Journal of Chromatography, 168 (1979) 395–400
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CHROM. 11,352

FRACTIONATION OF MANNOSE-LABELED NEUTRAL GLYCOPEPTIDES BY QAE-SEPHADEX CHROMATOGRAPHY

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(Received July 25th, 1978)

SUMMARY

Mannose-labeled cellular glycopeptides derived from human diploid fibroblasts (KL-2) were separated into two classes by QAE-Sephadex chromatography. High-voltage paper electrophoresis and Sephadex G-50 chromatography were used to characterize the glycopeptides further. At least five distinct neutral fractions were isolated with molecular weights ranging from 1050 to 2000 daltons. A linear gradient of ammonium acetate eluted a heterogeneous population of acidic glycopeptides. The use of QAE-Sephadex enables a single-step fractionation of both neutral and acidic glycopeptides on one column. The technique appears to be sensitive enough to distinguish growth-dependent alterations between growing and non-growing cells.

INTRODUCTION

Cellular glycoproteins constitute a very heterogeneous population of molecules[1,2]. Extensive proteolytic digestion has been used to reduce this material to oligosaccharides with relatively few amino acids[3]. Among the techniques used to isolate and characterize glycopeptides have been high-voltage paper electrophoresis (HVPE)[4,5], gel filtration[6,7], affinity chromatography[8], and ion-exchange chromatography. Anionic exchangers have been used to separate net negatively charged molecules containing sialic acid[9–11] or, possibly, charged amino acids. Cation exchangers have been used to separate neutral mannose-rich glycopeptides[12,13]. This communication describes an alternative method of fractionating both acidic and neutral species of glycopeptide with one column whereas, otherwise, a combination of the above-mentioned techniques would be required. The technique permits the large-scale purification of glycopeptides with essentially 100% recovery.

MATERIALS AND METHODS

Cell labeling and partial glycopeptide purification

Human diploid fibroblasts (KL-2) were cultured in Eagle's minimum essential media supplemented with 5% calf serum and 5% fetal calf serum, and buffered to

maintain pH at optimal growth conditions[14]. The cells were tested for mycoplasmal infection and found to be negative[15]. Cells were labeled for 20–24 h either during exponential growth or in the non-growing state with D-[2-^{3}H]mannose (5 μCi/ml, specific activity 1 Ci/mmol, Amersham–Searle Radiochemical Centre, Arlington Heights, Ill., U.S.A.) or D-[^{14}C]mannose (0.5 μCi/ml, specific activity 240 mCi/mmol, Schwartz-Bioresearch, Orangeburg, N.Y., U.S.A.). Cell surface material was removed by mild Pronase digestion, and the remaining material was designated as cellular glycopeptides. The cell material was extensively digested with Pronase and partially purified by Sephadex G-50 chromatography[14].

Characterization of sugars in labeled glycopeptides

Hydrolysis of any glycopeptide sample under conditions to hydrolyze mannose and galactose (1 *N* HCl for 4 h at 100°) released radioactivity migrating with authentic mannose. Descending paper chromatography using Whatman 3MM paper was carried out with butanol–pyridine–water (6:4:3), developed for 15 h. Less than 10% radioactivity was found to migrate with authentic fucose when hydrolyzed with 0.1 *N* HCl at 80° for 45 min.

High-voltage paper electrophoresis

Cellular glycopeptides were lyophilized either as partially purified from Sephadex G-50 chromatography or fractionated on QAE-Sephadex, redissolved in 1–2 ml of water and loaded (approximately 160 μl) onto 1.5-in.-wide strips of Whatman 1MM paper. Electrophoresis was carried out at 4000 V for 3.5 h in pyridine–acetic acid–water (10:0.4:89.6), pH 6.5. Strips were dried, cut into 1-cm fractions, eluted with 0.5 ml of water for 30 min and counted aqueously in a liquid scintillation counter.

QAE-Sephadex chromatography

QAE-Sephadex A-25 (Pharmacia, Piscataway, N.J., U.S.A.), 3.0 $\pm$ 0.4 mequiv./g, was charged with 1 *M* ammonium acetate brought to pH 8.5 (at room temperature) with ammonium hydroxide and washed repeatedly with 5 m*M* ammonium acetate pH 8.5 until conductivity readings were stable. Care should be taken to avoid excessive stirring of the beads as this will affect the flow-rate. A column (42 $\times$ 1.5 cm I.D.) was prepared and thereafter all operations were done at 4°. Glycopeptides partially purified by Sephadex G-50 chromatography were initially eluted with 5 m*M* ammonium acetate pH 8.5 until only background radioactivity was detected. Five-milliliter samples were collected at a flow-rate of 20 min per tube. Thereafter a linear ammonium acetate gradient, pH 8.5, from 5 m*M* to 300 m*M* was used to elute the material remaining on the column. At completion of the gradient, elution by 1 *M* ammonium acetate recovered no significant radioactivity.

