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**The Acid Activatable Cysteine Proteinases of the Dictyosteliaceae and
Other Lower Eukaryotes**

by

Keith Edward Gale

A Thesis

**Submitted to the Faculty of Graduate Studies and Research
through the Department of Biological Sciences in Partial Fulfillment of the Requirements
for the Degree of Master of Science
at the University of Windsor**

Windsor, Ontario, Canada.



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ABSTRACT

The cysteine proteinases of *Dictyostelium discoideum* are unique enzymes in that they are found to be reversibly activated by pH shuffles in their lysosomal/endosomal microenvironments. The activation of these enzymes is unique as it does not coincide with a cleavage in the pro-sequence, alternatively a reversible conformational change is believed to occur (Cotter, 1997).

Previous work has hypothesised that proteinases were present in the extracellular matrix (ECM) of the pseudoplasmodium of *D. discoideum*. This work shows that acid-activatable cysteine proteinases are secreted during the aggregation of myxamoebae and the migration of the pseudoplasmodium through the use of gelatin-SDS-PAGE as well as with a novel *in situ* technique.

When assayed by gelatin-SDS-PAGE during the course of multicellular development in *D. discoideum*, the intracellular level of proteinases was found to decrease as development proceeded. However, this work finds that there is an increase in external levels of cysteine proteinases as multicellular development proceeds.

The lower eukaryotes, *Achlya ambisexualis*, *Chironomus*, and *Euglena gracilis* were found to lack the pH regulated cysteine proteinases akin to those in the Dictyosteliaceae. Alternatively, in the ciliate *Tetrahymena pyriformis*, the cysteine proteinase tetrairin was found to be activated by acid treatment, deactivated by a base treatment, and reactivated by an acid treatment when assayed by gelatin-SDS-PAGE. This is the only organism, other than the Dictyosteliaceae, currently known to have cysteine proteinases that are reversibly activated and deactivated in acid or alkaline

conditions. This may represent a new class of pH regulated cysteine proteinases that occur in lower eukaryotic organisms which undergo the vesicular pH shuffles which mammalian cells apparently lack.

The disulfide bonding of *D. discoideum* spore proteinases ddCP43 and ddCP48 were characterized and were observed to lack interdisulfide bonds. Native-PAGE analysis reveals two separate bands when the same sample were analyzed. These results indicate that ddCP43 and ddCP48 are independent enzymes and not part of a larger multimeric structure.

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LIST OF ABBREVIATIONS

Abs	absorbance
AP	aspartyl proteinase
ATCC	American Type Culture Collection
AX	axenic
cAMP	adenosine 3' 5'-cyclic monophosphate
cDNA	complementary deoxyribonucleic acid
CP	cysteine proteinase
ddCPx	<i>Dictyostelium discoideum</i> cysteine proteinase with apparent molecular mass of x
DNA	deoxyribonucleic acid
DTT	dithiothreitol
E-64	L-trans-epoxysuccinyl-leucylamide-(4-guanidino)-butane
ECM	extracellular matrix
g	gravimetric unit
kDa	kilodalton
KPi	inorganic potassium phosphate buffer
min	minute
M	molar
Mr	relative mobility
mM	millimolar
mRNA	messenger ribonucleic acid
O.D.	optical density

pH	negative log hydronium concentration
R_f	relative to the front
rRNA	ribosomal ribonucleic acid
RNA	ribonucleic acid
SDS-PAGE	sodium dodecyl sulfate - polyacrylamide gel electrophoresis
v/v	volume percent
V-H⁺-ATPase	vacuolar hydronium adenosine triphosphatase
w/v	weight/volume percent

INTRODUCTION

The division of the Myxomycota, otherwise known as the Dictyosteliaceae, or Dictyostelids but more commonly referred to as the cellular slime molds, are excellent tools in the study of developmental biology. Members of this genus have been found to enter three separate developmental pathways depending on environmental parameters. The asexual or 'developmental' pathway involves the proliferation of individual myxamoebae and the formation of a fruiting body as seen in *Dictyostelium discoideum* (Figure 1). The sexual or macrocyst life cycle exists in two variants, either homothallic reproduction occurs, as seen in *Dictyostelium mucoroides*, or heterothallic reproduction occurs, as seen in *D. discoideum*. In cases of environmental stress a third asexual pathway has also been observed in the cellular slime mold *Polysphondylium pallidum* in which stress resistant microcysts are formed (Raper, 1984).

Asexual Life Cycle of the Dictyosteliaceae

(*Dictyostelium discoideum*).

