The Properties of Extracellular Enzymes of *Venturia inaequalis* and Their Association with Loss of Virulence of the Fungus in Culture

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Ribonuclease, deoxyribonuclease, acid phosphatase and phenoloxidase were detected in preparations of extracellular melanoprotein isolated from cultures of *Venturia inaequalis*. The high initial levels of activity produced per g fungus declined within a few days of inoculation, to an approximately constant level. About six times as much activity was released by conidia germinating in medium enriched with wood extract than was released in basal medium. After 5 d incubation, the rate of production of enzymically active melanoprotein reflected the rate of growth of the fungus. Isolated melanoprotein stored at 4 °C for 3 months showed up to 190% more hydrolase activity than was measured originally, but longer storage caused a subsequent decrease in activity. Only phenoloxidase decreased continuously during storage. The apparent stability of the bond between melanin and protein under dissociating conditions contrasted with the reversible formation of complexes between the fungal product and polyelectrolytes.

A set of re-isolates of different ages produced randomly variable levels of hydrolase activity when cultured repeatedly over a period of 16 months. On each occasion, however, the specific and total activity of each hydrolase was (with one marginal exception) lower in cultures of the oldest re-isolate than in those of the later re-isolates. In contrast, the specific activity of phenoloxidase was highest in cultures of the oldest re-isolate. These changes were associated with the decline in virulence observed in stored re-isolates of clone E₁ of *V. inaequalis*.

INTRODUCTION

Extracellular enzymes associated with melanoprotein produced by cultures of *Venturia inaequalis* were studied with a view to elucidating the biological stability of the pathogen. An attempt was also made to correlate the various levels of enzyme activity produced in culture by different clones with the corresponding levels of virulence observed on test plants.

METHODS

Growth of V. inaequalis and preparation of extracellular melanoproteins. Stock cultures of each isolate were maintained under paraffin oil at 4 °C (Kirkham, 1957). Using the methods of Hignett *et al.* (1977), extracellular melanoprotein was isolated from 40 ml shake cultures of *V. inaequalis* and fractionally precipitated with ethyl alcohol giving two fractions (N50 and N75). The cultures were usually grown on basal medium augmented with diffusible neutral wood extract (NWED) (Hignett *et al.*, 1977). For cultures grown on basal medium without NWED, 0.05% (w/v) asparagine was added as an alternative nitrogen source. Growth curves were obtained by taking duplicate 40 ml shake cultures at intervals up to 18 d after inoculation. The mycelium was filtered off, washed exhaustively and dried to constant weight over phosphorus pentoxide. Non-diffusible material in the culture fluid was isolated by dialysis at 4 °C against five changes of 1 l water

over a period of 72 h and estimated (without further purification) by drying samples to constant weight at 105 °C. Omission of the alcohol precipitation step left a small proportion of polymerized phenolic material from the culture fluid in the non-diffusible melanoprotein preparation. This was tolerated in some experiments to minimize handling losses.

Gel electrophoresis and gel filtration. Polyacrylamide gel electrophoresis was carried out on 5% (w/v) 0.5 × 6 cm gel rods (Cyanogum 41; BDH) made up in 1% (w/v) Ampholine (LKB). After polymerization with 2-dimethylaminoethyl cyanide (BDH) (0.2%, v/v) and ammonium persulphate (0.2%, w/v), the gels were pre-run at 5 mA each for 1 h to remove persulphate before use. The lower and upper electrolytes were 2 M-acetic acid and 3% (v/v) ethanolamine, respectively. The acid mixture of Stenman & Gräsbeck (1972) was used as an alternative to Ampholine in the preparation of pH gradient gels. Measurements of pH were made on gel segments (3 mm) after maceration in 0.3 ml water.

In some experiments, melanoprotein (pigment) solutions were mixed with solid urea to a final concentration of 8 M before electrophoresis. Gel filtration was done with 8 M-urea, 4 M-NaCl or 1% (w/v) polyethylene glycol 4000 (Hopkin & Williams) as solvent (Hignett *et al.*, 1977).

Determination of enzyme pH optima. Buffer solutions at pH 4.0 and 5.6 were prepared with 0.1 M-citric acid, adjusted with 10 M-NaOH. Solutions at pH 7.5 and 8.6 were prepared with 0.1 M-Tris, adjusted with 2 M-HCl. The hydrolase activities (acid phosphatase, ribonuclease and deoxyribonuclease) of a typical preparation of extracellular fungal pigment were determined in these buffers, using the following methods. All enzyme assays were duplicated; the difference between duplicates was always less than 10%.

