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ENDOPOLYGALACTURONASE FROM *RHIZOCTONIA FRAGARIAE*

PURIFICATION AND CHARACTERIZATION OF TWO ISOENZYMES

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Summary

An electrophoretically homogeneous preparation of endo-polygalacturonase (poly(1,4-α-D-galacturonide)glycanohydrolase, EC 3.2.1.15) from culture filtrates of *Rhizoctonia fragariae*, a pathogenic agent in strawberry plants, was resolved into two isoenzymes when subjected to isoelectrofocusing in a narrow pH range. The isoelectric points of the two isoenzymes were 6.76 ± 0.03 and 7.08 ± 0.05. The two polygalacturonases exhibited similar substrate specificity, pH optimum and pattern of degradation of sodium polypectate. The two enzymes consisted of a single polypeptide chain which had an apparent molecular weight of 36 000 as determined by gel filtration on Sephadex G-100.

Introduction

The first stage of the infectious process of phytopathogenic microorganisms consists in many cases of the production of host cell-wall-degrading enzymes [1].

Degradative effects of some plant diseases, such as loss of electrolytes, cell death and tissue maceration, may be caused by pectolytic enzymes [2]. These enzymes, mainly lyases and hydrolases, are classified according to specificity toward their natural substrates, pectin and pectic acid; they can also be distinguished according to their mode of action upon the substrate, which can be degraded either beginning from the free ends (exo-enzymes) or at random (endo-enzymes).

Rhizoctonia fragariae Husain et McKeen, pathogenic agent of strawberry plants, has an extracellular pectolytic activity in vitro [3]. An endo-polygalac-

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turonase (poly(1,4-α-D-galacturonide)glycanohydrolase, EC 3.2.1.15) activity is present in culture filtrates of the fungus [4].

In this paper we report the purification and some molecular properties of two endo-polygalacturonase isoenzymes from *R. fragariae*.

Materials and Methods

Fungus culture. *R. fragariae*, CF strain, was a kind gift from Professor V. D'Ambra, Institute of Plant Pathology, University of Padua. The fungus was surface-cultured in Czapek-Dox liquid medium containing 1% pectin. The mycelium was grown in conical flasks for 19 days at 23°C and then removed by filtration through four layers of cheesecloth. The culture filtrate could be stored at −20°C for several months without decrease in enzymic activity.

Chemicals. Pure citrus pectin was obtained from NBC Corp. Sodium polypectate, polygalacturonic acid, xylan, araban, mannan, galactan, arabinogalactan, galactomannan and D-galacturonic acid were obtained from K & K Laboratories. Dinitrosalycilic acid and thiobarbituric acid were from Merck; DEAE-cellulose, CM-cellulose and 3 MM chromatographic paper from Whatman, Cellogel strips were from Chemetron (Milan) and Ampholine carriers from LKB, Sweden. All other chemicals were reagent grade.

Polygalacturonase assay. Enzyme activity was determined by two different methods. The first consisted in measuring the decrease in relative viscosity of a 0.6% solution of sodium polypectate in 35 mM citrate/phosphate buffer, pH 5.0; incubations and viscosity determinations were performed in Cannon-Fenske No. 300 viscometers at 30°C. One relative viscosimetric unit (U_v) was defined as the amount of enzyme which caused in 1 min 50% reduction of viscosity of 6 ml of the reaction mixture at the conditions of the assay. Enzyme activity was found to be proportional to the inverse of the time required to obtain a 50% reduction of viscosity. Enzyme activity was also found to be proportional to protein concentration when 0.005—0.15 U_v were present in the assay mixture. In the second method, the enzyme was incubated at 30°C in 35 mM citrate/phosphate buffer, pH 5.0 in the presence of 0.6% sodium polypectate and the reducing groups were determined by the method of Miller [5], using galacturonic acid as standard. One activity unit (U_r) was defined as the amount of enzyme producing 1 μequiv. of reducing groups per min.

For identification of the reaction products by paper chromatography 10 U_v of each enzyme were allowed to react in 1 ml of a reaction mixture containing 0.5% sodium polypectate, 50 mM acetate buffer, pH 5.0. The products were identified by descending paper chromatography, according to the method of Nasuno and Starr [6], using 1-butanol/acetic acid/water (4 : 2 : 3) as eluant.

