Phytochemistry, 1976, Vol. 15, pp. 1465–1470. Pergamon Press. Printed in England.

PURIFICATION OF A PHOSPHOLIPASE FROM *BOTRYTIS* AND ITS EFFECTS ON PLANT TISSUES

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(*Revised received* 3 *April* 1976)

Key Word Index—*Botrytis cinerea*; fungi; phospholipase; enzyme purification; lysosomes; acid phosphatase.

Abstract—A phospholipase from *Botrytis cinerea*, grown in pure culture, was purified more than 1000-fold. Whilst it possessed no acyl hydrolase activity toward a variety of *p*-nitrophenyl fatty acyl derivatives, phosphatidyl choline (lecithin) acted as a substrate; the enzyme being of either type 'A' or 'B' specificity. When the purified enzyme was applied to washed beetroot discs, betacyanin leakage was induced. Loss of substances which absorb at 260 nm also occurred when washed potato tuber discs were incubated with the enzyme. Incubation with a lysosome-enriched fraction from potato sprout tissues resulted in increased acid phosphatase activity in the incubation supernatant. The phospholipase had no macerating effect on a variety of plant tissues, nor did it cause disruption of isolated protoplasts from these tissues. Studies with mitochondria from mung beans revealed no effects of the enzyme upon the respiratory activity of these organelles. The result suggested that a major site of action of the *B. cinerea* phospholipase was the lysosomes.

INTRODUCTION

The object of the present work was to purify a fungal phospholipase, examine its effects on higher plant tissues and to evaluate the potential role of phospholipases during plant pathogenesis. It has previously been suggested [1–4] that certain pectic enzymes alone may be responsible for tissue maceration, membrane disorganisation and cell death in plant disease, and that phospholipases play a very limited role in pathogenesis. Since some of the work involved the use of enzyme extracts of uncertain purity, it was possible that the effects observed were not due solely to pectic enzymes. Other studies have employed pectic enzymes of high purity which have been tested upon intact plant tissues [1, 4–6]. There is, however, very little information on the effects of purified phospholipases or proteases and the role these enzymes may play in pathogenesis. Work using purified enzymes of *Erwinia carotovora* [7] has indicated that endopolygalacturonate *trans*-eliminase (endo-PGTE) has no apparent effect on the membrane structure of cucumber protoplasts although it is capable of causing tissue maceration. Phospholipase and protease of *E. carotovora* caused disruption of isolated protoplasts however, but cell death occurred only in potatoes if endo-PGTE were also present. This work with a bacterial pathogen suggested that phospholipases have a role in disease development following initial maceration of the cell wall by endo-PGTE which otherwise forms a barrier to attack of the protoplast by phospholipase and protease of the pathogen.

It has been demonstrated [8] that homogenisation of potato tissue results in a rapid breakdown of endogenous lipid materials and this was shown to be due, in part, to the action of a lipolytic enzyme claimed to possess wide specificity, which was released from a sub-cellular location. It is also possible that phospholipases from pathogens could induce host autolysis by initially disrupting the cellular location of host hydrolytic enzymes as suggested by other workers [9,10].

In this paper, we describe a purification scheme for a phospholipase from *Botrytis cinerea* and report its effects on selected plant tissues and organelles.

RESULTS

Isoelectric focussing of crude extracts

When the fractions of isoelectrically focussed crude extracts of *B. cinerea* were assayed for several enzyme activities, the profiles were as shown in Fig. 1. Three phospholipase peaks were obtained at pI 3.0, 8.5 and pI 11.2 which were closely associated with four peaks of protease activity at pI 2.5, 4.5, 8.4 and pI 12.4. In addition, two peaks of polygalacturonase activity were detected at pI 2.5 and 5.5 together with a broad low-activity band of diffuse isoelectric value between pH 7.5 and the top of the gradient. No pectin methyl *trans*-eliminase or acyl hydrolase activity was detectable in the isoelectrically focussed crude extract.

