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OLIGO-D-GALACTOSIDURONIC ACID—SEPARONS: SELECTIVE BIOAFFINITY ADSORBENTS OF ENDOPOLYGALACTURONASE

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SUMMARY

Oligo-D-galactosiduronic acids of different degrees of polymerization (DP 2, 4 and 5) were O-glycosidically bound to a poly(hydroxyethyl methacrylate) gel, Separon. Their ability to function as biospecific ligands of endopolygalacturonase was studied by affinity chromatography. Endopolygalacturonases of *Aspergillus niger* and from tomato were selectively adsorbed by Separon derivatives with tetra- and pentagalactosiduronic acids, whereas digalactosiduronic acid—Separon retained only a fraction of the enzyme. Adsorbed endopolygalacturonase was quantitatively displaced from the adsorbents either by changing the pH or by bioelution with oligogalactosiduronides of DP > 2. The dissociation constants of the affinity complexes (K_L) as determined by zonal analysis were close to the values for the soluble complexes (K_I) with counter-ligands of the same DP.

INTRODUCTION

At present, the method most commonly employed for separation of microbial endopolygalacturonases [poly(1,4-α-D-galacturonide)glycanohydrolase, EC 3.2.1.15] from other pectic enzymes is based on biospecific interaction of the enzyme with pectic acid cross-linked by epichlorhydrin [1, 2]. Owing to the polyelectrolytic character of the cross-linked pectate, however, nonspecific ionic interactions may interfere with biospecific binding [3]. The potential for application of this method is therefore reduced.

Recently, we have described a new selective affinity adsorbent of endopolygalacturonase prepared by O-glycosidic coupling of trigalactosiduronic acid to a poly(hydroxyethyl methacrylate) gel, Separon [4, 5]. Adsorption of the enzyme is based on an active-site-directed interaction with bound trigalactosiduronic acid. The glycosylation of poly(hydroxyethyl methacrylate)

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increased the hydrophilicity of the matrix and consequently suppressed unspecific hydrophobic interactions with endopolygalacturonase and other proteins. This provides the possibility of using this affinity sorbent not only for selective separation but also for studying its interaction with the endopolygalacturonase binding site. For the latter purpose, it was of interest to find out the ability of other oligogalactosiduronic acids to function as biospecific ligands and to characterize the optimum length of the affinity ligand.

The present paper deals with chromatography of endopolygalacturonase of *Aspergillus niger* and from tomato on Separon derivatives containing oligogalactosiduronic acids with degree of polymerization (DP) 2, 4 and 5, respectively, and furthermore evaluates the affinity and biospecificity of the interaction.

EXPERIMENTAL

Materials for preparation of affinity sorbents

The poly(hydroxyethyl methacrylate) gel, Separon H 1000 (particle size 125—200 μm), was the product of Laboratory Instrument Works (Prague, Czechoslovakia). Oligogalactosiduronic acids used as affinity ligands were prepared from a partial acid hydrolysate of sodium pectate by repeated gel chromatography on Sephadex G-25 [6], followed by desalting on Sephadex G-10 and freeze-drying. The preparations were homogeneous as determined by thin-layer chromatography on silica gel [7].

Glycosylation of Separon with oligogalactosiduronic acids

The method and experimental conditions used for the binding of oligogalactosiduronic acids to Separon were the same as described previously [4]: Separon beads were left to swell for 2 h at room temperature (23°C) in dry 1,4-dioxane freshly saturated with dry hydrogen chloride gas. The respective sodium oligogalactosiduronate was then added and the suspension was shaken for 48 h at room temperature. The beads were then washed successively with several portions of dry 1,4-dioxane, 0.05 *M* phosphate buffer (pH 7.9) until the washings gave a negative reaction for carbohydrates [8], dry acetone and diethyl ether and finally dried at 45°C. The content of carbohydrate covalently bound to the Separon matrix was determined by the method of Dubois et al. [8] (Table I).

