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Extracellular Acid Proteases from *Neurospora crassa*

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Three electrophoretically distinct acid proteases appear in culture filtrates of *Neurospora crassa*. Like the previously investigated alkaline and neutral proteases, these enzymes require induction by an exogenous protein. But in contrast to alkaline and neutral proteases, which are synthesized and secreted in response to limitation of any one of three nutrilites (carbon, nitrogen or sulfur), extracellular elaboration of the acidic proteases is more specifically a function of the missing nutrilite. AcP, a pepstatin-inhibitable enzyme similar to other fungal carboxyl proteases, was secreted in large amounts when protein was the sole source of sulfur. Only trace amounts were secreted when nitrogen was the limiting nutrilite, and it was undetectable under carbon limitation. M-1, a chelator-sensitive protease, was secreted when nitrogen or carbon was limiting. M-2, also chelator sensitive, was present only when nitrogen or sulfur was limiting. The evidence presented suggests that the differential regulation of the acidic proteases with respect to nutrilite deprivation may not occur at the level of transcription. AcP and M-2 were partially purified from nitrogen-derepressed cultures by ultrafiltration, cation-exchange chromatography, and gel filtration. AcP has a molecular weight of 66,000, is stable from pH 3.0 to 6.0, and is optimally active toward bovine serum albumin at pH 4.0. M-2 has a molecular weight of 18,000, is stable from pH 1.6 to 5.5, and has optimal activity at pH 4.5.

Neurospora crassa has been used in the study of eucaryotic regulation and has been examined previously relative to the regulation of extracellular protease biosynthesis. This process is of interest because synthesis requires both catabolite derepression and substrate induction. Induction is effected by the addition of an exogenous protein to the growth medium (7, 10), but the mechanisms of the inductive process are not known. Derepression of gene expression can be effected by limiting any one of the three nutrilites derivable from protein substrate, carbon, nitrogen, or sulfur (6, 11). The metabolic requirements of this process have been well studied (5, 8, 9), showing that the same two proteases, one alkaline and one neutral, are synthesized and secreted in all three cases. Evidence indicates a cross-pathway regulation in which three positive regulatory genes (one each for C, N, and S) control the protease structural genes. Two of the regulatory genes have been identified: the *nit-2* gene for N and the *cys-3* gene for S (11).

The existence of extracellular acid protease activity has been predicted from pH-stability profiles of *N. crassa* crude culture filtrates (7), but until now the enzymes have not been described. An intracellular acid protease has been reported (18), but it was produced under conditions of growth that preclude comparison with any inducible extracellular proteases. One would assume that production of extracellular acid protease would be controlled coordinately with that of alkaline and neutral proteases. The data presented here do not support this assumption. In this communication we report preliminary observations of the regulatory requirements and the properties of *N. crassa* extracellular acid proteases.

MATERIALS AND METHODS

Growth of organism. *N. crassa* (wild-type strain 74A [FGSC 262]) was maintained and harvested as described by Turner and Matchett (21). Media were prepared, and midexponential-growth-phase mycelia were transferred to the desired medium as described previously (6). Induction was effected by the addition of 1% bovine serum albumin (BSA; Miles Pentex; fraction V, fatty acid poor), and derepression was accomplished by the omission of a particular nutrilite (C, N, or S) from Vogel minimal medium (22). Thermolysin was added to C-limited cells to effect induction (8). Culture filtrates were sampled, filtered through Whatman no. 42 paper, and stored at 0°C until assayed.

Cellulose acetate electrophoresis. Electrophoresis and visualization of protease activity were carried out

by the method of Cohen (2), with the following modifications. Samples (3.0 μl) were spotted 3.1 cm from the cathode end of a cellulose acetate strip (78 by 150 mm; Shandon Southern Instruments, Inc.). Samples were allowed to migrate for 40 min at a constant current of 4.2 mA in 0.06 M Tris and 0.32 M boric acid, pH 7.0. The cellulose acetate was placed on glass plates containing 1% agar and 1% skim milk as substrate, previously adjusted to desired pH with 0.1 M buffer (succinate or Tris). The plates were incubated at 50°C for 1 h and stained for protein with naphthol blue black. Protease activity resulted in clearing zones on the plate.

