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ORIGINAL PAPER

# Chymostatin can combine with pepstatin to eliminate extracellular protease activity in cultures of *Aspergillus niger* NRRL-3

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**Abstract** *Aspergillus* strains are being considered as potential hosts for recombinant heterologous protein production because of their excellent extracellular enzyme production characteristics. However, *Aspergillus* proteases are problematic in that they modify and degrade the heterologous proteins in the extracellular medium. In previous studies we observed that media adjustments and maintenance of a filamentous morphology greatly reduced protease activity and that a low concentration of the aspartic protease inhibitor pepstatin inhibited the latter protease activity to the extent of approximately 90%. In this paper we report that when the serine protease inhibitor chymostatin is used in combination with pepstatin 99–100% of total protease activity in *Aspergillus* cultures is inhibited. In protease assays a concentration of 30 μM chymostatin combined with 0.075 μM pepstatin was required for maximum inhibition. Inhibitor concentrations of chymostatin and pepstatin of 120 and 0.3 μM, respectively, when added to *Aspergillus* cultures, has no significant effect on biomass production, glucose utilization or culture pH pattern. The potential of using these protease inhibitors in cultures of recombinant *Aspergillus* strains producing heterologous proteins will now be investigated to determine if the previously observed recombinant protein denaturing effects of *Aspergillus* proteases can be negated.

**Keywords** *Aspergillus niger* · Aspartyl protease · Pepstatin · Culture medium · Inhibition

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## Introduction

*Aspergillus* species have been used as workhorses in commercial enzyme production processes because high extracellular enzyme productivities can be consistently achieved [14, 34, 35]. For example some industrial *Aspergillus* strains are reported to secrete up to 25 g/l of glucoamylase. These extraordinary enzyme yields have encouraged biotechnologists to investigate the potential for these strains to be used as hosts for recombinant heterologous protein production. Unfortunately, *Aspergillus* species also secrete proteases into the extracellular medium, which cause degradation of heterologous proteins produced by recombinant *Aspergillus* strains [9, 24, 36, 37]. *Aspergillus niger* produces two extracellular aspartic proteases, a pepsin type protease PEPA and a non-pepsin type protease, PEPB [32, 33]. In addition, *A. niger* produces an intracellular vacuolar aspartic pepsin-type protease PEPE as well as other intracellular proteases.

Because cell lysis occurs to a greater or lesser extent during *Aspergillus* fermentations there is a need to consider the impact of both extracellular and intracellular proteases on *Aspergillus* production of heterologous proteins. Strains deficient in extracellular proteases typically exhibit better production of heterologous proteins [12, 15, 31]. While mutant strains deficient in the acid protease, aspergillopepsin, exhibited improvements in the production of chymosin as compared to the wild-type strain, other proteases still degraded some proteins [5, 6]. However, *A. nidulans*, for example, has around 80 protease genes in its genome and there are concerns that development of multiple protease-deficient mutants

in *Aspergillus* production strains would produce phenotypic strains unsuitable for fermentation processes [22].

We have previously used culture-based strategies focused on nitrogen nutrient type and maintenance of filamentous rather than pelleted morphology to reduce protease activity in *A. niger* NRRL-3 [22]. While the latter morphological effects may be explained in terms of greater cell lysis and intracellular protease release occurring in inner pellet locations, an *A. niger* gene encoding pepsin-like protease activity can be differentially expressed during pelleted but not during filamentous growth [13].

The aspartyl proteases of *Aspergillus* species are reported to be strongly inhibited by pepstatin (isovaleryl-val-val-statine-ala-statine where statine = [3S, 4S]-4-amino-3-hydroxy-6-methylheptanoic acid), which was first isolated from streptomyces [16–20, 29, 30], whereas PEPB is known to very insensitive to pepstatin [18]. By incorporation of pepstatin into the *Aspergillus* culture media designed to support filamentous growth protease activity was reduced by 80–90%, indicating a residual of 10–20% of activity was pepstatin resistant [1, 2]. In this paper we have investigated the potential to completely eliminate extracellular protease activity by adding additional protease inhibitors to the pepstatin-supplemented culture medium.

