Variability of Polygalacturonase and Protein Isoelectric Focusing Patterns in *Botrytis cinerea* Isolates

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(Received 7 December 1979; revised 28 February 1980)

Acetone precipitates from culture filtrates of three isolates of *Botrytis cinerea* were resolved by isoelectric focusing on polyacrylamide gels to detect differences in the polygalacturonase and protein patterns. Only a few bands – four in the protein patterns and two in the polygalacturonase patterns – were common to all the isolates. Differences were also detected in polygalacturonase and protein patterns of the same isolate at different ages of culture (7, 14 and 21 d). Identical polygalacturonase patterns were obtained when isoelectric focusing was applied to an acetone precipitate either directly or after further purification by ion-exchange chromatography.

INTRODUCTION

Recently, polygalacturonase (PG) isoenzymes of *Botrytis cinerea* Pers. ex Fr. have been described by Zalewska-Sobczak & Urbanek (1975) for *B. cinerea* E-200 and by Drawert & Krefft (1978) for *B. cinerea* CBS 144.55 using, respectively, ion-exchange chromatography and isoelectric focusing as resolution techniques. However, the number of isoenzymes found was different: the former isolated only two PG peaks (PG1 and PG2) whereas the latter isolated four isoenzyme bands in the region of pI 4. These results suggest either that the isoenzyme pattern of extracellular PG is not a characteristic of the species but differs from one isolate to another, or that different purification techniques may have led to different results. We therefore decided to check both hypotheses experimentally.

Acetone precipitates from culture filtrates of three isolates of *B. cinerea* were resolved by isoelectric focusing on polyacrylamide gels (i) to detect any differences in the isoenzyme and protein patterns, (ii) to determine whether these patterns vary with the age of cultures and (iii) to determine whether the isoenzyme patterns of PG obtained by focusing the crude extract differed from those resulting after a further purification step by ion-exchange chromatography.

METHODS

Isolates. Shoots showing infection by *B. cinerea* were collected from Venetum (N.E. Italy) vineyards and some 30 single-spore isolates were obtained. Each isolate was cultured on potato-dextrose agar (PDA) and stored under liquid paraffin at 15 °C. From among these isolates, stored in our Institute collection, three (designated H, BB and PP) were chosen at random and used for the present study.

Enzyme preparation. The isolates were surface cultured in Czapek's liquid medium (pH 5) containing 1 % (w/v) citrus pectin as sole carbon source and thiamin (0.1 µg ml⁻¹). The inoculum was one agar disc (3 mm diam.) cut from the edge of a 3 d non-sporulating culture on PDA inoculated from the original cultures under liquid paraffin. The mycelium was grown at 20 °C in 250 ml Erlenmeyer flasks containing 50 ml medium. Culture media were collected and precipitated with cold acetone between 20 and 66 % (v/v) as reported by Zalewska-Sobczak & Urbanek (1975). Acetone precipitates from 7, 14 and 21 d cultures of isolate H were

used to test the effect of age on PG patterns, and the three isolates were compared using samples from 14 d cultures. Before isoelectric focusing, samples were exhaustively dialysed against distilled water for 24 h at 4 °C. Before ion-exchange chromatography on DEAE-cellulose, samples were equilibrated against 10 mM-sodium phosphate buffer (pH 6.4) for 12 h at 4 °C. Protein was determined by the method of Lowry.

Isoelectric focusing on polyacrylamide gels. Isoelectric focusing in 7.5 % (v/v) polyacrylamide gels (8 cm long) was carried out as described by Wringley (1971) with 2.5 % (v/v) of carrier ampholite pH 3.5 to 10 (LKB). Focusing was performed for 5 h at 4 °C, using a commercial apparatus (Bio-Rad), by applying 1.0 mA per gel and gradually raising the voltage to 200 V. This voltage was maintained until the end of the run. Standard samples, containing about 200 µg protein and 10 % (w/v) sucrose, were layered on to the top of the gels (anode). The cathode solution was 0.4 % (v/v) 2-aminoethanol and the anode solution 0.2 % (v/v) sulphuric acid.

