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Multiple Forms of Polygalacturonase in Two Strains of *Rhizoctonia solani*

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An unspecialized strain of *Rhizoctonia solani* (FC895) produced *in vitro* seven isoenzymes (four major and three minor peaks) which were separated by isoelectric focusing over a narrow pH range. The same isoenzyme pattern was detected when hypocotyls of cauliflower seedlings inoculated with the fungus were used as the enzyme source. A specialized strain (FC1241) produced two isoenzymes (one major and one minor peak). The four major isoenzymes of FC895 and the major isoenzyme of FC1241 had the same pH optimum and mode of action on sodium polypectate.

INTRODUCTION

Rhizoctonia solani Kuhn occurs as many strains, some of which have a wide host range, whereas others are more specialized parasites. There are several reports that *R. solani* produces pectic enzymes *in vivo* and *in vitro* (Ayers *et al.*, 1966; Bateman, 1963*a, b*; 1964; Bateman *et al.*, 1969; Sherwood, 1966; Van Etten *et al.*, 1967). To explore physiological differences between strains of *R. solani*, we investigated the production of polygalacturonase by an unspecialized strain (FC895) and by a strain specialized for potato (FC1241). The importance of polygalacturonase in pathogenesis is now well established in certain diseases of plants in which it causes cell separation, increase in permeability and death of protoplasts (Bateman, 1968, 1972; Bateman & Millar, 1966). Recently, a role of polygalacturonase in specificity has been claimed (Mussel & Strand, 1977; Cervone *et al.*, 1978; Fanelli *et al.*, 1978).

This paper describes the separation and characteristics of polygalacturonase isoenzymes of strains FC895 and FC1241.

METHODS

Chemicals. Citrus pectin was obtained from NBC, Cleveland, Ohio, U.S.A.; sodium polypectate and D-galacturonic acid were from K & K Laboratories, Plainview, N.Y., U.S.A.; dinitrosalicylic acid and thio-barbituric acid were from Merck; CM-cellulose and 3MM chromatography paper were from Whatman; Ampholine carriers were from LKB. Other chemicals used were reagent grade.

Fungus cultures and inoculation. *Rhizoctonia solani* (FC895), from cauliflower, was obtained from the Stock Culture Collection of the Institute of Plant Pathology, Portici, Italy. Strain FC1241, isolated from potato, was obtained from the Stock Culture Collection of the Department of Botany, Imperial College, London.

The fungi were grown in 300 ml Czapek-Dox liquid medium, in which sucrose was replaced by 1% (w/v) pectin, in 1 l flasks at 23 °C. The mycelium was removed by filtration through four layers of cheesecloth and dried at 80 °C to constant weight. The culture filtrate was stored at -20 °C. Pathogenicity of the two strains was checked by inoculating young seedlings of cauliflower, tomato, potato, beet, carrot, bean and pea grown in a glasshouse for 2 weeks. The inoculum consisted of sterilized wheat seeds on which the fungus had been grown for 6 d at 22 °C. One infected seed was placed beside the hypocotyl of each seedling and the seedlings were placed in a growth room at 25 °C with a 12 h photoperiod. Uninoculated seedlings were used as controls. Pathogenicity of the fungi was also checked by placing potato tubers in a mixture of 1 kg sterilized soil and 5 g millet seeds on which the fungus had been grown for 20 d. Black scurf symptoms were then recorded after 3 months on newly formed tubers.

Polygalacturonase assay. Enzyme activity was determined by measuring the decrease in relative viscosity of a 0.6% (w/v) solution of sodium polypectate in 35 mM-citrate/phosphate buffer, pH 5.0, in Cannon-Fenske no. 300 viscometers, at 30 °C. One relative viscometric unit (RVU) was defined as the amount of enzyme causing a 50% reduction in the viscosity of 6 ml of the reaction mixture in 1 min under the conditions of the assay.

Thiobarbituric acid test. The products formed by the action of polygalacturonase on sodium polypectate were examined by the thiobarbituric acid procedure of Neukom (1960) as modified by Ayers *et al.* (1966).

Reducing groups determination. Hydrolysis of glycosidic bonds was followed by the reducing end-group analysis using the dinitrosalicylic acid procedure (Miller, 1959). As complete hydrolysis of the substrate produces reducing groups corresponding to the nominal monomer concentration, this latter value was assumed to correspond to 100% of substrate degradation.

Paper chromatography. The products formed by the action of polygalacturonase on sodium polypectate were examined by descending paper chromatography, using the method of Nasuno & Starr (1966), with 1-butanol/acetic acid/water (4:2:3, by vol.) as eluant. The reaction mixtures were incubated at 30 °C and contained 0.6% (w/v) sodium polypectate, 35 mM-citrate/phosphate buffer, pH 5.0, and 2.5 RVU enzyme ml⁻¹.

