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Analysis of serine/threonine-linked oligosaccharides derived by alkaline-borohydride treatment of mucin glycoproteins electroblotted onto membranes: comparison of the saccharide profiles of the 390 kDa and 350 kDa forms of epitectin

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Alkaline borohydride treatment is widely used for the release of carbohydrate moieties from O-glycosylated glycoproteins and mucins. We have adapted this procedure to micro quantities of glycoproteins blotted on membranes. After electrophoresis and transfer to nitrocellulose, nylon or polyvinylidene difluoride membrane, alkaline borohydride treatment was done directly on glycoprotein containing areas of membrane which were cut out with the aid of guide strips stained with Coomassie Blue or lectin-digoxigenin. In combination with standard saccharide fractionation techniques, this procedure can be used to characterize the oligosaccharides of mucins or mucin-type glycoproteins that are separated by gel electrophoresis from crude sources. Using this approach we have characterized the saccharides derived from the two species of epitectin, a malignancy-associated mucin type glycoprotein, isolated from metabolically labelled H.Ep2 cells.

Keywords: alkaline borohydride treatment, electroblotting, O-linked saccharides, mucin glycoproteins

Introduction

Glycosylation of the hydroxyl groups of serine and threonine (O-glycosylation) is a wide-spread modification of proteins with important biological functions [1]. Many of these glycoproteins exist in multiple glycoforms some of which are present only in minute quantities. Hence, the conventional methods of purification of the individual glycoforms followed by the release and structural analysis of the saccharides are complicated and not very productive. This is particularly relevant for the malignancy-associated mucin glycoproteins which are plasma membrane components of cancer cells [2, 3]. Thus, a procedure for examining the O-linked saccharides of minute quantities of individual glycoproteins would be extremely useful for structure-function studies.

Sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) is a powerful technique for the separations of proteins and glycoproteins. Therefore, gel electrophoresis followed by electrotransfer to membranes is a widely used approach for characterizing picomole quantities of (glyco)proteins. Antibodies and lectin probes are useful for the detection and immunological identification of the electrotransferred material. Methods have been developed for obtaining amino acid composition [4], amino acid sequence [5] and monosaccharide composition [6] of (glyco)proteins transferred to various membranes. The release of asparagine-linked oligosaccharides by gas-phase hydrazinolysis of asialo α_1-acid glycoprotein transferred to Immobilon membrane has been reported by Kawashima *et al.* [7]. A recent report described the use of endoglycosidases to release and analyse the asparagine-linked oligosaccharides from electroblotted recombinant glycoproteins tissue plasminogen activator and erythropoietin [8].

This report describes the electrotransfer of mucin glycoproteins to blots, the release of O-linked saccharides of the membrane bound glycoprotein by reductive beta-elimination and the analysis of the released

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products by gel filtration. This method was applied to the analysis of O-linked oligosaccharides from fetuin, glycophorin and epitectin [9, 10], a malignancy-associated mucin glycoprotein. A preliminary abstract of this work has been published [11].

Materials and methods

Fetuin, glycophorin A, and sodium borohydride were purchased from Sigma Chemical Co. (St Louis, MO, USA), Polyvinylidene di fluoride (PVDF) transfer membrane (trade name Immobilon) of pore size 0.45 μm was from Millipore (Bedford, MA, USA). Nytron nylon membrane S was from Schleicher & Schuell (Keene, NH, USA). Nitrocellulose (Hybond™ extra) of pore size 0.45 μm was from Amersham (Arlington Heights, IL, USA). High and low molecular weight protein standards, acrylamide, *N*, *N'*-methylene-bisacrylamide, ammonium persulfate, *N*, *N*, *N'*, *N'*,-tetramethylethylenediamine of electrophoretic grade and Bio-Gel P2 were from Bio Rad Laboratories (Richmond, CA, USA). Peanut agglutinin (PNA) and wheat germ agglutinin (WGA) conjugated with digoxigenin and anti-digoxigenin conjugated alkaline phosphatase were from Boehringer Mannheim Co. (Indianapolis, IN, USA). The tetrasaccharide, NeuNAc → Gal → (NeuNAc) → GalNAcOH, the trisaccharide NeuNAc → Gal → GalNAcOH and the disaccharide, Gal → GalNAcOH, were isolated from fetuin and glycophorin by treatment with alkaline sodium borohydride as described [12]. The sialic acid residues of fetuin and glycophorin were modified to the seven- and eight-carbon analogues and labelled with tritium as described by Van Lenten and Ashwell [13]. Eagle's minimum essential medium was from Flow Laboratories Ltd (McLean, VA, USA). Fetal calf serum, glutamine, sodium pyruvate and non-essential amino acids were from Gibco BRL (Grand Island, New York, USA). D-[6-^{3}H] Glucosamine hydrochloride (26.8 Ci mmol^{-1}) was purchased from ARC (St Louis, MO, USA).

