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POLYGALACTURONASE OF *BOTRYTIS CINEREA* E-200 PERS

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Summary

A polygalacturonase (poly(1,4-α-D-galacturonide)glycanohydrolase, EC 3.2.1.15) was purified from the culture fluid of *Botrytis cinerea*. The polygalacturonase preparation, homogeneous on the basis of disc-gel electrophoresis also showed pectinesterase activity. Some properties of the purified polygalacturonase were studied. It had a molecular weight about 69 000. It was inactivated by *p*-chloromercuribenzoate, tetranitromethane and urea. A 50% loss in viscosity of sodium polypectate solution occurred when 4.6% of the glycosidic bonds were hydrolyzed. The only end product of sodium polypectate and oligogalacturonides hydrolysis was monogalacturonic acid.

Introduction

Botrytis cinerea, like many other fungal phytopathogens, produces pectolytic enzymes. These enzymes have been considered to function in aiding pathogens to spread within the host plants. Several workers have demonstrated a heterogeneity of pectolytic enzymes in crude preparations from *B. cinerea* [1—3]. However, no data has been found in literature about homogeneous pectolytic preparations from this fungus. The physical, chemical and enzymatic properties of these enzymes are also little known. The present paper deals with purification and characterization of *B. cinerea* extracellular polygalacturonase (poly(1,4-α-D-galacturonide)glycanohydrolase EC 3.2.1.15).

Material and Methods

Botrytis cinerea E-200 Pers, obtained from the Institute of Breeding and Acclimatization of Plants, Bydgoszcz, Poland was the strain of fungus used in the investigation presented below. The fungus was surface-cultured in the liquid Czapek's medium with pectin. After 10 days of culturing, the mycelium was centrifuged out. Acetone was added to the culture fluid to 20% and resulting

precipitate was centrifuged out. Supernatant was adjusted with acetone to 66% and the precipitate was dissolved in 0.01 M acetate buffer, pH 4.0. This solution was passed through a column of Sephadex G-50. Active fraction was applied to a column of DEAE-cellulose. Elution was done by linear gradient of 0—0.2 M NaCl in the phosphate buffer, pH 6.4. Enzymatic fraction having been eluted in 0.075 M/0.1 M NaCl was finally purified by filtration on Sephadex G-100 column, washed with the acetate buffer, pH 4.0. The purified preparation of polygalacturonase thus obtained, was further characterized. Its homogeneity was checked using polyacrylamide gel electrophoresis.

The polygalacturonase activity was determined by estimating the increment of reducing groups with 3,5-dinitrosalicyclic acid method [4]. The reaction medium contained 1 ml of 0.5% sodium polypectate or pectin N.F. (68% esterification) in 0.01 M acetate buffer, pH 4.0 and 1 ml of enzyme solution. One unit of polygalacturonase activity was defined as that amount which liberated 1 μmol of galacturonate equivalents per minute at 30° C.

Pectinesterase activity was measured according to Wang and Keen, at pH 5.0 [5]. The protein was determined by the Lowry et al. [6]. The molecular weight was estimated with a column of Sephadex G-100 according to the Andrew's method [7].

Hydrolytic products were examined by chromatography on Whatman No. 1 paper with the mixture, ethyl acetate/acetic acid/water (3 : 3 : 1) as a solvent. After development, the paper was sprayed with phthalate anisidine reagent and heated at 90° C for 5 min.

Results

Table I summarizes results obtained at each step of purification procedure. Two peaks exerting polygalacturonase activity have been found during separation on DEAE-cellulose (Fig. 1). Peak II was the subject of this paper. This peak after additional purification on Sephadex G-100 was quite homogeneous when checked on disc gel electrophoresis at pH 8.4 (Fig. 2). The position of the enzyme activity appeared to be identical with a protein band.

TABLE I

PURIFICATION OF POLYGALACTURONASE FROM *B. CINEREA*

The total enzymatic activity was measured with sodium polypectate.