Potato tuber discs were cut and washed for 3 hr in running tap water. Following this, six discs were introduced into diluted aliquots (1 ml of dialysed fraction: 9 ml of H_2O) of each of the dialysed electrofocussed fractions and the mixtures were incubated with gentle agitation at 27° for 2.5 hr. Conductivity of the diluted fractions was measured before disc introduction and after their removal at the end of the incubation period. Figure 2 shows electrolyte release from potato tuber discs into the incubation medium caused by the fractions. An essentially similar profile of electrolyte release was obtained when washed beetroot discs were substituted for potatoes. These experiments demonstrated some correlation between the fractions containing hydrolytic

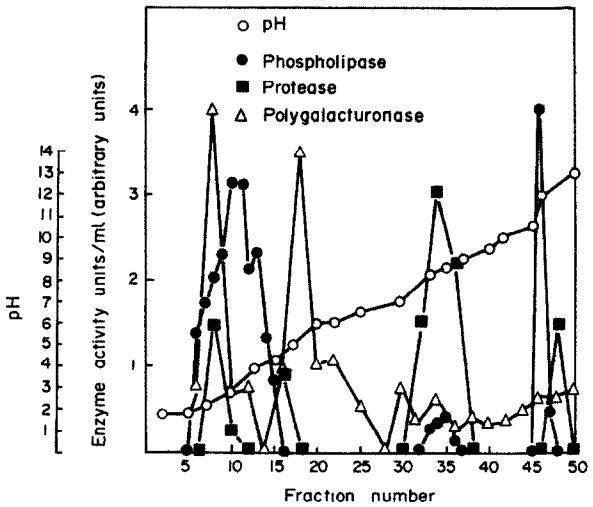

Fig. 1. Enzyme activity in fractions of crude isoelectrically focussed extracts of *Botrytis cinerea*. ○ pH, ● phospholipase (arbitrary scale × 10^3 units/ml), ■ protease (arbitrary scale × 0.05 units/ml), △ polygalacturonase (arbitrary scale × 8.3 units/ml).

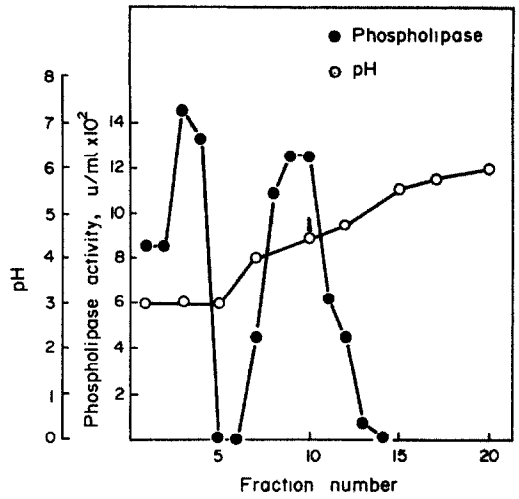

Fig. 3. Isoelectric focussing of *Botryti* [illegible] phospholipase. The extract was dialysed against 1% glycine following gel-filtration before application to the isoelectric focussing column (pH range 3.5–10, focussing time 72 hr, 5 W). ● phospholipase, ○ pH.

enzyme activity and those causing electrolyte release from the tissue discs. However, in view of the overlapping of some of the enzyme activity peaks, it was not possible to draw any firm conclusion with regard to the relative action of individual enzymes on the tissues.

Purification and characterisation of a phospholipase from Botrytis cinerea

Two peaks of phospholipase activity were detected following the gel-filtration stage of enzyme purification. Upon re-combining these peaks and electrofocussing, two peaks of activity of pI 3.0 and 4.3 were obtained (Fig. 3). The peak of lower pI was unstable following dialysis to remove ampholines and no further work was carried out upon it. It was difficult to conclude which of the three enzymes in the crude isoelectrically focussed extract corresponds to the stable pI 4.3 peak. Purification resulted in a loss of one enzyme present in the crude extract, a decreased stability of another and general modifications to structure resulting in pI values different to those seen in the crude impure extract.