Protease assays. Acidic protease activity was determined with a modification of Schwabe's (17) fluorescamine assay. The reaction mixture contained the following: 0.5 ml of 1% BSA in 0.1 M citrate (pH 4.0) for AcP (acid protease) and 0.1 M acetate (pH 4.5) for M-2; an appropriate sample size (containing, at most, 0.1 unit of activity for AcP or 0.01 unit for M-2); and an amount of buffer to bring the final volume to 0.625 ml. Controls were immediately precipitated with 0.375 ml of 10.7% trichloroacetic acid; experimentals were incubated for 1 h at 37°C before being precipitated. After centrifugation, samples from the supernatant fluid were treated with fluorescamine and read on a spectrofluorometer, as described previously (14). One unit of activity (Lu) is defined as the amount of enzyme that produces trichloroacetic acid-soluble free amines equivalent to 1 μmol of L-leucyl-L-leucine per h by action on BSA at 37°C. Pepstatin-inhibitable activity was determined by treating samples with pepstatin (1/10 with 10^{-3} M in 95% ethanol) or with equivalent volumes of ethanol and incubating on ice for 1 h before assaying at pH 3.4. The difference between the activity of pepstatin-treated samples and that of alcohol-treated samples was defined as pepstatin-inhibitable activity. For alkaline protease activity, the succinylated casein assay described previously was used (14).

Partial purification of AcP and M-2. Twelve liters of culture filtrate from nitrogen-starved, gelatin-induced mycelium was obtained as described previously (14). Sodium azide (0.02%) was added to the filtrate as a preservative, and the pH was lowered to 3.4 by the addition of 1 M citric acid. The preparation was maintained at 4°C for the remainder of the purification. The filtrate was concentrated to ~300 ml on a Pellicon cassette ultrafiltration system (Millipore Corp.) with a 10,000-molecular-weight limit. The concentrate was dialyzed against 0.01 M citrate (pH 3.4) containing 0.02% sodium azide. It was then applied to a carboxymethyl cellulose column (2.5 by 16 cm; Whatman CM52) that had been equilibrated with the same buffer. After washing with 0.05 M KCl (in buffer), the column was eluted with a 500-ml, 0.05 to 0.15 M KCl gradient. Two peaks of activity (at pH 5.0) were eluted. The electrophoretic assay and the use of inhibitors showed that the first peak contained AcP and the second peak contained M-2. The fractions from each peak were pooled separately, concentrated to ~10 ml on a Diaflo PM-10 membrane, and applied to a Sephadex G-75 column (1.6 by 100 cm). The resultant peak of activity from each chromatograph was pooled and used for characterization.

Enzyme characterizations. Enzyme preparations were treated with known protease inhibitors, incubated on ice for 1 h, and assayed for remaining activity. The inhibitors used (and final concentrations) were phenylmethylsulfonyl fluoride (2 mM), EDTA (3 mM), pepstatin (0.1 mM), *p*-chloromercuribenzoate (1 mM), 2-mercaptoethanol (5 mM), and *o*-phenanthroline (55 mM).

The pH optima of AcP and M-2 were determined by performing the standard assay at several pH levels. For pH stability, enzyme preparations were diluted in 0.1 M buffer, allowed to stand at room temperature for 24 h, and assayed for remaining activity. Buffers used were: citrate (pH 1 to 6) and phosphate (pH 5.5 to 7.5) for AcP and acetate (pH 1 to 6) and phosphate (pH 5.5 to 7.5) for M-2.

Preparation of extracts. Mycelia were collected by filtration and immediately frozen on dry ice. After lyophilization and grinding (21), the powder was extracted for 2 h on ice with 0.025 M succinate (pH 4.5), 0.002 M $CaCl_2$, and 0.02% sodium azide (1 ml/45 mg of cells). The extract was centrifuged at 4°C for 20 min at 15,000 rpm, and the supernatant was collected.

RESULTS

Identification of the proteases. Alkaline and neutral proteases are secreted when exponential-phase mycelia of *N. crassa* are transferred to media containing protein inducer and lacking C, N, or S or any combination of these nutrilites (6, 11). Electrophoresis on cellulose acetate of the filtrates of such cultures and visualization of protease activity at pH 5.5 or less revealed three previously unreported extracellular proteases of *N. crassa* (Fig. 1). These enzymes were differentiated from one another by electrophoretic mobility and the use of inhibitors. One or two

