Regulation of Exocellular Proteases in *Neurospora crassa*: Metabolic Requirements of the Process

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Received for publication 3 February 1975

To induce exocellular proteolytic enzyme from carbon-starved exponential-phase cells of *Neurospora crassa*, both a protein substrate and an activating protease of certain specific properties must be present at the same time. The cells must be capable of protein synthesis, since cycloheximide inhibits the process, but cell growth, as determined by increase in cell mass, does not appear to be required. Both soluble (bovine serum albumin, myoglobin) and insoluble protein substrates (collagen, corn zein) will affect protease induction, although certain soluble, globular proteins (egg white globulin, bovine gamma globulin) will not. In most cases, rates of protease induction are proportional to protein concentration, regardless of the nature of the inducing protein. All activating proteases capable of affecting induction in a manner similar to that of *N. crassa* exocellular protease were of bacterial origin and were exoproteases. Mammalian proteases and peptidases had little or no effect on the induction process.

The biosynthesis of exocellular proteolytic enzymes has been of practical concern for a number of years. Factors such as substrate (16, 20), ionic strength and milieu (23), and incubation conditions (21) have been manipulated to achieve maximum yields of proteolytic enzyme from fungi and *Streptomyces*.

These studies, however, have yielded little information on the mechanism of protease biosynthesis and, thus, have provided no real basis for systematic analyses of the role of variables of culture in the process. Recently, series of studies on the regulation of exocellular protease biosynthesis in the fungi *Aspergillus nidulans* (1, 2, 3) and *Neurospora crassa* (4, 5, 8) have appeared. Using synthetic media and cells in exponential phase, the authors have delineated the general mechanisms of protease regulation in these two organisms. In *A. nidulans*, protease biosynthesis is repressed by substrate quantities of nitrogen, carbon, and sulfur. Starvation for any one of these nutrients results in derepression and protease biosynthesis. Regardless of missing nutrient, *A. nidulans* appears to produce at least two exocellular proteolytic activities (alkaline and neutral), as judged by electrophoretic analyses. The kinetics of secretion for these enzymes does not appear to be a function of the nutrient missing from the milieu.

In *N. crassa*, protease biosynthesis is inducible by a protein substrate when cells are starved for either carbon, nitrogen, or sulfur; substrate concentrations of carbon, nitrogen, or sulfur repress enzyme biosynthesis, with catabolite repression occurring when substrate levels of a carbon source are provided. As judged by immunological reactivity, electrophoresis, response to specific inhibitors and pH stability, *Neurospora* produces an acid, an alkaline, and a neutral protease activity under all conditions of induction. As is the case with *Aspergillus*, kinetics of secretion for two of these activities does not appear to be a function of limiting nutrient (B. Cohen, J. E. Morris, and H. Drucker, manuscript in preparation).

These two systems employ exponential-phase cells. As such, parameters of substrate and culture condition can be manipulated over short periods of time, and general analyses of optimal conditions can be performed quickly, perhaps yielding a mechanistic basis for future studies. Work from this laboratory has shown that protease biosynthesis from exponential-phase cells of *N. crassa*, placed into a medium in which the protein bovine serum albumin (BSA) is sole carbon source, requires catalytic quantities of added protease. In this communication, further delineation of the metabolic requirements of this process (in terms of specificity for inducing protein, added protease, etc.) will be analyzed in exponential-phase cells of *N. crassa*.

**MATERIALS AND METHODS**

**Assay of proteolytic activity.** The proteolytic activity assay method of McDonald and Chen (10), as utilized previously (4), was employed. One unit of
activity is defined as the amount of enzyme that produces one microgram-equivalent of non-trichloroacetic acid-precipitable tyrosine per minute by action on casein (7).

**Preparation of proteolytic enzymes.** All proteolytic enzymes employed (unless otherwise indicated) were dialyzed against 0.1 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (pH 8.0) plus 0.002 M Ca and assayed for activity in this buffer.

**Thermolysin.** Thermolysin (Calbiochem, A grade, crystallized three times) crystals were dissolved as described previously (6). The specific activity of the enzyme as assayed here was 1,620 proteolytic units (PU) per mg of protein.

**Pronase.** Pronase (Calbiochem, A grade, lot 023054) under the assay conditions employed here had 306 PU/mg of protein.

**Subtilisin.** Subtilisin (Schwarz/Mann, crystalline, lot X3866) under the assay conditions employed here had a specific activity of 275 PU/mg of protein.

