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Regulation of Exocellular Proteases in *Neurospora crassa*: Role of *Neurospora* Proteases in Induction

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Cells of *Neurospora crassa* strain 74A, grown on sucrose for 12 h and transferred to a medium containing protein as sole carbon source, would not produce exocellular protease in significant amounts. When a filtrate from a culture induced to make protease by normal growth on a medium containing protein as principal carbon source was added to an exponential-phase culture in protein medium, exocellular protease was made in amounts similar to those made during normal induction. The material in the culture filtrate that participated in the induction process was identified as protease by its heat lability, molecular weight, and the dependence of induction rate on units of proteolytic activity added to the exponential-phase culture. Induction of the formation of exocellular protease by exponential-phase cells appears to require a protein substrate, added proteolytic activity, and protein synthesis. The protease produced by induced exponential-phase cells was as efficient in promoting induction as normally induced enzyme, whereas constitutive intracellular enzyme was only 50% as efficient. The bacterial protease thermolysin was able to induce exocellular protease at 90.7% of the rate observed with added *N. crassa* exocellular protease.

A recent series of experiments (1) has demonstrated the ability of *Neurospora crassa* to grow on a protein as principal carbon source with the concomitant production of exocellular proteases. These enzymes were shown to be inducible by the protein carbon source, unlike the constitutive proteases reported in *Pseudomonas* and *Arthrobacter* (4, 7). Furthermore, in the presence of easily metabolizable carbon sources such as sugars, amino acids, or peptides (in addition to protein), the synthesis of exocellular protease is repressed. Synthesis of protease by *Neurospora* has been reported previously by Matile et al. (6).

This early phase of the study used conidia of *Neurospora* inoculated into medium containing a protein carbon source. Because protease synthesis started during the early exponential-phase of growth, the initial events of induction could not be analyzed due to the relatively small numbers of vegetative cells found during this period and the small amount of protease that is, therefore, produced. The inducing substrate in this case is a macromolecule which should not be capable of entering the cell intact (8), which implies that the early events of induction, before transportable amino acids and peptides are produced, are undoubtedly the most crucial steps in the process and, as such, should be analyzed in depth. The work described in this communication deals with the early phases of exocellular protease synthesis and may be useful in developing a model for the regulation of early steps in this induction process.

MATERIALS AND METHODS

Preparation of starved exponential-phase cells of Neurospora. Conidia of *N. crassa* strain 74A were inoculated into a medium consisting of 1% sucrose and Vogel minimal salts (11) as described by Turner and Matchett (10). After 12 h of growth (mid-exponential phase), the cells were harvested by filtration and suspended in an amount of Vogel minimal salts medium equivalent to the volume of the original cultures. The suspended cells were then incubated at 30 C, with aeration for 30 min, to deplete the endogenous sugar pool. Cells of this type will be referred to as starved exponential-phase cells throughout this communication. Protease induction of starved exponential-phase cells was performed in a culture medium containing 2% bovine serum albumin (BSA; Pentex fraction V) in Vogel salts. A volume of cells was mixed with an equivalent volume of the induction medium,

both maintained at 30 C, resulting in a culture containing 0.5 to 1 mg of *N. crassa* cells per ml in 1% BSA.

Preparation of N. crassa exocellular proteolytic activity. Conidia of *N. crassa* strain 74A were inoculated into a medium consisting of 0.1% sucrose, 1% BSA, and Vogel minimal salts. The cells were allowed to grow for 12 h at 30 C, and culture filtrate containing protease (2-3 PU/ml; for definition of PU, see below) was obtained by filtration of the culture. This material was used without further purification. Conditions for production of proteolytic activity by this method and a partial characterization of the enzyme mixture have been described previously (1). Cells of this type will be referred to as normally induced cells. This is an operational definition and does not imply that the enzyme induced from starved exponential-phase cells is different in any respect from that of normally induced cells. They are identical, as will be seen, with respect to the criterion investigated in this communication.

