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# THE ISOLATION OF OLIGOGALACTURONIC ACIDS BY COLUMN CHROMATOGRAPHY*

C. W. NAGEL AND T. M. WILSON
*Food Sciences Program, Department of Horticulture, Washington State University, Pullman, Wash. 99163 (U.S.A.)*
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SUMMARY

A series of oligogalacturonic acids, including di-, tri-, tetra-, penta-, hexa-, hepta- and octa-galacturonic acids and unsaturated di-, tri-, tetra- and pentagalacturonic acids, were produced by enzymic cleavage of pectic acid and separated by ion exchange column chromatography. Evidence for their purity was presented.

## INTRODUCTION

In order to do more definitive studies on the action of different pectic enzymes, it was necessary to obtain large quantities of well characterized oligogalacturonides. Therefore, an investigation was made of possible methods that could be utilized to obtain pure polymers containing two to eight galacturonides.

DEUEL AND STUTZ[6] in their review of 1958 have summarized the progress made up to that time. In all cases, enzymes were utilized to digest the pectic acid and produce the oligogalacturonides. The oligogalacturonides, di-, tri- and tetragalacturonic acids, have been isolated by precipitation of their salts. Thus, PHAFF AND LUH[19] were able to separate the dimer and trimer by selective precipitation of their lead salts. Subsequently, DEMAIN AND PHAFF[4] isolated the tetramer by precipitation of the copper salt. The three same oligogalacturonides were isolated by McCREADY AND McCOMB[14] with the use of paper chromatography.

The glycol esters of dimer and trimer have been separated by ASHBY *et al.*[1] by charcoal column chromatography. Anion column chromatography has been used to separate dimer, trimer and tetramer[1,5,20]. More recently HATANAKA AND OZAWA[10] utilized DEAE cellulose column chromatography to separate oligogalacturonides up to hexamer.

In the case of the unsaturated oligogalacturonic acids (4,5-dehydrogalacturonosyl unit on the non-reducing end) produced by lyases, the unsaturated dimer has been isolated by precipitation of its strontium salt[17] while the unsaturated dimer, trimer and tetramer have been isolated by Dowex-1 column chromatography[16].

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Of all the techniques used in the past, column chromatography appeared to be the most practical means of obtaining the desired oligomers. Therefore, this paper reports the isolation of both saturated and unsaturated oligogalacturonic acids from enzyme digests of pectic acid with the use of column chromatography.

## MATERIALS AND METHODS

### *Enzymes*

The endopolygalacturonase (YPG) of *Saccharomyces fragilis* No. 351 was prepared according to the procedure of LUH AND PHAFF[11] except that 3% (w/v) glucose was used in the medium. The endopectic acid transeliminase (endo PATE) of a *Bacillus sp.* described by HASEGAWA AND NAGEL[9] was used for the preparation of unsaturated uronide digests. A fungal polygalacturonase preparation, Pectinol 10-M (Lot 346), was kindly supplied by Rohm and Haas, Philadelphia, Pa. This preparation was free of lyase activity and after dialysis against water was used without further purification.

### *Substrates*

All of the oligogalacturonides were prepared from polygalacturonic acid (No. 3491, Sunkist Growers, Corona, Calif.) by enzymic digestion. The method of McCREADY[13] was used for preparation of galacturonic acid except that Pectinol 10-M was substituted for Pectinol 100-M. Paper chromatograms revealed only monomer after 7 days' incubation. The monomer was converted to the free acid with Dowex-50 ($H^+$) and crystallized while being concentrated under reduced pressure. A hot, saturated aqueous solution of monomer was decolorized with carbon and recrystallized by the addition of acetone. The monomer was dried in a vacuum over calcium chloride. The equivalent weight, based on reducing groups, was 226.

The saturated uronides were prepared by hydrolyzing pectic acid at 30° for various reaction times and at different pH values with YPG. A typical reaction mixture contained 3% (w/v) pectic acid, 0.075 *M* acetate buffer and 10% (v/v) YPG. For preparative work, 120 g of pectic acid were degraded at one time. The reaction was terminated by heating in a boiling water bath for 5–7 min. After cooling the solution was stored in a refrigerator. Prior to addition to the column the reaction mixture was filtered through filter aid.

