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The elimination of *O*-linked glycans from glycoproteins under non-reducing conditions

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A simple procedure is described for the elimination of *O*-linked glycans from bovine submaxillary mucin under non-reducing conditions, using triethylamine in aqueous hydrazine. The glycans were isolated as the hydrazones, which were converted to the reducing glycans by exchange with acetone in neutral aqueous solution. The glycan alditols obtained after reduction corresponded to those obtained by the reductive β-elimination of *O*-glycans.

Keywords: *O*-linked sugars; mucin; glycoprotein; β-elimination; sugar hydrazones; reducing sugars

Introduction

The glycans of glycoproteins are typically either *N*-linked (attached to asparagine) or *O*-linked (attached to serine or threonine) [1]. In order to carry out their structural analysis, it is desirable to release them from the protein backbone. To this end, several enzymic and chemical methods are available. Enzymic release of *N*-glycans can be achieved with peptide *N*-glycosidases and endoglycosidases [2], but fewer enzymes are available for removal of *O*-glycans [3]. Chemical treatment with anhydrous hydrazine [4] has been used to remove glycans of both classes, and has been adapted, using milder conditions, to the selective removal of *O*-glycans [5]. The usual method for the release of *O*-glycans is by digestion of the glycoprotein in mild alkali, usually in the presence of borohydride [6]. A significant disadvantage of the method is the reduction of the glycans to the alditols. Moreover, it is not truly specific for *O*-glycans, as there is a significant release of *N*-glycans [7, 8], because of the inclusion of the borohydride [9].

The purpose of the present study was to define and evaluate simple experimental conditions for the specific release of *O*-linked glycans from a glycoprotein, and for their isolation in an unreduced form suitable *inter alia* for chemical derivatisation, reductive incorporation of tritium label or the attachment to polymer supports.

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Materials and methods

Bovine submaxillary mucin type 1S was obtained from Sigma Chemical Co (USA), hydrazine monohydrate (98%) from Aldrich (USA), BioRad resin AG50W-X8 (H^+) and Chelex-100 (Na^+) from BioRad (USA). All other chemicals were of analytical quality.

High-performance anion-exchange chromatography

High-performance anion-exchange chromatography at high pH was performed using a Waters Model 625 LC system, with a Model 464 amperometric detector operating in pulsed mode (E1 = 80 mV, E2 = 733 mV, E3 = −675 mV; T1 = 0.400 s, T2 = 0.400 s, T3 = 0.200 s). Separation was carried out with a Dionex CarboPac PA1 column (4 × 250 mm), with the following eluents: Monosaccharide analysis: isocratic elution at 1 ml min^{-1} with 0.015 M sodium hydroxide for 20 min, followed by a 10 min wash with 0.4 M sodium hydroxide (Program 1); Oligosaccharide and neuraminic acid analysis: a linear gradient of sodium acetate (0.02–0.25 M in 0.1 M sodium hydroxide over 50 min), followed by a wash with 0.4 M NaOH for 10 min (Program 2) [5].

Monosaccharide analysis

Samples were hydrolysed in 2 M trifluoroacetic acid for 4 h at 100 °C. The acid was removed by evaporation, the residue dissolved in water (0.100 ml), 2-deoxy-D-glucose (2 μg) was added as an internal standard and aliquots

(0.050 ml) analysed by high-performance anion-exchange chromatography.

Neuraminic acid analysis

Samples were hydrolysed in 0.1 M trifluoroacetic acid for 40 min at 80 °C, evaporated in a stream of nitrogen and analysed by high-performance anion-exchange chromatography.

Reversed-phase chromatography of hydrazones

A mixture of protein and glycan hydrazones was dissolved in water (0.3 ml), applied to an Alltech C_{18} Extract Clean disposable column (500 mg) and eluted at 0.25 ml min^{-1}. Three successive fractions were collected on elution with water (10 ml), 5% aqueous acetonitrile (5 ml) and 10% aqueous acetonitrile (5 ml).