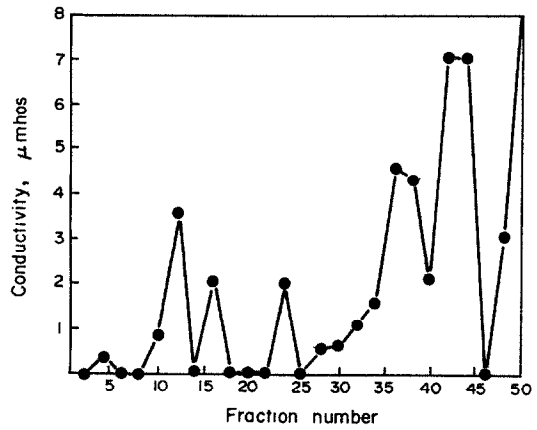

Fig. 2. Electrolyte release from washed potato discs incubated with fractions of the crude isoelectrically focussed *Botrytis cinerea* extract. Six potato discs each were incubated with diluted (1 ml extract: 9 ml dist H_2O) electrofocussed fractions at 27°. The difference in conductivity of the incubation mixtures over 2·5 hr was measured.

It is not possible to quote a value for the final purification factor of the pI 4.3 peak in terms of sp. act. since the protein values were too low to be recorded by the standard method used [11]. It would appear that a sp. act. increase in excess of 1000-fold has been achieved. No pectin methyl *trans*-eliminase activity was detectable in the purified extract.

With refined soybean lecithin as substrate (2.75 mM), the extract before final electrofocussing caused a linear decrease in acyl ester content over 3 hr of 0.083 ± 0.013 μmol/mg protein/hr at pH 5 and 30°. An assay for phospholipase 'C' [12, 13] proved negative. No acyl hydrolase could be detected in the extract at any stage of purification when *p*-nitrophenyl fatty acyl derivatives other than acetate were employed as substrates. In the case of *p*-nitrophenyl acetate, the activity was minimal and of *ca* 130 units/ml in crude extracts.

The results suggested that the phospholipase separated was of either type 'A' or 'B' specificity. Although we have not characterised the specificity further, other workers [14] using GLC techniques, state that crude *B. cinerea* phospholipase is of type 'B'.

Betacyanin leakage from beetroot discs

When beetroot discs, (*Beta vulgaris* var. rubra) washed for 1 hr in H_2O, were transferred to 10 ml aliquots of H_2O containing enzyme extracts and incubated with gentle agitation at 30°, leakage of betacyanin, measured as the increase of *A* at 530 nm in the medium, was observed as indicated in Fig. 4. A greater efflux of pigment occurred from discs incubated with the active *B. cinerea* enzyme extract (1500 units/ml) than from the other treatments. Commercial phospholipase 'A' had no effect upon efflux. It was apparent that the betacyanin pigment was destroyed after *ca* 100 min in solution since the *A* at 530 nm diminished after this time in some treatments. In the case of the active *B. cinerea* treatment however, there was a levelling-off of *A* which possibly indi-

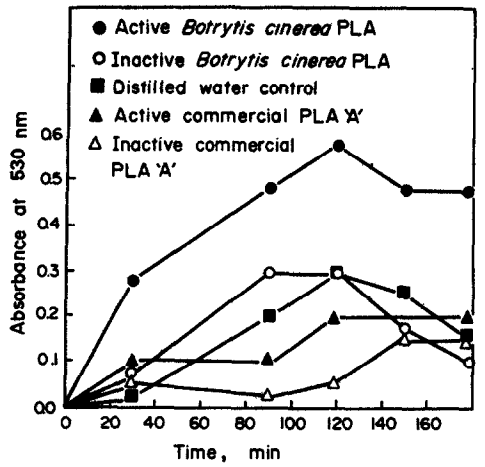

Fig. 4. Leakage of betacyanin from washed beetroot discs incubated with enzymes. ● Active *Botrytis cinerea* purified phospholipase; ○ inactive *Botrytis cinerea* phospholipase (boiled for 1.5 hr); ▲ active commercial phospholipase 'A'; △ inactive commercial phospholipase 'A' (boiled for 1.5 hr); ■ dist H_2O control. Six beetroot discs each, washed overnight in running tap H_2O, were incubated in 10 ml mixtures as indicated. The A at 530 nm of the mixtures was read over 3 hr.

cated that efflux of the pigment was balancing the rate of breakdown in this case. The incubation time required to achieve maximum efflux varied between experiments from 75 to 180 min. Following this treatment, discs were examined under the microscope in an attempt to detect any obvious tissue damage but this was never observed.

