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Note

Two-dimensional separation of glycopeptides and charged oligosaccharides on silica thin layers

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The composition of the carbohydrate moieties of the glycoproteins is subject to variation under environmental influences, while the amino acid sequence of their polypeptide chain is genetically determined. Variations of the carbohydrate chains are characteristic for some pathological alterations of serum glycoproteins [1]. The glycans of cell-surface glycoproteins are modified during malignant transformation [2]. These glycans seem to be involved in cell association phenomena and in tissue differentiation [3, 4]. Thus, the study of the carbohydrate chains of glycoproteins has gained increasing importance in biomedical research.

With few exceptions the carbohydrate portion of each pure glycoprotein is a mixture of heteroglycans. The proteolytic hydrolysis of a glycoprotein produces a mixture of glycopeptides, from which fractions can usually be isolated differing in their carbohydrate composition. Several methods have been described for the separation of glycopeptides, involving ion-exchange [5] or exclusion chromatography [6, 7], paper [7] or thin-layer electrophoresis [8]. The latter excepted, these methods are insufficiently sensitive for the detection of glycopeptides with less than 1 μg of sugar constituents, and their resolving power is often insufficient. No thin-layer chromatographic (TLC) methods were described for the separation of glycopeptides containing more than 3—4 sugar residues. Such glycopeptides and oligosaccharides could be separated by paper chromatography [9], but the method is time-consuming (migration times of several weeks or months).

In the present study, a rapid micro-method is described for the separation of glycopeptides obtained by the pronase digestion of glycoproteins, containing ten or more sugar units. The same method can also be used for the two-dimensional separation of charged oligosaccharides.

MATERIAL AND METHODS

TLC sheets of "Selecta 1500" (Schleicher and Schüll, Dassell, G.F.R.) were used for the separations. Fibrinogen and pronase were obtained from Sigma (St-Louis, Mo., U.S.A.), ovalbumin from Koch-Light (Colnbrook, Great Britain) and [1-^{14}C]acetic anhydride (25.5 mCi/mole, in 20% benzene solution) from CEA (Gif-sur-Yvette, France). Insoluble gastric mucin [10] from donors of blood group A was a gift of Prof. D. Waldron-Edward, McGill University, Montreal, Canada.

Preparation of the glycopeptides

Crude pronase digests were prepared as follows: 1 mg of glycoprotein was suspended in 400 μl of water containing 20 μg of pronase and 2 μl of 25% ammonium hydroxide solution, the mixture was incubated for 48 h at 50°. The solution was dried in a vacuum desiccator and dissolved in 100 μl of water, 500 μl ethanol was added, the mixture was allowed to stand for 20 h at 4° and the glycopeptides precipitated were centrifuged. The precipitate was redigested with pronase as described above. A 100-μl volume of 50% trichloroacetic acid was then added, the mixture centrifuged, the supernatant extracted three times with 3 ml diethyl ether and lyophilized.

Glycopeptides were prepared by pronase digestion and purified by Sephadex G-25 and G-50 column chromatography [11, 12]. The glycopeptide mixture obtained by the pronase digestion of the fibrinogen (fibrinoglycopeptides) was treated with 0.1 *N* sulfuric acid at 80° [12] to remove the sialic acid groups.

Chromatographic separation of glycopeptides and oligosaccharides

TLC was performed on silica thin-layer sheets (Selecta 1500: 20 × 20 cm) in the following solvent systems:

(A) *n*-Propanol—nitromethane—acetic acid—water (7:2:2:2);
(B) *n*-Propanol—nitromethane—water (4:3:3);
(C) Ethanol—nitromethane—acetic acid—water (5:3:3:3);
(D) Ethanol—*n*-butanol—0. 1 *N* HCl (10:1:5) [13];
(E) Ethanol—*n*-butanol—0. 5 *N* triethylamine borate buffer pH 8.5 (6:1:3).

Fingerprinting

Crude pronase digests, purified glycopeptides or oligosaccharide mixtures containing 0.2—2 μg of carbohydrate constituents were applied 2—3 cm from the low edge of the 20 × 20 cm sheets, in the proximity of the median line. It is possible however to realize two fingerprints on the same plate, applying the samples 3 and 12 cm respectively for the anodic side of the sheet. Dinitrophenyl (DNP)-alanine was used to indicate the electrophoretic migration rate. The electrophoretic step was carried out in acetic acid—pyridine—water (10:1:89) at pH 3.8 [8], and 10—20 V/cm for 2—3 h. DNP-alanine migrates 6—7 cm under these conditions.