Isoelectric focusing. Isoelectric focusing experiments were performed on an LKB 8100 column, cooled at 5°C, containing 3 ml of 40% Ampholine pH 6—8 in a stabilizing linear sucrose gradient (0—40%). Focusing was attained in 24 h, applying a constant voltage of 1000 V. 2.1-ml fractions were collected from the column for pH and activity determinations.

Protein determination. Protein concentration was measured according to the method of Lowry et al. [7] or by determining the ratio of the absorbance at 260 nm to that at 280 nm [8].

Results

Enzyme purification. Culture filtrates of *R. fragariae* from 30 flasks were concentrated by ultrafiltration through Amicon UM-10 Diaflo membranes to a final volume of 164 ml and centrifuged at 15 000 × *g* for 20 min. The supernatant was dialyzed against 10 mM sodium acetate buffer, pH 5.0 and adsorbed on a 2.7 × 20 cm CM-cellulose column equilibrated with the same buffer. The column was eluted with 50 ml of the dialysis buffer, followed by 200 ml of a linear 0—0.3 M NaCl gradient. The elution profile is shown in Fig. 1, a single peak of activity being eluted at about 0.2 M NaCl. The fractions with the highest specific activity were pooled and dialyzed against 10 mM acetate buffer, pH 5.0. The pooled fractions were adsorbed on a second CM-cellulose column 0.9 × 12 cm, equilibrated with the same buffer and eluted with 70 ml of a linear 0—0.4 M NaCl gradient. The activity was eluted as a single peak coinciding with absorbance at 280 nm (Fig. 2). The fractions having a specific activity higher than 230 U_v/mg were pooled and dialyzed against 1% glycine.

The dialyzed fraction was then subjected to isoelectric focusing. The two distinct enzyme peaks obtained (Fig. 3), focusing in the gradient at pH 6.76 ± 0.03 and 7.08 ± 0.05, were called polygalacturonase 1 and polygalacturonase 2, respectively. (The accuracy of p*I* values is given as the difference between the highest and lowest figure of four experiments). The fractions corresponding to the two activity peaks were pooled and dialyzed extensively against 20 mM acetate buffer, pH 5.0. Table I shows the purification procedure from 12.5 l of culture filtrate. Both polygalacturonase 1 and polygalacturonase 2 were purified more than 50-fold with recoveries of 9.7% and 8.1%, respectively.

Enzyme homogeneity. Only one peak of activity was obtained when each isoenzyme was separately refocused under the conditions outlined above. One single protein band was obtained when both polygalacturonase 1 and polygalacturonase 2 were subjected to Cellogel electrophoresis [9]. The two enzymes

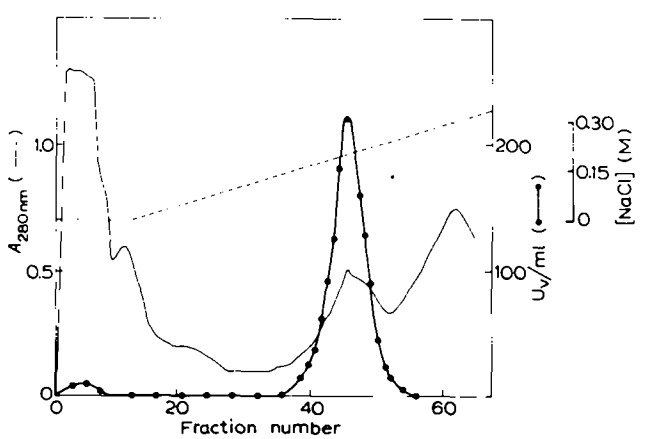

Fig. 1. Elution profile of polygalacturonase activity from the first CM-cellulose column. Sample volume was 164 ml. 3.9-ml fractions were collected and assayed for activity with the viscosimetric method. •, polygalacturonase activity; ———, absorbance at 280 nm; - - - - - -, sodium chloride gradient. Other experimental details are given in the text.