Preparation of other proteolytic enzymes. A solution of thermolysin was prepared by addition of 10 mg of 3× crystals (A grade, Calbiochem) to 5 ml of 0.02 M $CaCl_2$. The crystals were dissolved (2) by raising the pH of the suspension to 11.3 by addition of 0.1 N NaOH (approximately 0.8 ml). The pH of the solution was then lowered to 7.0 by addition of 0.1 N HCl. A 1.0-ml amount of 0.1 M tris(hydroxymethyl)aminomethane (Tris) (pH 8.0) plus 0.02 M $CaCl_2$ was added, and the solution was made to 10 ml with distilled water. Solutions containing 100 μg of enzyme per ml in 0.01 M Tris, pH 8.0, plus 0.002 M $CaCl_2$ were used in experiments requiring thermolysin addition. The thermolysin used in these studies had a specific activity of 1,370 PU/mg.

Trypsin and chymotrypsin (Calbiochem A grade, bovine pancreas) were dissolved in 0.01 M Tris, pH 8.0, plus 0.002 M $CaCl_2$. Solutions containing 100 μg of enzyme per ml were assayed for proteolytic activity and contained 412 PU of enzyme per mg of trypsin and 409 PU of enzyme per mg of chymotrypsin.

Assay of proteolytic activity. The assay method of McDonald and Chen (5) as utilized previously (1) was employed. One unit of activity (PU) is defined as that amount of enzyme which produces one microgram equivalent of non-trichloroacetic acid-precipitable tyrosine per minute by action on casein.

Preparation of extracts of N. crassa cells. Crude extracts of starved exponential-phase cells and normally induced cells were prepared from ground lyophilized organisms (10). The ground cells were extracted with 0.1 M Tris, pH 8.0, plus 0.002 M $CaCl_2$, and the extract fraction was separated from the pellet by centrifugation at 9,750 × *g* for 30 min at 3 C. The yellow-brown crude extract was used directly in the assay for proteolytic activity. The pellet was resuspended in 2 ml of 0.1 M Tris, pH 8.0, plus 0.002 M $CaCl_2$ and washed by centrifugation. This was repeated, and the washed pellet was then suspended in 1 ml of Tris buffer and assayed for protease.

Sephadex chromatography of cell-free filtrate. A column of Sephadex G-25 (1 by 30 cm) washed with 0.01 M Tris, pH 8.0, plus 0.002 M $CaCl_2$ was calibrated for void volume with 2 ml of blue Dextran 2000 (Pharmacia). A 2-ml sample of exocellular filtrate from normally induced cells containing protease activity equivalent to 1.55 PU/ml was placed on the column, and the sample was eluted with 0.01 M Tris, pH 8.0, plus 0.002 M $CaCl_2$. A fraction emerging in the void volume contained 100% of the original proteolytic activity. Fractions equivalent to two and three times void volume were also collected.

RESULTS

When starved exponential-phase cells are dispersed in a medium in which the sole carbon source is the protein bovine serum albumin, they produce little exocellular proteolytic enzyme through a test period of 8 h (Fig. 1). If culture filtrate from normally induced cells is added to starved exponential-phase cells at the time of their addition to BSA-containing medium, there is an immediate appearance of proteolytic activity in the culture filtrates, and the amount of this activity increases linearly for 6 h (Fig. 1). At 6 h, protease synthesis appears to stop, but the level of activity in the medium is equivalent to the highest levels of protease found in normally induced cells after 14 to 16 h of growth (1).

The amount of protease produced from starved exponential-phase cells at 4 h, and thus the rate of enzyme production, is dependent upon the amount of protease present in the filtrate from normally induced cells (Fig. 2). Addition of boiled filtrate (clotted protein not removed) to starved exponential-phase cells in the presence of BSA results in no induction of exocellular protease regardless of the amount of material added. The boiled filtrate is void of proteolytic activity (Table 1). Induction of enzyme from starved exponential-phase cells appears to require protein substrate, added proteolytic activity, and protein synthesis (Table 1), since addition of normally induced culture filtrate to starved exponential-phase cells in the absence of protein substrate does not result in enzyme induction. Cycloheximide, a known inhibitor of protein synthesis, in the presence of all other components of the inducing system prevents synthesis of exocellular protease from starved exponential-phase cells.

