Journal of Chromatography, 603 (1992) 139–143
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 090

Separation of oligogalacturonic acids by high-performance gel filtration chromatography on silica gel with diol radical

J. Naohara

Environmental Resources Research Centre, Okayama University of Science, Okayama 700 (Japan)

M. Manabe

Faculty of Agriculture, Kagawa University, Kagawa 761-02 (Japan)

(First received November 1st, 1991; revised manuscript received February 6th, 1992)

ABSTRACT

Oligogalacturonic acids (OLGAs) ranging from two to nineteen residues in length were separated using high-performance gel filtration chromatography on a silica gel with diol radical. The optimum conditions (eluent, column temperature) for separation of OLGAs by high-performance gel filtration chromatography were investigated. The column used in this experiment allowed a high pressure of 4900 p.s.i. and a flow-rate of 2 ml/min. The stationary phase of silica gel stabilized the separation of OLGAs. The peaks of OLGAs separated using this column were assigned by comparing retention times with standards, and the molecular weights of the corresponding OLGAs were determined by fast atom bombardment mass spectrometry.

INTRODUCTION

The separation of oligogalacturonic acids (OLGAs) from chemically and enzymatically degraded polygalacturonic acid is important in the structural analysis of the cell wall in plants. It is also important for studying the mechanism of action of pectin-degrading enzymes. The oligomers are recognized to be capable of regulating a number of physiological responses in plants, including the endogenous elicitation and induction of phytoalexin [1–3], an increase in the activity of phenylalanine ammonia lyase [4], the production of a proteinase inhibitor [5], lignification, and the induction of chitinase and β-1,3-glucanase [6]. OLGAs are involved in the fixation of extesin and lignin into the cell wall in plants [7]. It is reported that OLGAs have antimicrobial action [8]. Therefore, it is necessary to be able to separate OLGAs rapidly and to detect OLGAs with a wide range of degree of polymerization (DP) in these investigations.

OLGAs have been analysed by thin-layer chromatography [9–11], ion-exchange chromatography [12–21], gel filtration chromatography [22,23], reversed-phase chromatography [13,17,24–26], electrophoresis [27], ultracentrifugation [27] and gas–liquid chromatography [28]. However, these analyses have the disadvantages of a long analysis time, a low distribution coefficient and poor sensitivity of longer OLGAs.

High-performance gel filtration (HPGF) chromatography is one of the best methods of analysing oligosaccharides, especially longer-DP oligomers, in a short time. HPGF chromatography has the advantage that no sample derivatization is required. In the present work, HPGF chromatography was per-

Correspondence to: Dr. J. Naohara, Environmental Resources Research Centre, Okayama University of Science, Okayama 700, Japan.

0021-9673/92/$05.00

formed using a column packed with hydrophilic and porous spherical silica gel of 5 μm particle size covered with a high degree of alcoholic OH radicals. This column allows the continuous use of high pressure and high flow-rate, and the separation of samples is stabilized as a result of the fact that silica gel is harder than polymer gels. The column can endure pressures as high as 4900 p.s.i. The pH of eluents can be between 5 and 8. The temperature of the column can range from 4 to 40°C.

The optimum conditions for the separation of OLGAs were investigated and the molecular weights of OLGAs were determined by fast atom bombardment mass spectrometry (FAB-MS).

EXPERIMENTAL

Materials

α-D-Galacturonic acid, digalacturonic acid, trigalacturonic acid, polygalacturonic acid and endo-polygalacturonase (endo-PG) (EC 3.2.1.15) were from Sigma (St. Louis, MO, USA).

Preparation of OLGAs

A mixture of OLGAs was produced by enzyme digestion of polygalacturonic acid at 30°C for 40 min with endo-PG. The reaction mixture was heated at 100°C for 10 min to terminate the reaction. Ethyl alcohol was added to the reaction mixture at a final concentration of 65% after cooling. The resulting precipitates of OLGAs separated by centrifugation (4530 *g*, 20 min) were dissolved in water and lyophilized. The OLGAs were treated with 0.05 *M* sodium hydroxide at 0°C for 90 min. The de-esterified OLGAs were precipitated and separated by centrifugation (4530 *g*, 20 min). They were dissolved in water and lyophilized.

