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Note

Improved method for the sequential purification of polysaccharidases by affinity chromatography

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Affinity chromatography is widely used for enzyme purification. The chromatographic support is generally an insoluble matrix on which a suitable ligand has been covalently linked. In the case of a polysaccharidase, the macromolecular substrate can be cross-linked leading to a matrix which is specifically recognized by the corresponding endopolysaccharidase. Rexová-Benková and Tibensky[1] introduced this affinity technique for the purification of *Aspergillus niger* polygalacturonase on cross-linked pectic acid. We have been able to generalize this method to other endopolygalacturonases[2,3] and to other types of endopolysaccharidases, mainly amylases[4] and cellulases[5,6].

In this report we describe the use of this affinity technique for the sequential purification of some polysaccharidases (amylases, polygalacturonases, cellulases) from a complex biological extract rich in each of these enzymes, *i.e.*, an extract from the larvae of a xylophagous insect, *Phoracantha semipunctata.*

MATERIALS AND METHODS

The polysaccharides comprised wheat starch, soluble starch (Merck, Darmstadt), insoluble cellulose (Whatman), carboxymethylcellulose (Blanose R 195, Novacel) and sodium polygalacturonate (Sigma).

The crude enzyme extract was obtained from *Phoracantha semipunctata* larvae as described previously[7].

Protein concentrations were estimated by the method of Bradford[8] using bovine serum albumin as standard.

Chromatographic supports

Polysaccharides were cross-linked by epichlorhydrin under alkaline conditions. The experimental conditions were adapted to each polysaccharide–polysaccharidase pair.

Starch and cellulose. A modification of the technique of Kuniak and Marchessault[9] was used. Each polysaccharide was treated with a 10% aqueous solution of sodium hydroxide and epichlorhydrin. The molar ratio sodium hydroxide/poly-

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saccharide (expressed in anhydroglucose units) was 1 for starch and 5 for cellulose. The molar ratio epichlorhydrin/polysaccharide was 1.25 for starch and 0.6 for cellulose.

After continuous stirring, first at room temperature and then at 50°C, the mixture was neutralized by acetic acid, after which the polysaccharide was washed, dried and ground to fine particles (Table I).

Polygalacturonic acid. This was cross-linked according to the technique of Rombouts *et al.*[10]. Sodium polygalacturonate was treated in an ethanolic medium (95°) with epichlorhydrin and 5 *M* sodium hydroxide. The molar ratio sodium hydroxide/polysaccharide (expressed in anhydrogalacturonic acid) was 1 and the molar ratio epichlorhydrin/polysaccharide was 1.5. After continuous stirring at 40°C, the mixture was washed with distilled water and neutralized with acetic acid. After filtration, the cross-linked polysaccharide was washed with ethanol–water (3:1) and with ethanol. It was then air dried (Table I).

The experimental conditions used for the cross-linking of each polysaccharide are summarized in Table I.

Enzyme assays

All enzyme assays were performed at 37°C.

α-Amylase. The amylolytic activity in column effluents was located using the iodine–potassium iodide reagent[11].

For all other assays, the Nelson–Somogyi colorimetric method was used as previously described[12,13] to determine the liberated reducing groups using wheat starch as a substrate.

Polygalacturonase. The polygalacturonase activity was estimated by the Nelson–Somogyi method as previously described[3] using sodium polygalacturonate as a substrate.

Cellulase. The cellulolytic activity was estimated by the Nelson–Somogyi method as previously described[6] using insoluble cellulose or carboxymethylcellulose as substrates.

In column effluents, the cellulolytic activity was located using agarose gels containing carboxymethylcellulose (1%). An aliquot (10 μl) of each effluent was

TABLE I

EXPERIMENTAL CONDITIONS FOR CROSS-LINKING OF POLYSACCHARIDES

Polysaccharide	*Molar ratio*		*Continuous stirring*
	*Sodium hydroxide/ polysaccharide**	*Epichlorhydrin/ polysaccharide*	
Starch	1	1.25	Room temperature: 90 min 50°C: 2 h
Cellulose	5	0.6	Room temperature: 15 min 50°C: 1 h
Sodium polygalacturonate	1	1.5	40°C: 4 h

* Expressed in anhydrohexose units.