Acid phosphatase. A 10% (w/v) aqueous solution of 4-nitrophenyl disodium orthophosphate (0.1 ml), 0.1 ml pigment (N50 at 5.0 mg ml⁻¹ or N75 at 10.0 mg ml⁻¹) and 1.0 ml citrate buffer pH 5.5 were placed in a dialysis sac (8/32 Visking, Scientific Supplies, London, was used in all experiments) and dialysed against 5.0 ml buffer at 30 °C. After 2 h, a 1.0 ml sample of diffusate was removed and replaced by 1.0 ml fresh buffer. The sample was shaken with 0.1 ml 10 M-NaOH after which the absorbance at 405 nm was determined. After 4 h, a further 1.0 ml sample of diffusate was similarly assayed. The same procedure was applied to reagent control solutions which contained 0.1 ml 4-nitrophenyl disodium orthophosphate solution and 1.1 ml buffer. The results are expressed as the difference in absorbance at 405 nm between test and control solutions after 4 h incubation.

Tests on wheat-germ acid phosphatase (BDH), with or without the use of dialysis sacs, showed that under the conditions described the results were not significantly affected by the presence of the membrane.

Ribonuclease. Reprecipitated yeast RNA (BDH; 100 mg) was dissolved in 10 ml water and autoclaved (15 min at 121 °C) in a dialysis sac immersed in water. After cooling, the product was dialysed at 4 °C against several changes of water for 72 h. The absorbance at 257 nm of the retained RNA solution was measured.

The RNA solution (0.5 ml), 0.1 ml pigment (N50 at 5.0 mg ml⁻¹ or N75 at 10.0 mg ml⁻¹) and 0.5 ml Tris buffer pH 7.5 were placed in a dialysis sac and dialysed against 5.0 ml buffer at 37 °C on a shaker. The absorbance of the diffusate at 257 nm in a 1.0 cm cell was determined after 2 and 4 h. Reagent control sacs containing 0.5 ml RNA solution and 0.6 ml buffer were similarly treated. The results are expressed as the difference in absorbance at 257 nm between test and control solutions after 4 h incubation.

The results of tests on bovine pancreatic ribonuclease (BDH) by the method described above showed that virtually no further diffusion of enzymically degraded RNA occurred when the reaction was stopped by the addition of 0.1 vol. 50% (w/v) trichloroacetic acid. Hence the presence of the membrane did not significantly affect the results of the assay.

Deoxyribonuclease. DNA (20 mg; prepared from yeast by the phenol method) was dissolved in 10 ml water, placed in a dialysis sac immersed in water and autoclaved (15 min at 121 °C). After cooling, the sac was dialysed against several changes of water for 72 h at 4 °C. The absorbance of the retained DNA solution was measured at 257 nm and the solution was used to detect deoxyribonuclease activity in pigment by the method described for ribonuclease measurement.

Phenoloxidase. Assays were carried out using the mixture of plant phenolics contained in NWED, or with catechol. (The results showed that the choice of substrate was not critical.) In the time-course experiments with NWED, phenoloxidase was assayed using reaction mixtures consisting of 0.2 ml non-diffusible material (at 10 mg ml⁻¹) and 0.1 ml NWED (30%, w/v), made to 1.5 ml with water. In the time-course experiments with basal medium, the enzyme was assayed using reaction mixtures consisting of 0.5 ml non-diffusible material (at 10 mg ml⁻¹) and 0.1 ml 100 mM-catechol, made to 2.0 ml with water. In other phenoloxidase assays, 0.1 ml 10 mM-catechol (or 30%, w/v, NWED) was added to samples of solution containing 0.25 mg pigment, then made up to 1.5 ml with water before incubation. The reagent controls contained the equivalent amount of water instead of enzyme solution. The absorbance of solutions was measured at 425 nm on mixing and again after incubation at room temperature for 4 h. Results are given as the difference between the changes in absorbance of the test and control solutions.

Stability of the enzyme activity of pigments during storage. N75 pigments from three re-isolates of clone E₁ grown in October 1976 were filter-sterilized, assayed for enzyme activity and kept at 4 °C for 3 months. On removal from storage, the pigments were dialysed against several changes of water for 48 h at 4 °C before their enzyme activities were redetermined. Pigments obtained in March 1977 from a variety of re-isolates of clone E₁ were filter-sterilized, assayed, and kept at 4 °C for 6 months before re-assay as above.