Purification step	Total protein (mg)	Total activity (units × 10^3)	Specific activity (units/mg)	Yield (%)
Culture supernatant	2312.0	189.7	82.0	100
Acetone 20% (supernatant)	1420.6	179.0	126.0	94.3
Acetone 66% (precipitate)	900.0	138.5	153.8	73.0
Sephadex G-50	301.1	119.2	395.8	62.8
DEAE-cellulose, peak II	23.4	42.4	1811.9	22.3
Sephadex G-100	18.4	37.7	2048.9	19.8

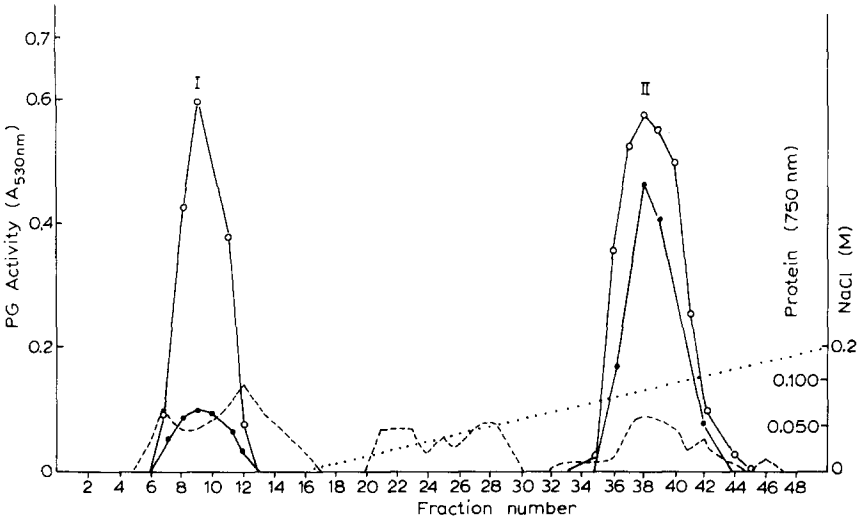

Fig. 1. DEAE-cellulose column chromatography. Column size: 1.5 cm × 30 cm, 0.1 M sodium phosphate buffer, pH 6.4, NaCl gradient is shown in the figure, 5.8 ml fractions were collected. Fractions 35—44 were combined. ○———○, activity (substrate, sodium polypectate); ●———●, activity (substrate, pectin N.F.); - - - - - -, protein; . . . . . , NaCl.