Cell culture

The human laryngeal carcinoma (H.Ep 2) cells were maintained as monolayer cultures in minimum essential medium supplemented with glutamine, sodium pyruvate, non-essential amino acids and 10% (v/v) heat inactivated fetal calf serum as described [14]. After reaching confluency, the cells were harvested by treatment with versene, followed by centrifugation at 1000 × g for 15 min and washed with phosphate buffered saline (PBS). Metabolic labelling of the glycoproteins was carried out by growing the cells in 5 ml of medium containing [6-^{3}H]-glucosamine (10 μCi ml^{-1}) for 48 h prior to harvesting.

Purification of epitectin from H.Ep 2 cells

The extraction and purification of the radio-labelled epitectin from H.Ep 2 cells was carried out as described previously [10]. Briefly, [^{3}H]glucosamine labelled or unlabelled cells were suspended in 10 ml of 10 mM Tris-HCl, pH 8.0, 0.2% sodium deoxycholate, 0.02% sodium azide, 1 mM PMSF, and 5 mM EDTA. The cells were broken by forcing the suspension through a 19 gauge syringe needle several times. The mixture was stirred at 4 °C for 1 h, the extract centrifuged at 12 000 × **g** for 30 min and the supernatant placed in boiling water bath for 10 min. The solution was cooled and centrifuged again to remove insoluble proteins. The supernatant was applied to a column (1 × 5 cm) of PNA-Sepharose 4B equilibrated with PBS containing 0.1% CHAPS (cholamidopropyl dimethyl ammonio-1-propane sulfonte). The column was washed with at least 10 bed volumes of the same buffer and the bound glycoproteins eluted with 4% galactose in PBS containing 0.1% CHAPS. This eluate was exhaustively dialysed against distilled water at 4 °C and lyophilized. The dry material was reconstituted by dissolving in 1 ml of 10 mM Tris-HCl, pH 8.0, containing 0.1% sodium deoxycholate and chromatographed on a Sepharose CL 6B column (1 × 50 cm) using the same buffer. Fractions of 1.0 ml were collected and analysed for radioactivity and for epitectin by enzyme-linked immunosorbant assay (ELISA). Pure epitectin was recovered from the pooled fractions by dialysis followed by lyophilization.

SDS-PAGE and electroblotting

Fetuin and glycoprotein, unlabelled or [^{3}H]-labelled, were electrophoresed on a 10% polyacrylamide mini gel. [^{3}H]glucosamine-labelled epitectin was subjected to electrophoresis according to Laemmli [15] on a 3.5–12% gradient gel without stacking gel. Electroblotting was performed by the method of Towbin *et al.* [16] using PVDF membrane and Tris-glycine buffer (23 mM and 192 mM, respectively, pH 8.9 and 10% methanol) for low molecular weight glycoproteins such as fetuin. Electrotransfer of high molecular weight and highly glycosylated mucin glycoproteins, such as glycophorin and epitectin was done using nitrocellulose or nylon membrane and 10 mM Tris-HCl buffer, pH 8.9. The membranes were soaked in transfer buffer; in the case of PVDF membrane after wetting with 100% methanol. The gel was sandwiched between two sheets of membranes followed by several layers of Whatman chromatography papers and subjected to electrotransfer at 120 mA for 18 h using the Transblot cell system (Bio Rad Laboratories, Richmond, CA). The protein bands on PVDF and nitrocellulose were detected with 0.1% Coomassie Blue R205 in 50% methanol or with 0.1% amido black in methanol: acetic acid: water (25:7:67). The glycoproteins on membranes were detected with lectins such as wheat germ agglutinin(WGA)-conjugated with digoxigenin according to manufacturers instructions (Boehringer Mannheim Co.)