Time-course experiments. Three time-course experiments were set up with clone E₁ re-isolated in 1976: (1) 15 × 40 ml cultures with NWED were grown at 18 °C on an orbital shaker, and triplicate cultures were removed at intervals of 2 to 3 d over a period of 14 d; (2) 8 × 40 ml cultures with NWED were grown as above, and pairs of cultures were removed at intervals over a period of 6 d; (3) 30 × 40 ml cultures in basal medium alone were grown as above and sampled in triplicate at intervals of 2 to 3 d over a period of 35 d. The mycelium in the pooled samples was filtered off, thoroughly washed with water and the dry weights were recorded. The culture fluids were concentrated under vacuum at 37 °C to 0.25 vol. The concentrated fluids from experiment (1) were dialysed against several changes of water at 4 °C for 48 h. Those from experiments (2) and (3) were dialysed against 4 l water at 20 °C for 2 h using a 'Bio-Fiber 50' hollow fibre device (Bio-Rad Laboratories). The results of the two methods did not differ significantly. The non-diffusible material was concentrated under vacuum at 37 °C to 10 mg ml⁻¹. The precipitation step with ethanol was omitted. The enzyme activities of the non-diffusible material were assayed as previously described, using 0.2 ml samples per test for the hydrolases. Phenoloxidase was assayed as described earlier.

Investigation of the virulence of four different clones of V. inaequalis, the respective enzyme activities in culture fluid and the effects of storage at 4 °C on clone E₁. Two re-isolates of clone E₁ (E₁74 and E₁77) and three other clones (W₂, W₈ and W₃₆) were each grown in six replicate 40 ml shake cultures. The non-diffusible material produced by each isolate was prepared by concentrating the culture fluid to 0.25 vol., followed by dialysis for 48 h against several changes of water at 4 °C. The retained material was concentrated to 9.0 mg ml⁻¹. Then 3 vol. ethanol at -10 °C were added to each preparation. After 3 h at 0 °C, the precipitated whole pigments were collected by centrifugation and redissolved in 0.5 ml portions of water at 0 °C, overnight. Insoluble material was centrifuged off and the concentrations of the solutions were adjusted to 10 mg ml⁻¹. Enzyme activities were then assayed using 0.01 ml samples.

The inoculum for use on test plants was prepared by gently, but exhaustively, washing the mycelium with water to remove adherent medium, then shaking in water to release the conidia. These were separated from the mycelium by filtration through muslin. The absorbance of each conidial suspension was adjusted to 0.02 (Kirkham, 1957) (except for clone W₈, which was adjusted to 0.01) and applied to test plants as described below.

Inoculation of test plants. MM109 apple rootstocks were inoculated as described by Kirkham (1957), with certain modifications. Inoculum was sprayed on to individual plants at the rate of 6 ml per 20 plants. A glass scent sprayer was used with compressed air (at 10 lbf in⁻², 69 kPa), at approximately 20 cm from the plant tips. The ambient temperature was maintained between 18 and 20 °C whilst the room was kept at about 80% relative humidity. The inoculation chamber walls (made of double cotton muslin) were covered on the outer surfaces with aluminium foil to deflect sunlight and reduce evaporation, and were kept wet with recycled tap water. An overhead mist of distilled water (lasting 15 to 20 s) was generated inside the chamber every 20 min during the 24 h infection period. The internal humidity was thereby maintained above 90% relative humidity as recorded by a hair hygograph. Electrical conductivity measurements confirmed that this procedure kept the leaves wet continuously during the infection period.

RESULTS AND DISCUSSION

The production of non-diffusible extracellular material in NWED-augmented basal medium increased 5 d after inoculation, in parallel with the growth of the fungus. Growth was very poor in basal medium without NWED and very little extracellular material was produced (Fig. 1).

