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Two-dimensional poly(acrylamide) electrophoresis of fluoresceinated glycopeptides. Resolution and structural characterization of ovalbumin glycans *,**

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ABSTRACT

The microheterogeneous mixture of fluoresceinated glycopeptides (FGPs) obtained from the single site of glycosylation of chicken ovalbumin was resolved by a combination of discontinuous electrophoresis in a high-density poly(acrylamide) gel (PAGE) for sizing, in conjunction with borate–PAGE. Two FGPs of similar size but with different mobilities in borate–PAGE were purified and characterized by sequential exoglycosidase digestion and sizing on the discontinuous PAGE system, as well as by methylation analysis. The two FGPs of identical size are distinct and have structures β-D-Glc*p*NAc-(1 → 2)-α-D-Man*p*-(1 → 3)-[β-D-Glc*p*NAc-(1 → 4)]-[β-D-Glc*p*NAc-(1 → 2)-α-D-Man*p*-(1 → 6)]-β-D-Man*p*-(1 → 4)-β-D-Glc*p*NAc-(1 → 4)-β-D-Glc*p*NAc-1 → R and α-D-Man*p*-(1 → 2)-α-D-Man*p*-(1 → 3 or 6)-{α-D-Man*p*-(1 → 3)-[α-D-Man*p*-(1 → 6)]-α-D-Man*p*-(1 → 6 or 3)}-β-D-Man*p*-(1 → 4)-β-D-Glc*p*NAc-(1 → 4)-β-D-Glc*p*NAc-1 → R (R = Asn-(amino acids)-fluorescein). The results demonstrate that two-dimensional PAGE is applicable to the separation and characterization of complex mixtures of FGPs. The procedure is rapid, sensitive, and convenient for glycopeptide mapping, and for the purification and structural characterization of glycans. Furthermore, the FGPs can be characterized with affinity matrices, such as lectins, and by methylation analysis.

INTRODUCTION

Chicken ovalbumin possesses a single site of *N*-glycosylation[1]. The microheterogeneity observed for the glycan at this location is extensive. Oligomannose, hybrid, and "bisected" complex structures displaying an array of branch patterns[2–5], and oligosaccharides containing sialic acid[6] and sulfate[6] have been reported. Identifi-

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cation and structural analysis of each component in this varied mixture of glycans poses a challenge to the analyst.

Carbohydrate-sequence analysis of oligosaccharides from glycoproteins is performed by the use of chemical, enzymic, and physical methods[7]. All approaches require the isolation of chemically homogeneous glycans. To date, the major methods of purification of the isolated glycan or glycopeptide are associated with its size, charge, composition[7], or reactivity with specific reagents, such as lectins[8]. Discontinuous, poly(acrylamide) gel electrophoresis (PAGE) can separate fluorescein-derivatized, Pronase-limit glycopeptides (FGPs) of different sizes or charge densities (or both)[9]. It has been used to purify and characterize the oligosaccharides of glycopeptides from murine immunoglobulin G[10], *Sophora japonica* agglutinin[11], and the hemagglutininating lectin from *Wistaria floribunda*[12]. To broaden the applications of PAGE for a complex mixture of FGPs, a convenient and reliable separation method for FGPs of similar charge densities and size is required. Borate complexation of neutral saccharides, resulting in anionic products, has been used for the electrophoretic separation of glycopeptides on paper [13] and poly(acrylamide) gels[14]. The resolving power of this approach is due to the differential binding of borate ion to carbohydrates of different structures[15]. The borate ion complexes best with *cis*-oriented, vicinal hydroxyl groups[16]. We have taken advantage of the differential reactivity of the borate ion with FGPs of the same size, but with different glycan structures, to add a second dimension of resolution to PAGE for the purification and analysis of a complex mixture of FGPs. The successful application of our approach was demonstrated with the purification and structural characterization of two ovalbumin FGPs possessing the same number of monosaccharide residues but differing in structure.

EXPERIMENTAL

Materials. —FGPs were prepared from ovalbumin (25 mg; Sigma Chemical Co., St. Louis, MO) after extensive Pronase (Sigma) digestion in the manner described for fetuin[9]. FGPs were separated from fluorescein isothiocyanate reaction products and fluoresceinated amino acids by gel filtration through Sephadex G-10[9].