TABLE I

BINDING CAPACITIES OF $(GalA)_n$—SEPARONS FOR ENDOPOLYGALACTURONASE OF *A. NIGER*

Affinity ligand		Adsorbed enzyme (mg protein per g carrier)
DP	Content (%, w/v)	
2	8.9	0.6
3	4.6	3.1
4	4.7	7.3
5	3.1	7.0

Enzymes. A crude enzyme preparation of *A. niger*, containing a mixture of pectic enzymes, was prepared from the culture filtrate by a procedure which included salting-out of proteins with ammonium sulphate (0.9 saturation), precipitation with 75% ethanol and desalting by gel filtration on Sephadex G-25 [1]. Endopolygalacturonase was prepared from the crude preparation by affinity chromatography on cross-linked pectic acid [1]. A crude preparation of tomato pectic enzymes [9] was received from Dr. O. Markovič from the Institute of Chemistry, Slovak Academy of Sciences (Bratislava, Czechoslovakia).

Substrates. Sodium pectate (D-galacturonan content 89.8%) was prepared from citrus pectin (Genu Pectin, Kφbenhavns Pektinfabrik, Denmark) by repeated alkaline de-esterification with 0.1 *M* sodium hydroxide, followed by precipitation with hydrochloric acid at pH 2.5 and by neutralization of the acid with sodium hydroxide. Oligogalactosiduronic acids, up to DP 9, used as affinity counter-ligands, were prepared by the same procedure as described for the preparation of affinity ligands.

Enzyme assay. Endopolygalacturonase activity was assayed spectrophotometrically at pH 4.2 (0.1 *M* acetate buffer) by the method of Somogyi [10]. The activity is expressed in μmol of reducing groups liberated within 1 min per 1 mg of protein, and determined by means of a standard graph for D-galactopyranuronic acid. The same method, with digalactosiduronic acid as substrate, was also employed for an exopolygalacturonase (EC 3.2.1.67) activity assay. The protein content was determined by the method of Lowry et al. [11], with human serum albumin as standard. The activity of pectinesterase was determined by titration (0.1 *M* sodium hydroxide) of carboxyl groups released at pH 4.4 (*A. niger* pectinesterase) or pH 7.0 (tomato enzyme) in the course of 30 min from a 0.5% solution of citrus pectin (66.4% esterified).

Analysis of the reaction products

The products of enzymic degradation of pectic and oligogalactosiduronic acids were analysed by thin-layer chromatography performed on silica sheets (Silufol, Kavalier, Czechoslovakia) in 1-butanol—formic acid—water (2:3:1) [7].

Affinity chromatography

Chromatography on $(GalA)_n$—Separons* was performed in columns (4 × 1.4 cm I.D.) equilibrated with 0.1 *M* acetate buffer (pH 4.2). The enzyme sample was applied onto the column within 30 min. The column was first eluted with the equilibrating buffer until the unadsorbed proteins had been eluted. Adsorbed endopolygalacturonase was eluted with 0.1 *M* sodium acetate (pH 7.0). The absorbance at 280 nm and polygalacturonase activities were measured for all fractions. Before the next run, the adsorbents were re-equilibrated with 0.1 *M* acetate buffer (pH 4.2).

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*$(GalA)_n$ = oligogalactosiduronic acid of DP *n*.

Determination of the binding capacity of $(GalA)_n$—Separons

The column of the respective $(GalA)_n$—Separon was successively charged with aliquots of endopolygalacturonase (0.3 mg) in 1 ml of acetate buffer (pH 4.2) until the effluent showed constant activity. The bound endopolygalacturonase was then eluted with 0.1 *M* sodium acetate (pH 7.0) and protein content and enzyme activity at pH 4.2 were determined.

Determination of dissociation constants of endopolygalacturonase with immobilized and soluble oligogalactosiduronic acids

The dissociation constants of the soluble complex (K_I) and insoluble complex (K_L) were determined by elution chromatography using zonal analysis according to Dunn and Chaiken [12], performed in 0.1 *M* acetate buffer (pH 4.2) containing the respective oligogalactosiduronic acid at various concentrations. With soluble oligouronic acids, kinetic measurements were also performed and the kinetic constants were computed using the program of Wilkinson [13].

Electrophoresis

Ultrathin-layer isoelectric focusing in polyacrylamide gels on silanized polyester films was performed according to Radola [14]. Polygalacturonase activity was detected by ruthenium red staining of gels [15], which were immersed in 1.3% sodium pectate (pH 4.2) for 10—30 min after electrophoresis.

RESULTS AND DISCUSSION

Oligogalactosiduronic acids used for the glycosylation of Separon differ in affinity and mode of complexing with *A. niger* endopolygalacturonase. While di- and trisaccharide are the final products of the enzyme action, the former no longer interacting with the enzyme [16], tetra- and pentagalactosiduronides represent its lowest substrates. Starting with the trisaccharide, the affinity toward endopolygalacturonase increases with increasing DP [16].

