

Protein Degradation and Protease Activity During the Life Cycle of *Blastocladiella emersonii*

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Analysis of protein degradation during the life cycle of *Blastocladiella emersonii* showed that (i) protein degradation is especially high during two phases of differentiation (sporulation, 12%/h and germination, 5%/h) in contrast with a much smaller degradation rate in the other phases (growth and zoospores, less than 1%/hr); (ii) protein degradation during germination in growth medium, as well as most of the germination process, is quantitatively unaffected by cycloheximide; (iii) a caseinolytic protease (pH optimum 5.5, apparent molecular weight 55,000 to 60,000) is present in extracts of zoospores and germinating cells; (iv) this protease activity is very low (perhaps absent) in extracts of late growth phase cells, but reappears during induced sporulation; (v) a different class of caseinolytic protease activity (pH optima 7 and 10; apparent molecular weight 25,000 to 30,000) is found in cellular extracts of late growth phase and early phases of sporulation; (vi) the latter class of enzyme activity is released into the medium during later phases of sporulation and is replaced in the cells by the former class. Speculations as to the roles of protein degradation in cell differentiation are discussed.

The water mold *Blastocladiella emersonii* begins each new asexual life cycle as a zoospore, a motile uninucleate cell which can be maintained for relatively long periods in the absence of exogenous organic nutrients (31, 35). When appropriately stimulated, populations of zoospores convert to sessile round cells and then to vegetative cells (germlings) in semisynchronous fashion. Germlings are then capable of prolific coenocytic growth (30, 34, 37). After the growth period, vegetative cells can be induced to sporulate semisynchronously (25, 30).

Of the two cell differentiation phases of the life cycle (sporulation and germination), germination is the one studied in greatest detail. During this phase, pre-existing structures of the zoospore become rearranged and/or disappear and at least two new structures, the cell wall and germ tube, are formed (30, 32, 37). Most of the sequence of known structural changes during germination appears not to require concomitant protein synthesis (32, 33), and none of it appears to require ribonucleic acid (RNA) synthesis (21, 32, 33). Thus, molecular mechanisms not directly related to controls at the level of gene expression are presumably involved.

In this investigation, we begin to explore one

possible class of mechanisms, namely, protein degradation. Degradation rates for different phases of the life cycle are reported, as are initial characterizations of protease activity through the life cycle.

MATERIALS AND METHODS

Measurement of in vivo protein degradation. Maintenance of stocks and growth conditions were identical to those described by Soll et al. (30). Zoospores were harvested from first generation cultures grown on peptone-yeast extract-glucose (PYG) agar and transferred to the defined liquid growth medium, DM₂, under one of the following conditions.

(i) For germination experiments, 2×10^6 to 4×10^6 cells were inoculated in tissue culture dishes (Falcon no. 3003) containing 10 ml of DM₂ plus 3 μ Ci of ¹⁴C-leucine (final specific activity 1.49 μ Ci/ μ mol) per dish. After 12 h at 20 C, growth was terminated by decanting this medium, washing the dishes extensively with sporulation solution (SS) (10^{-3} M CaCl₂, 10^{-3} M tris[hydroxymethyl]aminomethane (Tris)-maleate buffer, pH 6.7), and incubating the adhering vegetative cells at 27 C in 4 ml of SS per dish. After sporulation, the zoospores were washed and centrifuged three times with unlabeled used sporulation solution (uSS), derived from the cell-free supernatant of cultures which were grown and sporulated in the absence of label. Such washed, prelabeled zoospores were induced to germinate in siliconized, water-jacketed Bellco spinner flasks (Siliclad, Clay Adams, Inc.)

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containing either germination solution (GS) (5×10^{-2} M KCl, 10^{-2} M $MgCl_2$, 10^{-3} M $CaCl_2$, 10^{-3} M Tris-maleate buffer, pH 6.7) or DM_2 , plus 2×10^{-4} M unlabeled leucine. The germination flasks were maintained at 27 C by means of a circulating water bath. All cultures were exposed to ordinary room illumination.

