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# Sensitive blotting assay for the detection of glycopeptides in peptide maps

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## ABSTRACT

A dot blotting assay using digoxigenin hydrazide (Glycan detection kit, Boehringer Mannheim Biochemicals) was used to screen an endoproteinase Lys-C peptide map of ribonuclease B for the presence of glycopeptides. The carbohydrate content of the identified glycopeptide fraction was then further characterized by monosaccharide analysis using high-pH anion-exchange chromatography with pulsed amperometric detection (HPAE–PAD). The tandem use of a hydrazide dot blotting technique to screen peptide maps for glycopeptides and subsequent use of HPAE–PAD to identify the monosaccharide composition of glycopeptide hydrolyzates proved to be a quick, sensitive and reliable method for identifying glycopeptides and analyzing their glycan composition without derivatization of the carbohydrate.

## INTRODUCTION

Electrophoretic separation of proteins and/or peptides resolved by sodium dodecyl sulfate–polyacrylamide electrophoresis (SDS-PAGE) and followed by Western blotting[1] has become a widely used technique for analysis of complex protein and/or peptide mixtures[2–4]. The antibody probes used in Western blotting to identify particular epitope(s) can be substituted with reagents which recognize proteins containing specific functional groups such as amino and sulfhydryl groups[5]. Additionally, reagents which recognize carbohydrate moieties have been used as probes in solid-phase blotting procedures to identify and characterize glycoproteins. Solid-phase blotting and detection procedures for identifying and characterizing glycoproteins immobilized on membranes have utilized two approaches.

The first approach for detection of glycoprotein carbohydrate moieties on membranes has utilized radioactively labeled, fluorescently labeled or enzyme-labeled lectin probes[6–8]. Because lectins recognize and bind to particular sugar groups on glycoproteins, this approach is selective. A second, more general approach towards solid-phase detection of carbohydrate residues of glycoproteins has been achieved by using hydrazide probes. Hydrazide probes react with oxidized sugars on immobilized glycoproteins. Thus, glycoproteins are treated with sodium periodate which reacts with the vicinal hydroxyl groups in the glycan chains forming aldehyde groups. After

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oxidation, the aldehydes generated thus can be detected with the hydrazide probe. Many generalized hydrazide procedures for detection of glycoproteins have been developed[9–14]. These procedures differ with respect to the particular hydrazide probe, oxidation conditions and visualization reagents.

Because many proteins are glycosylated at multiple sites, thorough structural characterization of glycoproteins requires localization of glycosylation sites of the glycoprotein. This has been achieved by specific chemical or enzymatic cleavage of glycoproteins into a mixture of peptides and glycopeptides that can be subsequently separated and characterized using high-performance liquid chromatographic (HPLC) methods and ultraviolet (UV) detection. Unfortunately, UV detection alone does not permit discrimination between glycopeptides and peptides. In this report, a hydrazide detection method previously used for identifying glycoproteins in Western blots[14] has been used to simultaneously screen in one step all reversed-phase chromatography peptide fractions for the identification of glycopeptides. Briefly, all fractions in a reversed-phase endoproteinase Lys-C (cleaves proteins at lysine residues) peptide map of ribonuclease B (RNase B) were subjected to periodate treatment, labeled with digoxigenin hydrazide, blotted onto a nitrocellulose membrane and simultaneously probed with an enzyme labeled anti-digoxigenin Fab fragment to quickly identify the glycopeptides in the peptide map. Glycopeptide fractions were then further characterized by monosaccharide analysis using high-pH anion-exchange chromatography with pulsed amperometric detection (HPAE–PAD)[15].