Gel chromatography

Hydrazine-treated bovine submaxillary mucin (2–3 mg) in water (0.1 ml) was applied to a column (1.5 × 100 cm) of Fractogel HW-50(S) and eluted with water at 0.5 ml min^{-1}, with UV detection at 206 nm and collection of fractions (2 ml).

Desalting of glycans by gel chromatography

The sample (0.2–0.5 ml) was applied to a Sephadex G-10 column (1.0 × 9.5 cm) and eluted with water at 0.25 ml min^{-1}, with UV detection at 206 nm and collection of fractions (0.25 ml). Fractions 16–22, which typically contained the glycans, were batched and freeze dried.

Elimination of O*-glycans*

Bovine submaxillary mucin (2–3 mg) was dissolved in 50% (v/v) aqueous hydrazine hydrate (0.20 ml), with or without 0.1 M sodium hydroxide or 0.2 M triethylamine, and incubated at 45 °C for 18 h. When sodium hydroxide had been used, 0.1 M hydrochloric acid (0.20 ml) was then added. The solution was dried under a stream of nitrogen and the last traces of hydrazine removed by evaporation with toluene (3 × 0.2 ml).

N*-acetylation of glycan hydrazones*

Glycan hydrazones (5–50 μg) were dissolved in saturated sodium hydrogen carbonate solution (0.040 ml) and acetic anhydride (2 μl) added. The mixture was kept at room temperature for 20 min, with occasional mixing, and desalted by gel chromatography.

Deblocking of glycan hydrazones

Aqueous acetone method. Hydrazones (10–200 μg) were dissolved in 20% aqueous acetone (0.2 ml) and incubated at 55 °C for 24 h. The mixture was evaporated in a stream of nitrogen, redissolved in water and freeze dried.

Copper acetate method. Hydrazones (10–200 μg) were dissolved in copper acetate solution (1 mM, 0.5 ml) and incubated at 27° for 30 min. The mixture was applied to a mixed-bed ion-exchange column (0.5 × 5.0 cm), consisting of Chelex 100 (Na^+ form) and BioRad resin AG50W-X8 (H^+), and eluted with water at 0.25 ml min^{-1}. The eluate (10 ml) was freeze dried.

Reduction of glycans

Reducing oligosaccharides (25–250 μg) were dissolved in 0.01 M sodium hydroxide (0.04 ml) and solid sodium borohydride (2 mg) added. The mixture was kept at 4 °C overnight, acidified by dropwise addition of 1 M acetic acid, freed of borate by repeated evaporation with methanol in a stream of nitrogen and desalted by gel chromatography.

Reductive elimination of O*-linked glycans*

Bovine submaxillary mucin (2–3 mg) was dissolved in a solution (0.30 ml) of sodium borohydride (1.6 M) in sodium hydroxide (0.08 M) and incubated at 45 °C for 18 h. The solution was cooled in ice, acidified with 1 M acetic acid and applied to a column (4 × 0.5 cm) of Bio-Rad AG50W-X8 (H^+). The eluate (10 ml) was freeze dried and freed of borate as before.

Results

Elimination of glycans

Bovine submaxillary mucin was treated with 50% aqueous hydrazine and the mixture fractionated by gel chromatography. Aliquots of the eluate were hydrolysed with trifluoroacetic acid and examined by high-performance anion-exchange chromatography. Only fractions 18–45, corresponding to high molecular weight material, contained sugars, indicating that no significant release of sugars had taken place.

When this experiment was repeated using 50% aqueous hydrazine containing 0.1 M sodium hydroxide, no sugars were associated with the high molecular weight material but fractions 55–75, corresponding to oligosaccharides, contained galactose and galactosamine, indicating the effective release of the glycans. This experiment was repeated using 0.2 M triethylamine in place of the sodium hydroxide, so that a desalting step could be avoided. The release of glycans was equally effective.