The asexual life cycle begins with the germination of a single capsule-shaped dormant spore, with dimensions of approximately 3x7 μm (Figure 1). Spore germination is the process in which a dormant spore becomes activated and germinates, releasing a single, nascent myxamoeba. Germination of the dormant spore occurs in four

Figure 1. Asexual life cycle of *Dictyostelium discoideum*. The following stages are diagrammed: (a) spore dispersal; (b) spore germination; (c) vegetative growth; (d) nutrient starvation to preaggregation, time 0-5 hours; (e) beginning of aggregation, time 6 hours; (f) middle of aggregation, time 8 hours; (g) late aggregation, time 9 hours; (h) tipped aggregation, time 11 hours; (i) standing slug, time 13 hours; (j) initiation of pseudoplasmodium (slug) migration, time 16 hours; (k) end of short slug migration period, time 18 hours; (l) re-establishment of vertical polarity in preparation for culmination, time 19 hours; (m) initiation of culmination, time 20 hours; (n) early culmination, time 21 hours; (o) middle culmination, time 22 hours; (p) culmination complete, time 24 hours; and (q) fruiting body and spore maturation, time 1-10 days (Cotter *et al.*, 1992).

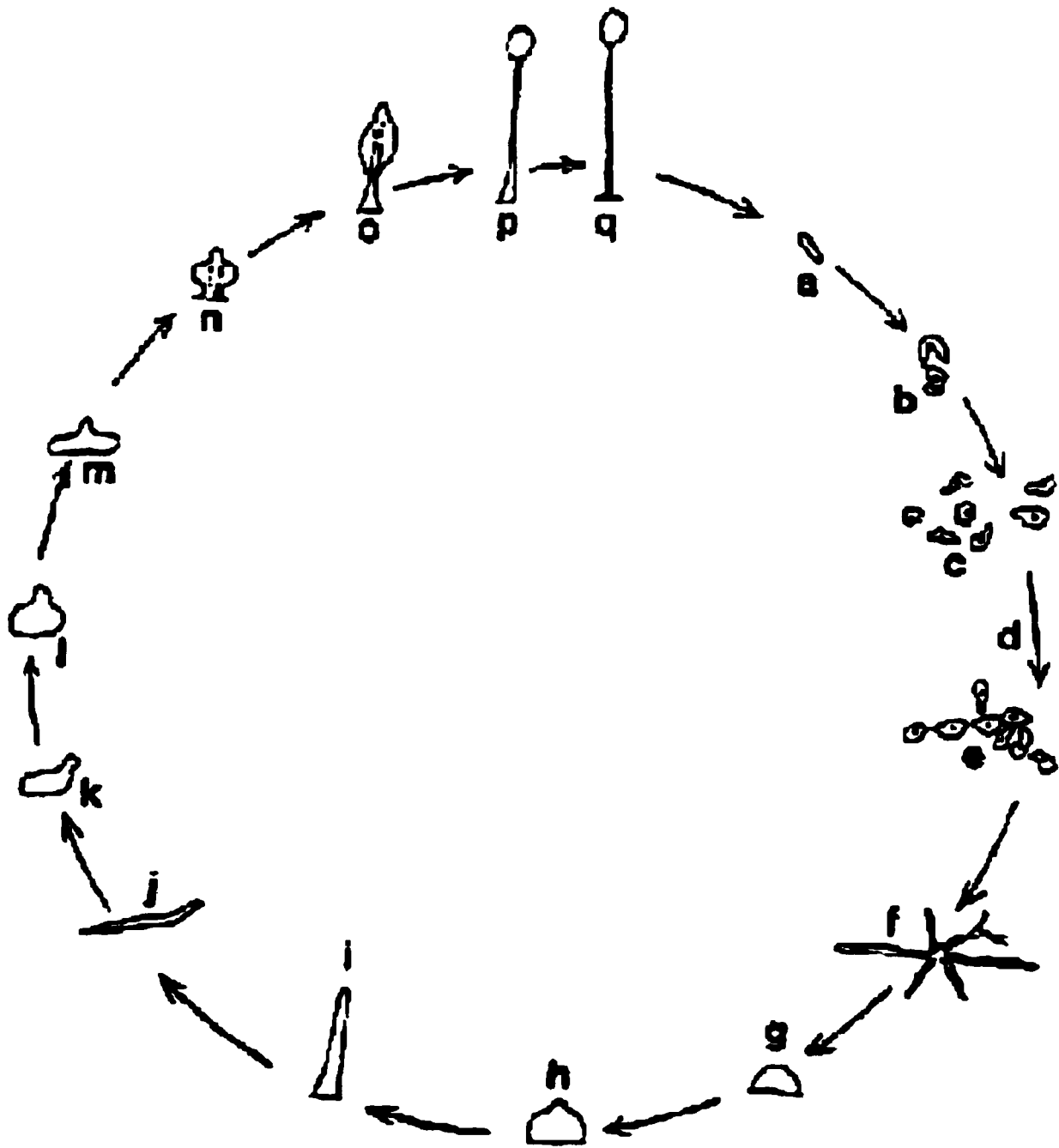


Figure 1.