(ii) For experiments where cells were maintained as zoospores rather than induced to germinate, the zoospores were obtained exactly as in (i) but were then resuspended in spinner flasks with uSS plus 2×10^{-4} M unlabeled leucine.

(iii) For experiments with growing or sporulating cultures, 3×10^8 to 5×10^8 zoospores were inoculated into spinner flasks containing 800 ml of DM_2 plus 20 μCi of ^{14}C -leucine (final specific activity, 0.11 $\mu Ci/\mu mol$). The cultures were grown under constant aeration (0.5 liters of oxygen and 2.8 liters of air per min) at 27 C for 6 h. The cells were collected on Whatman no. 541 filter paper, and were thoroughly washed with unlabeled medium. For growth experiments, the cells were washed with DM_2 ; for sporulation experiments, the cells were washed with SS. After washing, the cells were resuspended in appropriate spinner flasks containing either DM_2 or SS (both media containing 2×10^{-4} M unlabeled leucine).

(iv) The release of trichloroacetic acid-soluble radioactive polypeptides, or amino acid from prelabeled proteins, was monitored as follows. At intervals, 2.0-ml samples (cells plus medium) were added to 0.1 volume of ice cold 50% trichloroacetic acid-10% Cas-amino Acids (CA), incubated overnight at 4 C, and then filtered (0.45 μm pore diameter; Millipore filters, prewashed with 5% acid-1% CA). The filtrates were collected and then the filters were washed with 20 ml of cold 5% trichloroacetic acid-1% CA and dried for 2 h at 60 C in glass scintillation vials.

Aqueous samples were counted in a solution containing 4 g of 2,5-diphenyloxazole (PPO), 200 mg of 1,4-bis-2-(5-phenyloxazolyl)benzene (POPOP), 60 g of naphthalene, 60 ml of methanol, 20 ml of propylene glycol, and dioxane to complete 1 liter. The dried filters were counted in a solution containing 4 g of PPO and 100 mg of POPOP per liter of 20:80 absolute ethanol-toluene. All samples were counted in a Beckman liquid scintillation spectrometer.

Characterization of proteases by molecular weight estimation. Columns were constructed as follows: Sephadex G-100 (Pharmacia) was swollen and washed extensively with KCl-Tris buffer (0.1 M KCl; 0.05 M Tris-hydrochloride, pH 7.5). The columns (1.8-cm diameter) were packed under low pressure (10 g/cm² at the top) to a height of 50 cm and equilibrated in the same buffer. The columns were developed at 4 C at a flow rate of 14 ml/h. Fractions (2.4 ml) were collected and analyzed for enzyme activity (0.5-ml portions per fraction) or absorbancy at 280 nm. Columns were calibrated according to the method of Andrews (1) with the following molecular weight markers: bovine plasma albumin, molecular weight 67,000 (Mann); albumin dimer, molecular weight 134,000; chymotrypsinogen A, molecular weight 25,000 (Schwartz). A 2-ml solution containing 5 mg each of albumin and chymotrypsinogen A

was fractionated, and the fractions exhibiting peak absorbancies at 280 nm for the three molecules were plotted as log molecular weight versus elution volume (see Fig. 5).

Protease assay. Extracts were prepared as follows. First generation zoospores were obtained from PYG agar cultures. Round cells and germlings were obtained after incubation of such cells in GS for 20 and 60 min, respectively. Vegetative cells (8 h old), as well as cells induced to sporulate, were obtained exactly as in section (iii) above except that no label was added.

All harvested samples were washed with SS, either by centrifugation or filtration, and were disrupted in a French pressure cell (Aminco Corp., 8,000 psi). Samples were then centrifuged at $35,000 \times g$ for 30 min, and the supernatants were assayed for proteolytic activity.