In view of the time required for gel chromatography, the glycan-containing components were isolated by reversed-phase chromatography in subsequent experiments. The recoveries of galactosamine in the three fractions were 72, 22 and 5%, respectively, corresponding to 99% total recovery of the galactosamine in the mucin. Fraction 1 was selected for further study, as pilot experiments had indicated that it contained all of the acidic glycans.

Conversion of glycan hydrazones to reducing glycans

Acetone method. The glycan hydrazones were incubated in 50% aqueous acetone for 24 h at 55 °C. After evaporation under reduced pressure, they were analysed by high-performance anion-exchange chromatography. The chromatogram (Figs. 1b, 2a) differed from that of the glycan hydrazones (Fig. 1a), but it was concluded that the free glycans had not been formed, as subsequent treatment with sodium borohydride produced chromatographic peaks (Fig. 2b) which were not the same as those from the reductive elimination method (Fig. 2d). Coinjection of mixed samples confirmed this conclusion. The hydrazones were subjected to *N*-acetylation, followed by desalting, and once again incubated with aqueous acetone. This time, the altered chromatogram (Fig. 1c) corresponded to the desired reducing glycans, as reduction with borohydride gave a chromatogram (Fig. 2c) which corresponds to that obtained after reductive elimination (Fig. 2d). A mixture of the samples also gave peaks which are indistinguishable from those of the individual samples. The slight quantitative differences between these chromatograms are attributed to differences in the fractionation of the glycans in the reversed-phase separation of the glycan hydrazones.

The two groups of peaks at approximately 7 and 17 min (Figs. 2c, 2d) correspond to acidic glycans containing *N*-acetylneuraminic acid and *N*-glycolylneuraminic acid,

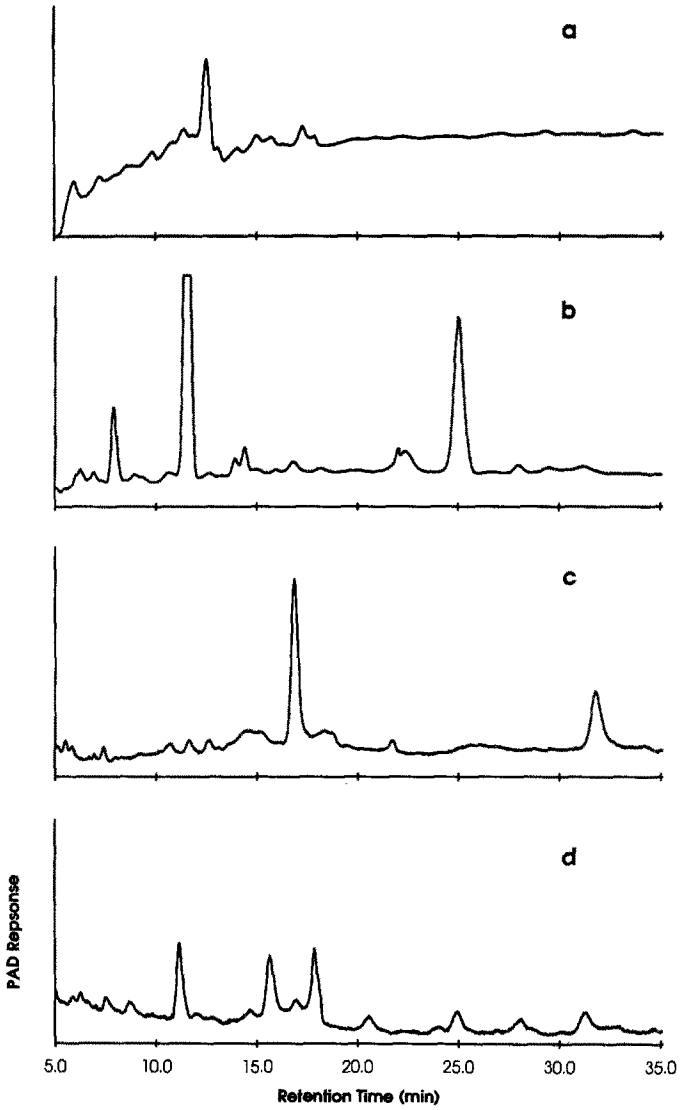

Figure 1. High-performance anion-exchange chromatograms of glycans and glycan hydrazones from bovine submaxillary mucin. (a) Hydrazones obtained by elimination in triethylamine-hydrazine. (b) Acetone azines from treatment of hydrazones with acetone. (c) Reducing glycans from treatment of acetohydrazones with acetone. (d) Glycans from treatment of hydrazones with copper acetate.

