Extracellular proteases produced by the Quorn® myco-protein fungus *Fusarium graminearum* in batch and chemostat culture

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***Fusarium graminearum* was grown in batch and continuous (chemostat) culture on a glucose-mineral salts medium in the presence and absence of casein. In the absence of casein no protease activity was detected in the culture filtrate from either batch or chemostat culture. For batch cultures grown on medium containing casein, most of the proteolytic activity detected in the supernatant during exponential growth had an optimum at ca pH 5.0. However, as the cultures passed from late exponential into stationary phase, the pH profile of the protease activity broadened until most of it was in the alkaline pH region. For glucose-limited chemostat cultures grown on media containing casein, protease activity had a narrow pH optimum with maximum activity at pH 5.0. For all concentrations of casein examined, protease activity was greater in chemostat culture than in batch culture. Extracellular proteases from batch and chemostat cultures were purified by bacitracin–Sepharose affinity chromatography. At least seven proteins were purified from batch cultures but chemostat cultures contained only a single aspartic protease with a molecular mass of 40 kDa.**

Keywords: *Fusarium graminearum*, acid protease, aspartic protease, bacitracin, fungal proteases

INTRODUCTION

Fusarium graminearum A3/5 (the Quorn® myco-protein fungus) is potentially a good host for the production of recombinant proteins. Importantly, it can be grown at maximum specific growth rate ($\mu_{\max}$) in 155 m³ continuous flow cultures which can be maintained for several weeks (Trinci, 1994) and, recently, systems have been developed for its transformation and for expression of a heterologous protein (Royer *et al.*, 1995; Wiebe *et al.*, 1997). The advantage of growing *F. graminearum* at or near $\mu_{\max}$ in continuous flow culture, is that, theoretically, greater productivity can be achieved for growth-related recombinant proteins in such systems than in a series of batch cultures or in fed-batch cultures.

A highly branched (colonial) mutant of *F. graminearum*, CC1-5, was used in this study as it is better adapted for growth in submerged culture than the parental strain (A3/5). The compact nature of this strain also makes it more suitable for transformation studies (Wiebe *et al.*, 1997).

Amongst other factors, the yield of recombinant protein produced in a culture system is influenced by exogenous proteases produced by the host. However, although much is known about proteases produced by fungi in batch cultures (van Noort *et al.*, 1991), the effects of culture conditions on protease production in continuous flow cultures of filamentous fungi has not previously been studied. The present study shows that the number and type of extracellular proteases produced by *F. graminearum* in batch and chemostat culture differ markedly.

METHODS

Strains and culture conditions. *F. graminearum* Schwabe strain CC1-5 is a highly branched (colonial) mutant isolated from a glucose-limited chemostat (Wiebe *et al.*, 1992). It was maintained on the defined medium of Vogel (1956) with 10 g glucose l⁻¹ as the carbon source and 15 g Taiyo agar l⁻¹ (Lucas Meyer Colloids Ltd). Vogel's mineral salts solution was prepared at 50 × final concentration, sterilized by membrane filtration (0.2 µm diam.) and added to the sterile glucose solution (autoclaved at 121 °C for 15 min). Macroconidia for

inocula were harvested in sterile distilled water (10 ml) from 10–14-d-old plates and inoculated (to give a final concentration of approximately 5×10^4 macroconidia ml⁻¹) into liquid Vogel's medium containing 5 g glucose l⁻¹ and varying concentrations of casein. Cultures were grown in batch (1.2 l) and chemostat (2.1 l) culture as described by Wiebe & Trinci (1991). All cultures were grown in a Braun Biostat M fermenter at 25 ± 0.1 °C, 1000 r.p.m. stirrer speed, pH 5.8 ± 0.1 , $0.6\text{--}0.9$ l air (l culture)⁻¹ min⁻¹ aeration, with foam control by periodic addition of polypropylene glycol (mixed molecular weight, Foamaster, Henkel-Nopco).