phases, activation, post-activation lag, swelling and emergence (Cotter, 1981). This process is regulated by various factors such as pH, temperature, oxygen tension, osmotic pressure, discadenine, ammonia and also by an undefined biochemical designated as the autoactivator (Cotter *et al.*, 1979, Cotter, *et al.*, 1992). When a spore is activated the dormancy of the spore is interrupted; however, deactivation of the spores can occur, allowing the spores to revert to dormancy (Cotter, 1977). Deactivation can occur only during lag phase, before spore swelling (Cotter, 1977). Once spores have germinated, the amoebae enter the growth phase of the life cycle and become vegetative cells if a food source is present; if food is not present, the cells quickly exit the vegetative phase and enter the migratory phase of multicellular development (Cotter *et al.*, 1990). As vegetative cells, they utilize bacteria as a food source and undergo numerous cell divisions. Approximately 6 to 8 hours after all bacteria are consumed, some of the myxamoebae acquire the ability to secrete pulses of cyclic adenosine monophosphate (cAMP) (Bonner, 1967). The cAMP serves as an extracellular messenger. Other cells migrate toward these high levels of cAMP. These cells then begin to secrete cAMP to attract more cells. Once the cells reach one another, they bind linearly to one another and form streams of cells spiraling around the cAMP signaling centre to form tight aggregates of approximately 10^5 cells (Loomis, 1982). It is now believed that cells initiate development prior to aggregation (Jermyn, *et al.*, 1989). The aggregated myxamoebae form a mound of cells and continue to differentiate. As cells differentiate, they enter the developmental phase of the life cycle as a multicellular organism. Cells located at the tip of the mound begin to show characteristics of prestalk cells whereas cells found throughout the rest of the mound reveal prespore cell characteristics (Jermyn *et al.*,

1989). Prestalk and prespore cells will terminally differentiate into the final two cell types found in the mature organism; that is, stalk and spore cells respectively. As multicellular development proceeds the tip of the mound will lengthen upwards and form an upright slug which will topple over and migrate (Raper, 1940). The prestalk cells move towards the anterior portion (20%) of the slug whereas the prespore cells are located towards the posterior portion (80%) of the slug (Takeuchi, 1969). It should be noted that there are also prestalk cells located in the prespore region of the slug. These cells are termed anterior like cells and they will give rise to the basal disc of the mature fruiting body (Sternfield and David, 1982). Recently accumulating evidence and corresponding hypotheses have been suggesting that the orientation in the migrating slugs is governed by the internal concentration of NH_3 within the mass of cells (Cotter, 1992, Bonner *et al.*, 1986). As the slug is migrating it also behaves positively to phototaxis, thermotaxis, and rheotaxis, all of these forms of taxis might be related to NH_3 but some of the evidence is tenuous (Bonner, 1993). Light and low humidity can stimulate the next stage of development, culmination (Raper, 1984). During culmination, a hollow cellulose vertical tube is constructed whereby prestalk cells enter and differentiate into stalk cells, through a process involving cell swelling and programmed cell death (Bonner, 1967; Cornillon *et al.*, 1994). As this is occurring, the prestalk cells are lifting prespore cells up towards the top of the mature stalk cells where the prespore cells will differentiate into mature encapsulated spores. Thus, the final structure of the asexual life cycle is the mature fruiting body of dead stalk cells supporting dormant, viable spores.

Tetrahymena pyriformis

Tetrahymena pyriformis is a ciliated protozoon, which lives in fresh water and grows to a length of 40-50 μm along the anterior-posterior axis. *Tetrahymena*'s versatile biology provides extremely useful experimental tools. With a minimum doubling time below 2 hours, it is among the fastest growing eukaryotes. It readily grows in a wide range of media such as totally defined synthetic culture medium, rich nutrient broth, or monobacterial culture. It has powerful active transport mechanisms and can be labeled with virtually any small molecule. The sexual stage can be induced at will, with high efficiency and synchrony.

The life cycle consists of an alteration of haploid and diploid stages (haplophase and diplophase, respectively). Cell reproduction is exclusively by binary fission; it is exclusively asexual and occurs only in the diplophase. Cell division is accompanied by a variety of morphogenetic events that result in the development of duplicate sets of cell structures, one for each daughter.

Conjugation is the sexual stage of the *Tetrahymena* life cycle, which includes no cell reproduction. During conjugation, two cells pair, form a temporary junction, exchange gamete nuclei and generate and differentiate the nuclear apparatus of their sexual progeny (Nanney, 1980).

Achyla ambisexualis

Achyla ambisexualis is a filamentous, usually saprophytic water mold belonging to the Class Oomycetes. The Oomycetes fall into a continuum between the plants and fungi, although they have in the past been classified as true fungi and exhibit various characteristics of this kingdom, they have no close phylogenetic relationship with the fungi. *Achyla* is generally found in aquatic habitats or on moist soil and hence has been grouped into a class of organisms commonly referred to as the water molds. Several species of *Achyla* have been found to be parasitic on fish or fish eggs and may be pathogenic to these organisms (Smith *et al.*, 1994).

Achyla is readily identified as coenocytic aggregations of hyphae. The vegetative mycelium consists of two components, the substrate-penetrating rhizoidal system and the emergent hyphal system. Septa are normally formed in the hyphae only to delimit the organs of asexual reproduction, zoosporangia and gemmae, and the sexual reproductive bodies, the oogonial and antheridial cells (Johnson, 1956).