The plates were dried overnight at room temperature and developed perpendicularly to the direction of the electrophoretic migration by solvent A or C. Plates are chromatographed with solvent A by the ascending method,

until the solvent reaches the upper edge of the sheet. Solvent C is used for the fingerprinting of the glycopeptides in a continuous flow chamber [14]. Migration time 18—30 h.

Detection of the carbohydrate constituents was carried out by the sulphuric acid char, or using the orcinol—sulphuric acid reagent [8].

The plates were exposed for 2—3 days to Kodak Kodirex films and developed with Kodak LX14 developer.

Partial alkaline degradation of the gastric mucin

A 10-mg amount of insoluble gastric mucin from blood group A individuals [10] was suspended in 1 ml of 0.33 *M*, 1% KBH_4 containing sodium hydroxide solution. The mixture was stirred for 6 days at room temperature, neutralized with Amberlite CG-120 (H^+) and centrifuged. The supernatant was lyophylized.

Radioacetylation of glycopeptides

A 0.5-mg amount of the lyophylized pronase digest was dissolved in 200 μl water, and 100—200 μCi [^{14}C]acetic anhydride in 100 μl acetone were added (about 1 μl of the benzene solution of the anhydride). The mixture was shaken vigorously, allowed to stand 1 h at room temperature, and evaporated to dryness in a vacuum desiccator over potassium hydroxide and phosphorus pentoxide. N-Acetylation realized under these conditions is sufficient for radioactive labelling but is not quantitative. The reaction can be completed by non-labelled acetic anhydride as described [12].

RESULTS AND DISCUSSION

The R_F values of some oligosaccharides and glycopeptides in the solvents described are shown in Table I. The separation of oligosaccharides for 2—8 sugar units can be realized with solvents A, B and E. Solvents C and D may be used for the separation of the glycopeptides. The best results were obtained with solvent C using a continuous-flow chamber. Most of the glycopeptides studied have a tendency for tailing in solvent D.

The fingerprints of the fibrinoglycopeptides and of the desialofibrinoglycopeptide are shown in Fig. 1. The carbohydrate in the fibrinogen is linked to two different segments of the polypeptide chains. The pronase digestion of this protein yields glycopeptides with Asp-Lys and Gly-Gly-Asp-Arg sequences [15, 16]. The oligosaccharide chains linked to the aspartic acid residues of these peptides are heterogenous. The average heterosaccharide is branched and contains 4 to 5 residues of N-acetylglucosamine, 4 residues of mannose, 3 residues of galactose and 1 to 2 residues of sialic acid linked through 2—3 and 2—6 linkages [16, 17]. Fibrinoglycopeptides contain the same structural elements as the heterosaccharide chains of the other serum glycoproteins [18].

The fingerprint of the desialofibrinoglycopeptides shows two major sugar-containing spots (Fig. 1a) indicating the presence of two major glycopeptides with different electrophoretic and chromatographic mobilities. The difference in the electrophoretic behaviour of these glycopeptides can be explained by

TABLE I

R_F VALUES OF OLIGOSACCHARIDES AND GLYCOPEPTIDES ON SILICA THIN-LAYERS (SELECTA 1500)

Chromatographed by the ascending method, migration: 18 cm. nm = No migration, n.t. = not tested.

Sample	Solvent				
	A	B	C*	D*	E
Maltose	0.42	0.52	0.85	n.t.	n.t.
Lactose	0.39	0.46	0.79	n.t.	n.t.
Raffinose	0.26	0.41	0.71	0.82	0.53
Stachiose	0.11	0.26	0.61	0.70	0.35
Bovine fibrino-glycopeptides	n.m.	n.m.	(0.02—0.07) 0.12	(0.2—0.28) 0.32	n.m.
Bovine desialo-fibrinoglycopeptides	n.m.	n.m.	(0.03—0.1)	(0.29—0.38)	n.m.
Bovine fibrinoglyco-peptides N-acetylated	n.m.	n.m.	(0.10—0.14) 0.22	(0.25—0.32) 0.43	n.m.
Ovalbumine glyco-peptides	n.m.	n.m.	(0.10—0.19)	0.21; 0.29; 0.32	n.m.

