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Aspergillus niger ENDOPOLYGALACTURONASE. I. STUDIES ON PURIFICATION BY AGAROSE GEL CHROMATOGRAPHY

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INTRODUCTION

Pectins are carbohydrate polymers, mainly composed of galacturonic acid residues joined by $\alpha 1 \rightarrow 4$ linkages. In order to determine their fine structure, Talmadge et al. (1) have recently used the specific action of one pectolytic enzyme on sycamore cell walls and studied the composition of the liberated oligogalacturonides by chemical methods.

Pectolytic enzymes exist commercially as a mixture produced by microorganisms. Their isolation, purification, and properties have been studied for more than 10 years, and the work on endopolygalacturonase [poly(1,4αD-galacturonide)glycanohydrolase, endoPG, E.C. 3.2.1.15.] has been reviewed by Rombouts and Pilnik (2).

EndoPG has been obtained by the usual conventional methods of ethanol, acetone, or ammonium sulfate precipitation, chromatography on ion exchangers, or gel filtration. In order to speed and simplify the purification procedure, several original methods have been developed. For instance, Lineweaver et al. (3) purified endoPG seven-fold from a commercial preparation of fungal pectolytic enzymes by adsorption on alginic acid, but this method was ineffective for yeast endoPG purification (4). Patel and Phaff (5) and Phaff and Demain (6) used pectic acid gel for purification of tomato polygalacturonase and yeast endoPG, respectively. Rexova-Benkova and Tibensky (7) purified an endoPG from *Aspergillus niger* by affinity chromatography on a column of crosslinked pectic acid. This last method was also successfully used by Foglietti et al. (8) for a 140-fold purification of an endoPG from *Helix pomatia* juice.

During chromatography on agarose gel (Bio-Gel A 0.5 m), English et al. (9) observed that *Colletotrichum lindemuthianum* endoPG was eluted after the total volume of the column. In view of the resemblance between the anhydrogalactose residues of the agarose and the anhydrogalacturonic ones of endoPG substrate, these workers suggested that biospecific interactions between agarose and endoPG were occurring.

The present communication reports further investigations on the purification of endoPG from *Aspergillus niger* by chromatography on agarose gel. In an analytical procedure, the effect of dialysis, pH, and molarity of the elution buffer on the binding of the enzyme has been studied, the best recovery of the activity being obtained with a NaCl gradient. The results led to the description of a preparative procedure.

MATERIAL AND METHODS

Material

Pectinase (USK 5933), an impure commercial preparation of pectolytic enzymes synthesized by *Aspergillus niger*, was used. This preparation was a gift of Rapidase Company (Seclin, France).

Methods

EndoPG Activity Evaluation. The extent of polygalacturonic acid (Schuchardt) hydrolysis by endoPG was measured by either the formation of reducing groups or the decrease in viscosity. The reaction mixture was made of polygalacturonic acid (2 ml, 0.5% wt/vol) solubilized by disodium ethylene diaminetetraacetate (2% wt/vol) and neutralized to pH 4.2 by 1 N sodium hydroxide, 100 mM acetate buffer (2 ml, pH 4.2), and enzyme solution (1 ml, suitably diluted).

Reducing groups formed were measured by the method of Nelson (10), using galacturonic acid as standard. EndoPG activity was then expressed in nkatals, where 1 nkatal is defined as the amount of enzyme which liberates 1 nmol reducing group (or 1 nmol galacturonic acid) per second at 30°C. Activity was linearly related to the concentration of enzyme used.

For viscosity measurements, the reaction mixture (4 ml) was pipetted into an Ostwald viscometer (solvent flow time, 114 sec) in a water bath at 30°C. The reciprocal of the specific viscosity of the reaction mixture was graphed versus the reaction time (taken as the sum of the starting time and the half of the flow time). This relation was linear during the initial reaction period. EndoPG activity was expressed as the slope of the straight line $d(1/\eta_{sp})/dt$ in min^{-1}. One viscosity unit (VU) is defined as the amount of

enzyme increase in 1 min^{-1} at 30°C, the reciprocal of the specific viscosity of the reaction mixture. Activity was linearly related to the concentration of enzyme used.

In the analytical study, the percentages of bound activity (VU) and of bound protein were calculated after elution of the bound proteins at pH 6.0 (200 mM acetate buffer). Degree of purification is the ratio between the specific activity (VU/mg) of the bound activity and the specific activity of the crude preparation.

