Journal of Industrial Microbiology & Biotechnology (1999) 22, 71–77

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Inactivation of polycaprolactone depolymerase (cutinase) in *Fusarium* cultures by an extracellular protease

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Cultures of *Fusarium moniliforme* grown on polycaprolactone (PCL) or on cutin as a sole source of carbon and energy had low levels of detectable PCL depolymerase (cutinase) activity in the supernatant medium. A small peak of depolymerase activity was observed after hyphal accumulation had ceased, but this activity soon declined. The low level of the peak of activity and its decline were attributable to proteolytic inactivation of the depolymerase. A decrease in the pH of cultures coincided with the appearance of protease activity in the supernatant at about the same time as the appearance of the transient peak of depolymerase activity. Addition of protease substrates (bovine serum albumin, casein) to the culture at this time caused a dramatic although temporary increase in PCL depolymerase activity. The same effect was seen for cultures of *F. solani pisi*. Use of a different buffer system for the medium prevented a drop in pH and resulted in higher and stable levels of PCL depolymerase activity.

Keywords: *Fusarium moniliforme*; cutinase; polycaprolactone depolymerase; acid protease; biodegradable polymer

Introduction

The disposal of plastic waste has become a problem, due to the rapid increase in use of plastics and the decreasing landfill capacity for disposing of them. Developing biodegradable plastics has been proposed as one way to deal with this problem [8,19]. Several aliphatic polyesters are biodegradable, including polyhydroxyalkanoates, which are natural microbial polyesters, and polycaprolactone (PCL), a synthetic polyester, but they are expensive to produce and/or have physical properties that make them unsuitable for most applications [8,19]. New biodegradable plastics with improved properties are needed, and an understanding of degradative enzymes, or depolymerases, will be useful in their development.

A variety of bacteria and fungi secrete depolymerases that degrade PCL [2,3,22,27,28], including phytopathogenic fungi of the genus *Fusarium* [21]. We have shown that the natural substrate of *Fusarium* PCL depolymerases is cutin, the polyester structural component of plant cuticle [21]. Oligomers of PCL resemble the natural inducers of cutinase, and PCL is both an inducer and a substrate of cutinase. Although *Fusarium* strains show wide zones of clearing of PCL on agar [21], indicating effective secretion of active cutinase, our initial efforts to obtain enzyme from liquid cultures of *F. moniliforme* and *F. solani pisi* grown on PCL or cutin as carbon and energy source resulted in low and unstable activities. We report here on experiments suggesting that the low yields and instability were due to proteolytic inactivation of cutinase by an extracellular aspartyl (commonly called acid) protease appearing during a drop in culture pH. This finding was unexpected, because naturally secreted fungal enzymes are usually relatively resistant to attack by proteases secreted by the same strain at the same time [5,30,31], and *Fusarium* cutinases have been described as highly resistant to proteolytic inactivation [13,15]. A different buffer system prevented the decline in culture pH and gave higher activities of stable enzyme.

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Received 1 July 1998; accepted 6 December 1998

Materials and methods

Fungal strains and media

Fusarium moniliforme was from the University of Connecticut culture collection. *Fusarium solani* f sp *pisi* (ATCC 38136) was obtained from the American Type Culture Collection, Rockville, MD, USA. Cultures were maintained on potato dextrose agar (Difco Laboratories, Detroit, MI, USA) or V8 juice agar (V8 juice, 200 ml; $CaCO_3$, 3 g; agar, 25 g per liter). Basal mineral salts medium (BMS) contained (per liter): K_2HPO_4, 0.7 g and KH_2PO_4, 0.7 g sterilized separately from $MgSO_4 \cdot 7H_2O$, 0.7 g; NH_4Cl, 1 g; $NaNO_3$, 1 g; and 1 ml of a trace salts solution consisting of, per ml: NaCl, 5 μg; $FeSO_4 \cdot 7H_2O$, 2 μg; $ZnSO_4 \cdot 7H_2O$, 2 μg; and $MnSO_4 \cdot H_2O$, 7 μg [2,3]. A second, more highly buffered mineral medium (MM) contained (per liter): $(NH_4)_2SO_4$, 2 g; KH_2PO_4, 4 g; Na_2HPO_4, 6 g; $MgSO_4$, 0.2 g; $FeSO_4 \cdot 7H_2O$, 1 mg; $CaCl_2$, 1 mg; H_3BO_3, 10 μg; $MnSO_4$, 10 μg; $ZnSO_4$, 70 μg; $CuSO_4$, 50 μg; MoO_3, 10 μg [17].

