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ELECTROPHORESIS OF URONIC ACIDS, NEUTRAL SUGARS AND HYDROLYSATES OF ACIDIC POLYSACCHARIDES ON SILYLATED GLASS-FIBRE PAPER IN ELECTROLYTES OF BIVALENT CATIONS

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SUMMARY

The separation of different uronic acids, oligogalacturonic acids and neutral sugars by electrophoresis on silylated glass-fibre paper is described. As electrolytes 0.1 *M* solutions of barium, zinc and calcium acetate were used. In these solutions the separation of acid constituents was possible without interference from neutral sugars. Depending on the electrolytes, a mixture of galacturonic, mannuronic, glucuronic and guluronic acid was separatad within 90–165 min. The partial characterization of acid polysaccharides such as pectin, alginate and mixtures thereof is described.

INTRODUCTION

Different workers have described the electrophoretic separation of uronic acids or glycosaminoglycans, having uronic acids as monomeric constituents, in electrolytes containing bivalent metal ions, using cellulose or derivatives thereof as supporting materials[1–7]. A review of previous work was published by Deyl[8].

In our previous work[9], electrophoresis on silylated glass-fibre paper was used to separate neutral sugars and polysaccharide hydrolysates. Compared with supporting materials such as cellulose acetate and cellulose paper, the chemically inert glass-fibre paper exhibits different advantages, such as small electroendoosmotic flow and little adsorption of the sample at the surface, and permits the staining of the separated substances even with aggressive chemical reagents. Further, electrophoresis on glass-fibre paper allows the simultaneous separation of low- and high-molecular-weight substances. The 0.05-*M* borate buffer (pH 9.2) used gives good separations of neutral sugars. However, this buffer system is not suitable for the separation of uronic acids.

The aim of this investigation was to develop an electrophoretic method that could be used for the identification of uronic acids and other hydrolysis products of acidic polysaccharides without interference from occasionally present neutral sugars.

In the experiments described here, electrophoresis on silylated glass-fibre paper was used for this purpose. Various electrolyte systems were tested (as described by

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Deyl[8]), in order to achieve optimal separations. Further, the possibility of characterizing acidic polysaccharides after hydrolysis without previous fractionation and/or derivatization processes is demonstrated.

EXPERIMENTAL

Equipment

For electrophoresis an LKB (Bromma, Sweden) 2117 Multiphor instrument equipped with a cooling plate was used.

Chemicals

Zinc acetate dihydrate, calcium acetate and barium acetate, all of analytical-reagent grade, were purchased from Fluka (Buchs, Switzerland). The following reference substances were used: oligogalacturonic acids (prepared by enzymatic degradation of sodium pectate and separated according to Hasegawa and Nagel[10]), galacturonic acid (GalA), glucuronic acid (GlcA), mannuronic acid (ManA) lactone and myo-inositol (Sigma, St. Louis, MO, U.S.A.), guluronic acid (GulA) [not available commercially; a hydrolysate of sodium alginate or propylene glycol alginate with 2 *M* trifluoroacetic acid (TFA) containing sufficient GulA was used[9]]. Apple pectins, degree of esterification (*DE*) 37% and 73% and amidated pectin, degree of amidation (*DA*) 28.6% and 18.4% were obtained from Obipektin (Bischofszell, Switzerland), sodium alginate and propylene glycol alginate from Kelco (San Diego, CA, U.S.A.), Protan (Drammen, Norway) and Fluka (Buchs, Switzerland).

Hydrolysis of polysaccharides

The preparation of polysaccharide hydrolysates was performed as described in the accompanying paper[9].

Electrolytes

Solutions (0.1 *M*) of zinc, barium and calcium acetate in deionized, deaerated water were used as electrolytes for the electrophoretic experiments.

