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# SELECTIVE ISOLATION OF ENDO-D-GALACTURONANASE OF *Aspergillus niger* BASED ON INTERACTION WITH TRI(D-GALACTOSIDURONIC ACID) COVALENTLY BOUND TO POLY(HYDROXYALKYL METHACRYLATE)

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## ABSTRACT

A selective affinity-adsorbent for the extracellular endo-D-galacturonanase (E.C. 3.2.1.15) of *Aspergillus niger* was prepared by covalent coupling of tri(D-galactosiduronic acid) to Separon, a poly(hydroxyalkyl methacrylate) gel. Complexing of the enzyme with the adsorbent is pH dependent; maximal interaction occurs at the optimum pH for enzyme activity. The enzyme was quantitatively displaced from the adsorbent either by changing the pH or by bioelution with soluble tri(D-galactosiduronic acid) or other substrate. Within the range of substitution of Separon examined [content of tri(D-galactosiduronic acid) 1.7–6.7%] the amount of endo-D-galacturonanase retained was proportional to the content of affinity ligand. Under the same conditions, unsubstituted carrier did not complex with endo-D-galacturonanase. The dissociation constant of the affinity complex, as determined by zonal analysis, kinetic measurements, and by means of the adsorption isotherm $K_L$ (0.54 mmol.L$^{-1}$), is close to the value ($K_I$ 0.44 mmol.L$^{-1}$) obtained by the two first methods with soluble tri(D-galactosiduronic acid). The results show that adsorption of endo-D-galacturonanase on tri(D-galactosiduronic acid)–Separon is due exclusively to active-site-directed interaction with bound affinity-ligand.

## INTRODUCTION

Isolation of endo-D-galacturonanase (poly[(1→4)-$\alpha$-D-galactosiduronate] glycanohydrolase, E.C. 3.2.1.15) from a cultivation medium of *Aspergillus niger* requires its separation from such pectic enzymes as exo-D-galacturonanase (E.C. 3.2.1.67), pectinesterase (E.C. 3.1.1.11), pectin lyase (E.C. 4.2.2.10), some non-pectolytic enzymes, and other contaminating substances. A method previously elaborated[1] for selective purification of endo-D-galacturonanase of *A. niger* is based on biospecific interaction[2] of the enzyme with pectic acid crosslinked by

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epichlorhydrin. The method has also been applied in the purification of other endo-D-galacturonanases[3,4], and it seems that crosslinked pectic acid functioning simultaneously as carrier and affinity ligand could be used under appropriate conditions as a general biospecific adsorbent of any endo-D-galacturonanase, regardless of its action pattern or molecular properties. In addition to bioaffinity, the polyionic character of crosslinked pectate may, in some cases, cause nonspecific ionic interactions with the enzyme or some other proteins[3]. In such cases, the purification potential of the method, and the possibility of its application in the study of the active site of the enzyme, are considerably restricted. For such instances, and particularly for the last purpose, an adsorbent containing a low-molecular-weight affinity ligand bound to an inert polymer-matrix that interacts specifically with the active site of the enzyme would be more suitable.

Recently, we have found that endo-D-galacturonanase of *A. niger* interacts with tri(D-galactosiduronic acid) preferentially in a nonproductive complex[5]. Other pectic enzymes, with the exception of exo-D-galacturonanase, do not interact with this uronic acid oligosaccharide. It was, therefore, of interest to find out whether tri(D-galactosiduronic acid) covalently bound to an insoluble carrier would function as a biospecific ligand for endo-D-galacturonanase. The trisaccharide was attached by *O*-glycosylic linkage[6] to a synthetic sorbent based on poly(hydroxyalkyl-methacrylate) matrix [7] commercially available under the names Separon or Spheron*.

## EXPERIMENTAL

*Materials for preparation of the affinity sorbent.* — The poly(hydroxyalkyl methacrylate) gel Separon H 1000, of particle size 125–200 $\mu$m, was a product of Laboratory Instrument Works, Prague. Tri(D-galactosiduronic acid) was isolated from the enzymic hydrolyzate of sodium pectate produced by endo-D-galacturonanase immobilized on poly(ethyleneterephthalate)[8] in a column (1.4 × 2.5 cm) at pH 4.2 (0.1M acetate buffer), 23°, flow rate 1.2 mL.min$^{-1}$, enzyme content 0.5 mg per mL of carrier. The products were separated by gel chromatography on Sephadex G-25 (Fine)[9], desalted on Sephadex G-10, and freeze-dried. The preparation was homogeneous by t.l.c. on silica gel[10].

