

Separation of Oligogalacturonic Acids by Dextran Gel Chromatography

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From a mixture of oligogalacturonic acids obtained by partial acid hydrolysis or by enzymatic digestion of pectic acid the first five homologues of D-galacturonic acid were isolated by gel chromatography on Sephadex G-25 — fine with 0.05 M phosphate buffer, pH 7 as eluents. When water or 0.01 M phosphate buffer were used as eluents the oligogalacturonic acids were excluded from the column with minimum differences in elution volumes and consequently no separation of the oligogalacturonic acids was achieved. With 0.05 M phosphate buffer Sephadex G-25 — fine acts as molecular sieve where the elution volumes of the oligogalacturonic acids are approximately linear functions of the logarithm of their molecular weights.

Low molecular oligogalacturonic acids produced by enzymatic hydrolysis of pectic acid have been fractionated so far by chromatography on ion exchangers. Nagel and Hasegawa [1] have reported the separation of the reaction products of hydrolysis of pectic acid catalyzed by pectic acid endo-*trans*-eliminase on Dowex 1 using the concentration gradient of the acetate buffer as eluents. Derungs and Deuel [2] have used essentially the same method with formic acid as eluents. Hatanaka and Ozawa [3] separated oligogalacturonides liberated by the action of yeast endopolygalacturonase on pectic acid by chromatography on DEAE-cellulose with the concentration gradient of NaHCO₃. The first four homologues have been separated by this method.

In the course of our studies aimed at the elucidation of the mechanism of *Aspergillus niger* endopolygalacturonase action, it was necessary to prepare substantial quantities of lower oligogalacturonic acids, mainly of the first five homologues of D-galacturonic acid which could be used as substrates. As neither the chromatography on Dowex 1 nor the chromatography on DEAE-cellulose did not give satisfactory separation of the five lowest oligogalacturonic acids we investigated the applicability of gel chromatography to the separation of oligogalacturonic acids obtained by partial acid hydrolysis or by enzymatic hydrolysis, since recently this method was used in the separation of homologous series of neutral oligosaccharides [4] as well as of acidic oligosaccharides obtained from acid glycosaminoglycans by the action of hyaluronidase [5].

Experimental

Material and methods

Pectic acid (degree of esterification 0%; content of polygalacturonide 75.5%) prepared from Czechoslovak commercial preparation of apple pectin by repeated alkaline deesterification with 0.1 N-NaOH (pH 10; 22°C) and by following precipitation at pH 2.5 was used as the starting material for the preparation of oligogalacturonic acids.

Endopolygalacturonase (poly- α -1,4-D-galacturonide glycanohydrolase — E.C. 3.2.1.16) used for the enzymatic hydrolysis of pectic acid was isolated from the culture filtrate of *Aspergillus niger* according to the method developed in our laboratory [6].

Paper chromatography in the solvent system ethyl acetate—acetic acid—water (18 : 7 : 8) was used for the identification of oligogalacturonic acids. D-Galacturonic acid served as standard. The oligogalacturonic acids were identified on the basis of the values of $\log(R_F/1 - R_F)$ representing linear functions of DP of the oligogalacturonic acids [7].

Acid hydrolysis

The partial acid hydrolysis of pectic acid was carried out in water solution adjusted by sulfuric acid to pH 3.0—3.5 on boiling water bath under reflux. After 15 hours' hydrolysis the insoluble material was filtered off and the supernatant was neutralized by solid BaCO₃. The Ba²⁺ ions were removed by filtration through a Zerolit 225 (H⁺) column. The unhydrolyzed highmolecular fraction was precipitated from the filtrate by four volumes of ethanol. Neutral saccharides were separated from the supernatant by filtration through Dowex 1X8 (100—200 mesh) in acetate form using water as solvent. The oligogalacturonides were eluted from the ion exchanger by 4 and 6 M acetic acid. The acetic acid was removed from the eluates by repeated evaporation with water in the vacuum at 30°C. The pure mixture of oligogalacturonides was freeze-dried.

Enzymatic hydrolysis

The enzymatic hydrolysis was carried out in McIlvaine buffer pH 4.2 at room temperature. The reaction was stopped by heating the reaction mixture on boiling water bath for 10 minutes. The insoluble fraction was removed by centrifugation and the supernatant was partially concentrated in a rotatory evaporator at 30°C. The salts and the D-galacturonic acid were separated from the mixture of oligogalacturonic acids by gel filtration through Sephadex G-25 — medium with water as eluents. Fractions containing oligogalacturonides were pooled and freeze-dried.

Separation of oligogalacturonic acids

Two chromatographic columns 4.4 cm in diameter and 120 cm in length were used for the separation of oligogalacturonic acids. The columns were connected in such a manner that the outlet of the first column was attached to the inlet at the top of the second column by a polyethylene capillary tube with the inner diameter of 1.5 mm. The sample of the hydrolysate in 10 ml of 0.05 M phosphate buffer, pH 7, was applied on the top of the first column. The columns were eluted by the same buffer at a flow rate of 0.4 ml/min. The peaks of the oligogalacturonic acids were localized in the effluent from the column by determining the reducing groups by photocolometric method with 3,5-dinitrosalicylic acid [8] as well as by paper chromatographic analysis. The fractions of each peak were pooled, concentrated in a rotatory evaporator and desalted on a 1.4 × 120 cm Sephadex G-15 column. Finally the solutions of the desalted products were freeze-dried.