Proteolytic activity from starved exponential-phase cells induced for protease synthesis was equivalent to proteolytic activity from normally induced cells in its ability to serve as an inducer of enzyme (Table 2). Intracellular enzyme from normally induced cells and induced starved exponential-phase cells is identical in specific activity. This is a constitutive activity (1) and does not depend upon growth conditions. Addi-

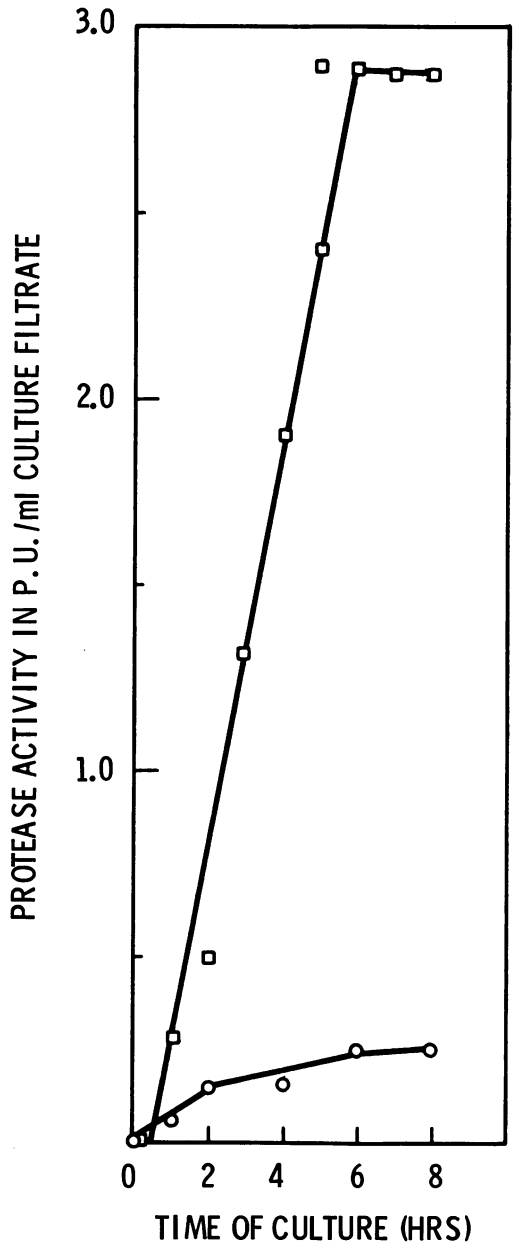

FIG. 1. *Induction of exocellular protease synthesis in starved exponential-phase cells of N. crassa by addition of culture filtrate of normally induced cells. Starved exponential-phase cells were prepared as described in Materials and Methods. A 25-ml amount of such a cell suspension was mixed with 25 ml of Vogel salts containing 2% BSA. At the intervals shown, 5-ml fractions of cell suspension were removed from the culture and filtered. The cell-free filtrate was assayed for proteolytic activity. Symbols: O, protease activity from starved exponential-phase culture receiving no additions; □, protease activity from culture filtrate from culture of starved exponential-phase cells to which 2 ml of culture filtrate from normally induced cells containing 3.0 PU of protease activity per ml was added. Protease produced is corrected for this amount of enzyme.*

