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MAPPING AND SPECIFIC DETECTION OF GLYCOPEPTIDES ON THIN-LAYER PLATES

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SUMMARY

A thin-layer electrophoretic method is used for the separation of the glycopeptides. Specific detection methods permit the differentiation of the sugar constituents.

Carbohydrate-containing and carbohydrate-free peptides are distinguished by the successive application of ninhydrin and orcinol–sulphuric acid reagents on to the thin-layer peptide maps of glycoprotein hydrolysates.

INTRODUCTION

Electrophoresis is one of the most useful methods for the separation of peptides and glycopeptides. However, the specific detection of the non-reducing glycans of the glycopeptides is difficult on carbohydrate-containing beds, such as paper-, cellulose-, starch- or dextran-containing thin layers. Several methods have been published for the detection of non-reducing carbohydrates under such conditions[1–7], but most of them lack specificity and are insufficiently sensitive for the detection of less than 1-μg amounts of the sugar constituents.

In this paper a specific and sensitive method for the separation and detection of glycopeptides by thin-layer electrophoresis is described. The differentiation of carbohydrate-containing and carbohydrate-free peptides is achieved by the successive application of ninhydrin and orcinol–sulphuric acid reagents. Peptides and glycopeptides can be distinguished on the same chromatographic plate after two-dimensional separations.

MATERIAL AND METHODS

Materials

Plates or sheets of "Selecta 1500" pre-coated with silica gel were purchased from Schleicher and Schüll, Dassel, G.F.R.

The glycopeptides were obtained by collagenase–pronase digestion[8,9] of the proteoglycan–glycoprotein–collagen complexes (CSC, crude soluble collagen)[9] prepared from calf cornea as described by ROBERT AND DISCHE[10].

The structural glycoprotein of the pig cornea was prepared according to ROBERT *et al.*[11].

Pig thyroglobulin was purchased from Mann Research Laboratories Inc., New York[12].

Polysaccharide of *B. cereus* W. was prepared in the laboratory of Prof. IVANOVICS (Szeged, Hungary). Chondroitin-4-sulphate was a gift from Dr. MORISSON (Culver City, Los Angeles, U.S.A.). Hydroxylysine glucosidogalactoside was isolated from calf cornea and purified by preparative paper electrophoresis[8]. Amylose was obtained from Prolabo (Paris).

Electrophoretic separation

In the electrophoretic apparatus, the plates or sheets were deposited on a metal block, cooled with flowing tap water. Plates of 20 × 20 cm were used.

For one-dimensional electrophoresis, the experiments were carried out with a pyridine–acetate buffer at pH 3.8 (acetic acid–pyridine–water, 10:1:8). Other pyridine acetate or formate containing buffers may also be used.

Samples containing 0.2–1.5 μg of sugars were applied on the median line of the plates. A potential gradient of 10–20 V/cm was applied for 2–3 h.

Fingerprinting

The two-dimensional separations of the trypsic and chymotrypsic hydrolysates of glycoproteins were carried out on silica thin layers by a modification of the method described by WIELAND AND GEORGOPOULOS[13].

Proteolytic digests, containing 0.2–1.5 μg of carbohydrate constituents, were applied 2–3 cm from the lower edge of the plates (20 × 20 cm), or on the median line or two-thirds of the way along the plate on the anodic side for electrophoresis. DNP-alanine or DNP-serine was used to indicate the electrophoretic migration rate. The electrophoresis was carried out as described above.

The plates were dried overnight at room temperature and developed perpendicularly to the direction of the electrophoretic migration with one of the following solvents:

(1) *n*-butanol–pyridine–acetic acid–water (68:10:14:25)[13]

(2) isoamyl alcohol–pyridine–water (7:8:6).

The solvent front should reach the upper edge of the plates.

Detection of peptides

The plates are dipped in a 0.1% solution of ninhydrin in acetone, dried and heated to 50–60°.

Detection of carbohydrate constituents

Resorcinol reaction. The plates were sprayed with the resorcinol–HCl reagent of SVENNERHOLM AND SVENNERHOLM[14] and kept in a moist atmosphere for 15 min at 100°.

Acidic hydrolysis. The plates were heated at 100–105° in a tight glass chamber containing 4 *N* HCl.

Morgan–Elson reaction. The hydrochloric acid-treated plates were thoroughly dried, sprayed with acetylacetone reagent in 0.5 *N* Na_2SO_3 solution, heated for 20 min at 100° in a water-saturated atmosphere, and sprayed with Ehrlich's reagent[15].