Immunoblotting

After transfer, the membrane was cut out into several strips corresponding to the number of protein samples loaded for electrophoresis. One strip containing the sample was used for visualizing the glycoprotein of interest by immunoblotting and a duplicate strip was used for treatment of alkaline borohydride. Other strips were stained with either Coomassie blue or Amido black to detect all the protein bands in the sample. The strips for immunoblotting were washed with PBS, blocked with 3% bovine serum albumin in PBS for 1 h and finally incubated with WGA- or PNA-digoxigenin (10 μg ml^{-1}) for 2 h. The strips were washed three times with PBS containing 0.1% Triton X100 (PBS-T) and then incubated with rabbit anti-digoxigenin IgG, Fab for 1 h. The strips were washed three times with PBS-T and developed by addition of NBT and BCIP reagents.

Alkaline borohydride treatment

The glycoprotein band(s) on the unstained strip was located using as a guide the strip stained with lectin-digoxigenin. The area of the membrane containing the glycoprotein of interest was excised, cut into small ($\sim 5 \times 5$ mm) pieces and placed in an ampoule. A 1.0 M solution of $NaBH_4$ in 0.05 M NaOH (200 ml) was added, the ampoule sealed under N_2 and incubated at 37 °C for 72 h [12]. The mixture was cooled in an ice bath, neutralized by dropwise addition of 4 M acetic acid and evaporated to dryness under vacuum (Speed-Vac). The residue was repeatedly treated with methanol to remove volatile methyl borate, dissolved in 1 ml water and aliquots analysed for radioactivity.

Gel permeation chromatography

Gel permeation chromatography of oligosaccharides was carried out on columns of Bio gel P2 (200–400 mesh) eluted with 0.1 M pyridine acetate, pH 5.0. Reference ^{3}H-labelled tetra- tri- and di- oligosaccharides, [^{14}C] *N*-acetylneuraminic acid and [^{14}C] glucose were used to calibrate the column.

Results and discussion

SDS-PAGE and electrotransfer

Initial experiments were performed to compare the recovery of O-linked oligosaccharides from [^{3}H] sialic acid labelled fetuin and glycophorin after SDS-PAGE and electrotransfer to various membranes followed by β-elimination. The test glycoprotein (6000–10000 cpm) was subjected to SDS-PAGE, electroblotted onto membranes and the position of the glycoprotein located with the aid of stained guide strips. The appropriate portion of the membrane was cut out, subjected to β-elimination and radioactivity in the supernatant determined. Typically, about 23% and 26% of the radioactivity in [^{3}H]-labelled fetuin applied on the gel could be recovered in the β-eliminated saccharides from PVDF and nitrocellulose membranes, respectively. Since about 74% of the radioactivity in our preparation of [^{3}H]-sialyl labelled fetuin is in N-linked saccharides this corresponds close to quantitative yields of the O-linked saccharides. In the case of [^{3}H] sialic acid labelled glycophorin the corresponding recovery of saccharides from PVDF membrane was only 8%. In contrast, the yield of the released O-linked saccharides was in the 30–32% range when glycophorin was transferred to nitrocellulose or nylon membranes. When the tritium-labelled glycophorin was applied directly to membrane pieces, allowed to dry and the pieces subjected to alkaline borohydride treatment the radioactivity recovered in the supernatant was about 82%. This suggests that the low recovery is due to inefficient electrotransfer rather than to incomplete β-elimination. In previous studies, we have noted that the efficiency of the transfer of glycophorin and other mucin glycoproteins using methanol containing Tris-glycine transfer buffers according to Towbin *et al.* [16] was extremely low but was better in Tris-HCl buffers [unpublished results]. Hence in this study we used Tris-HCl buffers without methanol for transfer of mucin glycoproteins. The reason for the poor electrotransfer of glycophorin compared to fetuin cannot be readily explained but could be due to a combination of factors. These glycoproteins differ in the quantity and nature of saccharides. Glycophorin has a total carbohydrate content of about 55%, the major portion of which is O-linked (15 O-linked and one N-linked) [17] in contrast fetuin has a lower carbohydrate content of about 22% with only three O-linked and an equal number of N-linked saccharides [18]. It is possible that the higher carbohydrate content results in poorer transfer and binding to membranes, for example less SDS will be associated with highly glycosylated glycoproteins. In fact, glycophorin is known to behave anomalously in SDS-PAGE [19]. For unknown reasons the binding and retention of glycophorin appears to be somewhat better on nitrocellulose and nylon membranes compared to PVDF membrane. This is not due to differences in the pore sizes of the membranes since the nitrocellulose and PVDF membranes were of the same (0.45 μm) pore size. It should be noted that the yield of asparagine-linked oligosaccharides released by hydrazinolysis of asialo α_1-acid glycoprotein transferred to membrane was only 14–16% [7].