The unsaturated uronides were prepared by degrading pectic acid at 30° with the endo PATE of the soil *Bacillus*. The reaction mixtures contained 3% (w/v) pectic acid, 0.001 *M* calcium, 0.05 *M* glycine (pH 9.4) and 10% (v/v) of the dialyzed crude enzyme preparation. The reaction was terminated by titrating to pH 4.0 with Dowex-50 ($H^+$) and then the entire mixture was passed over a column of Dowex-50 ($H^+$). This converted the uronides to the free acid and removed calcium, glycine and protein. The Dowex-50 was washed with distilled water until the effluent was neutral. The preparation was then stored in the refrigerator. If necessary, the solution was filtered through filter aid prior to use.

### *Column chromatography*

For the separation of a digest equivalent to 30 g of pectic acid, a 3.0 × 100 cm Dowex-1 X8 (100–200 mesh) column in the formate form was used. This has a volume

of approximately 750 ml and represents approximately 1000 mequiv. Since 30 g of pectic acid digest contained approximately 170 mequiv. of carboxyls, the column load was 17 % of capacity. The column was loaded and washed with 500–700 ml of distilled water prior to elution of the oligogalacturonides. During elution 20 ml fractions were collected. After each run the column was recycled by the passage of 1 *N* sodium chloride followed by 1 *N* sodium formate until the effluent was chloride free. Occasionally the columns had to be repacked. The resin was screened and suspended in water, followed by decantation several times to remove suspended insoluble matter.

After analysis, the fractions representing individual peaks were pooled, barium acetate or strontium chloride was added in 50 % excess and the salts were precipitated by the addition of ethanol (final concentration of 70 % and 60 % for the unsaturated and normal dimers, respectively, and 50 % for longer chain uronides). More efficient precipitation could be obtained if acetone were substituted for all or part of the ethanol. The precipitates were collected by filtration, washed with 95 % ethanol followed by acetone and dried in vacuum over calcium chloride.

To ensure purity of the individual uronides, the salts from several column runs were combined, converted to the free acid by treatment with Dowex-50 ($H^+$) and rechromatographed. For the pooling of the fractions from these further purified uronides, particular care was used in detecting impurities which co-chromatographed with the front of the peak. Only those fractions which were shown by paper chromatography to contain the desired oligouronide were pooled. The salt of the uronide was then precipitated as previously described. It was then converted to the free acid by passage over a Dowex-50 ($H^+$) column after which the aqueous solution was lyophilized.

*Paper chromatography*

The uronides were identified by paper chromatography on Whatman No. 4 paper using the epiphase from ethyl acetate–acetic acid–water (2:1:2, solvent system 1)[4] or pyridine–ethyl acetate–acetic acid–water (5:5:1:3, solvent system 2)[7] for irrigation. The individual compounds were revealed by a periodate–benzidine reagent[8].

*Analyses*

Total galacturonic acid was determined by the carbazole procedure[12]. Carboxyl groups were titrated to a phenolphthalein endpoint with 0.01 *N* sodium hydroxide. Reducing groups were determined by the modified hypoiodite method of PATEL AND PHAFF[18]. A modification of the carbazole method (0.01 ml sample) was used for the semi-quantitative determination of normal uronides in the fractions obtained from the columns. The unsaturated uronide concentration was determined by measuring the absorbance of a 1/100 dilution of each sample at 232 mμ in a 1 cm silica cuvette with a Beckman DU spectrophotometer.

The $R_M$ values of the saturated uronides were calculated from $R_{gal}$ values obtained from chromatograms developed in solvent system No. 1.

## RESULTS AND DISCUSSION

Since DEMAIN AND PHAFF[3] demonstrated that the pH optimum for YPG attack of trimer and tetramer (pH 3.5) was one pH unit below that for attack of pectic acid

(pH 4.5) it was concluded that maximal yield of longer chained oligogalacturonides would be obtained if the pH of the reaction were maintained above the optimum for pectic acid. Thus the following reaction times and pH values were used to obtain maximal yield of the indicated products: pentamer to octamer, 30–60 min at pH 6.0; dimer to pentamer, 16 h at pH 6.0; dimer to tetramer, 40 h at pH 6.0; dimer and trimer, 40 h at pH 6.0, followed by adjustment to pH 3.5 and incubation for 54 h; dimer, 3–5 days at pH 3.5.

In the case of the unsaturated uronides the activity of PATE was followed by measuring the absorbancy of a proper dilution at 232 m$\mu$. The reaction was stopped when approximately 20–25 % of the bonds had been cleaved.