The substrate for the assay was 1 ml of 1% casein (Hamersteen) denatured in 4 M urea, in a total reaction volume of 2 ml (substrate, enzyme extract, buffer, and other additives). All buffers were used at a final concentration of 0.05 M. Except where otherwise noted (Fig. 4a), 0.5 ml of enzyme extract was used throughout. After 2 h of reaction at 28 C, 2 ml of 10% trichloroacetic acid was added to each reaction mixture. After filtration (Whatman no. 42 filter), 1 ml of filtrate was assayed for soluble peptides and amino acids by the McDonald and Chen (23) modification of the Lowry method. For each reaction mixture, a corresponding blank was prepared by adding trichloroacetic acid at zero time. Enzyme activity was calculated as the difference in absorbancy at 700 nm between filtrates of the 2-h reaction mixtures and filtrates of the corresponding zero time blanks. All reactions and blanks were performed in duplicate. The values for replicate determinations were generally well within 10% of the average between them, except occasionally when the differences between reactions and blanks were very small (low enzyme activity).

Protein here and elsewhere was estimated by the microbiuret method of Itzaki and Gill (11) by using bovine serum albumin, fraction V (Sigma), as the standard. Specific enzyme activity was calculated as absorbancy at 700 nm per mg of protein per h. For plots of relative enzyme activity (Fig. 3 and 4), constant volumes of enzyme extract were used and relative enzyme activity was expressed as the percentage of maximum activity.

RESULTS

Measurement of in vivo protein degradation. ^{14}C -leucine was chosen as a workable label for proteins for our purposes since it was shown previously (D. R. Soll, Ph.D thesis, U. of Wisc., 1970) that about 95% of the radioactivity incorporated into acid-precipitable material, at least during germination, could be recovered as leucine in chromatograms of protein hydrolysates of vegetative cells. To maximize the measurements of protein degradation, an excess of unlabeled leucine (2×10^{-4} M) was added after the termination of the labeling period.

Even with this precaution, the results in Fig. 1a document that the release of counts into acid-soluble material (cells plus medium) during growth is very small (0.5 to 1%/h, if at all). If this measurement of degradation were a gross underestimate due to the rapid reutilization of labeled amino acid (i.e., rapid breakdown and resynthesis of protein without exchange of labeled amino acid with cold amino acid in the medium), then inhibitors of protein synthesis should block the reutilization and lead to a greatly elevated measurement of degradation. However, when doses of cycloheximide (20 $\mu\text{g}/\text{ml}$) sufficient to inhibit protein synthesis 65 to 70% were added to such growing cultures, a significantly higher level of degradation was not observed.

In contrast to the situation during growth, the release of counts into trichloroacetic acid-soluble material during sporulation is impressively higher (Fig. 1a). The rate of release is roughly linear throughout the course of sporulation and has a net value of about 12%/h. By 150 min, a period prior to the first release of zoospore progeny, some 35% of the counts in the pre-labeled vegetative cell proteins have become acid-soluble.

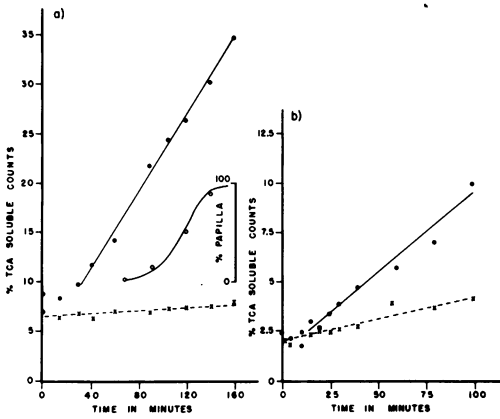


FIG. 1. Protein degradation during different phases of the life cycle. At the indicated times, samples were processed for trichloroacetic acid-soluble radioactivity. Acid-soluble counts are expressed as percentages of acid-precipitable counts in the zero time samples. a, Pre-labeled vegetative cells were resuspended in DM_2 (x) for growth or SS (●) for sporulation at 5×10^5 cells/ml. The percentages of sporulating cells (sporangia) with discharge papillae (○) in the latter culture were scored after fixing 1-ml samples in formaldehyde at the indicated times. b, Pre-labeled zoospores were resuspended in GS (●) for germination or μ SS (x) to maintain zoospores at 1.45×10^5 cells/ml. All incubation solutions contained 2×10^{-4} M unlabeled leucine.