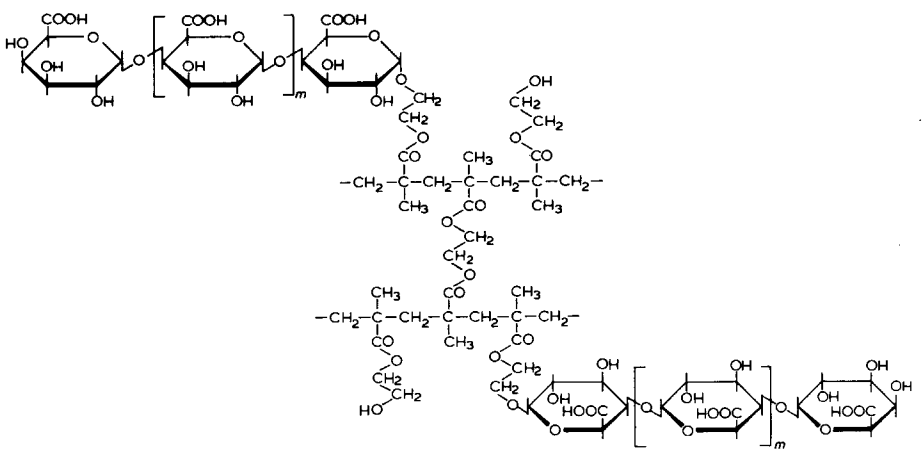

Fig. 1. Partial tentative structure of $(GalA)_n$—Separons; m = 0—3.

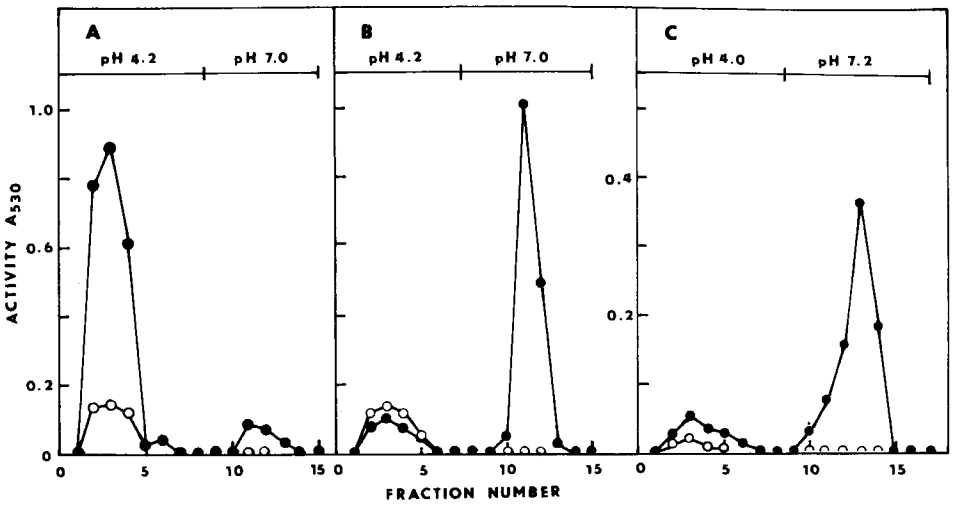

Fig. 2. Chromatography of *A. niger* endopolygalacturonase on columns of $(GalA)_2$—Separon (A), $(GalA)_4$—Separon and $(GalA)_5$—Separon (B) and tomato polygalacturonase on $(GalA)_4$—Separon (C). Crude enzyme preparation (10 mg) was chromatographed on columns (4 × 1.4 cm I.D.) equilibrated in acetate buffer (pH 4.2). The first peak was eluted with the equilibrating buffer, the second peak with 0.1 *M* sodium acetate (pH 7). (•) Activity on sodium pectate; (○) activity on digalactosiduronic acid.

All oligogalactosiduronides are, however, substrates of exopolygalacturonase.