Cutin isolation

Cutin was isolated from apples as described [32], with modifications. Peels from Cortland and McIntosh apples were boiled in oxalate buffer (4 g L^{-1} oxalic acid and 16 g L^{-1} ammonium oxalate) for 2–4 h. Cuticle was collected by filtration through cheesecloth, washed several times with distilled water, dried, and ground in a Thomas Wiley mill

(60 mesh followed by 100 mesh; Fisher Scientific, Pittsburgh, PA, USA). Powdered cuticle was extracted with chloroform/methanol (2:1, v/v) overnight with mild stirring. A second extraction was done with chloroform in a Soxhlet apparatus (Fisher Scientific) for 24 h. The cuticle was dried at 80°C, then washed several times with distilled water. Pectin and cellulose were removed by treatment with *Rhizopus* sp pectinase (1 g L^{-1}) and *Aspergillus niger* cellulase (5 g L^{-1}) (Sigma Chemical Co, St Louis, MO, USA) in pH 4.0 acetate buffer (50 mM) at 22°C for 48 h. Cutin was recovered by filtration, washed with distilled water, and dried at 105°C.

Depolymerase production by F. moniliforme grown on PCL or cutin

F. moniliforme was grown in BMS broth with polycaprolactone (PCL) 787 (molecular weight 80000, Union Carbide, Bound Brook, NJ, USA) or 0.2% cutin as the sole source of carbon and energy. For PCL-grown cultures, 5 g of PCL 787 was dissolved in 50 ml of chloroform, then glass microscope slides were dipped into the PCL solution. The chloroform was allowed to evaporate, and the slides were placed in a slide holder. The PCL-coated slides were sterilized in 70% ethanol for 1 h, then the holder containing the slides was placed into a sterile beaker, and the ethanol was allowed to evaporate. Sterile BMS broth (500 ml) was aseptically poured into the beaker, and inoculated with 2 ml of an overnight culture of *F. moniliforme* grown in trypticase soy broth. The culture was incubated at 22°C with aeration by mild stirring. For the cutin-grown cultures 0.2 g of cutin was added to 100 ml of BMS broth in a 250-ml Erlenmeyer flask and then autoclaved. The flask was inoculated with 2 ml of an overnight culture of *F. moniliforme* grown in trypticase soy broth, then incubated at 22°C with shaking at 220 rpm. Supernatants of cultures, clarified by centrifugation in a microfuge, were assayed daily for esterase and/or PCL depolymerase activity.

Esterase activity was determined spectrophotometrically with *p*-nitrophenyl caproate (PNPC, Sigma Chemical Co) as substrate, using a procedure adapted from Purdy and Kolattukudy [24]. PNPC was used as a model substrate to detect cutinase activity; PNP esters are good substrates for cutinase [14,17]. The assay mixture (1 ml total volume) contained 5 mM PNPC (100 μl), 50 mM potassium phosphate buffer, pH 6.8 (700–875 μl), and culture supernatant (25–200 μl). The absorbance at 405 nm was recorded for 3 min. Activity was expressed as the increase in A_{405} min^{-1} ml^{-1} of supernatant $\times 10^3$.

PCL depolymerase activity was measured by a densitometric assay using a suspension of PCL crystals, as described [7]. PCL (Cellomer Associates, Webster, NY, USA) with a molecular weight of about 20000 was used. A 1% suspension of PCL crystals was prepared by precipitating the polymer from an acetone solution with water, as described [9]. The assay mixture (1.5 ml total volume) contained 100 μl of 1% PCL suspension, 50 mM potassium phosphate buffer, pH 6.8 (0.4–1.15 ml), and culture supernatant (0.25–1 ml). The optical density at 600 nm was recorded for 3 min, during which time active preparations showed a linear decrease. Activity was expressed in units of decrease in OD_{600} min^{-1} ml^{-1} of supernatant $\times 10^3$.