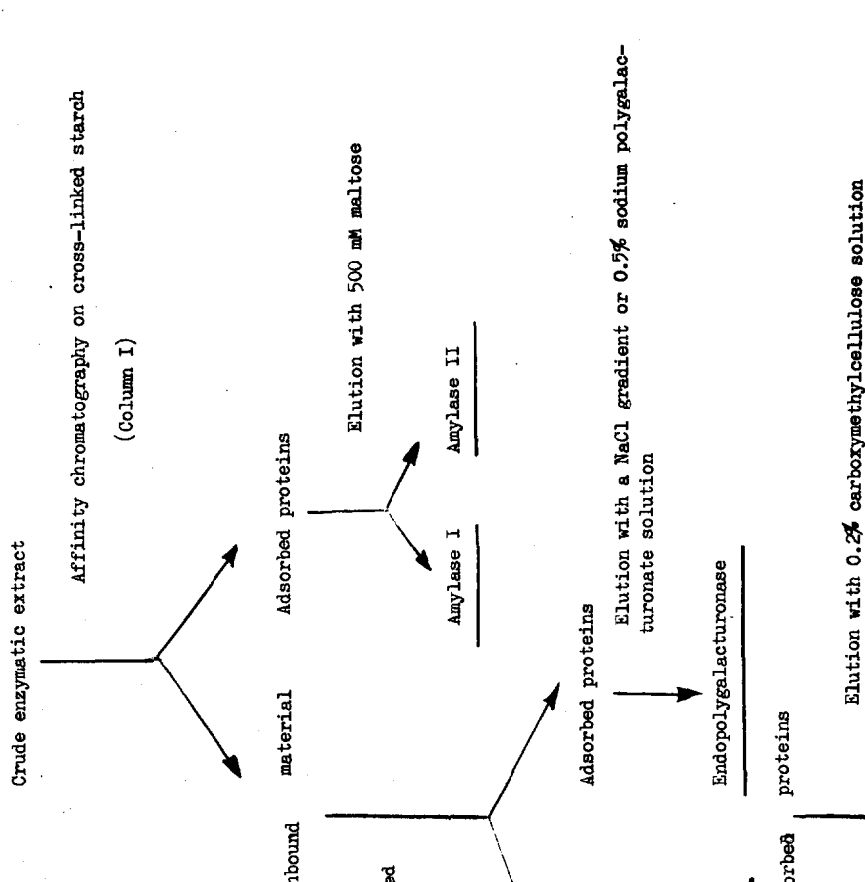

Fig. 1. Scheme for the sequential purification of polysaccharidases from *Phoracantha semipunctata* larvae.

placed in a well. After incubation for 1 h at 37°C and washing with 0.9% sodium chloride the gels were stained with an aqueous solution of Congo Red (0.1%). Activities were revealed as colourless zones on the red plate.

Identification of products of enzymatic hydrolysis

Hydrolysis products were separated by thin-layer chromatography on silica gel plates.

For the separation of oligosaccharides from starch and cellulose hydrolysates, the chromatograms were developed with propanol-1–ethyl acetate–ethanol–acetic acid–pyridine–water (7:3:3:2:2;4, v/v). For the separation of oligogalacturonates, the solvent system was butanol-1–formic acid–water (2:3:1, v/v).

In all cases, spots were revealed by heating the plates after spraying with a solution of sulphuric acid in acetone (5%).

Isoelectric focusing

Amylase and polygalacturonase. Analytical isoelectric focusing was performed using 5% acrylamide plates (LKB Ampholine PAG plates) containing 2.4% ampholytes (pH 3.5–9.5). Electrofocusing was performed as previously described[7].

Cellulose. An agarose gel I.E.F. (Pharmacia) aqueous solution (1%, w/v) containing 2.4% ampholytes (pH 3.5–9.5) was bedded on a gel-bound plate (LKB).

The anodic and cathodic solutions were 0.05 *M* sulphuric acid and 1 *M* sodium hydroxide, respectively. The electric power was maintained constant at 7 W for 90 min. Electrofocusing was performed at 4°C.

RESULTS AND DISCUSSION

The crude enzymatic extract from *Phoracantha semipunctata* larvae contains high levels of different polysaccharidase activities, mainly amylase, polygalacturonase and cellulase. So it can be used as a model for the sequential isolation of endo-polysaccharidases.

The purification of each polysaccharidase was performed sequentially by affinity chromatography on the corresponding cross-linked polysaccharide as described in Fig. 1.

Column I (cross-linked starch)

The first step was the purification of amylase on cross-linked starch according to a modification of the procedure previously described[4] (Fig. 2).