Enzymes detected in the non-diffusible fraction of culture filtrates showed pH optima at 4.0, 7.5 and 7.5 for acid phosphatase, ribonuclease and deoxyribonuclease, respectively. Activities were subsequently assayed at or near these values. Phenoloxidase was assayed in water at about pH 5.0, no significant pH change being observed during the reaction, and higher values being excluded by the onset of atmospheric oxidation. Activity was detected immediately after germination of the inoculum, but the initial high levels of activity (per g mycelium) declined rapidly within a day or two. As mycelial growth entered the exponential phase, the parallel increase in non-diffusible melanoprotein was accompanied by an

Fig. 1. Growth of *V. inaequalis* and production of non-diffusible material in 40 ml shake cultures. ●, Non-diffusibles in NWED culture fluid; □, dry weight of mycelium in NWED culture; △, non-diffusibles in basal culture fluid; ○, dry weight of mycelium in basal culture. The term 'non-diffusible' includes a small proportion of polyphenolic material with the total melanoprotein, as mentioned in Methods.

increase in enzyme activity which, after some fluctuations, kept the specific activities approximately constant (Table 1). The results also show that the initial release of enzymes by conidia was approximately six times greater in the NWED augmented medium than in basal medium alone. Extracellular enzymes were produced without associated melanin by cultures grown in basal medium alone, but the total yields were very poor compared with those in the enriched medium. Even so, the enzyme activities per g fungus after several days' growth in the two media were of similar magnitude.

Except for the phenoloxidase activity, which declined continuously, the enzyme activity of the isolated pigments (N75 fractions) remained constant during storage at 4 °C in sterile aqueous solution over a period of 1 to 2 weeks. However, after 3 months' storage the specific activities (in all but two cases) had increased significantly ($\geq 20\%$) above the original levels (Table 2). This effect indicated that inactive enzyme was slowly activated during storage. In contrast, further work with both N50 and N75 fractions showed that after 6 months storage many of the enzymes had activities close to or less than the original values, indicating that normal deterioration of protein eventually predominated (Table 3). Three of the 16 samples had lost significant ($\geq 20\%$) amounts of activity, eight showed little apparent change ($\leq 20\%$) and only five still had activities considerably above those measured 6 months previously (in March 1977). Table 3 also shows a strong tendency for each activity to increase in one fraction and to decrease in the other. The strength of the bond between the melanin and the protein components of the fungal product was indicated by the lack of effect of dissociating agents, and the failure of the separation techniques employed to isolate protein. The evidence suggests that enzyme protein is tightly bound in the fungal pigment, but that cryptic activity is slowly revealed without total dissociation occurring.

The formation of complexes in mixtures of melanoprotein and polyelectrolytes in commercial ampholyte solutions was observed during electrophoresis. Gel electrophoresis

Table 1. *Production of extracellular enzymes during growth of V. inaequalis on basal medium or NWED-augmented basal medium*

The figures show the activity (per g mycelium) of unfractionated non-diffusible material. Phenoloxidase in basal medium was measured with catechol as substrate; NWED was used to assay the enzyme in the augmented media. Separate 14 and 6 d experiments were done with NWED.

Time after inoculation (d)	Basal medium				NWED medium					
	Deoxyribo-nuclease	Ribonuclease	Acid phosphatase		Ribonuclease		Acid phosphatase		Phenoloxidase	
					14 d	6 d	14 d	6 d	14 d	6 d
1	240.0	2950.0	710.0	—	—	17500.0	—	4550.0	—	512.0
2	230.0	3850.0	470.0	56.0	—	4250.0	—	920.0	—	394.0
3	45.0	950.0	260.0	—	950.0	1550.0	760.0	430.0	782.0	352.0
5	—	—	—	—	290.0	—	230.0	—	208.0	—
6	110.0	660.0	130.0	8.0	—	510.0	—	140.0	—	70.0
7	—	—	—	—	270.0	—	230.0	—	86.0	—
8	60.0	520.0	70.0	18.0	—	—	—	—	—	—
10	—	—	—	—	230.0	—	290.0	—	98.0	—
13	70.0	355.0	80.0	34.0	—	—	—	—	—	—
14	—	—	—	—	260.0	—	270.0	—	80.0	—
17	50.0	255.0	80.0	42.0	—	—	—	—	—	—
21	17.0	100.0	60.0	34.0	—	—	—	—	—	—
28	22.0	100.0	50.0	22.0	—	—	—	—	—	—
35	33.0	110.0	80.0	34.0	—	—	—	—	—	—

—, Not determined.

Table 2. *Stability of extracellular enzymes during storage at 4 °C for 3 months*

The specific activities are expressed as the difference in absorbance between sample and control solutions, per mg melanoprotein.

Pigment	Enzyme assayed	Date of re-isolation	Specific activity		
			Date of culture and 1st assay Oct. 1976	2nd assay Jan. 1977	Percentage change
N75	Acid phosphatase	1974	0.98	0.93	-5
		1975	0.52	1.52	+192
		1976	0.81	1.00	+23
N75	Ribonuclease	1974	2.58	3.46	+34
		1975	2.34	3.30	+41
		1976	2.88	3.36	+17
N75	Deoxyribonuclease	1974	0.39	0.66	+69
		1975	0.34	0.68	+100
		1976	0.44	0.78	+77

Table 3. *Stability of extracellular enzymes during storage at 4 °C for 6 months*

The results are derived as in Table 2.