When the electrotransfer of epitectin, a malignancy-associated mucin glycoprotein, was tested by examining blots with the lectin-dioxigenin system, we found that the efficiency of these glycoproteins was also low–similar to that of glycophorin. Based on lectin-dioxigenin staining nylon appeared to be better than PVDF and nitrocellulose membranes for the transfer of epitectin. This is not surprising in view of the higher molecular weight of epitectin, compared to glycophorin, in addition to its high carbohydrate content of 50% comparable to glycophorin ([10] Zhu and Bhavanandan, unpublished results). We have noted that the efficiency of electrotransfer of epithelial mucins such as human and monkey tracheobronchial mucins and bovine submaxillary mucin from acrylamide-agarose composite gels are also extremely poor (Bhavanandan *et al.*, unpublished results). It is not surprising that high molecular weight, highly glycosylated proteins do not efficiently transfer since these molecules probably do not migrate out of the gel readily and in addition may not bind strongly to the 'hydrophobic' membranes. However, in all cases sufficient transfer can be obtained in order to obtain information on the profile of the O-linked saccharides. It should be pointed out that other investigators have reported difficulties in the transfer to membranes of high molecular weight, albeit non-glycosylated, proteins for sequencing studies [5, 20].

Examination of saccharides released from electroblotted glycoproteins

Glycophorin (50 μg) was subjected to SDS-PAGE, electroblotted to nylon membrane and the appropriate portion of membrane treated with alkaline borohydride. The released oligosaccharides were recovered after gel filtration on a calibrated Bio Gel P-2 column and the monosaccharide composition determined by gas chromatography after methanolysis, re-*N*-acetylation and trimethylsilylation [21]. The ratio of GalNAc(OH): GlcNAc: Gal: Sialic acid was 1.00:0.16:1.17:1.41 compared to 1.00:0.20:1.04:1.55 obtained when a sample of the same glycophorin was directly treated with alkaline borohydride and analyzed similarly. The elution profile on a column of Bio Gel P-2 of the radioactive saccharides released from [^{3}H]-labelled glycophorin electroblotted on nitrocellulose is shown in Fig. 1A. The profile is very similar to that of the same glycophorin preparation subjected directly, that is in solution, to beta elimination (Fig. 1B). The slight differences could at least partly, be attributed to the presence of minor contaminant glycoproteins in glycophorin preparation which would be absent in an electrophoretically purified blotted sample. In other words, the profile in Fig. 1A could be considered the true oligosaccharide profile of highly purified glycophorin A. Overall, these profiles are similar to that reported by Fukuda *et al.* [22]. Based on comparison of the elution position to reference saccharides and to the results of Fukuda *et al.* we can tentatively identify the peak as follows: peak I is a mixture of the penta saccharides [NeuNAc → NeuNAc → Gal (NeuNAc →) GalNAcOH and NeuNAc → Gal (NeuNAc → NeuNAc) → GalNAcOH]; peak II is the tetrasaccharide [NeuNAc → Gal(NeuNAc) → GalNAcOH] and peak III, the trisaccharide [NeuNAc → Gal → GalNAcOH]. The distribution of radioactivity in the penta-, tetra- and trisaccharides derived from electroblotted [^{3}H]-labelled glycophorin is 13%, 62% and 24%, respectively compared to the mass distribution of 5%, 78% and 17% for the same peaks reported by Fukuda *et al.* [22]. The elution positions of peaks IV and V coincide with that of the disaccharide [Gal → GalNAcOH] and the monosaccharide [GalNAcOH], respectively.