Leakage of substances absorbing at 260 nm from potato discs.

Potato discs, (*Solanum tuberosum* cv. Majestic) were cut (6.5 mm diameter) and washed in H_2O for 10 hr. Ten were then transferred to each incubation mixture containing either 8 ml of 0.05 M citrate buffer pH 5 or 8 ml of 0.1 M Pi buffer pH 7 together with 1 ml of electrofocussed *B. cinerea* enzyme pI 4.3 (1500 units/ml) or 1 ml of H_2O. The mixtures were incubated at 30° with gentle agitation and at 15 min intervals, a 3 ml aliquot of each solution was removed and the *A* measured at 260 nm before being returned to its respective incubation vessel [15]. *A* readings were taken over 3 hr (Table 1). The results reveal a small stimulation of efflux at pH 5 due to the enzyme extract compared with the corresponding control. At pH 7, the efflux in the enzyme treatment is slightly less than its corresponding control. The *A* recorded for both controls at the two pH values were very similar. There is thus a factor within the enzyme extract which appears to slightly stimulate efflux of substances absorbing at 260 nm when the incubation is carried out at pH 5 but not at pH 7.

Microscopic investigation of potato discs following this experiment failed to reveal any differences between the various treatments or between freshly cut or washed discs. When crude commercial pectinase was added to discs with gentle agitation under the microscope, cell maceration became evident after *ca* 10 min at room temperature.

Leakage of ions from washed potato discs

Six potato discs each (6.5 mm diameter) which had been previously washed for 3 hr in H_2O were introduced into incubation media consisting of 9 ml of H_2O plus either 1 ml of active *B. cinerea* phospholipase extract or 1 ml of heat inactivated enzyme (boiled for 1.5 hr). The conductivities of the solutions were measured immediately before and after incubation for 3 hr at 30° with gentle agitation. In some experiments, conductivities were also measured at intermediate times. After initial incubation, the discs of each treatment were transferred to H_2O only (10 ml aliquots) and subsequent ionic efflux over the next hr was measured at 15 min intervals. The results of a typical experiment are shown in Table 2 and indicate that any enhanced efflux occurring from tissues treated with the enzyme extracts was not the result of the active enzyme since boiled extracts elicited similar effects to non-boiled samples. It was of interest to note that the increased efflux occurring in treatments incubated with either active or boiled enzyme extracts was not apparent until discs were transferred from the initial medium containing the extract to H_2O.

Experiments with protoplasts

Cucumber and tomato protoplasts were viewed under

Table 1. Leakage from washed potato tuber discs of substances having absorbance at 260 nm incubated with or without purified *Botrytis cinerea* phospholipase. The nett increase of A_{260nm} in incubation mixtures was calculated by subtracting values of controls containing no enzyme from those possessing active enzyme. Incubations were done in either citrate buffer 0.05 M pH 5 or Pi buffer 0.05 M pH 7.

	Absorbance increase at 260 nm					
	pH 5			pH 7		
Time min	+Enzyme	−Enzyme	Nett Increase	+Enzyme	−Enzyme	Nett Increase
0	0	0	0	0	0	0
15	0·03	0·01	0·02	—	—	—
30	0·10	0·05	0·04	0·07	0·06	0·02
45	0·15	0·09	0·06	—	—	—
60	0·18	0·11	0·06	0·11	0·13	−0·02
90	0·25	0·20	0·07	0·15	0·20	−0.05
120	0·27	0·26	0·01	0·19	0·24	−0.05
180	0·35	0·31	0·04	—	—	—
210	—	—	—	0·29	0·38	−0·08

Table 2. Ionic conductivities in incubation media of washed potato tuber discs treated with active and denatured *Botrytis cinerea* phospholipase.