Euglenophycophyta- The Euglenoids

The green euglenoids, or Euglenales, are single celled, biflagellated protozoa with a longitudinal length of approximately 130 μm . These unique organisms are capable of photosynthesis, having chlorophylls a and b, however excess carbohydrate is stored as the glucose polymer paramylon, not starch as in higher plants. The Euglenales can be sustained with sunlight as their sole energy source, however they are also able to take up

their food by phagocytosis, and can absorb sugars and even proteins through their plasma membrane. It has been found that when members of this species are being grown photosynthetically they will grow even faster when supplied with organic compounds (Mauseth, 1991).

The Euglenoids reproduce asexually by longitudinal binary fission. In cases of environmental stress dormant cysts are formed by all members of the order (Bold and Wynne, 1978).

Chironomus

The genus *Chironomus* of the family Chironomidae is ubiquitous and usually the most abundant insect group in all types of freshwater environments. *Chironomus* is a holometabolous insect; it has four life stages; egg, larva, pupa, and adult. Furthermore, the larval stage passes through four larval instars. During each successive stage the larva becomes increasingly red due to the presence of hemoglobin. The possession of hemoglobin is correlated with the ability of *Chironomus* and related larvae to function in environments with low oxygen concentration (Hudson, 1994).

Proteinases

Proteinases are enzymes that catalyse the hydrolysis of peptide bonds within proteins. Proteinases have been the focus of research in many areas of biology ranging from development to apoptosis. Physiologically, these enzymes have been found to play two general roles; the digestion and the modification of pre-existing proteins.

Four distinct groups of proteinases have been classified, these groups are: the cysteine, serine, aspartic, and metalloproteinases. Each of these groups are differentiated with respect to their catalytic sites, substrate specificities, cofactors and specific natural inhibitors (Bond and Butler, 1987). In spite of the differences in catalytic mechanisms, the enzymes of the four classes share a common property of going through an intermediate (or transition state) in which the normally trigonal carbon of the peptide bond becomes tetrahedral due to the temporary addition of a nucleophile. In the case of the serine and cysteine proteinases, the nucleophile is the serine hydroxyl or the cysteine thiol at the active site. The aspartic and metallo class proteinases utilise a water molecule which acts as the nucleophile in hydrolysis of the peptide bond. The proteolytic reaction proceeds directly in aspartic and metallo proteinases. Alternatively, in the serine and cysteine proteinases an acyl-enzyme intermediate must be hydrolysed by water in a second step during proteolysis (Creighton, 1993).

Cysteine Proteinases

Cysteine proteinases are identified as those proteases which contain cysteine, histidine, and aspartic acid or asparagine residues in the catalytic site (Twining, 1994). These enzymes are commonly found to be lysosomal which catalyze the hydrolysis of peptide, amide, ester and thiol ester bonds. They are active at pH 4.0 to 8.0 with pH 6.0 being their optimal pH. At this pH, a thiolate-imidazolium ion pair is formed between the cysteine and histidine residues, and the aspartic or asparagine residue acts as a stabilizer (Barrett *et al.*, 1994). The classic activation of the cysteine proteinases occurs after a cleavage of their pro-peptide region either by acid hydrolysis or proteolysis by other proteinases. The substrate specificity of the enzymes is for peptides that contain bulky hydrophobic amino acids in their P2 site (Twining, 1994). *In vivo*, the main inhibitor of the cysteine proteinases are the cystatins, however, synthetic inhibitors include iodoacetate, dithiodipyridine, mercuric chloride, and 1-trans-epoxysuccinyl-leucylamido(4-guanidino)butane (E64). An important characteristic of E64 and the related epoxide inhibitors is that they are almost unreactive with low molecular mass thiols such as cysteine and dithiothreitol, so may safely be used in the buffer that activates the cysteine proteinase (Salvesen and Nagase, 1989).

The lysosomal cysteine proteases can be divided on the basis of their amino acid sequence into three groups: the papain, bleomycin hydrolases and the calpains (Berti and Storer, 1995). Most of the cysteine proteinases are endopeptidases, but there are also some exopeptidases as well as some proteins without known catalytic activity. The papain group is the largest and most studied group. Amongst the 90 known members of the family are enzymes from bacteria, fungi, protozoa, and plants, as well as animals.

Notable members of the papain group include, the plant protease papain, *D. discoideum* CP1 and CP2, mammalian cathepsins B, H, and L (Bond and Butler, 1987).

The main *in vivo* function of the cysteine proteinases in lysosomes is the degradation of proteins that have been taken up by the cell or originated from other compartments of the same cell. The end-products of the acidic overall proteolysis by cysteine proteinases and all other lysosomal proteolytic enzymes are amino acids and dipeptides which diffuse through the lysosomal membrane and are available again for protein synthesis in the cell.

Proteinases of the Dictyosteliaceae

In cellular slime moulds proteinases are likely to play a number of key roles during the life cycle. These include participation in the digestion of nutrients during the vegetative phase, degradation of endogenous proteins to release amino acids for developmental protein synthesis and energy metabolism, and possibly during germination.