## Materials and methods

### Culture

*Aspergillus niger* NRRL-3, obtained from the Northern Regional Research Laboratory, Peoria, IL, was used as a model strain in this study. Agar slopes, inoculated with this culture, were incubated at 30°C for 3–4 days (until good sporulation had occurred) and stored for up to 3 months at 4°C.

### Sources of protease inhibitors

Pepstatin-A [isovaleryl-val-val-AHMAH-Ala-AHMAH where AHMAH = (3S, 4S)-4-amino-3-hydroxy-6methylheptanoic acid], chymostatin {*N*-(*N*α-cabonyl-cpd-x-pheal)-phe(cpd = capreomycidine) (capreomycidine = [S,S]-α-(2-iminohexahydro-4-pyrimidyl) glycine}, E-64 [trans epoxysuccinyl-L-leucylamido-(4-guanidino)butane] and DAN (diazo acetyl-DL-norleucine methyl ester) were obtained from Sigma-Aldrich, St. Louis, MT. Alpha-2-macroglobulin (from bovine plasma) was obtained from Roche Applied Science, Mannheim, Germany.

### Media

Agar slopes: glucose 10 g/l, yeast extract (Sigma, St. Louis, MT) 3 g/l, peptone (Fisher Biotech, Fair Lawn, NJ) 3 g/l, and technical agar bactoagar (Difco, Sparks, Madison) 20 g/l.

Yeast nitrogen base-CMC medium (g/l): glucose 10, yeast nitrogen base (Sigma, St Louis, MT) 6.7, carboxymethylcellulose (sodium salt, high viscosity, Sigma, St. Louis, MT) 20, $NaH_2PO_4$ 1.55, $Na_2HPO_4{\cdot}7H_2O$ 4.85, and $KH_2PO_4$ 4, and pH 6.5.

### Culture conditions

The standardized spore inoculum prepared by suspending the contents of the agar slope in 10 ml of distilled water containing 1% Triton-X100 (Fisher Scientific, Orangeburg, NJ). A high concentration of the above aqueous spore inoculum ($5 \times 10^8$) was added into 250 ml Erlenmeyer flask containing 50 ml medium and the inoculum flasks were incubated for 24 h on an orbital shaker at 37°C and 200 rpm as indicated, and 3 ml of the resulting culture was used to inoculate experimental cultures containing the same medium which were then incubated under the same conditions.

Culture supernatants were prepared by centrifugation at 3,000*g* for 6 min followed by filtration using Whatman no. 1 paper to remove residual carboxymethylcellulose.

### Analytical methods

Glucose was measured in culture supernatants as reducing sugars using the dinitrosalicylic acid method [23]. Protease assay was carried out by incubating an aliquot of culture filtrate with buffered casein (bovine milk, Sigma, St. Louis, MT) solution (2.5 mg/ml in 0.05 M sodium phosphate buffer) at pH 6.5 and 37°C in a final assay volume of 2 ml. The reaction was stopped and residual protein precipitated by addition of 4 ml trichloroacetic acid. After standing for 1 h, supernatant was obtained by centrifugation at 3,000*g* for 6 min. To 1 ml of supernatant, 5 ml of 0.4 M sodium carbonate was added followed by 0.5 ml Folin-phenol reagent. After 10 min tyrosine liberated by the action of protease was measured at 660 nm. One unit of protease activity liberates 1 μg tyrosine per minute under the assay conditions.

## Results

The effect of pepstatin and various other inhibitors on inhibition of *Aspergillus* protease activity present in

**Table 1** Effect of supplementary inhibitors on pepstatin inhibition of protease activity in culture filtrates from *A.niger* grown in the YNB medium

| Inhibitor [concentration in assay] | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Pepstatin [0.075 μM] | – | + | – | – | – | – | + | + | + | + |
| α-2-Macroglobulin [10 μM] | – | – | + | – | – | – | + | – | – | – |
| DAN [12 mM] | – | – | – | + | – | – | – | + | – | – |
| Chymostatin [60 μM] | – | – | – | – | + | – | – | – | + | – |
| E-64 [10 μM] | – | – | – | – | – | + | – | – | – | + |
| Culture incubation time (h) | Protease activity (% of uninhibited control) | | | | | | | | | |
| 48 | 100 | 8.2 | 126 | 217 | 54.1 | 115 | 39.3 | 78.4 | 0 | 48.9 |
| 72 | 100 | 4.4 | 68 | 109 | 84 | 65 | 12.9 | 22.1 | 0 | 29.8 |
| 96 | 100 | 4.3 | 76 | 63 | 88 | 92 | 10.7 | 8.7 | 0.6 | 41.5 |