Time (min)	Conductivity μmhos: Water control	Denatured enzyme	Active enzyme
	(a) Conductivities in first medium (see text)		
0	2·35	56	42
150	30·00	70	59
	(b) Conductivities following transfer of discs to freshly distilled water*		
0	5·5	11·0	8·0
15	9·5	23·5	18·5
30	12·0	32·5	25·0
45	14·5	39·0	32·5
60	17·0	45·0	37·5

* Initial conductivity of dist H_2O before disc introduction was 2.25 μmhos.

the phase-contrast microscope during irrigation on the slide with commercial preparations of phospholipases 'A', 'C' or 'D' or the *B. cinerea* extract at various stages of purification. For both types of protoplast, only commercial phospholipase 'A' promoted protoplast bursting. Although all protoplasts ultimately collapsed, this could always be attributed to heating effects or osmotic stress except in the case of phospholipase 'A'. It was thus concluded that *B. cinerea* phospholipase had no effect upon the plasmalemmae of the cells examined.

Leakage of acid phosphatase from a lysosome-enriched fraction from potato sprouts

Experiments in which lysosome-enriched fractions were incubated at 37° with equal volumes of either H_2O or the active purified *B. cinerea* phospholipase indicated that a nett rise in supernatant acid phosphatase activity occurred in samples containing the active enzyme. Typical results are presented in Table 3 and show an increase in acid phosphatase of the supernatant in all treatments following incubation. The active *B. cinerea* phospholipase, however, caused considerably greater acid phosphatase release compared with the boiled enzyme or control. It would appear that this enzyme is capable of causing damage to potato sprout lysosomes.

Experiments with mung bean mitochondria

Using a suspension of mitochondria from mung beans, it was confirmed that the addition of Triton-X-100 caused a rapid decrease in turbidity of the suspension as a result of organelle lysis [16]. It was postulated that a similar effect might be observed by addition of phospholipases to suspensions if they also attacked mitochondrial membranes. It was seen however, that the rate of decrease of turbidity (measured as the decrease of *A* at 300 nm) in such experiments was not appreciably different from that of buffer incubated controls and mitochondrial suspensions containing either commercial phospholipase 'A' or the *B. cinerea* enzyme at various stages of purity. These turbidity changes occurred at a much slower rate than those produced by incubating with Triton-X-100.

Experiments on mitochondrial respiration rates using classical Warburg manometric techniques, failed to demonstrate any effect of the purified *B. cinerea* phospholipase, nor was there any effect upon cytochrome *C* oxidase or NAD-dependent *l*-glutamate dehydrogenase activities. It is thus apparent that this fungal phospholipase does not have any direct effect upon mitochondria.

DISCUSSION

Purified endopolygalacturonate *trans*-eliminase from *E. carotovora* caused electrolyte loss, tissue maceration and cellular death of potato tissue [1]. Although no specific mechanism was proposed to explain cell death it was suggested that a substrate for endo-PGTE resided in the plant cell membrane or within the protoplast. The present work using relatively crude isoelectrically focussed extracts from *B. cinerea* has shown that phospholipases, each variously associated with polygalacturonate or proteolytic enzymes, can also induce leakage of ions from potato and beetroot tuber discs. Further, when one of these phospholipases was purified to exclude polygalacturonate and proteolytic activity, certain effects were still observed when it was applied to a variety of plant tissues.

Work with the antibiotic, filipin, demonstrated [15] that it was capable of causing efflux from washed potato tuber discs of substances. claimed to be nucleotides. absorbing at 260 nm in a similar manner to that observed here with purified fungus phospholipase. It had previously been established that the site of action of filipin was at the membrane [17, 18] and it is possible therefore, that the *B. cinerea* phospholipase may have a similar site of action to that of the antibiotic. It is noteworthy that the action of the phospholipase upon leakage occurs only at pH 5, the optimum pH value determined for *B. cinerea* phospholipase of culture filtrates [14].