Dormant spores of *D. discoideum* appear to contain only small amounts of cysteine proteinase activity compared to that found in extracts of nascent amoebae using gelatin-SDS-PAGE, or *in vitro* assays using nitroanilide or peptidyl amidomethylcoumarin substrate (North *et al.*, 1990 a,b, 1996; North and Cotter, 1991).

During the spore swelling stage of germination, extracts of *D. discoideum* begin to show a major cysteine proteinase activity with an apparent molecular mass of 48 kDa

(ddCP48), and minor activity in bands identified as ddCP43 using gelatin SDS-PAGE (North *et al.*, 1990b). Substrate specificity tests reveal that ddCP48 of spores more closely resembles mammalian cathepsins L and S, rather than cathepsin B, since the enzyme is very active against the fluorogenic substrate, Boc-Val-Leu-Lys-NMec, but inactive against Z-Arg-Arg-NMec (North *et al.*, 1996).

A 1 minute treatment of the minigel slab with 10% (v/v) glacial acetic acid, after electrophoresis but before incubation at pH 4.0, reveals that the cysteine proteinase (ddCP48) of germinating spores is present also in dormant spores (North *et al.*, 1996). Thus, spores of *D. discoideum* possess one or more cryptic cysteine proteinases, packaged at some point during the sporulation process, which are capable of being acid activated in a polyacrylamide gel. Use of two-dimensional gel electrophoresis before and after acid-activation, does not reveal any change in the apparent molecular mass of the enzymes (North *et al.*, 1996). This suggests that a conformational change in the enzymes is responsible for the activity changes detected (Cotter *et al.*, 1992; North *et al.*, 1996). Additional treatments, such as incubation of an acid treated gel with ammonium chloride at pH 9.5 – 10.5 for 1 minute, are capable of deactivating the cysteine proteinases of the dormant and germinating spores (Cotter *et al.*, 1997). Following this treatment, the gel can be treated with 10% acetic acid for a second time resulting in the reappearance of the cysteine proteinase activity (Cotter *et al.*, 1997).

Using the irreversible cysteine proteinase inhibitor E64 (Cotter *et al.*, 1997) provides further evidence that indicates conformational change is responsible for the change in observed activity. This suicide inhibitor inhibits cysteine proteinases that are already active; yet extracts of dormant spores do not react with this inhibitor until after

their cysteine proteinases are acid activated. The inhibitor can be used to titrate the relative amount of active versus inactive cysteine proteinase in any cell extract (Cotter, *et al.*, 1997).

After emergence of amoebae begins (3 hours), additional proteinases (ddCP38 and ddCP30) are observed. These post-emergence cysteine proteinases are similar to the vegetative enzymes, and also demonstrate the phenomenon of reversible activation with acetic acid/ammonia treatments. The enzyme activity is a mixture of cathepsin L/S-like activity and yeast *kex2-like* activity (North *et al.*, 1990b; 1996). The timing and intensity of enzyme activity is related to the method of spore activation, suggesting that these enzymes may not have a developmental role to play in spore germination (North *et al.*, 1990b). In support of this it has been shown that spore wall protein degradation does not occur during germination of wild type spores (West and Erdos, 1990).

Contrary to cysteine proteinase activity, an aspartyl proteinase (ddAP58) present in dormant spore extracts (North *et al.*, 1990b) is unaffected by acetic acid (North *et al.*, 1996) or ammonia treatments (Cotter *et al.*, 1997). Its activity decreases during spore germination, but in general, aspartyl proteinase activity does not change greatly throughout the entire life cycle of *D. discoideum* (North *et al.*, 1990b). The developmental roles of the aspartyl proteinases remain unclear at this time.

Giorda and Ennis (1987) analyzed the life cycle for ubiquitin expression using a cDNA probe (pLK229). They found six different species of mRNA with the highest level of expression during spore germination. The next highest level of expression was during multicellular differentiation at 16 and 20 hours of development. Surprisingly, mRNA for ubiquitin was low during vegetative growth.

Giorda and Ennis (1987) concluded that there may be more protein degradation occurring (in the cytosol) during spore germination than during other developmental stages. The proteasomes required to degrade ubiquitinated proteins were isolated recently (Schauer *et al.*, 1993). The proteasomes were found in both the cytoplasm and in higher concentrations in the nucleus. There was neither evidence for subunit nor proteolytic activity changes in the proteasomes during development.

Experiments with classical proteinase inhibitors did not reveal the mechanism of proteolytic activity unequivocally. However, it was suggested that the proteasome might contain atypical serine or cysteine proteinases, or possibly an entirely new mechanism (Schauer *et al.*, 1993). A 26S protease subunit homolog has been cloned and partially disrupted in *D. discoideum* (Cao and Firtel, 1995). The homolog is part of a very large family of genes having many different functions. Complete elimination of its activity is lethal. It is not certain at this time if the gene codes for a component of the *D. discoideum* proteasome (Cao and Firtel, 1995).