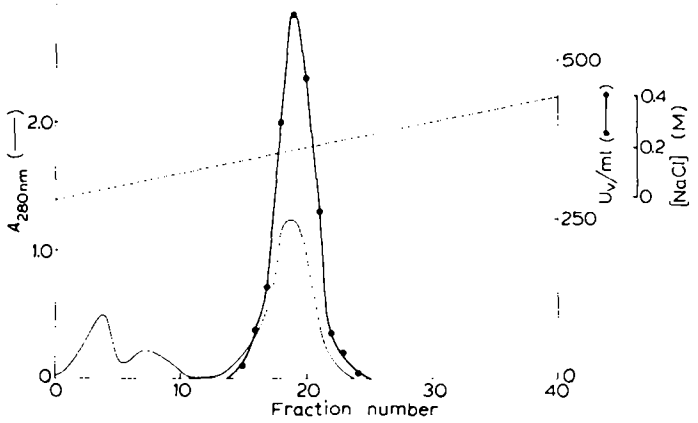

Fig. 2. Elution profile of polygalacturonase activity from the second CM-cellulose column. Sample volume was 46 ml. 1.7-ml fractions were collected and assayed for activity with the viscosimetric method. •, polygalacturonase activity; ———, absorbance at 280 nm; - - - - - -, sodium chlorid gradient. Other experimental details are given in the text.

also migrated as a single band upon electrophoresis in polyacrylamide gel in the presence of SDS [10].

Molecular weight. The molecular weights of polygalacturonase 1 and polygalacturonase 2 were estimated by gel filtration [11] on a Sephadex G-100 column (106 × 1.5 cm), equilibrated in 100 mM phosphate buffer pH 7.5. The elution volumes of the two enzymes did not differ when they were applied to the column either separately or in a 1 : 1 mixture. An apparent molecular weight of 36 000 was calculated for both isoenzymes. The enzyme recovery after the gel filtration experiments was higher than 90% in both cases.

Effect of pH. The rate of hydrolysis of sodium polypectate by polygalacturonase 1 and polygalacturonase 2 as a function of pH was studied using the viscosimetric assay. The curves for the two isoenzymes were similar and in both cases optimal activity was observed at pH 5.0.

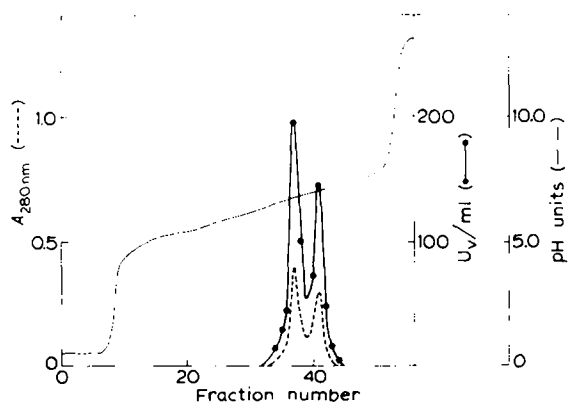

Fig. 3. Isoelectric focusing of polygalacturonase isoenzymes. 8.5 ml from the second CM-cellulose column were mixed with the lighter component of the sucrose gradient. 2.1-ml fractions were collected for pH and enzymic activity determinations. •, polygalacturonase activity; - - - - -, absorbance at 280 nm; ———, pH gradient.

TABLE I

PURIFICATION OF POLYGALACTURONASE FROM *RHIZOCTONIA FRAGARIAE*

Fraction	Volume (ml)	Total activity (U_V)	Total proteins (mg)	Specific activity (U_V/mg)	Yield (%)	Purification (-fold)
Filtrate	12 500	11 300	2400	4.7	100	—
UM-10 concentration	164	7 500	220	34.1	66	7
CM-cellulose I	46	5 250	77	68.2	46	14
CM-cellulose II	5.7	2 850	11.7	244	25	52
Electrofocusing:						
polygalacturonase 1	8.2	1 100	4.4	250	9.7	53
polygalacturonase 2	7.5	920	3.6	255	8.1	53

Substrate specificity and analysis of the reaction products. Sodium polypectate and, to a lesser extent, citrus pectin are substrates of the enzymes, whereas CM-cellulose, xylan, araban, mannan, galactan, arabinogalactan and galactomannan are not. Also, the paper chromatography patterns of the products of hydrolysis of sodium polypectate were identical showing, in both cases, a mode of action typical of "endo" digestion (Fig. 4). The activities obtained using the two different assays are reported in Table II. The viscosimetric to reducing activity ratios were very close indicating that the two isoenzymes behave similarly in hydrolyzing the internal glycosidic bonds of sodium polypectate [4].