HPGF chromatography

HPGF chromatography was performed on a Waters isocraic manual system (Waters, Division of Millipore, Tokyo, Japan) with a Waters R-401 refractive index (RI) detector. For the DP analysis of OLGAs, a YMC Diol 120 column (500 × 8 mm I.D.) and a precolumn (30 × 8 mm I.D.) (YMC, Kyoto, Japan) were used. A column oven was used to maintain the temperature at 25–50°C (Sugai U620, Sugai, Wakayama, Japan). An acetate buffer and a phosphate buffer were used in elution. Volumes of 25 μl of the liquid oligomers were injected. Chromatographic data were plotted and integrated using a Waters 714 data module.

Measurement of the fractionation parameter

The distribution coefficient, K_{av}, was calculated from the retention time of each peak as follows:

$$K_{av} = (V_e - V_0)/(V_t - V_0)$$

where V_e is elution volume of OLGAs or standards, V_0 is the void volume (verified by polygalacturonic acid) and V_t is the total bed volume.

Preparation of authentic OLGAs

The OLGAs were separated according to the modified method af Nothnagel *et al.* [1]. High-performance ion-exchange chromatography was performed using a Waters automatic gradient system with an SF-2120 Advantec fraction collector (Advantec Toyo, Tokyo, Japan). For the separation of the oligomers QAE-Sephadex A-25 in a Waters AP-1 glass column (600 × 10 mm I.D.) was used. The mixture of OLGAs (*ca.* 500 mg) was dissolved in 20 ml of 0.2 *M* imidazole–hydrochloric acid buffer, pH 7.0, and the solution was injected into the column. The OLGAs were eluted with a linear gradient of 0.2 *M* to 1.0 *M* imidazole–hydrochloric acid buffer, pH 7.0. The flow-rate was 2 ml/min and the volume of the collected fractions was 10 ml. The collected fractions were concentrated and desalted by a Microacilyzer S1 (Asahi Kasei, Tokyo, Japan), an automatic desalting device using AC-210-10 ion-exchange films. Each purified OLGA fraction was lyophilized.

FAB-MS analysis

The molecular weights of fractionated and purified OLGAs were analysed by FAB–MS. The device used was a Jeol JMS-GX 303HF (Jeol, Tokyo, Japan). Xenon was used as the bombarding gas, and an atom gun was operated at 6 kV, 20 mA. Samples were dissolved in methanol–water (1–5 μg/μl) and 1 μl of each sample solution was added to a drop of glycerol on the stainless-steel target.

RESULTS AND DISCUSSION

Effect of the ionic strength of the eluent

The effect of ionic strength (sodium chloride) on

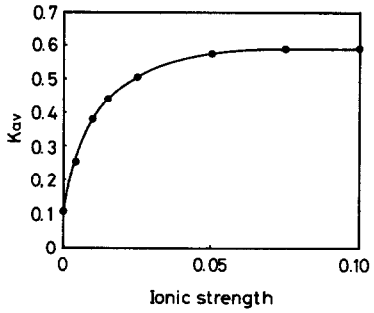

Fig. 1. Variation in the K_{av} value of galacturonic acid (amount injected: 25 μl of a 10 mg/ml solution) with the ionic strength of eluent.

the K_{av} of galacturonic acid (10 mg/ml, 25 μl) is shown in Fig. 1. The K_{av} of galacturonic acid increased with an increase in the ionic strength of the eluent, and the K_{av} of galacturonic acid attained an almost constant value of 0.56 when the ionic strength of the eluent was higher than 0.05. This tendency was the same as that of the effect of ionic strength on the K_{av} of galacturonic acid using the Bio-Gel P2 column [18].