Pigment	Enzyme assayed	Date of re-isolation	Specific activity		
			Date of culture and 1st assay Mar. 1977	2nd assay Sept. 1977	Percentage change
N50	Acid phosphatase	1974	0.76	0.81	+7
		1975	0.27	0.36	+33
		1976a	0.10	0.175	+75
		1976b	0.08	0.115	+44
N75	Acid phosphatase	1974	0.24	0.205	-15
		1975	0.26	0.22	-15
		1976a	0.13	0.10	-23
		1976b	0.16	0.14	-12.5
N50	Ribonuclease	1974	2.39	1.88	-21
		1975	2.35	0.28	-88
		1976a	3.93	4.90	+25
		1976b	3.13	2.92	-7
N75	Ribonuclease	1974	1.76	2.04	+16
		1975	2.85	3.34	+17
		1976a	2.21	2.70	+22
		1976b	2.01	2.21	+10

of N75 or N50 in 1 % (w/v) Ampholine pH 3 to 5 gave four major bands, which were cut out and eluted with water. On re-running these separately in the same system, patterns similar to the original were produced in each case, suggesting that complexes had formed. This was confirmed by using the alternative pH gradient system, which gave a single band with the melanoproteins. Further confirmation was afforded by mixing the melanoprotein (0.5 mg in 0.05 ml water) with 5.0 μ l 40 % (w/v) Ampholine pH 3 to 5 or pH 5 to 8 before electrophoresis on the mixed-acid gel (Fig. 2). In the latter case, the resulting sharply resolved bands lying between pH 3.3 and pH 4.3 were well below the lower limit of the Ampholine pH range 5 to 8, the pattern being different from that given by the low pH range Ampholine. Similar effects were observed during sucrose density gradient experiments. These effects showed that the melanoprotein possessed considerable reversible complexing capacity. This behaviour is similar to that of humic acids and proteins noted by Righetti

Fig. 2. Complex formation during polyacrylamide gel electrophoresis on a mixed-acid pH gradient. The melanoprotein (0.5 mg N75 in 50 μ l water) was mixed before loading with 5 μ l 40% (w/v) Ampholine pH 5 to 8 (a) or pH 3 to 5 (b).

et al. (1977), and contrasts with the apparent stability of the melanin-protein bond discussed previously.

Clone E_1 of the fungus is comparatively stable, retaining colony morphology and some declining virulence over a period of 2 to 3 years in contrast with other clones which lose virulence in a matter of weeks, in culture. Enzyme levels in the material isolated from culture filtrates of growths started at intervals of 6 months are unpredictable, but a pronounced long-term trend is superimposed upon this short-term instability. Table 4 shows the differences in enzyme activity of the pigments produced by a set of re-isolates of clone E_1 cultured simultaneously. The total and specific activities of the hydrolases were not re-produced by re-isolates cultured at intervals of several months. Consistent differences can be seen, however, between the activities of enzymes in cultures of re-isolates taken 4 or more years apart. Except for phenoloxidase, the total and specific activities of enzymes produced by the oldest (1970) re-isolate were, with one marginal exception, much lower than those of the younger re-isolates, in many cases by a factor greater than 10. In contrast, the specific activity of the phenoloxidase produced by the 1970 re-isolate was considerably greater than that of the more recent re-isolates. The 1970 re-isolate had virtually lost the capacity to sporulate in culture, and was avirulent. An inverse relationship between virulence and phenoloxidase production is also shown (for two other isolates of E_1) in Table 5.

Thus loss of virulence during storage is associated with decreased ribonuclease, deoxyribonuclease and acid phosphatase production in subsequent cultures, and an increase in the specific activity of phenoloxidase. Phenoloxidase has been implicated in disease resistance generally (Kljajić *et al.*, 1964; Tomiyama & Stahmann, 1964; Stahmann & Demorest, 1973). The role of phenoloxidase in disease is usually correlated with the generation of

Table 4. *Production of extracellular enzymes by re-isolates of clone E₁ after storage under oil at 4 °C*

The figures show the activities per g mycelium after 14 d growth; specific activities (per mg pigment) are given in parentheses.

