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SEPARATION OF α-D-GALACTURONIC ACID OLIGOMERS BY CHROMATOGRAPHY ON POLYACRYLAMIDE GEL

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SUMMARY

The separation of oligogalacturonic acids (up to nonamer) on Bio-Gel P-2 has been studied. The effects of eluent ionic strength, pH and temperature have been investigated. Ionic exclusion of galacturonic acid was shown when the column was eluted by water. The best separations of oligomers have been obtained with acetate buffer pH 3.6 ($I = 0.1$) at 65°C.

INTRODUCTION

The separation of oligogalacturonic acids is necessary for following an enzymatic or chemical degradation of pectic acid or for studying the mechanism of action of pectolytic enzymes on oligomers or polymers of α-D-galacturonic acid. This fractionation is generally achieved by thin-layer[1,2] or paper chromatography[3–5], but the estimation of each oligomer is difficult and higher oligomers are poorly separated. For these reasons, gel chromatography has been used.

For preparative purposes, Nagel and Wilson[6] and Liu and Luh[7] separated oligogalacturonic acids on Dowex 1-X8 and DEAE-Sephadex, respectively, while Rexova-Benkova[7] used gel filtration on Sephadex G-25 with 0.05 *M* phosphate buffer pH 7 as eluent.

In order to fractionate oligogalacturonic acids after enzymatic hydrolysis of pectic acid, some workers used gel filtration on Bio-Gel P-2 in 0.02 *M* NaCl[8], in 0.05 *M* acetate buffer pH 5.2[9] or on Sephadex G-25 in 0.1 *N* acetic acid[10], with apparently poor resolutions. Nevertheless, excellent separations have been obtained on Bio-Gel P-2 for homologous oligomers of glucose[11–15], mannose[16] or xylose[17,18]. Therefore, for analytical purposes, the fractionation of oligogalacturonic acids on this gel was studied by investigating the effects of eluent ionic strength, pH and temperature.

EXPERIMENTAL

Oligomers and polymer of galacturonic acid

α-D-Galacturonic acid (GA) was obtained from Sigma (St. Louis, MO,

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U.S.A.). A mixture of oligogalacturonic acids was obtained by hydrolyzing polygalacturonic acid using an endopolygalacturonase (E.C. 3.2.1.15)[19,20]; the percentage of hydrolysis, as determined by Nelson's method[21], was 8.5%. Pure oligogalacturonic acids (degree of polymerization, DP, 2–6) were prepared according to Nagel and Wilson[6]. Polygalacturonic acid (PGA) was purchased from ICN (Cleveland, OH, U.S.A.).

Gel chromatography

A jacketed column (203 × 2 cm) was filled with Bio-Gel P-2 (minus 400 mesh, Lot No. 171954; Bio-Rad Labs., Richmond, CA, U.S.A.) as recommended by John *et al.*[15]; chromatography was carried out at a flow-rate of 50 ml/h (Milton-Roy pump) under a pressure of 2–5 kg/cm². Before application of sample, the column was equilibrated by the passage of 2–3 column volumes of appropriate eluent or equilibrated for at least 14 h at the desired temperature. Polyacrylamide matrix is known to be stable in the pH range 1–12 and at high temperature.

Eluents

Distilled water, NaCl solutions and acetate buffers were used as eluents. Before use, they were degassed under vacuum. The acetate buffers were prepared by titrating sodium acetate to the appropriate pH with concentrated acetic acid. The final molarity of sodium acetate was 0.1 *M* and the ionic strengths of the buffers were taken to be 0.1. The pH was measured at room temperature.

Sample application and detection

The sample, up to 100 μl of a 20 mg/ml solution, was injected onto the column via a septum and the eluate was analyzed automatically by the *m*-hydroxydiphenyl method[22]; an aliquot (6 ml/h) of the effluent was introduced into the analytical line.

Determination of the fractionation parameters

The partition coefficient $K_{av.} = (V_e - V_0)/(V_t - V_0)$ is determined for each peak[18]; V_e, V_0 and V_t are the solute elution volume, void volume (as polygalacturonic acid elution volume, determined for each set of experimental conditions of ionic strength, pH and temperature) and total volume (calculated volume, 358 ml), respectively.

The relationships describing the performance parameters[23] are

$$N = 16\left(\frac{V_e}{w}\right)^2, \qquad R = 2\,\frac{V_{e1} - V_{e2}}{w_1 + w_2}$$

where N is the number of theoretical plates, R is the resolution and w is the peak width at the base.