Methods. —Sizing PAGE of the FGPs was performed by discontinuous electrophoresis in 20% acrylamide gels (20 × 20 cm) containing 1.25% *N,N'*-methylenebis(acrylamide) in a Tris–glycine buffer as described earlier[10]. Borate–PAGE was performed in 7.5% acrylamide gels (20 × 20 cm) containing 0.38% *N,N'*-methylenebis(acrylamide), prepared with 0.1 M sodium borate, pH 9.1. No separate stacking or sample gel layer was utilized. The same buffer was employed as electrode solution and electrophoresis was accomplished at a constant 200 V. Glycosidase digestion of FGPs and photography of the PAGE gels were performed as described earlier[9]. Jack bean α-D-mannosidase and *N*-acetyl-β-D-hexosaminidase were obtained from Sigma. FGPs were treated with snail β-D-mannosidase (a gift from Dr. J. Tkacz) at 37° for 4 h.

Lectin analysis was accomplished on FGP (1 nmol, 150 μL) with packed

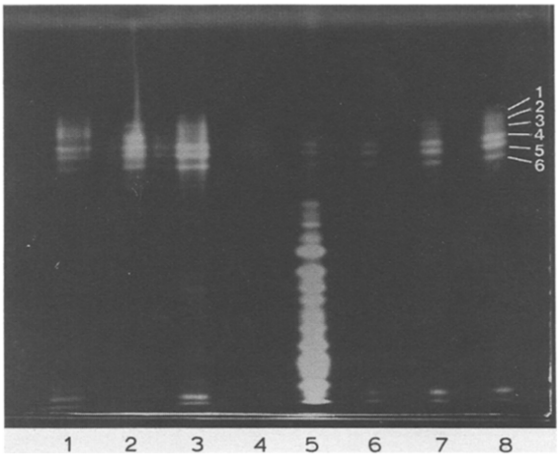


Fig. 1. Sizing PAGE of fluoresceinated glycopeptides from ovalbumin: Lane 1, FGPs unretained by concanavalin A-Sepharose; lane 2, concanavalin A-Sepharose-bound FGPs; lane 3, FGPs unretained by RCA I-Sepharose; lane 4, RCA I-Sepharose-bound FGPs; lane 5, fluoresceinated, Pronase-limit digest of ovalbumin; lane 6, lead peak from Sephadex G-10 filtration of the fluoresceinated, Pronase-limit digest of ovalbumin (diluted 1:400); lane 7, same as lane 6 (diluted 1:20); and lane 8, same as lane 6 (neat).

lectin-Sepharose (50 μL) in 0.05 M Tris buffer, pH 7.2, containing 0.1 M NaCl and mM $CaCl_2$. After incubation in the cold for 1 h, the suspension was centrifuged at 10000g for 2 min. The supernatant was removed and the lectin adsorbant was washed thrice by suspension in cold buffer (500 μL), followed by centrifugation. The resulting pellet was suspended in fresh buffer (50 μL) and applied to a poly(acrylamide) gel. Alternatively, the bound FGPs were eluted at 25° from the lectin adsorbant for 30 min with a solution of the appropriate saccharide inhibitor.

Methylation analysis of the individual FGPs was performed as described elsewhere[10].

RESULTS AND DISCUSSION

Pronase digestion of ovalbumin and fluoresceination of the total mixture resulted in numerous fluorescent components on sizing PAGE (Fig. 1, lane 5). The faintly detected FGPs of slower mobility can be separated from the more abundant nonglycopeptide constituents by gel filtration. Lane 8 shows the enrichment of six resolved FGP regions contained in the exclusion volume of the Sephadex G-10 column. Early elution from the gel filtration column and slow mobility on PAGE are properties expected for the higher molecular weight FGPs as compared to the other fluoresceinated compounds.