The effect of the injected volume (5–100 μl) on the retention time of galacturonic acid (10 mg/ml) was examined. When a 0.05 *M* sodium chloride solution was used as the eluent, the retention time of galacturonic acid lengthened a little as the volume injected increased, but the increase was negligible under the experimental conditions. The retention times were between 19.1 and 19.2 min.

Effect of the pH of the eluent

The effect of the pH of the acetate buffers on the retention time of the OLGAs is shown in Fig. 2. The retention times of OLGAs with different DP values increased as the pH of the acetate buffer solution was increased. The retention times of OLGAs in 0.2 *M* acetate buffer were longer than those in 0.05 *M* acetate buffer.

Effect of the temperature of the eluent

The effects of the column temperature on the relationship between the DP of OLGAs and $-\log K_{av}$ are shown in Fig. 3. The relationship between the DP of OLGAs and $-\log K_{av}$ was linear at column temperatures in the range 25–50°C. The observed DP was highest at 40–45°C. The $-\log K_{av}$ value of

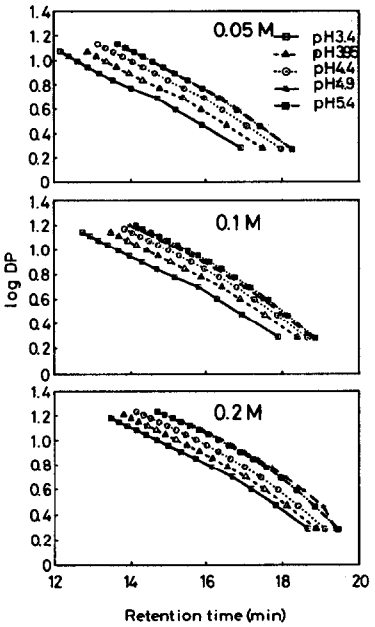

Fig. 2. Effect of pH of acetate buffers on retention time.

OLGAs with the Bio-Gel P2 column [18] increased with the increase in the column temperature. This column was stable under these temperature conditions.

Quantification of oligogalacturonic acid

The monomer, dimer and trimer standards (purchased from Sigma) could be quantified in the 2000-μg region in a given sample applied to the column. When the signal-to-noise ratio was 3, the detection limits of the monomer, dimer and trimer standards were 1.0 ng. It is thought that oligomers

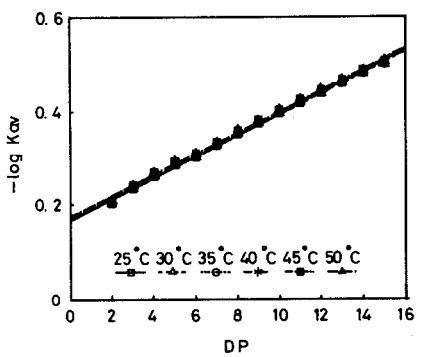

Fig. 3. Relationship between $-\log K_{av}$ and DP.

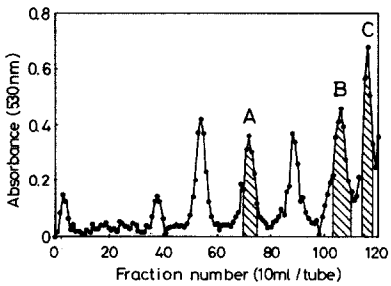

Fig. 4. HPLC of oligogalacturonic acid on QAE-Sephadex A-25.

with DP values larger than 4 may be quantified in the 2000-μg region in a standard solution even though those oligomers are not commercially available.

This column can be used for the identification and quantification of OLGAs in the isocratic elution mode. In the reports that described the separation of OLGAs by ion-exchange column [13,16,21] and reversed-phase columns [13,25,26], the highest DP value of the OLGAs separated in the isocratic elution mode [16] was 10. Since the pressure used for the (cation-exchange) column was limited to only 300 p.s.i. and a high flow-rate led to high back-pressures and to the loss of resolution, it seemed this method usig a cation-exchange column was not practical.

