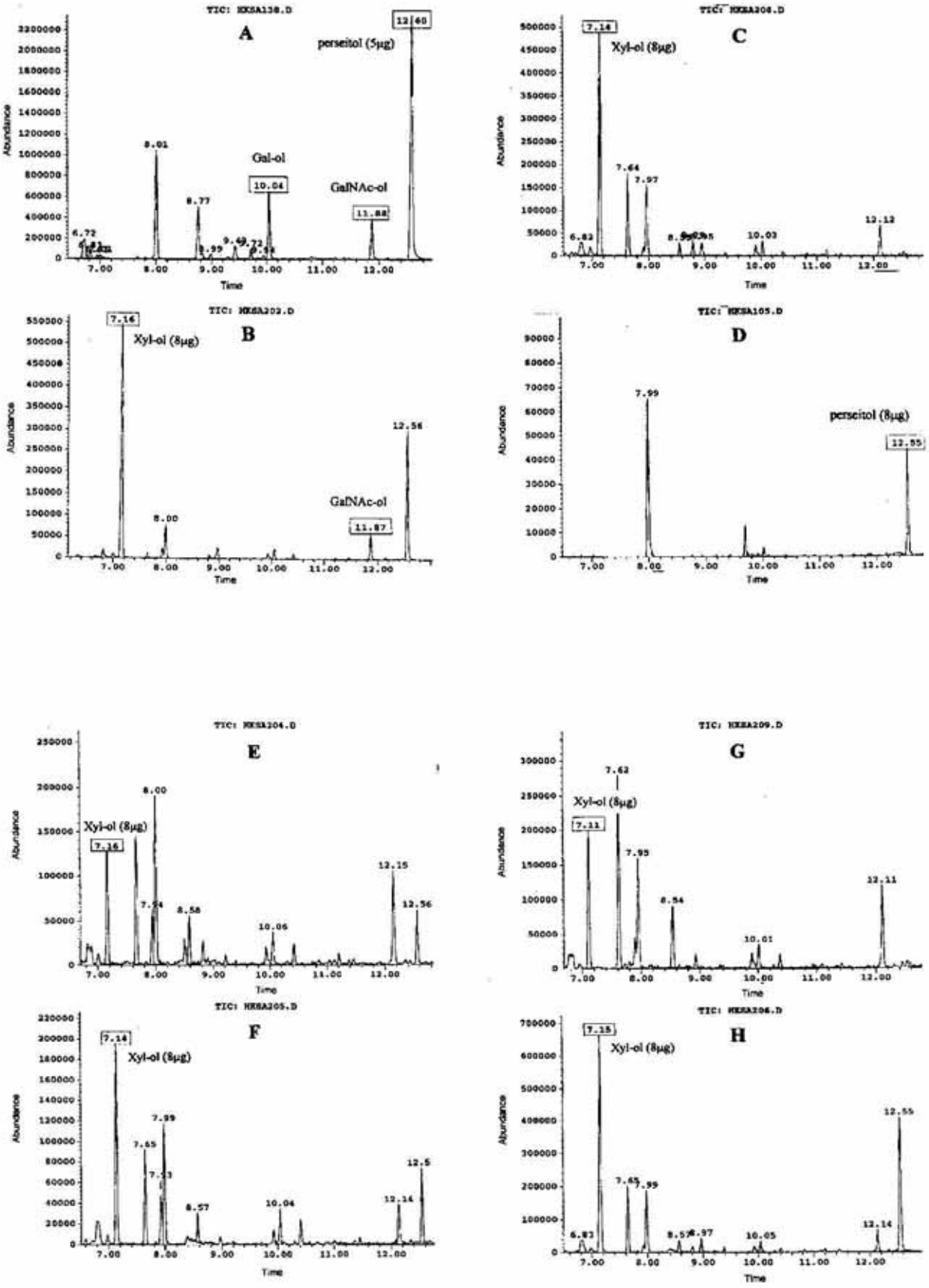
Several glycoproteins were treated under the conditions of the β-elimination reaction, Table 1. The monosugar alditols were detected in two out of nine glycoproteins tested (Fig. 1). One of them was human glycophorin A, a major sialoglycoprotein of human erythrocyte membranes [20], containing one N-glycan and 15 O-glycans. In this case two monosugars were detected: Gal-ol and GalNAc-ol, (Fig. 1A). The second glycoprotein was OSM (Fig. 1B), which is known to possess multiple sialyl Tn structures [10]. Our result, based on a sensitive analytical method, shows that in OSM molecule an unsubstituted Tn antigens are also present. Two reduced monosugars, detected here in human glycophorin, were previously reported in the literature as the products of mild alkaline degradation of this glycoprotein [13]. Nevertheless, for a long time they were considered an artifact, originating from further degradation of the released O-linked chains. In this investigation three different glycophorins were analyzed: human, murine and chicken. Despite the fact that all of them contain multiple O-linked oligosaccharides and show similar structural features, we found reduced galactose in the products of β-elimination only in the case of human glycophorin. This result strongly suggests that galactose residues exist as single units, bound directly to the peptide, at least in some human glycophorin native molecules. If the reduced galactose was a degradation product of the O-linked chains, one could expect its presence in the β-elimination products of all three glycophorins. Therefore, the above result indicates that biosynthetic pathways of the O-linked chains in three analyzed glycophorins, originating from different species, are different. One can assume that in human hematopoietic tissues a Gal transferase, introducing this sugar to Ser/Thr, is present or, more likely, that GalNAc transferase can also use UDP-Gal as a substrate. The presence of another reduced sugar, GalNAc-ol, has been well documented, both by immunochemical [12] and chemical [13] methods: it originates from single GalNAc residues