Filtrates were recovered from cultures at 24 h intervals and were assayed at the inhibitor concentrations indicated. Results are expressed as a percentage of the activity present in the corresponding control, which contained no inhibitors

culture filtrates harvested after different culture periods was investigated (Table 1). Inhibitors were used at concentrations reported in the literature to give maximum protease inhibition and were tested individually and in combination with pepstatin. The pepstatin concentration used was based on our previous work (0.075 μM). Pepstatin as expected inhibited protease activity to the extent of 90–95%. None of the other inhibitors when used alone substantially inhibited proteolytic activity. However, when used in combination with pepstatin, one inhibitor, chymostatin *N*-(*N*-α-carbonyl-cpd-x-phe-al)-
phe(cpd = capreomycidine)(capreomycidine = [S,S]-α-(2-iminohexahydro-4-pyrimidyl) glycine appeared to inhibit the pepstatin-insensitive protease activity, such that the combination completely eliminated protease activity. When two of the other test inhibitors were used in combination with pepstatin, percentage inhibition was less than that observed with pepstatin alone. These inhibitors appeared to interfere with the inhibitory activity of pepstatin.

The effect of chymostatin concentration on inhibition of pepstatin insensitive protease is presented in Table 2. A small inhibitory effect on pepstatin insensitive protease was observed at chymostatin concentrations of 15 and 30 μM. A chymostatin concentration of 60 μM in combination with 0.075 μM pepstatin exhibited a 100% inhibition of total protease activity in 72 h cultures and approximately a 99% inhibition of protease activity in 96 h filtrates. This result indicate an apparent K*i* value of between 30 and 60 μM for chymostatin against pepstatin-insensitive protease activity in *Aspergillus* culture filtrates.

We have previously shown that pepstatin, incorporated directly into the culture medium does not affect culture parameters and retains its protease inhibitory effect. A similar experiment was carried out incorporating chymostatin in the culture medium together with pepstatin (Table 3). The concentration of pepstatin and chymostatin in the culture medium were 0.3 and 240 μM, four times the maximum protease inhibitory doses of 0.075 and 60 μM used in the prior assays, to insure inhibition during the subsequent protease assay when the enzyme is diluted fourfold. Even at the higher chymostatin concentration chymostatin had no significant effect on *Aspergillus* biomass production, glucose depletion or culture pH. When filtrates were recovered from the inhibitor-supplemented cultures and assayed for protease activity (diluted fourfold by other assay constituents) 48, 72 or 96 h cultures supplemented with pepstatin (0.3 μM) and chymostatin (240 μM) exhibited 100%, and >99% inhibition, respectively.

**Table 2** Effect of chymostatin concentration, used in combination with 0.075 μM pepstatin, on protease activity (U/l) in culture filtrates from *A. niger* grown in the YNB medium

| Inhibitor concentration in the assay (μM) | | | | | | | |
|---|---|---|---|---|---|---|---|
| Pepstatin | 0 | 0.075 | 0.075 | 0.075 | 0.075 | 0.075 | 0.075 |
| Chymostatin | 0 | 0 | 3.75 | 7.5 | 15 | 30 | 60 |
| Culture incubation time (h) | Protease activity (U/l of culture) | | | | | | |
| 24 | 29.3 | 0 | 0 | 0 | 0 | 0 | 0 |
| 48 | 79.3 | 7.9 | 7.9 | 7.9 | 6.4 | 5.7 | 0 |
| 72 | 98.6 | 8.6 | 8.6 | 8.6 | 7.9 | 7.1 | 0 |
| 96 | 120.8 | 10.7 | 10 | 9.3 | 8.6 | 7.9 | 0.7 |

Filtrates were recovered from cultures at 24 h intervals and were assayed at the inhibitor concentrations indicated. Results are expressed as a percentage of the activity present in the corresponding control, which contained no inhibitors