*R_F values in parentheses are the lower and the upper limits of incompletely separated zones, otherwise they correspond to well-defined spots.

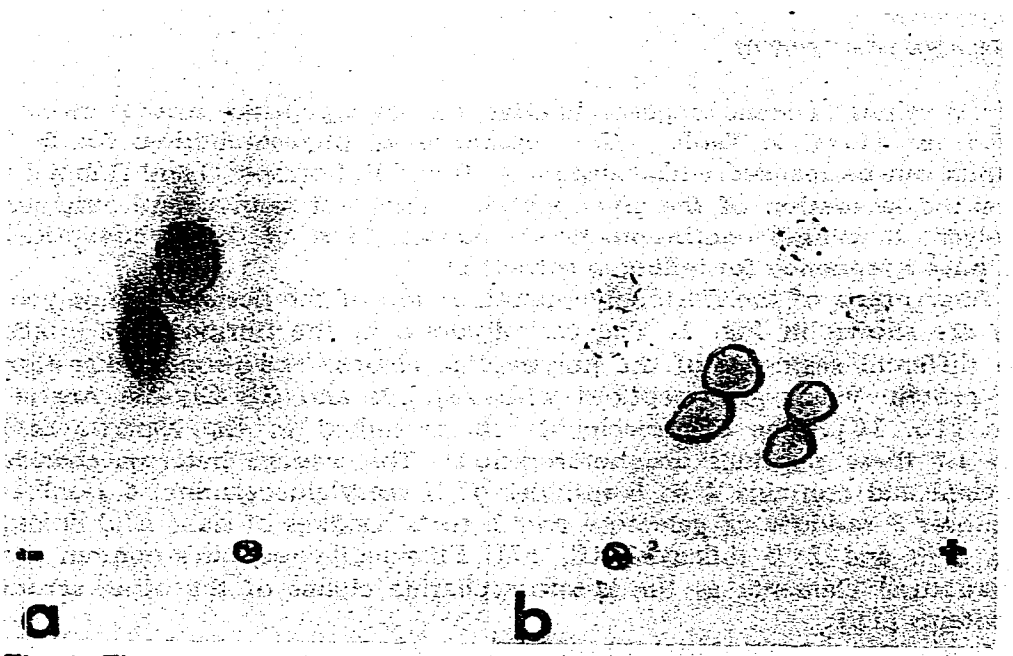


Fig. 1. Fingerprint of (a) desialo and (b) "native" fibrinoglycopeptides on Selecta 1500 sheets 20 × 20 cm. Electrophoresis: pyridine-acetate pH 3.8, 10V/cm. 2.5 h. Chromatography: solvent C, 24 h in a horizontal continuous flow chamber. Detection: 0.1% orcinol in 1 N H_2SO_4 in 80% ethanol, heating at 110° for 5 min.

the difference of the two peptide chains, hence the sialic acid was removed.

The fingerprint of the "native" fibrinoglycopeptide (Fig. 1b) is more complex. It exhibits the presence of 4 major carbohydrate-containing components indicating the increased heterogeneity of the heterosaccharide chains, explained partly by the variation of their degree of sialysation. This finding is consistent with the actual concept of the microheterogeneity of the carbohydrate moieties of glycoproteins [19].

Several minor carbohydrate-containing peptides can be detected on the fingerprint of both native and desialysed, fibrinoglycopeptides. This phenomenon can be explained by the presence of incomplete sugar chains [19] or of incompletely digested glycopeptides [16].

The fingerprint of the radioacetylated fibrinoglycopeptide mixture and its radioautogram are presented in Fig. 2. Each sugar-containing spot is radioactive. The electrophoretic as well as the chromatographic migration properties of the glycopeptides are changed by the N-acetylation. The sensitivity of the method can be 10—100-fold improved by the radioacetylation technique.