Electrophoresis

Electrophoresis was carried out at a constant current of 50 mA. Depending on the electrolyte used, a potential gradient of 16–26 V/cm was built up. The system was cooled with tap water at 8–10°C and equilibrated for 15 min prior to sample application. The time needed for optimum separation of the samples was dependent on the electrolyte–buffer used. Electrophoresis in zinc and calcium acetate solution was carried out for 90–120 min and in barium acetate solution for up to 165 min. The current was increased to 70 mA in this buffer system. After each electrophoretic run, the platinum electrodes were cleaned in 1 *M* hydrochloric acid. With calcium acetate buffer, the electrodes had to be cleaned from deposits during the electrophoretic run to prevent a sudden increase in the potential gradient. With glucose (Glc) taken as zero, the mobilities were measured and expressed relative to the mobility of glucuronic acid (GlcA) and reported as M_{GlcA}.

The migration zones were stained with 1,3-naphthalenediol reagent[9]. For the detection of cyclitols, the glass-fibre paper was sprayed with a 0.5% (w/v) solution of potassium permanganate in 1 *M* sodium hydroxide.

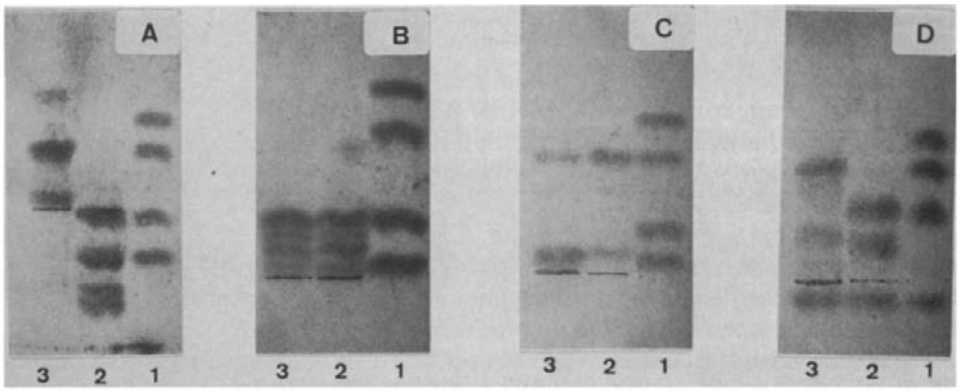

Fig. 1. Electrophoresis of uronic acids and neutral sugars. 0.1 *M* electrolytes of bivalent cations; detection with 1,3-naphthalenediol reagent. (A) 0.1 *M* barium acetate (70 mA, 125 min): 1 = uronic acid mixture (GalA, ManA and GlcA)–Glc; 2 = 2,3,4,6-tetra-O-methyl-D-Glc; 3 = Rha; 4 = Ara. (B) 0.1 *M* barium acetate (70 mA, 140 min): 1 = uronic acid mixture–Glc; 2 = 2,3,4,6-tetra-O-methyl-D-Glc; 3 = Rib; 4 = Frc. (C) 0.1 *M* zinc acetate (50 mA, 120 min): 1 = uronic acid mixture–Glc; 2 = 2,3,4,6-tetra-O-methyl-D-Glc; 3 = Man; 4 = Gal. (D) 0.1 *M* calcium acetate (50 mA, 120 min): 1 = uronic acid mixture–Glc; 2 = 2,3,4,6-tetra-O-methyl-D-Glc; 3 = Rib; 4 = Frc.

RESULTS AND DISCUSSION

Migration properties of uronic acids

In the electrophoresis, a reference mixture composed of Glc (mobility taken as zero), ManA, GalA and GlcA (mobility marker) was applied. The separation of the examined acids was complete in all three electrolytes used (Fig. 1). The mobility values determined in the zinc and calcium acetate solutions corresponded to those obtained by St. Cyr[2] (Table I). However, the mobility values in the barium acetate solution are

TABLE I

ELECTROPHORETIC MOBILITIES (M_{GlcA}) OF URONIC ACIDS ON SILYLATED GLASS-FIBRE PAPER

0.1 *M* electrolytes of bivalent cations, 50 mA for zinc and calcium acetate and 70 mA for barium acetate, 90–165 min.