In earlier studies, formic acid was used to elute the uronic acids. NAGEL AND ANDERSON[16] used sodium acetate, pH 6.0 rather than acetic acid to elute the uronides from Dowex-1 in order to avoid the extreme pH and higher concentrations of acetic acid required to elute the same compounds. Since formic acid is a stronger acid (pH 3.7) it was felt that sodium formate at pH 4.7 should be a better eluant than sodium acetate at pH 6.0. This was found to be true. Therefore, sodium formate, pH 4.7, was used.

The elution pattern obtained for a mixture of monomer through tetramer with a stepwise gradient is shown in Fig. 1. Elution was accomplished with the following order of addition of sodium formate solutions: 0.2 $M$, 900 ml; 0.3 $M$, 3400 ml; 0.4 $M$, 2500 ml; 0.5 $M$, 2500 ml; 0.6 $M$, 2500 ml. It was subsequently shown that a 7 l (total) linear gradient (0.2–0.8 $M$) would produce sharper peaks, require less time and less eluant and result in more concentrated solutions of uronides. For the separation of dimer to octamer, an 8 l linear gradient (0.2–0.9 $M$) was used.

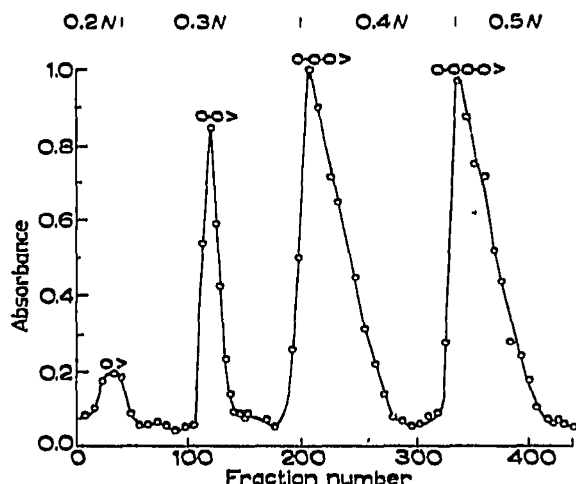



Fig. 1. Elution pattern of monomer (O>), dimer (O-O>), trimer (O-O-O>), and tetramer (O-O-O-O>) on a Dowex-1 (formate) column.

Table I summarizes the yields obtained when a reaction mixture was treated in order to obtain a high yield of trimer. The high yield of trimer is apparent. On a molar basis it represents 2.4 times the amount of dimer. However, from the data reported by DEMAIN AND PHAFF[3] the results are to be expected. Under optimum conditions, a yield of approximately 40 % was obtained for tetramer.

TABLE I

RECOVERY EXPERIMENT FOR THE OPTIMUM PRODUCTION OF TRIMER

| *Compound* | *Yield* | |
|---|---|---|
| | *g* | % |
| Monomer | 3.3 | 9.8 |
| Dimer | 5.9 | 17.5 |
| Trimer | 21.6 | 63.9 |
| Total | 30.8 | 91.1 |
| Original | 33.8 | 100 |

Fig. 2 shows a typical separation obtained with unsaturated oligogalacturonides. The sodium formate elution schedule was as follows: 0.06 *M*, 1500 ml; 0.08 *M*, 1500 ml; 0.1 *M*, 1500 ml; 0.2 *M*, 900 ml; 0.3 *M*, 1500 ml; 0.4 *M*, 3000 ml; 0.5 *M*, 2800 ml; 0.6 *M*, 2000 ml; 0.7 *M*, 2000 ml. No satisfactory linear gradient was devised for the elution of the unsaturated uronides.

In order to ensure purity each compound was rerun over a second column. The following elution sequence was used for the four unsaturated and four saturated (D.P. 2–5) uronides on Dowex-1 (3 × 100 cm): 0.06 *M*, 1000 ml; 0.08 *M*, 1000 ml; 0.1 *M*, 1000 ml, followed by increasing the formate concentration stepwise by increments of 0.1 *M*. At the concentration which eluted the uronide, sufficient eluant was used to elute the peak. The uronides were eluted at the following concentrations: unsaturated dimer to pentamer, 0.4, 0.5, 0.6 and 0.7 *M*, respectively; normal dimer to pentamer, 0.3, 0.4, 0.5, and 0.6 *M*, respectively. For the smaller quantities of hexamer, heptamer and octamer, a smaller Dowex-1 column was used (3.5 × 50 cm). Elution was accomplished as before but only 0.5 l of the stepwise concentrations was used. The uronides were eluted at the following concentrations of sodium formate: hexamer, 0.6 *M*; heptamer, 0.7 *M*; and octamer, 0.8 *M*.