Sample Preparation. All procedures were carried out at 4°C. For the analytical procedure, the appropriate acetate buffer (10 ml) was added to the enzyme preparation (1 g). The suspension was centrifuged (12,000 *g* for 15 min) to remove insoluble particles, and the supernatant liquor was examined directly by chromatography after overnight dialysis against the same buffer. A longer dialysis time was not desirable because of the presence of cellulases in the enzyme preparation. For the preparative procedure, the enzyme preparation (5 g) was suspended in the appropriate acetate buffer (25 ml). After centrifugation as described above, the supernatant (10 ml) was dialyzed against the same buffer for 7–8 hr.

Chromatography Procedures. Chromatography procedures were also carried out at 4°C. During analytical chromatography, a K16/40 column (Pharmacia) was packed with Sepharose 6B (lot 0385, Pharmacia) to a total volume of 40–42 ml. Samples of enzyme preparation (minimum 16 mg when dialyzed, maximum 33 mg when crude) in the appropriate buffer (0.5 ml) were applied and eluted at a pump rate of 20 ml/hr. Fractions (5–6 ml) were collected and analyzed. Between runs, the column was equilibrated by the passage of seven or eight column volumes of new buffer before the application of the new sample.

For preparative chromatography, a K26/100 column (Pharmacia) was filled with Sepharose 6B (lot 0385 or 6274) to a total volume of about 420 ml and samples (containing up to 1 g of protein in 10 ml acetate buffer) were applied and eluted under the same conditions as above. Fractions of 10–11 ml were collected and analyzed.

Other Procedures. Extinction coefficients for the enzyme solution were measured at 280 nm using a 1-cm cell. Protein concentration were taken to be proportional to extinction, assuming $OD^{0.1\%}_{280\,nm} = 1$.

Acetate buffers were formulated according to Gomori (11) and the pH was measured at room temperature.

RESULTS

EndoPG from a crude enzyme preparation, whether dialyzed or not, has been observed to be eluted or delayed or bound to the agarose gel. The

binding depends essentially on three factors: dialysis treatment of the preparation, molarity, and pH of the elution buffer.

Analytical Study

Conditions of the Binding of endoPG Activity: Effects of Dialysis. As shown in Table 1, when samples of impure and dialyzed enzyme preparation were chromatographed on Sepharose 6B, equilibrated with 20 mM acetate buffer, pH 4.24, and eluted with the same buffer, although the total applied activities were similar, 46% of endoPG activity was bound to the Sepharose 6B from the crude and 91% from the dialyzed preparation. The enzyme is therefore purified 5.6-fold in the first case and thirteen-fold in the second case; i.e., dialysis which eliminates 40–50% of 280-nm absorbing substances and leads to a 1.4- to twofold purification seems to be a necessary step for higher binding of enzyme activity.

Effect of Elution Buffer pH. Figure 1 shows the variation with elution buffer pH of the percentage of bound activity, the percentage of bound protein, and the degree of purification. About 90% of endoPG activity is bound to the agarose gel when the elution pH is varied between 4.05 and 4.42. Beyond this pH, this percentage rapidly decreases to zero at pH 5.2. In this latter case, endoPG is not bound to the agarose gel but slightly delayed; these results confirm those of English et al. (9) where purified endoPG was eluted after the total volume of a Bio-Gel A 0.5 m equilibrated with 40 mM acetate buffer, pH 5.2.

The percentage of bound protein remains constant between elution pH of 4.05 and 4.25, then decreases to zero at pH 5.2. The corresponding degree of purification is therefore not constant; up to an elution pH of 4.15, endoPG is purified tenfold, then it increases to a maximum value of

TABLE 1. **Influence of Dialysis of the Enzyme Preparation on the Binding of Endopolygalacturonase Activity to the Sepharose 6B Column.**[a]

	Applied proteins (mg)	Applied activity (VU)[b]	Bound proteins (mg)	Bound activity (%)	Degree of purification
Crude enzyme preparation	32.8	722	2.1	46	5.6
Dialyzed enzyme preparation	16.3	671	2.3	91	13

[a]Experimental conditions are given in the text.
[b]VU, viscosity units.