Uronic acid	*Barium acetate*		*Zinc acetate*		*Calcium acetate*	
	M_{GlcA}[a]	*Cyr*[b]	M_{GlcA}[a]	*Cyr*[b]	M_{GlcA}[a]	*Cyr*[b]
Glucuronic acid	1.00	1.00	1.00	1.00	1.00	1.00
Mannuronic acid	0.74	0.79	0.76	0.75	0.81	0.77
Galacturonic acid	0.24	0.42	0.30	0.30	0.55	0.50
Guluronic acid[c]	—[d]	0.26	0.41	0.44	0.41	0.40

[a] Mobility of Glc taken as zero.

[b] Electrophoretic mobilities (M_{GlcA}) determined by St. Cyr[2]. Mobility of myo-inositol taken as zero.

[c] No reference substance available. Mobility determined by electrophoresis of an alginate hydrolysate.

[d] Mobility in the range of neutral sugars.

different. As will be discussed below, this cannot be explained by the fact that St. Cyr used a different marker as zero (myo-inositol). Miyamoto and Nagase[3] reported a poor resolution of uronic acids using barium acetate buffer. This finding conflicts with our results obtained with electrophoresis on silylated glass-fibre paper (Fig. 1A and B), which gave a clear separation of uronic acids.

It is important that all lactones of the uronic acids be transformed into the acidic form prior to electrophoresis. Treatment with sodium hydroxide to split lactones must be done according to the method described previously[11]. Excess of sodium hydroxide should be avoided, and the pH should not be higher than 8.

Migration properties of neutral sugars

The high-voltage electrophoresis experiments of Angyal and Mills[7] with sugars, sugar derivatives and cyclitols showed that an axial–equatorial–axial sequence of three hydroxyl groups on a six-membered ring or three consecutive *cis*-hydroxyl groups on a five-membered ring form a favourable arrangement for complex formation with metal cations. Carbohydrates lacking this sequence exhibit only poor mobility. The common sugars in polysaccharides do not form strong complexes. Ribose, a frequent sugar in nature, has the required sequence for complex formation. However, this sugar is not a constituent of polysaccharides. In electrophoresis, complexed neutral sugars move towards the cathode and therefore do not interfere with uronic acids, which move towards the anode.

For the calculation of mobility values, the mobility of Glc was taken as zero. To compare the mobility values with literature data, the cathodic migration of Glc relative to the stationary marker 2,3,4,6-tetra-O-methyl-D-Glc was determined in all three electrolytes used. In addition, the migration properties of some common constituents of polysaccharides, galactose (Gal), rhamnose (Rha), mannose (Man) and arabinose

TABLE II

ELECTROPHORETIC MOBILITIES (M_{GlcA}) OF NEUTRAL SUGARS ON SILYLATED GLASS-FIBRE PAPER IN 0.1 *M* BARIUM ACETATE (70 mA, 120–165 min) WITH RESPECT TO 2,3,4,6-O-TETRAMETHYL-D-GLUCOSE AND GLUCOSE

Mobility taken as zero	M_{GlcA}	
	2,3,4,6-Tetra-O-methyl-D-glucose	*Glucose*
Glucose	−0.08	0.00
Arabinose	−0.12	−0.03
Mannose	−0.12	−0.04
Galactose	−0.09	−0.01
Rhamnose	−0.07	0.00
Fructose	−0.20	−0.11
Ribose	−0.35	−0.26
Myo-inositol	—[a]	−0.10
2,3,4,6-Tetra-O-methyl-D-glucose	0.00	−0.08

[a] No experimentally determined mobility value. Simultaneous staining of 2,3,4,6-tetra-O-methyl-D-Glc and myo-inositol is not possible with the detection reagent. Referring to eqn. 3 a mobility of M_{GlcA} −0.19 is calculated.