*O-Glycosylation of Separon by tri(D-galactosiduronic acid).* — Separon beads (5 g) were left to swell for 2 h at room temperature (23°) in 50 mL of dry 1,4-dioxane freshly saturated with dry hydrogen chloride gas. Sodium tri(D-galactosiduronate) (800 mg) was then added and the suspension was shaken for 48 h at room temperature. The beads were then washed several times on a sintered-glass funnel with dry 1,4-dioxane and then transferred into 0.05M phosphate buffer, pH

*Separon is produced by Laboratory Instrument Works, Prague 6, Czechoslovakia; Spheron by Lachema, Brno, Czechoslovakia. The materials, chemically identical, are distributed by Chemapol, Prague 4, Czechoslovakia.

7.9. The sorbent was washed with the same buffer until the washings gave negative reaction for carbohydrates[11]. Then the beads were washed successively with dry acetone and ether, and dried at 45°. The Separon derivative obtained contained 6.3% (w/w) of tri(D-galactosiduronic acid). Under given conditions, the amount of the tri(D-galactosiduronic acid) incorporated may be regulated by the ratios of Separon and acid used in the reaction, and by the time of reaction. The content of carbohydrate covalently bound to the Separon matrix was determined by titration of carboxyl groups and spectrophotometrically by the method of Dubois *et al.*[11].

*Enzyme.* — A crude enzyme-preparation of *A. niger* containing the mixture of pectic enzymes was prepared from the filtrate of a surface culture, growing on nutrient medium containing 1.5% of citrus pectin, by salting-out the protein fraction with ammonium sulfate (0.9 saturation) followed by precipitation with 76% ethanol and desalting by gel filtration on Sephadex G-25. Endo-D-galacturonanase was prepared from the crude preparation by affinity chromatography on cross-linked pectic acid[1]. The same procedure was used also for preparation of endo-D-galacturonanase from the commercial pectinase Rohament P (Röhm GmbH, FRG).

*Substrates.* — Sodium pectate (D-galacturonan content 89.8%) was prepared from citrus pectin (Genu Pectin, Københavns Pektinfabrik, Denmark) by repeated alkaline deesterification with sodium hydroxide (0.1 $ml.L^{-1}$), followed by precipitation with hydrochloric acid at pH 2.5 and by neutralization of the acid with sodium hydroxide. Oligo(D-galactosiduronic acids) of degrees of polymerization 2 and 4–7 were prepared by the same procedure as described for tri(D-galactosiduronic acid).

*Enzyme assay.* — Endo-D-galacturonanase and exo-D-galacturonanase activities were assayed spectrophotometrically at pH 4.2 (0.1M acetate buffer) by the reducing-group method of Somogyi[12]. Sodium pectate (0.5% solution) was used as the substrate for endo-D-galacturonanase, and di(D-galactosiduronic acid) (1 $mmol.L^{-1}$) for exo-D-galacturonanase. Endo-D-galacturonanase activity is expressed in mol reducing groups liberated within 1 s per kg of protein and determined by means of a standard graph for D-galactopyranuronic acid. The protein content was determined by the method of Lowry *et al.*[13] with human serum albumin as standard. The activity of pectinesterase was determined by titration (0.1 $mol.L^{-1}$ sodium hydroxide) of carboxyl groups released within 1 h from a 0.5% solution of citrus pectin (66.4% esterified) at pH 4.4 (0.1M acetate buffer).

*Adsorption of endo-D-galacturonanase on $(GalA)_3$–Separon.** — $(GalA)_3$–Separon (500 mg) containing 6.3% of tri(D-galactosiduronic acid) was suspended in 9.5 mL of acetate buffer (pH 4.2) and mixed with 0.5 mg of endo-D-galacturonanase in the same buffer (0.5 mL). The suspension was incubated with constant stirring at room temperature. The enzyme activity of the supernatant solution

*$(GalA)_3$–Separon: Tri(D-galactosiduronic acid)–Separon.

was determined at various time-intervals. The same experiment was performed in 0.05M acetate buffer and with the crude enzyme preparation in 0.1M buffer.