Table 1. Glycoproteins degraded under modified Carlson degradation conditions and analyzed for the presence of reduced monosugars.

The status of N- or O-glycosylation is indicated.

Glycoprotein	N-glycosylation	O-glycosylation	Monosugar alditol
Human glycophorin	+	+	GalNAc-ol Gal-ol
Murine glycophorin	+	+	n.d.
Chicken glycophorin	+	+	n.d.
MoAb IgM (B006)	+	+	n.d.
Fetuin	+	+	n.d.
Ovalbumin	+	–	n.d.
MoAb IgA (A008)	+	–	n.d.
Haptoglobin	+	–	n.d.
Ovine submaxillary mucin (OSM)	–	+	GalNAc-ol

n.d., not detected by g.l.c.-m.s; MoAb, monoclonal antibody.


A
perseitol (5μg)
Gal-ol
GalNAc-ol
B
Xyl-ol (8μg)
GalNAc-ol
C
Xyl-ol (8μg)
D
perseitol (8μg)
E
Xyl-ol (8μg)
F
Xyl-ol (8μg)
G
Xyl-ol (8μg)
H
Xyl-ol (8μg)
Abundance
Time

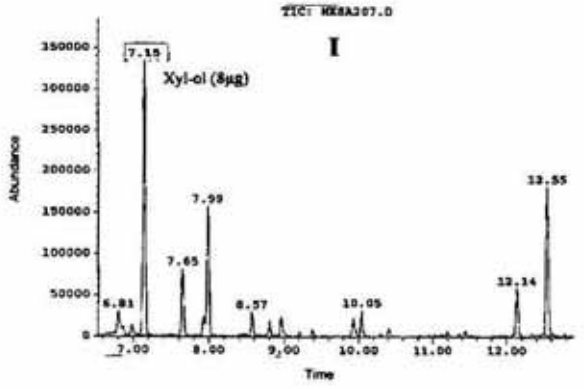


Figure 1. Gas chromatography of the reaction mixtures after Carlson degradation of the following glycoproteins.