Protease assay. Protease activity was determined by the method of Brock *et al.* (1982). Briefly, 10 ml samples of culture were filtered through pre-weighed Whatman No. 1 filters and the resulting filtrate was either assayed immediately or frozen at -20 °C prior to assaying. Filters were dried to constant weight at 60 °C for biomass dry weight determinations. Freezing of the culture filtrate had no effect on protease activity and samples remained stable at -20 °C for at least 11 months. Protease activity was determined by adding 50 µl culture filtrate to 1 ml 150 mM buffer containing 5 mg azocasein (Sigma) ml⁻¹. Samples were maintained at 25 °C for 2 h before stopping the reaction by adding trichloroacetic acid to give a final concentration of 6% (w/v). The samples were centrifuged at 16000 g in a MSE microfuge for 3 min; 0.5 ml of the resulting supernatant was transferred to a cuvette containing an equal volume of 1 M NaOH and the OD₄₅₀ was determined in a CE 292 Digital ultraviolet spectrophotometer (Cecil Instruments). One unit of enzyme activity was defined as the amount of enzyme necessary to give an absorbance of 1.0 under the assay conditions. Specific activity refers to units of enzyme activity (mg protein)⁻¹. The following buffers (van Noort *et al.*, 1991) were used to determine the pH optimum of the proteases: 150 mM sodium citrate (pH 3–4), 150 mM sodium acetate/acetic acid (pH 4–6), 150 mM piperazine-N,N'-bis(2-ethanesulfonic acid) (pH 6–7.5), 150 mM Tris/HCl (pH 7.5–9) and 150 mM sodium borate (pH 9–12.5).

Protein determination. Protein concentrations were determined using a bicinchoninic acid (BCA) protein assay kit (Pierce & Warriner) with bovine serum albumin as the standard (Pierce & Warriner).

SDS-PAGE. To determine the number and molecular mass of proteins secreted into the culture filtrate, and to monitor the depletion of casein from the medium, SDS-PAGE was carried out according to the method of Laemmli (1970) except that SDS was not included in the stacking gel. Gels (5%, w/v, acrylamide stacking gel and 12%, w/v, acrylamide separating gel) were prepared and run in a Bio-Rad Mini gel apparatus, with low-range protein molecular size markers (Bio-Rad). Gels were stained with 0.1% (w/v) Coomassie brilliant blue to visualize the proteins.

Bacitracin–Sephacryl affinity chromatography. Bacitracin, a broad spectrum peptide antibiotic, was used to purify proteases secreted into the culture filtrate by *F. graminearum*. Bacitracin–Sephacryl affinity chromatography was carried out essentially according to the method of van Noort *et al.* (1991). Samples of culture filtrate (10 ml) were concentrated by freeze drying, then brought to a volume of 4 ml by the addition of sterile distilled water before desalting using Econo-Pac 10 DG disposable chromatography columns (Bio-Rad). The concentrated, desalted culture filtrate was then passed down a bacitracin–Sephacryl affinity column. The bacitracin–Sephacryl affinity columns were either run with 25 mM sodium acetate buffer (pH 5.0) or 25 mM sodium

borate buffer (pH 9.5). Samples were routinely passed down a bacitracin–Sephacryl affinity column twice, so effectively doubling the length of the column. Bound proteins were eluted in 10 ml 1 M NaCl, 100 mM sodium acetate buffer (pH 5.0) or 100 mM sodium borate buffer (pH 9.5) (depending on the sample) and 25% (v/v) propan-2-ol. The same buffer and pH were used to elute the proteins as had been used to bind them to the bacitracin–Sephacryl affinity column. This resulted in total recovery of the bound proteins in an active form that could be subsequently assayed for protease activity. Samples were removed at each stage of the purification procedure and assayed for protease activity; prior to assays, the previously bound proteases were freeze-dried and desalted as described above.