The glycan nature of many of the fluoresceinated components in the exclusion volume is demonstrated by the ability of concanavalin A-Sepharose to specifically bind them. Lane 1 shows the pattern of fluoresceinated components not adsorbed

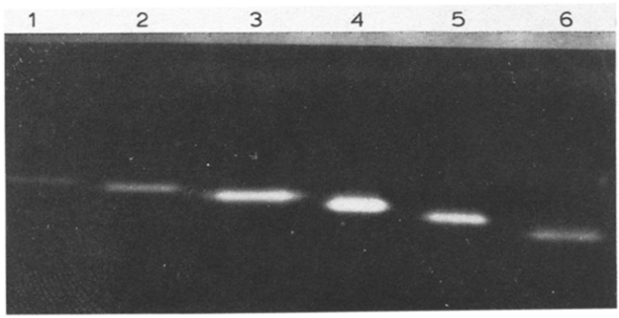


Fig. 2. Sizing PAGE of preparatively isolated FGPs from ovalbumin: Lane 1, FGP 1; lane 2, FGP 2; lane 3, FGP 3; lane 4, FGP 4; lane 5, FGP 5; and lane 6, FGP 6.

by concanavalin A-Sepharose. After extensive washing of the adsorbant, the beads were applied to the gel. It is evident (lane 2) that the electrophoretic conditions released the FGPs bound to the beads and caused them to move in a normal fashion. The FGPs so resolved are a subset of the fluorescent constituents separated in lane 8. PAGE of the FGPs specifically eluted from the adsorbant with 0.1 M methyl α-D-mannopyranoside produced the same pattern of fluorescent bands as shown in lane 2 (data not shown). In contrast, *Ricinus communis* agglutinin (RCA) I-Sepharose is unable to bind any FGPs. The nonadsorbed fluorescent components in the supernatant of the batch adsorption (lane 3) appear to be identical, in mobility and quantity, to those in the original solution (lane 8). As expected, the washed adsorbant did not yield any fluorescent material when subjected to PAGE (lane 4).

To further study the structure of individual FGPs, each of the six fluorescent regions was obtained in electrophoretic homogeneity by preparative PAGE (Fig. 2). The nature of the components in band 5 was examined further. The complexity of the mixture of FGPs is demonstrated, and insight into the structure of the glycan is gained by the ability of α-D-mannosidase and *N*-acetyl-β-D-hexosaminidase to act on specific FGPs. Fig. 3 shows that the action of either α-D-mannosidase (lane 8) or *N*-acetyl-β-D-hexosaminidase (lane 7) yielded products which indicate the presence of both enzyme-susceptible, and enzyme-resistant FGPs in band 5 (lane 9). That is, in both cases, fluorescent material of greater electrophoretic mobility was produced. Components exhibiting the mobility of the original sample, however, were also evident. Poretz and Pieczenik[9] had shown that the logarithm of the relative difference in mobility of two related FGPs is proportional to the difference in the number of neutral sugar units they possess. Based upon differences in mobility of the original components, and that of the product with greatest mobility from each enzyme digestion, it was estimated by reference to a standard curve that at least one constituent possesses three nonreducing, terminal or sequential (or both) 2-acetamido-2-deoxyglucose units, and the other component contains five nonreducing, terminal or sequential (or

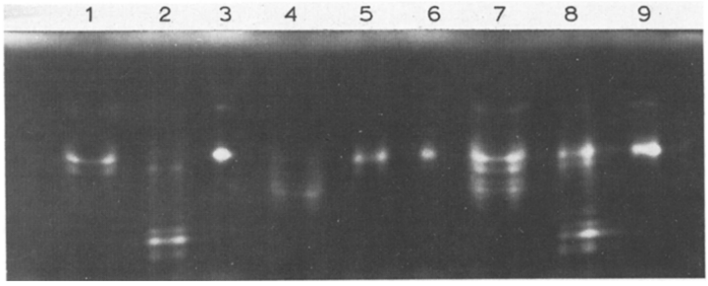


Fig. 3. Sizing PAGE of purified FGPs from ovalbumin: Lane 1, FGP 5-4 treated with *N*-acetyl-β-D-hexoaminidase; lane 2, FGP 5-4 treated with α-D-mannosidase; lane 3, FGP 5-4; lane 4, FGP 5-2 treated with *N*-acetyl-β-D-hexosaminidase; lane 5, FGP 5-2 treated with α-D-mannosidase; lane 6, FGP 5-2; lane 7, FGP 5 treated with *N*-acetyl-β-D-hexosaminidase; lane 8, FGP 5 treated with α-D-mannosidase; and lane 9, FGP 5.

both) α-D-mannose units. Confirmation of the probable presence of an oligomannose FGP in band 5 was obtained by the susceptibility of a portion of the material in this band to endoglucosaminidase H. This enzyme produced a single, rapidly moving fluorescent band with a mobility corresponding to the loss of 7 units of hexose from approximately 40% of the original FGPs in band 5 (data not shown). Approximamtely 60% of the sample was resistant to the endoglycosidase and traveled with the same mobility as the untreated material. The observation of only one fast moving product of endoglucosaminidase H activity indicated that the oligomannose FGPs are homogeneous in regard to their amino acid or peptide components.