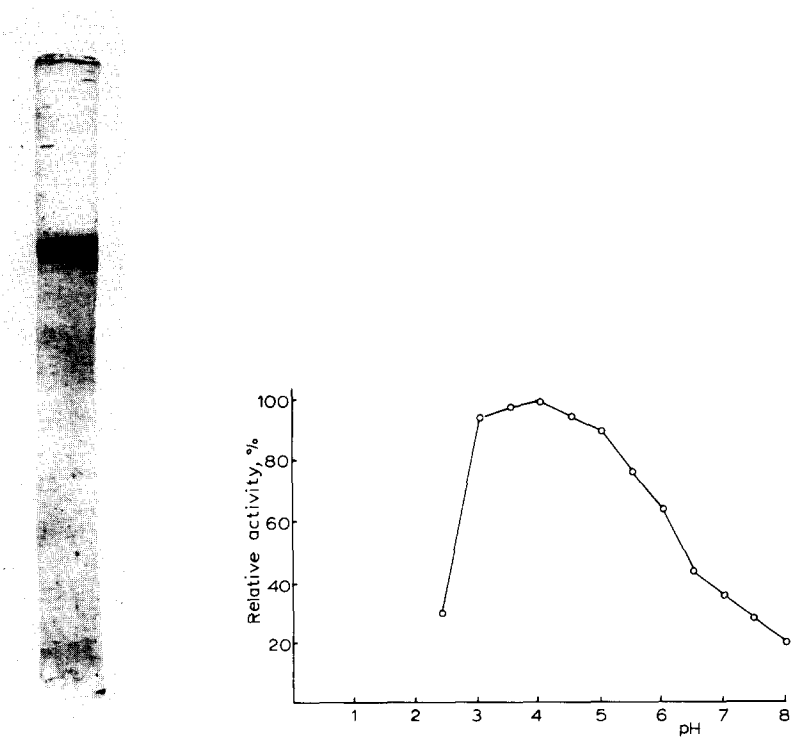

Fig. 2. Disc-gel electrophoresis of *B. cinerea* polygalacturonase. Separation was carried out in polyacrylamide gel (7.5%) at 5.0 mA per tube for 120 min. Tris/glycine buffer, pH 8.4 was used. The gels were stained with 0.5% amido black in 7.0% acetic acid.

Fig. 3. Effect of pH on polygalacturonase activity. The pH of sodium polypectate substrate was adjusted with McIlvaine buffer. Incubation time was 10 min at 30° C.

TABLE II

Polygalacturonase activities with sodium polypectate and pectin N.F. and pectinesterase activity in the mixture of pectolytic enzymes on several purification steps. PG, polygalacturonase; PE, pectinesterase; PMG, polymethylgalacturonase.

Purification step	Activity (units × 10^3)			Activity ratio	Activity ratio
	PG*	PMG**	PE	PMG/PG	PE/PG
Culture supernatant	189.6	132.2	59.8	0.69	0.31
Acetone 66%	138.5	95.1	46.4	0.68	0.33
Sephadex G-50	119.2	72.9	35.7	0.61	0.29
DEAE-cellulose, peak II	42.4	34.1	20.2	0.80	0.47
Sephadex G-100	37.7	30.7	18.5	0.81	0.49

* Activity with sodium polypectate.
** Activity with pectin N.F.

Purified polygalacturonase as well as crude preparations hydrolyzed preferentially sodium polypectate. The high ability of polygalacturonase to degrade pectin N.F. could have been clearly visible, too (Table II). The degradation of pectin was probably connected with its previous deesterification by pectinesterase, present in preparations. The experiments showed pectinesterase activity in the crude preparations and in the purified polygalacturonase (Table II). It is very interesting that the single protein band, obtained during disc-gel electrophoresis of the polygalacturonase preparation, being active with sodium polypectate and pectin N.F. as the substrates, exhibited the PE activity, too.

The molecular weight of polygalacturonase was about 69 000 according to the gel filtration method. The optimum pH for polygalacturonase activity with sodium polypectate as a substrate was 4.0 (Fig. 3). The polygalacturonase activity was strongly inhibited by *p*-chloromercuribenzoate, tetranitromethane (pH 7.0) and urea (Table III). *p*-Chloromercuribenzoate-inactivated polygalacturonase was reactivated by cysteine. During disc-gel electrophoresis of urea-

TABLE III

EFFECT OF SOME REAGENTS ON POLYGALACTURONASE ACTIVITY

The enzyme polygalacturonase was preincubated at 30°C for 30 min with chemicals at pH 7.0 (in the case of tetranitromethane also at pH 4.0), then the remaining activity was assayed at pH 4.0. The final concentration of chemicals was 10^{-3} M, only the concentration of urea was 8.0 M.

Chemicals	Remaining activity (%)
p-Chloromercuribenzoate	14
DFP	98
Tetranitromethane (pH 4)	69
Tetranitromethane (pH 7)	15
Urea	9
H_2O_2	82
EDTA	100
Cysteine	117