Protease inhibition assay. Inhibition assays were carried out as described above for protease assays except that one of four irreversible protease inhibitors (final concentration 1 mM) was added to 1 ml 150 mM sodium acetate buffer (pH 5.0) containing 50 µl purified protease. This mixture was incubated at 25 °C for 1 h before adding azocasein to a final concentration of 5 mg ml⁻¹ and incubating at 25 °C for a further 1 h (Barrett, 1994). The Sigma protease inhibitors used were 3,4-dichloroisocoumarin (serine protease inhibitor), *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane (E-64) (cysteine protease inhibitor), pepstatin A (aspartic protease inhibitor) and 1,10-phenanthroline (metalloprotease inhibitor).

RESULTS

Production of proteases by *F. graminearum* CC1-5 in batch culture

The pH of the culture was maintained at 5.8 ± 0.1 for all batch cultivations. When *F. graminearum* was grown with glucose as the sole carbon source, no protease activity was detected in the culture filtrate, even in stationary phase. During early exponential phase (when the fungus was growing predominantly on glucose) no significant proteolytic activity was detected in the filtrate of cultures grown on 5 g glucose l⁻¹ and 1, 5 or 10 g casein l⁻¹. For each concentration of casein used, the pH profile of the extracellular proteases produced was similar, with proteolytic activity in the culture filtrate increasing with increasing concentrations of casein (results not shown). However, only the results obtained for one cultivation are described below.

For batch cultures grown on 5 g glucose l⁻¹ and 5 g casein l⁻¹, an increase in proteolytic activity was detected at 30 h post-inoculation during the late exponential phase of growth (Fig. 1). At this time the protease(s) ($2.5\text{--}2.8$ U ml⁻¹) in the culture filtrate had a pH optimum from pH 4.0 to 5.0, with no significant protease activity detectable above pH 6.5. By 48 h post-inoculation (i.e. in early stationary phase) the pH profile of the proteolytic activity in the culture filtrate had changed. The increase in proteolytic activity observed at 30 h post-inoculation coincided with the degradation of casein in the medium as observed by SDS-PAGE (Fig. 2, lane 3). Two broad peaks of protease activity were detected, one with an alkaline pH optimum (at ca pH 8.0–8.5), the other with an acidic pH optimum (at ca pH 5.0). This

Fig. 1. Change over time in pH profile of proteases secreted by *F. graminearum* CC1-5 grown at 25 °C in batch culture (1.2 l) in a Braun Biostat M fermenter (for conditions, see Methods) on modified Vogel's medium containing 5 g glucose l⁻¹ and 5 g casein l⁻¹. Protease activity was assayed using azocasein as substrate at 25 °C. ○, 0 h; ■, 6.5 h; △, 23 h; ▲, 30 h; ◇, 48 h; ●, 54 h; □, 71 h post-inoculation.

Fig. 2. SDS-PAGE of culture filtrate from *F. graminearum* CC1-5 grown at 25 °C in batch culture (1.2 l) in a Braun Biostat M fermenter (for conditions, see Methods) on modified Vogel's medium containing 5 g glucose l⁻¹ and 5 g casein l⁻¹. An equal volume (10 ml) of culture filtrate from each time point sampled was analysed. Lanes: 1, 6.5 h; 2, 23 h; 3, 30 h; 4, 48 h; 5, 54 h; 6, 71 h post-inoculation. The size of protein molecular mass standards are indicated down the left-hand side of the figure.

broad range of protease activity persisted for the remaining duration of the experiment, except that at 71 h post-inoculation a more pronounced peak in proteolytic activity (7.6 U ml⁻¹) was observed at pH 8.5. Between 30 and 48 h post-inoculation the number of protein bands detected by SDS-PAGE increased. This coincided with an increase in protease activity above pH 5.0. From 48 to 70 h post-inoculation the protein profile of the culture filtrate did not change significantly, even though the pH profile of protease activity did (Fig. 1 compared to Fig. 2).

When culture filtrate from each of the batch cultures was analysed by SDS-PAGE, a similar result was obtained in each case. The filtrate harvested from the beginning of each batch culture contained undigested casein (seen in Fig. 2 as two heavy-staining protein bands at 31 and 35 kDa). Subsequently, as the casein became depleted, at least seven extracellular proteins ranging in size from 28 to 65 kDa were detectable in the culture filtrate (Fig. 2); these bands were of approximately equal intensity.