In view of the implied effects of the enzyme upon internal membranes of host tissues—the tonoplast in the case of betacyanin leakage from washed beetroot discs, the nuclear membrane in the case of nucleotide leakage from potato tuber discs, and lysosome membranes resulting in acid phosphatase leakage to the supernatant—it is curious that no visible effect was seen upon the plasmalemmae of host protoplasts incubated with the enzyme

Table 3. Release of acid phosphatase from potato sprout lysosomes incubated for 30 min with active or denatured purified *Botrytis cinerea* phospholipase. Acid phosphatase was assayed in the supernatants of incubation mixtures containing equal volumes of fresh lysosome-enriched fraction (pH 7.1) with purified active or heat denatured (boiled for 1.5 hr) phospholipase at zero time and after 30 min. The nett release of acid phosphatase was calculated with reference to a control incubation containing distilled water instead of phospholipase. Incubations were at 37°.

Treatment	Acid phosphatase activity units/ml supernatant: Zero time	30 min incubation	Activity increase
Water control	1200	1670	471
Denatured enzyme	1200	1840	642
Active enzyme	1120	2220	1100
	Nett water control-corrected increases of acid phosphatase in the supernatant		
	Denatured enzyme 177		
	Active enzyme 631		

preparation. Whilst the effect on lysosome preparations was observed *in vitro*, the betacyanin and nucleotide leakages were both observed *in vivo*. It appears therefore, that the phospholipase is capable of permeating the barrier of the outer cell membrane without causing its disruption and has a specific effect upon intra-cellular membranes only. The plasmalemma presumably presents no barrier to betacyanin or nucleotide leakage once these have been released from their normal intracellular locations.

The effect on lysosomes is interesting for it suggests a possible mechanism whereby host autolysis in disease may be induced by phospholipase of fungus origin. Provided a pathogen possesses the enzymic capability to initiate host autolysis, there is logistically no need for it to possess enzymes capable of degrading all structural components of the host since many of these can be hydrolysed by the lysosomal enzymes of the host.

Other workers [7], using purified phospholipases and pectinases from *E. carotovora*, stated that potato protoplasts were disrupted by phospholipase but that cell death occurred only if endo-PGTE were also present. Their work suggested that the action of bacterial phospholipase could not be realised upon intact tissue until the barrier of the cellulose cell wall had been removed by endo-PGTE. The phospholipase isolated from *B. cinerea* in this present study appears capable of permeating host cell walls and plasmalemmae and its action therefore appears to be different from that of the *E. carotovora* enzyme. Although phospholipase may affect the integrity of cells, the present work does not exclude the possibility that similar effects may be achieved by other components of fungal origin which have been removed in the purification process. Also, the biochemical mechanism of attack of fungal pathogens is likely to vary with the nature of the host and further research would be necessary to establish if the phospholipase isolated in the present work has different actions upon other plant tissues. Thus, whilst the work of others [4] clearly shows that endopectate lyase from *E. chrysanthemi* causes cell death indirectly owing to plasmalemma collapse following enzymic degradation of the cell wall, the present work suggests that a phospholipase from *B. cinerea* may have a potential for sub-cellular disorganisation in host–parasite interaction.

EXPERIMENTAL

Organisms. B. cinerea was maintained on slopes of potato dextrose agar. Sporing was induced in cultures grown on potato agar in Petri dishes under a 12 hr alternating black light régime after intitial growth in the dark of the cultures for *ca* 5 days at 25°. Potato agar was produced from the filtrate of freshly-boiled potatoes (400g diced tuber material boiled with 800 ml of dist H_2O, filtered and then made up to 1 l.) and contained 4% Oxoid agar No. 3. For enzyme purification, *B. cinerea* was grown in 500 ml conical flasks containing 300 ml of potato broth with 1% soybean lecithin (Sigma type 11s-commercial grade). Medium was seeded with spores giving a final concn of 10^6 spores/ml. Mycelial mats were harvested after 3 days of shaking on a rotary incubator at 140 rpm. at 25°. Potatoes (*Solanum tuberosum* cv. Majestic) were grown in the University experimental field, tubers being lifted in September and stored at 8° in the dark until required. Mung bean (*Phaseolus aureus*) seeds were purchased locally. Seeds were surface sterilised by soaking for 10 min in a soln of $Ca(OCl)_2$ (0·5%) and then transferring to H_2O for 1 hr. They were then sown in wet vermiculite and placed in the dark for 90 hr at 30°. Cucumbers and beetroots were obtained from local suppliers.