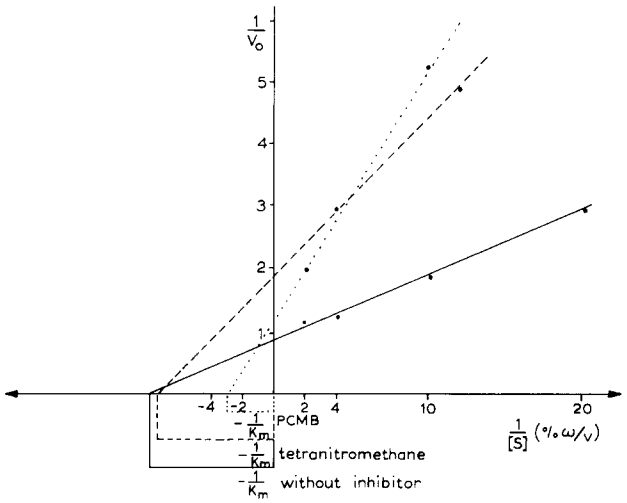

Fig. 4. K_m and V determinations in the presence of inhibitors. . . . . ., *p*-chloromercuribenzoate; - - - - - -, tetranitromethane; ———, without inhibitor. The final concentrations of inhibitors were $7 \cdot 10^{-4}$ M. The substrate was sodium polypectate used in the following concentrations: 0.05, 0.1, 0.25 and 0.5% w/v.

treated polygalacturonase, two protein bands were obtained instead of a single band in the case of untreated enzyme. Both of the bands were inactive.

The K_m and maximum velocity for the polygalacturonase acting on sodium polypectate were 0.12% (w/v) and 2352 μmol reducing groups/min/mg protein, respectively (Fig. 4). The K_m in the presence of tetranitromethane was very similar (0.12%), but the maximum velocity was diminished to 1052 μmol/min/mg protein. In the presence of *p*-chloromercuribenzoate the values 0.34% sodium polypectate and 1666 μmol/min/mg protein were obtained for K_m and V, respectively.

During the breakdown of sodium polypectate 50% loss in viscosity occurred when no more than 4.6% of glycosidic bonds were hydrolyzed (Fig. 5).

Changes undergone during the action of polygalacturonase on sodium polypectate and oligogalacturonides were detected by paper chromatography.

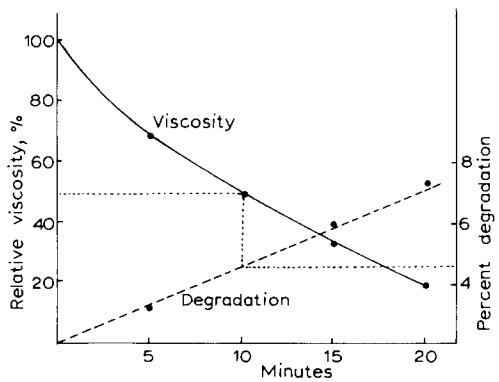

Fig. 5. Viscosity changes and percent degradation of sodium polypectate by polygalacturonase as a function of time. The reaction mixture contained 0.5% sodium polypectate in the acetate buffer pH 4.0 and enzyme solution. The temperature was 30° C.

TABLE IV

RELATIVE RATES OF *B. CINEREA* POLYGALACTURONASE ACTIVITY ON SODIUM POLYPECTATE AND OLIGOGALACTURONIDES

The reaction mixture consisted of enzyme and 0.5% substrate in acetate buffer at pH 4.0.

Substrate	Aldehyde groups released (μmol/min/mg protein)	Relative rate
Sodium polypectate	1953	100
Tetragalacturonic acid	1542	79
Trigalacturonic acid	1015	52
Digalacturonic acid	273	14

At earlier stages of hydrolysis, sodium polypectate was broken down mainly into monogalacturonic acid and to a lesser degree into oligogalacturonides (Fig. 5). The spots corresponding to oligogalacturonides gradually disappeared with the reaction time, so the monogalacturonic acid remained as the only product of the hydrolysis.