Isoelectric focusing. Isoelectric focusing experiments were done at 4 °C in a LKB 8100 column, containing 3 ml 40% Ampholine pH 5 to 8 in a stabilizing linear sucrose gradient (0 to 40%, w/v). Focusing was complete in 30 h, applying a constant voltage of 1 kV; 1.8 ml fractions were collected from the column for pH and activity determinations.

Protein determination. Protein concentration was measured by the Folin method of Lowry, using bovine serum albumin as standard.

RESULTS

Fungal specificity

When hypocotyls of young plants were inoculated with strain FC895, cauliflower, beet, carrot and pea plants were killed in less than 1 week, whereas after 2 weeks tomato, potato and bean plants were still alive. None of the plants was affected by strain FC1241. For both strains, newly formed potato tubers showed typical black scurf symptoms 3 months after placing tubers in a mixture of soil and millet seeds on which the fungus had been grown.

Enzyme production

Strain FC895 produced more polygalacturonase than did strain FC1241. The maximum level of extracellular polygalacturonase in culture filtrates was 3.0 RVU ml⁻¹ after 11 d growth for FC895, but only 0.11 RVU ml⁻¹ for FC1241 after 22 d growth (Fig. 1).

Enzyme purification

Three litres of filtrate from '10 d-old' cultures of FC895 and '21 d-old' cultures of FC1241 were concentrated by ultrafiltration through Amicon UM-10 Diaflo membranes to a final volume of 250 ml and then centrifuged at 10000 g for 20 min. The supernatants were dialysed against 10 mM-sodium acetate buffer, pH 5.0, and adsorbed on two separate CM-cellulose columns (2.7 × 20 cm) equilibrated with the same buffer. The columns were eluted with 60 ml of the dialysis buffer, followed by 260 ml of a linear 0 to 0.7 M-NaCl gradient. Two peaks of activity, eluted at about 0.2 and 0.3 M-NaCl, were obtained from the FC895 culture filtrate whereas only one peak, at about 0.2 M-NaCl, was obtained from the FC1241 culture filtrate (Fig. 2). The fractions from the two columns with the highest activity were pooled separately and dialysed against 1% (w/v) glycine. The pooled fractions were then subjected to isoelectric focusing. Strain FC895 gave seven distinct peaks focusing at pH 5.55, 5.98, 6.66, 6.78, 7.05, 7.32 and 7.66. Strain FC1241 gave only two peaks, PG-1 and PG-2, focusing at pH 5.50 and 6.01, respectively (Fig. 3). The possibility that one of the forms observed in isoelectric focusing experiments could consist of a complex between substrate and/or degradation products and the enzyme(s) was excluded by refocusing each peak. Refocusing of each single enzyme always gave a single peak banding at the same pH value. Moreover, the ratio of the different isoenzymes was essentially constant in different

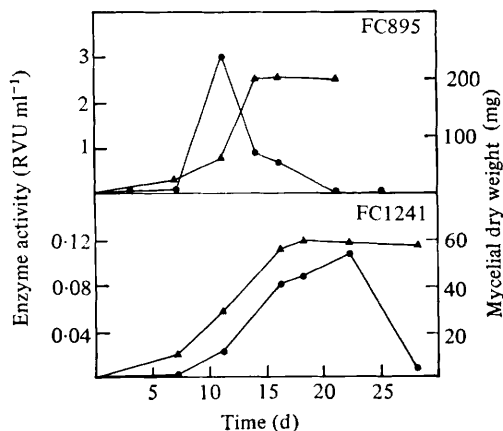


Fig. 1. Polygalacturonase production by *R. solani* growing in Czapek-Dox liquid medium with 1% (w/v) pectin replacing sucrose. ●, Polygalacturonase activity; ▲, mycelial dry weight.

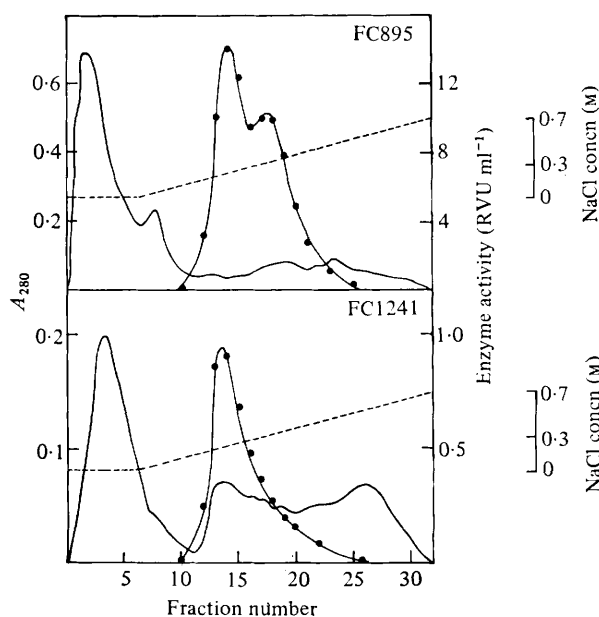


Fig. 2. Elution profile of polygalacturonase activity from a CM-cellulose column; 10 ml fractions were collected and assayed for activity. ●, Polygalacturonase activity; —, absorbance at 280 nm; ---, NaCl concentration. Other experimental details are given in the text.