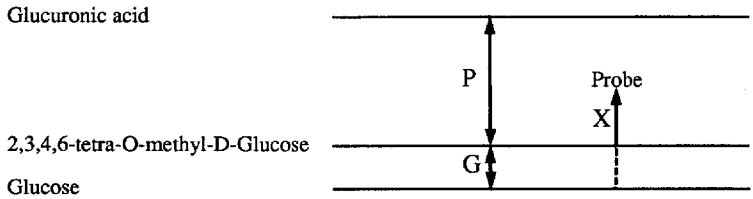

Fig. 2. Conversion of M_e values.

(Ara), were investigated. Ribose (Rib), a sugar forming strong complexes, and fructose (Frc) were also examined.

In 0.1 *M* barium acetate, Glc shows M_{GlcA} −0.08 relative to the stationary marker 2,3,4,6-tetra-O-methyl-D-Glc (Table II, Fig. 1A and B). For comparative purposes, all mobilities in the Tables I–III can be converted to values relative to 2,3,4,6-tetra-O-methyl-D-Glc, as follows (Fig. 2):

$$M_{e1} = (X + G)/(P + G) \tag{1}$$

$$M_{e2} = X/P \tag{2}$$

where $M_{e1} = M_e$ value with Glc taken as zero mobility and $M_{e2} = M_e$ value with 2,3,4,6-tetra-O-methyl-D-Glc as stationary marker. Extension of eqn. 1 with P/P gives

$$M_{e1} = (X/P + G/P)/(P/P + G/P) = (M_{e2} + G/P)/(1 + G/P)$$

leading to

$$M_{e2} = M_{e1}(1 + G/P) - G/P \tag{3}$$

The ratio G/P was determined experimentally and has a value of 0.08 in 0.1 *M* barium acetate.

In the experiments of St. Cyr[2], the mobility of myo-inositol was taken as zero. In 0.1 *M* barium acetate, different mobility data were obtained for the uronic acids. To check whether this is a consequence of the different markers taken as zero, the mobility of myo-inositol was also determined. Myo-inositol shows a mobility of M_{GlcA} −0.10 relative to glucose. Even taking into consideration the higher cathodic movement of myo-inositol, the mobility values of St. Cyr are not comparable to those obtained in our experiments. Ribose, as expected, has the highest mobility in 0.1 *M* barium acetate (Fig. 1B). In contrast to other sugars, ribose migrates as a diffuse zone. The distance for the determination of the electrophoretic mobility of ribose was measured in the zone centre. For the mobilities of the other neutral sugars, see Table II.

In 0.1 *M* zinc acetate, the tested neutral sugars had no measurable cathodic movement (Fig. 1C).

In 0.1 *M* calcium acetate, only a slight migratory difference was observed between the stationary marker 2,3,4,6-tetra-O-methyl-D-Glc and unsubstituted Glc. All sugars examined showed a small cathodic movement. Ribose exhibits the highest mobility as on electrophoresis in a barium acetate electrolyte (Fig. 1D).