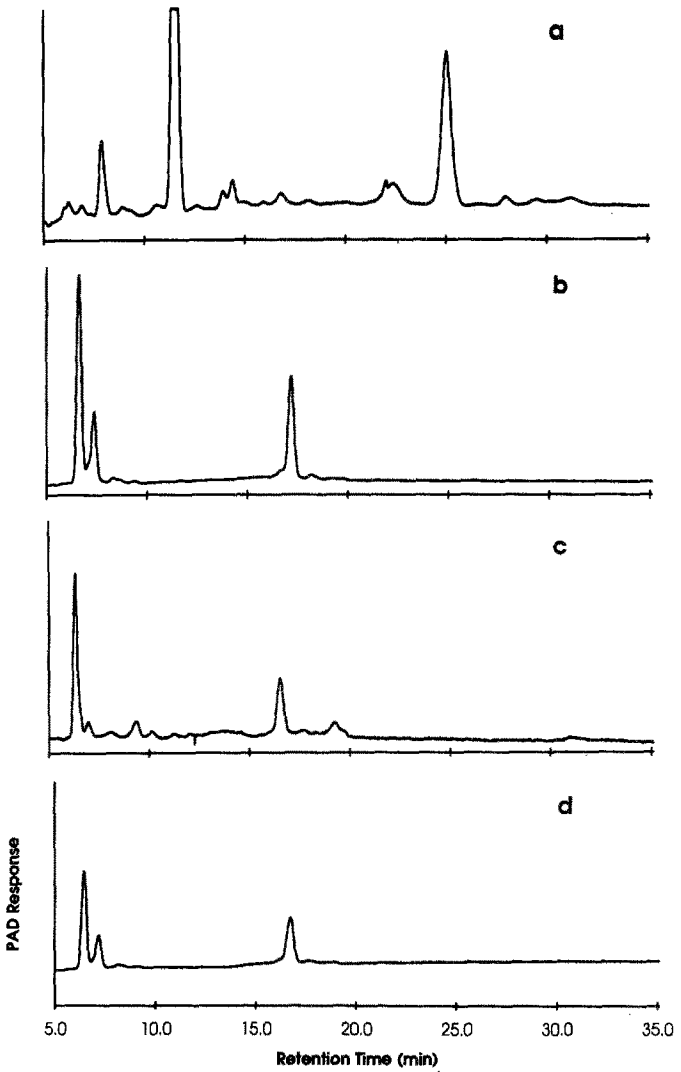

Figure 2. High-performance anion-exchange chromatograms of glycan hydrazones and glycan alditols from bovine submaxillary mucin. (a) Acetone azines from treatment of hydrazones with acetone. (b) Acetone azines after reduction with sodium borohydride. (c) Glycan alditols from reduction of reducing glycans. (d) Glycan alditols from reductive elimination.

respectively [10, 11]. Assuming equal response factors for the glycans, glycans containing these acids are estimated to be present in the ratio of 1.98:1. The ratio of the neuraminic acids obtained by mild hydrolysis of the starting glycoprotein was 1.48:1.

Copper acetate method. When the glycan hydrazones were treated with copper acetate, followed by cation-exchange chromatography, the product mixture was complicated and contained the expected reducing glycans in disappointing yield, because of incomplete removal of the hydrazone groups and the associated loss of the basic hydrazones on the column (Fig. 1d). When the hydrazones were *N*-acetylated and desalted before the copper acetate treatment, the glycans were obtained in good yield (data not shown).