Preparatively purified bands 1–6 from the sizing gels were each subjected to borate–PAGE in order to determine the complexity of each band. Borate–PAGE employing a gel of low sieving ability could allow separation of structurally dissimilar FGPs which exhibit a coincident mobility on sizing PAGE. Fig. 4 demonstrates that each band was resolved into two or more constituents by

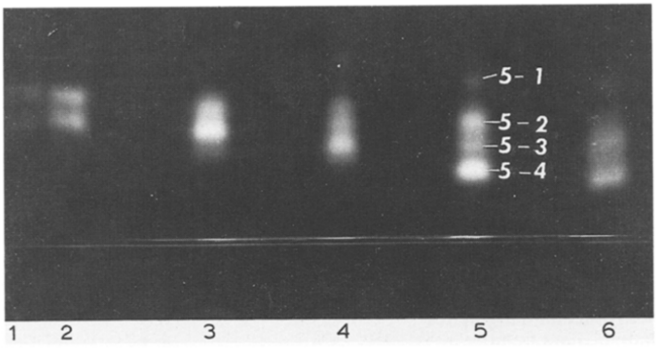


Fig. 4. Borate–PAGE of FGPs from ovalbumin. Lane 1, FGP 1; lane 2, FGP 2; lane 3, FGP 3; lane 4, FGP 4; lane 5, FGP 5; and lane 6, FGP 6.

borate–PAGE. Attempts to employ the discontinuous Tris–borate–PAGE procedure of Weitzman et al.[14] to resolve different components of bands 1–6 were unsuccessful. Band 5 clearly displays a minimum of four borate–PAGE resolvable FGPs. Preparative borate–PAGE was performed on band 5 in order to separate the individual FGPs, so that a more complete structural analysis may be performed on each. The sizing PAGE, following enzyme digestion of purified bands 5-2 and 5-4, shows that each fluorescent band behaved essentially as a pure FGP. *N*-Acetyl-β-D-hexosaminidase treatment had no effect on the major (> 90%) component of band 5-4 (compare lanes 1 and 3, Fig. 3). A minor contaminant of less than 10% of the fluorescent material was a substrate for the enzyme. This enzyme, however, caused cleavage of three hexose equivalents from band 5-2 (compare lanes 4 and 6, Fig. 3). Similarly, α-D-mannosidase altered the mobility of the major component of band 5-4 to a degree related to the loss of five mannose units (compare lanes 2 and 3, Fig. 3) but had no effect on band 5-2 (compare lanes 5 and 6, Fig. 3). α-D-Mannosidase treatment of the product of *N*-acetyl-β-D-hexosaminidase degradation of band 5-2 caused removal of two α-D-mannose units (data not shown). Similarly, β-D-mannosidase was capable of removing one hexose unit from the α-D-mannosidase product of band 5-4 (data not shown). These data suggest that FGP 5-2 is an N-linked glycan possessing three nonreducing, terminal 2-acetamido-2-deoxy-β-D-glucopyranosyl groups and FGP 5-4 is an *N*-linked oligomannose structure containing five α-D-mannose units linked to a β-D-mannosyl-di-*N*-acetylchitobiose core.

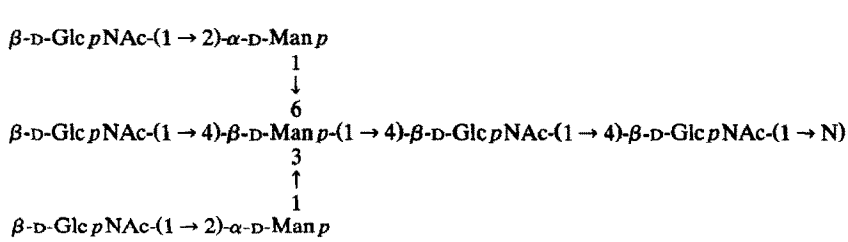
Methylation analyses of FGPs 5-2 and 5-4 (Table I) are consistent with these conclusions. The presence of a 2-*O*-methylmannitol derivative and absence of 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methylmannitol signify the probable presence of a "bisecting" 2-acetamido-2-deoxy-D-glucopyranosyl group on the β-D-mannosyl residue of FGP 5-2. This result, the finding of approximately three molar proportions of nonreducing, terminal aminodeoxyglucose per monomethylmannitol, and the presence of 1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methylmannitol as the only other mannose derivative clearly indicate that the structure of FGP 5-2 glycan is **1**.