Detection of the non-glycosylated peptides by ninhydrin reagent, as well as the localisation of the non-glycosylated N-acetyl peptides on the autoradiograms, indicate that these peptides migrate in the chromatograms much faster

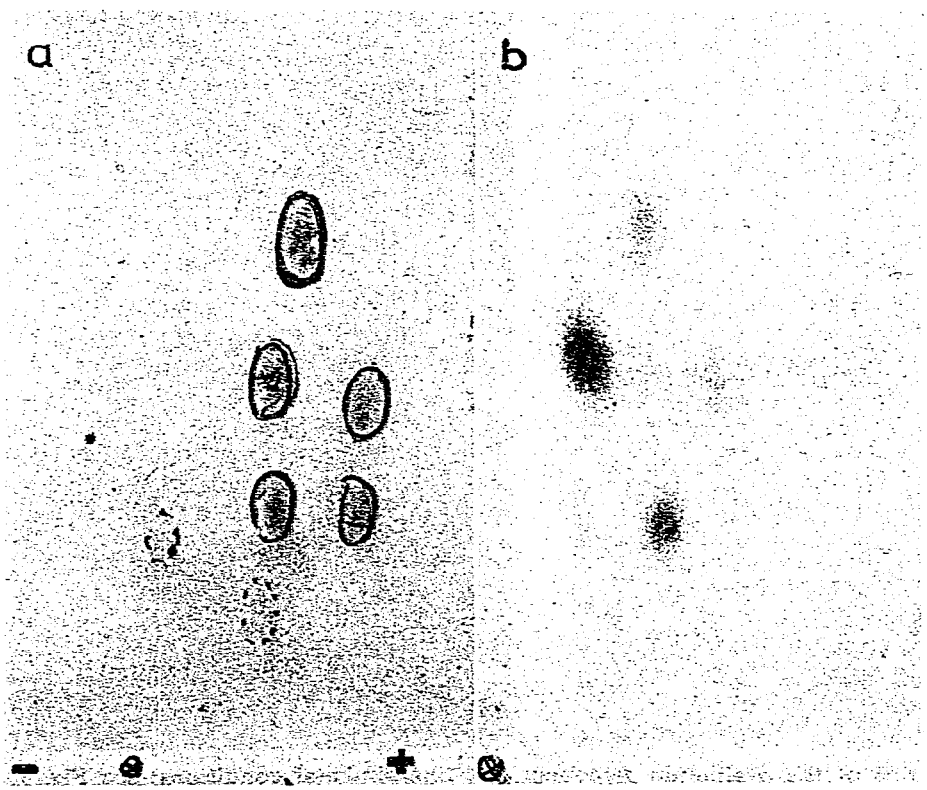
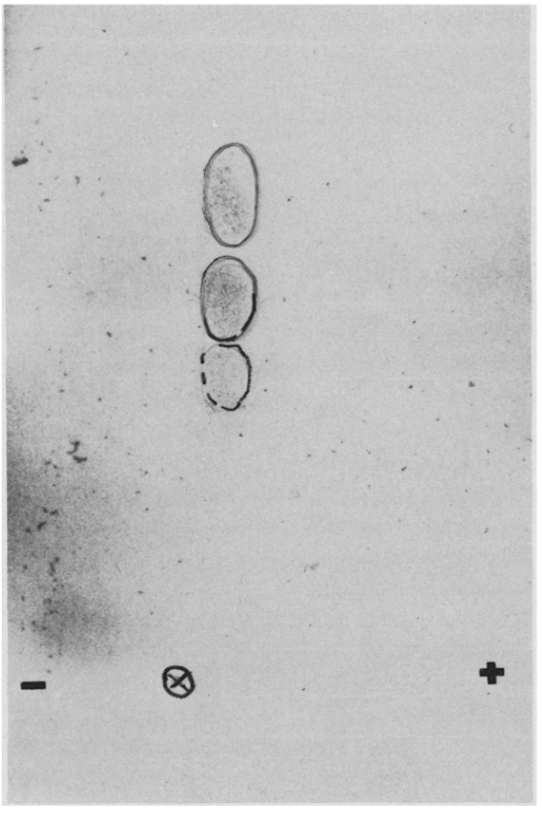


Fig. 2. Fingerprint of the N-acetylated ([1-^{14}C]acetic anhydride) "native" fibrinoglycopeptide (a) and its autoradiogram (b). Electrophoresis, chromatography and detection: see Fig. 1. Autoradiography on Kodirex films, exposure 3 days.

than glycopeptides (R_F between 0.8—1.0, in solvent C).