## EXPERIMENTAL

### *Materials*

RNase B (Type III-B; Lot 17F8170) from bovine pancreas was obtained from Sigma (St. Louis, MO, U.S.A.). Monosaccharides used for standards were from Sigma or Pfanstiehl (Waukeegan, IL, U.S.A.). The 50% (w/w) NaOH solution was purchased from Fisher Scientific (San Francisco, CA, U.S.A.). Endoproteinase Lys-C (Lot 11718220-17) as well as the Glycan detection kit (Lot 11907520-03) were obtained from Bochringer Mannheim Biochemicals (Indianapolis, IN, U.S.A.). Trifluoroacetic acid (TFA) was purchased from Pierce (Rockford, IL, U.S.A.). HPLC-grade acetonitrile (optima) was purchased from Fisher Scientific (Rockville, MD, U.S.A.). Nitrocellulose membranes (0.45 $\mu$m) were purchased from Schleicher and Schuell (Keene, NH, U.S.A.). All other reagents used were of the highest quality commercially available. Columns used were Selectispher Concanavalin A (10 cm × 5 cm I.D.), generously provided by Perstorp Biolytica (Lund, Sweden), Vydac 218TP54 purchased from The Separations Groups (Hesperia, CA, U.S.A.) and Dionex CarboPac PA1 (250 mm × 4 mm I.D.) (Dionex, Sunnyvale, CA, U.S.A.).

### *Reduction and carboxymethylation of ribonuclease B*

Reduction and carboxymethylation was carried out according to the procedure described by Allen[16].

### *Purification of ribonuclease B by Concanavalin A chromatography*

The commercial preparation of RNase B was found to be contaminated with significant amounts of ribonuclease A (non glycosylated). To enrich for the

glycosylated form, 10 mg of the carboxymethylated commercial preparation were chromatographed on a Selectispher Concanavalin A column. The lyophilized glycoprotein was dissolved in binding buffer: 5 m*M* sodium acetate buffer (pH 5.2) containing 0.1 *M* NaCl, 1 m*M* $CaCl_2$ and 1 m*M* $MnCl_2$. The sample was injected onto the column in binding buffer at a flow-rate of 1 ml/min. Glycosylated RNase B was recovered from the column in a single broad peak by gradient elution (0–100 m*M* methyl mannoside over 30 min in the presence of binding buffer) at a flow-rate of 1 ml/min. Bound and unbound fractions were separately pooled, exhaustively dialyzed against water and lyophilized on a SpeedVac A290 concentrator.

*SDS-PAGE*

SDS-PAGE (8–25%) followed by Coomassie blue staining was performed using the Phast System (Pharmacia, Uppsala, Sweden) to compare Concanavalin A-unbound and -bound fractions to the unfractionated RNase B.

*Endoproteinase Lys-C digestion*

Concanavalin A-purified, reduced and carboxymethylated RNase B was dissolved in digestion buffer (25 m*M* Tris–HCl, pH 8.5, 1 m*M* EDTA). Endoproteinase Lys-C was added at a substrate-to-enzyme ratio of 100:1 (w/w). The digestion was allowed to proceed at 37°C for 18 h, at which time the digestion was terminated by freezing.

*Peptide separation*

The system used for peptide separations consisted of a Dionex BioLC protein system (incorporating a gradient pump module and a variable-wavelength UV–VIS detector) and an Isco Foxy fraction collector. Eluents were sparged and pressurized with helium. Peptides from an endoproteinase Lys-C digest of 750 μg of Concanavalin A-purified, reduced and carboxymethylated RNase B were eluted from the Vydac 218TP54 reversed-phase column at a flow-rate of 1.5 ml/min with a linear gradient from 0 to 40% acetonitrile containing 0.1% TFA over 1 h. Fractions of 1.5 ml were collected every minute. Every individual fraction was subsequently lyophilized using a SpeedVac A290 concentrator and stored at −20°C until reconstituted for further analysis.

*Glycan detection blotting procedure for identification of glycopeptides*

Individual lyophilized fractions from the peptide separation were reconstituted in 100 μl of 0.1 *M* sodium acetate buffer. From each fraction 20-μl aliquots were then probed for the presence of carbohydrate. Periodate oxidation and subsequent digoxigenin-succinyl-amidocaproic acid hydrazide labeling of the fractions were carried out in solution, prior to blotting, exactly as described by the manufacturer (Boehringer-Mannheim Biochemicals). An appropriately sized piece of nitrocellulose membrane was then cut and completely submerged in a trough of water and shaken on an orbit shaker (Lab-Line Instruments, Melrose Park, IL, U.S.A.) to allow complete wetting. Every sample fraction (already treated with periodate and digoxigenin hydrazide) was diluted to a volume of 500 μl with 10 m*M* sodium phosphate buffer, pH 8.0. The wetted nitrocellulose membrane was securely clamped between the filter support and the O-rings of the incubation chambers of a reusable microfold blotting