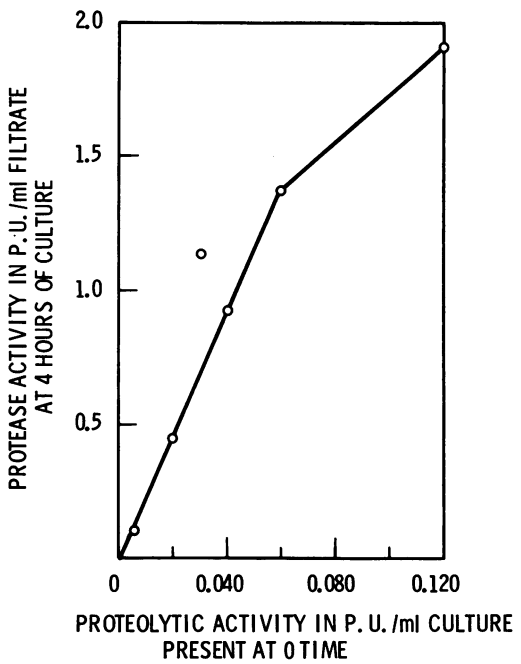

FIG. 2. *Protease induction from starved exponential-phase cells as a function of units of normally induced N. crassa exocellular protease added. A 25-ml amount of exponential-phase cells prepared as described in Fig. 1 was added to 25 ml of 2% BSA-Vogel salts. Normally induced exocellular protease of N. crassa containing 3.0 PU/ml of culture filtrate was added to the cells in an amount sufficient to give the indicated amount of proteolytic activity per milliliter of experimental culture. At 4 h, 5-ml samples were removed and assayed for protease.*

tion of intracellular protease from either type of culture induces synthesis of exocellular protease. The rate of production of induced enzyme per unit of proteolytic activity was, however, approximately 50% less than that observed when exocellular enzyme was employed (Table 2).

On the basis of heat stability and the dependence of induction rate on concentration of added proteolytic activity, we have assumed that the active material in culture filtrate from normally induced cells, induced starved exponential-phase cells, and cell extract is proteolytic enzyme. Further support for this hypothesis was obtained by chromatography of normally induced culture filtrate on Sephadex G-25. The

TABLE 1. *Requirements for synthesis of exocellular protease from starved exponential-phase cells of N. crassa*

Medium	Protease in PU/ml at 4 h of culture
Complete[a]	1.8
– Bovine serum albumin	0
– Cell filtrate of normally induced cells	0.2
1% BSA + boiled normally induced filtrate	0.2
Complete + 2 μg of cycloheximide per ml	0

[a] Complete medium contains 1% bovine serum albumin + 6.5 PU of added *N. crassa* exocellular protease from culture filtrate of normally induced cells. Preparation of culture is described in text.

TABLE 2. *Rates of exocellular protease induction by intracellular and exocellular protease of N. crassa*[a]

Source of enzyme	Units of protease added to culture	Rate of protease synthesis in starved exponential-phase cultures[b] (in PU/ml/h)
Normally induced exocellular enzyme[c]	6.5	1.0
Intracellular enzyme from normally induced cells[c]	6.5	0.50
Induced starved exponential-phase exocellular enzyme[d]	6.5	1.2
Intracellular enzyme from induced starved exponential-phase cells[d]	6.5	0.40

[a] Samples of intracellular enzyme and exocellular enzyme were added in amounts shown to 50 ml of medium containing 2% BSA-Vogel salts, and 50 ml of exponential-phase cells were added. Cells were then incubated at 30 C as previously described.

[b] Protease production was measured for 0 to 5 h, and this is rate of production in the time interval 2 to 4 h.

[c] Exocellular enzyme was culture filtrate from normally induced cells of *N. crassa* strain 74-A. Intracellular enzyme was extracted from normally induced cells as described in text.

[d] Exocellular enzyme was culture filtrate from induced starved exponential-phase cells after 6 h of culture. Intracellular enzyme was extracted from these cells as described in text.

only chromatographic fraction capable of inducing protease from starved exponential-phase cells was the fraction appearing in the void volume consisting of high-molecular-weight material containing all of the added proteolytic activity. Fractions appearing in 2 and 3× void volume, which possessed no proteolytic activity, did not induce protease (Fig. 3). Fractions of boiled normally induced culture filtrate prepared in the same fashion were void of protease-inducing ability.