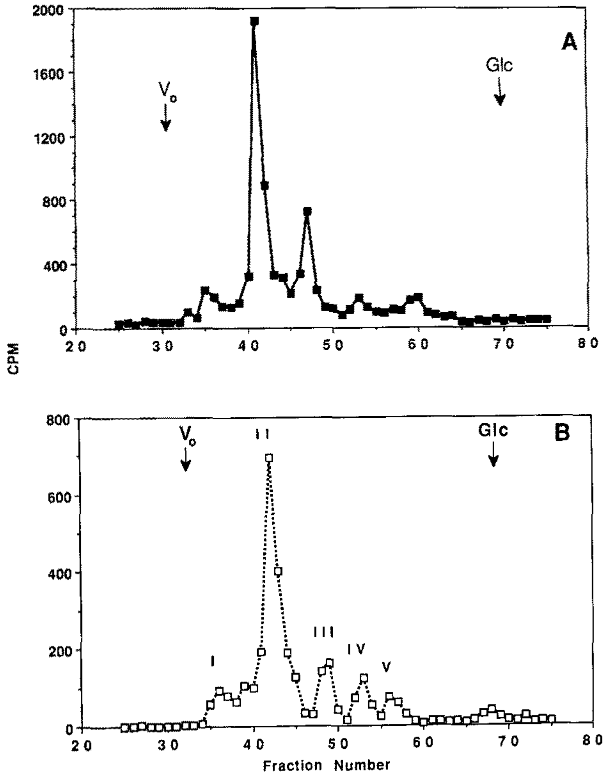

Figure 1. Gel filtration on Bio Gel P2 of oligosaccharides derived by alkaline borohydride treatment of [NeuNAc-^{3}H] glycophorin. A, electroeluted onto nitrocellulose membrane as described in the text and B, in solution. The column (1 × 110 cm) was eluted with 0.1 N pyridine acetate, pH 5.0, 1 ml fractions were collected and analysed for radioactivity. The positions of the void volume (V$_0$) and [^{14}C] glucose are indicated by arrows.

Examination of saccharides released from electroblotted [^{3}H] epitectin

We tested the utility of the procedure for analysing mucin glycoproteins in crude cell extracts. Extracts of unlabelled and [^{3}H]glucosamine labelled H.Ep 2 cells were subjected to SDS-PAGE on 3.5–12.0% gradient gels and the separated protein/glycoprotein components electroblotted onto nylon membrane using a Tris-HCl buffer, pH 8.9. Strips corresponding to the unlabelled cell extracts were cut out and treated with WGA-dioxigenin to visualize the sialo glycoprotein bands. The stained strips were used as guides to cut out the epitectin bands from the strip containing the [^{3}H]-labelled glycoprotein. The elution profiles on Bio Gel P-2 of the [^{3}H]-labelled oligosaccharides released from the membrane segments containing the labelled epitectin and a purified sample of [^{3}H]epitectin are illustrated in Fig. 2. The similarity of the profiles proves the applicability of the technique for the structural analysis of electrophoretically separated mucin glycoproteins.