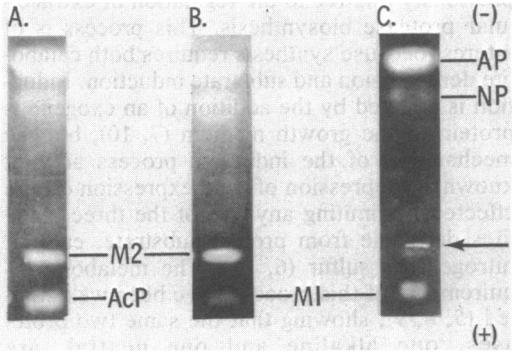

FIG. 1. Electrophoresis of culture filtrates for *N. crassa*. *N. crassa* mycelia were transferred to media containing 1% BSA and lacking a nutrilite (C, N, or S). Filtrates were electrophoresed on cellulose acetate, which was then placed on 1% skim milk-agar at pH 5.0. Proteolytic activity appears as clearing zones in the protein: A, 6 h after transfer to medium in which protein is the sole source of sulfur; B, 3 h after transfer to medium in which protein is the sole source of nitrogen; C, 4 h after transfer to medium in which protein is the sole source of carbon. AcP, Acid protease; AP, alkaline protease; NP, neutral protease; M-1, M-2, metalloproteases. The arrow indicates the origin.

acid proteases were secreted, depending upon which nutrilite was limiting. If nitrogen was limiting (−N), two electrophoretically distinct acid proteases were secreted, both EDTA inhibitable: metalloproteases 1 and 2 (M-1, M-2). Under conditions of sulfur limitation, (−S), we also observed two new bands: M-2 and another (AcP) which, although similar in electrophoretic mobility to M-1, was inhibitable by pepstatin rather than by EDTA. Carbon-limited (−C) cells secreted only M-1, although a trace amount of M-2 activity was occasionally present under these conditions.

Regulation of the acid proteases. The acidic proteases of *N. crassa* were found to be regulated similarly to the high-pH-optimum proteases (6) with respect to induction and repression. The addition of cycloheximide during induction eliminated secretion of the acid proteases, indicating that, like alkaline and neutral proteases, they are probably synthesized de novo in response to simultaneous derepression and induction.

The extracellular appearance of *N. crassa* proteases (levels of which were detectable by the qualitative electrophoretic assay) in response to the three states of derepression and combinations thereof is shown in Table 1. Although alkaline protease and neutral protease were elaborated in all cases, appearance of the acid proteases was more specifically regulated by nutrilite deprivation. The results of the double-limitation experiments imply that control of the extracellular appearance of the acid proteases was not due to differing responses of the structural genes to the various states of derepression. For example, if the AcP gene was derepressed in the −S case, it is also likely that it was derepressed in the −S,−N and the −S,−C cases; however, it was not present extracellularly, implying that control is post-transcriptional. When C was limiting, only M-1 was detectable, no matter what other nutrilite was lacking. Similarly, N derepression seemed to dominate when both N and S were absent. From these data it appears that the nonappearance of an acid protease, under some conditions, was not due to lack of response of the gene to the positive regulatory elements (i.e., *nit-2* or *cys-3* proteins).

Time course of induction. Secretion of the acidic proteases followed a time course similar to that reported for alkaline protease (6), showing a lag time of ~2 h for −S and ~1 h in −N or −C inductions. A quantitative assay specific for M-1 or M-2 was not developed, but AcP could be assayed without interference from other proteases with pepstatin, an inhibitor specific for carboxyl proteases. High levels of AcP were present in culture filtrates from −S inductions, whereas low levels of AcP were present in −N inductions, and no activity was pepstatin inhibitable when a carbon source was lacking. The low levels under −N conditions were undetectable on the electrophoretic assay. M-1 and M-2 appeared to follow the same pattern in the electrophoretic assay, with the exception that in the −N inductions, M-1 had no activity after 5 h (Fig. 2).

TABLE 1. Presence of extracellular proteases of *N. crassa* as a function of limiting nutrilite[a]

Limiting nutrilite	Presence of protease[b]				
	AcP	M-1	M-2	AP	NP
−S	+	−	+	+	+
−N	−	+	+	+	+
−C	−	+	−	+	+
−S,−N	−	+	+	+	+
−S,−C	−	+	−	+	+
−C,−N	−	+	−	+	+

[a] Presence of protease, detectable in culture filtrates by electrophoretic assay, is signified by +.

[b] AcP, Acid protease; M-1, M-2, metalloproteases; AP, alkaline protease; NP, neutral protease.