The silica gel column used in this experiment allowed the use of high pressure, 4900 p.s.i., and a high flow-rate, 2 ml/mn. The separation of OLGAs of DP 19 could be possible (see Fig. 6), by using porous (120 Å) spherical silica gel of 5 μm particle size covered with a high degree of alcoholic OH radicals. This simple and rapid method by which OLGAs with DPs of 19 may be separated has not yet been reported.

Molecular weight of each fraction

The OLGAs in the mixture were separated by high-performance ion-exchange chromatography using a QAE-Sephadex A-25 column. Each peak fraction was pooled, desalted with the Microacilyzer S1 and lyophilized. The molecular weights of lyo-

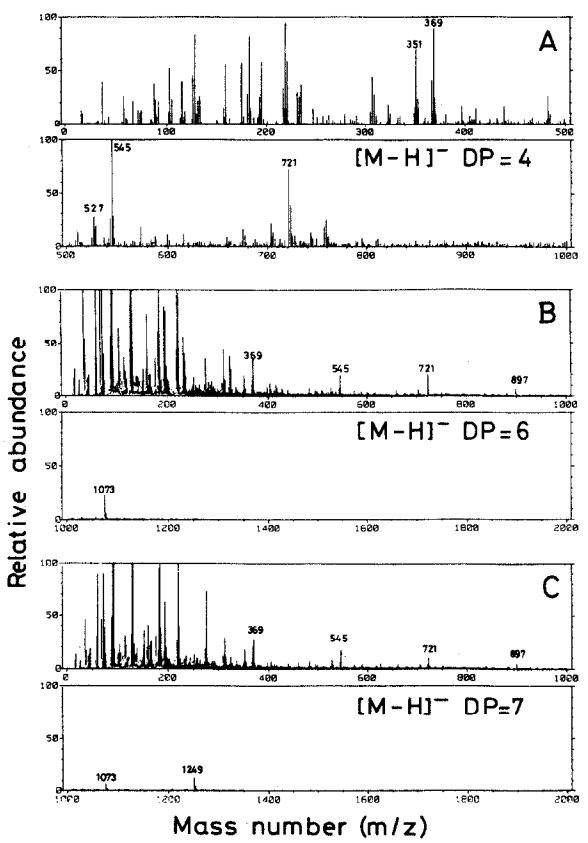

Fig. 5. FAB-MS analysis of oligogalacturonic acid.

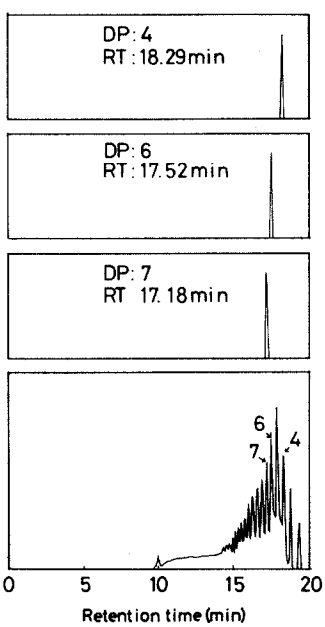

Fig. 6. High-performance gel filtration chromatographic analysis of oligogalacturonic acid. RT = Retention time.

philized OLGAs were maesured by FAB-MS. The results obtained are shown in Fig. 4. These fractions gave distinct molecular ions ($[M - H]^-$). Fraction A had a molecular ion at m/z 721 that corresponds to a molecular weight of 722, as shown in Fig. 5A. This signal should represent a tetragalacturonic acid. Consecutive loses of galacturonosyl residues from $[M-H]^-$ gave signals at m/z 545 and 369. As shown in Fig. 5B and C, the similar spcetra of the oligogalacturonic acids in fractions B and C, having molecular ions at m/z 1073 and 1249, correspond to hexa- and heptagalacturonic acid, respectively. These results are in agreement with the results obtained by Komae *et al.* [19]. The retention times of tetra-, hexa- and heptagalacturonic acid analysed by the HPGF chromatography with the YMC Diol 120 column were 18.31, 17.52 and 17.19 min, respectively (Fig. 6). Some peaks could be detected when the mixture of OLGAs was analysed; the peak at a retention time of 18.29 min was possibly tetragalacturonic acid, that at 17.52 min hexagalacturonic acid and that at 17.18 min heptagalacturonic acid.