The elution of an unsaturated uronide required a higher eluant concentration than the normal uronide of the same chain length. This was true in both stepwise elution and linear gradient elution, although no completely satisfactory gradient was developed for the unsaturated uronides. Although the double bond increased the

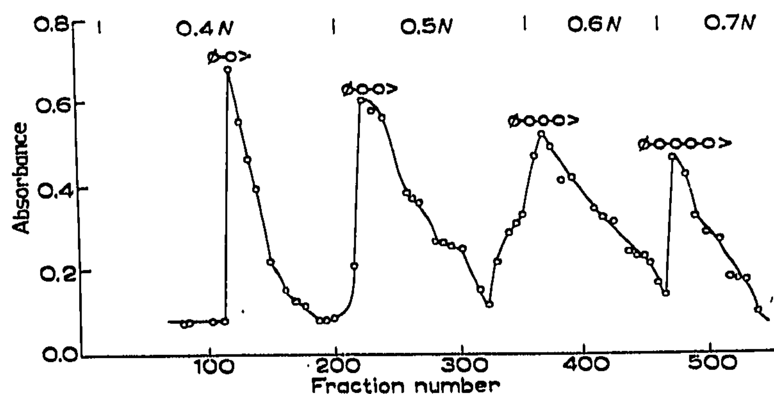



Fig. 2. Elution pattern of u-dimer (∅-O>), u-trimer (∅-O-O>), u-tetramer (∅-O-O-O>), and u-pentamer (∅-O-O-O-O>) on a Dowex-1 (formate) column.

apparent affinity of the uronide for the resin, the unsaturated uronides could still be separated. With the same column and elution conditions, the unsaturated uronides were eluted as broader peaks.

The properties of the normal and unsaturated uronides are listed in Table II. The equivalent weight was determined from the end group assay (hypoiodite). The water content was estimated from the difference between the equivalent weight and calculated anhydrous molecular weight. The equivalent weights for hexamer, heptamer and octamer were determined on free acids precipitated from aqueous solutions by the addition of ethanol and acetone and dried under vacuum over calcium chloride. The equivalent weights of the original lyophilized acids were 1146, 1316 and 1415, respectively. The agreement of the data for these compounds indicate the relative constancy of the water content even when they are isolated under different conditions.

The fourth and fifth columns (Table II) show determinations of chain length (D.P.). The determinations are simply the ratios of carboxyl to aldehyde group or monomer to aldehyde group content. Titration of the carboxyl groups in the longer uronides (D.P. $\geqslant 6$) resulted in values much smaller than expected from other data. The galacturonide content as determined by the carbazole method gave more consistent results.

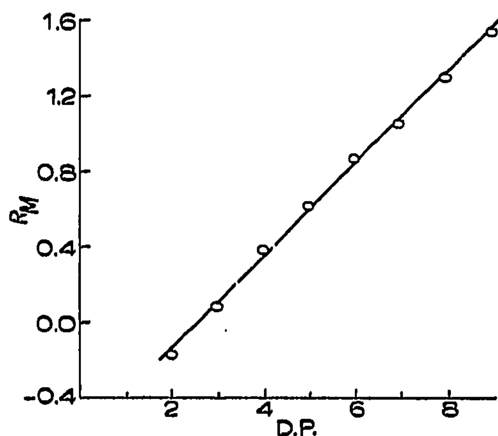
TABLE II

PROPERTIES OF THE OLIGOGALACTURONIC ACIDS

| *Compound* | *Equiv. Wt.* | *Moles $H_2O$ / Mole Cpd.* | *COOH*[a] / *CHO* | *Monomer / CHO* |
|---|---|---|---|---|
| Monomer | 226 | 1.6 | 1.04 | — |
| Dimer | 462 | 5.1 | 1.92 | — |
| Trimer | 615 | 3.8 | 2.94 | — |
| Tetramer | 790 | 3.8 | 4.09 | — |
| Pentamer | 965 | 6.3 | 5.02 | — |
| Hexamer | 1160 | 4.8 | — | 5.90 |
| Heptamer | 1306 | 3.1 | — | 6.78 |
| Octamer | 1469 | 2.4 | — | 7.80 |
| ASPA[b] | 3000 | 28.8 | — | 14.20 |
| U-dimer[c] | 439 | 4.8 | 1.87 | — |
| U-trimer | 611 | 4.6 | 3.00 | — |
| U-tetramer | 803 | 5.5 | 4.07 | — |
| U-pentamer | 951 | 3.9 | 4.98 | — |

[a] COOH = carboxyl groups; CHO = aldehyde groups.
[b] ASPA = acid-soluble pectic acid prepared by the procedure of McCREADY AND SEEGMILLER[16].
[c] U = unsaturated.