To determine which proteins present in the culture filtrate were proteases, the culture filtrates from three batch cultures were purified by bacitracin-Sepharose affinity chromatography using two buffers of differing pH (because there appeared to be two distinct pH optima for the proteases present in the culture filtrate). Filtrate harvested from cultures growing exponentially (30 h post-inoculation) was purified at pH 5.0 and filtrate harvested from stationary phase culture (71 h post-inoculation) was purified at pH 9.5. Only one protein was found to bind to, and be eluted from, bacitracin when the culture was growing exponentially. This protein had a molecular mass of 40 kDa (data not shown). In contrast, seven proteins were found to bind to, and be eluted from, bacitracin when the culture was in stationary phase (Fig. 3). Only three of the proteins purified by bacitracin-Sepharose affinity chromatography had been detected by SDS-PAGE of the crude culture filtrate (presumably because they were present at low concentrations).

Production of proteases by *F. graminearum* CC1-5 in carbon-limited chemostat culture

Fig. 4 shows that little or no protease activity was detected in the filtrate of cultures of *F. graminearum* CC1-5 grown at a dilution rate of 0.16 h⁻¹ (doubling time, 4.3 h) in a glucose-limited chemostat culture fed

Fig. 3. SDS-PAGE of proteases purified by bacitracin-Sepharose affinity chromatography at pH 9.5 from culture filtrate from *F. graminearum* CC1-5 grown for 71 h at 25 °C in batch culture (1.2 l) in a Braun Biostat M fermenter (for conditions, see Methods) on modified Vogel's medium containing 5 g glucose l⁻¹ and 5 g casein l⁻¹. An equal concentration of protein was analysed for each sample. Lanes: 1, proteins eluted from bacitracin; 2, proteins not bound to bacitracin; 3, crude culture filtrate. The arrowhead indicates the putative 40 kDa protease. The size of protein molecular mass standards are indicated down the left-hand side of the figure.

with Vogel's medium containing 5 g glucose l⁻¹. When the medium feed for this chemostat culture was changed to Vogel's medium containing 5 g glucose l⁻¹ and 1 g casein l⁻¹, protease activity was observed in the culture filtrate and Fig. 4 shows that it had an optimum pH of 5.0 (7.8 U ml⁻¹). Protease activity in the culture filtrate

Fig. 5. SDS-PAGE of culture filtrate from *F. graminearum* CC1-5 grown at 25 °C in a chemostat (2:1; for conditions, see Methods) with 5 g glucose l⁻¹ and three different concentrations of casein. An equal volume of culture filtrate from each time point sampled was analysed. Lanes: 1, 25 h post-inoculation (0 g casein l⁻¹); 2, 49 h post-inoculation (0 g casein l⁻¹); 3, 73.5 h post-inoculation (1 g casein l⁻¹); 4, 96 h post-inoculation (1 g casein l⁻¹); 5, 121 h post-inoculation (1 g casein l⁻¹); 6, 145 h post-inoculation (5 g casein l⁻¹); 7, 167 h post-inoculation (5 g casein l⁻¹); 8, 173 h post-inoculation (5 g casein l⁻¹). The arrowhead indicates the 40 kDa protease. The size of protein molecular mass standards are indicated down the left-hand side of the figure.

increased to 9.9 U ml⁻¹ when the concentration of casein in the medium feed was raised to 5 g casein l⁻¹, though the pH profile of the protease essentially remained unchanged (Fig. 4). During this experiment, protease activity and protein concentration were measured in culture filtrate harvested from the fermenter vessel and the overflow; no difference was detected between the two samples.

Analysis of the proteins present in the culture filtrate harvested from chemostat cultures (Fig. 5) showed a very different protein profile from those obtained from batch culture (Fig. 2). As can be seen in Fig. 5, a single

Fig. 4. pH profile of proteases secreted by *F. graminearum* CC1-5 grown in a chemostat (2:1; for conditions, see Methods) with 5 g glucose l⁻¹ and three different concentrations of casein. Samples were taken at 49 h post-inoculation (0 g casein l⁻¹, ◆), 96 h post-inoculation (1 g casein l⁻¹, ■) and 167 h post-inoculation (5 g casein l⁻¹, ▲). Protease activity was assayed using azocasein as substrate at 25 °C.