Determination of thermodynamic parameters

The thermodynamic parameters involved in the fractionation process, ΔG^0 (free energy), ΔH^0 (enthalpy) and ΔS^0 (entropy), were calculated by:

$$\Delta G^0 = -RT \ln K_{av.}$$

$$-\ln K_{av.} = \frac{\Delta H^\circ}{RT} - \frac{\Delta S^\circ}{R}$$

The ΔH^0 and ΔS^0 values were obtained by regression analysis from the dependence of ln $K_{av.}$ on $1/T$.

RESULTS AND DISCUSSION

Effects of eluent ionic strength at 65°C

Void volume is independent of the eluent ionic strength, in contrast with the elution volume of galacturonic acid (GA). Fig. 1 shows the variation of the $K_{av.}$ of GA (amount injected, 10 μl of a 20 mg/ml solution): it increases from 0.05 in distilled water to 0.46 at $I = 0.075$ and then remains constant. Under the same conditions, the $K_{av.}$ values for D-galactose are 0.63 and 0.62 when the column is eluted by distilled water and 0.1 *M* NaCl, respectively.

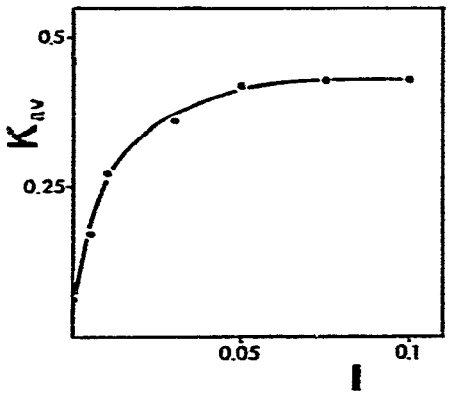

Fig. 1. Variation of the $K_{av.}$ value of galacturonic acid (amount injected, 10 μl of a 20 mg/ml solution) with the eluent ionic strength (I), fixed by addition of NaCl at 65°C.

The early elution of GA could be explained by ionic exclusion between GA and the few negatively charged groups of Bio-Gel P-2[24]; the pK value for GA is 3.4–3.5[25]. This was demonstrated by injecting increasing amounts of GA on the column eluted by distilled water. The elution profiles (Fig. 2) are characterized by a skewed leading edge starting at the void volume, and a sharp tail; with an increase in the amount injected, the peak maximum is displaced towards greater elution volumes but the slope of the leading edge is constant. Such elution profiles are typical of the ion-exclusion mechanism[26].

The oligomer mixture was chromatographed with 0.1 *M* NaCl as eluent. The logarithms of the $K_{av.}$ values of the oligogalacturonic acids are linearly related to the DP; fractionation parameters ($K_{av.}$ and resolution R) are given in Table I.

Effects of eluent pH

The effect of eluent pH (0.1 *M* acetate buffer) at 65°C on the separation of the oligomer mixture was investigated. Over the range of eluent pH used (3.6–5.6) a linear relationship was found between log $K_{av.}$ and DP, and peak widths were independent of the amount injected. At pH 5.6, oligomers containing 1–5 GA units are separated. When the elution pH is decreased, peaks of higher oligomers appear:

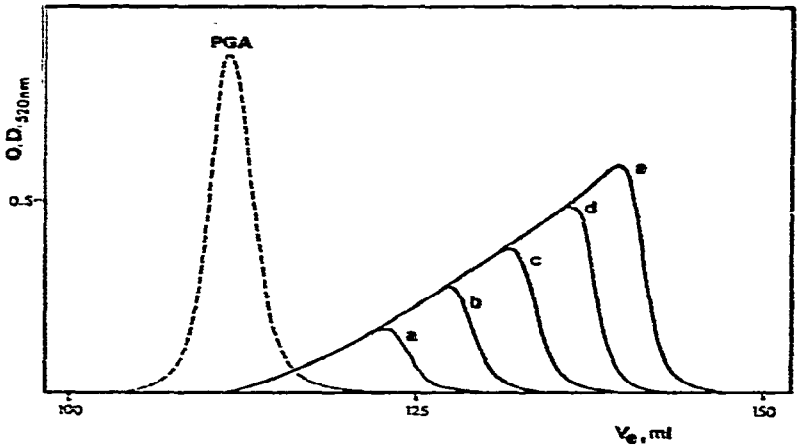

Fig. 2. Elution profiles, at 65°C, of galacturonic acid in water at different concentrations. Amount injected of a 20 mg/ml solution: a, 5 μl; b, 10 μl; c, 20 μl; d, 30 μl; e, 40 μl. PGA = Elution volume of polygalacturonic acid, as void volume.