Properties of the acid proteases. AcP and M-2 were partially purified from culture filtrates obtained from nitrogen-derepressed, gelatin-induced mycelia. The preparations were not homogeneous, but only one band of protease

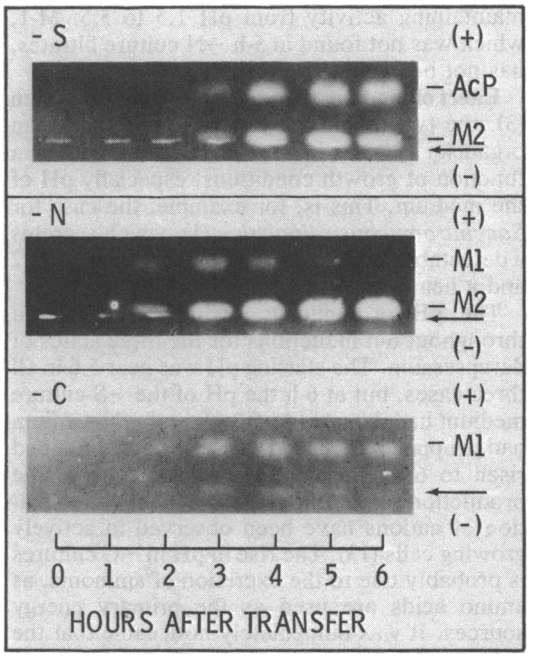

FIG. 2. Time course of extracellular acid proteases induced from *N. crassa*. Procedures were as described in the legend to Fig. 1. Culture filtrates were sampled through 6 h after transfer to medium lacking sulfur, nitrogen, or carbon. (Abbreviations used in the figure are defined in the legend to Fig. 1.)

activity was observed with the electrophoretic assay. Furthermore, the M-2 preparation was 100% inhibited by EDTA, and the AcP preparation was 96% inhibited by pepstatin, indicating that, in each case, only one acid protease species was present in active form. M-2 was also inhibited 100% by *o*-phenanthroline but was not affected by phenylmethylsulfonyl fluoride, *p*-chloromercuribenzoate, 2-mercaptoethanol, or pepstatin. As mentioned, AcP was 96% inhibited by pepstatin and 35% inhibited by *p*-chloromercuribenzoate but was not affected by the other inhibitors mentioned. Molecular weights of 66,000 for AcP and 18,000 for M-2 were obtained by chromatography on a calibrated G-75 column.

AcP was found to have maximal activity toward BSA at pH 4.0. This value is slightly higher than that of most fungal carboxyl proteases; however, the acid protease from *Penicillium notatum* has an identical pH activity profile toward BSA (15). M-2 had a narrow pH optimum toward BSA, with a maximum at about pH 4.5. This is considerably lower than that of other cation-requiring microbial proteases which generally fall under the category of neutral proteases. Investigation of pH stability showed that the stability range of AcP resembles that of many other fungal carboxyl proteases: from pH 3.0 to 6.0. M-2 was stable at very low pH, maintaining activity from pH 1.5 to 5.5. M-1, which was not found in 5-h −N culture filtrates, has not been characterized.

Effect of medium pH. As pointed out by Cohen (3), the type of protease (acidic or alkaline) an organism secretes in active form often may be a function of growth conditions, especially pH of the medium. This is, for example, the case for *Saccharomycopsis lipolytica* (1), which secretes a detectable acid protease when grown at pH 3.2 and a neutral protease when grown at pH 7.0.

The pH of inducing media was measured throughout 6-h inductions for the three states of derepression. The starting pH was near 5.6 in all three cases, but at 6 h the pH of the −S culture medium had dropped to 5.2, that of −N medium had dropped to 5.4, and that of −C medium had risen to 6.3. The decreases in pH due to the production of organic acids and unequal utilization of cations have been observed in actively growing cells (13). The rise in pH in −C cultures is probably due to the excretion of ammonia, as amino acids are used as the primary energy sources. It was immediately noticeable that the pH in −C cultures was above the stable range for AcP and M-2. To test whether these enzymes were being secreted but had become inactivated, −C cultures were kept titrated to pH 5.2 ± 0.2 throughout the induction with 1 N HCl or with Vogel medium previously adjusted to pH 1.5 with concentrated HCl. In both cases, alkaline and neutral proteases were induced normally, but no AcP was secreted (Fig. 3). In replicate experiments carried out with −N cultures, AcP activity showed no increase over the levels secreted in standard inductions. The metalloproteases M-1 and M-2 were not quantitatively investigated as a function of the medium pH. However, by the electrophoretic assay, it appears that in high-pH media (6.0 or above), M-1 is present under all three states of derepression and that at low pH (5.4 or below), M-2 is present. The M-2 protease, which was not detected extracellularly whenever carbon was limited (Table 1), was present under carbon starvation conditions when the inducing media had been previously titrated to a lower pH (Fig. 4). At an intermediate pH level (~5.5), both M-1 and M-2 were present, as shown on plates for the 2- to 5-h samples of −N culture filtrates in Fig. 2.