**Elastase.** Elastase (Schwarz/Mann, crystalline, lot Y4266, 17 Sacher units per mg of crystal) was not directly soluble in the dilute Tris buffer employed in this study. It was dissolved in identical fashion to thermolysin (5) and had specific activity of 235 PU/mg of protein.

**Collagenase.** Clostridium histolyticum collagenase (Schwarz/Mann, lot X-3860) was assayed by the procedure of Mandl et al. (11), with ninhydrin reagent (12) employed to determine amino acid release. The preparation of collagenase used in this study had 2.75 collagenase units per mg and a specific activity of 16 PU/mg in the caseinolytic assay.

**Papain.** Papain (crystalline suspension containing 28 mg of protein per ml, Schwarz/Mann, lot V4358) was dissolved in a diluent containing 1.12 mM ethylenediaminetetraacetic acid, 5.6 mM L-cysteine-hydrochloride, and 7.2 μM mercaptoethanol. Proteolytic activity of papain was measured on 2% casein dissolved in 0.2 M KPO₄ buffer (pH 7.0) plus 1 mM ethylenediaminetetraacetic acid. Thermolysin, the protease routinely employed in these investigations, was also assayed at pH 7.0 with omission of ethylenediaminetetraacetic acid from the substrate solution. Papain had a specific activity of 120 PU/mg of protein under these conditions and thermolysin had specific activity of 1,220 PU/mg of protein.

**Leucine aminopeptidase.** Hog kidney leucine aminopeptidase type IV (Sigma Chemical Co. crystalline suspension, lot 122B-2810) was activated as described by Hill et al. (9). The suspension (200 μg) was dissolved in a solution containing 21.8 mM Tris buffer (pH 8.0) plus 1 mM MnCl₂ for 2 h at 40 C. The assay was as described by Roncari and Zuber (17) and employed leucine-p-nitroanilide as substrate in 21.8 mM Tris buffer (pH 8.0) plus 6 mM MgCl₂. The enzyme had 4.4 U of leucine aminopeptidase activity per mg of protein (1 U equals 1 μM leucine released per min). There was no protease activity in the aminopeptidase preparation as judged by the caseinolytic assay.

**Carboxypeptidase A.** Bovine pancreatic carboxypeptidase A was assayed as described by Folk and Schirmer (6), with hippuryl-L-phenylalanine as the substrate (0.001 M in 0.025 M Tris buffer [pH 7.5] plus 0.5 M NaCl). The enzyme had 16.7 carboxypeptidase units per mg and 0.113 PU/mg in the caseinolytic assay.

**Protein substrates.** The following protein substrates were employed in this study: bovine serum albumin, Pentex fraction V (Calbiochem); horse heart myoglobin (Sigma Chemical Co., lot 92C-7761-9); bovine achilles tendon collagen (Sigma, lot 112C-8200); swine skin gelatin, type I (Sigma, lot 32C-1810); ovalbumin, crystalline grade III (Sigma, lot 19B-8132); bovine blood fibrin, grade II (Sigma, lot 91C-1410); corn zein, grade II (Sigma, lot 39B-1270); egg white globulin (Sigma, lot 90C-80001); and bovine gamma globulin, Cohn fraction II (Sigma, lot 112C-1700).

Insoluble protein substrates (collagen, fibrin, zein) were washed prior to use by vacuum filtration and air dried. Gelatin suspensions were stirred at 60 C until all protein was dissolved and brought from gel to solution by shaking at 30 C prior to experiments.

**Conditions of protease induction.** The protease induction procedure has been described previously (5). Conidia of N. crassa 74A were inoculated into a medium consisting of 1% sucrose and Vogel salts (22). After 12 h of growth (mid-exponential growth phase), the cells were harvested by vacuum filtration and suspended in an amount of Vogel minimal salts medium equivalent to the volume of the original culture. The cells were then incubated at 30 C with aeration for 30 min to deplete the endogenous sugar pool. Cells treated in this fashion will be referred to as starved, exponential-phase cells throughout this communication. Protease induction of starved, exponential-phase cells was performed by mixing equivalent volumes of cells with twice the desired concentration of protein in Vogel salts, with both the cells and protein solution at 30 C. This results in a culture containing 0.5 to 1 mg (dry weight) of N. crassa cells per ml at the desired protein concentration. Activating protease was then added (from 0.03 to 0.16 PU/ml of culture) and the cells were then incubated at 30 C for the duration of an experiment. Protease in the culture filtrate was assayed as described previously. Under the conditions described here, both the neutral protease activity and the alkaline protease activity of Neurospora are being measured (Cohen et al., manuscript in preparation).