*Affinity chromatography.* — Chromatography on $(GalA)_3$–Separon was performed in a column (1.5 × 3.5 cm) equilibrated with acetate buffer, pH 4.2. The enzyme sample, dissolved in the same buffer, was adsorbed onto the column within 30 min. The column was then eluted with the equilibrating buffer until the unadsorbed proteins had been eluted. Adsorbed endo-D-galacturonanase was released with 0.1 $mol.L^{-1}$ sodium acetate. The absorbance at 280 nm and D-galacturonanase activities were measured for all fractions.

The effect of pH on the formation and dissociation of the endo-D-galacturonanase–$(GalA)_3$–Separon complex was examined by changing the pH of the first and second eluting buffer, respectively. In both instances, the activity of endo-D-galacturonanase after its elution from the column was determined at pH 4.2 and constant volume.

*Determination of the binding capacity of $(GalA)_3$–Separon.* — The column of $(GalA)_3$–Separon was successively charged with aliquots of endo-D-galacturonanase (0.5 mg) in 0.5 mL of acetate buffer, pH 4.2 until the effluent showed constant enzyme activity. The bound endo-D-galacturonanase was eluted with sodium acetate and its activity and protein content were determined. The experiment was performed with Separon derivatives containing 1.7, 4.6, 6.2, and 6.7% of tri(D-galactosiduronic acid), respectively.

*Determination of the dissociation constants of endo-D-galacturonanase with bound and soluble tri(D-galactosiduronic acid).* — The dissociation constants of the soluble complex ($K_1$) and insoluble complex ($K_1$) were determined (*a*)by affinity chromatography, using zonal analysis according to Dunn and Chaiken[14], performed in series of columns (1.4 × 9.5 cm) of $(GalA)_3$–Separon in 0.1M acetate buffers pH 4.2 containing tri(D-galactosiduronic acid) at concentrations varying from 0.69 to 4.12 $mmol.L^{-1}$; (*b*) by means of the adsorption isotherm obtained by treating at pH 4.2 the enzyme (0.2 mg) with $(GalA)_3$–Separon the quantity of which was varied from 23 to 155 mg; and (*c*) by measuring the effect of tri(D-galactosiduronic acid) (soluble and bound, respectively) on the kinetics of endo-D-galacturonanase in mixtures containing 0.03 mg of enzyme, 0.66 μmol of tri(D-galactosiduronic acid), and 0.16–3.3 mg of sodium pectate per mL. The kinetics for endo-D-galacturonanase in the presence of unsubstituted Separon were simultaneously measured. The data were treated according to Lineweaver and Burk[15].

*Analysis of the reaction products.* — The products of enzymic degradation of pectic acid were analyzed by t.l.c. performed on silica sheets (Silufol, Kavalier, Czechoslovakia) in 2:3:1 1-butanol–formic acid–water[10].

*Discontinuous poly(acrylamide) gel electrophoresis.* — This procedure was performed in Tris–glycine buffer, pH 8.3 at 4° and a potential gradient 3 V/cm (refs. 16, 17).

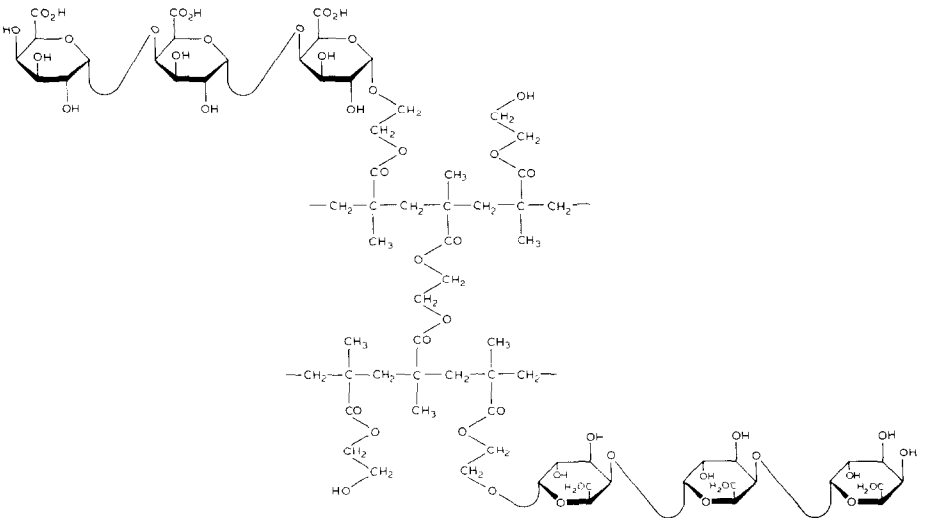

Fig. 1. Partial tentative structure of $(GalA)_3$–Separon.