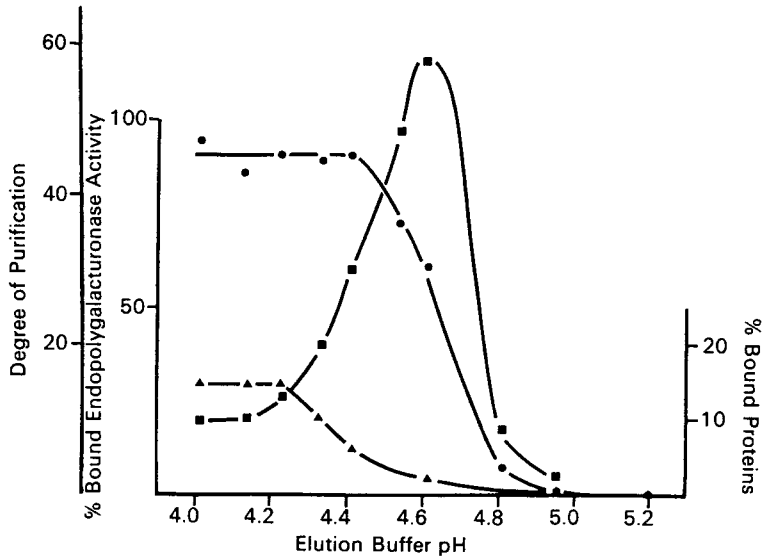

FIG. 1. Influence of elution buffer pH (20 mM acetate) on the binding to the Sepharose 6B column of endoPG activity (●) and total proteins (▲) and on the degree of purification of the enzyme (■). Experimental conditions are given in the text.

sixtyfold when the elution pH is 4.60, and beyond this pH it diminishes and is 2.5-fold at pH 4.95.

Consequently, to obtain the highest degree of purification, the elution must be done at pH 4.6, where only 60% endoPG activity is retained.

In contrast, in order to collect the quasitotality of endoPG activity, the elution must be carried out at a pH range between 4.3 and 4.4. In this latter case, 90% endoPG activity and 7–12% protein are bound to the agarose gel, leading to twenty- to thirtyfold purification.

Effect of Elution Buffer Molarity. The study of elution buffer molarity was carried out at pH 4.34. Figure 2 shows that, up to a 20 mM molarity, 90% of endoPG activity is bound to the column. Beyond this molarity, the percentage decreases; 7.5% of activity is bound with 100 mM buffer molarity. The percentage of bound proteins decreases rapidly, from 17.5% with 10 mM buffer to 2% with 100 mM buffer.

Figures 1 and 2 show that the bound proteins are not homogeneous: endoPG is still contaminated with inert proteins. A differential elution technique must be carried out.

Elution from Sepharose 6B of Bound Activity. Application of a solution of sodium polygalacturonate, stepwise or with a gradient (0–0.6% wt/vol), did not elute the enzyme.

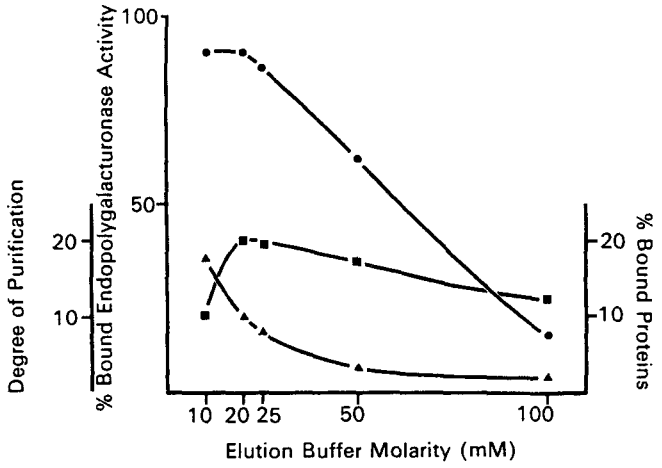

FIG. 2. Influence of elution buffer molarity (acetate buffer pH 4.34) on the binding to the Sepharose 6B of endoPG activity (●) and proteins (▲) and on the degree of purification of the enzyme (■). Experimental conditions are given in the text.

When the bound protein to the agarose column was successively washed with acetate buffer of the same molarity but increasing pH, as shown in Fig. 3, some endoPG activity was eluted at pH 4.4–5.3. The total activity was eluted at pH 5.3. This technique of elution is therefore unsatisfactory since there is no elution pH value which could separate endoPG from the other proteins.

Again, when the bound endoPG protein was eluted with a continuous pH gradient made from 20 mM acetate buffer, pH 4.24 (100 ml), and 20 mM acetate buffer, pH 6.00 (100 ml), a poor separation of activity was observed. In contrast, the technique of elution with NaCl gradient between 0 and 0.15 mM gave satisfactory purification, and this technique was used on a preparative scale.

