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COMPARISON OF ANALYTICAL TECHNIQUES FOR SEPARATION OF N-LINKED OLIGOSACCHARIDES IN A MODEL GLYCOPROTEIN (RIBONUCLEASE B)

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Abstract

Methods for separating oligosaccharides have always been limited by the detection system utilised. Carbohydrates do not naturally contain any chromophores or flourophores and therefore un-derivatised glycans cannot be sensitively detected by the usual methods of UV absorbance. Therefore high pH anion exchange chromatography with pulsed amperometric detection (HPAEC/PAD), flourophore assisted carbohydrate electrophoresis (FACE), and derivatisation of the oligosaccharides using 1-(p-methoxy)phenyl-3-methyl-5-pyralozone (PMPMP) and separation by reverse phase-high performance liquid chromatography (RP-HPLC) were studied using a model N-linked glycoprotein (ribonuclease B). Results show the most sensitive method for separating N-linked glycan from the glycoprotein is HPAEC/PAD. Separated glycans can be analysed using FACE and Electrospray mass spectrometry (ES/MS). Derivatisation of oligosaccharides with PMPMP did not adequately provide a superior method of separating the N-linked glycans.

keywords :HPAEC/PAD, FACE, PMPMP, Separation of N-linked glycans

1 Introduction

The separation and detection of complex carbohydrate mixtures by HPLC and electrophoresis is difficult owing to the polar, hydrophilic nature of this class of analytes and the fact that they do not possess an ultra violet (UV) chromophore. The problem is further complicated by the need to separate closely related structural isomers that can differ only by a single linkage position or their geometry. Therefore the following different N-linked oligosaccharides separation methods were studied. They are high pH anion exchange chromatography with pulsed amperometric detection (HPAEC/PAD), flourophore assisted carbohydrate electrophoresis (FACE) and derivatisation of the oligosaccharides with 1-(p-methoxy)phenyl-3-methyl-5-pyralozone (PMPMP) and subsequent separation by reverse phase-high performance liquid chromatography (RP-HPLC). Electrospray mass spectrometry (ES/MS) was used to confirm the results of these various techniques. Ribonuclease B was used as a model glycoprotein to test the three different methods. There are five known glycoforms of ribonuclease B ($Man_5GlcNAc_2$, $Man_6GlcNAc_2$, $Man_7GlcNAc_2$, $Man_8GlcNAc_2$, and $Man_9GlcNAc_2$). Each is different by one extra mannose residue. They have already been extensively studied and characterised therefore making the protein an ideal model.

2 Materials and Methods

2.1 High pH anion exchange chromatography with pulsed amperometric detection (HPAEC/PAD)

In this method carbohydrates were separated from a mixture of oxanions at high pH (>12) by high pH anion exchange chromatography (HPAEC) and detected by pulsed amperometry. The elution order of the oligosaccharide is dependent on their molecular size, sugar composition, linkages of monosaccharides and the acidity of the substituted hydroxyl groups. The highly basic conditions necessary for the separation of carbohydrates also enable pulsed amperometric detection (PAD).
The chromatograph system (Dionex) used consists of a gradient pump, a detector (PAD-11), and an eluent degas model (EDM). The EDM is used to sparge and pressurise the eluents. The system is controlled and data collected using Dionex AI450 software. Oligosaccharides were separated using a carbopac PA100 column (4×250 *mm*) (Dionex) equipped with a carbopac guard column (4×50 *mm*). Peptide-N4-(N-acetyl-β-D-glucosaminyl) asparagine amidase (PNGase F) digestion of ribonuclease B were analysed according to the method of Townsend *et.al.*, 1995[1].

2.2 Flourophore assisted carbohydrate electrophoresis (FACE)

2.2.1 Sample preparation

Recombinent PNGase F (50 μl of 1 mg/ml) was added to ribonuclease B (1 ml of 1 mg/ml) and left overnight to digest at 37^0C. The digest was monitored by Polyacrylamide gel electrophoresis (PAGE), and when the reaction was completed the protein was precipitated by adding 3 volumes of cold 100% ethanol and left to stand for 10 *minutes* at 0^0C. The precipitated protein was removed by centrifugation and supernatant containing the released glycans retained and supernatant was then dried by evaporation under vacuum (Speed Vac, Savant, SVC 100H).

