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Communication

Application of aqueous hydrazine solution for β-elimination of O-glycans from gastric mucin

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O-Glycans from pig gastric mucin were released by β-elimination in 0.2 M triethylamine and 50% aqueous hydrazine solution. The released glycan hydrazides were isolated using Centricon 10 separators, brought to their reducing form and reductive by labelled with *p*-aminobenzoic acid ethyl ester (ABEE). Labelled products were fractionated into neutral and acid fractions on a Bio-Gel P4 column, calibrated with a mixture of dextran oligosaccharides, labelled according to the same procedure.

During isolation and fractionation of the glycans released from glycoproteins, it is necessary to use a sensitive method for their monitoring during separation by column chromatography. Chemical labelling of N-glycans to introduce a chromophore or fluorophore into the molecules, is a common procedure, as their liberation in reducing form can be simply performed by an enzymic method. There is no such procedure for O-glycans, released by reductive β-elimination in a form excluding chemical labelling by reductive amination. In our experiments, we followed the recently described method of β-elimination of O-glycans [1] in the presence of aqueous hydrazine solution, which, after hydrazide de-blocking reaction, allows to restore the released glycans in their reducing form. Glycans with reducing *N*-acetylgalactosamine were chemically la-

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Abbreviations: ABEE, *p*-aminobenzoic acid ethyl ester.

belled and their fractionation was monitored with a HPLC- UV detector of high sensitivity.

MATERIALS AND METHODS

Pig gastric mucin was isolated from pig stomachs as described previously [2]. The mucin was purified by gel exclusion chromatography on a Bio-Gel A15m column in buffered urea solution, digested with pronase, and glycopeptide was isolated on a Bio-Gel A 0.5 m column. The preparation (10 mg of glycopeptide lyophilizate for each method to be applied) was, in different conditions, subjected to β-elimination according to the hydrazine method [1] or to the classical procedure by alkaline/borohydride treatment [3, 4]. For either method, appropriate conditions were elaborated to obtain maximum oligosaccharide recovery. Two millilitres of 50% aqueous hydrazine solution containing 0.2 M triethylamine was used, and the elimination reaction was performed at 45°C for 48 h. After evaporation of volatile post-reaction components, the dry sample was dissolved in 6.4 ml of saturated $NaHCO_3$ and 0.32 ml of acetic anhydride was added. After 20 min of incubation, sodium ions were removed on Dowex 50 × 8 column (H^+ form).

The β-elimination products were centrifuged in the Centricon 10 separators (4000 r.p.m. for 2 h) and the under-membrane fluid was collected. The extent of oligosaccharide hydrazones or oligosaccharide polyols recovery was determined by the phenol-sulfuric acid method [5].

Oligosaccharide hydrazones were converted to reducing oligosaccharides with acetone (24 h at 55°C in 2.0 ml final volume of 20% acetone) and acetone was evaporated. To separate mucin oligosaccharides into neutral and acid fractions aqueous solution of oligosaccharide was applied to a DEAE-Sephadex A-25 column (10 × 1 cm) in acetate form [6] and neutral oligosaccharides were eluted with 5 column volumes of water. Acid oligosaccharides were eluted with three column volumes of pyridinium acetate buffer (pH 5.4).

For Bio-Gel P-4 column calibration, dextran 40 000 was hydrolysed in 1 M HCl at 100°C for 5 h, and HCl was removed on Dowex 1 column in acetate form.

Both pools of mucin oligosaccharides and malto-oligosaccharides from dextran hydrolysate, were submitted to reductive amination with *p*-benzoic acid ethyl ester (ABEE), according to Matsuura & Imaoka [7]. To the dried oligosaccharide samples in reacting vials, 10 μl of water was added, followed by 40 μl of freshly made labelling reagent (35 mg of ABEE, 3.5 mg $NaBH_3CN$, 350 μl of methanol and 41 μl of glacial acetic acid). Vials were tightly closed and heated at 80°C for 1 h with stirring at 5 min intervals. Then 1 ml of water was added and excess of ABEE was removed by 5 cycles of extraction with 1 ml of diethyl ether. The ABEE was removed from the aqueous phase by solid-phase extraction with the use of silica C-18 tubes (Supelco), and the UV-absorbing labelled oligosaccharides were eluted with water and 10% acetonitrile water solution [7].