The pH of the medium was also found to have an effect on the induction of *N. crassa* alkaline and neutral proteases in −C cells. Carbon-depressed inductions at normal pH required the

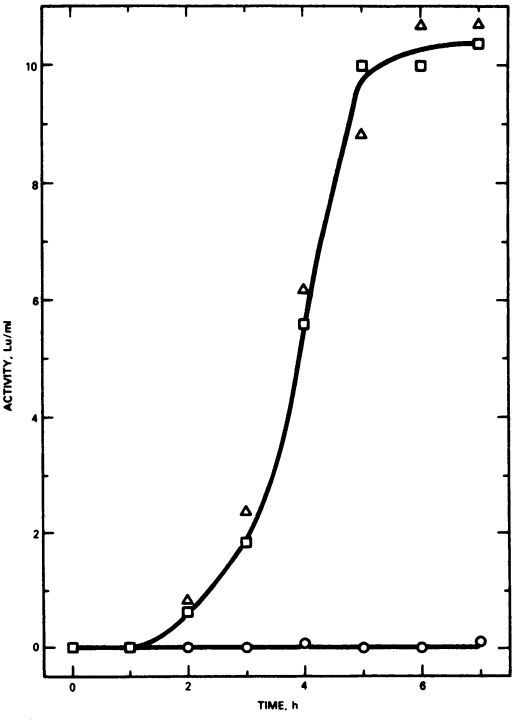

FIG. 3. Effect of low pH on secretion of extracellular proteases. Symbols: □, protease activity at pH 8.0 from a −C induction; △, protease activity at pH 8.0 from a −C induction with pH maintained at 5.2; ○, pepstatin-inhibitable activity from the same pH 5.2 culture.

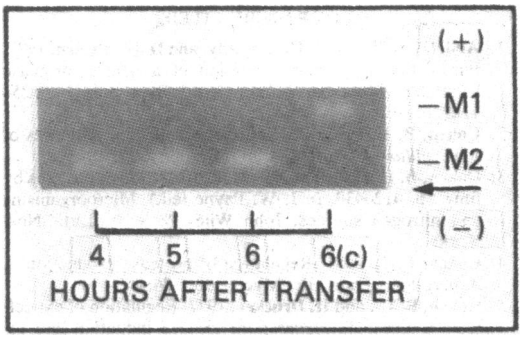

FIG. 4. Effect of medium pH on the presence of M-2 protease. Samples were from a −C induced culture with a starting pH of 4.5 and a 6-h sample from a normal-pH culture [6(c)]. (Conditions and abbreviations were as described in the legend to Fig. 1.)

addition of an exogenous protease for synthesis and secretion of these proteases (8, 9). Active M-2 was not present in these cultures. However, in low-pH (~4.5) cultures, induction of alkaline and neutral proteases occurred without exogenous protease, and M-2 was present. *N. crassa* also induced without added protease if a small amount of carbon source was included in the medium (e.g., 0.1% sucrose) (7, 11). Under these conditions, normal growth occurred until the carbon source was depleted, lowering the medium pH, which resulted in secretion of active M-2. The exogenous protease was required for induction only where the medium pH was not compatible with the stability of M-2.

Activity in mycelial extracts. The data in Table 1 suggest that the appearance of AcP extracellularly is not controlled at the level of transcription. If control is post-translational (i.e., by modification or secretion or both), intracellular levels should increase with derepression regardless of lack of secretion. Preliminary experiments showed this to be the case. Pepstatin-inhibitable activity was measured in mycelial extracts collected during −C and −S inductions (Fig. 5). The kinetics of intracellular AcP accumulation for −S mycelia were similar to those previously found for alkaline protease (manuscript in preparation), showing a lag time followed by rapid synthesis and, after 5 h, decreasing levels due to slowed synthesis and continued secretion. In the −C experiment, AcP levels increased similarly to those of alkaline protease in −C mycelia; however, AcP levels remained high. These data clearly indicate that a pepstatin-inhibitable protease is derepressed and synthesized in response to carbon limitation, but is not secreted. Total activity (intracellular plus extracellular) was about 20-fold higher in the −S culture. At the 6-h time point, the −C culture had 96 Lu/g of cell weight, all intracellular; the −S culture had 69 Lu of intracellular activity per g and 1,848 Lu/g extracellularly.