Effect of additions on PCL depolymerase production

Maximum PCL depolymerase activity was seen in stationary phase, followed by a decrease. Additions were tested for their effect on depolymerase activity. Cultures (100 ml) of *F. moniliforme* were grown in BMS with 0.2% cutin, at 22°C with shaking at 220 rpm. Bovine serum albumin (BSA, Sigma Chemical Co, essentially fatty acid free) was added to 0.02% final concentration (1 ml of a 20 mg BSA ml^{-1} solution in 50 mM phosphate buffer, pH 6.8) after 63 h of incubation, at which time depolymerase activity had begun to drop after reaching a maximum. A parallel control culture received 1 ml of the buffer. Depolymerase activity was measured 1 h after the addition of BSA, and at several times after this.

Two other proteins, casein and gelatin (1 ml of 4.7 mg casein ml^{-1} or 3.3 mg gelatin ml^{-1} added to 100 ml), were also tested. Differences in concentration of these proteins (Bio-Rad Protein Assay, Bio-Rad Laboratories, Hercules, CA, USA), were due to differences in their solubility in phosphate buffer, pH 6.8. To test the possibility that the effect of proteins was due to their use as a nitrogen source, amino acids (0.02% casamino acids) or $(NH_4)_2SO_4$ (5 mM) were added to cultures. All additions were made after 63 h of incubation, when maximum PCL depolymerase activity was observed. The effect of BSA on the PCL depolymerase activity of *F. solani pisi* ATCC 38136 was tested as above, except that BSA was added after 52 h of incubation.

Protease activity in the culture supernatant of F. moniliforme grown on cutin

F. moniliforme was grown in BMS with 0.2% cutin at 22°C with shaking at 220 rpm. At 63 h, the culture was divided into two equal parts, and BSA (0.02%) was added to one half, while the other half received an equal volume of buffer, as above. Supernatants were tested for protease activity using azocasein (Sigma Chemical Co) as the substrate [4]. Culture supernatant was microcentrifuged, then 1 ml was combined with 0.1 ml of azocasein stock solution (10 mg azocasein ml^{-1} 50 mM phosphate buffer, pH 6.8). The initial absorbance was determined by immediately removing 0.25 ml and adding it to 1 ml of trichloroacetic acid (TCA) in a microfuge tube, followed by centrifugation for 2 min and measurement of the absorbance of the supernatant at 340 nm. A_{340} after 60 min of incubation of the azocasein/supernatant mixture at room temperature was determined by the same procedure. Background absorbance was determined by replacing culture supernatant with buffer. Protease activity was expressed as ΔA_{340} min^{-1} $ml^{-1} \times 10^3$.

The pH, packed cell volume, and depolymerase activity of the *F. moniliforme* cultures were determined along with protease activity. Packed cell volume was determined by removing 10 ml of culture at intervals, centrifuging it in a graduated centrifuge tube at $550 \times g$ for 5 min, and reading the level at which the cells settled [6].

Classification of proteases present in the culture supernatant of F. moniliforme grown on cutin

The types of proteases present in the culture supernatant of *F. moniliforme* grown on cutin were determined using

protease inhibitors [20,31]. Supernatants from cultures with and without added BSA were examined. Pepstatin in methanol, EDTA, or phenylmethylsulfonyl fluoride (PMSF) in isopropanol were added (1 μg pepstatin ml^{-1}; 20 mM EDTA; 100 μM PMSF final concentrations) to the protease assay mixture described above, and A_{340} was read at 0, 15, 30, and 60 min. Controls included: the protease assay mixture: (i) without inhibitors; (ii) with 50 mM phosphate buffer, pH 6.8 instead of culture supernatant; (iii) with isopropanol; and (iv) with methanol.

Results

PCL depolymerase production by F. moniliforme grown on PCL and on cutin

When *F. moniliforme* was grown in BMS mineral medium with PCL as carbon and energy source, the PCL depolymerase activity in culture supernatants was very low until after 7 days of incubation, then showed an increase to a modest peak over 2 more days, followed by a decline (Figure 1). Our discovery that *Fusarium* PCL depolymerase is cutinase [21], suggested that the natural substrate, cutin, might give higher depolymerase activity than PCL. Cultures grown on cutin showed an earlier rise of depolymerase activity, beginning at 3 days of incubation, but the peak activity was not significantly higher than that seen in PCL-grown cultures, and instability was again seen (Figure 2). Cutinase can be assayed by its esterase activity [24], and PCL depolymerase and esterase activities showed the same rise and fall in cutin-grown cultures (Figure 2), consistent with the presence of one unstable enzyme having both activities. Several repetitions of these experiments gave similar results, although the time course of enzyme production varied slightly.