Discussion

The standard procedure [6] for the release of *O*-glycans from a glycoprotein involves the digestion of a glycoprotein with dilute alkali. The glycans are eliminated as reducing sugars, which are unstable in the alkali, and sodium borohydride is added in high concentration to convert them to the stable alditols before degradation occurs. The inclusion of the borohydride, however, is somewhat problematic, as it leads to significant release of *N*-linked glycans [7–9] and some fragmentation of the protein chain [12, 13]. Tritium label is often added at this stage [4], using labelled borohydride, but its incorporation is inherently inefficient because of the high molarity of reducing agent which is required (typically 0.8–1 M).

Both *O*- and *N*-linked glycans are released from glycoproteins by heating with anhydrous hydrazine at 95° or above [5], but the *O*-linked glycans can be released selectively by using milder conditions [5]. The release of the *N*-glycans results from the hydrazinolysis of the amide linkages of asparagine, but the removal of the *O*-glycans probably involves a β-elimination process, promoted by the basicity of the hydrazine and analogous to that which occurs in aqueous alkali. An important advantage of using hydrazine is that, as the sugars are released, they are converted to the hydrazones and protected from degradation under the basic conditions.

It seemed possible that aqueous hydrazine might be sufficiently basic to promote β-elimination, and that the glycans might be converted to the base-stable hydrazones as they are released. In exploratory experiments, we observed no release of glycans from bovine mucin after extended incubation in 50% aqueous hydrazine, but the addition of 0.1 M sodium hydroxide to the hydrazine led to a smooth release of the glycans. After neutralization of the alkali, the sample was fractionated by gel or reversed-phase chromatography. It was necessary to desalt the sugar-containing fractions by gel chromatography before analysis by high-performance anion-exchange chromatography. An improvement to the method used a volatile organic base, triethylamine, instead of sodium hydroxide. The elimination was equally effective, but a desalting step was no longer required.

For the complete success of any method which uses hydrazine for the release of glycans, it is essential to have available a method for regenerating the reducing glycans from the hydrazones. Sugar hydrazones are somewhat resistant to acidic hydrolysis, but can be hydrolysed to the parent glycans under mildly acidic conditions [14] if they are first converted to the acetohydrazones (Fig. 3). A disadvantage of such an acidic treatment is the concomitant loss of a small proportion of the labile neuraminic acid residues [15]. We suspected that it might be possible to achieve deblocking of the hydrazones more conveniently and safely under neutral conditions, by employing ketone exchange of the hydrazine component with an excess of acetone. Accordingly, the mucin hydrazones were incubated with aqueous acetone. A change was observed in the chromatographic profile (compare Figs. 1a and 1b), but this did not correspond to conversion to the reducing glycans, and probably indicates the conversion of the hydrazones to the acetone azines (Fig. 3). When the hydrazones were *N*-acetylated to the acetohydrazones (Fig. 3) before the treatment with acetone, however, complete conversion to the reducing glycans was observed (Fig. 1c). On reduction, these were converted to the glycan alditols (Fig. 2c) which corresponded to the products of the standard reductive elimination method (Fig. 2d).

Another mild procedure for the deblocking of glycan hydrazones [5] involves a brief treatment with copper acetate, followed by removal of cations on a mixed-bed cation-exchange column. When we applied the method to the mucin hydrazones, the yield of reducing glycans was low (Fig. 1d). After conversion to the acetohydrazones, however, the yield of deblocked glycans was almost as high as with the acetone method. An *N*-acetylation step is a recognized part of the published protocol using anhydrous hydrazine [4, 5], because of the complete loss of the *N*-acyl substituents in the hydrazine. Although some of our observed losses can be attributed to the loss of *N*-acyl substituents during the elimination, it appears that prior conversion of the hydrazones to the acetohydrazones is important to the success of the deblocking using copper acetate, as well as to the acetone method. Overall, incubation in aqueous acetone is experimentally simpler and avoids the necessity for subsequent chromatographic purification.