If *Neurospora* protease is the agent responsible for induction of proteolytic activity from exponential-phase cells of *Neurospora*, it is possible that other proteolytic enzymes may be able to perform the same function. Equivalent amounts in terms of units of proteolytic activity of the mammalian proteases trypsin and chymotrypsin and the *Bacillus thermoproteolyticus* protease thermolysin were added to exponential-phase cells of *N. crassa* in a protein-containing medium (Table 3). The bacterial protease thermolysin was almost identical to *Neurospora*'s own exocellular protease in stimulating the induction of proteolytic activity. The mammalian protease trypsin had less than 30% of the inducing ability of *Neurospora* protease

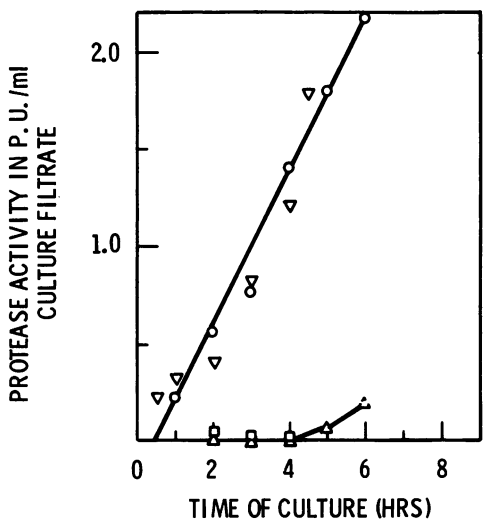

FIG. 3. *Results of Sephadex fractionation of culture filtrate from normally induced cells of N. crassa strain 74-A. Culture filtrate was chromatographed on Sephadex G-25 as described in Materials and Methods. Material before fractionation had 1.55 PU/ml, and sample emerging in void volume of column had 1.04 PU/ml. Total protease recovered in void volume was 99% of starting material. Sephadex fractions were added to 50 ml of 2% BSA-Vogel salts immediately before addition of 50 ml of exponential-phase cells. Proteolytic activity was measured at the intervals shown. Symbols: O, addition of one ml of untreated normally induced culture filtrate; ∇, addition of 2 ml of Sephadex void volume fraction; Δ, addition of fraction emerging at 2× void volume and containing no protease activity; -□-, no addition to culture.*

TABLE 3. *Substitution of N. crassa protease in the induction process by other proteases*[a]

Enzyme added	Protease added (PU/ml of culture)	μg of enzyme/ml of culture	Enzyme[b] (μM)	Protease synthesis[c] in PU/ml/h	*N. crassa* control (%)
N. crassa normally induced exocellular protease	0.064			1.00	100
Thermolysin[c]	0.064	0.040	1.2×10^{-3}	0.907	90.7
Trypsin[c]	0.064	0.160	6.7×10^{-3}	0.277	28
Chymotrypsin[c]	0.064	0.155	6.6×10^{-3}	0	0

[a] Starved exponential phase cells (100 ml) were added to 100 ml of 2% BSA-Vogel salts and equivalent amounts (in terms of PU/ml) of the proteases were added to each culture. Solutions of trypsin, chymotrypsin, and thermolysin at a concentration of 100 μg/ml were employed. The culture filtrate of normally induced *N. crassa* cells contained 2 PU/ml.

[b] Based on molecular weights of 34,800 for thermolysin (9), 24,000 for trypsin (12), and 24,800 for chymotrypsin (3).

[c] Rate of enzyme synthesis in the interval 2 to 6 h of induction.

and chymotrypsin was without effect. It is of interest to note the very small amounts of thermolysin (1.2×10^{-9} M) required to achieve induction of protease from *Neurospora*.

To eliminate the possibility that gross cell disruption plays a role in the observed enzyme induction, electron micrographs of starved exponential-phase cells before and after 6 h of incubation in the BSA-containing medium, plus added proteolytic activity, were made. There was no evidence of gross cell disruption. (We are indebted to James Hampton and Roy Adee of this laboratory for the electron microscopy.)