Carbazole reaction. The uronic acid-containing glycopeptides can be made visible as described recently by LIPIELLO AND MANKIN[16]. The plates were sprayed with concentrated H_2SO_4 containing 0.025 *M* sodium tetraborate, heated for 3 min at 100°, and sprayed again with a 0.125% solution of carbazole in ethanol, then heated at 100° until the purple spots appeared.

Cysteine–sulphuric acid reaction[17]. The layers were sprayed with 2 *N* H_2SO_4, dried for 30 min at room temperature, heated for 4 min at 100° and sprayed again with

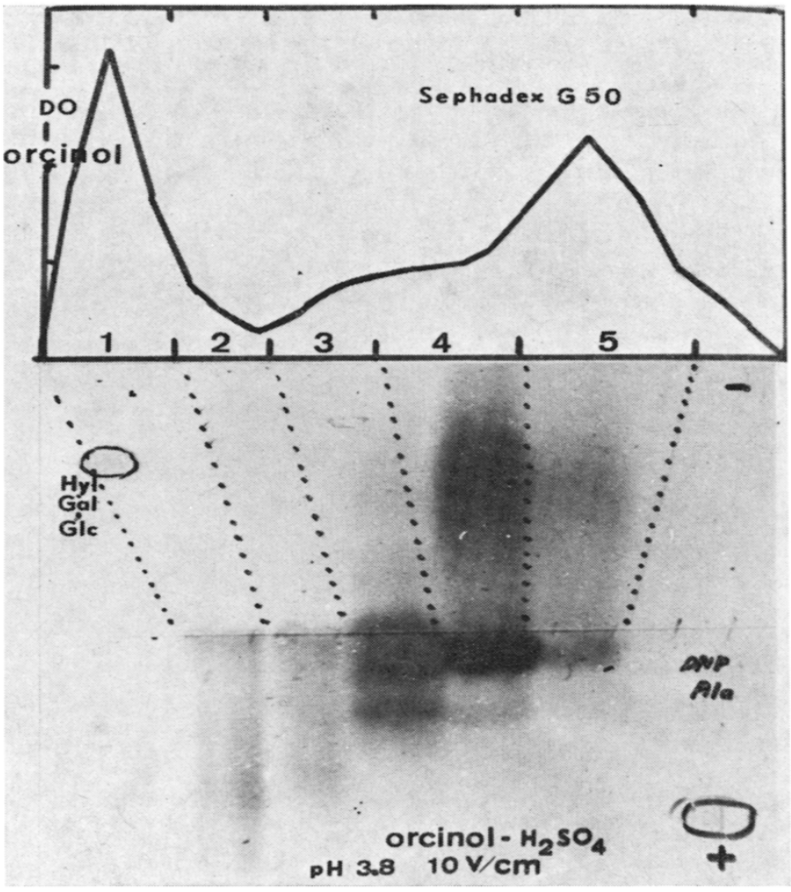

Fig. 1. *Above*: elution diagram (Sephadex G 50 column, 2 × 80 cm, eluted with 0.1 *M* acetic acid) of glycopeptides of different molecular weight[9,18] obtained from the collagenase–pronase-digested proteoglycan–glycoprotein–collagen complex of the calf cornea ("crude soluble collagen", CSC[10,11]). Hexoses are determined by the orcinol reaction; galactose–mannose (1:1) mixture was used as standard.

Below: electrophoretic pattern of the elution groups (on silica layers; pyridine–acetate, pH 3.8, 10 V/cm, 2.5 h); the glycopeptides are detected by the orcinol–sulphuric acid method (see text).

0.1% solution of cysteine in 80% ethanol. The colour was developed by heating at 100° for 1–3 min.

Orcinol–sulphuric acid reaction. The plates were sprayed with a 0.1% solution of orcinol in 2 *N* H_2SO_4 and the colour was developed by heating at 100° for 10–15 min.

RESULTS AND DISCUSSION

Fig. 1 shows the electrophoretic pattern of glycopeptide mixtures of different molecular weights, obtained from the collagenase–pronase-digested proteoglycan–glycoprotein–collagen complex of the calf cornea (crude soluble collagen, CSC)[10,11] by exclusion chromatography.

This complex contains three types of carbohydrate chain bearing proteins: proteoglycans, structural glycoproteins and collagen[9,18]. The proteolytic digest of this substance was particularly suitable for demonstrating the electrophoretic separation of the different types of glycopeptides.

The acidic glycosaminoglycans are separated into two main bands in fractions 1 and 2. Experiments with authentic samples indicate that part of the sulphated glycosaminoglycans remains adsorbed on the starting line. The neutral and weakly acidic (containing sialic acid) glycopeptides derived from the glycoproteins, as well as the basic glycopeptides, containing the hydroxylysine glycosides of collagen, are clearly separated (compare fractions 3 with 4 and 5). Fraction 5 contains large amounts of peptides and amino acids, but these are not detected by the orcinol–sulphuric acid reagent.