2.2.2 Labeling and Polyacrylamide Gel Electrophoresis (PAGE) running conditions

The protocol used in this study was based on the procedure described previously (Jackson, 1994)[2][3]. Typically 4μl of 8-amino-napthalene-1,3,6 trisulphonic acid solution (ANTS) (0.15 M in 15% acetic acid) was added to 10-20 μg of oligosaccharides followed by 4 μl of cyanoborohydride solution (74 mg/ml in dimethyl sulphoxide) and the reaction mixture incubated at 37^0C overnight. After incubation the reaction mixture was dried in a speed-vac then re-suspended in an appropriate volume of loading buffer (80% glycerol, 20% water). Oligosaccharides (1-2 μg) were loaded. A 4 μl of a standard glucose ladder containing linear glucose polymers from 2-8 glucose units was used as the marker. Electrophoresis was performed at 5-8^0C for about 1.5 *hours* with running buffer (192 mM Glycine, 25 mM Tris, pH 8.5). This was achieved by immersing the electrophoresis chamber in ice. After running the gel, the bands were visualised under UV light, and the image recorded using an Alpha Innotech gel documentation system.

2.3 Separation of 1-(p-methoxy)phenyl-3-methyl-5-pyralozone (PMPMP) labeled oligosaccharides using RP-HPLC

2.3.1 Preparation of 1-(p-methoxy)phenyl-3-methyl-5-pyralozone (PMPMP)

Preparation of PMPMP was performed by modification of the method of Kakehi *et.al.*,(1991)[4]. An ethanolic solution (40 ml) of p-methoxy-phenylhydrazine hydrochloride (5.6 g, 32 mM), sodium acetate trihydrate (5.45 g, 40 mM), and ethyl acetoacetate (4.16 g, 32 mM) was refluxed for 2 *hours*. After cooling, the mixture was evaporated to dryness, and the residue dissolved in 10 ml of ethanol. The insoluble material was removed by filtration, and the filtrate was evaporated to dryness. The residue was dissolved in a small volume of 4:1(v/v) toluene/ethyl acetate and fractionated on a column of silica gel (150 g, Silica gel 60, Merck) pre-equilibrated

with the same solvent. In total, 100 fractions of 10 *ml* were collected and analysed by TLC (Silica gel 60 F 254, Merck) using the same solvent fractions 40 - 70, which showed a single component on TLC (R_f 0.41, detected by UV absorbance), were collected and evaporated to dryness. Crystallisation from methanol yielded 24.5% (w/w) of the compound with a UV λ_{max} at 249 *nm*. PMPMP was analysed by mass spectrometry and nuclear magnetic resonance (NMR).

2.3.2 Derivatisation and RP-HPLC of oligosaccharides with PMPMP

Oligosaccharide samples obtained by PNGase F digestion of glycosylated proteins were derivatised as follows: To a sample of oligosaccharide (cleaved from 1mg of protein) in a screw capped polypropylene tube 0.3 *M* NaOH (20μl) and 0.5 *M* PMPMP in methanol (20 μl) were added. The pH of the mixture was about 8.3 (as measured on universal indicator paper). The mixture was incubated for 20 *minutes* at 70^0 C to allow derivatisation to occur, and was then cooled and neutralised with 0.3 *M* HCl (20 μl). To the mixture 200 μl of water-saturated ethyl acetate and 200 μl of water were added and the mixture shaken vigorously. The organic layer was carefully removed using a syringe, and the extraction was repeated four more times. The aqueous layer was evaporated to dryness, and the residue was dissolved in 200 μl of a mixture of water and acetonitrile (85:15, v/v). 100 μl of this solution was used for HPLC analysis. A column (250 $\times$4.6*mm*) (Vydac, C_{18}) was eluted with a mixture of 100 *mM* phosphate buffer (pH 7.0) and acetonitrile (85:15, v/v) at a flow rate of 0.6 *ml/minute*. The eluent was monitored at 249 *nm*.

2.3.3 Purification of derivatised olgosaccharides

The separated derivatised oligosaccharides had to be desalted to obtained accurate ES/MS data. This was accomplished using a nylon syringe filter disk (0.45, 25 *mm* dia., Nylon Acrodisc, Gelman Science). A 5 *ml* disposable plastic syringe was cut at the 2 *ml* mark to use it as a funnel. The nozzle was primed with a mixture of acertonitrile and water (95:5 v/v) using a syringe and the pre cut syringe was attached to the filter. The filter was rinsed with 2$\times$1 *ml* mixture of acetonitrile and water (95:5 v/v) using the gravity flow method. Derivatised samples were diluted with 1 *ml* mixture of acetonitrile and water (95:5 v/v) and mixed. The diluted reaction mixture was transferred as such without any centrifugation onto the filter and allowed to flow through. The filter was washed with 2 $\times$1 *ml* mixture of acetonitrile and water (95:5 v/v) or 1-2 *ml* in sample aliquots. Both flow through and washes were discarded, and the bound oligosaccharides were eluted with 2$\times$0.5 *ml* mixture of acetonitrile and water (20:80 v/v) into a clean test tube and mixed.