enzyme preparations. The fractions corresponding to PG-2, PG-4, PG-6 and PG-7 from FC895 and PG-2 from FC1241 were pooled separately, dialysed extensively against 20 mM-sodium acetate buffer, pH 5.0, and characterized. The analysis of polygalacturonase produced in diseased tissue was carried out by isoelectric focusing of aqueous extracts of cauliflower hypocotyls inoculated with FC895. The isoenzyme pattern obtained was similar to that obtained using culture filtrate.

Enzyme characterization

Enzyme characterization was carried out according to a procedure previously used to characterize polygalacturonase from *Rhizoctonia fragariae* (Cervone *et al.*, 1977a, b).

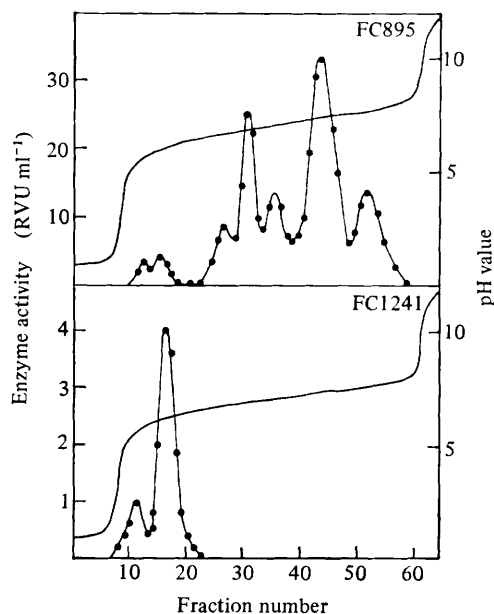


Fig. 3. Isoelectric focusing of polygalacturonase isoenzymes.
●, Polygalacturonase activity; —, pH.

The pH-dependence curves of the five isoenzymes were similar, with optimal activity around pH 5.0.

The absorption spectra of the chromogens formed in the reaction between isoenzymes and thiobarbituric acid exhibited a maximum at 515 nm. This indicated that they were hydrolases. The absence of a peak at 550 nm suggested that the preparations had no lyase activity (Albersheim *et al.*, 1960).

The ratio of viscosity decrease to reducing group increase during degradation of sodium polypectate was almost the same for each of the isoenzymes. A 50% loss in viscosity corresponded to only 3% hydrolysis of the glycosidic bonds. This meant that hydrolysis of the glycosidic bonds of sodium polypectate was at random.

Paper chromatography showed that the products of hydrolysis of sodium polypectate were similar. For each isoenzyme, the end products were mono-, di- and trigalacturonate.

From these results, the isoenzymes were classified as endopolygalacturonases [poly(1,4- α -D-galacturonide) glycanohydrolase; EC 3.2.1.15].

DISCUSSION

In the relationship between host and pathogen there are several levels of specificity (Brian, 1975). Some plant pathogens are restricted to a single species, or to a few closely related species, while others are able to infect a very wide range of hosts. *Rhizoctonia solani* is highly variable genetically and exists as a large number of strains many of which are specialized in the sense that they each attack only a restricted range of host species. Others, such as strain FC895 used during this study, are less specialized. Strain FC895 attacked potato tubers, cauliflower, beet, carrot and pea plants; in contrast, strain FC1241 was specialized for potato tubers.

A previous paper reported that polygalacturonase from *R. fragariae* was absorbed only by the tissue of hosts susceptible to the pathogen. If specificity is determined, at least in part, by polygalacturonases, a polyphagous pathogen, such as strain FC895, would be

expected to produce a large number of polygalacturonases. In contrast, a specialized parasite, such as strain FC1241, would be expected to produce only a few.

The mechanism of host-parasite specificity is still unclear as our knowledge of the molecules involved in the phenomenon is very limited. In this connection it seems fruitful to explore the physiological differences between different plant pathogens or between different strains of the same pathogens. Thus, we have found that two strains of *R. solani* which exhibit a different host specificity also exhibit different polygalacturonase isoenzyme patterns. The extent to which the different patterns are involved in the different specificity of the two strains remains for further study.

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