Mannose-containing glycopeptides were routinely checked for the presence of free mannose before application to QAE-Sephadex by descending paper chromatography[14].

RESULTS AND DISCUSSION

Mannose-containing cellular glycopeptides partially purified by Sephadex G-50 chromatography contain a mixture of acidic species (whose carbohydrate por-

tion terminates with sialic acid) and neutral species (terminating with neutral sugars)[4]. As shown below, when a mixture of these glycopeptides is placed on QAE-Sephadex, neutral species have a weak affinity, since they rapidly elute at low salt concentration, while acidic species (carrying a net negative charge) are retained but elute with a linear gradient of higher salt concentrations.

Glycopeptides collected by Sephadex G-50 chromatography were lyophilized, redissolved in a minimal amount of water, and applied to a column of QAE-Sephadex. The mannose-labeled glycopeptides were initially eluted from the QAE-Sephadex with 5 m*M* ammonium acetate pH 8.5. As shown in Fig. 1a, they separated into at least five distinct peaks. The molecular weight of each peak was estimated by Sephadex G-50 chromatography based on their mobilities compared to known standards.

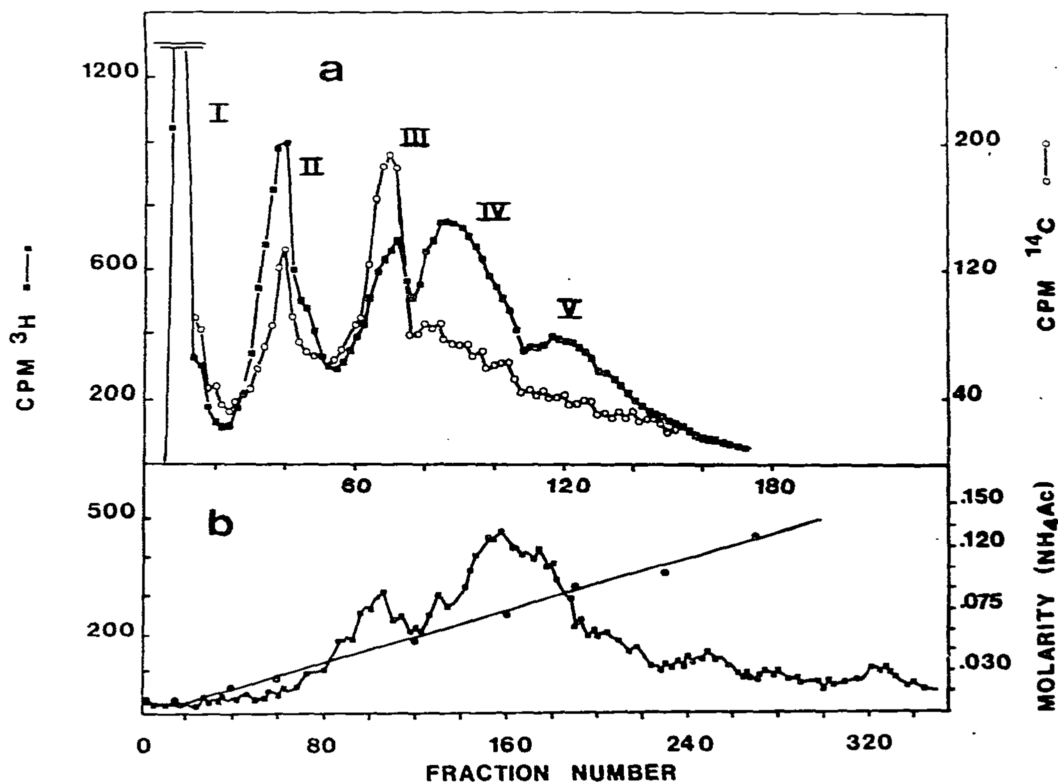

Fig. 1. QAE-Sephadex fractionation of cell material. Exponentially growing cells were labeled with D-[2-^{3}H]mannose (5 μCi/ml, 1 Ci/mmol) and non-growing cells with D-[^{14}C]mannose (0.5 μCi/ml, 240 mCi/mmol) as described under Materials and methods. The mannose-containing glycopeptides eluted and pooled from Sephadex G-50 columns were lyophilized and further chromatographed by QAE-Sephadex. (a) The sample (1.1×10^{6} ^{3}H cpm and 3.0×10^{5} ^{14}C cpm) was applied to a column (42×1.5 cm I.D.) and eluted with 5 m*M* ammonium acetate, pH 8.5. Five-milliliter fractions were collected at a flow-rate of 20 min per tube. Elution was continued until only background radioactivity was detected. (b) Glycopeptides eluted with a linear gradient, 5 m*M* to 300 m*M* ammonium acetate, pH 8.5. 1500 ml of 5 m*M* ammonium acetate in the mixing chamber and 1500 ml of 300 m*M* ammonium acetate in the reservoir. No significant radioactivity was recovered by subsequent elution with 1 *M* ammonium acetate. For simplicity, only the growing material is plotted in 1b, but is similar for the non-growing.