DISCUSSION

Two inducible and derepressible extracellular acid proteases have been partially purified from *N. crassa*. One of them, M-2, has properties that make it unique when compared with proteases of other microorganisms. Chelator-sensitive proteases generally fall into the neutral protease category and are similar to *N. crassa* neutral protease (16). These enzymes are optimally active at neutral pH and have molecular weights of 30,000 to 40,000. The M-2 protease has a molecular weight of 18,000 and a narrow pH optimum for BSA hydrolysis at 4.5. The specificity of the enzyme is not yet known; however, it showed no activity toward the carboxypeptidase or aminopeptidase substrates used by Siepen et al. for *N. crassa* intracellular peptidases (18).

N. crassa is unusual in that induction is a strict requirement for synthesis of extracellular protease. Of 22 strains of *Aspergillus* sp. tested by Cohen, none was inducible (4); furthermore, many organisms reported to be inducible have not met the criteria for induction and may not actually be inducible (3). Evidence presented here suggests that the unique requirement of induction and the unique protease, M-2, may not be mutually exclusive. Our data, which show that added protease activity is required for induction under conditions only when M-2 is not

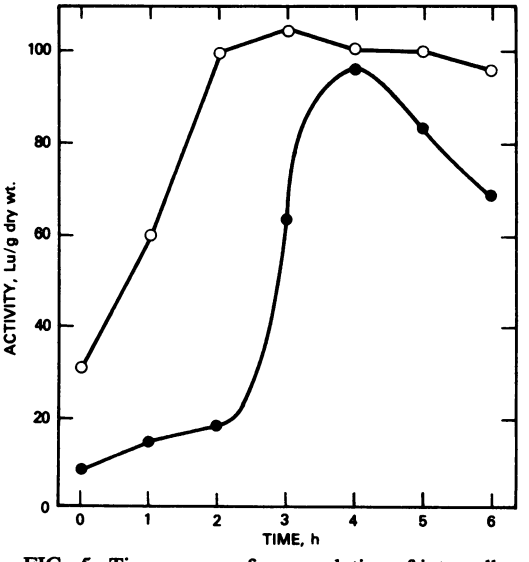

FIG. 5. Time course of accumulation of intracellular pepstatin-inhibitable activity. Mycelia were harvested hourly after transfer to inducing medium. Extracts were prepared and assays were performed as described in the text. Symbols: ●, extracts from −S BSA-induced mycelia; ○, extracts from −C BSA-induced mycelia.

present in active form, imply a possible role for M-2 in the inductive process. We are conducting further experimentation and a complete purification of M-2 to test this hypothesis.

The extracellular carboxyl protease (AcP) was found to be similar to other fungal acid proteases with the exception of its molecular weight (66,000). It has been reported that the molecular weight of *Aspergillus oryzae* acid protease ranges from 32,000 to 63,000, depending on the amount of carbohydrate attached (19, 20). The presence of attached carbohydrate may also account for the relatively high observed molecular weight of the *N. crassa* carboxyl protease.

N. crassa acid proteases are regulated similarly to the alkaline and neutral proteases, except that the former are found in culture filtrates in various combinations, depending on the conditions of derepression. It is difficult to compare them with the proteases of other fungi because many of the proteases reported are derived from stationary-phase culture filtrates or mycelia. Furthermore, even in the few organisms where regulation has been studied, rarely have both alkaline and acidic proteases been investigated. Cohen has recently reviewed pertinent literature (3).

The metalloprotease M-2 may not be detectable in −C cells because of *Neurospora*-induced pH changes in the medium. Further investigation is needed; at the present time it is likely that the appearance of both metalloproteases may be a function of pH or of the buffering capacity of the medium (M-1 being present at pH levels greater than 5.5 and M-2 being present at lower levels). The two enzymes have the same pH optima and inhibitor sensitivities and may be different forms of the same enzyme. A pH-dependent precursor-product relationship is one possibility. Precursors of extracellular proteases have been reported for other microorganisms (12).

In contrast to M-2, AcP (which appears to be rigidly controlled) is secreted in significant amounts only under conditions of sulfur derepression, regardless of medium pH. Carbon-starved cells show derepression of an intracellular pepstatin-inhibitable protease, but it is not secreted. Secretion would, in fact, be inefficient in the media tested since carbon-starved cells raise the pH of their own environment. Control may be exerted at the level of secretion, but this alone would not explain the reduction in total synthesis of AcP under carbon limitation compared with synthesis under sulfur limitation.

ACKNOWLEDGMENTS

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