It should be noted that the classical procedure [4] for the isolation of *N*-glycans after hydrazinolysis does not include a specific step for the deblocking of the hydrazone groups. It does, however, require an extended preparative paper chromatography fractionation step, designed to remove the amino acid hydrazides. The time spent in such an aqueous environment probably also achieves a significant

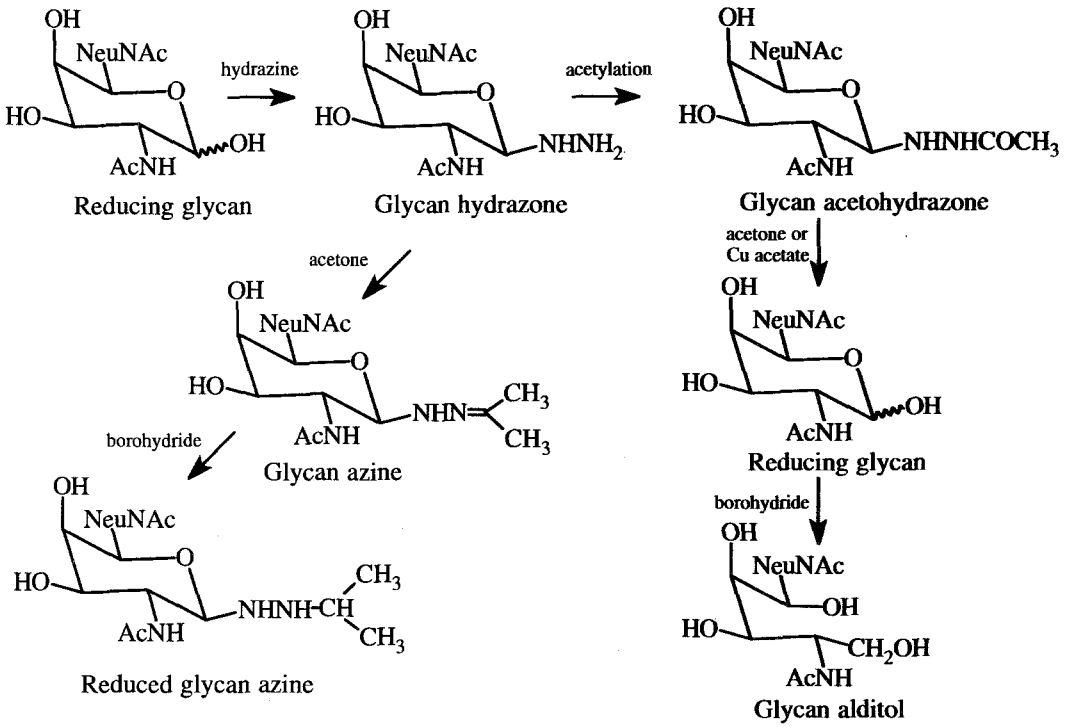

Figure 3. Hydrazone chemistry of a typical *O*-glycan from bovine submaxillary mucin.

degree of hydrolysis of the hydrazones, which would explain why the step is essential for the effective incorporation of tritium label [4].

In common with the sodium hydroxide-sodium borohydride procedure [10, 11], the glycans were isolated with the *N*-acyl groups intact. Two classes of acidic glycans were obtained: those containing *N*-acetylneuraminic acid, which eluted first using high-performance anion-exchange chromatography, and those containing *N*-glycolylneuraminic acid, which are more retained (Figs. 2c, 2d). The two classes were present in the ratio of 1.98:1, whereas the corresponding neuraminic acids in the starting glycoprotein were in the ratio of 1.48:1. A proportion of the *N*-acyl substituents had therefore been released under the elimination conditions, representing a loss of important structural information. This loss is complete when anhydrous hydrazine is used [4, 5]. Studies are continuing to establish the minimal conditions for elimination, in order to overcome this shortcoming.

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