Over a period of weeks, it was noted that the rates (and thus the final levels) of protease synthesis were decreasing under the conditions of culture described here. This was found to be a function of the age of agar-butt stock used in initial culture preparation and of the number of times that a given stock of N. crassa 74A (obtained from the Fungal Genetic Stock Center, Humboldt, Calif.) was transferred from one agar butt to another. The reason for this variation is not known. However, when experiments were repeated with new stock cultures obtained from the Center and compared to previous experiments with older stock cultures, the effect of varying parameters was the same, regardless of the total activity produced by a given culture of carbon-starved, exponential-phase cells.
That is, the kinetic experiments (data as in Fig. 3), when performed with older stock cultures as the initial inocula, result in data giving the same dependence on thermolysin for both the initial rate of protease synthesis and the release of enzyme from cells. The second (fast) rate of synthesis is considerably slower, and these data are thus not used in this communication. After making these observations, we took the conidia employed in the preparation of carbon-starved, exponential-phase cells from agar butts that were less than 3 weeks old, and every 3 months a new fungal culture collection stock of N. crassa 74A was started. This resulted in cultures producing protease at rates that did not vary by more than 25% over a 3-month period. Variability of this type has been reported before (W. R. Wiley, personal communication).

RESULTS

Growth and protease biosynthesis in exponential-phase cells of N. crassa. Previous studies (5) have implied that the exocellular proteases produced from normally induced cells of N. crassa (cells grown from conidia on a medium containing protein as sole carbon source [5]) were involved in the induction of enzyme from starved, exponential-phase cells transferred to a medium containing protein as the sole carbon and energy source. When starved, exponential-phase cells were placed in a medium containing 1% BSA-salts alone, very little exocellular protease was produced over an 8-h period. If 0.06 PU of N. crassa exocellular protease per ml of culture was added to the cells, there was an immediate induction of proteolytic enzyme. This enzyme, produced by starved, exponential-phase cells was equivalent in its inducing ability to the normally induced enzyme. One other protease (the Bacillus thermodigynificus enzyme thermolysin) was almost equivalent to the Neurospora proteases; the mammalian proteases trypsin and chymotrypsin had little or no effect on the induction process.

Since the relationship of protease biosynthesis to cell growth has not been established, measurements of cell growth and protease levels in culture filtrate were performed. Thermolysin (0.06 PU/ml) was added to starved, exponential-phase cells in a medium containing BSA (1%) and salts. Induction of protease began after 1.5 to 2 h (Fig. 1). Two hours after protease reached a maximal rate of induction, growth of the cells reentered exponential phase. The specific activity of the induced protease (in terms of PU per milligram of cells) reached a constant value after initiation of growth and decreased after 3 h of exponential-phase growth.

It would appear that the rate of protease biosynthesis from starved, exponential-phase cells (in a 3- to 6-h interval) is considerably larger than that of normally induced cells in exponential growth (Table 1, rate expressed as PU per milliliter of culture per hour). However, when expressed in terms of specific activity change (PU per milligram of cells per hour), rates of protease synthesis in normal induction and in starved, exponential-phase cell induction are remarkably similar. The growth rates of the two types of culture differ to some extent, but this may be due to the high starting concentration of cells in the exponential-phase induction, resulting in an oxygen deficiency or depletion of some other nutrile in the medium.

The above experiment demonstrates that normal growth and protease induction appear to occur in starved, exponential-phase cells that have been induced to make exocellular protease by a protein substrate and added thermolysin. Furthermore, it would appear that growth in itself is not required for protease biosynthesis and that 4.0 PU/ml of cell filtrate protease will hydrolyze protein in quantity sufficient to provide amino acids for utilization as carbon and energy source.

Protease biosynthesis as a function of protein and protein concentration. Rate of protease synthesis appears to be proportional to
concentration of protein substrate (Fig. 2). There seems to be a threshold for response to the protein BSA over the range 0 to 0.4%, followed by a linear increase in rate with BSA concentration to 0.8%. Saturation of the cells with BSA appears to occur at 1% protein in the medium.