The standards used were two [^{14}C]acetylated ovalbumin glycopeptides of known structure[16], namely [^{14}C]acetylAsn(GlcNAc)$_2$(Man)$_5$ (molecular weight 1393) and [^{14}C]acetylAsn(GlcNAc)$_2$(Man)$_6$ (molecular weight 1555); and calf thyroglobulin unit A (molecular weight about 2000). All peaks eluted within the glycopeptide region and no radioactivity was recovered in the region of free mannose. The apparent molecular weight for the components ranged from approximately 1050 to 2000 daltons; they were neutral (see below). In general, neutral glycopeptides contain a simple oligosaccharide chain, (Man)$_n$(GlcNAc)$_2$, linked to asparagine[17,18]. The difference in molecular weight between each peak reported here (*cf.* Fig. 1a) can be accounted for by one or two monosaccharide residues. These results are consistent with the "oligomannosyl cores", ranging from 3–7 or 8 mannose residues, proposed by Muramatsu *et al.*[19] to exist in neutral glycopeptides obtained from diploid fibroblasts. However, recent studies on peak I (Fig. 1a) suggest that it may contain both glycopeptide and oligosaccharide material.

When a linear gradient of 5–300 m*M* ammonium acetate was applied to the QAE-Sephadex column, a heterogeneous mixture of acidic mannose-labeled material was obtained between 30 m*M* and 150 m*M* salt (Fig. 1b). With the present material it was not possible to detect distinct species in the eluted material. However, a similar analysis of cell surface material from exponentially growing and non-growing cells, to be reported separately, gave three distinct major peaks and several minor ones[20].

Glycopeptides partially purified by Sephadex G-50 chromatography can also be separated into acidic and neutral populations by HVPE (Fig. 2a). Peaks I–V (Fig. 1a) were also analyzed by HVPE. All five migrated as neutral glycopeptides (Fig. 2b–f). Of particular interest is the fact that the five species, while differing significantly in molecular weight, migrated only 3–4 fractions apart, and thus would be virtually indistinguishable from each other by HVPE. It was noted that the cellular glycopeptide profile (Fig. 2a) contains an apparent basic mannose-labeled species, comprising about 3–4% of the total material. Only peak I (Fig. 2b) was found to contain this component. With respect to the order of elution from the column, the neutral peaks appear to become progressively more contaminated with acidic material, ranging from no contamination in peak I to 33% contamination in peak V.

The heterogeneous peak of mannose-labeled glycopeptides eluted from the column between 30 m*M* and 90 m*M* salt concentration was collected (fractions 80–116, 120–140, 150–160 and 170–190) and an aliquot of each fraction was analyzed by HVPE; 83–98% of the radioactivity migrated as acidic glycopeptides. In general it was noted that each of the four fractions contained decreasing amounts of neutral material ranging from 17% in fractions 80–116 to 2% in fractions 170–190. The apparent cross contamination of neutral and acidic glycopeptides observed is not likely to be due to overlapping peaks on the QAE-Sephadex column since elution of the neutral glycopeptides is routinely continued to fraction 240 (only background radioactivity is recovered beyond fraction 180) before the linear gradient is applied.

A comparison of the neutral glycopeptides derived from non-growing and growing cellular material suggested growth-dependent alterations (Fig. 1a). The neutral glycopeptides derived from non-growing cellular material were markedly reduced in the high-molecular-weight species (IV and V) relative to those derived from growing cellular material. A similar result has previously been reported by Muramatsu *et al.*[19]. They used two specific endo-β-N-acetylglucosaminidases[21,22] to