After spore germination, the pattern of cysteine proteinases depends upon the growth medium (North, 1988). For instance, when AX2 cells are grown in axenic medium, the major cysteine proteinases observed by gelatin-SDS-PAGE (the A pattern) are ddCP54, ddCP51, ddCP45, ddCP42, ddCP41 and ddCP30 (North, 1988; North *et al.*, 1988; 1990a; 1996); a minor activity of ddCP38A is also present. The addition of bacteria shifts the A pattern to a B pattern over several hours, to reveal predominantly ddCP48, ddCP43, ddCP38B, and ddCP30 in AX2 cells (North, 1988). The ddCP30 activity, found in both patterns A and B, is present in relatively high amounts. However, its resolution is poor on gelatin containing gels. It was purified by North and Whyte

(1984) and shown to be similar in its activity profile to mammalian cathepsins L and S. A mutant strain, HL244, expresses only the A protein pattern, and does not respond to the presence of bacteria by switching to the B pattern (North, unpublished results). The changes in banding pattern in AX2 cells do not affect total cysteine proteinase activity levels (North and Cotter, 1991), and were initially believed to result from a possible post-translational modification of glycosylation.

It is during vegetative growth that the net activity of the multiple cysteine proteinases reach their highest observable levels (North, 1988; North *et al.*, 1988, 1989, 1991; Mehta *et al.*, 1995). This suggests that the major function for many of the enzymes is nutritional. Axenic cell growth is accompanied by the secretion of a number of known lysosomal enzymes, such as acid phosphatase, α -mannosidase, β -glucosidase, trehalase, and β -N-acetylglucosaminidase (Dimond *et al.*, 1981; Seshadri *et al.*, 1986). The cysteine proteinases synthesized in axenically growing cells are differentially secreted.

It is ddCP42 which accumulates to the greatest extent in axenic medium (North, 1982; North *et al.*, 1990a; Champion, 1995). This enzyme is most effective in hydrolyzing Bz-Pro-Phe-Arg-Nan, and would appear to be similar to mammalian cathepsins L and S in activity. Enzymes, possibly cysteine proteinases, with activity towards the substrate Z-Tyr-Lys-Arg-Nan (yeast *kex2*-like) are not secreted during vegetative growth (North *et al.*, 1990a; North and Cotter, 1991). Addition of the bacteria shifts the A pattern to the B pattern with the secretion of a portion of the newly formed ddCP38B. The sequencing of the N-termini of ddCP42 and ddCP38B, by Champion (1995) in K. Williams' laboratory clearly demonstrates that, like CP4 (*cprD*) and CP5 (*cprE*), they are products of two separate genes related to the CP1 (*cprA*), CP2 (*cprB*),

and CP3 genes, transcribed during multicellular development (Williams *et al.*, 1985; Pears *et al.*, 1985; Presse *et al.*, 1986a,b). The ddCP38B proteinase secreted by cells growing on bacteria may be one of the isoform products of the cysteine proteinase genes (*cprF* or *cprG*). Antibodies recognizing GlcNAc-1-P clearly show that the products of CP4 (*cprD*), CP5 (*cprE*), CP6 (*cprF*), and CP7 (*cprG*) are four separate, but related, cysteine proteinases (Souza *et al.*, 1995; Ord *et al.*, 1997).

A role for cysteine proteinases in the processing of vegetative lysosomal enzymes has been investigated in axenic cells by Cardelli *et al.* (1990). Raising the average pH of lysosomal/endosomal compartments to pH 6.4 with NH₄Cl results in the accumulation of newly synthesized intermediate forms of α -mannosidase and β -glucosidase which are very slowly processed to mature forms (see Cardelli *et al.*, 1990). Use of inhibitors such as leupeptin and antipain, prevents processing of precursor and intermediate forms of α -mannosidase and β -glucosidase. The cysteine proteinase inhibitor, Z-Phe-AlaCHN₂ prevents the processing of intermediate forms to mature forms of the enzymes (Cardelli *et al.*, 1990). These results suggest that complete processing of lysosomal enzymes depends on an acidic environment, and that it is carried out by two classes of cysteine proteinases residing in different intracellular compartments (Cardelli *et al.*, 1990).

Studies by Dr. Michel Satre and Dr. Hud Freeze strongly suggest that specific classes of lysosomal enzymes may be segregated into different lysosomal/endosomal compartments, and may make contact with endocytosed or phagocytosed material at different rates. Satre and coworkers show that a 34 kDa cysteine proteinase and a 46 kDa aspartyl proteinase are already localized in endocytic compartments after a 3-minute iron dextran pulse. Analysis of vesicles after a 10 to 40 minute chase period shows that the

amount of the 34 kDa cysteine proteinase decreases along the endocytic pathway, but the 46 kDa aspartyl proteinase is still present in post-lysosomal compartments. The cysteine proteinase level is high in vegetative cells, but progressively disappears during development. In contrast, the amount of the aspartyl proteinase remains constant throughout the life cycle. This pattern correlates with the regulation of cysteine and aspartyl proteinase activities mentioned earlier (North *et al.*, 1988, 1996; North and Cotter, 1991).