TABLE I

FRACTIONATION PARAMETERS OF OLIGOGALACTURONIC ACIDS AT 65°C IN 0.1 *M* NaCl AND 0.1 *M* ACETATE BUFFERS AT DIFFERENT pH VALUES

The calculation of $K_{av.}$ and R is described in the Experimental section.

Oligomer	*NaCl 0.1 M*		*pH 3.6*		*pH 3.8*		*pH 4*		*pH 4.5*		*pH 5*		*pH 5.6*	
	$K_{av.}$	R	$K_{av.}$	R	$K_{av.}$	R	$K_{av.}$	R	$K_{av.}$	R	$K_{av.}$	R	$K_{av.}$	R
GA_1	0.46		0.66		0.62		0.59		0.52		0.48		0.45	
		3.9		3.0		3.3		3.8		4.5		4.8		57
GA_2	0.28		0.52		0.47		0.43		0.34		0.30		0.26	
		2.3		2.5		2.6		2.8		3.0		2.9		3.1
GA_3	0.17		0.40		0.35		0.31		0.23		0.19		0.16	
		1.4		2.0		2.0		2.1		2.0		1.8		2.1
GA_4	0.11		0.31		0.26		0.22		0.15		0.12		0.10	
		0.8		1.5		1.4		1.5		1.3		1.0		1.0
GA_5	0.07		0.24		0.20		0.16		0.10		0.07		0.06	
				1.1		0.8		1.0		0.7		—		—
GA_6			0.19		0.15		0.12		0.06		0.05		—	
				0.7		0.6		0.5		—				
GA_7			0.14		0.11		0.08		0.04					
				0.4		—		—		—				
GA_8			0.11		0.08		0.06		—					
				—*		—		—						
GA_9			0.08											

* No accurate measurement can be made.

Ga_6* at pH 5, GA_7 at pH 4.5, GA_8 at pH 4, GA_9 at pH 3.8 and GA_{10} at pH 3.6, but precise determinations of $K_{av.}$ values for these peaks were not possible. Typical chromatograms at pH 5 and 3.6 are shown in Fig. 3A and 3C.

* GA_n = oligogalacturonic acid, n = degree of polymerization.

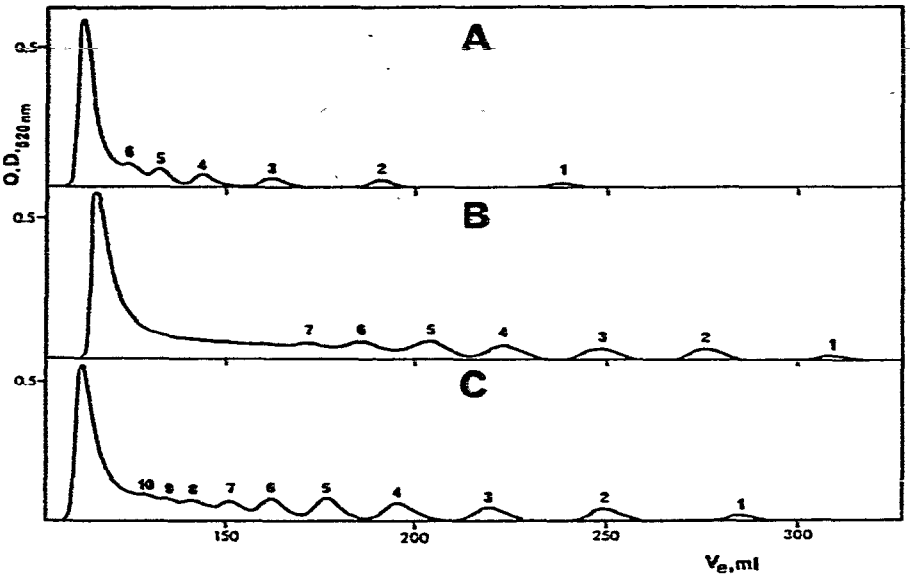

Fig. 3. Elution profiles of oligogalacturonic acids on a Bio-Gel P-2 column (203 × 2 cm). A, 0.1 *M* acetate buffer pH 5 at 65°C; amount injected, 30 μl of a 20 mg/ml oligomer solution. B, 0.1 *M* acetate buffer pH 3.6 at 25°C; amount injected, 50 μl of a 20 mg/ml oligomer solution. C, 0.1 *M* acetate buffer pH 3.6 at 65°C; amount injected, 60 μl of a 20 mg/ml oligomer solution. The numbers 1, 2, 3, ... 10 refer to the degree of polymerization.