Sparing effects of added protease substrates on PCL depolymerase activity

Instability of PCL depolymerase activity might have been due to attack by an extracellular protease. In that case,

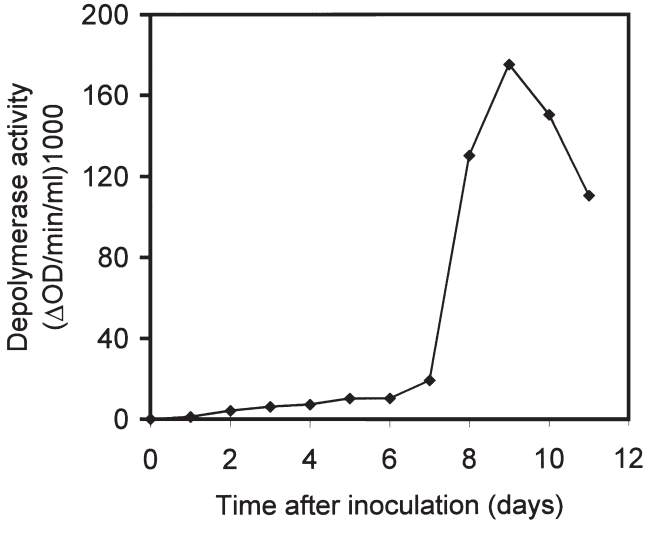

Figure 1 PCL depolymerase activity in the culture supernatant of *Fusarium moniliforme* grown in BMS with PCL as the sole source of carbon and energy.

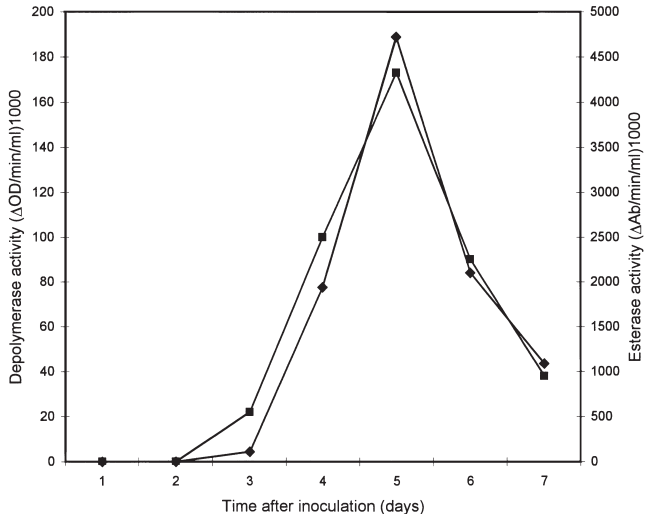

Figure 2 PCL depolymerase (–◆–) and esterase activity (–■–) in the culture supernatant of *Fusarium moniliforme* grown in BMS with cutin as the sole source of carbon and energy.

addition of proteins to the culture might provide competing protease substrates and protection for the depolymerase. Addition of bovine serum albumin (BSA) to cutin-grown cultures when at their usual low peak of PCL depolymerase activity resulted in a dramatic increase in activity within an hour, to a level 10- to 20-fold higher than that of a parallel control culture (Figure 3). The increase in activity was temporary, followed by a rapid decrease. Several repetitions of this experiment gave similar results, and a similar sparing effect of BSA on PCL depolymerase activity was also seen in cutin-grown cultures of another *Fusarium* species, *F. solani pisi* (Figure 3). Other proteins had an effect similar to