The labelled products were separated on two series-connected Bio-Gel P-4 Superfine columns (1.6 × 100 cm), eluted with water at 55°C, at a flow rate of 0.2 ml per minute, and monitored with both refractometric and UV detectors.

The P-4 columns were calibrated with chemically labelled isomaltooligosaccharides mixture, giving the accuracy of a single glucose unit.

RESULTS AND DISCUSSION

The β-elimination reaction in alkaline conditions, using triethyl amine and in the presence of 50% aqueous hydrazine, yields a slightly lower recovery of the oligosaccharide pool in comparison with the classical method (Table 1). The chemical reactions involved in the release and labelling of oligosaccharides

Table 1. Comparison of recovery of oligosaccharides

Neutral carbohydrates in:	Reductive β-elimination	Hydrazine β-elimination
Starting glycopeptide	1250 μg	1250 μg
β-Elimination products	540 μg (43.2%)	690 μg (55.2%)
Oligosaccharide fraction in Centricon 10 ultrafiltrate	432 μg (34.5%)	360 μg (28.8%)

Neutral carbohydrates were determined by the phenol-sulphuric acid method [5]

are presented in Fig 1. β-Elimination in aqueous hydrazine solutions is easier to perform and more convenient, than the hydrazinolysis procedure in strictly anhydrous conditions