Preparative Purification of Endopolygalacturonase

For preparative purification of endoPG, the sample was applied, after dialysis, to a Sepharose 6B column (87 × 2.6 mm diameter) equilibrated with 20 mM acetate buffer, pH 4.34, and the column was eluted with the same buffer until absorbance of the eluate at 280 nm was zero. This washing removed 80–90% of the protein and also 10% of polygalacturonase activity. The main endoPG activity was eluted by applying a linear gradient of 20 mM acetate buffer containing 0–0.15 M NaCl, respectively (500 ml of each). Finally, the proteins still bound to the column (5–6%) were removed by

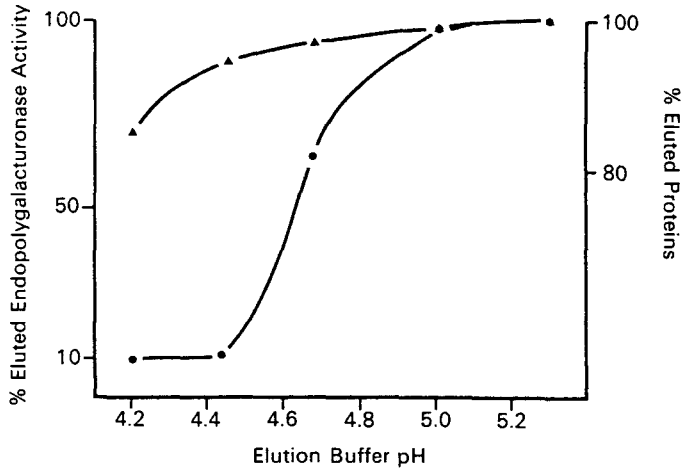

FIG. 3. Elution of the proteins bound to the Sepharose 6B column (20 mM acetate buffer, pH 4.24) with 20 mM acetate buffer of increasing pH. ●, EndoPG activity; ▲, proteins eluted.

elution with 20 mM acetate buffer containing 2 M NaCl (600 ml); about 4–5% polygalacturonase activity was detectable in this eluate.

A typical elution profile is shown in Fig. 4 and the corresponding purification in Table 2. The combined active fractions represent a 62-fold purification in terms of viscosity units (corresponding to 52% recovery) and 36-fold in terms of nkatals (corresponding to 36% recovery), the corresponding yield of protein being 0.8%.

The activity of the eluted enzyme solution was not stabilized by addition of bovine albumin serum, either 0.1 or 1 ml of a 0.5% wt/vol solution or 1 ml of a 1% wt/vol solution.

DISCUSSION

One endoPG from a commercial mixture can be extensively purified by a method based on chromatography on Sepharose 6B. Other activities observed during the purification may represent exopolygalacturonase activity or other endoPG activity.

Before this chromatography, an essential preliminary step is dialysis of the impure preparation, which doubles the specific activity of the bound protein. This fact may be explained by elimifiation, during dialysis, of small molecules which could hinder interactions between agarose and endoPG.

pH and molarity of the elution buffer strongly influence the binding of endoPG to agarose gel (compare Fig. 1 and Fig. 2). Pure agarose is