**Table 3** Effect of chymostatin concentration, added in combination with 0.3 μM pepstatin to the *A. niger* YNB culture medium, on protease activity (U/l) in culture filtrates recovered and assayed at 24 h intervals

| | | | | | |
|---|---|---|---|---|---|
| Inhibitor concentration in the culture (μM) | | | | | |
| Pepstatin | 0 | 0.3 | 0.3 | 0.3 | 0.3 |
| Chymostatin | 0 | 0 | 60 | 120 | 240 |
| Inhibitor concentration in the assay (μM) | | | | | |
| Pepstatin | 0 | 0.075 | 0.075 | 0.075 | 0.075 |
| Chymostatin | 0 | 0 | 15 | 30 | 60 |
| Culture incubation time (h) | Protease activity (U/l of culture) | | | | |
| 24 | 30.7 | 0 | 0 | 0 | 0 |
| 48 | 60 | 7.9 | 7.1 | 6.4 | 0 |
| 72 | 82 | 8.6 | 7.9 | 7.1 | 0.7 |
| 96 | 107 | 9.3 | 8.6 | 7.9 | 0.7 |

Given the culture filtrate is diluted 4-fold during the assay procedure, the maximum concentrations of inhibitors present in the assay are 25% of their concentrations in the culture medium

## Discussion

We have previously noted that low concentrations of pepstatin inhibit protease activity in culture to an extent of 80–90% and concluded that this activity was primarily due to inhibition of a combination of the *Aspergillus* extracellular and of intracellular aspartyl proteases present in the culture filtrate as a result of cell lysis. We speculated that the protease activity observed in the presence of pepstatin is likely due to some of the non-aspartyl proteases, which have been described in *Aspergillus* species, including the serine proteases and serine carboxypeptidades [3, 4, 16, 17, 25–27]. We have now demonstrated that essentially complete inhibition of protease in *Aspergillus* culture filtrates is achieved using a combination of inhibitors, namely pepstatin and chymostatin at optimized inhibitor concentrations. Chymostatin is a well-characterized inhibitor of serine proteases or chymotrypsin-like proteases [20].

It was previously demonstrated that supplementation of the culture medium with pepstatin appeared to have no effect on fungal growth or on metabolism as reflected by glucose consumption or pH changes and we have now confirmed that chymostatin even at much higher concentrations (120 μM/l) does not interfere with these fermentation parameters. Use of a natural peptide-containing inhibitor rather than a reactive organic or metal inhibitor is advantageous in that this inhibitor appears to have protease specific rather than general protein-denaturing properties. The concern that the fungal mycelial biomass might adsorb chymostatin by non-specific binding or that enzymes might even biodegrade the inhibitor appear unlikely, given that inhibitor-supplemented cultures still retained protease-inhibition capability throughout the culture time course. A similar observation was previously made for pepstatin.

Serine alkaline proteases from *Aspergillus fumigatus* and *Aspergillus terreus* are inhibited by chymostatin [11, 28] and hence at least part of the inhibitory effect of chymostatin in *Aspergillus niger* cultures is likely due to inhibition of *A. niger* serine proteases. Serine proteases from *A. fumigatus* appear to play a role in the diverse respiratory disorders of this pathogen [28].

Serine proteases from some other fungi and bacteria have also been found to be inhibited by chymostatin. Serine protease from the filamentous fungus *Scedosporium apiospermum* is inhibited by chymostatin [19]. The serine protease from the nematophagous fungus, *Drechmeria coniospora*, inhibited by chymostatin, appears to be responsible for the infective process involving the nematode, *Panagrellus redivivus* [16]. A chymostatin sensitive protease produced by the bacterium *Xenorhabdus menatophilia* is involved in insect immunosuppression and associated bacterial pathogenicity [10].

Chymostatin has been found to have a role as a chemotherapeutic agent against protease-mediated disease states including malaria [8]. Indeed, therapeutic research strategies have also exploited use of combinations of protease inhibitors, including both petstatin and chymostatin [21]. Chymostatin also suppresses dimethylhydrazine-induced colon carcinogenesis [7].

We will now test the applicability of the best conditions for minimizing protease activity to production of heterologous proteins by recombinant *Aspergillus* hosts to minimizing proteolytic degradation of the heterologous proteins produced in these cultures.

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