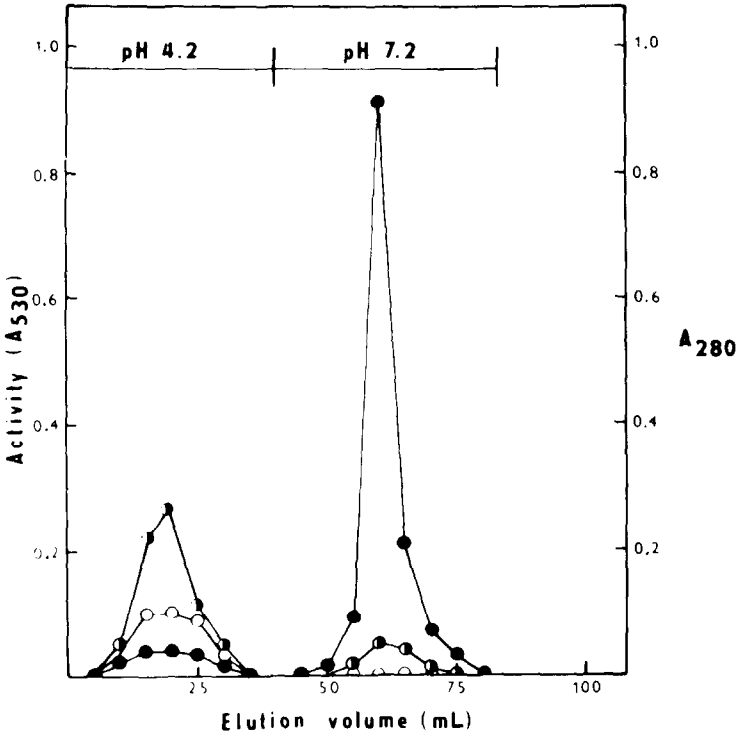


Fig. 2. Chromatography of endo-D-galacturonanase on a column of $(GalA)_3$–Separon. Crude enzyme of *A. niger* (10 mg) was chromatographed on a column (1.5 × 3.5 cm) pre-equilibrated in acetate buffer pH 4.2. The first fraction was eluted with the equilibrating buffer, the second fraction with 0.1M sodium acetate. Symbols: ● activity on sodium pectate, ○ activity on di(D-galactosiduronic acid), ◑ $A_{280}$.

## RESULTS

Covalent binding of tri(D-galactosiduronic acid) to Separon resulted in a glycosylated carrier (for a tentative structure see Fig. 1) that adsorbed selectively and reversibly the endo-D-galacturonanase of *A. niger*. The affinant was not attacked by the enzyme or other pectic enzymes produced by the microorganism. Incubation of the crude enzyme preparation of *A. niger* with $(GalA)_3$–Separon at pH 4.2 (pH optimum of endo-D-galacturonanase[18]) led to a decrease of endo-D-galacturonanase activity in the supernatant solution whereas the activities of exo-D-galacturonanase and pectinesterase remained unchanged. Equilibrium was established after ~20 min of incubation. Adsorption of the enzyme was not substantially affected by the concentration of buffer. Formation of the complex of endo-D-galacturonanase with $(GalA)_3$–Separon and its dissociation were affected by pH in the same manner as found with soluble substrates[18], maximum interaction occurring at

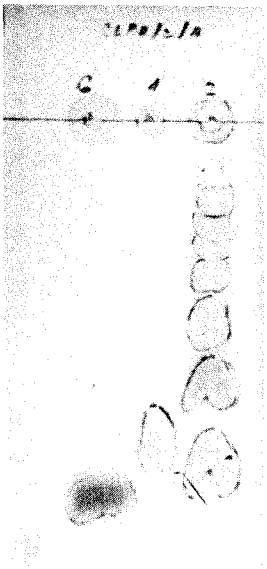

Fig. 3. T.l.c. of products of degradation of sodium pectate by unadsorbed (*1*) and adsorbed (*2*) fractions. G, Standard of D-galactopyranuronic acid; solvent system 2:3:1 1-butanol–formic acid–water.