A, human glycophorin; B, ovine submaxillary mucin; C, murine glycophorin; D, chicken glycophorin; E, mouse monoclonal antibody IgA; F, mouse monoclonal antibody IgM; G, human haptoglobin; H, fetuin; I, ovalbumin. Xyl-ol and perseitol were used as internal standards. Relative abundance of the peaks is presented in the TIC (total ion current) mode and the time is given in minutes.

linked to the polypeptide in human glycophorin molecule.

In an additional experiment human glycophorin A and OSM were desialylated before starting the β-elimination reaction. In this case the content of Gal-ol and GalNAc-ol in the reaction products was approximately 8 and 2 times higher, respectively, in the glycophorin-derived sample, and GalNAc-ol content was about 8 times higher in the OSM-derived sample (Fig. 2), when compared with the two native glycoproteins (Fig. 1A and 1B, respectively). This result is in agreement with the known fact that sialyl-GalNAc is the major O-glycan in OSM [10] and shows that, in human glycophorin A, the respective monosugars exist also as sialylated units.

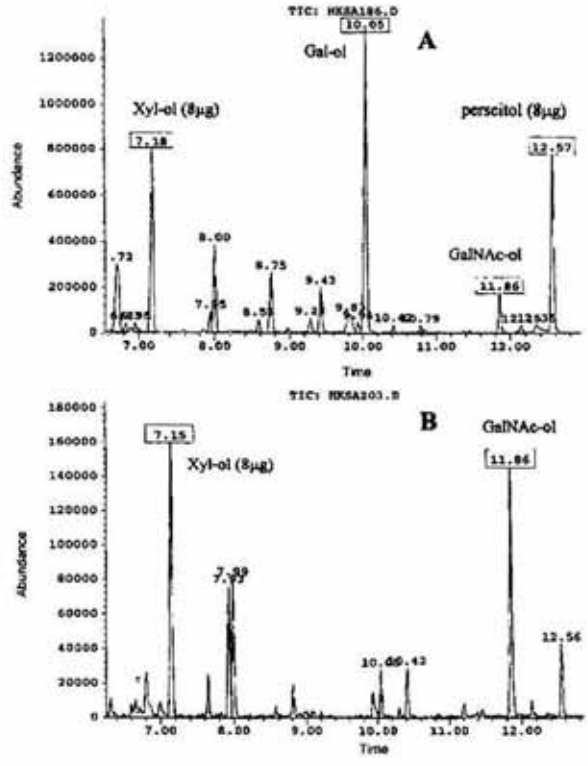


Figure 2. Gas chromatography of the reaction mixtures of two glycoproteins, desialylated with acid before Carlson degradation.

A, human glycophorin; B, ovine submaxillary mucin. Xyl-ol and perseitol were used as internal standards. Relative abundance of the peaks is presented in the TIC (total ion current) mode and the time is given in minutes.

In all analyses of the Carlson degradation products where reduced monosugars were found, their identification was performed on

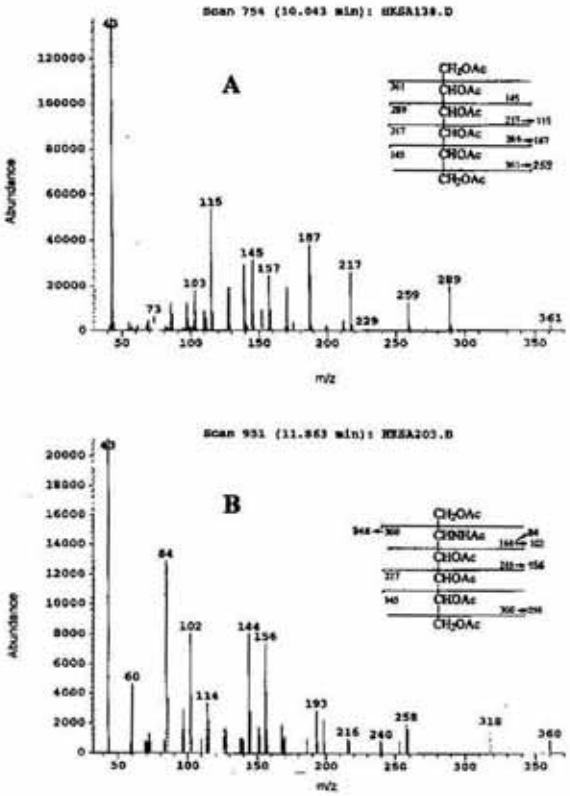


Figure 3. Mass spectra of two monosugar peracetylated alditols and their fragmentation patterns.