apparatus (V & P Scientific, San Diego, CA, U.S.A.). Diluted fractions were loaded into the top incubation chambers. Vacuum was slowly applied to trap the (glyco)-peptides onto the nitrocellulose membrane. After all the liquid had been drawn through the membrane, the membrane was removed and placed in a trough containing 10 m*M* phosphate buffer, pH 8.0 for 3 min. After 3 min, the membrane was incubated in blocking solution (Glycan detection kit) and gently shaken on an orbit shaker for 30 min. The membrane was then repeatedly washed with Tris-buffered saline, pH 6.5 (3 × 10 min) while shaking. Incubation of the membrane with the alkaline phosphatase-conjugated sheep anti-digoxigenin Fab and the staining reaction were carried out according to the manufacturer's protocol.

*Hydrolysis of (glyco)peptide fractions for monosaccharide analysis*

Acid hydrolysis of samples to be analyzed by HPAE–PAD was carried out in screw-cap sealed 1.5-ml polypropylene microfuge tubes as follows. To 20-μl aliquots of each reconstituted reversed-phase HPLC fraction were added 20 μl of 4 *M* TFA. Sample volume was brought up to 100 μl with 2 *M* TFA. The samples were hydrolyzed in a heating block for 6h at 100°C. The tubes were cooled and the acid was removed by lyophilization in a SpeedVac A290 concentrator.

*HPAE–PAD monosaccharide analysis*

The system used for the HPAE–PAD monosaccharide analysis consisted of a Dionex BioLC carbohydrate system (incorporating a gradient pump module and Model PAD-II detector). The Dionex eluent degas module was employed to sparge and pressurize the eluents with helium. In these experiments, eluent 1 was 200 m*M* NaOH and eluent 2 was water. These eluents were prepared by suitable dilution of 50% NaOH solution with high-purity water. Sample injection was via a Rheodyne 7125 valve equipped with a 50-μl sample loop and a Tefzel rotor seal to withstand the alkalinity of the eluents.

Monosaccharides [monosaccharide control samples as well as (glyco)peptide hydrolyzates] were separated on a column (250 mm × 4.6 mm I.D.) of Dionex CarboPac PA1 pellicular anion-exchange resin, equipped with an CarboPac guard column, using a flow-rate of 1 ml/min at ambient temperature. The analysis of monosaccharides[15] was carried out at an isocratic NaOH concentration of ca. 16 m*M* (*i.e.*, 8% eluent 1 and 92% eluent 2) for 17 min. A 5-min column wash with 200 m*M* NaOH (100% eluent 1) followed by a 10-min equilibration with the starting eluent was required to yield highly reproducible retention times for the monosaccharides with a *ca.* 45-min run time.

Detection of the separated monosaccharides was by PAD using a gold working electrode[13]. In order to minimize baseline distortion around the amino sugar peaks, 500 m*M* NaOH was added to the post-column effluent via a mixing tee at a flow-rate of 0.7 ml/min using the Dionex postcolumn reagent pump. The following pulse potentials and durations were used for monosaccharide analysis: $E_1 = 0.05$ V ($t_1 = 420$ ms); $E_2 = 0.60$ V ($t_2 = 120$ ms); $E_3 = -0.6$ V ($t_3 = 60$ ms). The response time of the PAD 2 was set to 3 s.

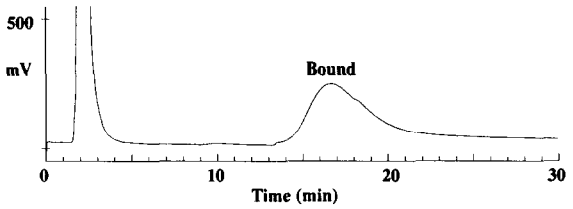


Fig. 1. Concanavalin A purification of ribonuclesase B. S-Carboxymethylated ribonuclease B (10 mg) was injected at 0.0 min. Column, high performance SelectiSpher Concanavalin A, 10 cm × 4.6 mm I.D. (Perstorp Biolytica) From 0 to 7 min, isocratic wash with binding buffer (see Experimental), followed by a 30-min linear gradient from 0 to 100 m*M* methyl mannoside at a flow-rate of 1 ml/min.