Protein degradation during germination, while not as great as during sporulation, is significant (about 5%/h; see Fig. 1b and 2). After a short lag, the rate is roughly linear for at least two hours (Fig. 2) and is comparable when germination takes place in GS or in the growth medium (Fig. 2).

Figures 1b shows also that the rate of protein degradation for zoospores is clearly lower (about 1.2%/h) than during germination. This lower value must be considered as an overestimate because, in the experiment reported, 13% of the cells were no longer zoospores by 60 min. Assuming that this latter cell contingent was degrading its proteins at the germination rate (5%/h), the actual value for zoospores should be very close to that observed during growth ($1.2 - (13 \times 5/100) = 0.75\%/h$). Using colorimetric methods for bulk protein and with zoospores obtained and maintained under very different conditions, Suberkroop and Cantino (35) have reported somewhat higher values for protein breakdown in zoospores.

Effects of cycloheximide on protein degradation during germination. In the presence of cycloheximide, zoospores induced to germinate all reach a reversible morphological block point: they fail to form germ tubes and the retracted internal flagellar axoneme fails to disappear; other known structural events accompanying the conversion of zoospores to germlings occur on schedule (32, 33). We previously reported that the degree of inhibition of incorporation of amino acid into acid-precipitable material by cycloheximide differs depending upon whether zoospores are induced to germinate in GS or in growth medium. Inhibition was virtually complete in growth medium, but only some 75 to 80% complete in GS (32, 33).

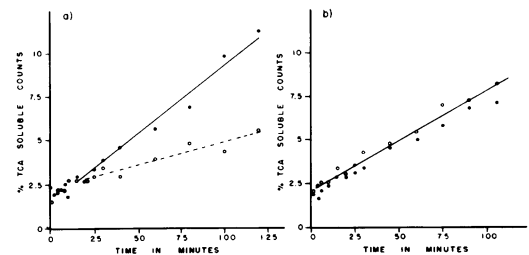


FIG. 2. Effect of cycloheximide on protein degradation during germination. Samples were processed and data are expressed as in Fig. 1. Both GS and DM_2 contained 2×10^{-4} M unlabeled leucine. Pre-labeled zoospores were resuspended in: a, GS, without (●) or with (○) 20 μg of cycloheximide per ml at 1.45×10^5 cells/ml; b, DM_2 , without (●) or with (○) 20 μg of cycloheximide per ml at 1.9×10^5 cells/ml.

We now find that there is also a difference with respect to protein degradation, albeit in the opposite direction. Cycloheximide had no detectable effect on the rate of degradation in growth medium (Fig. 2b); it has a significant, though not complete, inhibitory effect on degradation in GS (Fig. 2a).

The fact that cycloheximide does not affect the rate of protein degradation under conditions where it apparently completely inhibits protein synthesis (i.e., in growth medium) suggests that the enzymatic machinery for accomplishing this degradation pre-exists in the zoospore. Moreover, since the rate of degradation is significantly higher during germination than it is in zoospores, this pre-existing machinery is presumably activated in some way during germination. The explanation for the partial inhibition of degradation by cycloheximide in GS, as opposed to growth medium, remains unknown.

pH curves of proteolytic activity at different stages of the life cycle. Extracts of zoospores, round cells, and germlings exhibit pH profiles of proteolytic activity which are indistinguishable and show a pH optimum at about pH 5.5 (Fig. 3a-c). On the other hand, extracts of 8-h-old vegetative cells (near the stationary phase of growth under the present conditions) exhibit a completely different pH profile (Fig. 3d). In these extracts, maximum activity occurs at around pH 7 with a broad shoulder extending up to pH 10.

During sporulation, proteolytic activity is increasingly recovered in the cell-free medium (Fig. 4a). The pH profile of this activity is very similar, and probably identical, to that present in the cells at the end of vegetative growth (compare Fig. 4b, activity in the sporulation medium, with Fig. 3d, activity in extracts of vegetative cells). Again, this profile is decidedly different from that of the enzyme activity found in the cells (zoospores) at the end of sporulation (Fig. 3a). Figure 4a demonstrates that the activities measured at pH 7 and at pH 10 are released into the medium during sporulation with indistinguishable time courses, and that the bulk of the release occurs between the completion of papilla formation and zoospore release.