TABLE I

Methylation analysis of fluoresceinated glycopeptides 5-2 and 5-4

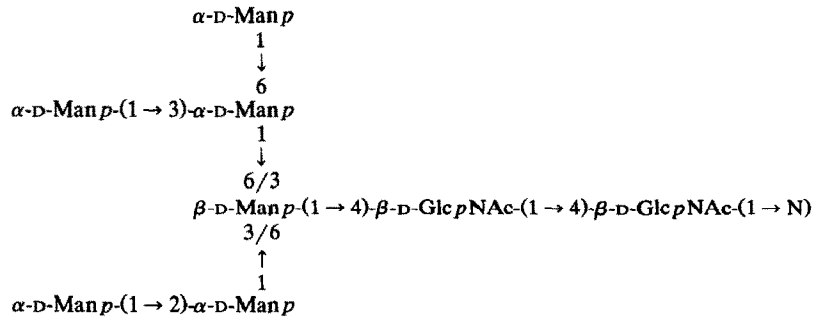
Partially methylated alditol peracetate	Molar ratio	
	FGP 5-2	FGP 5-4
2,3,4,6-Tetra-*O*-methylmannitol	0.0	3.0 [a]
3,4,6-Tri-*O*-methylmannitol	2.0 [b]	1.3
2,4-Di-*O*-methylmannitol	0.0	2.0
2-*O*-Methylmannitol	1.3	0.0
2-Deoxy-3,4,6-tri-*O*-methyl-2-(*N*-methyl)acetamidoglucitol	2.5	0.0
2-Deoxy-3,6-di-*O*-methyl-2-(*N*-methyl)acetamidoglucitol	1.6	1.7

[a] 2,3,4,6-Tetra-*O*-methylmannitol peracetate normalized to 3.0. [b] 3,4,6-Tri-*O*-methylmannitol peracetate normalized to 2.0.



1

Methylation analysis of FGP 5-4 demonstrated that this glycan possesses two molar equivalents of *O*-4-linked 2-acetamido-2-deoxy-D-glucose units per three nonreducing, terminal D-mannosyl groups. The detection of one molar proportion of 1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methylmannitol, and two molar proportions of the 2,4-di-*O*-methylmannitol derivative supports the conclusion that FGP 5-4 is most probably one, or a mixture of FGPs with the following isomeric glycan structures **2**.



2

Alternative structures, however, possessing the α-D-mannopyranosyl-(1 → 2) unit attached to any one of the nonreducing, terminal α-D-mannopyranose units may be justified.

The suggested structures of FGPs 5-2 (**1**) and 5-4 (**2**) are consistent with their observed electrophoretic mobilities in borate–PAGE. The oligomannose glycopeptide terminating in D-mannopyranosyl residues would be expected to bind more borate ions than the "bisected", complex glycan of FGP 5-2[16]. This would result in a greater anionic charge on FGP 5-4 than on FGP 5-2 and, hence, a greater electrophoretic mobility. Both the oligomannose and "bisected" complex structures described above have been reported previously as components of ovalbumin[2,5,13]. The earlier studies required ion-exchange chromatography, paper electrophoresis, and gel filtration; whilst the structural characterization employed gel filtration analysis of exoglycosidase-treated glycopeptides and methylation analysis. More recently, the application of two sequential HPLC chromatographic procedures on derivatized oligosaccharides of ovalbumin resulted in the separation of 12 individual components[17]. Two of these components, which exhibited elution characteristics similar to standard oligosaccharides with structures identical to those we report here, were not characterized further.

Our results demonstrate that the combined use of two-dimensional PAGE of anionic FGPs to separate and characterize complex mixtures of glycopeptides allows for a rapid, sensitive, and convenient approach to glycopeptide mapping and structural characterization. Furthermore, the FGPs so identified are amenable to characterization with affinity matrices, such as lectins, and by methylation analysis.

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