It is conceivable that starved exponential-phase cells, even though apparently intact and not lysed by the *N. crassa* proteases, may "leak" some form of inactive intracellular protease into the medium which may be activated by the added proteases. It is also possible that the cell pellet may contain inactive proteases which, when acted upon by the added enzyme activity, would release active protease into the medium.

To test these possibilities, crude extracts of starved exponential-phase cells were prepared from cells prior to their addition to medium containing protein and added proteolytic activity and 5 h after incubation in the medium plus protease. Proteolytic activity from the culture filtrate of normally induced cells was added to both crude extract and pellet fraction of the samples. Protease was measured before and after addition of enzyme, and in all cases (Table 4) there was no significant increase over the sum of proteolytic activities in the samples containing added protease.

The period of starvation used throughout this study was shown to be optimal by the following experiment. Cells were grown on 1% sucrose as described in Materials and Methods and suspended in Vogel salts medium. The protein substrate and *N. crassa* exocellular protease were immediately added to one 50-ml sample of cells. Five other 50-ml samples were placed at 30 C and allowed to starve. At 30-min intervals for 3 h, samples were removed and bovine serum

TABLE 4. *Effect of normally induced exocellular protease of N. crassa on protease activity of cell fractions*[a]

Fraction[b]	Protease activity in PU/ml	Observed sum of activity in fraction + exocellular protease[c]	Calculated sum of activity in fraction + added exocellular protease	Observed/calculated × 100
Crude extract, 0 time	2.575	3.364	5.046	67
Pellet from extraction, 0 time	0.272	3.191	2.74	116
Crude extract, 5 h	2.717	4.000	5.188	77
Pellet from extraction, 5 h	0.545	3.414	3.016	113

[a] A 150-ml amount of starved log-phase cells was added to 150 ml of 2% BSA; 7.4 PU of N. crassa exocellular protease was added to the culture; and immediately after this addition, 150 ml of cell slurry was harvested by filtration and lyophilized. The remaining 150 ml was harvested at 5 h and lyophilized.

[b] An 85-mg amount of 0 to 5-h lyophilized cultured cells was extracted as described in the text, and soluble and insoluble centrifuged fractions prepared.

[c] Exocellular protease (2.47 PU) was added to the fractions.

albumin plus *N. crassa* exocellular protease were added to one of the starved-cell flasks. The rates of protease induction from the cells were measured, and protease induction appeared to be optimal after 30 min of starvation (Fig. 4). At zero time of starvation, there appeared to be a slower rate of induction. Since previous work had shown that sugars repress protease biosynthesis (1), this could be due to a large pool of intracellular sugar present at this interval. After 1 h of starvation, the rate of protease induction fell markedly, reaching a level of ½ of the 30-min rate after 2.5 h of starvation.

DISCUSSION

The data presented here show that starved exponential-phase cells of *N. crassa*, when transferred to a medium containing a protein substrate, are not capable of immediate synthesis of exocellular protease unless proteolytic activity is added to the culture. Culture filtrates from normally induced cells and induced starved exponential-phase cells were employed as the source of inducing activity with equal effect. The agent in the filtrate responsible for induction would appear to be proteolytic enzyme for the following reasons. (i) It is of high molecular weight; (ii) it is heat labile; (iii) induction rate is proportional to the amount of proteolytic activity present in added culture filtrates; and (iv) a pure proteolytic enzyme of defined specificity can substitute with almost equal efficiency for the culture filtrate from normally induced cells.