Table 1. Purification of 40 kDa aspartic protease from culture filtrate of *F. graminearum* grown in a chemostat fed with a medium containing 5 g glucose l⁻¹ and two different concentrations of casein (for conditions, see Methods)

Protease was purified at pH 5.0.

Sample assayed	Specific protease activity [U (mg protein) ⁻¹] (percentage of total activity)	
	1 Casein concn (g l ⁻¹): Sample time (h post-inoculation):	5 167
Original culture filtrate	4.5 (100)	7.0 (100)
Bacitracin non-binding fraction	0	5.6 (15)
Bacitracin eluate	86.8 (73.5)	62.3 (85)

Table 2. Effect of protease inhibitors on a 40 kDa acidic protease purified by bacitracin–Sepharose affinity chromatography from culture filtrate from *F. graminearum* CC1-5 grown in a chemostat with 5 g glucose l⁻¹ and 5 g casein l⁻¹ (for conditions, see Methods)

Treatment	Normalized protease activity (%) [SEM]
Fresh sample	100 [2.5]
Pre-incubated sample	98.3 [8.4]
DMSO	103.5 [8.5]
3,4-Dichloroisocoumarin	99.8 [1.7]
E-64	105.4 [3.3]
Pepstatin A	17.6 [0.94]
1,10-Phenanthroline	87.3 [5.3]

high molecular mass (97 kDa) protein (of unknown function, but lacking protease activity) was present in culture filtrate throughout the chemostat culture, first appearing during glucose-limitation (lanes 1 and 2, Fig. 5). No protein of this molecular mass was detected in culture filtrates from batch cultures. When 1 g casein l⁻¹ (final concentration in the in-flowing medium) was added to the medium feed, a lower molecular mass protein (40 kDa) appeared in the culture filtrate (lanes 3–5, Fig. 5); a protein of similar molecular mass was detected in the culture filtrate from batch fermentations (arrowhead in Fig. 3). On increasing the casein concentration in the medium feed to 5 g l⁻¹, the concentration of the 40 kDa protein increased (lanes 6–8, Fig. 5) and a number of other proteins appeared. At no time was undigested casein detected in the culture filtrate of any of the chemostat cultures (however, there was a gap of 18 h between the onset of casein addition and the first sample being taken). The detection of protease activity in the culture filtrate coincided with the appearance of the 40 kDa protein in the culture filtrate for both concentrations of casein tested. Protease activity was higher in culture filtrates from chemostat, 1.656 U (mg dry wt)⁻¹ (Fig. 4), than from batch cultures, 1.19 U (mg dry wt)⁻¹ (Fig. 1).

Proteases produced in chemostat cultures fed with casein were purified by bacitracin–Sepharose affinity chromatography at pH 5.0. Table 1 gives results of the degree of purification obtained. When the various column fractions were separated by SDS-PAGE, only a single protein was found to bind to bacitracin (arrowhead in Fig. 5). Thus, in contrast to culture filtrates from batch cultures, those from chemostat cultures grown in the presence of casein, contained only one protein of 40 kDa which bound to immobilized bacitracin.

Type of protease produced in chemostat culture

The type of protease produced during chemostat culture was determined by incubating the 40 kDa bacitracin-purified acidic protease with a range of protease inhibitors. Since 3,4-dichloroisocoumarin, E-64 and 1,10-phenanthroline had no effect on the proteolytic activity of the 40 kDa protein whilst pepstatin A reduced its proteolytic activity by over 80%, it was concluded from the results shown in Table 2 that the 40 kDa protein was an aspartic acidic protease. It is possible that the protein was not pure and a small amount of a metalloprotease was also present as 1,10-phenanthroline did reduce the proteolytic activity slightly and pepstatin

A did not completely inhibit proteolytic activity. It is also possible that the acidic protease was not completely inhibited by pepstatin, as pepstatin A inhibits pepsin-like proteases but not all acidic proteases.