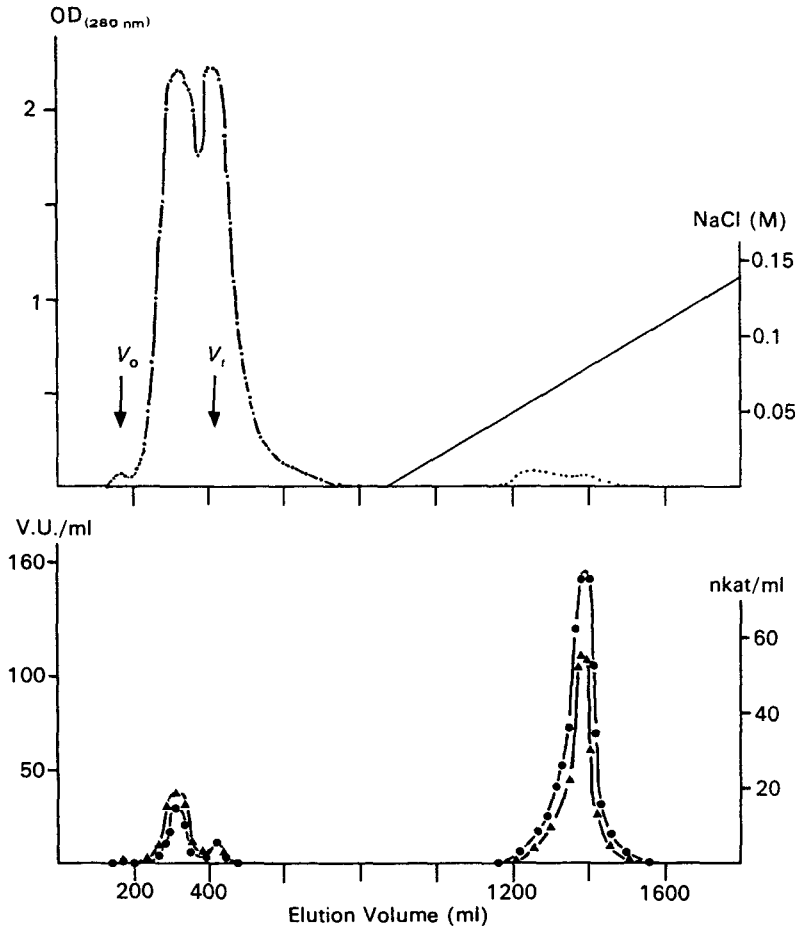

FIG. 4. Fractionation of enzyme preparation on Sepharose 6B at 4°C. 980 mg proteins is loaded onto a Sepharose 6B column (2.6 × 80 cm) equilibrated with 20 mM acetate buffer, pH 4.34. After washing with the same buffer, the column is eluted with a linear concentration gradient of NaCl (0–0.15 M) in the same buffer and 10.5-ml fractions are collected. (a) Proteins (●) and NaCl concentration (——). (b) Enzyme activities: ●, viscosimetric units (VU); and ▲, nkatals.

electrically neutral, but commercial agarose is still contaminated by some traces of agaropectin and the sulfate and carboxyl groups of this component (12) give the Sepharose 6B slight properties of a cation exchanger.

The fact that endoPG is eluted after the total volume of an agarose column was explained by English et al. (9) as being due to biospecific interactions between agarose and the enzyme. However, we found that a

TABLE 2. **Purification of *Aspergillus niger* Endopolygalacturonase**

	Crude preparation	After dialysis	After Sepharose 6B chromatography
Volume (ml)	10	13.5	298
Proteins (mg)	1,500	980	12.6
Protein yield (%)		65.3	0.8
Total activity (VU)	33,000	33,750	17,080
Activity yield (VU %)		102.3	52
Total activity (nkat)	22,000	21,600	6,700
Activity yield (nkat %)		98.2	30
Specific activity (VU mg)	22	34.4	1,355
Degree of purification (in VU)		1.6	62
Specific activity (nkat/mg)	14.7	22	530
Degree of purification (in nkat)		1.5	36

solution of sodium polygalacturonate—the preferential substrate of endoPG—does not elute the enzyme, suggesting the existence of noncompetitive interactions between endoPG and agarose.

EndoPG chromatography on agarose may therefore be an ion-exchange effect. But the low density of charges of the Sepharose, which were estimated by Crone (13) to be 2.6×10^{-6} eq/ml of gel, does not rule out other kinds of interactions to explain the preferential binding of endoPG to the agarose.

Several techniques were tried to elute the proteins bound to the Sepharose 6B column. Elution with a pH stepwise gradient gave poor results in our case, whereas Foglietti et al. (8) obtained a good separation of endoPG from contaminating proteins after binding of endoPG to a cross-linked pectic acid. Application of a pH continuous gradient has led to less satisfactory results than application of a linear NaCl gradient. By such a gradient (0–0.15 M NaCl), a good separation of endoPG was obtained. All these techniques of elution are compatible with ionic interactions between agarose and endoPG.

During preparative experiments, instability of the enzyme was detected, for only 67–70% activity was collected. This instability has also been observed for other preparations (9, 14, 15). All workers have reported apparent stabilization by the addition of bovine serum albumin, but in our experience this agent does not significantly improve the activities recovery.

In contrast to the method of Rexova-Benkova and Tibensky (7), our proposed purification procedure does not need a special gel and is simple, rapid, and reproducible. Over 18 months, several purifications on the same column and on two different batches of Sepharose 6B led to the same results.

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