A number of protein substrates were compared to BSA in terms of their effectiveness as inducers of exocellular protease. Five concentrations of the test protein were employed in the induction process, and thus estimates of rates of protease induction as a function of protein concentration could be made. The proteins were then compared relative to BSA (Table 2).

**Table 1. Comparison of normal protease induction with starved, exponential-phase cell induction**

<table>
<thead>
<tr>
<th>Type culture</th>
<th>Rate</th>
<th>Doubling time of cells (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PU/ml</td>
<td>PU/mg of cells per h</td>
</tr>
<tr>
<td>Normally induceda</td>
<td>0.38</td>
<td>0.500</td>
</tr>
<tr>
<td>Carbon-starved, exponential-phase cells† induced with thermlysin</td>
<td>1.35</td>
<td>0.736</td>
</tr>
<tr>
<td>Carbon-starved, exponential-phase cells† induced with N. crassa exocellular protease ††</td>
<td>1.00</td>
<td></td>
</tr>
</tbody>
</table>

- † See reference 5.
- † Data determined from Fig. 1. Rates are for time interval 2.5 to 4.5 h.
- †† See reference 5. Specific activity was not determined, but at time of enzyme addition cell concentration was 0.7 mg of cell per ml.

**Fig. 2. Rate of protease induction from N. crassa as a function of concentration of the protein BSA.** A 50-ml volume of starved, exponential-phase cells was added to two times the given concentration of BSA. Six PU of thermlysin was added and the cells were incubated for 5 h at 30 C. Exocellular protease was assayed at 30-min intervals during the period of incubation, and rates of protease synthesis were determined for the 2- to 5-h interval.

**Table 2. Effect of protein substrates on protease induction from N. crassa***

<table>
<thead>
<tr>
<th>Protein</th>
<th>Conc (wt/wt) (%)</th>
<th>Rate in test protein/rate in BSA</th>
<th>Optimal protein* concn</th>
<th>Rate (PU/ml per h) at optimal concn</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myoglobin</td>
<td>0.2</td>
<td>1.19</td>
<td>0.46</td>
<td>1%</td>
</tr>
<tr>
<td>Collagen</td>
<td>0.6</td>
<td>0.92</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Collagen</td>
<td>1.0</td>
<td>0.46</td>
<td>1%</td>
<td>0.455</td>
</tr>
<tr>
<td>Gelatin</td>
<td>0.2</td>
<td>21.78</td>
<td>0.2%</td>
<td>0.92</td>
</tr>
<tr>
<td>Fibrin</td>
<td>0.6</td>
<td>2.25</td>
<td>0.6%</td>
<td>0.413</td>
</tr>
<tr>
<td>Zein</td>
<td>0.2</td>
<td>3.12</td>
<td>0.2%</td>
<td>0.981</td>
</tr>
<tr>
<td>Bovine Serum</td>
<td>0.6</td>
<td>0.42*</td>
<td>0.6%</td>
<td>0.413</td>
</tr>
<tr>
<td>Albumin</td>
<td>1.0</td>
<td>1.0*</td>
<td>1.2%</td>
<td>1.62</td>
</tr>
<tr>
<td>Ovalbumin</td>
<td>0.2</td>
<td>0*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Egg White Globulin</td>
<td>0.6</td>
<td>0*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bovine Gamma</td>
<td>0.2</td>
<td>0*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Globulin</td>
<td>0.6</td>
<td>0*</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>0*</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Thermlysin was added to a concentration of 0.06 PU/ml at initiation of culture. Protease assays were then performed at 30-min intervals for 5 h during culture.
* Concentration where greatest rate of protease induction observed.
* Average rate in three experiments.

At low concentrations (0.2 to 0.6%), myoglobin appears to be similar to BSA in terms of inducing ability. However, at the optimal concentration of myoglobin (1%), the rate was approximately half that observed at the same concentration of BSA. Curves of rate of protease synthesis versus myoglobin concentration illustrated a threshold effect for the protein similar to that observed for BSA.

Collagen, fibrin, and zein are insoluble proteins. *N. crassa* mycelia, cultured on these proteins as the sole carbon source, appear to "ball up" around the materials; that is, most of the mycelial strands appear to be associated with the particulate protein. This was most marked in the case of collagen, which was present in the medium as minced strips of approximately 0.5 by 1-cm linear dimension. Proteolysis of these insoluble substrates as a
function of time of culture was most apparent in the case of fibrin, where (after 5 h) little insoluble protein remained (initial protein concentration 0.2 to 0.6%).