3 Results

3.1 High pH anion exchange chromatography with pulsed amperometric detection (HPAEC/PAD)

Figure 1 shows the oligosaccharide map obtained for the oligosaccharides of ribonuclease B. The PNGase digest mixture contained ribonuclease B, enzyme and a mix-

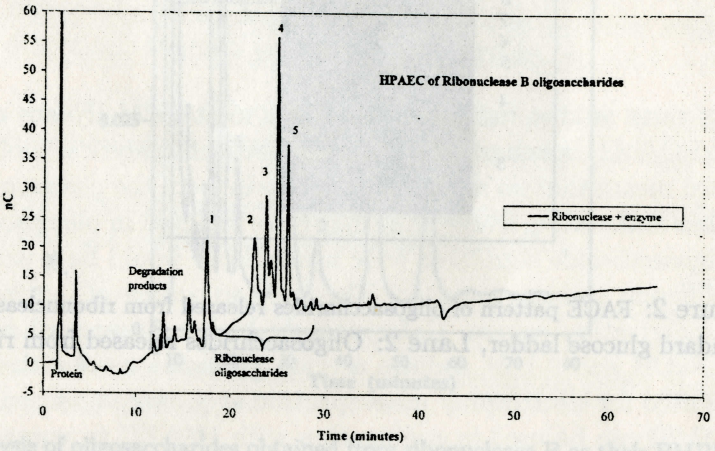

Figure 1: Oligosaccharides map obtained from the PNGase-released oligosaccharides of Ribonuclease B

ture of oligosaccharides. When this mixture is loaded (dissolved in water) onto the Carbopac PA-100 column it allows clean separation of these diverse molecules. The first to elute are the proteins (ribonuclease B and PNGase F) within the first 5 *minutes*. The oligosaccharides elute later according to the their size and the conformation. Identification of these was done by a comparison of the elution times with a previous study[4]. There are 5 main peaks. The first reasonably significant peak, which eluted at approximately 18 *minutes*, is $Man_5GlcNAc_2$ (peak 1) the last peak is $Man_9GlcNAc_2$ (peak 5). Between these peaks are $Man_6GlcNAc_2$, $Man_7GlcNAc_2$ and $Man8GlcNAc_2$ respectively. $Man_7GlcNAc_2$ eluted as two peaks, because of the different branch positions of the terminal mannose residues [4]. Other small peaks that eluted before these oligosaccharides may be due to degradation products.

3.2 Flourophore assisted carbohydrate electrophoresis (FACE)

The FACE pattern of oligosaccharide released from ribonuclease B is shown in Figure 2, lane 2 shows the size-based separation of neutral oligomannose oligosaccharides released from ribonuclease B, $Man_5GlcNAc_2$ is the lowest major band, with $Man_5GlcNAc_2$ to $Man_9GlcNAc_2$ forming the ladder.

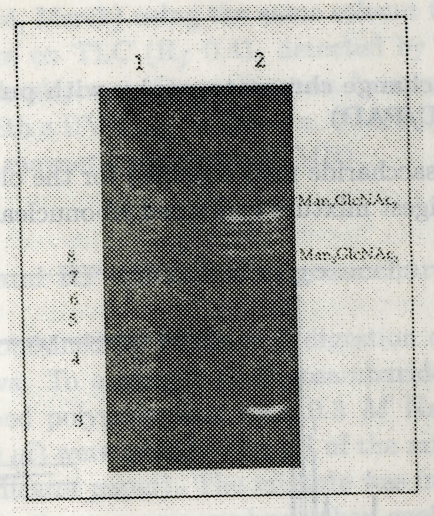

Figure 2: FACE pattern of oligosaccharides released from ribonuclease B
Lane 1: Standard glucose ladder, Lane 2: Oligosacchrides released from ribonuclease B

The band seen at the bottom of the lane is believed to be a degradation product of ribonuclease oligosaccharides. A standard ladder containing linear glucose polymers from 2-8 glucose units was run as a marker in the other lane (lane 1).

3.3 Separation of 1-(p-methoxy)phenyl-3-methyl-5-pyralozone (PMPMP) labeled oligosaccharides using RP-HPLC

3.3.1 Preparation of 1-(p-methoxy)phenyl-3-methyl-5-pyrazolone

PMPMP was prepared according to the method described in methods section. PMPMP was purified and re-crystallised from methanol. The yield was 24.5% (w/w) of the compound and gave a UV λ_{max} at 249 *nm*.