A, Gal-ol; B, GalNAc-ol.

the basis of retention time in g.l.c separation and mass spectra (Fig. 3).

Unlike the previously described procedure [15], in which the products of β-elimination of human glycophorin were separated by gel filtration and the content of reduced monosugars was analyzed in the salt fraction, the presented method is based on direct analysis of the reaction mixture after β-elimination of glycoproteins, with one derivatization step and without any gel filtration. According to the literature the mild alkaline degradation, which permits β-elimination, splits all kinds of oligosaccharides O-glycosidically linked to a peptide. Susceptibility to reducing β-elimination is diminished only in these cases where the O-linked chains are bound to hydroxyamino acids, which occupy terminal positions in the peptide [21]. The released O-linked sugars are present in the reaction mixture as free, reduced structures, they undergo peracetylation and are extractable with chloroform. When such a sample is injected to a column of gas chromatograph and analyzed under standard temperature conditions, only monosugar peracetylated alditols are eluted from the column and analyzed in a mass selective detector. Peracetylated di- and trisaccharides can also be analyzed by g.l.c.-m.s., but much higher temperatures, up to 330°–350°C, are needed. Therefore, under the conditions applied, only peracetylated monosugar alditols are analyzed, and this decides on specificity of the method.

Carbohydrates, as the molecules forming the very outer structures of the cells, create characteristic antigens. In tumor cells the so-called tumor-associated carbohydrate antigens, among others Tn and sialyl Tn structures have been recognized [22]. These structures may be examined using lectins or specific monoclonal antibodies [23]. The present paper describes a chemical method, which may be very useful in analysis of glycoproteins from transformed cells, by supplying direct evidence for the presence of Tn and/or sialyl Tn antigens.

The authors thank Professor E. Lisowska for critical reading of the manuscript.

REFERENCES

1. Kornfeld, R. & Kornfeld, S. (1976) Comparative aspects of glycoprotein structure. *Annu. Rev. Biochem.* **45**, 217–237.

2. Krusius, T., Finne, J. & Rauvala, H. (1978) The poly(glycosyl) chains of glycoproteins. Characterisation of a novel type of glycoprotein saccharides from human erythrocyte membrane. *Eur. J. Biochem.* **92**, 289–300.

3. Kubelka, V., Altman, F. & März, L. (1995) The asparagine-linked carbohydrate of honeybee venom hyaluronidase. *Glycoconjugate J.* **12**, 77–83.

4. Duk, M., Krotkiewski, H., Forest, E., Rodriguez, J., Kochman, M. & Lisowska, E. (1996) Evidence for glycosylation of the juvenile hormone-binding protein from *Galleria mellonella* hemolymph. *XXXII Meeting of the Polish Biochemical Society, Cracow, September 17–20*, Abstracts, p. 245 (in Polish).

5. Takahashi, N., Nakagawa, H., Tsukamoto, Y., Masuda, K., Lee, K.B. & Lee, Y.C. (1996) Structural analysis of N-linked oligosaccharides of horse radish peroxidase and stem bromelain as related to the difference in substrate specificity between glycoamidases A and F. *XVIII International Carbohydrate Symposium, Milano*, Abstracts, p. 179.

6. Montreuil, J. (1980) Primary structure of glycoprotein glycans. Basis for the molecular biology of glycoproteins. *Adv. Carbohydr. Chem. Biochem.* **37**, 157–223.