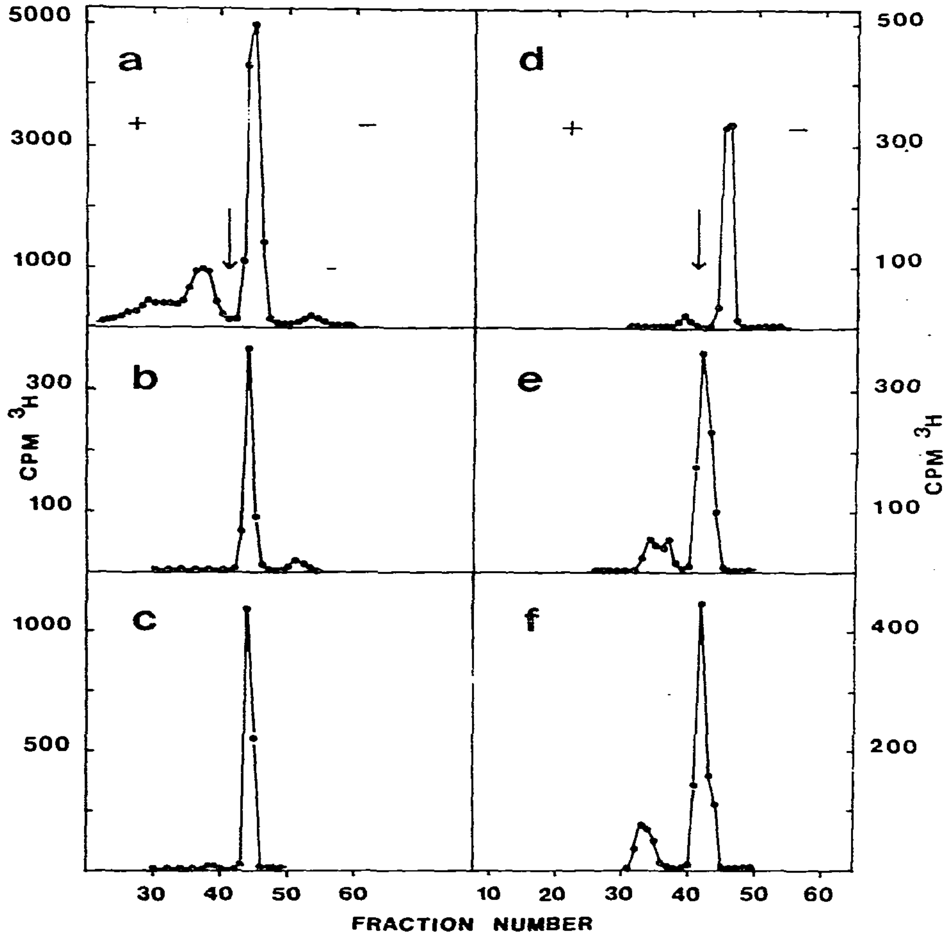

Fig. 2. Electrophoretic analysis of neutral glycopeptides. Mannose-labeled glycopeptides were prepared as outlined in the legend of Fig. 1 and further characterized by HVPE at pH 6.5.(a) Cell material was partially purified by Sephadex G-50 chromatography and analyzed by HVPE, at 4000 Volts for 3.5 h. Acidic glycopeptides migrate to the positive pole (fractions 20–40) and neutral glycopeptides move slightly to the negative pole (fractions 41–46). Neutrality is defined by the characteristic migration of neutral sugars under identical conditions. The arrow indicates the origin (fraction 41). (b) Species I eluted with 5 mM ammonium acetate; (c) species II; (d) species III; (e) species IV; and (f) species V. Apparent acidic contamination for each peak was as follows (measured as percent acidic cpm of total radioactivity recovered from paper): Species I, 0%; species II, 0%; species III, 5%; species IV, 22%; and species V, 33%. For simplicity, only the ^{3}H-labeled glycopeptides from growing cellular material are shown. The ^{14}C-labeled non-growing cellular material gave similar results.

convert the mixture of cellular neutral glycopeptides isolated by HVPE into an array of oligosaccharides, and paper chromatography to identify different neutral species of glycopeptides. HVPE separates cellular glycopeptides into two heterogeneous classes based on charge differences[4] (*cf.* Fig. 2a). It should be stressed that the present technique, however, permits the separation of cellular glycopeptides into acidic material and at least five distinct neutral peaks whose presence could not be readily

predicted without prior glycosidase digestion. Thus the technique has the advantage of making direct comparisons of intact glycopeptides derived from two different cell populations. Thereafter, fractions of interest may be further studied using specific enzymatic probes and the digested products analyzed by paper chromatography.

ACKNOWLEDGEMENTS

The [^{14}C]acetyl ovalbumin glycopeptides and calf thyroglobulin unit A were made available as a generous gift from Dr. Takashi Muramatsu. We wish to express gratitude to Drs. S. Anand Kumar and Benedetto Terrana for helpful suggestions. Finally, our thanks to Dr. Irwin Herskowitz for reviewing the manuscript. This work was supported by grants from NIH (GM24071) and the City University of New York (11614).

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