Determination of single monosugars bound to a peptide

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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 glycoporphin A and ovine submaxillary mucin, additionally free galactitol was detected in the degradation products of glycoporphin. This result suggests that some single galactose units, O-glycosidically linked to the peptide are present in human glycoporphin 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-acetylgluco-
samine 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(glyco-
syl) 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-acetylgalactosamine; 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$_5$GlcNAc$_2$, 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 Galβ1-3GlcNAc (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 *Motulacea laevis* lectin and its receptor residing on the human glycoporin 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 glycoporin 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 glycoporin 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 glycoporins 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. J. Kątynik 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 NaN$_3$H$_4$ (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 (xylose or peroside), 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 chromato-graph was used, equipped with a mass selective detector 5971A and a capillary column HP-1 (0.2 mm × 12 m); the temperature program 150°C–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 (xyitol 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 determined 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 results, based on a sensitive analytical method, show that in OSM molecule an unsubstituted Tn antigens are also present. Two reduced monosugars, detected here in human glycoporphin, were previously reported in the literature as the products of the O-linked chains, one could expect its presence in the β-elimination products of all three glycoporphins. Therefore, the above result indicates that biosynthetic pathways of the O-linked chains in three analyzed glycoporphins, 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.

<table>
<thead>
<tr>
<th>Glycoprotein</th>
<th>N-glycosylation</th>
<th>O-glycosylation</th>
<th>Monosugar alditol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human glycophorin</td>
<td>+</td>
<td>+</td>
<td>GalNAc-ol, Gal-ol</td>
</tr>
<tr>
<td>Murine glycophorin</td>
<td>+</td>
<td>+</td>
<td>n.d.</td>
</tr>
<tr>
<td>Chicken glycophorin</td>
<td>+</td>
<td>+</td>
<td>n.d.</td>
</tr>
<tr>
<td>MoAb IgM (8006)</td>
<td>+</td>
<td>+</td>
<td>n.d.</td>
</tr>
<tr>
<td>Fetuin</td>
<td>+</td>
<td>+</td>
<td>n.d.</td>
</tr>
<tr>
<td>Ovalbumin</td>
<td>+</td>
<td>–</td>
<td>n.d.</td>
</tr>
<tr>
<td>MoAb IgA (A008)</td>
<td>+</td>
<td>–</td>
<td>n.d.</td>
</tr>
<tr>
<td>Haptoglobin</td>
<td>+</td>
<td>–</td>
<td>n.d.</td>
</tr>
<tr>
<td>Ovine submaxillary mucin (OSM)</td>
<td>–</td>
<td>+</td>
<td>GalNAc-ol</td>
</tr>
</tbody>
</table>

n.d. = not detected by g.l.c.-m.s.; MoAb, monoclonal antibody.
linked to the polypeptide in human glycoporphin molecule.

In an additional experiment human glycoporphin 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 glycoporphin-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 glycoporphin A, the respective monosugars exist also as sialylated units.

In all analyses of the Carlson degradation products where reduced monosugars were found, their identification was performed on the basis of their characteristic mass spectra. The mass spectra of the monosugar peracylated alditols and their fragmentation patterns (Fig. 3) are very similar to those of the standards. The mass spectra of the monosugar peracylated alditols and their fragmentation patterns (Fig. 3) are very similar to those of the standards. From these spectra, it can be seen that the monosugar peracylated alditols have a characteristic fragment at m/z 103, which is diagnostic for the presence of a monosugar peracylated alditol. The mass spectra of the monosugar peracylated alditols and their fragmentation patterns (Fig. 3) are very similar to those of the standards. From these spectra, it can be seen that the monosugar peracylated alditols have a characteristic fragment at m/z 103, which is diagnostic for the presence of a monosugar peracylated alditol.
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 degradations, 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 hydroxyaminos, 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 330º–350ºC, 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.

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REFERENCES