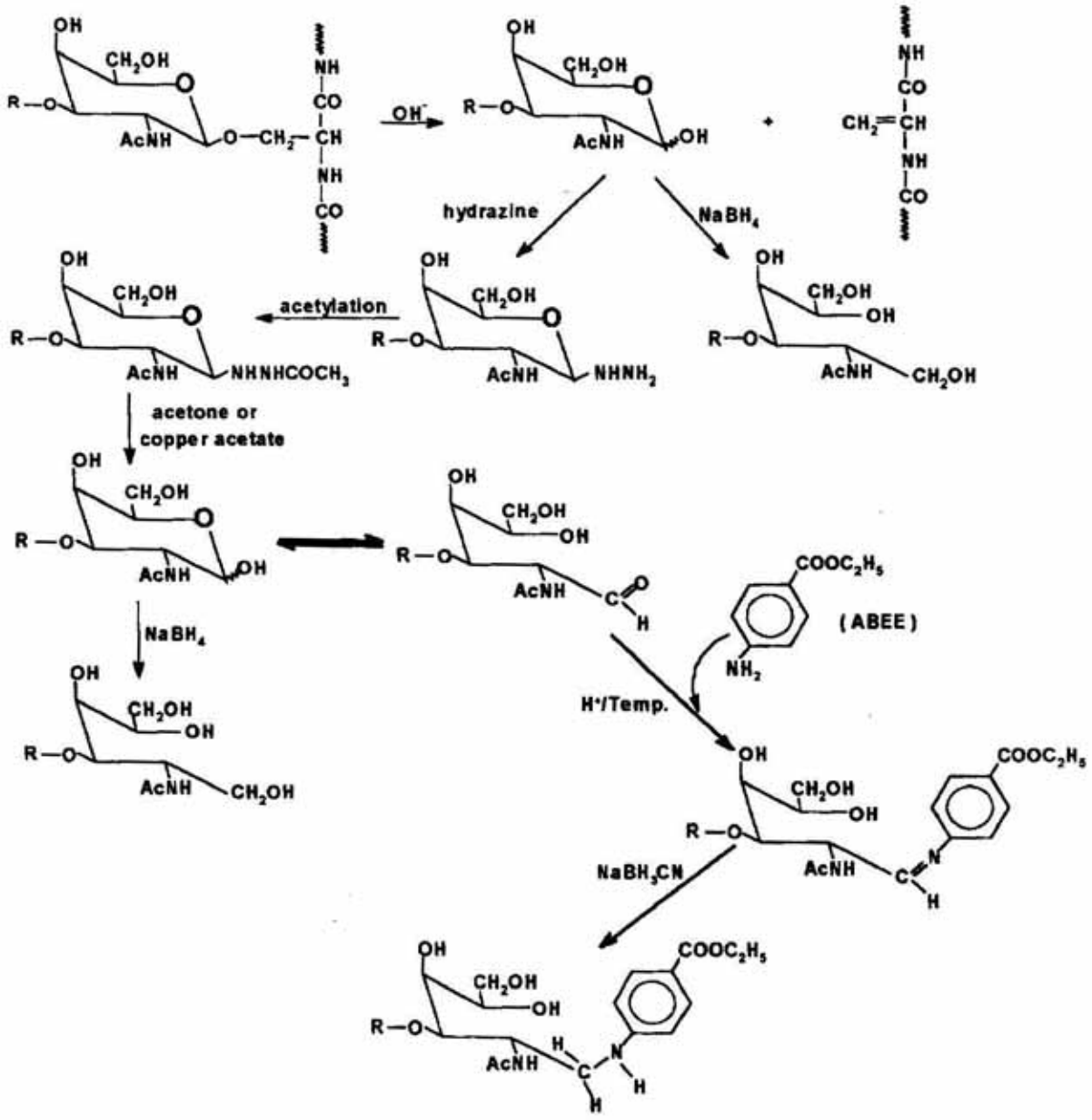

Figure 1. Reactions occurring during cleavage and labelling of oligosaccharides.