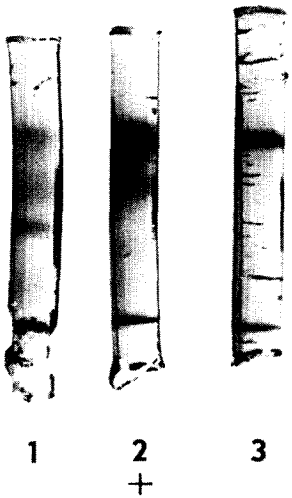


Fig. 4. Electrophoresis on poly(acrylamide)gel. *1*, Crude enzyme preparation of *A. niger*; *2*, endo-D-galacturonanase separated by affinity chromatography on crosslinked pectic acid; and *3*, endo-D-galacturonanase isolated by affinity chromatography on $(GalA)_3$–Separon. Buffer:Tris–glycine pH 8.3; potential 3 V/cm.

pH 4.0–4.2 and total displacement of the enzyme from the adsorbent at pH >6.0.

The column-chromatographic procedure involved adsorption of endo-D-galacturonanase at pH 4.2 and its displacement from the adsorbent by sodium acetate; the elution pattern for the crude enzyme preparation is shown in Fig. 2. Approximately 85% of the protein, including pectinesterase and ~10% of the D-galacturonanase activity, was eluted unretarded by the equilibrating buffer, in the first peak. The remaining 90% of the D-galacturonanase, corresponding to 15% of the protein applied to the column, appeared in the second peak eluted after the pH change. The enzyme activity towards di(D-galactosiduronic acid) recorded in Fig. 2, and t.l.c. of the products of degradation of pectic acid by both separated frac-

TABLE I

SEPARATION OF THE CRUDE PECTOLYTIC PREPARATION OF *A. niger* ON $(GalA)_3$–SEPARON

| *Enzyme* | *Volume mL* | *Enzyme activity U/mL* | *Protein mg/mL* | *Specific activity U/mg protein* | *Purification* |
|---|---|---|---|---|---|
| Crude enzyme | 1 | 0.335 | 5.480 | 0.061 | 1 |
| Unadsorbed fraction | 22 | 0.002 | 0.159 | 0.013 | |
| Adsorbed fraction | 24 | 0.015 | 0.017 | 0.845 | 13.8 |

U = $\mu$Mol reducing groups.$s^{-1}$.

tions (Fig. 3), show that the D-galacturonanase activity not retained in the column corresponds exclusively to an enzyme catalyzing the cleavage of terminal glycosidic linkages and thus producing D-galacturonic acid, namely, exo-D-galacturonanase, whereas the enzyme in the second peak corresponds to endo-D-galacturonanase. The chromatographic procedure increased the specific activity of endo-D-galacturonanase 13.8 times (Table I). Endo-D-galacturonanase was also eluted in a single, sharp peak when a pH gradient of 4.2–7.2 for the elution buffers was used for its release from the complex. The chromatography also permitted separation of an enzymically inactive protein-fraction, inseparable from endo-D-galacturonanase by affinity chromatography on crosslinked pectic acid[1] (Fig. 4). The specific activity of the enzyme thus increased by 21%. The purified endo-D-galacturonanase showed a single protein and activity zone in discontinuous electrophoresis on poly(acrylamide) (Fig. 4).

Quantitative displacement of endo-D-galacturonanase from the column could also be achieved by biospecific elution at pH 4.2 with any substrate, polymeric or oligomeric, as well as by tri(D-galactosiduronic acid). At the same concentration of oligo(D-galactosiduronic acids) (1 mmol.L$^{-1}$) used as soluble affinity-counter-ligands, the elution volume of the enzyme decreased with increasing degree of polymerization up to the hexasaccharide level; a most pronounced difference occurred between the tri- and tetra-saccharide. The elution volume of endo-D-galacturonanase in the presence of counterligands of d.p. $\geq 7$ was the same as that of the noninteracting enzymes. Endo-D-galacturonanase chromatographed on unmodified Separon H 1000 was eluted without retardation, with other enzymes.

The binding capacities of four $(GalA)_3$–Separons differing in their content of tri(D-galactosiduronic acid) are summarized in Table II. Within the range of substitution of Separon examined, the amount of adsorbed endo-D-galacturonanase was proportional to the content of affinity ligand.