Epitectin isolated from H.Ep 2 cells contains two distinct species of apparent molecular weights 390 kDa and 350 kDa [9, 10]. It is of interest to determine whether the two species differ in their oligosaccharide profiles. However, previous attempts to separate the two species by conventional separation techniques such as gel permeation chromatography in various matrices and buffer systems including those containing SDS, FPLC, lectin affinity chromatography, ion exchange chromatography and isoelectric focusing have been unsuccessful (Bhavanandan, unpublished results). In this study we took advantage of β-elimination on membrane to compare the oligosaccharides of the 390 kDa and 350 kDa epitectin bands. The results illustrated in Fig. 3 show that the profiles of the oligosaccharides in both species are very similar. Small differences were noted in the proportions of the oligosaccharides however, these differences may not be significant.

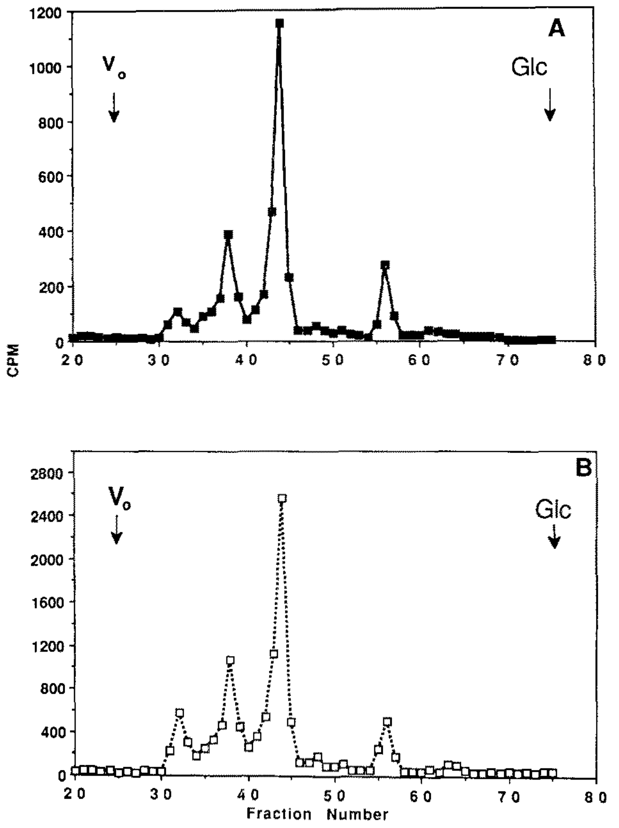

Figure 2. Gel filtration on Bio Gel P2 of O-linked saccharides derived from [^{3}H] epitectin. Extract of H.Ep.2 cells metabolically labelled with [^{3}H] glucosamine was subjected to SDS-PAGE, electrotransferred to nylon membrane and portion of the membrane containing the [^{3}H] epitectin was localized and cut out as described in the text. Elution profiles of saccharides released by alkaline borohydride treatment of A, the membrane pieces containing [^{3}H] epitectin and B, a solution of a purified preparation of [^{3}H] epitectin. Chromatography conditions are described in the legend of Fig. 1.

In conclusion, we demonstrate the capability of releasing and partially characterizing serine/threonine-linked saccharides of glycoproteins which are present in minute amounts in complex mixtures and therefore difficult to sufficiently purify for structural analysis. Advantage can then be taken to obtain sufficient material by high resolution electrophoretic technique followed by transfer to 'inert' membranes for further manipulation. The released saccharides can be analysed by gel filtration as in these studies and by other techniques such as HPLC [23] or high pH anion exchange chromatography [24, 25]. The application of the method to analyse or compare the saccharide profiles of glycoforms of mucin glycoproteins is valuable as illustrated by the examinations of the two species of epitectin. It has been impossible to separate the two forms of epitectin by any other technique. An important finding is that the efficiency of electrotransfer of mucin glycoproteins is variable and is influenced by several factors including molecular mass, carbohydrate content, type of membrane and buffer system used. Even though it appears that nylon and nitrocellulose are better than PVDF for this class of glycoproteins, our experience indicates that if maximum transfer is desired it is best to carry out preliminary testing with a variety of membranes and buffer systems. All three membranes tested were found to be stable to the alkaline borohydride treatment conditions used. Our strategy, in combination with approaches described by others to obtain monosaccharide composition [6] and information of asparagine-linked oligosaccharides [7], allows complete structure characterization of electroblotted glycoprotein compared to lectin staining methods (for example, the first choice glycoconjugate analysis system from Boehringer Mannheim Biochemicals) which only provide tentative information about the nature of the oligosaccharides present.