Fractionation parameters ($K_{av.}$, resolution R) are summarized in Table I. $K_{av.}$ values increased with decreasing pH. When the pH is decreased, resolutions between GA and GA_2, and between GA_2 and GA_3, decrease and the resolution between GA_3 and GA_4 is constant, while resolution between higher oligomers is increased. The number of theoretical plates varied in a similar way.

Better separations could be achieved if the elution pH was lower than 3.6 but,

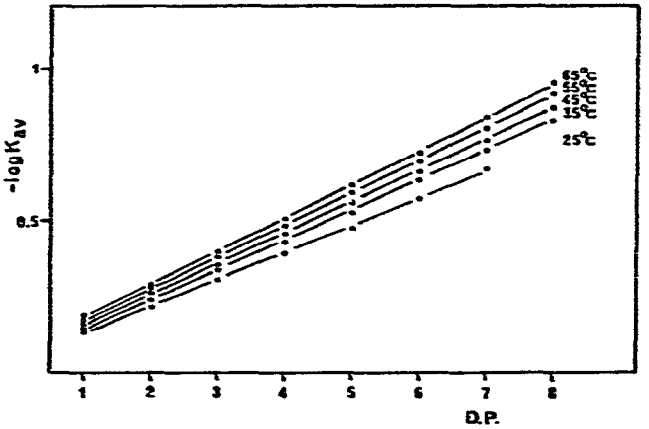

Fig. 4. Relationship between $-\log K_{av.}$ and the degree of polymerization (DP) of oligogalacturonic acids at different elution temperatures (0.1 *M* acetate buffer pH 3.6).

TABLE II

EFFECT OF ELUTION TEMPERATURE, IN 0.1 *M* ACETATE BUFFER pH 3.6, ON THE FRACTIONATION PARAMETERS OF OLIGOGALACTURONIC ACIDS

The calculation of $K_{av.}$, R and V_0 (void volume) is described in the Experimental section.

Oligomer	*25°C*		*35°C*		*45°C*		*55°C*		*65°C*	
	$K_{av.}$	R	$K_{av.}$	R	$K_{av.}$	R	$K_{av.}$	R	$K_{av.}$	R
GA_1	0.74		0.72		0.69		0.67		0.66	
		2.0		2.2		2.7		2.9		3.0
GA_2	0.62		0.58		0.56		0.54		0.52	
		1.6		1.8		2.1		2.3		2.5
GA_3	0.51		0.47		0.44		0.42		0.40	
		1.3		1.6		1.7		1.8		2.0
GA_4	0.41		0.38		0.35		0.33		0.31	
		0.9		1.2		1.3		1.4		1.5
GA_5	0.33		0.30		0.28		0.26		0.24	
		0.6		0.8		1.0		1.1		1.1
GA_6	0.27		0.24		0.22		0.20		0.19	
		0.3		0.5		0.6		0.7		0.7
GA_7	0.21		0.19		0.17		0.16		0.14	
		—		—		—		0.3		0.4
GA_8	—*		0.15		0.13		0.12		0.11	
		—		—		—		—		—
GA_9	—		—		—		0.09		0.08	
V_0 *(ml)*	116		114		113		112.5		112	

* No accurate measurement can be made.

in this case, some degradation in the solute structure, such as depolymerization or saponification, could occur if pectin hydrolysate is chromatographed[27].

Effects of eluent temperature

The effect of eluent temperature was investigated in 0.1 *M* acetate buffer (pH 3.6). The log $K_{av.}$ values were linearly related to DP in the temperature range 25–65°C (Fig. 4) and the peak widths are independent of the oligomer concentration. Typical chromatograms at 25°C and 65°C are shown in Fig. 3B and C.

TABLE III

THERMODYNAMIC PARAMETERS FOR OLIGOGALACTURONIC ACIDS ON POLYACRYLAMIDE GEL P-2

Degree of polymerization of oligomer	*$\Delta G°$ (at 65°C) (cal/mole)*	*$\Delta H°$ (cal/mole)*	*$\Delta S°$ (cal/mole·K)*
1	279	−612	−2.6
2	437	−855	−3.8
3	605	−1124	−5.1
4	777	−1341	−6.3
5	947	−1579	−7.5
6	1117	−1766	−8.5
7	1289	−1888	−9.4
8	1468	−2032	−10.4

As shown in Table II, the K_{av} and V_0 values decrease with increasing temperature in contrast to the increase in resolution. However, the resolution can be considered satisfactory at each temperature tested. These results are in good agreement with those reported by other workers for neutral oligosaccharides[11–13,28].