Further indications of a homogeneous series of uronides are obtained from the data as presented in Fig. 3 and 4. BATE-SMITH AND WESTALL[2] have shown by paper chromatography that the $R_M$ value is a linear function for a homologous series of phenolic compounds [$R_M = \log(1/R_F - 1)$]. The data in Fig. 3 were determined using monomer as the reference compound ($R_{gal}$) and plotting the $R_M$ value against the theoretical D.P. of each uronide. The linear relationship indicates the homogeneity



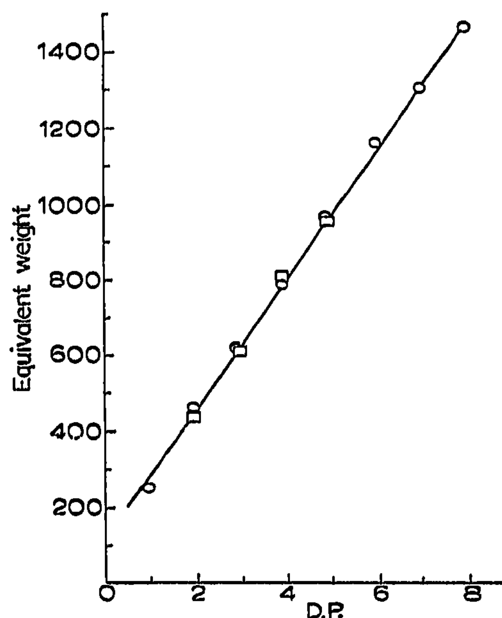




Fig. 3. Relationship between the D.P. and the $R_M$ values of normal oligogalacturonides.

Fig. 4. Relationship between the D.P. and equivalent weight. ○ = saturated uronides; □ = unsaturated uronides.

of the homologous series of saturated uronides. Fig. 4 shows the linear relationship between the theoretical D.P. and the experimental equivalent weight for both the unsaturated and saturated oligouronides.

## REFERENCES

1 J. Ashby, J. Brooks and W. W. Reid, *Chem. Ind. (London)*, (1955) 360.
2 E. C. Bate-Smith and R. G. Westall, *Biochim. Biophys. Acta*, 4 (1950) 427.
3 A. L. Demain and H. J. Phaff, *J. Biol. Chem.*, 210 (1954) 381.
4 A. L. Demain and H. J. Phaff, *Arch. Biochem. Biophys.*, 51 (1954) 114.
5 R. Derungs and H. Deuel, *Helv. Chim. Acta*, 37 (1954) 657.
6 H. Deuel and E. Stutz, *Advan. Enzymol.*, 20 (1958) 341.
7 M. Gee and R. M. McCready, *Anal. Chem.*, 29 (1957) 257.
8 H. T. Gordon, W. Thornburg and L. N. Werum, *Anal. Chem.*, 28 (1956) 849.
9 S. Hasegawa and C. W. Nagel, *J. Food Sci.*, 31 (1966) 838.
10 C. Hatanaka and J. Ozawa, *Ber. Ohara Inst.*, 13 (1966) 89.
11 B. S. Luh and H. J. Phaff, *Arch. Biochem. Biophys.*, 48 (1954) 23.
12 E. A. McComb and R. M. McCready, *Anal. Chem.*, 24 (1952) 630.
13 R. M. McCready, in R. L. Whistler and M. L. Wolfrom (Editors), *Methods in Carbohydrate Chemistry*, Vol. II, Academic Press, New York, 1963, p. 27.
14 R. M. McCready and E. A. McComb, *Agr. Food Chem.*, 1 (1953) 1165.
15 R. M. McCready and C. G. Seegmiller, *Arch. Biochem. Biophys.*, 50 (1954) 440.
16 C. W. Nagel and M. M. Anderson, *Arch. Biochem. Biophys.*, 112 (1965) 322.
17 C. W. Nagel and R. H. Vaughn, *Arch. Biochem. Biophys.*, 94 (1961) 328.
18 D. S. Patel and H. J. Phaff, *J. Biol. Chem.*, 234 (1959) 237.
19 H. J. Phaff and B. S. Luh, *Arch. Biochem. Biophys.*, 36 (1952) 231.
20 W. W. Reid, in R. L. Whistler and M. L. Wolfrom (Editors), *Methods in Carbohydrate Chemistry*, Vol. I, Academic Press, New York, 1962, p. 309.