As can be seen from the data, fibrin was an excellent inducer of protease at concentrations as low as 0.2%. No threshold value for fibrin concentration was observed, and rate of protease synthesis as a function of protein concentration decreased at higher fibrin levels. The optimal rate of protease biosynthesis achieved at 2% fibrin was almost equivalent to the rate of biosynthesis occurring at 1% BSA.

At low protein concentrations, corn zein appears to effect a greater degree of induction than BSA. However, at concentrations of zein greater than 0.6%, the rate of protease biosynthesis appears to decrease; at the optimum zein concentration, rate of protease induction is less than half that achieved on BSA. No threshold value for zein concentration was observed.

Of the insoluble proteins, collagen appears to be the poorest inducer. No protease biosynthesis occurs until collagen levels rise above 0.8%, and optimal collagen concentration appears to be above 1%. At this last concentration, protease biosynthesis occurs at a rate 17% that of the same concentration of BSA.

Four other soluble proteins were evaluated as inducers of exocellular protease. Three (ovalbumin, egg white globulin, bovine gamma globulin) were without effect in the induction of enzyme. Neither activating protease (thermolysin) nor induced Neurospora protease was significantly inhibited by these substrate proteins, so simple inhibition of newly synthesized proteases by these materials appears to be unlikely. In the case of ovalbumin, incubation under conditions of induction results in denaturation of the protein and formation of a flocculent precipitate which, unlike the other insoluble proteins employed in these experiments, floats on the surface of the culture. This physical separation of protein substrate from mycelia may be a factor in the inability of this protein to induce exocellular protease.

Gelatin appears to be an excellent inducer of exocellular protease from N. crassa. The rate of protease biosynthesis at 0.2% gelatin is equivalent to the rate of protease biosynthesis at 1% BSA. There appears to be no threshold concentration and there is little variance of rates for protease synthesis over the concentration interval 0.2 to 1%.

It would appear from these data that both soluble and insoluble proteins are capable of serving as inducers for exocellular protease biosynthesis from N. crassa. Globular-structured proteins such as BSA and myoglobin, as well as random-coiled proteins (under conditions of incubation) such as gelatin, appear to effect induction. Since all of the proteins employed in this study are digestible by either thermolysin or N. crassa exocellular protease, digestibility of the protein substrate may also not be a key factor in the induction process.

**Induction as a function of thermolysin concentration.** After the protein BSA was established as an effective inducer of protease biosynthesis, a series of experiments was performed in which BSA was held at an optimal concentration (1%) and thermolysin concentrations were varied over a range of 0 to 0.28 PU/ml of culture.

The kinetics of protease induction from carbon-starved, exponential-phase cells of N. crassa appears to be a function of the number of units of thermolysin added to the culture (Fig. 3). This has also been demonstrated for the normally induced N. crassa exocellular enzyme, although not to the same degree (5). By sampling protease activity in the culture filtrate at 10-min intervals over the first 150 min after

![Fig. 3. Kinetics of synthesis of exocellular protease by starved, log-phase cells of N. crassa with increasing concentrations of thermolysin. A 2,200-ml volume of 1% sucrose-Vogel salts was inoculated with conidia of N. crassa 74A, and the cultures were grown for 12 h at 30°C with aeration by shaking. The cells were then harvested by filtration, suspended in 2,200 ml of Vogel salts, and incubated for 30 min at 30°C. A 150-ml volume of cells at a concentration of 1 mg/ml was added to each of 14 flasks containing 150 ml of 2% BSA-Vogel salts, and a 100 μg/ml solution of thermolysin was added in amount sufficient to give the desired concentration of enzyme. Symbols: O, 0.026 PU/ml of culture; Δ, 0.092 PU/ml of culture; □, 0.26 PU/ml of culture.](http://jb.asm.org/)

thermolysin addition, we could resolve the kinetics of enzyme synthesis into three components.

The initial appearance of protease in the media (level of enzyme remaining constant in the 0- to 80-min interval) is not inhibited by cycloheximide and, thus, may not require protein biosynthesis. Whether this is leakage of intracellular protease to the milieu or a release of a protease bound externally to the cells by the added thermolysin is not known at this time. After this initial increase in exocellular protease, a slow linear increase in enzyme levels, followed by a rapid linear phase of enzyme synthesis, is observed. These two kinetic components of the induction process are inhibited completely by cycloheximide and, therefore, appear to require protein synthesis. Previous studies have shown that both neutral and alkaline protease are produced in these intervals (Cohen et al., manuscript in preparation).