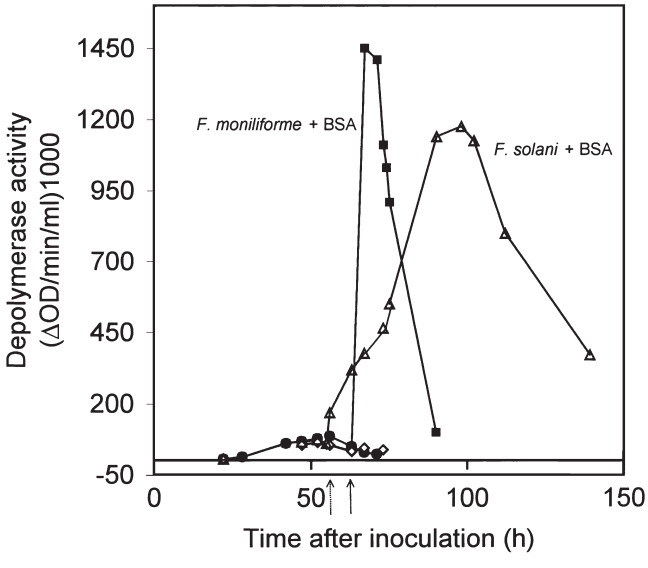

Figure 3 Effect of bovine serum albumin (BSA) on PCL depolymerase activity of *Fusarium moniliforme* and *F. solani pisi* ATCC 38136 grown in BMS with cutin. BSA dissolved in phosphate buffer, pH 6.8, or buffer only was added to the *F. moniliforme* culture at 63 h (solid arrow) and to the *F. solani* culture at 56 h (dotted arrow). *F. moniliforme*, BSA (–■–); *F. moniliforme*, buffer only (–◇–); *F. solani*, BSA (–△–); *F. solani*, buffer only (–●–).

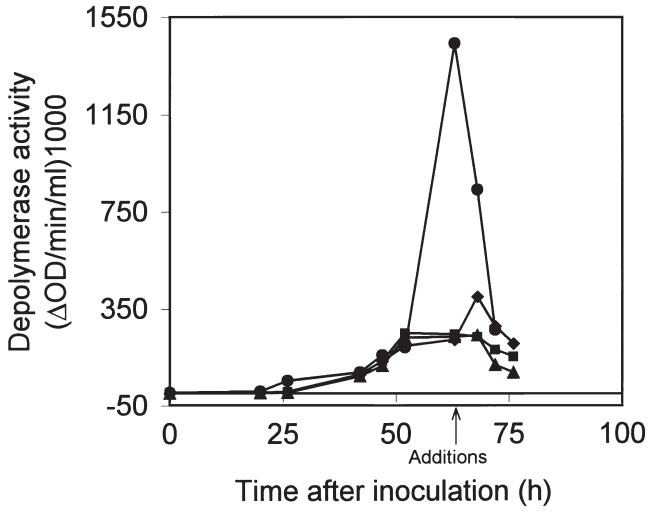

Figure 4 Effect of various additions on PCL depolymerase activity of *Fusarium moniliforme* grown in BMS with cutin. Gelatin (–◆–), casamino acids (–■–), ammonium sulfate (–▲–), sodium caseinate (–●–). Arrow indicates time of additions (63 h).

that of BSA. Addition of sodium caseinate and gelatin to a cutin-grown culture of *F. moniliforme* resulted in 7-fold and 1.6-fold increases in depolymerase, respectively (Figure 4). The differences in the magnitude of the effect of the added proteins may be due in part to differences in their final concentrations in the culture medium, which were 200 μg ml^{-1} for BSA, 47 μg ml^{-1} for casein, and 33 μg ml^{-1} for gelatin.

The effect of added proteins was not due to their serving as a source of carbon or nitrogen for growth. Addition of casamino acids or ammonium sulfate had no effect on depolymerase activity (Figure 4). Moreover, the cultures were well into stationary phase when the usual low peak of depolymerase occurred and proteins were added, and no further growth was seen after the addition of protein. Figure 5 shows this for BSA.