The changing distribution of enzyme activity in the cells during sporulation has also been examined. The activity resident in cell extracts at each of three points during sporulation has been monitored at the three crucial pH optima, 5.5, 7, and 10 (Table 1). As expected from the above results, the activity at pH 10 greatly decreases as sporulation proceeds. Also, the

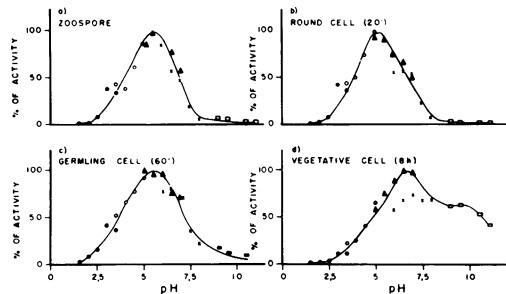


FIG. 3. pH profiles of protease activity in cell extracts. Extracts of zoospores (a), round cells (b), germlings (c), and 8-h vegetative cells (d) were prepared and assayed for enzyme activity using as buffers (0.05 M): ●, glycine-hydrochloride; ○, acetate-hydrochloride; ▲, cacodylate-hydrochloride; ×, imidazole-NaOH; □, glycine-NaOH. Relative enzyme activity is represented as the percentage of the highest value found in each extract. Extract protein per reaction tube was: zoospore, 173 μ g; round cell, 96 μ g; germling, 88 μ g; and vegetative cell, 132 μ g. Specific activities (absorbancy at 700 nm per mg of protein per h) were: zoospore, 0.97; round cell, 0.80; germling, 0.78 at pH 5.5, cacodylate-hydrochloride buffer; and vegetative cells, 0.98 at pH 7, cacodylate-hydrochloride buffer.

activity at pH 5.5 increases. Further interpretation of the data in Table 1 is complicated by the fact that the pH curves for enzyme activity measured at pH 5.5 (Fig. 3a-c) and at pH 7 (Fig. 3d and 4b) obviously overlap. Thus, the enzyme activity measured at pH 5.5 is "contaminated" by pH 7 enzyme activity and vice-versa. When the appropriate corrections are estimated and tabulated (Table 2, legend), a clearer picture of the changing distribution of enzyme activity is obtained. The vegetative cells contain very little pH 5.5 enzyme. Since these cells are nearing the stationary phase of growth and thus may have embarked upon some initial phases of sporulation, the question of whether exponentially growing cells contain even this low level of pH 5.5 enzyme must remain open. At any rate, there is an approximately 10-fold increase in the level of this enzyme activity in the cells during the first 140 min of induced sporulation. Conversely, there is about a sevenfold decrease over the same time period in the level of pH 7 to 10 enzyme activity.

Characterization of protease by molecular weight estimation. The proteolytic activity in zoospore extracts can be eluted from a Sephadex G-100 column as a reasonably symmetrical peak with maximum activity recovered in an elution position corresponding to a molecular weight of 55,000 to 60,000 for globular protein

(Fig. 5a). The eluted activity exhibits the typical pH profile of pH 5.5 enzyme activity (i.e., zoospore enzyme, Fig. 3a). On the other hand, the activity recovered from the sporulation medium exhibits a different elution profile with an estimated molecular weight of 25,000 to 30,000 (Fig. 5b). This enzyme activity exhibits the typical pH profile of pH 7 to 10 enzyme (i.e.,

vegetative cell enzyme, Fig. 3d). Enzyme activity with similar elution and pH profiles has been recovered from chromatography of vegetative cell extracts (J. Correa and W. Lodi, unpublished data).

Note especially that the two elution profiles depicted in Fig. 5 are virtually non-overlapping. In the zoospore extract, no activity is eluted at the position of the pH 7 to 10 enzyme. In the sporulation medium, if there is any activity eluted at the position of the pH 5.5 enzyme, it is a vanishingly small amount.