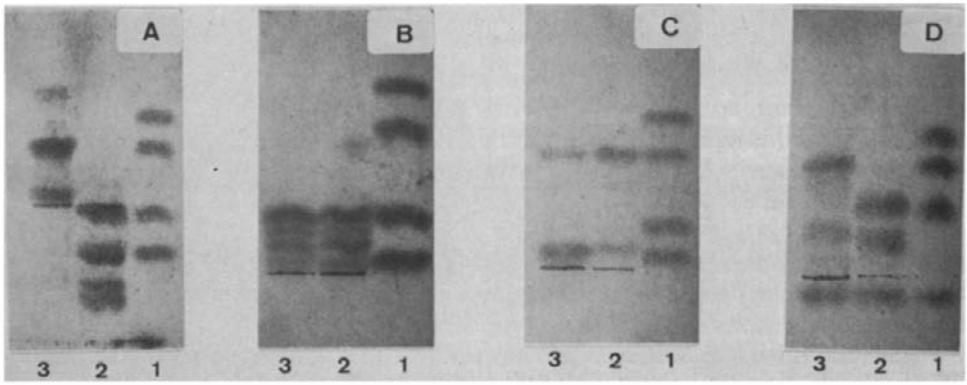

Fig. 3. Electrophoresis of polysaccharide hydrolysates. 0.1 *M* electrolytes of bivalent cations, detection with 1,3-naphthalenediol reagent. (A) 0.1 *M* zinc acetate (50 mA, 90 min): 1 = uronic acid mixture (GalA–ManA and GlcA)–Glc; 2 = hydrolysate of pectin (*DE* 37%); 3 = hydrolysate of propylene glycol alginate. (B) 0.1 *M* barium acetate (70 mA, 165 min): 1 = uronic acid mixture–Glc; 2 = hydrolysate of amidated pectin; 3 = hydrolysate of pectin (*DE* 37%). (C) 0.1 *M* barium acetate (70 mA, 120 min): 1 = uronic acid mixture–Glc; 2 = hydrolysate of propylene glycol alginate; 3 = hydrolysate of sodium alginate. (D) 0.1 *M* calcium acetate (50 mA, 120 min): 1 = uronic acid mixture–Glc; 2 = hydrolysate of pectin (*DE* 73%); 3 = hydrolysate of sodium alginate.

Migration properties of polysaccharide hydrolysates

The electrophoresis method described in this paper is especially suitable for the characterization of hydrolysates of acidic polysaccharides, as uronic acids are efficiently separated. The separation is not disturbed by neutral sugars, hence a selective analysis of the acidic constituents in polysaccharides without interference from neutral sugars is possible. Further, the method is also suitable for the characterization of partially hydrolysed acidic polysaccharides, the separation of low- and high-molecular-weight degradation products in the same run being possible.

As typical examples, the zone patterns obtained by electrophoresis of hydrolysates of pectins, alginates and propylene glycol alginates are discussed. These polysaccharides are easy to distinguish by their different uronic acid constituents (Fig. 3A–D). Hydrolysates of pectins show identical zone patterns independently of *DE* and *DA*. Propylated derivatives of alginates also have a zone pattern independent of *DE*.

TABLE III

ELECTROPHORETIC MOBILITIES (M_{GalA}) OF GALACTURONIC ACID OLIGOMERS ON SILYLATED GLASS-FIBRE PAPER

0.1 *M* electrolytes of bivalent cations, 50 mA for zinc and calcium acetate and 70 mA for barium acetate 90–165 min.

Electrolyte (0.1 M)	*Mono-GalA*	*Di-GalA*	*Tri-GalA*	*Penta-GalA*
Zinc acetate	0.30	0.00	−0.27	−0.40
Barium acetate	0.24	0.18	0.11	0.06
Calcium acetate	0.55	0.40	0.32	0.27

