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LARGE-SCALE PREPARATION OF GALACTURONIC ACID OLIGOMERS BY MATRIX-BOUND POLYGALACTURONASE

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ABSTRACT

endo-Polygalacturonase from *Saccharomyces fragilis* has been linked to a Sepharose-4B matrix and remains catalytically active. A continuous-flow procedure over an immobilized-enzyme column was developed to derive oligomeric break-down products of polygalacturonate. Further separation and purification of these oligomers was performed on columns of Biogel P2 and P4.

INTRODUCTION

Studies of the catalytic mechanism of pectolytic enzymes are complicated because the substrates are not very well defined. Pectin and pectic acid preparations are obtained by extraction from natural products and contain many impurities, amongst which are plant pigments. Moreover, natural pectin and pectic acid are heteropolymers. The saccharide back-bone is composed of α-(1→4)-linked D-galacturonic acid residues. Besides the galacturonic acid residues, rhamnose is also found in the back-bone, whereas neutral sugars like arabinose, xylose, and galactose form side-chains[1–3]. Another disadvantage in studying pectolytic enzymes is the variation in the degree of esterification of the carboxyl groups and the distribution of the ester groups along the back-bone[4]. To obtain kinetic details of the mechanism and binding characteristics, such as the existence of sub-sites, for such biopolymer-degrading enzymes, the usual approach is to prepare a series of oligomeric substrates. Well-known examples among the polysaccharide-cleaving enzymes, where such investigations proved to be successful, are lysozyme[5] and alpha-amylase[6,7]. The preparation of oligomers of galacturonic acid has been described on several occasions[8–11]. Generally, the route followed is one of enzymic breakdown by a polygalacturonase and subsequent separation of oligomeric products, for example, on ion-exchange columns using acetate or formate buffers[12,13]. We have developed a continuous procedure for the preparation of oligomers. The separation technique has been improved by applying a molecular-sieving procedure with Biogel P2 and P4, analogous to the separation of D-glucose and its oligosaccharides[14].

MATERIALS AND METHODS

Immobilization of polygalacturonase. — Some strains of *Saccharomyces fragilis* excrete a constitutive *endo*-polygalacturonase into the culture medium[15]. A freeze-dried, crude preparation of this enzyme was a generous gift from Dr. F. Rombouts. Sepharose-4B (Pharmacia) was activated by the procedure of Cuatracasas[16] after extensive washing with distilled water. For 10 ml of packed gel, 400 mg of cyanogen bromide were used for the activation reaction, which occurred at pH 10.5 during 15–20 min. The activated gel was washed with 500 ml of ice-cold 100mM sodium hydrogen carbonate buffer (pH 8.5). The gel was suspended in the same buffer (30 ml/10 ml of gel), followed by the addition of 0.5% (w/v) of polygalacturonic acid (Dr. T. Schuchardt GmbH. & Co., Hohenbrunn, Germany) having a uronide content of 85–87% and a d.p. of ~60 (personal cummunication by Dr. A. G. J. Voragen). Finally, protein (16 mg) was added (the real amount of polygalacturonase is unknown). The coupling reaction was continued for 24 h at 5°. Unreacted material was eluted from the gel in column form (2 × 14 cm), by 2 l of sodium acetate buffer (50mM, pH 6).

The activity of the Sepharose-bound polygalacturonase was tested by (*a*) incubation with substrate with subsequent viscosity determinations, or (*b*) substrate elution over a small enzyme–agarose column in combination with t.l.c. to analyse the products.

To 3 columns (2-cm diameter) of agarose–enzyme (52 ml), 3-ml samples of 1% sodium pectate were applied at 25° in 50mM sodium acetate buffer (pH 6) and eluted at 9.5, 20, and 28.5 ml/h, respectively. Fractions (2 ml) were collected, analysed by t.l.c. and by determination of optical activity, and appropriately combined.