Enzyme assays. (a) Phospholipase was assayed by measuring the decrease of acyl ester concn over a given time in a 1% aq emulsion of refined soybean lecithin at pH 5 using the method of ref. [19]. Suitable zero-time blanks were included. However, for routine analysis, the cup–plate technique [20] was adopted. Wells (8 mm diam) were cut in the 0.5% lecithin-containing agar of the plates using a cork borer. Aliquots (0.1 ml) of the enzyme extracts were introduced into the wells and incubated for 24 h+ at 25° when the diameters of any white haloes produced around the wells were measured. One unit of phospholipase activity was arbitrarily defined as that amount of enzyme causing 1 mm^2 of agar to become opaque under the conditions of the assay described above. (b) Acyl hydrolase using a variety of *p*-nitrophenyl fatty acyl derivatives as substrates was assayed by a method similar to that of ref [21] modified and described by us [22]. (c) Protease was assayed by the method of ref [23] as modified in ref [24], with ninhydrin as the chromogen. In addition, a cup–plate procedure similar to that used for phospholipase was devised in which milk casein (1% Sigma technical grade) was substituted for lecithin. Haloes developed following incubation after the addition of a few crystals of *l*-cysteine hydrochloride to the wells containing protease activity. (d) Polygalacturonase was assayed by the method of ref [25] (e) Pectin methyl *trans*-eliminase was assayed by the method of ref [26]. (f) Acid phosphatase activity was determined using *p*-nitrophenyl phosphate for substrate as described in ref [27]. Appropriate zero-time blanks were included. One unit of enzyme activity was defined as that amount of enzyme liberating 1 μmol of *p*-nitrophenol/min under the conditions of the assay. (g) Cytochrome *C* oxidase in mitochondrial extracts was assayed by a modified method of ref [28]. (h) Soluble protein was determined by the method of ref [11] and all activities of enzymes are quoted as sp. act. unless otherwise stated.

Enzyme purification. Shake culture flasks containing potato-lecithin broth seeded with *B. cinerea* spore suspension were harvested 72 hr after inoculation. The mycelial mass was separated from the supernatant by filtration and ground in a pestle and mortar containing sand with a minimal vol of Pi buffer (0.05 M pH 7). Homogenate was filtered and the filtrate together with the culture supernatant was separately clarified by centrifugation at 38000 *g* for 30 min at 2°. Both supernatants were combined and designated the crude extract. This was used without further treatment in the initial isoelectric focussing experiments. For further purification however, *ca* 2 l. of the crude extract was concentrated 4-fold by lyophilisation, followed by protein precipitation using $(NH_4)_2SO_4$. The majority of phospholipase activity was found in protein precipitating between 40–80% saturation. This procedure resulted in a *ca* 10-fold purification of phospholipase activity. The resulting fraction was dialysed against Pi buffer (0.05 M pH 7) containing 2 mM $CaCl_2$ and 2 mM $MnSO_4$ [29]. Such dialysed pellets were stable when stored at −20° and no activity loss was experienced over many months. The 40–80% dialysed $(NH_4)_2SO_4$ fraction was equilibrated with 0.05 M citrate buffer pH 5 and stirred for 2 hr with an equal quantity (w/v) of CM cellulose, pre-equilibrated with the same buffer. Following centrifugation of the mixture at 2000 *g* for 10 min, the majority of the phospholipase activity was found in the supernatant. This procedure produced a 2-fold sp. act. increase. After vol reduction against polyethylene glycol and re-dialysis against 0.05 M citrate buffer pH 5 containing 2 mM $CaCl_2$ and 2 mM $MnSO_4$, the phospholipase-containing extract was subjected to DEAE cellulose ion exchange column chromatography using a NaCl molarity gradient (0–0.5 M) to elute the protein. Phospholipase activity eluted as a single peak *ca* half-way along the NaCl molarity gradient and a *ca* 50-fold sp. act. increase of the combined fractions containing phospholipase was recorded. The vol of pooled fractions was again reduced, re-dialysed and equilibrated to the previous conditions before the extract was applied to a column of Sephadex