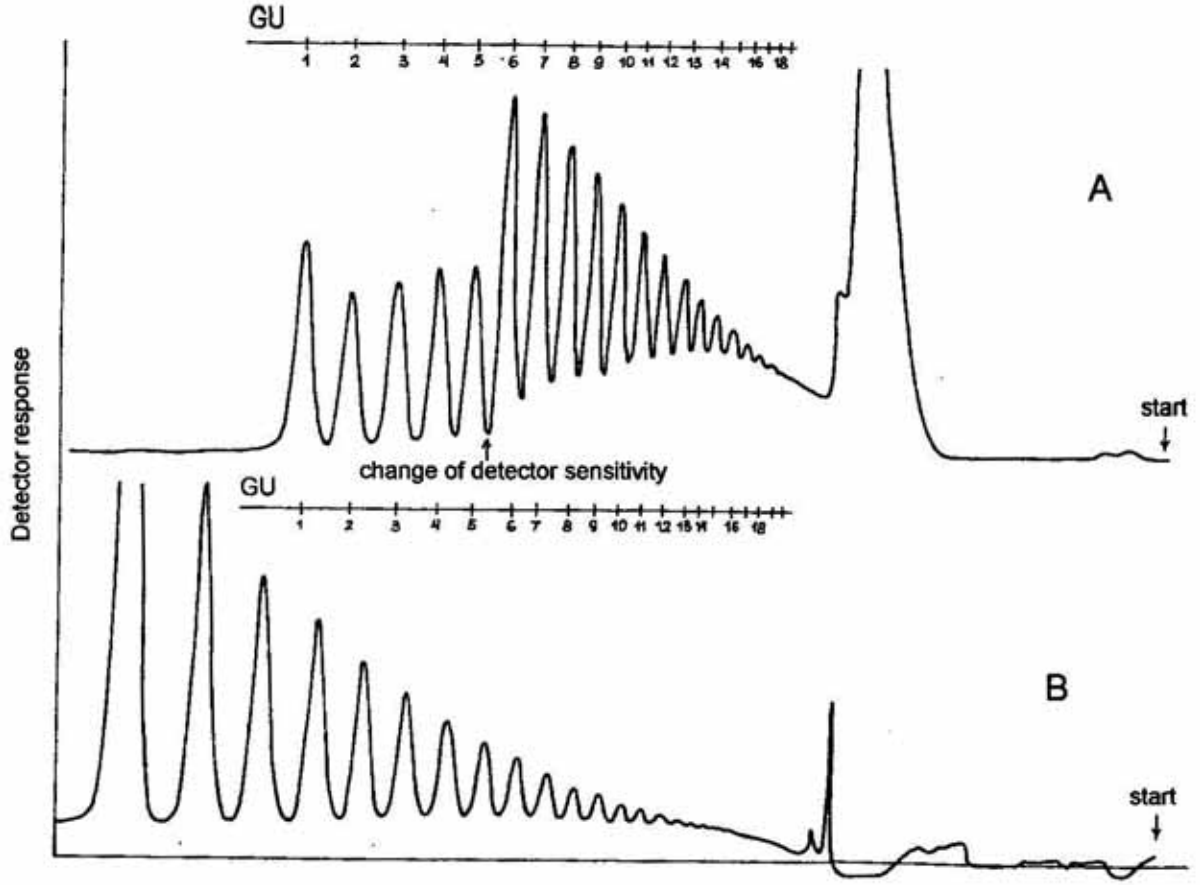

Figure 2. Separation of glucose oligomers of dextran hydrolysate on Bio-Gel P-4 Superfine.

A. Response of refractive index monitor with unlabelled oligosaccharides. B. UV-monitor response at 305 nm with labelled ABEE-oligosaccharides. GU, glucose units.

[8]. Moreover, all the reagents used can be simply removed by evaporation.

The chemical labelling of O-linked oligosaccharides is a technique rather rarely used for fractionation of glycans. The most frequently performed is the classical β-elimination reaction, with oligosaccharide reduction by a sodium borotritide/sodium borohydride mixture [9]. However, in addition an expensive radioactive reagent and a radioactivity detector must be used, for oligosaccharide monitoring.

In contrast to 2-aminopyridine labelled oligosaccharides (unpublished), ABEE labelled oligosaccharides could be effectively separated

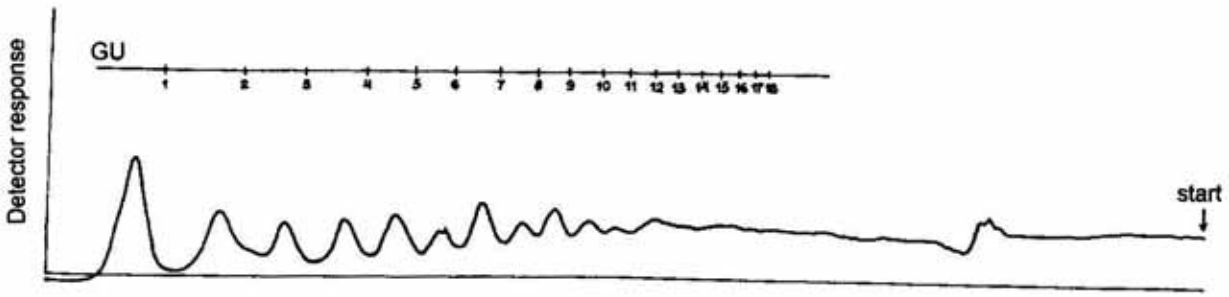

Figure 3. The separation of neutral ABEE-labelled oligosaccharides of mucin on Bio-Gel P-4 Superfine.

GU, glucose units.

on the Bio-Gel P-4 column, and isomaltooligosaccharides gave separate peaks for each additional glucose unit present (Fig. 2). In comparison to unlabelled oligosaccharides detected with a refractive index monitor, retention time of the labelled ones increased by about three and a half glucose units, and detection with the UV monitor increased the sensitivity by a factor of approx. 100. The results obtained are presented in Fig 3. We also attempted to label oligosaccharides with the 2-aminopyridine fluorescence label, but their separation on the P-4 column was unsuccessful, owing to 2-aminopyridine interaction with the column packing.

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