The results outlined above indicate that *R. fragariae* produced two polygalacturonases in cultures containing pectin as the sole carbon source. It is remark-

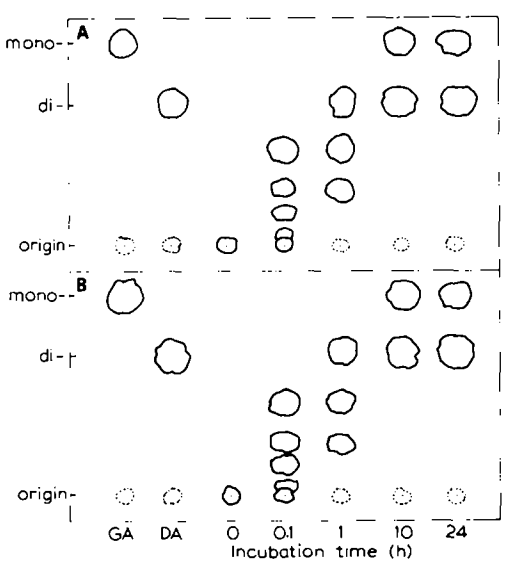

Fig. 4. Paper chromatogram of the products of reaction of (A) polygalacturonase 1 and (B) polygalacturonase 2 with sodium polypectate. 25 µl-aliquots from the reaction mixtures were directly spotted onto the chromatogram at the indicated times. GA and DA spots contained 25 µl of 75 mM galacturonic acid and 75 mM digalacturonic acid, respectively.

TABLE II

SUBSTRATE SPECIFICITY AND RATIOS OF REDUCING TO VISCOSIMETRIC ACTIVITY OF POLYGALACTURONASE 1 AND POLYGALACTURONASE 2

Enzymes were assayed for activity as indicated in Materials and Methods. When necessary, the assay mixture contained 0.6% citrus pectin.

Substrate	Enzyme activity			Activity ratio for sodium polypectate
	Citrus pectin	Sodium polypectate		
	(U_V)	(U_V)	(U_r)	(U_r/U_V)
Polygalacturonase 1	0.019	0.134	1.0	7.46
Polygalacturonase 2	0.018	0.123	0.89	7.26

able that, from the data of Table I, 2% of the protein present in the culture filtrate is polygalacturonase. The relative abundance of this protein may be related to the specific pathogenic role invoked for pectolytic enzymes of such fungi. The two polygalacturonases can be classified as endo-polygalacturonase according to the criteria suggested by Bateman and Millar [12]. Polygalacturonase 1 and polygalacturonase 2 were resolved and purified to homogeneity by means of isoelectrofocusing in a narrow pH range. Other techniques, such as electrophoresis and ion exchange chromatography on CM-cellulose or on DEAE-cellulose, were not effective in demonstrating the heterogeneity of enzyme preparations.

Polygalacturonase 1 and polygalacturonase 2 consist of a single polypeptide chain, as demonstrated by SDS gel electrophoresis. The apparent molecular weights, as determined by gel filtration, were identical. The enzymes from *R. fragariae* are similar in size to the polygalacturonases from other fungi [13—18]. The higher molecular weight value reported for the enzyme from *Botrytis cinerea* [19] is due, as suggested by the authors, to the occurrence of a pectinesterase-polygalacturonase complex.

The properties of the two isozymes are very similar. The pH optimum is the same for both enzymes and is close to those commonly observed for other fungal [20,21,23,24] and bacterial [6,22] polygalacturonases. The two isozymes exhibit the same substrate specificity and do not show differences in the pattern of reaction products released from sodium polypectate. The end products of hydrolysis, in both cases, are mono- and digalacturonate.

The possibility that one of the forms observed in isofocusing experiments could consist of a complex between substrate and/or degradation products and the enzyme(s) exists in principle. However, it is rendered unlikely by the result of the tests for homogeneity: refocusing of each single enzyme always gave a single peak banding at the same pH value. Similarly, gel filtration experiments on the two enzymes gave symmetrical elution patterns corresponding to an identical molecular weight. Moreover, the ratio of polygalacturonase 1 to polygalacturonase 2 was essentially constant in different enzyme preparations.

Preliminary experiments show that the two enzymes are glycoproteins having a different carbohydrate content, as measured by the anthrone method. The roles of the two different carbohydrate moieties in the catalytic function

and in the mechanisms of action of the enzymes will be the object of further investigation.

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