Acknowledgements

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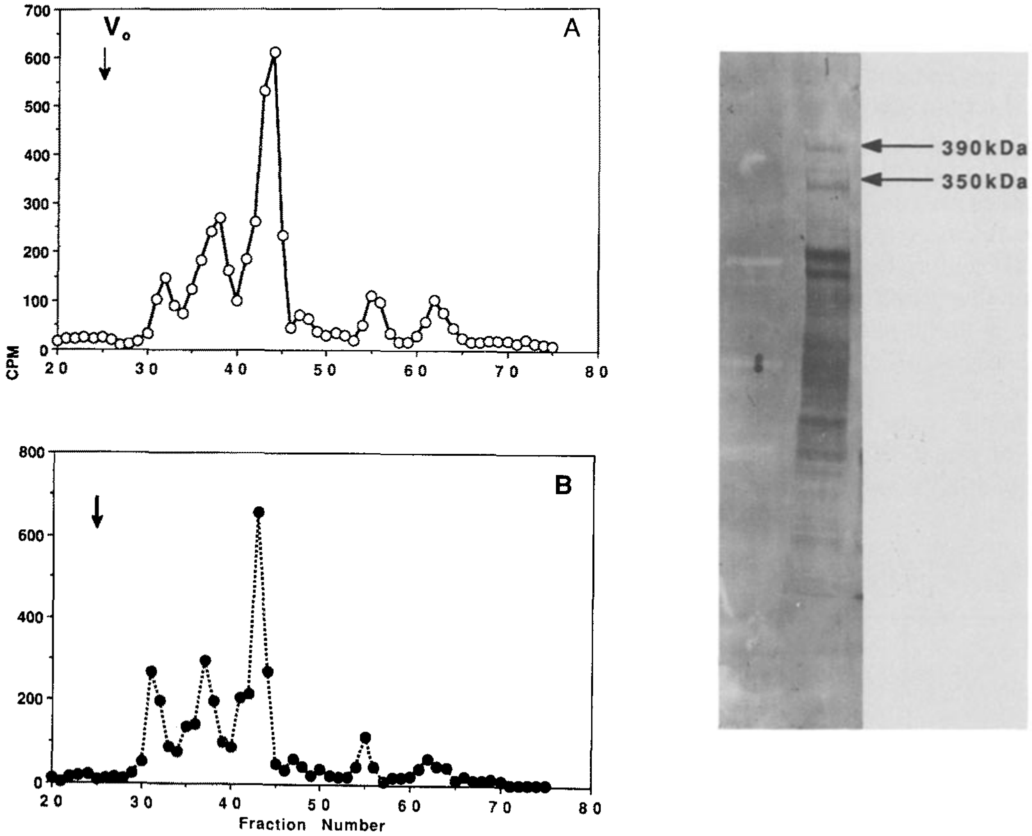

Figure 3. Gel filtration on Bio Gel P2 of the O-linked saccharides released from the 390 kDa (A) and 350 kDa (B) species of [^{3}H] epitectin by alkaline borohydride treatment. Experimental details are as described in Fig. 2, except that the 390 and 350 kDa bands were cut out separately. The guide strip of H.Ep.2 cell glycoproteins stained with WGA-digoxigenin shown on the right was used to locate the epitectin bands of interest. Based on previous structural analysis [10] the five major peaks, in the order of elution, are identified as: NeuNAc → Gal (NeuNAc → Gal → GlcNAc) → GalNAcOH, NeuNAc2 → 3Galβ → (NeuNAc2 →6) Gal NAcOH; NeuNAc2 → 3Galβ → GalNAcOH; Gal1 → 3 GalNAcOH and GalNAcOH.

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