Dr. Hud Freeze and coworkers present evidence that lysosomal proteins with different carbohydrate modifications sort to functionally distinct endo-lysosomal compartments. They have cloned four vegetative cysteine proteinase genes, and have shown that the enzyme products are phosphoglycosylated having GlcNAc-1-P linked to serine. All previously studied lysosomal enzymes, such as α -mannosidase, β -N-glucosidase, and β -N-acetylglucosaminidase, contain Man-6-P in an unusual methylphosphodiester linkage. Most important is the fact that each group of enzymes resides in distinct vesicles. Vesicles with the cysteine proteinases containing GlcNAc-1-P fuse with bacteria-loaded phagosomes in less than three minutes after ingestion of the bacteria, but vesicles containing lysosomal enzymes with Man-6-P fuse only after about fifteen minutes. After the bacteria are degraded, the two classes of enzymes are again segregated (Souza *et al.*, 1995).

At the end of vegetative growth, but before aggregation is well underway, lysosomal enzymes with cathepsin-like activity are secreted by the participating cells along with β -N-acetylglucosaminidase and α -mannosidase (Rossomando *et al.*, 1978). Incubation of washed cells in buffer is a convenient method to collect the secreted

intracellular components over a 3 hour time period; such conditioned medium was shown to stimulate the rate of cell aggregation in plastic petri dishes (Rossomando *et al.*, 1978).

After further experiments, Fong and Bonner (1979) also concluded that proteolysis is a necessary step in the early differentiation of cells in preparation for aggregation, and based on inhibitor studies with leupeptin and antipain, that proteinases with cathepsin-like activity were responsible. A recent study by Simms and Katz (unpublished results), has extended these findings to show that cysteine proteinase inhibitors temporarily block aggregation. The continued secretion of proteinases during starvation and development (North, 1982; North *et al.*, 1988; 1990a) might be the reason why aggregation eventually occurs in the presence of cysteine proteinase inhibitors.

The incorporation of carbohydrates, such as 100 mM sucrose, into a starvation buffer greatly enhances the final secretion yield of typical lysosomal enzymes, including some of the cysteine proteinases (Crean and Rossomando, 1979; Seshadri *et al.*, 1986; Klein *et al.*, 1989; North *et al.*, 1990a). The cysteine proteinase ddCP42 of axenic cells is the prominent enzyme secreted, but contributions to the extracellular BzPFRase activity also were made by ddCP41 and ddCP30 (North *et al.*, 1990a). On agar plates, Mehta *et al.* (1995) actually detected a burst in the multiple forms of cysteine proteinase I immediately after clearing of the bacterial lawn. The activity then declines 2 -20 fold after the onset of multicellular development.

Fong and Rutherford (1978) found a sharp decrease in cysteine proteinase activity between aggregation and pseudoplasmodia migration, and only a gradual decrease in aspartyl proteinase activity during this time. The decrease in cysteine proteinase activity was greater in cells destined to become spores, than in those that became stalk cells.

Starvation of cells in shaken suspensions in the presence of cAMP neither changed the pattern, nor the rate, of cysteine proteinase secretion observed without cAMP (North *et al.*, 1988). However, cAMP produced during aggregation induces transcription of two cysteine proteinase genes known as CP1 (*cprA*) (Williams *et al.*, 1985), and CP2 (*cprB*) (Pears *et al.*, 1985) also known as *pst-cath* (Datta and Firtel, 1987), by 10 – 12 hours.

A truncated third gene, CP3, is expressed at a later stage of development (18 hours) (Presse *et al.*, 1986a,b).

New cysteine proteinase activities are observed after aggregation, especially when axenic cells are allowed to aggregate on filter pads. The A pattern of axenic cells shifts to the B pattern so that a 48 kDa enzyme is observed after 6 to 8 hours of development (North *et al.*, 1988, 1996). A cysteine proteinase of approximately 45 kDa may persist, or be replaced, with an enzyme of similar size (North *et al.*, 1988). Nevertheless, the overall trend is a decrease in activity when SDS-PAGE is used to detect cysteine proteinase activity with gelatin hydrolysis or peptidyl amidomethylcoumarins (North *et al.*, 1988; 1996; North and Cotter, 1991). After 12 hours of development, a cysteine proteinase with a relative molecular mass of 60 kDa is detected by acid activation, whereas the activity of a 24 kDa is enhanced by acid activation (North *et al.*, 1996).

These secreted enzymes may be required to orient a slug toward the soil surface in preparation for culmination. Bonner (1993) has shown that the ammonia avoidance reaction of the prestalk region of a slug may be simulated by the addition of papain in a polyacrylamide bead to one side of a slug causing the slug to turn away from the enzyme diffusing from the bead. Antipain in a polyacrylamide bead causes a slug to turn toward the inhibitor (Bonner, 1993). This suggests that cysteine proteinases secreted from the

slug are involved in the turning. There is general agreement that the prestalk region of a slug contains acidic vesicles in contrast to the prespore region of the slug, and that the prime source of ammonia is degradation of protein by prestalk cells (see Gross, 1994). Internally, peptidases and amino acid degrading enzymes are active at this time, and may be responsible for the production of ammonia (Firtel and Brackenbury, 1972; Pamula and Wheldrake, 1996). The inhibition of aggregation, the slug avoidance reaction, and the blockage of culmination by ammonia/ammonium ion result from the diffusion of ammonia into an intracellular acidic compartment thereby raising its pH (Davies *et al.*, 1993).