Covalent binding of the oligogalactosiduronides to Separon resulted in glycosylated carriers that are stable under the experimental conditions used (Fig. 1). O-Glycosidic binding rendered the affinity ligands resistant to the action of both endopolygalacturonase and exopolygalacturonase. Their ability to function as biospecific adsorbents for endopolygalacturonase was tested in a column chromatography procedure, under the same reaction conditions used recently with trigalactosiduronic acid—Separon [4]. The crude enzyme preparation of *A. niger* (10 mg) containing a mixture of pectic enzymes was applied onto the column at pH 4.2, which is the optimum pH for endopolygalacturonase action. After elution of all substances not retained by the column, the adsorbed enzyme was displaced from the adsorbent by changing the pH of the eluting solution. For identification of endo- and exopolygalacturonase, the activity on pectic and digalactosiduronic acids was measured and the reaction products were analysed in both separated fractions. The elution patterns are shown in Fig. 2. The total activity toward digalactosiduronic acid, which corresponds to exopolygalacturonase, and that of pectinesterase were eluted unretarded from all three adsorbents with the equilibrating buffer. Endopolygalacturonase, manifested by its specific action on the polymeric substrate, was quantitatively adsorbed on Separon derivatives with immobilized tetra- and pentagalactosiduronic acids and, consequently, appeared in the second peak, whereas digalactosiduronic acid—Separon retained only a minute fraction of this enzyme. Similar differences were also found with respect to the capacities of the adsorbents when tested with the specific endopolygalacturonase (Table I) and also in chromatography of tomato pectic enzymes. Because of the low adsorption capacity of the disaccharide derivative, only

adsorbents with tetra- and pentagalactosiduronic acid were further characterized. The isolated endopolygalacturonase showed three multiple forms in ultrathin-layer isoelectrofocusing, with very close p*I*. Its activity increased fourteen-fold.

The retained endopolygalacturonase could be quantitatively displaced from the adsorbents by increasing the pH, but also by biospecific elution at pH 4.2 with any substrate (polymeric or oligomeric) and with trigalactosiduronic acid; digalactosiduronic acid was ineffective. At the same concentration of oligogalactosiduronic acid (0.5 mmol/l) used as soluble affinity counter-ligand, the elution volume (measured in the maximum of the elution peak) decreased with increasing DP up to the hexasaccharide level. The most pronounced difference occurred between the tetra- and pentasaccharide (Fig. 3). The elution volumes of endopolygalacturonase with oligosaccharides of DP 7–9 and with pectic acid (DP 153) were close to each other and to that of non-interacting substances. The eluted enzyme degraded all counter-ligands showing the action patterns and activities typical of soluble fungal endopolygalacturonase [16].

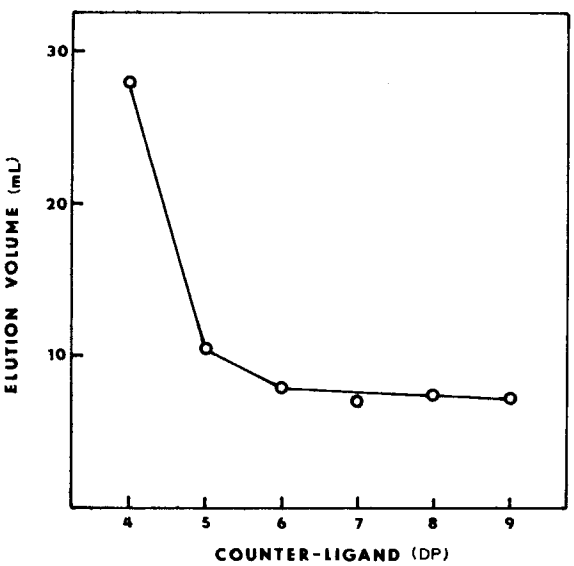

Fig. 3. Elution volumes of fungal endopolygalacturonase from $(GalA)_4$—Separon with oligogalactosiduronic acids of different DP.

The biospecificity of the interaction of endopolygalacturonase with Separon derivatives and its affinity for counter-ligands were evaluated by determining the dissociation constants of complexes of the enzyme with the affinity adsorbents (K_L) and with soluble counter-ligands (K_I), by means of elution chromatography using zonal analysis chromatography [12]. With both adsorbents, the elution volume of endopolygalacturonase (V_i) decreased with increasing concentration and increasing DP of soluble oligogalactosiduronide in the eluting buffer, whereas for a given counter-ligand this volume increased with increasing DP of the affinity ligand. The results are treated graphically in Fig. 4. During the elution of endopolygalacturonase, a degradation of the