7. Wu, A.M., Kabat, E.A., Pereira, M.E.A., Gruezo, F.G. & Liao, J. (1982) Immunochemical studies on blood groups: The internal structure and immunological properties of water-soluble human blood group A substance studied by Smith degradation, liberation and fractionation of oligosaccharides and reaction with lectins. *Arch. Biochem. Biophys.* **215**, 390–404.

8. Lisowska, E. (1995) Tn antigens and their significance in oncology. *Acta Biochim. Polon.* **42**, 11–18.

9. Shier, W.T., Lin, Y. & DeVries, A.L. (1975) Structure of the carbohydrate of antifreeze

glycoproteins from an Antarctic fish. *FEBS Lett.* **54**, 135–138.

10. Murty, V.L.N. & Horowitz, M.I. (1968) Alkaline-reductive cleavage of ovine submaxillary mucin. *Carbohydr. Res.* **6**, 266–275.

11. Haltiwanger, R.S., Kelly, W.G., Roquemore, E.P., Blomberg, M.A., Dennis Dong, L.-Y., Kreppel, L., Chou, T.-Y. & Hart, G.W. (1992) Glycosylation of nuclear and cytoplasmic proteins is ubiquitous and dynamic. *Biochem. Soc. Trans.* **20**, 264–269.

12. Duk, M., Mitra, D., Lisowska, E., Kabat, E.A., Sharon, N. & Lis, H. (1992) Immunochemical studies on the combining site of the A + N blood type specific *Moluccella laevis* lectin. *Carbohydr. Res.* **236**, 245–258.

13. Thomas, D.B. & Winzler, R.J. (1969) Structural studies on human erythrocyte glycoproteins. Alkali-labile oligosaccharides. *J. Biol. Chem.* **244**, 5943–5946.

14. Lisowska, E., Duk, M. & Dahr, W. (1980) Comparison of alkali-labile oligosaccharide chains of M and N blood-group glycopeptides from human erythrocyte membrane. *Carbohydr. Res.* **79**, 103–113.

15. Krotkiewski, H., Duk, M. & Lisowska, E. (1995) Subtle differences in glycosylation of blood group M and N type glycophorin A detected with anti-Tn lectins and confirmed by chemical analysis. *Acta Biochim. Polon.* **42**, 41–44.

16. Lisowska, E., Messeter, L., Duk, M., Czerwiński, M. & Lundblad, A. (1987) A monoclonal anti-glycophorin A antibody recognizing the blood group M determinant: Studies on the subspecificity. *Mol. Immunol.* **24**, 605–613.

17. Krotkiewski, H., Nilsson, B. & Svensson, S. (1989) Structural analysis of the carbohydrate chains of a mouse monoclonal IgM antibody. *Eur. J. Biochem.* **184**, 29–38.

18. Lipniunas, P., Grönberg, G., Krotkiewski, H., Angel, A.-S. & Nilsson, B. (1993) Investigation of the structural heterogeneity in the carbohydrate portion of a mouse monoclonal immunoglobulin A antibody. *Arch. Biochem. Biophys.* **300**, 335–345.

19. Likhosherstov, L.M., Novikova, O.S., Derevitskaya, V.A. & Kochetkov, N.K. (1990) A selective method for sequential splitting of O- and N-linked glycans from N,O-glycoproteins. *Carbohydr. Res.* **199**, 67–76.

20. Lisowska, E. (1988) in *Molecular Immunology of Complex Carbohydrates* (Wu, A.M., ed.) pp. 265–315, Plenum Press, New York, London.

21. Sharon, N. (1975) *Complex Carbohydrates. Their Chemistry, Biosynthesis and Functions,* p. 77, Addison-Wesley Publishing Company.

22. Hakomori, S-i. (1991), Possible functions of tumor-associated carbohydrate antigens. *Curr. Opin. Immunol.* **3**, 646–653.

23. Nakada, H., Inoue, M., Tanaka, N. & Yamashina, I. (1994) Coexpression of cancer-associated carbohydrate antigens, Tn and sialyl Tn. *Glycoconjugate J.* **11**, 262–265.