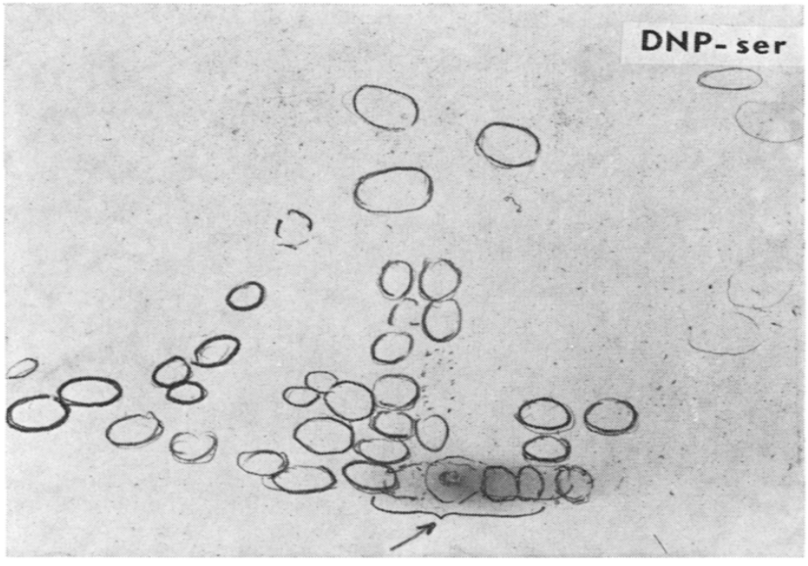

Fig. 2. Fingerprint of performic acid-treated trypsin-digested pig thyroglobulin on Selecta 1500 plates of 20 × 20 cm. Electrophoresis: pyridine–acetate, pH 3.8, 10 V/cm, 2.5 h. Chromatography: solvent 1. Detection: 0.1% ninhydrin in acetone, spots marked by pencil, followed by spraying with 0.1% orcinol in 2 *N* H_2SO_4 and heating at 100° for 10 min. Glycopeptides are indicated by arrows.

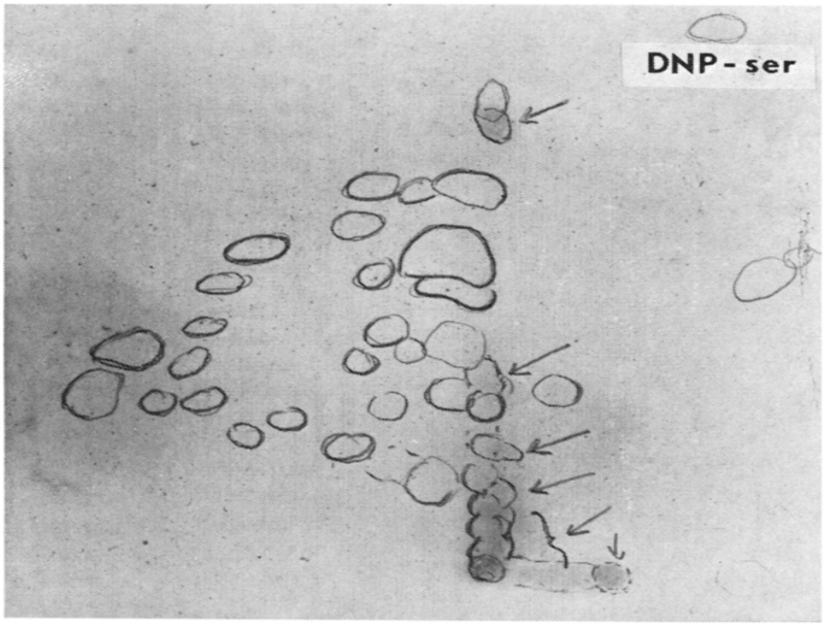

Fig. 3. Fingerprint of trypsin-digested pig thyroglobulin. Electrophoresis as described in Fig. 1. Chromatography: solvent 2.