Results and Discussion

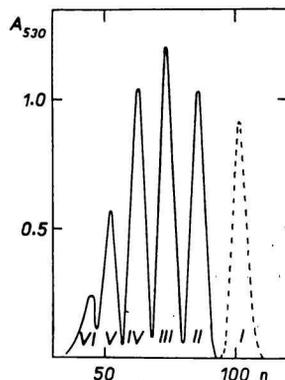
A mixture of oligogalacturonic acids with high content of lower homologues of D-galacturonic acid was produced by the partial acid hydrolysis of pectic acid. As the final products of the action of *Aspergillus niger* endopolygalacturonase on

pectic acid are mono-, di-, and trigalacturonic acids in our experiments, aimed at the isolation of the first five homologues. The enzymatic digestion was stopped when 60% enzymatic hydrolysis was achieved. This stage was determined on the basis of a preceding enzymatic hydrolysis accomplished under identical conditions as those of the preparative hydrolysis.

Fig. 1. Separation of oligogalacturonic acids by gel chromatography on Sephadex G-25 — fine, with 0.05 M phosphate buffer, pH 7, as eluents.

n — fraction number; A_{530} — absorbancy at 530 nm after the reaction of oligogalacturonic acids with 3,5-dinitrosalicylic acid.

Roman figures correspond to DP of oligogalacturonic acids. Dotted peak correspond to monogalacturonic acid.



The reaction mixture obtained after the enzymatic as well as the partial acid hydrolysis was separated by gel filtration through Sephadex G-25 — medium into two fractions. The fraction eluted from the column with the eluate volume corresponding to the void volume contained the mixture of all oligogalacturonic acids while the second, slower fraction contained D-galacturonic acid and salts.

Fractionation of oligogalacturonic acids by gel chromatography on Sephadex G-25 — fine carried out in 0.05 M phosphate buffer, pH 7, is shown in Fig. 1. On the chromatographic column 4.4 cm in diameter and 240 cm in length, the separation into five fractions was obtained. For the laboratory conditions inconveniently long column was replaced by two columns, 120 cm in length, connected with each other as mentioned formerly. Chromatographic analysis of the fractions showed that they correspond to di-, tri-, tetra-, penta-, and hexagalacturonic acids. The 0.05 M phosphate buffer was used on account of the very poor resolution of oligo-

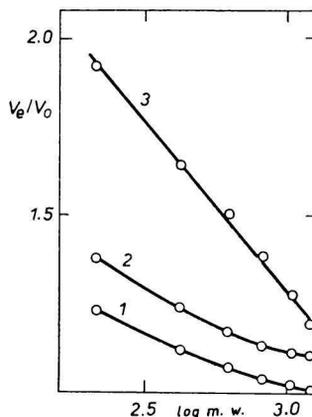


Fig. 2. Relative elution volumes (V_e/V_0) as functions of the logarithm of molecular weights of oligogalacturonic acids with water (1), 0.01 M phosphate buffer (2), and 0.05 M phosphate buffer (3) as eluents.

galacturonic acids obtained when water or phosphates of lower concentration were used as eluents. In water, the oligogalacturonic acids were eluted from the column in one broad peak with minimum differences in the elution volumes not enabling even their partial separation.

Partial separation was obtained when a 0.01 M phosphate buffer was used as eluents. In this case the oligogalacturonic acids were eluted in distinct peaks though not well separated; the last fractions of a peak coincided with the first fractions of the next one causing partial contamination by the nearest lower and higher homologues.

Optimum separation was obtained in a 0.05 M phosphate buffer where the elution volumes of the oligogalacturonic acids were approximately a linear function of the logarithm of their molecular weights (Fig. 2). The comparison of the dependence of values of the relative elution volumes V_e/V_0 (V_e — elution volume, V_0 — void volume) on the logarithm of the molecular weight [9] of the oligogalacturonic acids in water, 0.01 M phosphate, and 0.05 M phosphate buffer, shows that Sephadex acts as a molecular sieve for oligogalacturonic acids only with the 0.05 M phosphate buffer (Fig. 2). The presence of negatively charged carboxylic groups in oligogalacturonic acids obviously causes their exclusion from the Sephadex column in water as well as in 0.01 M phosphate buffer and consequently the differences in elution volumes of the oligogalacturonic acids are smaller than those corresponding to the differences in their molecular weights [10, 11]. With 0.05 M phosphate buffer as eluents this effect is eliminated and under these conditions the gel chromatography on Sephadex is very suitable method for the isolation of the first five homologues of the D-galacturonic acid.

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