The initial appearance of enzyme in the medium and the rate of the first (slow) synthesis of protease are simple functions of thermolysin concentration (Fig. 4), whereas the rate of the second (fast) synthesis of enzyme appears to be independent of thermolysin concentration (Fig. 4, inset). The onset of this second rate of protease biosynthesis is, however, a function of thermolysin concentration.

The rates of enzyme synthesis were determined from plots, derivative $dP/dt$ versus $t$ (time in minutes), and were the points at which rates reached plateau values. The initiation of the second rate of protease synthesis was also determined from these plots. It would appear that all of the thermolysin-dependent processes (release of enzyme from the cells, initial rate of protease synthesis, initiation of the second phase of protease synthesis) have approximately the same dependence on thermolysin concentrations, i.e., a limiting value for the phenomena is reached at thermolysin concentrations from 0.16 to 0.2 PU/ml of culture.

**Effect on protease induction of substitution of thermolysin by other proteases.** In addition to thermolysin, a number of proteolytic enzymes of bacterial, mammalian, and plant origin were employed as “activators” in the induction process. These studies were for the purpose of comparing proteases of differing specificity and origin to thermolysin. They involved fewer determinations of kinetics of protease biosynthesis as functions of added protease concentration and fewer times of assays during culture (assays performed at 30-min intervals during the course of induction). The range of enzyme concentrations employed, however, (from 0.04 to 0.240 PU/ml of culture medium) did allow determination of an approximate optimal concentration value for the added proteases. The number of assays performed during the interval of induction was sufficient for qualitative comparison of the kinetics of induction effected by the proteases listed in Table 3 versus thermolysin.

At optimal levels of added protease, all of the bacterial enzymes (Pronase, subtilisin, Clostridium histolyticum collagenase) were equivalent to thermolysin in terms of inducing ability (Table 3). With the exception of trypsin and carboxypeptidase, mammalian proteases and peptidases (chymotrypsin, elastase, leucine aminopeptidase) were without effect on the induction process. In addition, both trypsin and carboxypeptidase were poor inducers (Table 3). The inducing ability of carboxypeptidase may be due to the protease activity in the carboxypeptidase employed rather than to carboxypeptidase activity. Further purification of the carboxypeptidase used in this study would be required, however, to demonstrate this point clearly.

The plant protease papain was 59% as effec-
The enzymes capable of effecting induction (Pronase, subtilisin, collagenase, *N. crassa* normally induced protease) at levels approximately equivalent to those observed for thermolysin-activated induction were kinetically similar to thermolysin. That is, there was no added protease-dependent effect on the fast rate of protease biosynthesis, but rather an effect on the time (after addition of activating protease) of linear protease biosynthesis. The lag before linear protease biosynthesis occurs appears to be a function of the concentration of these enzymes. Regardless of concentration or type of bacterial enzyme employed, the linear (fast) rate of protease induction is the same, i.e., from 0.8 to 1.20 PU/ml per h. Given the limited intervals of protease assay during the course of induction, no statement can be made on the effects of the enzymes listed in Table 3 on the slow phase of protease biosynthesis.

Kinetically, papain, trypsin, and carboxypeptidase are qualitatively similar to thermolysin in the induction process. That is, they affect, in a concentration-dependent fashion, the time of onset of protease biosynthesis rather than the rate of synthesis. However, protease is synthesized at a low rate (0.3 to 0.5 PU/ml per h) relative to thermolysin.

Thermolysin is capable of serving in the induction process at 6.5-fold less concentration (in micrograms per milliliter of culture) than any of the other effective enzymes. Given this, and the low level of protease activity (0.04 to 0.08 PU/ml) needed for protease-dependent induction, thermolysin would appear to be the enzyme of choice in this experimental system.