DISCUSSION

In the absence of an exogenous protein source, no proteases were detected in the filtrate of batch or chemostat cultures of *F. graminearum*, indicating that proteases in this fungus are substrate-induced (Fig. 4).

In batch culture grown on a medium containing casein, casein was still present 23 h after inoculation. However, no casein was detected (as assessed by SDS-PAGE) in chemostat cultures 18 h after it had been added to the medium feed. Our results contrast with the results of Royer *et al.* (1995) who detected only very low levels of extracellular proteins in culture filtrate of *F. graminearum* A3/5 grown for 5 d in batch culture. Since a batch culture of *F. graminearum* would be in stationary phase after 5 d, we conclude that the majority of proteolytic activity in the filtrate of such cultures would be active at pH 8.5. Therefore, by assaying for protease activity at pH 7.5, Royer *et al.* (1995) may have underestimated the protease activity in their cultures.

The proteases produced by exponential phase cultures of *F. graminearum* grown in the presence of casein varied with culture conditions. During batch culture a broad pH optimum was observed (Fig. 1) for protease activity and this contrasted with the sharp peak of activity (optimum at ca pH 5.0) observed in chemostat culture (Fig. 4). In batch cultures, the increase in alkaline protease activity (Fig. 1) was correlated with the onset of stationary phase; this protease was not observed in steady-state chemostat cultures grown at 0.16 h^{-1} (doubling time 4.3 h). The presence of a broad pH optimum for protease activity in the exponential phase of batch culture was not expected as most investigations of fungal proteases report a single protease, although frequently little information is given about the growth phase in which the protease was produced.

The (major) protease detected in the culture filtrate of chemostat cultures of *F. graminearum* was a 40 kDa aspartic protease. Aspartic proteases have been identified in a number of fungi: *Rhizopus hangchow* (Ichishima *et al.*, 1995), *Rhizopus chinensis* (Takahashi, 1987), *Rhizopus niveus* (Kurono *et al.*, 1971; Horiuchi *et al.*, 1988), *Penicillium janthinellum* (Hsu *et al.*, 1977; James & Sielechi, 1983), *Cryphonectria parasitica* (Barkholt, 1987), *Rhizomucor pusillus* (Tonouchi *et al.*, 1986), *Rhizomucor miehei* (Christensen *et al.*, 1988), *Aspergillus saitoi* (Shintani & Ichishima, 1994), *Aspergillus niger* (Archer *et al.*, 1992; Mattern *et al.*, 1992; Calmers *et al.*, 1991; Inoue *et al.*, 1991; van Noort, 1991), *Aspergillus awamori* (Berka *et al.*, 1990; Hayashida & Flor, 1981), *Mucor miehei* (Gray *et al.*, 1986) and *Botrytis cinerea* (Movahedi & Heale, 1990a, b), all of which have a molecular mass between 34 and 43 kDa. Thus, the aspartic protease produced by

F. graminearum would appear to be typical of fungal aspartic proteases.

Royer *et al.* (1995) are currently developing this fungus as a host for recombinant protein production. Accumulation of recombinant protein in the medium of a *F. graminearum* culture would induce protease production; therefore, if this fungus is used as a host for recombinant protein production, a protease-deficient strain should be chosen. Since the 40 kDa aspartic protease was responsible for the majority of proteolytic activity secreted into the culture filtrate of chemostat-grown cultures, this enzyme would be the best candidate to delete or render inactive if continuous flow cultures of *F. graminearum* were used for recombinant protein production. However, the use of batch or fed-batch cultures of *F. graminearum* for recombinant protein production would necessitate the deletion of a number of proteases, including the alkaline protease.

ACKNOWLEDGEMENTS

The authors would like to thank the BBSRC for a ROPA award which made this work possible.

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Received 15 January 1997; revised 6 May 1997; accepted 14 May 1997.