Separation of the oligomers by gel chromatography. — Biogel P2 and P4 (200–400 mesh) were equilibrated in 20mM sodium chloride before use. After 3 days, two columns (2.6 × 100 cm) of the equilibrated gel were prepared. The columns were linked in series with an LKB peristaltic pump positioned after the second column, and the flow rate was maintained at 20 ml/h. The separation was performed at 45° with a 2-mm flow cell and a JASCO ORD/UV-5 spectrophotometer, coupled to an external recorder (Photovolt), for continuous determination of optical rotation at 230 nm. Fractions (10 ml) were also collected.

Desalting and esterification of the galacturonic acid oligomers. — The fractions containing individual oligomers were combined and the concentration of oligomer was determined by the carbazole procedure[17]. Strontium chloride was added (1:1, w/w) and the galacturonate salts were precipitated by the addition of 4 vol. of ethanol. The precipitates were collected by filtration, washed with ethanol (80%) until chloride-free, and dissolved in water, and the solutions were stirred for 1 day with Dowex-W x8(H^+) resin (20–50 mesh, 1.7 mequiv./ml) in order to remove cations. A twenty-fold excess of resin with respect to the carboxyl groups was used. The oligogalacturonic acids were analyzed by t.l.c., freeze-dried, and dried over P_2O_5 for several days *in vacuo*.

Esterification was effected[18] under dry air in closed reaction vessels by adding 50 ml of 0.02M methanolic hydrogen chloride[21] per g of oligomer. The reaction was continued with stirring for 10 days for the lower oligomers (up to 4), and for 15 days for higher oligomers. The mixtures were neutralised (to pH 6–7) by stirring overnight with silver carbonate, then filtered, concentrated, and analysed by t.l.c. on silica gel[17] with 1-propanol–water[18] (7:2). Reference di-, tri-, and tetra-saccharides were a generous gift from Dr. A. G. J. Voragen.

RESULTS AND DISCUSSION

endo-Polygalacturonase from *Saccharomyces fragilis*, which randomly hydrolyses pectic acid, was used because this enzyme is particularly suitable for the preparation of oligomers (up to the pentamer[19]). An additional advantage is the absence of lyase activity. The enzyme has been used[9] to prepare an oligomer series up to the tetramer. By varying the pH of the reaction mixture, the pattern of oligomer formation could be varied[9]. The efficiency of binding of the enzyme and the turnover number of the free and immobilized enzyme were not determined, because an active enzyme was the sole requirement. However, to diminish the possibilities of enzyme inactivation upon binding, sodium pectate was added during the coupling reaction. The breakdown pattern of the pectate was investigated on a column of agarose–enzyme by varying the flow rates instead of the pH to affect the size distribution of the oligomers. The results are shown in Fig. 1.

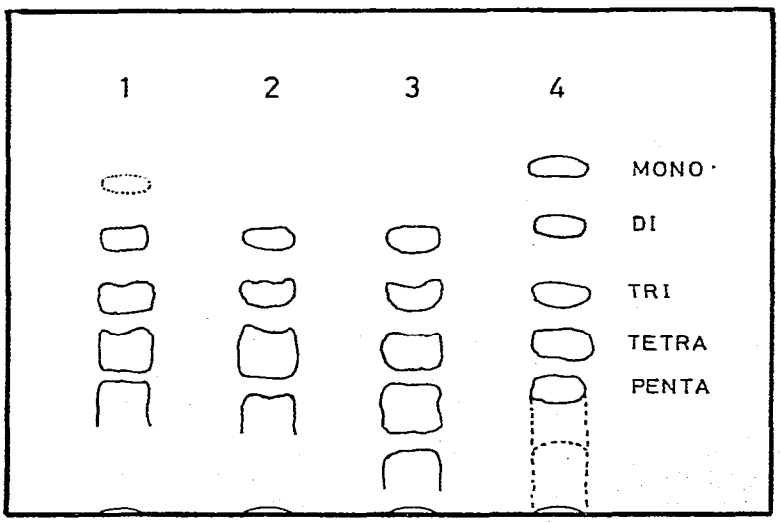

Fig. 1. T.l.c. of the products formed by Sepharose–galacturonase upon elution of a column with a 1% polygalacturonic acid solution. Flow-rates: 9.5 (1), 20 (2), and 28 ml/h (3); 4 is the reference mixture of oligogalacturonic acids.