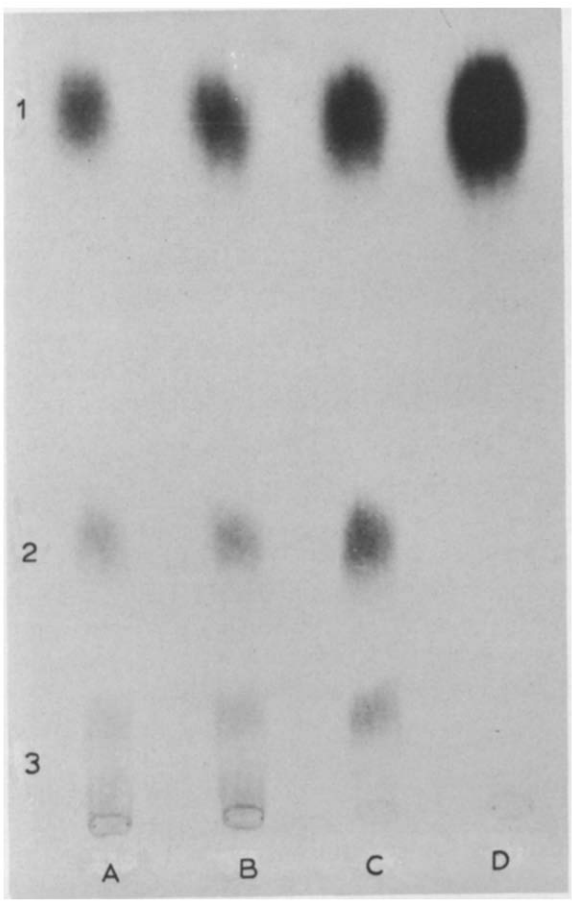

Fig. 6. Paper chromatogram of the hydrolysis products from the action of polygalacturonase on sodium polypectate. Time incubation: (A) 10 min, (B) 20 min, (C) 60 min, (D) 120 min. 1, monogalacturonic acid; 2, dimer; 3, trimer.

Tetragalacturonic acid was degraded first into mono- and tri-galacturonic acid; digalacturonic acid was detected further. The end product was only monogalacturonic acid.

Table IV presents relative rates of polygalacturonase activity on oligo- and polygalacturonides. The activity was maximal with sodium polypectate, followed by tetra-, tri- and digalacturonic acids.

Discussion

The ability of *B. cinerea* polygalacturonase to degrade pectin N.F. was connected with the presence of pectinesterase in the preparation. It is very probable that polygalacturonase and pectinesterase may exist as a complex. The following facts support the occurrence of this complex: (1) During disc-gel electrophoresis of polygalacturonase preparation, only a single protein band was obtained, but it showed pectinesterase activity apart from the polygalacturonase activity. (2) The polygalacturonase used here has relatively high molecular weight in comparison with the values obtained for other polygalacturonases of microorganisms [8,9]. The existence of pectinesterase-polygalacturonic acid lyase complex with high molecular weight was reported at *Clostridium multifermentans* [10]. The occurrence of two protein bands during disc-gel electrophoresis of polygalacturonase preparation previously treated with 8 M urea may indicate dissociation of polygalacturonase-pectinesterase complex.

Considering the rapid decrease of viscocity and the presence of oligogalacturonides during sodium polypectate hydrolysis, we conclude that our polygalacturonase attacks the glycosidic bonds in a random manner. Also, this mode of action is confirmed by the fact that the rate of substrate degradation diminished in parallel to the polymerization degree. However, it exhibits rather large preference for the hydrolysis of terminal bonds in the substrates. Therefore, we can explain why the principal product of sodium polypectate hydrolysis is monogalacturonic acid and why tetragalacturonic acid is cleaved initially to mono- and trigalacturonic acids.

The inhibition of polygalacturonase activity by *p*-chloromercuribenzoate suggest the presence of SH-groups in the active center of the enzyme. The decrease of polygalacturonase activity after incubation with tetranitromethane in the pH 7.0 indicates that tyrosine is also important for the activity of polygalacturonase. According to Riordan et al., tetranitromethane nitrates the tyrosine at pH 7.0, but at a lower pH, the solution oxidizes cysteine residues [11]. The proof of the role of SH-groups and tyrosine in the catalytic reaction of *B. cinerea* polygalacturonase will be the subject of further studies.

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