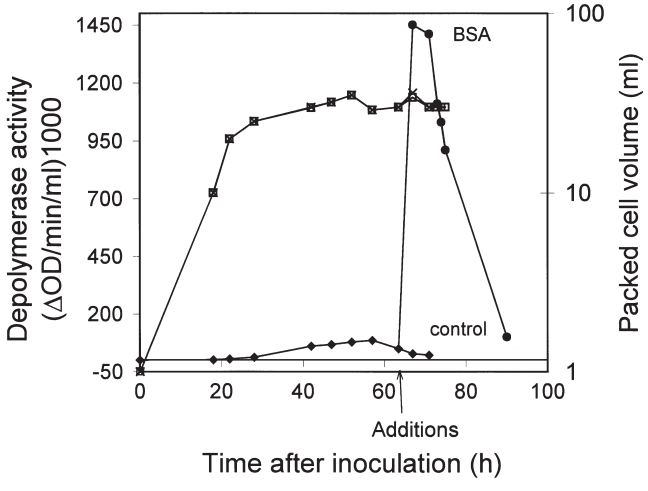

Figure 5 Effect of BSA addition on PCL depolymerase activity and growth (packed cell volume) of *Fusarium moniliforme* grown in BMS with cutin. After 63 h of incubation, the original culture was divided into two equal parts, then BSA dissolved in phosphate buffer, pH 6.8, was added to one half and buffer only was added to the other half. Depolymerase activity, BSA (–●–); depolymerase activity, buffer only (–◆–); growth, BSA (–□–); growth, buffer only (–X–).

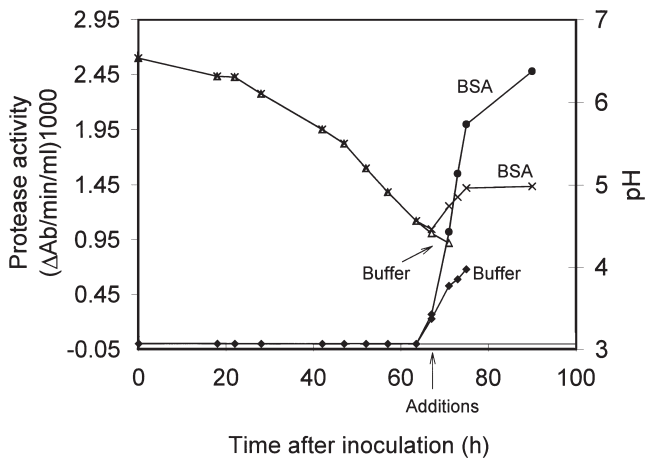

Figure 6 Protease activity and pH in the culture supernatant of *Fusarium moniliforme* grown in BMS with cutin. These data are from the same experiment as in Figure 5. Protease activity, BSA (–●–); protease activity, buffer only (–◆–); pH, BSA (–X–); pH, buffer only (–△–).

pH drop and appearance of protease activity in culture supernatants of cutin-grown F. moniliforme

The increase in PCL depolymerase activity resulting from the addition of proteins was only temporary, and was soon followed by a rapid decrease, much more rapid than the decrease in activity seen in cultures without additions (compare Figure 2 with Figure 3, data for *F. moniliforme*, and Figure 4). The pH of cultures decreased steadily during stationary phase, and protease activity was detected in the supernatant when it had dropped to 4.5, at about the same time that PCL depolymerase activity began to rise (Figure 6). The subsequent addition of BSA resulted in the appearance of increased protease activity over that seen in a parallel control culture without BSA (Figure 6), consistent with the more rapid decline in depolymerase activity seen after addition of protein being due to increased proteolytic attack. The type(s) of protease(s) present depended on whether exogenous proteins had been added. In supernatants of cultures without an addition only pepstatin, an inhibitor of aspartyl (acid) proteases, caused a decrease in protease activity (Table 1). The serine protease inhibitor

Table 1 Effect of protease inhibitors on protease activity in supernatants of cutin-grown *F. moniliforme* cultures with and without added bovine serum albumin (BSA)[a]

Addition to assay[b]	Protease activity as % of activity with no addition to assay	
	Culture without BSA	Culture with BSA
None	100	100
Pepstatin	50	60
PMSF	100	32
EDTA	112	54

[a]A BMS cutin culture was divided into two flasks after 63 h of incubation. BSA in buffer was added to 0.02% final concentration to one flask and buffer only to the other, incubation was continued for 10 h, then clarified supernatants were assayed for protease activity.
[b]Final concentrations: pepstatin 1 μg ml^{-1}, PMSF 100 μM, EDTA 20 mM.