G150 which was eluted with citrate buffer (0.05 M pH 5). Two well-defined peaks of phospholipase activity were obtained. These were combined and the vol of extract reduced against polyethylene glycol as before, after which the extract was dialysed against 1% glycine. The extract was finally subjected to isoelectric focussing (see below) between the pH range 3.5–10 using a column of 110 ml capacity. Two peaks of phospholipase were clearly discernable, one of pI 3, the other at pI 4.3. The enzyme of lower pI value was unstable and all activity was lost following dialysis to remove sucrose and ampholines from the fractions. The activity of the second phospholipase was stable however over a period of several weeks when stored at −20°.

Isoelectric focussing. In initial expts using a crude extract, isoelectric focussing was carried out with a column of 440 ml capacity and ampholine carrier ampholytes at 1% w/v concn within the range pH 3.5–10. Crude extract (120 ml) was distributed equally between the two component gradient solns prior to their introduction into the gradient former for mixing. Sucrose was used as the stabilising medium and the polarity was arranged with the anode at the bottom of the column. The electrofocussing time was 50 hr and the power input was maintained at 10 W throughout at 4°, with a water cooling temp of 4°. The column contents were fractionated at a rate of 60 ml/hr and fractions dialysed against two changes of 0.05 M citrate buffer pH 5 for 12 hr to remove ampholines. For the final electrofocussing stage of the phospholipase purification scheme, the smaller column of 110 ml capacity was used with an electrofocussing time of 72 hr and constant power input of 5 W.

Preparation of experimental tissues. Cucumber protoplasts were produced by the method of ref [7] except that protoplasts floating to the surface of the incubation medium were used directly and not subjected to the further purification described in this ref. The variable yield of protoplasts obtained may have depended on both the source and the time of year of purchasing the cucumbers. In preparing tomato fruit protoplasts, it was noted that the physiological condition of the fruits, determined by their treatment before protoplast preparation, affected yield and relative susceptibility of protoplasts to bursting. Green fruits were stored for 14 days at 8° following harvesting, then placed at 4° for 18 hr before use. Locular tissue from the fruits was incubated at room temp with occasional stirring for 1.25 hr in a medium of 0.05 M Pi buffer pH 7 containing 20% sucrose and 2% commercial pectinase. Protoplasts were harvested using a Pasteur pipette near the surface of the incubation mixture after it had been allowed to settle out.

Potato sprout lysosome-enriched fractions were obtained as follows. Potato tubers, stored at 8° in the dark for several months after harvesting were induced to sprout by transferring to a temp of 25° for several weeks in the dark. Sprouts were removed from the tubers, cut into segments and homogenised with buffer as described in ref. [30]. The brei was forced through two layers of muslin and the filtrate was centrifuged at 2000 *g* for 10 min to remove the larger debris. The supernatant from this treatment was further centrifuged at 25000 *g* for 30 min and the pellet was re-suspended in a minimal vol of the extraction buffer and called the crude lysosomal fraction. Mitochondria were obtained from mung bean hypocotyls which were harvested after 90 hr growth as described in ref. [31]. The preparations so obtained were deemed to be active since they specifically took up Janus green 'B' stain. Respiratory studies were conducted at 25° with a Warburg respirometer, each flask containing 0.5 ml of the mitochondrial extract, 0.5 ml of the enzyme preparation and 1 ml of a reaction mixture containing the following: sucrose, 0.3 M; glucose, 0.15 M; ATP, 1.5×10^{-3} M; $MgSO_4$ 1.5×10^{-3} M; Pi as the Na salt, 0.015 M; NaF 0.015 M; α-oxoglutaric acid, 0.03 M. The centre wells of flasks contained 20% KOH with filter paper wicks.

Conductivity measurements. Expts using test tissues were carried out on 6.5 mm diam discs of *ca* 1 mm thickness which were washed in running tap H_2O for times specified in the *Results* section. Conductivities were measured with a conductivity bridge.

Acknowledgement—This work was supported by a grant from the Agricultural Research Council.

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