**Effect of order of addition of protein substrate-activating protease on induction.** As demonstrated previously (5), protein substrate and added protease are essential for protease induction from starved, exponential-phase cells. Both of these agents appear to be necessary at the same time as demonstrated by the following experiment. Cells were incubated with thermolysin alone for a period of up to 90 min. After incubation, the cells were harvested by filtration, washed free of added protease, and then suspended in a medium containing the protein BSA. No protease appeared in the culture filtrates up to 6 h after the transfer (Fig. 5A). Another sample of starved, exponential-phase cells was incubated with 1% BSA for 90 min. The cells were then harvested by filtration, washed free of added protein, and added to a solution of Vogel salts plus 0.1 PU of thermolysin per ml of culture. No net synthesis of protease occurred (Fig. 5B). If cells were incubated for 90 min with either thermolysin alone or protein alone, followed by addition of the missing essential component, protease biosynthesis occurred (Fig. 5C). The low rates of protease biosynthesis in these cultures may be due to pronounced carbon starvation. The rates of protease synthesis observed in this experiment were similar to those recorded after 100 min of starvation. Indeed, changing the period of depletion for one of the inducing components results in curves of rate as a function of depletion similar to those generated by prolonged periods of carbon starvation prior to addition of both essentials for induction (protein and added protease [5]).

If cycloheximide, a known inhibitor of protein synthesis (19), is added to starved, exponential-phase cells at the time of their addition to a medium containing thermolysin plus a protein

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**Table 3. Effect of proteases on protease induction from *N. crassa***

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Protease units added per ml of culture</th>
<th>Enzyme (μg/ml)</th>
<th>Protease PU/ml at 5 h + enzyme/protease PU/ml at 5 h + thermolysin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pronase</td>
<td>0.080</td>
<td>0.26</td>
<td>0.970</td>
</tr>
<tr>
<td>Subtilisin</td>
<td>0.080</td>
<td>0.29</td>
<td>1.07</td>
</tr>
<tr>
<td>Elastase</td>
<td>0.235</td>
<td>1.0</td>
<td>0</td>
</tr>
<tr>
<td><em>C. histolyticum</em> collagenase</td>
<td>0.160</td>
<td>10</td>
<td>1.03</td>
</tr>
<tr>
<td>Leucine aminopeptidase</td>
<td>0</td>
<td>0.261</td>
<td>0</td>
</tr>
<tr>
<td>Papain</td>
<td>0.120</td>
<td>1.0</td>
<td>0.59</td>
</tr>
<tr>
<td>Carboxypeptidase</td>
<td>0.339</td>
<td>3.0</td>
<td>0.39</td>
</tr>
<tr>
<td>Chymotrypsin*</td>
<td>0.064</td>
<td>0.155</td>
<td>0</td>
</tr>
<tr>
<td>Trypsin*</td>
<td>0.064</td>
<td>0.160</td>
<td>0.28</td>
</tr>
<tr>
<td><em>N. crassa</em> normally induced enzymes</td>
<td>0.064</td>
<td>0.040</td>
<td>1.10</td>
</tr>
<tr>
<td>Thermolysin</td>
<td>0.064</td>
<td>1.0</td>
<td>0</td>
</tr>
</tbody>
</table>

*50 ml of starved, exponential-phase cells was added to 50 ml of 2% BSA in water. The desired amount of protease was added, and protease assays were done on the culture at 30-min intervals.

* Data from reference 5.
substrate, no protease is synthesized, although there appears to be some immediate release of enzyme from the cells. If cycloheximide is added after protease synthesis becomes linear with respect to time, protease secretion stops immediately (Fig. 6).

These last two experiments (effect of order of addition of components and effect of cycloheximide) imply that not only must the three conditions for induction be present (protein substrate, specific protease, protein synthesis) but all must be present at approximately the same point in time.

DISCUSSION

Protease biosynthesis in both Neurospora and Aspergillus appears to be a process contingent upon starvation for one or all of three main nutrilties derivable from protein (carbon, nitrogen, or sulfur). In one case, the process of biosynthesis is effected by induction (Neurospora [4, 5, 8]); in the other case, it is by derepression (Aspergillus [1–3]). Growth of cells in either case is not, per se, involved in the process. In fact, maximal rates of protease biosynthesis in Neurospora under conditions of carbon starvation appear to occur long before appreciable cell growth, with protease biosynthesis ceasing soon after growth begins. The same appears to be true in Aspergillus. These data would suggest that a selection procedure based upon the ability of a microbial species to grow at high rate on a protein substrate may not yield an organism capable of maximal protease biosynthesis in terms of final amounts of enzyme produced in a single culture. Rather, an organism capable of limited growth on a protein substrate may be more able to sustain maximal rates of protease biosynthesis over long periods of time. In point of fact, a series of experiments involving a Neurospora mutant defective in amino acid transport suggests that this is the case (H. Drucker, Abstr. Annu. Meet. Am. Soc. Microbiol. 1973, P33, p. 146).