The simplest interpretation of these results, together with those of preceding sections, is that: (i) the pH 7 to 10 enzyme exists in 8-h vegetative cells but is released into the medium during sporulation, and (ii) the pH 5.5 enzyme exists at very low levels, if at all, in 8-h vegetative cells. It appears in the cells during

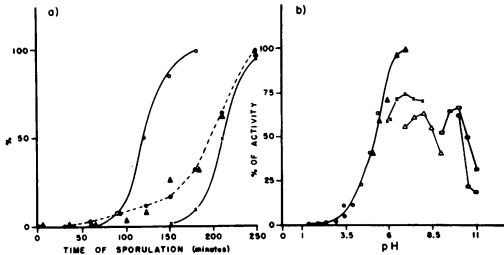


FIG. 4. a, Appearance of proteolytic activity in sporulation medium. Vegetative cells (5×10^8) were induced to sporulate in 1 liter of SS in spinner flasks at 27 C under constant aeration. The percentages of cells with discharge papillae (O) or which had released zoospores (x) were scored after fixing 1-ml samples in formaldehyde at the indicated times. The proteolytic activity released into the medium was assayed in supernatants after pelleting 100-ml samples at the indicated times: ▲, activity measured at pH 7, cacodylate-hydrochloride buffer (0.4 ml of medium); ●, activity measured at pH 10, glycine-NaOH buffer (0.5 ml of medium). Relative enzyme activities are expressed as percentages of the respective values at the end of sporulation (250 min). b, pH curve of the proteolytic activity released during sporulation. Sporulation medium obtained from the 250-min sample of Fig. 4a was assayed for proteolytic activity at different pH values with the following buffers: ●, glycine-hydrochloride; ○, acetate-hydrochloride; ▲, cacodylate-hydrochloride; ×, imidazole-NaOH; △, Tris-hydrochloride; □, glycine-NaOH; ■, carbonate-hydrochloride. Relative enzyme activities are expressed as percentages of the highest value found (pH 7, cacodylate-hydrochloride buffer).

TABLE 1. Protease specific activity in cell extracts from different times of sporulation, measured at different pH values^a

Cell extract	pH 5.5	pH 7	pH 10
Vegetative (8 h)	0.66	0.96	0.54
90 Min (SS)	1.38	0.96	0.17
140 Min (SS)	1.78	1.14	0.08

^a Uncorrected specific activities. Expressed so that a specific activity of 1 equals an increase in absorbance of 1.0 at 700 nm per mg of protein per hour by using the protease assay. The values of pH 5.5 and 7 are with cacodylate-hydrochloride buffer; the values at pH 10 are with glycine-NaOH buffer.

TABLE 2. Protease specific activity in cell extracts from different times of sporulation, measured at different pH values^a

Cell extract	pH 5.5	pH 7	pH 10
Vegetative (8 h)	0.16	0.83	0.54
90 Min (SS)	1.13	0.26	0.17
140 Min (SS)	1.71	0.12	0.08
Net change in sp. act.	10.6 (fold increase)		6.75 (fold decrease)

^a Corrected specific activities. Since the pH 5.5 enzyme exhibits negligible activity at pH 10 (Fig. 3a-c), it was assumed that activity measured at pH 10 was entirely due to pH 7 to 10 enzyme. The activities at pH 5.5 and at pH 7 (cacodylate-hydrochloride buffer) due to this amount of pH 7 to 10 enzyme were then calculated from the pH profile "calibration" curve of Fig. 4b. The calculated value at pH 7 is entered in the table, whereas the calculated pH 5.5 value was subtracted from the total activity measured at pH 5.5 (Table 1) to estimate the amount of activity at pH 5.5 due to pH 5.5 enzyme. That the estimates of pH 5.5 versus pH 7 to 10 enzyme activities are reasonable approximations is supported by: (i) the fact that the two enzyme activities can be physically separated by column chromatography (as in Fig. 5), and (ii) a further check on the calculations. From the calculated values of pH 5.5 enzyme entered in the table, the contribution of pH 5.5 enzyme to activity measured at pH 7 can be estimated (using the pH calibration curve of Fig. 3a). These estimates should approximate the differences in pH 7 values entered in Table 1 versus Table 2. The comparisons are: for the vegetative cells, 0.08 units calculated and 0.13 units by difference; for the 90-min sample, 0.85 units calculated and 1.02 units by difference.