## RESULTS AND DISCUSSION

The established technique of peptide mapping by reversed-phase HPLC was used to separate and isolate (glyco)peptides derived from an endoproteinase Lys-C digestion of purified bovine RNase B. Tandem use of a hydrazide dot blotting technique to screen the peptide map for glycopeptides and subsequent use of HPAE–PAD to identify the monosaccharide composition of glycopeptide hydrolyzates proved to be a quick, sensitive and reliable method for identifying glycopeptides and analyzing their glycan composition without derivitization of the carbohydrate.

Concanavalin A chromatography of reduced and carboxymethylated RNase B (Fig. 1) resulted in the identification of an unbound fraction and a bound fraction that could be eluted with methyl mannoside. Protein assay (data not shown) revealed that 30% of the applied RNase B was recovered in the bound fraction. HPAE–PAD analysis of the carbohydrate composition of the bound glycoprotein revealed a six-fold enrichment in mannose when compared to the starting glycoprotein (data not shown). SDS-PAGE and subsequent Coomassie blue staining (Fig. 2) of the commercial

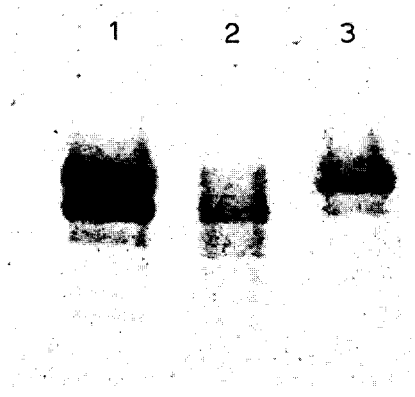


Fig. 2. Monitoring Concanavalin A purification of S-carboxymethylated ribonuclease B by 8–25% SDS-PAGE and Coomassie blue staining (Phast System, Pharmacia). Lanes: 1 = S-carboxymethylated ribonuclease B (note lower-molecular-weight band which is ribonuclease A "contamination"); 2 = pooled "unbound" fraction from Concanavalin A fractionation of S-carboxymethylated ribonuclease B; 3 = pooled "bound" fraction from Concanavalin A fractionation of S-carboxymethylated ribonuclease B.

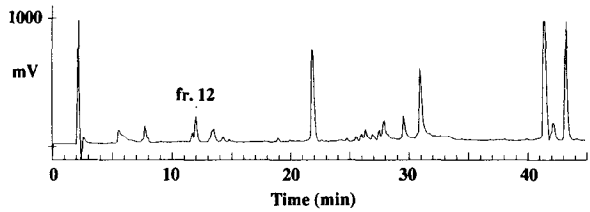


Fig. 3. Endoproteinase Lys-C reversed-phase HPLC map of Concanavalin A-purified, S-carboxymethylated ribonuclease B. A 750-μg amount of 18-h endoproteinase Lys-C digest of Concanavalin A-purified S-carboxymethylated ribonuclease B was loaded onto a Vydac 218TP54 (25 cm × 4.6 mm I.D.) column. Shown is the resultant peptide separation from a 60-min gradient of 0–40% acetonitrile in the presence of 0.1% TFA at a flow-rate of 1.5ml/min.

preparation, the Concanavalin A-bound fraction and the Concanavalin A-unbound fraction revealed a clear separation of the higher-molecular-weight glycosylated form (bound fraction) from the lower-molecular-weight RNase A contaminant (unbound fraction). Similar results have been reported by Bernard *et al.*[17].

Reversed-phase HPLC peptide mapping of reduced and carboxymethylated preparations of bovine RNase A has been reported by McWherter *et al.*[18] for cyanogen bromide, *Staphylococcus aureus* protease, tryptic and tryptic followed by chymotryptic digestions. Endoproteinase Lys-C cleavage of RNase B (cleaves specifically at lysine residues) reported here resulted in a peptide map that showed eleven major peaks with several minor peaks (Fig. 3). The theoretical sequence of the glycopeptide, based on cleavages at lysine residues, results in a predicted peptide-sequence containing six amino acid residues: Ser-Arg-Asn-Leu-Thr-Lys. Protein sequencing of the fraction 12 glycopeptide to confirm the predicted sequence remains to be determined.