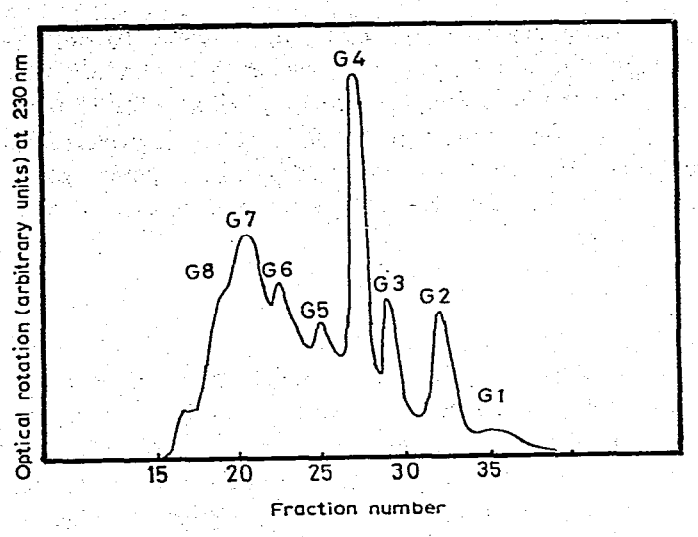

Fig. 2. Elution pattern of a mixture of oligogalacturonic acids (200 mg in 0.5 ml) on a column of Biogel P2 equilibrated and eluted with 20mM NaCl at 45°. The peaks G1–G8 correspond to monomer–octamer.

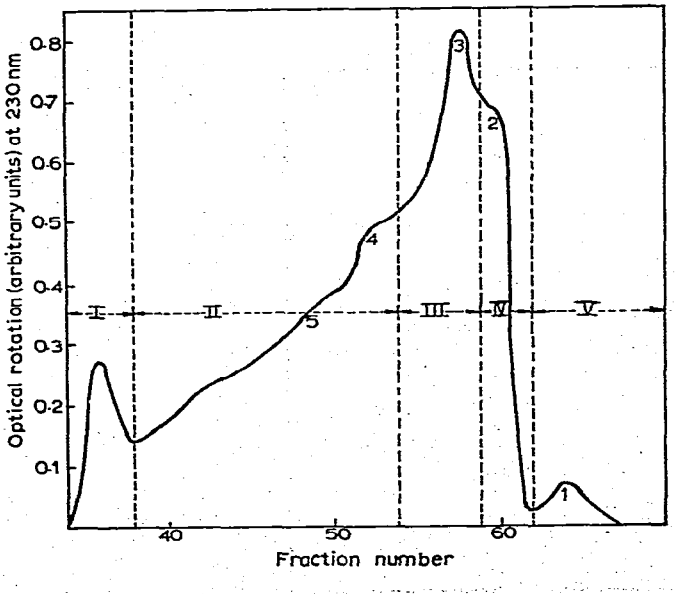

Fig. 3. Elution pattern of a mixture of oligogalacturonic acids (2 g in 5 ml) on two columns (2.6 × 100 cm) of Biogel P2 linked in series, and equilibrated and eluted with 20mM NaCl at 45°. The fractions combined are indicated by I–V, and 1–5 refer to the elution volumes of the oligomers (dimer-hexamer) of galacturonic acid.

The degradation was always incomplete since some starting material remained; the degree of degradation will, of course, depend on the content of methyl ester and foreign sugar, as well as on the degree of polymerization. With a flow rate of 9.5 ml/h, some monomer was formed. Although we have not determined the pH optimum of the immobilised polygalacturonase, the results are in agreement with those for batch preparations[15].