REFERENCES

1 E. A. Nothnagel, M. McNeil, P. Albersheim and A. Dell, *Plant Physiol.*, 71 (1983) 916.
2 D. F. Jin and C. A. West, *Plant Physiol.*, 74 (1984) 989.
3 K. R. Davis, A. G. Darvill, P. Albersheim and A. Dell, *Plant Physiol.*, 80 (1986) 568.
4 G. De Lorenzo, A. Ranucci, D. Bellincampi, G. Salvi and F. Cervone, *Plant Sci.*, 51 (1987) 147.
5 P. D. Bishop, G. Pearce, J. E. Bryant and C. A. Ryan, *J. Biol. Chem.*, 259 (1984) 13172.
6 C. A. Ryan, *Annu. Rev. Cell Biol.*, 3 (1987) 295.
7 B. Robertsen, *Physiol. Mol. Plant Pathol.*, 28 (1986) 137.
8 H. Yokotsuka, T. Matsudo, T. Kushida, N. Inamine and T. Nakashima, *Hakkou Kougaku*, 62 (1984) 1 (in Japanese).
9 Y. K. Liu and B. S. Luh, *J. Chromatogr.*, 151 (1978) 39.
10 O. Markovič and A. Slezárik, *J. Chromatogr.*, 312 (1984) 492.
11 L. W. Dower, P. L. Irwin and M. J. Kurantz, *Carbohydr. Res.*, 172 (1988) 292.
12 C. Hatanaka and J. Ozawa, *J. Agric. Chem. Soc. Japan*, 46 (1972) 417 (in Japanese).
13 A. G. J. Voragen, H. A. Schols, J. A. de Vries and W. Pilnik, *J. Chromatogr.*, 244 (1982) 327.
14 M. R. Hardy and R. R. Townsend, *Proc. Natl. Acad. Sci. U.S.A.*, 85 (1988) 3289.
15 A. T. Hotchkiss, K. B. Hicks, L. W. Doner and P. L. Irwin, presented at the *3rd Chemical Congress of North America, Toronto, 1989*, Abstract 95.
16 K. B. Hicks and A. T. Hotchkiss, Jr., *J. Chromatogr.*, 411 (1988) 382.
17 K. B. Hicks, *Adv. Carbohydr. Chem. Biochem.*, 46 (1988) 17.
18 M. O. Maness and A. J. Mort, *Anal. Biochem*, 178 (1989) 248.
19 K. Komae, A. Komae and A. Misaki, *Agric. Biol. Chem.*, 54 (1990) 1477.
20 A. T. Hotchkiss, Jr. and K. B. Hicks, *Anal. Biochem.*, 184 (1990) 200.
21 A. T. Hotchkiss, Jr., K. B. Hicks, L. W. Doner and P. L. Irwin, *Cabohydr. Res.*, 215 (1991) 81.
22 J. H. McClendon, *J. Chromatogr.*, 171 (1979) 460.
23 J. F. Thibault, *J. Chromatogr.*, 194 (1980) 315.
24 A. Heyraud and C. Rochas, *J. Liq. Chromatogr.*, 5 (1982) 403.
25 A. Heyraud and C. Leonard, *Carbohydr. Res.*, 215 (1991) 105.
26 J. F. Preston III and J. D. Rice, *Carbohydr. Res.*, 215 (1991) 137.
27 A. J. Barrett and D. H. Northcote, *Biochem. J.*, 94 (1965) 481.
28 W. R. Raymond and C. W. Nagel, *Anal. Chem.*, 41 (1969) 1700.