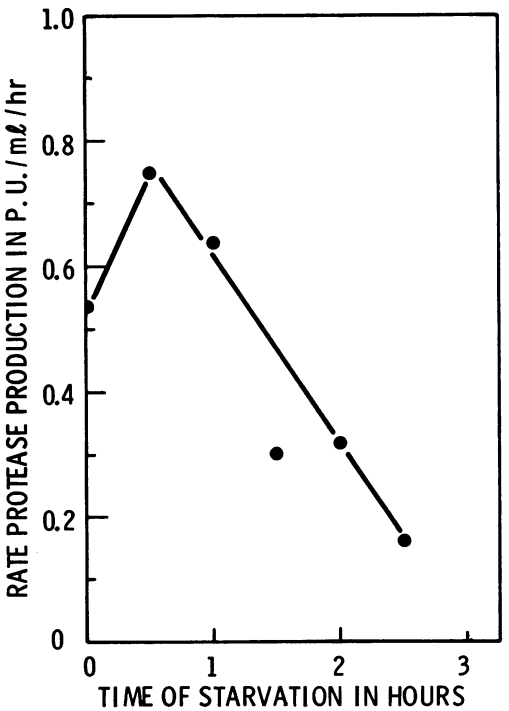

FIG. 4. *Induction of exocellular protease synthesis in exponential-phase cells of N. crassa as a function of period of starvation. Exponential-phase cells were prepared as described in Materials and Methods. A 50-ml amount of cell suspension was incubated in Vogel salts medium in the absence of carbon source for the intervals shown. As a function of time, 50 ml of 2% BSA and 4 ml of culture filtrate containing 3 PU of protease activity per ml from normally induced cells were added to the starved cells. Protease activity was measured after additions, and the rates of protease biosynthesis in the interval 2 to 8 h were determined.*

If we assume that induction of exocellular protease involves the action of protease, as is suggested by our data, a number of working models can be suggested.

It is possible that the added protease causes hydrolysis of the protein substrate and that the hydrolysis products (peptides and amino acids) serve as relatively nonspecific inducers for exocellular protease. This possibility appears unlikely for at least three reasons. (i) The amount of protease added is insufficient to cause any large scale hydrolysis of BSA within the time period of the first observed increase in protease. (ii) There is an element of specificity for added protease. Intracellular enzyme is 50% as efficient as induced exocellular enzyme in the induction processes; thermolysin is as efficient, and the mammalian proteases trypsin and chymotrypsin appear to have little (trypsin) or no (chymotrypsin) inducing ability. (iii) Peptide mixtures (tryptone, peptone) are not capable of inducing protease and indeed are excellent repressors of protease biosynthesis (1).

It may be argued that the exocellular proteases and thermolysin produce some peptide of unique properties by virtue of their specificity. No direct argument against this possibility can be made at this time, but this would mean that a similar peptide would be derived from any protein substrate capable of inducing protease. On an intuitive basis this would appear unlikely. Minimally, a model of this sort would suggest that protein substrates of different amino acid composition, and thus different sequence, would differ in terms of the kinetics of induction for protease. *Neurospora* is capable of growth on casein (unpublished observation) with identical doubling times and protease production, suggesting that specificity of added protease at the level of the protein substrate is not a factor in the induction process.

The observations recorded here might not involve induction per se, i.e., recognition of a

substrate by the cell and synthesis of enzymes specific for degradation of that substrate. Rather, the added enzymes might cause cell disruption over a period of time. If this were the case, protein synthesis might not be involved in enzyme production, and there might be some cytological signs of cell damage. Electron microscopy shows no evidence of cell disruption, and addition of cycloheximide to starved exponential-phase cells under inducing conditions prevents appearance of protease. In this same vein, one could postulate that the cells are continually leaking inactive protease from wall, membrane, or cytoplasm, which is activated by added enzyme. This is not the case since no material capable of activation to protease was found either in cell extracts or cell particulate fraction.

In the induction of enzymes for substrates capable of entering cellular cytoplasm, regulation is considered to involve some type of interaction between substrate and genetic material. The presence of a substrate is essential for the production of exocellular protease in *Neurospora*, but this substrate is incapable of transport into the cells, and thus, in itself, is incapable of effecting induction. A macromolecule, most probably a protease, produced by *Neurospora* during normal induction is, in addition to protein substrate, required to achieve a physiological rate of exocellular protease synthesis. The mechanism of this apparently autocatalytic phenomenon is not understood at this time, but its existence is worth further examination.

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