The biospecificity of interaction by endo-D-galacturonanase with $(GalA)_3$–Separon (6.3% substituted) was evaluated on the basis of the dissociation constants of the complexes of the enzyme with soluble tri(D-galactosiduronic acid) ($K_I$) and with $(GalA)_3$–Separon ($K_I$) (Table III). In zonal analysis, the elution volume of endo-D-galacturonanase ($V_i$) decreased with increasing concentration of tri(D-

TABLE II

BINDING CAPACITIES OF $(GalA)_3$–SEPARON CONTAINING DIFFERENT AMOUNTS OF AFFINITY LIGAND

| *Content of affinity ligand* | | *Adsorbed enzyme mg protein/g carrier* |
|---|---|---|
| *% (w/w)* | *μmol/g carrier* | |
| 1.7 | 31.13 | 1.07 |
| 4.6 | 84.55 | 3.11 |
| 6.3 | 115.40 | 4.00 |
| 6.7 | 122.70 | 4.17 |

TABLE III

DISSOCIATION CONSTANTS OF COMPLEXES OF ENDO-D-GALACTURONANASE WITH SOLUBLE ($K_I$) AND LINKED ($K_L$) TRI(D-GALACTOSIDURONIC ACID)

| *Method of determination* | $K_I$ *mmol.L*$^{-1}$ | $K_L$ *mmol.L*$^{-1}$ |
|---|---|---|
| Affinity chromatography | 0.453 ±0.038 | 0.542 ±0.039 |
| Adsorption isotherm | | 0.419 ±0.029 |
| Kinetics | 0.443 | 0.538 |

galactosiduronic acid) in the eluting buffer. The results are treated graphically (Fig. 5) and the $K_I$ (0.453 mmol.L$^{-1}$) and $K_L$ (0.542 mmol.L$^{-1}$) values determined by the standard method[14], using 10 mL for $V_o$, 8.5 mL for the void volume, and for the concentration of affinity ligand the values determined by chemical analysis. Similar values for $K_I$ (0.61 mmol.L$^{-1}$) and $K_L$ (0.56 mmol.L$^{-1}$) were obtained with $(GalA)_3$–Separon containing 4.6% of tri(D-galactosiduronic acid). In kinetic measurements, both soluble tri(D-galactosiduronic acid) and its Separon derivative behaved as competitive inhibitors of endo-D-galacturonanase, whereas unsubsti-

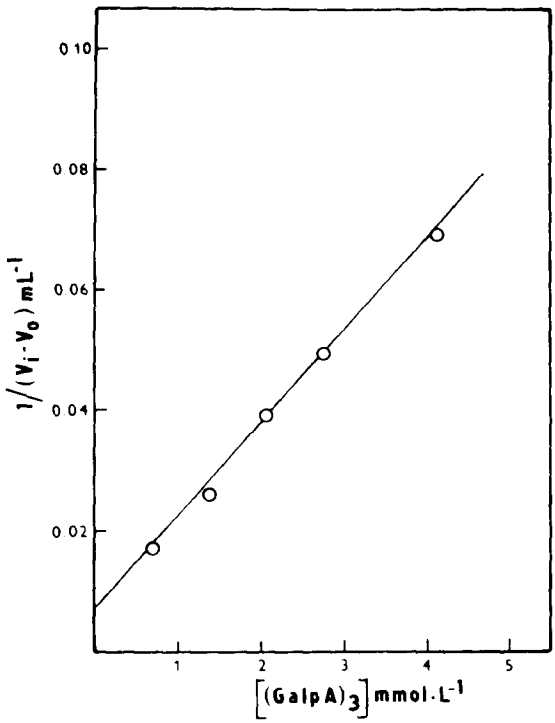


Fig. 5. Graphical illustration of the zonal-analysis data[14] used for determination of dissociation constants. Chromatographic conditions: column 1.4 × 9.5 cm; content of bound tri(D-galactosiduronic acid) 6.3%; $V_o$ = 10 mL; void volume $V_m$ = 8.5 mL.