Choice of protein substrate in the induction process appears to require empirical analyses. Earlier work from this laboratory on protease biosynthesis under conditions of carbon starvation (4) and work by Hanson and Marzluf (8) on protease induction under conditions of sulfur starvation have shown that amino acids and peptides do not effect induction and indeed, in the case of carbon-starved cells, repress protease biosynthesis in the presence of a protein

Fig. 5. Effect of the sequence of component addition on protease induction in N. crassa. (A) Starved, exponential-phase cells (50 ml, 1 mg of cells per ml) were incubated for 90 min with 0.1 PU of thermolysin per ml of Vogel salts. After incubation, the cells were harvested by filtration and suspended in 50 ml of 1% BSA-Vogel salts medium. Protease in cell filtrate was assayed at the intervals shown. (B) Starved, exponential-phase cells (50 ml, 1 mg of cells per ml) were incubated for 90 min with 1% BSA-Vogel salts. After incubation, the cells were harvested by filtration and suspended in Vogel salts (50 ml) containing 0.1 PU of thermolysin per ml of culture filtrate. Protease in cell filtrate was assayed at the intervals shown. (C) Starved, exponential-phase cells (50 ml, 1 mg of cells per ml) were incubated for 90 min in (D) 0.1 PU of thermolysin per ml of Vogel salts or in (E) 1% BSA-Vogel salts. After incubation, the cells were harvested by filtration and suspended in 50 ml of Vogel salts containing 1% BSA plus 0.1 PU of thermolysin per ml of medium.

Fig. 6. Effect of cycloheximide on protease induction from N. crassa. (A) Starved, exponential-phase cells (50 ml, 1 mg of cells per ml) were added to 50 ml of 2% BSA-Vogel salts. At zero time, 0.2 ml of a 2 mg/ml solution of cycloheximide was added to the culture, resulting in a culture containing 4 μg of cycloheximide per ml. Exocellular protease was assayed in the culture filtrates at the times indicated. (B) Starved, log-phase cells (50 ml, 1 mg of cells per ml) were added to 50 ml of 2% BSA-Vogel salts. After 5 h of culture, 0.2 ml of a 2 mg/ml solution of cycloheximide was added to the culture, resulting in a culture containing 4 μg of cycloheximide per ml. Exocellular protease was assayed in the culture filtrates at the times indicated.
inducer. Although Hanson and Marzluf expressed protein effect in terms of amount of enzyme produced after 20 h, rather than in terms of rate and optimal protein concentration as in this study, the relative effects of myoglobin and BSA reported in their study are similar to those reported here. Since BSA can be sterilized easily and Neurospora cells appear to grow fairly homogeneously in BSA-containing medium, it has been chosen as the substrate for further studies on the induction process.

For maximal protease biosynthesis under conditions of carbon starvation, enzymes of bacterial or fungal origin appear to be necessary. These enzymes have specificity for hydrophobic amino acids located at either the N terminus of a peptide bond, as is the case for the neutral protease thermolysin (13), or at the C terminus of a peptide bond, as is the case for the alkaline protease subtilisin (14). The specificity of these enzymes cannot, of itself, explain their effectiveness in the induction process. Neither chymotrypsin (18) nor elastase (15), which are of somewhat similar specificity, are capable of effecting induction. This would imply that factors other than catalytic activity of the activating protease per se may be involved in the induction process.

The induction of exocellular protease from carbon-starved cells of N. crassa does not (unfortunately) appear to be a simple process. Any model attempting to explain the process must consider the role and/or existence of the following: (i) the relative specificity of “activating” proteases; (ii) the relative lack of specific properties for protein inducers; (iii) the requirement for both protease “activator” and protein substrate; and (iv) the complex kinetics of the process. In particular, the fact that all kinetic components have the same reliance on concentration of activating protease implies some type of relationship between these kinetic events.

Studies in progress at this time will, we hope, lead to the delineation of a mechanistic model incorporating and resolving these riddles.

ACKNOWLEDGMENTS

I would like to thank Louise Neil for her excellent technical assistance and Dvara-Lee Felton for her editorial assistance. This work was done by Battelle, Pacific Northwest Laboratories, for the U.S. Atomic Energy Commission under contract AT(45-1)-1830.

LITERATURE CITED