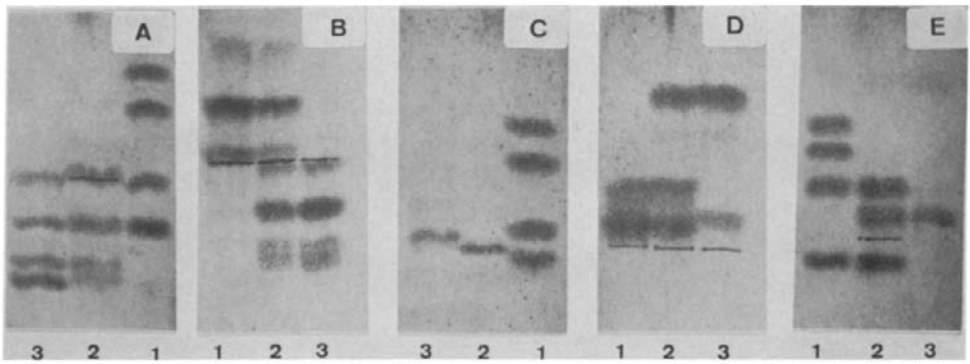

Fig. 4. Electrophoresis of hydrolysates of polysaccharide mixtures and oligo-GalA. 0.1 *M* electrolytes of bivalent cations, detection with 1,3-naphthalenediol reagent. (A) 0.1 *M* zinc acetate (50 mA, 115 min): 1 = uronic acid mixture (GalA, ManA and GlcA)–Glc; 2 = hydrolysate of amidated pectin; 3 = GalA oligomer mixture (mono-, di-, tri-, penta-GalA). (B) 0.1 *M* zinc acetate (50 mA, 120 min): 1 = hydrolysate of propylene glycol alginate; 2 = hydrolysate of mixture of propylene glycol alginate–pectin (*DE* 73%); 3 = pectin (*DE* 73%). (C) 0.1 *M* barium acetate (70 mA, 120 min): 1 = uronic acid mixture–Glc; 2 = penta-GalA; 3 = di-GalA. (D) 0.1 *M* barium acetate (70 mA, 120 min): 1 = hydrolysate of pectin (*DE* 73%); 2 = hydrolysate of mixture of pectin (*DE* 73%)–propylene glycol alginate; 3 = hydrolysate of propylene glycol alginate. (E) 0.1 *M* calcium acetate (50 mA, 95 min): 1 = uronic acid mixture–Glc; 2 = hydrolysate of pectin (*DE* 73%); 3 = GalA oligomer mixture (di-, tri-, penta-GalA).

In addition to the zones of galacturonic acid and neutral sugars, pectins form additional cathodic moving spots (Fig. 3A, Table III) in 0.1 *M* zinc acetate. The components of a mixture of oligo-GalA (mono-, di-, tri- and penta-GalA) are separated using zinc acetate as electrolyte (Fig. 4A). Di-GalA has almost zero mobility. Obviously, the zinc complexation shifts di-GalA to a nearly neutral form. The fractions with M_{GlcA} −0.27 and −0.34 to −0.45 have the mobilities of tri- and penta-GalA, respectively. Penta-GalA forms a distinct zone. The corresponding zone in pectin hydrolysates is broader. This is explained by the fact that tetra-GalA is not completely separated from penta-GalA. A reference sample of tetra-GalA was not available. Even in mixtures with pectins, alginates are easily detected by their uronic acid constituents (Fig. 4B). Alginates form an additional weak zone of M_{GlcA} 1.13 in addition to the two uronic acid fractions. This zone possibly originates from incomplete acid hydrolysis or subsequent reactions. Neutral sugar fractions are detectable in sugar-standardized samples. In addition to the formation of distinct zones, a diffuse smear is visible, probably also as consequence of subsequent reactions during incomplete hydrolysis. Only electrophoresis in zinc acetate produces fractions moving towards the cathode. In 0.1 *M* barium acetate solutions oligo-GalA shows a low mobility on electrophoresis (Fig. 4C, Table III). In pectin hydrolysates, oligo-GalA separates only after a prolonged analysis time. In the hydrolysates of alginates, distinction of GulA from the neutral sugar fraction is difficult. However, alginates are easily identified in mixtures with pectins by the constituent ManA (Fig. 4D). In 0.1 *M* calcium acetate solutions, GulA has a slightly higher mobility than oligo-GalA in pectin hydrolysates (Figs. 3D and 4E, Table III). An identification of the components of a pectin–alginate mixture is possibly by identification of GalA and ManA.

An analysis of acidic constituents by electrophoresis in electrolytes of bivalent cations improves the electrophoresis in borate solutions. Acidic polysaccharides are easily characterized by differences in their uronic acid constituents. In addition to the characterization of acidic polysaccharides, a possible application of this method is the analysis of gelling and thickening agents, especially those which cannot be differentiated by electrophoresis as intact polysaccharides[12].

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