For protein identification, gels were washed in 10 % (w/v) trichloroacetic acid and stained for 1 h with 0.0625 % (w/v) Coomassie Blue R 250 in 20 % (w/v) trichloroacetic acid as described by Wright *et al.* (1973). Excess stain was removed by repeated washing with 12.5 % (w/v) trichloroacetic acid.

PG isoenzyme identification was carried out as reported by Lisker & Retig (1974). For staining, gels were generally incubated for 30 min with 1.2 % (w/v) polygalacturonic acid (Sigma, grade III), washed with tap water and immersed for 4 to 5 min in 0.05 % Ruthenium Red solution. Longer incubation times were sometimes needed to enhance the staining of some bands. The pH gradient was determined by cutting gels (without samples) into 10 mm slices. The slices were soaked in 2 ml distilled water for 2 h and the pH was measured at 22 °C using a pH meter equipped with a microelectrode. Controls included gels incubated in substrate-deficient medium and heat-treated gels (100 °C for 5 min).

Ion-exchange chromatography. Ion-exchange chromatography was performed on extracts from isolate PP, since this had the simplest PG pattern. It was carried out on a refrigerated (4 °C) 1.6 × 30 cm DEAE-cellulose column equilibrated with 10 mM-sodium phosphate buffer (pH 6.4) followed by 400 ml of a linear 0 to 0.7 M-NaCl gradient in the same buffer. The flow rate was 0.5 ml min⁻¹ and 4.5 ml fractions were collected for protein and PG activity determinations. Protein content was determined by recording the absorbance at 280 nm. PG activity was tested by the procedure of Nelson (1944) with D-galacturonic acid as standard. The reaction mixtures, containing 4 ml 0.5 % (w/v) polygalacturonic acid in 0.1 M-sodium acetate buffer (pH 5) and 0.1 ml enzyme fraction, were incubated for suitable periods at 30 °C. Reaction mixtures containing boiled enzyme, or lacking substrate, were used as controls. Activity is expressed as units contained in each fraction. A unit is defined as the amount of enzyme which liberated 1 µmol galacturonate equivalent in 1 min under the condition of assay. Active fractions were pooled and subjected to isoelectric focusing.

RESULTS

Figure 1 shows the isoenzyme patterns of PG secreted by isolate H at different ages of culture (7, 14 and 21 d). At the first harvest, seven isoenzyme bands were detected; at the second and third, 16 bands were detected, with no differences in pI values for comparable bands. The only difference between 14 and 21 d cultures was in the staining intensity of some bands.

Protein patterns of the same isolate H at the same ages of culture are shown in Fig. 2. The protein bands showed variability both in number and in pI values. The greatest number of bands was detected in 14 d cultures, the least at 21 d. Throughout the whole experimental period only one band (pI 5.7) was consistently present.

When the isolates were compared using 14 d cultures (Fig. 3), they exhibited PG patterns with bands differing in number and in pI values, with the exception of those at pI 4.2 and 4.5 which were present in all isolates. The three isolates also showed marked differences in their protein patterns (Fig. 4): they exhibited only four common bands at pI 4.2, 7.0, 7.2 and 7.5.

Acetone precipitate from isolate PP (14 d cultures), subjected to ion-exchange chromatography, showed two peaks (A and B) of PG activity (Fig. 5). The A peak was bound to the resin and eluted with the NaCl gradient. When separately subjected to isoelectric focusing, the A and B forms were each resolved into two isoenzyme bands (Fig. 6). Together these accounted for the bands obtained by focusing the original acetone precipitate (Fig. 3).

Fig. 1

Fig. 2

Fig. 1. PG patterns after isoelectric focusing on polyacrylamide gels of *B. cinerea* isolate H at 7, 14 and 21 d of culture. Intensity of PG staining: ■, high; ▨, medium; □, low; ----, trace.

Fig. 2. Protein patterns after isoelectric focusing on polyacrylamide gels of *B. cinerea* isolate H at 7, 14 and 21 d of culture. Arrows indicate the bands common to all three patterns (----, only trace).