Values of $\Delta G°$, $\Delta H°$ and $\Delta S°$ are given in Table III. The large and negative $\Delta H°$ values reflect strong interactions between the oligomers and the gel which increase with DP[12].

CONCLUSIONS

Ionic exclusion between galacturonic acid and the polyacrylamide gel was demonstrated when the gel is eluted by water. This interaction was avoided by increasing the ionic strength of the eluent up to 0.1.

The separation of the oligogalacturonic acids was shown to be pH-dependent (0.1 *M* acetate buffers). Good fractionation of oligomers of DP 1–10 can be obtained by decreasing the elution pH to 3.6.

The higher the temperature, the better is the resolution and the negative temperature dependence of the elution volume indicates that a mechanism other than steric exclusion operates in the separation process of oligogalacturonic acids. This phenomenon has been also reported for neutral oligosaccharides.

The resolutions are better than those reported elsewhere on Bio-Gel P-2 or Sephadex G-25[7–10] and the separation is less time-consuming than with ion-exchange, but the chromatography on Bio-Gel P-2 is only suitable for analytical purposes.

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REFERENCES

1 A. Koller and H. Neukom, *Biochim. Biophys. Acta*, 83 (1964) 366.
2 Y. K. Liu and B. S. Luh, *J. Chromatogr.*, 151 (1978) 39.
3 R. J. Young and M. E. Corden, *Biochim. Biophys. Acta*, 83 (1964) 124.
4 R. Pressey and R. S. Allen, *J. Chromatogr.*, 16 (1964) 248.
5 O. T. Page, *Phytopathology*, 51 (1961) 337.
6 C. W. Nagel and T. M. Wilson, *J. Chromatogr.*, 41 (1969) 410.
7 L. Rexova-Benkova, *Chem. Zvesti*, 24 (1970) 59.
8 F. E. A. van Houdenhoven, P. J. G. de Wit and J. Visser, *Carbohydr. Res.*, 34 (1974) 233.
9 P. D. English, A. Maglothin, K. Keegstra and P. Albersheim, *Plant Physiol.*, 49 (1972) 293.
10 J. H. McClendon, *J. Chromatogr.*, 171 (1979) 460.
11 W. Brown, *J. Chromatogr.*, 52 (1970) 273.
12 A. Heyraud and M. Rinaudo, *J. Chromatogr.*, 166 (1978) 149.
13 N. K. Sabbagh and I. S. Fagerson, *J. Chromatogr.*, 120 (1976) 55.
14 K. Kainuma, A. Nogami and C. Mercier, *J. Chromatogr.*, 121 (1976) 361.
15 M. John, G. Trénel and H. Dellweg, *J. Chromatogr.*, 42 (1969) 476.
16 W. Brown and K. Chitumbo, *J. Chromatogr.*, 66 (1972) 370.
17 J. Havlicek and O. Samuelson, *Carbohydr. Res.*, 22 (1972) 307.
18 W. Brown and Ö. Andersson, *J. Chromatogr.*, 67 (1972) 163.

19 J. F. Thibault and C. Mercier, *J. Solid-Phase Biochem.*, 2 (1977) 295.
20 J. F. Thibault and C. Mercier, *J. Food Biochem.*, 2 (1978) 379.
21 N. Nelson, *J. Biol. Chem.*, 153 (1944) 375.
22 J. F. Thibault, *Lebensm. Wiss. Technol.*, 12 (1979) 247.
23 J. J. Kirkland, *Modern Practice of Liquid Chromatography*, Wiley, New York, 1971.
24 R. L. Pecsok and D. Saunders, *Separ. Sci.*, 3 (1968) 325.
25 R. Kohn and P. Kovac, *Chem. Zvesti*, 32 (1978) 478.
26 A. R. Cooper and S. van Derveer, *J. Liquid Chromatogr.*, 1 (1978) 693.
27 J. J. Doesburg, *Pectic Substances in Fresh and Preserved Fruits and Vegetables, I.B.V.T. Communication No. 25*, Institute for Storage and Processing of Horticultural Products, Wageningen, 1965, p. 44.
28 H. Dellweg, M. John and G. Trenel, *J. Chromatogr.*, 57 (1971) 89.