At a pH of 6, the desired mixture of oligomers (varying from dimer to octamer) was obtained, containing a satisfactory distribution of all the components. A continuous production was established by eluting a column with large volumes of a 1% solution of sodium pectate in 50mM acetate buffer (pH 6.0) at a flow rate of 20 ml/h. The eluate was collected at ~0°. After operation for one week, the column had to be cleaned with buffer of high ionic strength, in order to remove coloured substances.

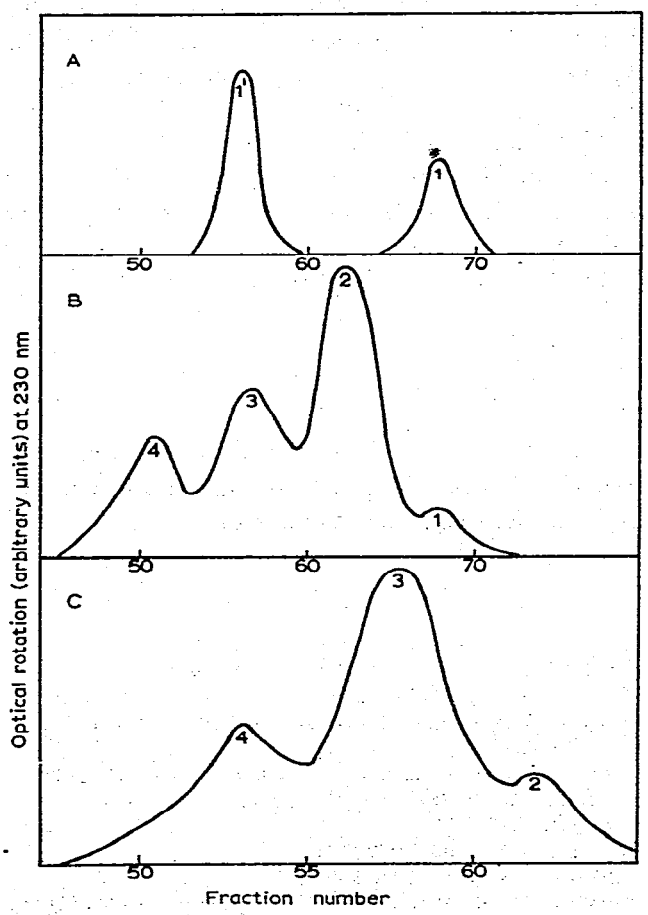

Fig. 4. Elution patterns (A–C) of fractions V, IV, and III, respectively, after esterification. Column dimensions and conditions as indicated in Fig. 3. Number 1′ indicates the elution volume of the polymerized dimer of galacturonic acid.

The described procedure has the advantage that the solution of hydrolysed substrate has a constant composition. The oligomer mixture was concentrated approximately 35-fold at 45°, and insoluble material, which contained only 1.5% of the original galacturonic residues, was discarded. The remaining solution was still coloured.

Fig. 2 shows the fractionation of a sample (0.5 ml) containing 0.2 g of a mixture of oligomers on one column of Biogel P2; eight components were detected. The resolution was temperature dependent[22]; 45° was chosen because the material of the "cooling" jacket was not resistant to the optimal temperature of 60°. The elution positions of the oligomers showed a linear relationship between the distribution coefficent (K_{av} and the log mol. wt. (M_w), which has been verified by t.l.c.

In order to obtain a large quantity of each esterified oligomer, fractions (5 ml, containing ~2 g of oligomers) were purified on two columns of Biogel P2 linked in series. The elution pattern is given in Fig. 3. Refractionation was necessary in order to obtain individual oligomers in a pure form. The fractions III–V in Fig. 3 were collected and esterified, and separately chromatographed on Biogel P2 (Fig. 4). Complete purification of the tri-, tetra-, and penta-mer required rechromatography of the central fractions of the corresponding peaks. The purification of the oligomers with a d.p. 5 or more can be achieved by using Biogel P4.

After esterification, polymerisation of the dimer ester (Fig. 3) and, to a lower extent, of the trimer ester was observed, which can be inferred from the respective elution positions. The structures of these polymers are unknown.

Kinetic studies on pectin transeliminase, using the lower oligomers as substrates. are in progress.

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