Fig. 3

Fig. 4

Fig. 3. PG patterns after isoelectric focusing on polyacrylamide gels of *B. cinerea* isolates H, PP and BB at 14 d of culture. Intensity of PG staining: ■, high; ▨, medium; □, low; ----, trace. Arrows indicate the bands common to all three patterns.

Fig. 4. Protein patterns after isoelectric focusing on polyacrylamide gels of *B. cinerea* isolates H, PP and BB at 14 d of culture. Arrows indicate the bands common to all three patterns (----, only trace).

DISCUSSION

Isolates H, PP and BB secrete several proteins into the culture medium, as found by Drawert & Krefft (1978) with *B. cinerea* CBS 144.55. When subjected to isoelectric focusing, these proteins were resolved into several bands widely distributed between pI 3.7 and 8.0 and not confined to the acid zone of the gels as reported for *B. cinerea* CBS 144.55. Moreover, the proteins from different isolates differed in number, pI and intensity of staining.

Fig. 5. Elution profile of PG activity by ion-exchange chromatography of *B. cinerea* isolate PP: —, PG activity; ····, A_{280} ; ---, NaCl concentration.

Fig. 6. PG patterns after isoelectric focusing on polyacrylamide gels of the peaks of activity (A and B, see Fig. 5) from ion-exchange chromatography of *B. cinerea* isolate PP. Intensity of PG staining: ■, high; ▨, medium.

At different culture ages, marked qualitative and quantitative changes in protein patterns also occurred in the isolates examined.

Reports of differences in protein patterns among different isolates of the same species are few, in part contradictory and usually obtained in attempts to use protein patterns as a taxonomic key for recognizing different species. Chang *et al.* (1962) found that a wild strain as well as a mutant strain of *Neurospora crassa* had distinctive protein patterns. Different conclusions were reached by Clare (1963) who stated that isolates of the same *Pythium* species from different localities were either indistinguishable or showed little difference. He further showed that filtrates of any one isolate harvested at three different ages showed identical electrophoretic patterns characteristic of each *Pythium* species.

Whitney *et al.* (1968), working with *Verticillium dahliae*, *Fusarium oxysporum* and *Verticillium albo-atrum*, found few intraspecific differences in protein patterns of two isolates of *V. albo-atrum*. Their results, however, showed that marked changes in protein bands of the three species occurred in the culture period examined. In more recent work, Chesson *et al.* (1978) found that one strain of *Cephalosporium diospyri* gave essentially the same soluble protein patterns during a culture period of 11 d.

That heterokaryosis in *B. cinerea* causes a high degree of natural phenotypic variability is well established (cf. Grindle, 1979) but our results also suggest that between random isolates of the fungus there exists variability in physiological features such as protein as well as enzyme patterns. Our results indicate that different isolates have PG patterns which differ one from another both in the number and in the pI values of the isoenzymes. The multiple forms of PG obtained from the *B. cinerea* isolate studied by Drawert & Krefft (1978) were localized at around pH 4, whereas the PG bands obtained from our isolates had pI values not only near 4 but also at other values.

Multiple enzyme forms could be the results of the evolution from simple enzymic patterns of an ancestor in response to changing hosts or habitat (Willettts *et al.*, 1977). From this point of view, more PG forms would indicate more opportunities for inducing disease on different hosts. Our results are therefore consistent with the well-known ability of *B. cinerea* to infect a wide host range.

In isolate H, the PG pattern was more constant with increasing culture age than was the soluble protein pattern. Between 14 and 21 d cultures there was a complete change in the protein pattern while in the PG pattern the only difference was in the staining intensity.

In conclusion, our results suggest that attempts to evaluate the qualitative and quantitative ability of *B. cinerea* to produce pectic enzymes must take into account the heterogeneity of the species and that comparisons between isolates must be made at the same age of culture and with the same resolution techniques.

The authors would like to thank Dr R. J. W. Byrde and Mr A. H. Fielding, Long Ashton Research Station, University of Bristol, for helpful discussion of the manuscript.

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