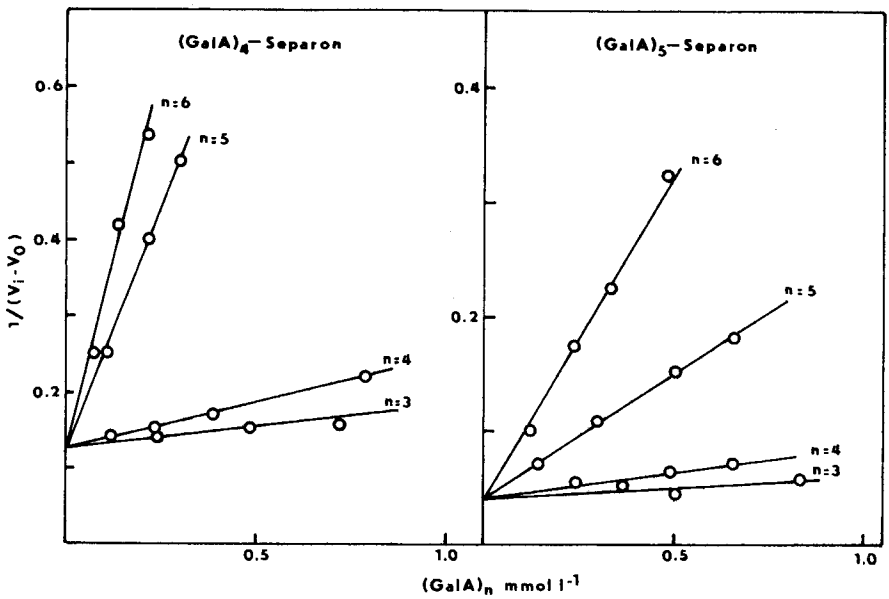

Fig. 4. Graphical illustration of the zonal analysis data [12] used for determination of dissociation constants. Chromatographic conditions: $(GalA)_4$—Separon: adsorbent volume 5.39 ml; V_0 3.5 ml; V_m 3.0 ml. $(GalA)_5$—Separon: adsorbent volume 6.16 ml; V_0 4.0 ml; V_m 3.5 ml. Flow-rate 1.5 ml/min. Enzyme: endopolygalacturonase of *A. niger*, 0.1 mg in 0.5 ml of equilibrating solution.

counter-ligands acting as substrate occurred. Its low degree, not exceeding 1.8% in tetragalactosiduronic acid, 4.3% in pentasaccharide and 5.9% in hexasaccharide, obviously did not substantially affect the elution profiles, as resulted from the linearity of the plots in Fig. 4. The values K_L and K_I (Table II) were calculated by the standard method [12], to determine the concentration of affinity ligands using the values determined by chemical analysis.

The results obtained with both adsorbents indicate that the adsorption of endopolygalacturonase is based on biospecific interaction with bound oligogalactosiduronic acid. This follows from: (a) identity of the dissociation constants of the complexes of the enzyme with both the ligand and the counter-ligand of a defined DP. This indicates further that the interaction

TABLE II

DISSOCIATION CONSTANTS OF COMPLEXES OF *A. NIGER* ENDOPOLYGALACTURONASE WITH AFFINITY LIGANDS (K_L) AND COUNTER-LIGANDS (K_I)

Ligand/counter-ligand DP	$(GalA)_4$—Separon		$(GalA)_5$—Separon		Kinetics
	K_L (mmol/l)	K_I (mmol/l)	K_L (mmol/l)	K_I (mmol/l)	K_I (mmol/l)
3		2.08		1.76	
4	1.06	1.03		0.84	0.86
5		0.10	0.18	0.18	0.21
6		0.057		0.071	0.08

of the enzyme with the affinity ligands is not affected by the support nor by the O-glycosidic binding of oligogalactosiduronides to the support; (b) the possibility of bioelution of the enzyme at the optimum pH of its activity with soluble oligogalactosiduronic acids forming catalytic complexes with endopolygalacturonase [16]; (c) the dependence of elution volumes on the counter-ligand concentration, which leads to a common intercept on the $1/(V_i - V_o)$ axis of the respective graphical plot for oligogalactosiduronic acids of different DP (Fig. 4).

The values of dissociation constants determined both by elution chromatography and kinetic measurement show that O-glycosidic binding of oligogalactosiduronic acids does not affect their interaction with endopolygalacturonase and, as a consequence, the interaction of the adsorbed enzyme with soluble substrates. This provides the possibility of employing these affinity sorbents to study the active-site-directed interactions of endopolygalacturonase.

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