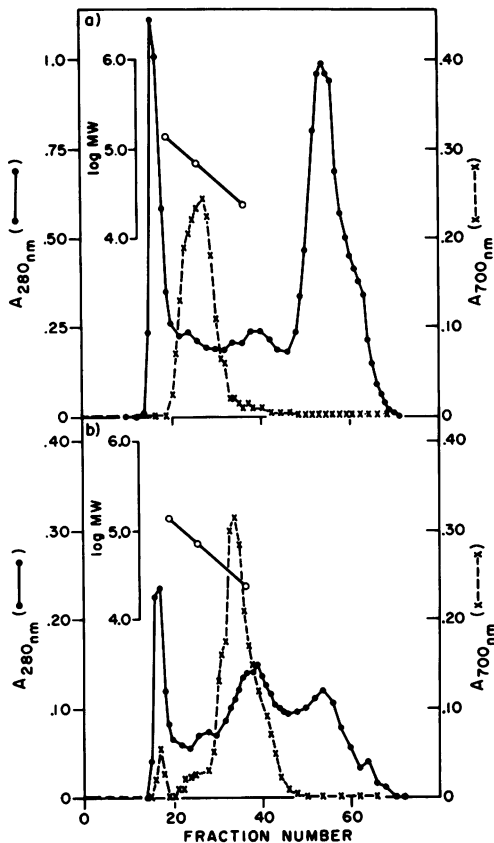


FIG. 5. Sephadex G-100 chromatography of the proteolytic activities present in zoospore extracts and in sporulation medium. (a), A 2-ml amount of zoospore extract (14 mg of protein) and (b) 2 ml of sporulation medium (5 mg of protein, obtained as a $10\times$ concentrate by ultrafiltration of the 250-min sample of Fig. 4a) were analyzed on a Sephadex G-100 column. Symbols: O, molecular weight calibration of the column, \bullet , absorbancy of column fractions at 280 nm; \times , enzyme activity in the protease assay (2-h incubations) of 0.5-ml portions of each 2.4-ml column fraction. Cacodylate-hydrochloride buffer at pH 5.5 (a) and pH 7 (b) was used in the enzyme assays.

sporulation, is not released into the medium, and is retained by the zoospores. It is also present during germination, in contrast to the pH 7 to 10 enzyme.

DISCUSSION

Correlations between enhanced rates of protein degradation and certain phases of cell differentiation—namely, those occurring under nongrowth conditions—have been reported for several microorganisms, e.g., *Dictyostelium* (38, 39), *Chlamydomonas* (13), *Bacillus* (15). In *B. emersonii*, nongrowth phases marked by drastic changes in cell architecture (germination and

sporulation) are also accompanied by sizable increases in previously low levels of cellular protein degradation (Fig. 1).

The biological significance of correlations between protein degradation rates and certain phases of cell differentiation remain ill-defined. The most obvious functions are to provide amino acids for new protein synthesis or simply to get rid of unneeded proteins, or both. Indeed, during *Blastocladia* sporulation, Murphy and Lovett (25) have reported a 25% net decrease in total protein during roughly the period when the excretion and replacement of proteases reported here take place.