		Date of culture and assay			
Enzyme and pigment	Date of re-isolation	Nov. 1975	Apr. 1976	Oct. 1976	Mar. 1977
Acid phosphatase					
N50	1970	— (0.15)	1.58 (0.53)	0.38 (0.075)	— —
	1974	— (1.68)	48.25 (3.60)	7.15 (0.88)	28.70 (0.76)
	1975	— (4.39)	61.50 (5.70)	23.70 (1.625)	5.18 (0.27)
	1976	— —	— —	23.10 (1.315)	1.72 (0.10)
N75	1970	— (0.20)	6.85 (0.325)	3.58 (0.12)	— —
	1974	— (1.12)	21.75 (0.26)	69.20 (0.97)	22.20 (0.24)
	1975	— (0.50)	29.50 (0.73)	48.30 (0.515)	27.95 (0.255)
	1976	— —	— —	78.00 (0.805)	13.80 (0.125)
Ribonuclease					
N50	1970	— (0.24)	1.13 (0.38)	2.55 (0.51)	— —
	1974	— (0.48)	48.30 (3.61)	45.50 (5.625)	90.10 (2.39)
	1975	— (0.52)	32.80 (3.05)	76.80 (5.26)	44.95 (2.35)
	1976	— —	— —	83.00 (4.75)	67.50 (3.93)
N75	1970	— (0.72)	31.20 (1.48)	43.65 (1.505)	— —
	1974	— (1.41)	175.0 (2.10)	182.9 (2.57)	162.0 (1.76)
	1975	— (1.27)	90.50 (2.27)	216.5 (2.325)	312.0 (2.85)
	1976	— —	— —	280.0 (2.89)	244.0 (2.21)
Deoxyribonuclease					
N50	1970	0.00	0.18 (0.06)	0.00 (0.00)	— —
	1974	0.00	6.45 (0.48)	6.44 (0.795)	11.70 (0.31)
	1975	0.00	4.00 (0.37)	10.00 (0.685)	5.75 (0.30)
	1976	—	— —	6.54 (0.37)	8.25 (0.48)
N75	1970	0.00	3.80 (0.18)	6.25 (0.214)	— —
	1974	0.00	21.70 (0.26)	28.00 (0.39)	25.70 (0.28)
	1975	0.00	14.10 (0.35)	31.80 (0.34)	50.30 (0.465)
	1976	—	— —	42.35 (0.44)	39.85 (0.36)
Phenoloxidase*					
		1976			
N50	1970	9.7 (1.92)			
	1974	— —	Sum of phenoloxidase activity in N50 and N75 fractions		
	1975	14.4 (0.99)			
	1976	14.4 (0.82)			
N75	1970	34.6 (1.18)	44.3		
	1974	22.8 (0.32)	—		
	1975	14.9 (0.16)	29.3		
	1976	23.2 (0.24)	37.6		

—, Not determined. * Assayed using 10 mM-catechol.

phenolic oxidation products toxic to the pathogen. Production of extracellular phenoloxidase by *V. inaequalis* would tend to enhance this effect, thereby inhibiting its growth. The fungus would also be deprived of phenolic substrates, which would hinder its capacity to synthesize melanoprotein which is active in the apple scab disease (Hignett & Kirkham, 1967; Hignett, 1973).

In view of the generally observed changes in virulence of isolates of *V. inaequalis* during long-term storage and the variation in the associated enzyme activity described previously, an attempt was made to correlate the two effects. The results show that the different clones and isolates varied widely in virulence, but no general correlation was seen between the enzyme activities generated in culture and the virulence of the different clones on MM109 apple rootstocks (Table 5).

Table 5. *Virulence of V. inaequalis* on MM109 apple rootstocks, and the enzyme activity of melanoprotein isolated from the cultures used to provide the inoculum

Phenoloxidase was assayed with NWED. Specific activities are expressed per mg unfractionated melanoprotein.

Clone	Specific activity			Mean no. of lesions per plant
	Phenol-oxidase	Acid phosphatase	Ribo-nuclease	
E ₁ 74	2.68	1.97	3.70	0.50
E ₁ 77	1.95	1.86	3.60	18.80
W ₂	0.98	3.03	3.20	2.20
W ₈	1.25	0.05	0.0	11.60
W ₃₆	1.25	0.73	0.70	2.00

The evidence shows that the levels of enzyme activity released into culture fluid correlate with changes in virulence of clone E₁ during long-term storage, but do not indicate the comparative levels of virulence of different clones.

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