Every collected fraction was evaluated for the presence of carbohydrate by performing hydrazide blotting as well as by performing HPAE–PAD analysis of hydrolyzates of each fraction. Glycan detection screening of all fractions from the endoproteinase Lys-C map revealed positive staining of a putative glycopeptide in fraction 12 (Fig. 4). The identification of a single glycopeptide fraction was consistent with the known structure of RNase B, which contains a single glycosylation site at asparagine in position 34 of its amino acid sequence[19,20]. This single glycosylation site is known to display microheterogeneity with respect to the attached oligosaccharide

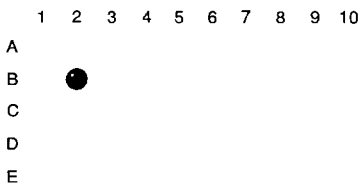


Fig. 4. Digoxigenin hydrazide blotting of periodate-oxidized reversed-phase HPLC fractions to identify glycopeptide fraction(s). Fractions treated with digoxigenin hydrazide were subsequently blotted onto a nitrocellulose membrane, probed with an alkaline phosphatase-conjugated anti-digoxigenin antibody and stained according to the manufacturer's protocol [Boehringer Mannheim Biochemicals). The only fraction found to stain positively was fraction 12 (B2); see also "fr. 12" glycopeptide peak in Fig. 3].

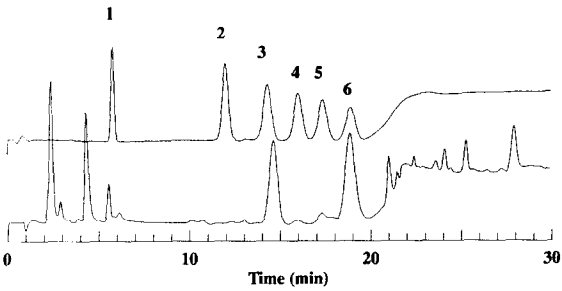


Fig. 5. High-sensitivity analysis of fraction 12 glycopeptide hydrolyzate. An aliquot of fraction 12 glycopeptide was hydrolyzed for 6 h at 100°C in 2 *M* TFA. The TFA was removed by lyophilization and the hydrolyzate was redissolved in 200 μl of water. Analysis of 150 μl of the fraction 12 hydrolyzate is shown in the lower tracing. The upper tracing is a chromatogram of 500 pmol monosaccharide standards. Peaks: 1 = fucose; 2 = galactosamine; 3 = glucosamine; 4 = galactose; 5 = glucose; 6 = mannose. Conditions for chromatography are as described under Experimental.

chains[21,22]. To verify the presence of carbohydrate in the single, positively stained peptide map fraction and the absence of carbohydrate in all other fractions, all fractions were evaluated by HPAE–PAD monosaccharide composition analysis for the presence of carbohydrate. Fraction 12 was the only fraction found by this technique to contain monosaccharides (Fig. 5). Additionally, the identification of mannose and glucosamine were consistent with the known carbohydrate monosaccharide composition of RNase B[21,22].

Other techniques for identifying attachment sites of Asn-linked sugars in glycoproteins using fast atom bombardment mass spectrometry have been described[23]. The present glycopeptide screening technique does not require expensive instrumentation. Possible problems with solid-phase hydrazide probe screening of HPLC peptide maps include the possibility that all peptides may not bind well to nitrocellulose membranes. While the quantitative evaluation of glycopeptide binding to nitrocellulose membranes remains to be evaluated; in the present report, we can state that the sensitivity of the technique minimally detected glycopeptides containing 1-nmol quantities of mannose (HPAE–PAD quantitation of mannose in an identical fraction 12 aliquot to the aliquot subjected to the hydrazide blotting procedure; data not shown).

Additionally, variations of the length and temperature of oxidation as well as the concentration of sodium periodate may affect the specificity of the hydrazide-labeling reactions the degree of labeling and ultimately the sensitivity of the procedure. None of these parameters have been examined with the present glycopeptide screening technique. Finally, complications may occur if behavior of glycopeptides in reversed-phase chromatography is influenced by carbohydrate microheterogeneity at single Asn glycosylation sites. According to Takahashi *et al.*[24], behavior of glycopeptides in reversed-phase chromatography is determined mainly by amino acid composition. Because it is known that RNase B displays microheterogeneity with respect to the oligosaccharides which are attached at asparagine residue 34 (refs. 21 and 22), future experiments will focus upon using HPAE–PAD to map these different oligosaccharides.

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