PMSF had no effect, while EDTA, an inhibitor of metalloproteases, stimulated activity slightly (Table 1). In contrast, total protease activity in cultures 10 h after BSA addition was most sensitive to PMSF, although inhibition by EDTA and pepstatin was also seen (Table 1), consistent with the presence of at least two different proteases [31].

Stability of PCL depolymerase in cutin-grown cultures of F. moniliforme with a more effective buffer system

If falling pH caused induction of a secreted protease [10,29] active against PCL depolymerase (or cell lysis, releasing an intracellular protease attacking it), and this resulted in the observed low levels and instability of the depolymerase in cutin-grown cultures in BMS medium, then the use of a medium with a buffer system preventing a decrease in culture pH during incubation should give higher and stable levels of activity. Use of a different mineral medium (MM) with higher buffering capacity (71 mM phosphate, compared to 9 mM for BMS) prevented a drop in pH and resulted in stable levels of PCL depolymerase that were twice as high as those seen in the original (BMS) medium at peak (Figure 7).

Discussion

Fusarium moniliforme showed strong clearing of PCL in minimal agar, but cultures grown on PCL in liquid medium with the same salts and buffer system showed low yields of PCL depolymerase (cutinase) in the culture supernatant, and an unexplained loss of activity after maximum activity was reached (Figure 1). Use of the natural substrate, cutin, instead of PCL gave only slightly higher depolymerase activity, and the loss of activity still occurred (Figure 2). Our cutin cultures were incubated on a shaker, and there have been reports of instability of secreted enzymes due to shaking. In one case, a filamentous fungus that showed good clearing of a lipase substrate in agar lacked detectable lipase activity in shaking liquid culture, due to inactivation at the air/medium interface [25]. However, we saw the same low production and instability of PCL depolymerase

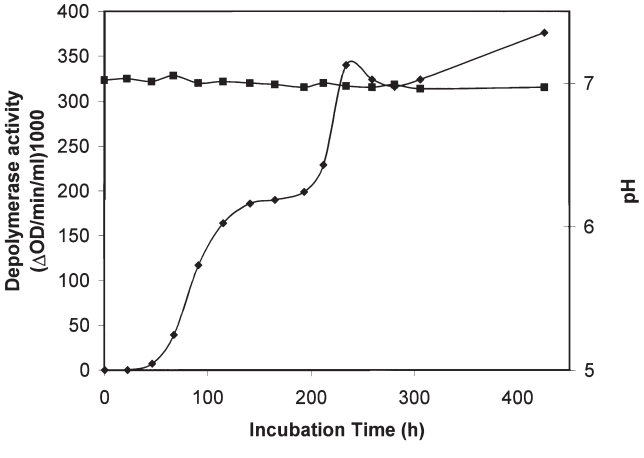

Figure 7 PCL depolymerase activity (–◆–) and pH (–■–) in the culture supernatant of *F. moniliforme* grown in a well-buffered medium (MM) with cutin as the sole source of carbon and energy.

in standing liquid cultures as in shaken cultures (unpublished results). Another possible explanation for our observations was proteolytic inactivation of the depolymerase. Although naturally secreted proteins are relatively stable to attack by extracellular proteases [30,31], there are exceptions. For example, a pectin lyase of *Aspergillus niger* was undetectable in liquid culture medium due to inactivation by a secreted acid protease [16].