3.3.2 Derivatisation of oligosaccharides with PMPMP and RP-HPLC

The oligosaccharide mixture from ribonuclease B was derivatised with PMPMP as described in methods section. The peaks between 3-17 *minutes* are due to the reagent blank and artifacts as these were also present in a control sample.

3.3.3 ES/MS results of PMPMP derivatives of separated ribonuclease B oligosaccharides

Five distinct peaks were collected from the ribonuclease B oligosaccharides profile of the PMPMP derivatives (Figure 3).

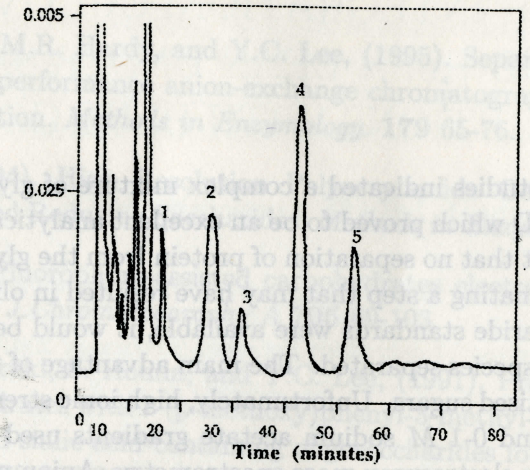

Figure 3: Analysis of oligosaccharides obtained from ribonuclease B as their PMPMP derivatives by RP-HPLC. The amount of sample injected corresponded to 1 *mg* of protein

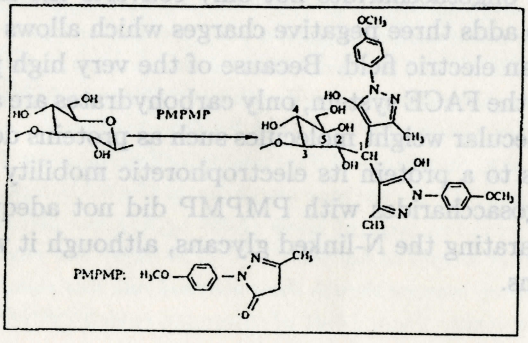

Figure 4: Derivatisation with PMPMP

Confirmation of this was achieved by the analysis of peak 4 by ES/MS. From ES/MS data, peak 4 isolated from the ribonuclease B showed an intense signal of M + H ion at m/z 1887.8 which gives evidence of the octasaccharide structure (2PMPMP + $Man_6GlcNAc_2$ + 2Na+) as two PMPMP molecules react with one molecule of

oligosaccharide as shown in Figure 4. The molecular weight of PMPMP is 204 but the ES/MS data for purified PMPMP gave an M+H ion at m/z 233. Therefore the molecular weight of PMPMP was considered as 232Da. This is probably due to additional ethyl group as suggested by NMR and ES/MS (Dr. David Officer-personal communication).

4 Discussion

The results of these studies indicated a complex mixture of glycans could be separated by HPAEC/PAD which proved to be an excellent analytical tool. The running conditions used meant that no separation of protein from the glycan component was necessary. Thus eliminating a step that may have resulted in oligosaccharide losses. If suitable oligosaccharide standards were available, it would be possible to quickly identify the different species separated. The main advantage of this method was the detection of underivatised sugars. Unfortunately, high ionic strength associated with 10-100 mM NaOH and 0-1 M sodium acetate gradients used with HPAEC/PAD are not well suited for electrospray mass spectrometry. Anion micromembrane suppression (AMMS) can be used in conjunction with HPAEC to remove Na^+ for the determination of oligosaccharides before ES/MS. However, this facility was not available at the time.

The principle of FACE separation of carbohydrates is based on size and charge. ANTS labeling of an oligosaccharides not only converts the molecule into fluorescent product but also adds three negative charges which allows a previously neutral sugars to migrate in an electric field. Because of the very high percentage (30-35%) of acrylamide used in the FACE system, only carbohydrates are able to move into the gel, whereas high molecular weight molecules such as proteins do not. Consequently, if carbohydrate binds to a protein its electrophoretic mobility should be retarded. Derivatisation of oligosaccharides with PMPMP did not adequately provide a superior method of separating the N-linked glycans, although it was successfully with ribonuclease B glycans.

5 Conclusion

The most sensitive method for separating N-linked glycan from the glycoprotein is HPAEC/PAD. Separated glycans can be analysed using FACE and ES/MS. Derivatisation of oligosaccharides with PMPMP did not adequately provide a superior method of separating the N-linked glycans.

Acknowledgement

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