The buffered crude enzyme extract (1 ml, 20 mg proteins) was applied on a column (25 × 1 cm) containing an homogeneous mixture of cross-linked starch (4 g) and Sephadex G-10 (4 g) equilibrated with 10 m*M* malate buffer pH 6.5 containing 10 m*M* calcium acetate and 20 m*M* sodium chloride. Protein contaminants and all enzyme activities except that of α-amylases were eluted by the equilibrating buffer. The two amylasic activities (amylases I and II) were then eluted by a 500 m*M* buffered maltose solution.

The homogeneity of the two fractions and their mode of action were studied on the lyophilizates obtained after maltose elimination (Sephadex G-25 chromatography). Homogeneity was tested by isoelectric focusing. Amylase I showed a major

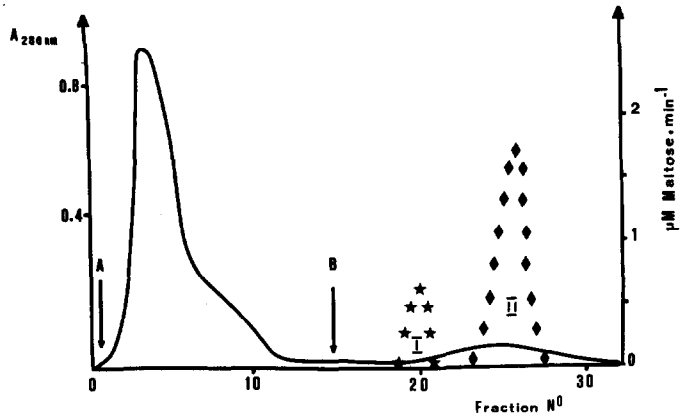

Fig. 2. Affinity chromatography of the crude extract of *Phoracantha semipunctata* larvae on cross-linked starch (column I). The column (25 × 1 cm) was equilibrated with 10 m*M* malate pH 6.5 containing 10 m*M* calcium acetate and 20 m*M* sodium chloride. Elution was carried out with (A) equilibrating buffer and (B) 500 m*M* maltose solution in the equilibrating buffer. The flow-rate was 10 cm/h and 2.5-ml fractions were collected. Proteins were measured estimated from the absorbance at 280 nm (———). Enzyme activity was estimated by the Nelson–Somogyi colorimetric method. Amylasic activity was eluted in two peaks: amylase I (★) and amylase II (◆).

protein band at p*I* 4.2. In addition, a faint band was detected but no amylase activity was revealed at this position. The enzyme activity was located only in the major protein band. Amylase II showed a single protein band at p*I* 4 with which was associated the amylasic activity. These two enzymes differed with respect to their optimum pH (6 for amylase I and 5.3 for amylase II).

The mode of action of the two enzymes was investigated as previously described[7]. Amylase I behaves as a maltotetraose-forming exoamylase, while amylase II is a typical endoamylase (α-amylase).

Column II (cross-linked polygalacturonic acid)

After dialysis against distilled water and lyophilization, the unbound material from column I was chromatographed on a cross-linked polygalacturonic acid column (Fig. 3). The buffered enzyme solution (1 ml) was applied on a column (20 × 1.2 cm) containing an homogeneous mixture of cross-linked polygalacturonic acid (5 g) and Sephadex G-10 (5 g) equilibrated with 100 m*M* acetate buffer pH 4.8.

Protein contaminants and all enzyme activities except that of endopolygalacturonase were eluted by the equilibrating buffer. The polygalacturonase was then eluted out by a linear gradient of 0–0.5 *M* sodium chloride in the equilibrating buffer. It can also be specifically eluted by a buffered 0.5% sodium polygalacturonate solution. The first type of elution was chosen to avoid chromatographic elimination of the excess of polygalacturonate and of oligogalacturonates present in the effluent.

The homogeneity and the mode of action of the enzyme were tested on the lyophilizates obtained after dialysis against distilled water. A single protein band at p*I* 5.8, which was associated with the polygalacturonasic activity, was revealed by isoelectric focusing. The optimum pH was 5. When the enzyme was incubated with sodium polygalacturonate, oligogalacturonates of various degrees of polymerization