Fusarium moniliforme and other *Fusarium* species secrete acid proteases in acidic mineral-protein medium [10,29]. We detected proteolytic activity in supernatants of *F. moniliforme* cultures grown on cutin after the pH dropped below 4.5 (Figure 6), and its appearance corresponded closely with a decrease in PCL depolymerase (cutinase) activity (Figure 5). This protease activity was inhibited by pepstatin alone of the standard protease inhibitors (Table 1) and may thus be classified as an aspartyl (acid) protease [20,31]. Secretion of acid proteases in response to decreasing pH is common among filamentous fungi [1,16], including *Fusarium* [10,29], but we cannot eliminate the possibility that low pH caused cell lysis and release of an intracellular protease. If secreted cutinase was being inactivated by a protease, then exogenous proteins might act as competitive inhibitors of the inactivation, and in fact addition of BSA or casein to stationary phase cutin-grown cultures at their usual low peak of PCL depolymerase activity not only prevented the usual loss of activity but actually resulted in a 10- to 20-fold increase in activity (Figures 3, 4, and 5). Stationary phase cutin-grown cultures of *F. solani pisi* showed a similar PCL depolymerase rebound upon addition of BSA (Figure 3). The rebound of depolymerase activity was temporary, presumably because proteolysis of the added protein soon left the depolymerase unprotected. Adding a source of nitrogen or sulfur or carbon can repress protease production in fungi [30,31,34], but proteolysis of the added proteins to yield repressing amino acids was not the basis for the protection of PCL depolymerase. The rise in depolymerase activity after addition of protein was too fast to be the likely result of repression of new protease secretion, at least in *F. moniliforme* (Figures 3, 4, and 5). More conclusively, addition of casamino acids (the product of casein hydrolysis) or addition of an inorganic source of nitrogen and sulfur, ammonium sulfate, had no effect on PCL depolymerase activity (Figure 4), eliminating protease repression as an explanation. In fact, addition of BSA resulted in the appearance of even higher proteolytic activity (Figure 6). This is consistent with induction by BSA of a new extracellular protease, including a serine protease and perhaps a metalloprotease. A comparison of data in Table 1 shows that protease activity in the supernatant of a culture following addition of BSA has become sensitive to inhibition by PMSF and by EDTA.

Inactivation of PCL depolymerase by an extracellular protease can explain the low peak of activity and its subsequent disappearance, but it doesn't explain why so little PCL depolymerase activity is seen in cultures during growth on PCL or cutin. Growth on cutin is complete by 40 h in our experiments with *F. moniliforme* (Figure 5), but no proteolytic activity is seen until after 60 h (Figure 6). There may be two factors accounting for very low

detectable PCL depolymerase activity during growth. First, cutinase is produced by *Fusarium* only in nutrient-limited slow growth, and then in low basal levels [13,15]. If cutin is present, basal levels of cutinase cleave it to release inducers, resulting in high levels of enzyme and facilitating penetration of the fungus into plant cells to obtain food [11,12]. Thus, although cutinase makes growth on cutin as a source of carbon and energy possible, it is an enzyme of secondary metabolism rather than a true catabolic enzyme, and its increased production in cutin medium after hyphal accumulation has stopped is not surprising. *Fusarium* cutinase, considered as a member of a set of penetration enzymes secreted under starvation conditions after the primary growth phase has ended [13], resembles lignin peroxidase secreted in the idiophase by the white-rot fungus *Phanerochaete chrysosporium* [5,33], although the peroxidase is resistant to idiophasic secreted proteases [5]. As a second contributing factor to low detectable depolymerase during growth, cutinase may remain associated with its insoluble substrate, cutin, until the enzyme is in excess [13]. Addition of excess cutin or PCL to supernatants containing cutinase results in an irreversible (in our hands) disappearance of activity in the PCL depolymerase assay (unpublished observations).

The susceptibility of cutinase to proteolytic attack is likely to be due to the acidic pH of the culture medium. Secreted cutinase has been said to be highly resistant to proteolysis by trypsin, chymotrypsin, proteinase K, elastase, and clostripain [13,15], but the conditions used for the tests were not reported. Many proteins secreted by fungi are glycosylated, which enhances their stability in the extracellular environment [23]. Cutinase secreted by *Fusarium* is glycosylated, and this may be important in providing stability and resistance to environmental factors such as heat and proteolytic attack [13,15]. However, conformational changes caused by SDS make cutinase susceptible to proteolysis [13], and a conformational change under acidic conditions, commonly seen in proteins [26], might make cutinase susceptible to proteolysis. Because the optimum pH for *Fusarium* cutinase activity is between 9 and 10, with activity dropping off very rapidly below pH 6 [18], there would be no expected natural function for the enzyme at low pH.

Acid protease attack on secreted pectin lyase B and secreted heterologous cloned proteins in *Aspergillus niger* cultures can be mitigated by use of more phosphate in the buffer system, which minimizes acid protease production [1,16]. Similarly, a medium with a more concentrated buffer system (71 mM in phosphate as compared to 9 mM for the original medium) gave higher and stable PCL depolymerase activities (Figure 7).

Acknowledgements

We acknowledge the US Department of Agriculture for financial support.

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