Pronase digestion of ovalbumin yields mannose and N-acetyl-glucosamine-containing oligosaccharides linked to aspartic acid [20]. The presence of two minor and three major carbohydrate chains was demonstrated in the glycopeptide mixture. The major fractions contained glucosamine and mannose in ratios of 4:6, 2:6 and 2:5, respectively [20]. The presence of three major carbohydrate-containing spots on the fingerprint of the ovalbumin glycopeptides (Fig. 3) is consistent with these results. The identity of the electrophoretic mobility of these substances can be explained by the identity of the amino acid moiety (aspartic acid only) and by the absence of sialic acid in the carbohydrate fraction.

The differences in the chromatographic migration of the sialic acid-containing and the sialic acid-free fibrinoglycopeptides (Table I, Fig. 2) as well as the galactose-free ovalbumin glycopeptides (Fig. 3) suggest that the chromatographic behaviour of the glycopeptides studied is strongly influenced by the structure and composition of the carbohydrate chains.



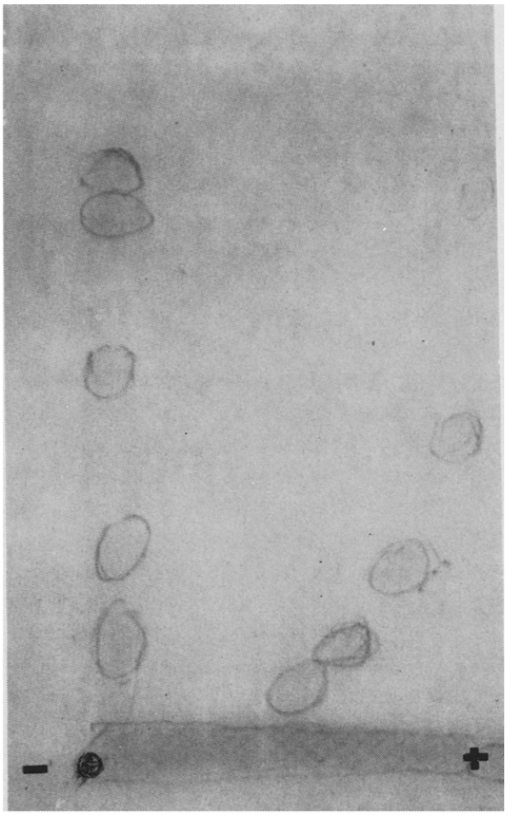


Fig. 3. Fingerprint of the ovalbumin glycopeptides. Experimental conditions: see Fig. 1.

Fig. 4. Fingerprint of the partial alkaline degradation products of the insoluble gastric mucin from blood group A individuals. Electrophoresis and detection as described in Fig. 1. Chromatography: solvent A ascending method, migration 18 cm.

However, much more work is needed with glycopeptides of exactly known structure before reliable correlations can be established between the structure of the sugar chain and the chromatographic mobility of glycopeptides.

Fig. 4 shows the fingerprint of the oligosaccharides obtained by the partial degradation of an insoluble gastric mucin in the presence of alkaline borohydride. Neutral and acidic oligosaccharides with a reduced aldehyde group were liberated from these mucins under the conditions used (peeling effect) [21]. A satisfactory two-dimensional separation of these oligosaccharides was obtained. The non-degraded macromolecular carbohydrate components exhibit electrophoretic mobility but they are not displaced by the solvents used in the chromatographic step.

The method is especially useful for studies of the variations of the carbohydrate chains of glycoproteins during development, aging, malignant transformations or other pathological conditions.

In such cases, the amino acid sequence of the polypeptide does not change, and in consequence, the peptide chains of the glycopeptides obtained by proteolytic digestion are identical.

Thus, only the composition of the carbohydrate moieties of these glycopeptides determines their electrophoretic and chromatographic mobilities.

As fingerprints prepared from the purified glycopeptide mixtures or from the crude pronase digest are identical, aliquots of these digests can be used directly for the characterization of the glycans of the glycoproteins.

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