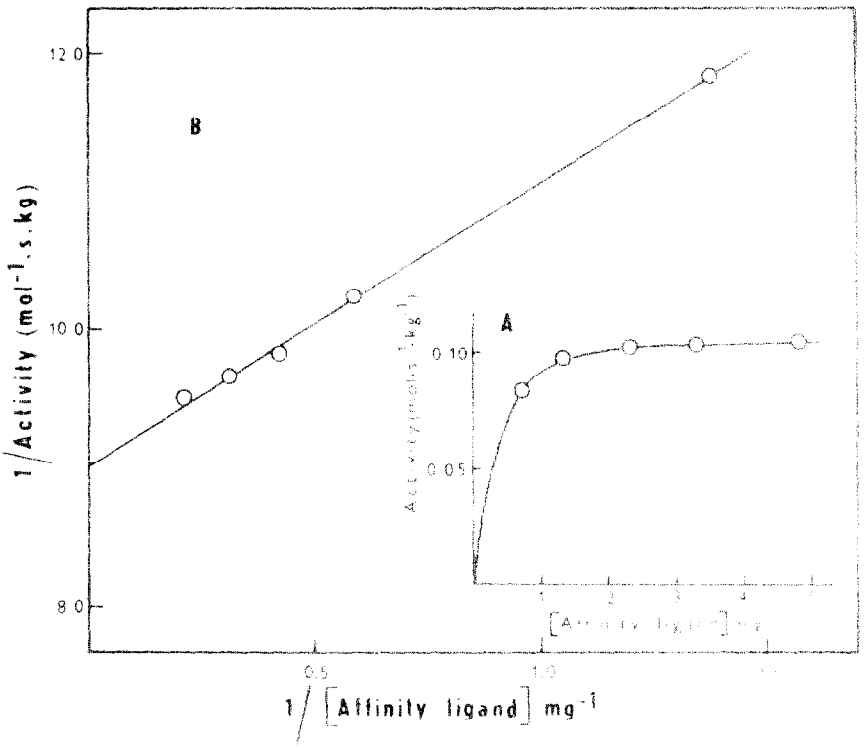


Fig. 6. Effect of the concentration of affinity adsorbent (A, expressed in the amount of affinity ligand) on the quantity of adsorbed endo-D-galacturonanase, and B, double reciprocal plot of the same data. Samples of endo-D-galacturonanase (0.2 mg) in 3 mL of acetate buffer (pH 4.2) were treated with the adsorbent in amounts from 23 to 155 mg.

tuted Separon H 1000 did not affect the enzyme activity. The dissociation constants $K_I$ (0.443 mmol.L$^{-1}$) and $K_I$ (0.538 mmol.L$^{-1}$) were close to each other as well as to the values obtained by the previous method. A similar value for $K_I$ (0.419 mmol.L$^{-1}$) was also obtained by means of a double-reciprocal plot of the adsorption isotherm (Fig. 6).

Whereas with extracellular endo-D-galacturonanase of *A. niger*, all activity was retained on the column of $(GalA)_3$–Separon and then eluted within one sharp, symmetrical peak, endo-D-galacturonanase from commercial pectinase (Rohament P, identified as a preparation of *Aspergillus* sp.) was found to contain multiple forms of endo-D-galacturonanase differing in affinity towards covalently bound tri(D-galactosiduronic acid). Under the same conditions as employed for the *A. niger* extracellular enzyme, a specific preparation of endo-D-galacturonanase obtained by affinity chromatography on crosslinked pectic acid and having two pH optima[8] was separated on $(GalA)_3$–Separon giving two fractions. One was minor, did not interact with the affinant, and was eluted within the equilibrating buffer; a main fraction containing ~90% of the activity was retained on the column