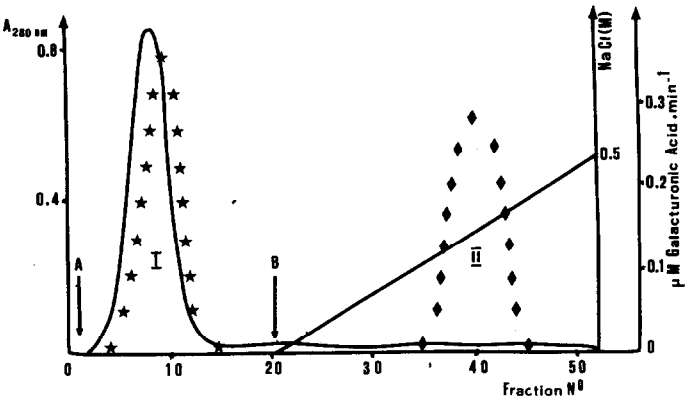

Fig. 3. Affinity chromatography on cross-linked polygalacturonic acid (column II). The column (20 × 1.2 cm) was equilibrated with 0.1 *M* acetate buffer pH 4.8. Elution was carried out with the equilibrating buffer (A) and with a linear gradient of 0–0.5 *M* sodium chloride (B). The flow-rate was 13 ml/h and 3-ml fractions were collected. Proteins were estimated from the absorbance at 280 nm (———). Enzyme activity was estimated by the Nelson–Somogyi colorimetric method: exopolygalacturonase (I) (★) and endopolygalacturonase (II) (◆).

(DP) were produced in the early stages of hydrolysis. The final product of the degradation was digalacturonate. This pattern is typical of an endopolygalacturonase which produces oligogalacturonates with various DPs.

Column III (cross-linked cellulose)

After dialysis against distilled water and lyophilization, the unbound material from column II was chromatographed on a cross-linked cellulose column (Fig. 4). The buffered enzyme solution (1 ml) was applied on a column (25 × 1.5 cm) con-

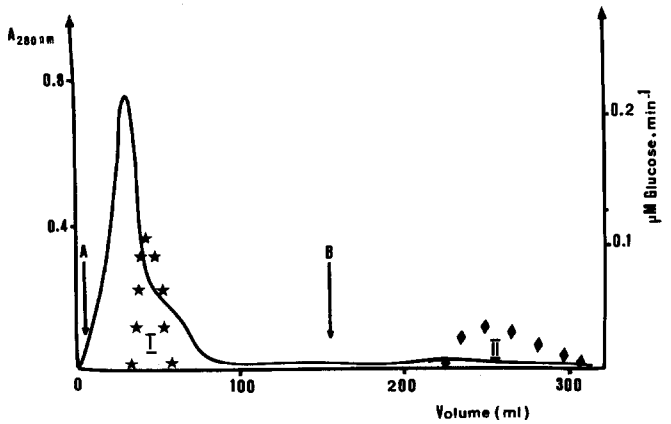

Fig. 4. Affinity chromatography on cross-linked cellulose (column III). The column (25 × 1.5 cm) was equilibrated with 50 m*M* acetate buffer pH 4.8. Elution was carried out with the equilibrating buffer (A) and a 0.2% carboxymethylcellulose solution (B) in the equilibrating buffer. The flow-rates were 2 ml/h (A) and 5 ml/h (B); 2-ml fractions were collected. Proteins were estimated from the absorbance at 280 nm (———) and enzymatic activity by the Nelson–Somogyi colorimetric method: exoglucanase (I) (★) and endoglucanase (II) (◆).

taining cross-linked cellulose (10 g) equilibrated with 50 m*M* acetate buffer pH 4.8. Protein contaminants and all enzyme activities except that of the endocellulase were eluted by the equilibrating buffer. The endocellulase activity was then specifically eluted by a 0.2% carboxymethylcellulose buffered solution.

Two protein bands at p*I* $\approx$ 4 were revealed by isoelectric focusing. Each of them corresponds to an endocellulasic activity leading to the liberation of cellodextrins of various DPs after incubation with crystalline cellulose.

Assays for differential elution of these two endocellulases were attempted with carboxymethylcellulose solutions of different concentrations, as previously described for *Trichoderma viride* endocellulases[5]. A good resolution cannot be achieved because of the poor stability of the cellulolytic activities of *Phoracantha semipunctata*. However, this chromatographic step allows the separation of endoglucanasic activities from exoglucanase eluted by the equilibrating buffer as shown previously[6].

CONCLUSIONS

Sequential affinity chromatography on cross-linked polysaccharidses allowed the isolation of the corresponding polysaccharidases.

Successive chromatography on cross-linked starch, cross-linked polygalacturonic acid and cross-linked cellulose led respectively to the purification of two amylases, one endopolygalacturonase and to the separation of two endocellulases from the exocellulasic activities contained in the crude extract of *Phoracantha semipunctata* larvae.

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