There are indications, however, that proteases serve other, more specific purposes. Among others, the following functions have been mentioned or documented, or both: (i) removal of proteins which would otherwise not allow the cell differentiation to take place (i.e., protein inhibitors; see ref. 22); (ii) selective cleavage of precursor proteins during assembly or disassembly processes, or both (e.g., during the intracellular production of small RNA animal viruses; see ref. 9, 12, 36; during the assembly of bacteriophage heads; see ref. 8, 10, 16; and during the post-synthetic insertion of a reticulocyte membrane protein; see ref. 20); (iii) selective modification of pre-existing forms of key enzymes. This latter function has been the subject of extensive investigation during sporulation in *Bacillus*. As compared to their respective vegetative forms, both aldolase (27) and RNA polymerase (18, 24, 26) have been reported to be subjected to highly specific proteolytic modifications during the transition to spore formation. Although the originally reported modification of RNA polymerase core enzyme has now been attributed to an in vitro artifact (19), the sigma subunit of the enzyme still seems to be removed or inactivated early during sporulation. In any case, genetic, physiological, and morphological studies (17, 18, 28) indicate quite convincingly that a certain serine protease is indeed required to progress through early stages of *Bacillus* sporulation. Implicit in all three of the above modes of protease action is the suggestion that modifications (or removals) of pre-existing proteins by enzymatic cleavage may furnish key control steps during the transitions from one cell phenotype to another.

With the exception of the reticulocyte membrane protein, the evidence referred to above concerning specific proteolytic modifications comes from examples involving viruses or prokaryotic cells. With respect to eukaryotic cells, many examples of the cleavage of secreted proteins (zymogens, pro-insulin, pro-collagen,

blood clotting enzymes, etc.) are well documented. However, the search for examples specifically related to changes in eukaryotic cell phenotype (i.e., cell differentiation) is in its infancy. The recent provocative work of Cabib's group concerning the activation of chitin synthetase at the site of yeast septum formation (2 to 5) should provide further impetus for this search.

During *Blastocladiella* zoospore germination, there are several candidates for proteolytic modification: (i) one of the early events of germination is cell wall formation. Chitin constitutes the major fraction of the cell wall (D. Allin, unpublished data) and the terminal enzyme, chitin synthetase, has been studied (6, 7). The enzyme is already present in zoospores (6, 7), but in vivo activity (chitin synthesis) appears not to begin until after germination is initiated (D. Allin, unpublished data). There are some interesting parallels between the *Blastocladiella* enzyme (6, 7) and the above-mentioned yeast enzyme (2, 5, 14). The inactive yeast enzyme can be activated by a factor, apparently packaged in and released from a vesicle fraction (5), whose action is mimicked by trypsin (4). During *Blastocladiella* germination, fusion of vesicles with the plasma membrane can be observed around the time of cell wall construction (30). In yeast, Cabib et al.'s interpretation (2, 5) is that chitin synthetase is attached to the plasma membrane; (ii) another step in germination involves the general "turn on" of protein synthesis. This is apparently prevented in the zoospore by a factor reversibly associated with the ribosomes of the zoospore (29); (iii) there are a number of discrete structural changes which take place during germination and which are likely to include local peptide bond cleavage (e.g., flagellar retraction and disappearance of the flagellar axoneme; vesicle opening, fusion with each other and with the plasma membrane, and disappearance; nuclear cap disintegration; mitochondrial fragmentation; germ tube formation, etc., see 30, 32, 37). Several of these structural changes take place at the normal rate and to the normal extent in the presence of protein synthesis-inhibiting doses of cycloheximide (32, 33). The fact that the measured protein degradation rate during germination is unaffected by the presence of cycloheximide in growth medium (Fig. 2) lends further credence to the suggestion that local peptide cleavages by preformed protease may be involved in at least some of these structural modifications. However, only further, more direct analyses can decide whether any of the above-mentioned steps in germination do,

in fact, utilize proteolytic cleavage as a mechanism of change.

Finally, the developmental controls over proteolytic activity itself are of some interest. The protease activity present in extracts of zoospores, as well as of germinating cells, is apparently formed during sporulation and is almost absent (if present at all) in extracts of vegetative cells. Conversely, the protease activity of vegetative cells is apparently released into the medium during sporulation (Fig. 3 to 5 and Tables 1 and 2). The reasons for the relative inactivity of germination protease in vivo in swimming zoospores (protein degradation rate) and for its turn on in vivo during germination are not at present known. In vitro, the activity of the germination enzyme with the zoospore's proteins as substrate can be stringently controlled by the pH of the incubation mixture (W. Lodi, unpublished data). This latter finding has obvious technical implications for in vitro studies of zoospore versus germinating cell proteins and may be of biological interest as well.

ACKNOWLEDGMENTS

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