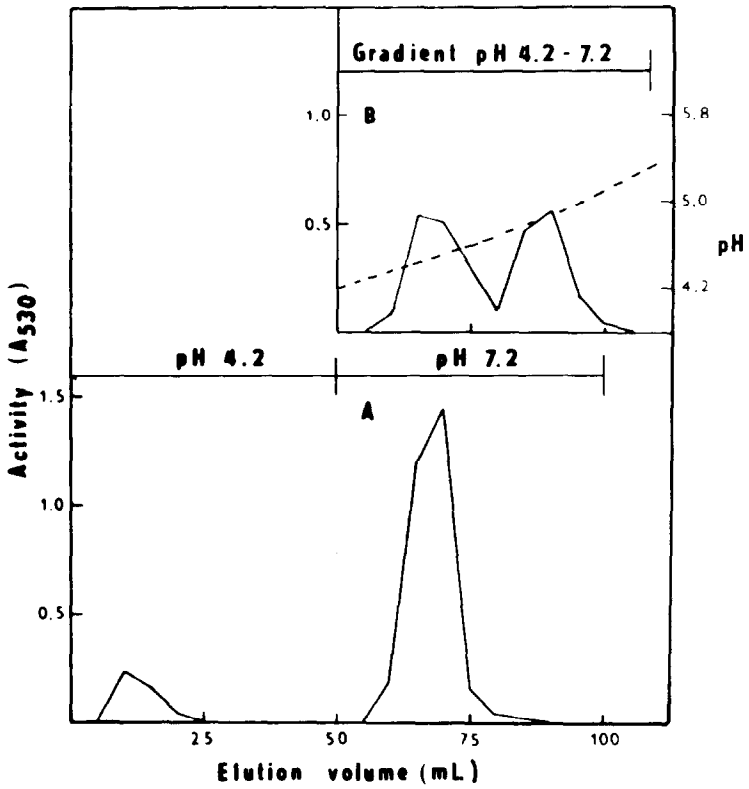


Fig. 7. Affinity chromatography of endo-D-galacturonanase (Rohament). A, chromatography under the conditions described in the legend to Fig. 2; B, elution of the adsorbed endo-D-galacturonanase by using a linear gradient pH 4.2–7.2.

and then liberated at higher pH (Fig. 7A). When retained fraction (Fig. 7B) was eluted with a pH gradient, two peaks of activity were obtained, the first, containing ~40% activity, at pH 4.5 and the second at pH 5.0. These two fractions most probably correspond to two forms differing in pH optimum[8] recently separated on ion-exchange derivatives of Spheron[19].

## DISCUSSION

Separon (Spheron) is a macroporous hydrophilic gel prepared in form of spherical particles by copolymerization of a hydroxyalkyl methacrylate and ethylene dimethacrylate[7]. Spheron has been successfully derivatized both for immobilization[20] and ion-exchange medium-pressure chromatography[19,21] of pectic enzymes of *A. niger*. *O*-Glycosylation of Separon with tri(D-galactosiduronic acid) provides a new functional derivative of poly(hydroxyalkyl methacrylate) which proved to be a specific sorbent for certain types of endo-D-galacturonanases.

A method originally devised for binding of monosaccharides was used for the synthesis of $(GalA)_3$–Separon, to the copolymer matrix[6]. The successful covalent coupling of a negatively charged, oligouronic acid highly insoluble in the glycosylating medium demonstrates the wide applicability of this procedure for preparation of various types of glycosylated carriers. The glycosylation of poly(hydroxyalkyl methacrylate) increases the hydrophilicity of the matrix and consequently suppresses unspecific hydrophobic interactions with endo-D-galacturonanase and other proteins.

It is evident from the results obtained with unsubstituted Separon, as well as from the proportionality between the content of affinity ligand and the adsorption capacity of the $(GalA)_3$–Separon, that the adsorption of endo-D-galacturonanase is because of its interaction with bound tri(D-galactosiduronic acid). This oligosaccharide is one of the final products of degradation of D-galacturonan by endo-D-galacturonanases, and for some of them it is the lowest oligo-D-galactosiduronate capable of nonproductive interaction[22]. The results obtained indicate that similar active-site-directed interaction also plays a role in the interaction of *A. niger* endo-D-galacturonanase with covalently bound tri(D-galactosiduronic acid). This assumption follows from (*a*) the identity of the pH effect on both enzyme activity[18] and formation of the complex with $(GalA)_3$–Separon; (*b*) the possibility of bioelution of the enzyme with soluble tri(D-galactosiduronic acid) or any substrate; (*c*) competitive inhibition of the enzyme by both soluble and linked tri(D-galactosiduronic acid); and (*d*) similarity of the dissociation constants of the soluble and insoluble complexes of endo-D-galacturonanase with the trisaccharide, showing that the interaction of the enzyme with affinity ligand is not affected by the support. Active-site-directed complexing, establishing high specificity towards certain type of endo-D-galacturonanase, provides the possibility for use of this affinity sorbent not only for selective separation but also in studies of the binding site of the enzyme.

Tri(D-galactosiduronic acid), which is one of the best substrates of exo-D-galacturonanase in terms of rate of degradation[23] when covalently bound to Separon, forms neither productive nor nonproductive complexes with this enzyme, although its nonreducing end (which is the site of attack) is not blocked. This inability to complex probably arises from steric hindrance caused by the matrix of the sorbent, which renders the sugar groups situated towards the *O*-glycosidically linked matrix inaccessible to the binding subsites of the enzyme.

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