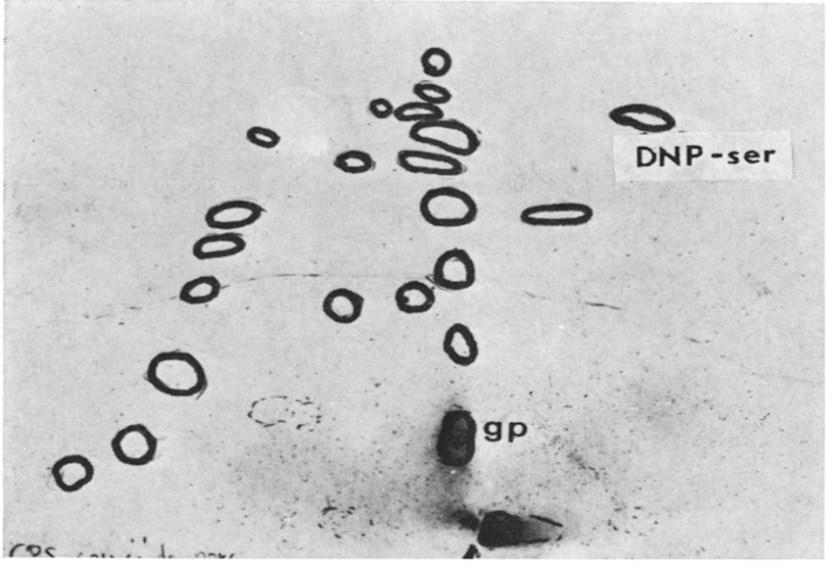

Fig. 4. Fingerprint of chymotrypsin-digested crude structural glycoprotein of corneal stroma. Electrophoresis and detection as described in Fig. 1. In solvent No. 2, used for this experiment, one of the glycopeptides (gp) migrates also.

TABLE I

DETECTION OF DIFFERENT SUGAR CONSTITUENTS OF VARIOUS GLYCANS ON SILICA THIN LAYERS

Treatment	*Sample*					
	Orosomucoide (human)	*Polysaccharide of B. cereus W*	*Hydroxylysine glucosylgalactoside*	*Chondroitin sulphate A*	*Amylose*	*Specificity of the reaction; detection limit of the constituents (µg)*
	Sugar constituents					
	Mannose, galactose, glucosamine, sialic acid	*Galactose, glucosamine*	*Galactose, glucose*	*Galactosamine, glucuronic acid*	*Glucose*	
Resorcinol–HCl[14]	Brownish green	—[a]	—	—	—	Sialic acid; 0.1–0.2
2 h at 100°, HCl vapour	Brown	—	—	—	—	Sialic acid; 0.2–0.3
2 h at 100°, HCl vapour, Elson–Morgan reaction	Brown	Pink	—	Pink	—	Hexosamines (pink); 1–2
H_2SO_4–borate + carbazole	—	—	—	Violet-red	—	Uronic acids; 0.2–0.3
H_2SO_4–cysteine	Brownish green	Violet	Violet-blue	—	Green	Hexoses; 0.2–0.3
H_2SO_4–orcinol	Violet	Blue	Violet	Bluish grey	Blue	Hexoses, hexosamines, uronic acids; 0.1–0.2

[a] — = no reaction.

Figs. 2, 3 and 4 show examples of the fingerprinting procedure. The plates were treated after electrophoresis and chromatography with the ninhydrin reagent, the spots corresponding to the peptides were marked with a pencil and the carbohydrate-containing peptides were made visible on the same plate by spraying with the orcinol reagent.

Thyroglobulin contains, per molecule (M.W. 669 000), fourteen sialic acid-containing sugar chains (M.W. about 3000) and nine "light" carbohydrate chains, which are devoid of sialic acid[12]. The fingerprints (Figs. 2 and 3) seem to be consistent with these results. Only peptides that are not bound to sugars migrate in the chromatographic step in solvent 1 (Fig. 2), while neutral and acidic glycopeptides are separated by electrophoresis. The electrophoretically neutral glycopeptides migrate in solvent 2, while the acidic ones are displaced only in the electrophoretic step. Similar results were obtained by fingerprinting the structural glycoprotein of pig cornea[10].

Peptides and amino acids give a violet colour with the ninhydrin reagent on the silica-coated plates, whereas they gave reddish and brownish colours on the Selecta 1500 sheets. The orcinol reaction is equally sensitive on Selecta 1500 sheets or plates.

Some examples of the specific staining of the different sugar constituents of glycans are given in Table I. Only the resorcinol and the carbazole reactions appear to be specific, for the sialic and uronic acids, respectively. The sensitivity of the Morgan–Elson reaction is poor; the pink colour characteristic of the hexosamines is weak, and could be covered by the brown decomposition products of neuraminic acids.

The colour obtained with the cysteine–sulphuric acid reaction turns to greyish brown in 10–20 min, even at room temperature.

The orcinol reaction can be used quite generally, it gives violet–bluish colours with hexoses, pentoses, hexosamines and uronic acids. Amino acids and peptides do not react. About 0.1 μg of hexoses can be detected with this reagent. The development of the colour is not influenced by the previous treatment of the silica layer with the ninhydrin reagent.

The method is recommended for the separation and preliminary characterisation of glycopeptides, as well as for structural studies on small amounts of glycoproteins.

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