Thus far, the CP1, CP2, and CP3 genes have not been assigned to the cysteine proteinase activities of the multicellular stages of development. A phylogenetic analysis of the major family of cysteine proteinases of eukaryotes reveals that this family consists of numerous gene duplications (Hughes, 1994). Certain cysteine proteinases from plants are homologous to CP1 (*cprA*) while some animal cysteine proteinases are homologous to CP2 (*cprB*) of *D. discoideum* (Williams *et al.*, 1985; Pears *et al.*, 1985; Presse *et al.*, 1986a,b). This indicates that the genes were duplicated prior to the divergence of *Dictyostelium* from plants and animals (Hughes, 1994). The prestalk CP2 gene is homologous to human cathepsins L and S in sequence (Hughes, 1994).

At the end of multicellular development, when the fruiting body is complete, mRNA for CP4 (*cprD*) and CP5 (*cprE*) is again detected, but in very low levels (Souza *et al.*, 1995).

Tetrahymena Proteinases

Previous work has shown the presence of three proteinases in *Tetrahymena pyriformis*, their requirement for cysteine and EDTA indicated that they were cysteine proteinases (Banno *et al.*, 1982). The majority of the cysteine proteinases in *Tetrahymena* has been localised within the subcellular particles (Muller, 1972; Levy *et al.*, 1976; Blum 1976; Banno *et al.*, 1982). As found in many other lower eukaryotic systems, the total internal proteinase activity in *Tetrahymena* at acidic pH was greater than that detected at alkaline pH (North and Walker, 1983). Recently, one of these cysteine proteinases has been isolated and purified, it is cathepsin-L like, has a molecular mass of approximately 28 kDa, and has been designated as tetrain (Suzuki *et al.*, 1997).

Achlya Proteinases

The proteinases of *Achlya* have been of interest because of their correlation with the facultatively parasitic nature of this organism. It has been hypothesized that secreted proteinases may play a role in the disease process caused by *Achlya* and related oomycetes.

Achlya ambisexualis produces extracellular proteinases only when a protein or related substrate is present as an inducer (Hill and Pott, 1997). A proteolytic pattern of these proteinases on gelatin-SDS-PAGE has also been performed that indicates activities with molecular masses of approximately 26, 48 and 58 kDa (Hill and Pott, 1997).

Proteinases of the Euglenales and Chironomus

Although it is widely believed that both the Euglenales and Chironomus utilize proteinases, no published work could be referenced in this area.

Objectives

A major objective of this work was to prove Dr. J.T. Bonner's hypothesis that the extracellular matrix of the pseudoplasmodium of *Dictyostelium discoideum* contains cysteine proteinases.

A second objective was to confirm the conservative nature of the acid activatable cysteine proteinase trait amongst the Dictyosteliaceae and to assess it in other lower eukaryotes in hopes of finding this phenomenon in another species.

A minor objective was to illustrate that the *D. discoideum* spore proteinases ddCP43 and ddCP48 are independent enzymes and not subunits of a multimeric structure.

METHODS AND MATERIALS

Growth of Members of the Dictyosteliaceae for Sorocarp Formation

Various members of the Dictyosteliaceae were utilized for this study. Spores taken from fruiting bodies were transferred by a sterile loop into 10 to 20 mL of autoclaved 10 mM potassium phosphate buffer at pH 6.5 (10 mM KPi). Two loops of *Escherichia coli* B/r were added to the spores and the suspension was mixed on a Vortex mixer. A 1.5 mL aliquot of the mixture was transferred to glucose-salts agar plates and the plates were swirled until the mixture covered the surface of the plates. Alternatively, SM/2 medium was also used in place of the glucose-salts medium. The plates were incubated at 23°C in the presence of light and after 3-4 days fruiting bodies formed.

The production of sorocarps of *Polysphondylium pallidum* is a modification of the above procedure, which is necessary to prevent the production of microcysts and to encourage the proliferation of fruiting bodies. *E. coli* bacteria were grown on a 1/10 LP plate until a full lawn covered the plate, next approximately 10 sori of *P. pallidum* were harvested and suspended in 1.0 mL of sterile, distilled water which was then vortexed. The spore suspension was then spread over the bacterial lawn, a high density of fruiting bodies was formed within 5-7 days.

To remove the mature sori a 1mm plastic tube connected to vacuum line was utilized which deposits the spores into a small volume of chilled water. Any stalks present with the spores were separated by light vortexing in a standard size glass test tube. This procedure allowed the spores to settle to the bottom while the stalks adhered to the sides of the test tube.

In situ Analysis Of Proteolytic Activity of the Pseudoplasmodia

D. discoideum spores (strain SG1) were harvested from glucose salts or SM/2 agar plates into a 25 mL beaker of room temperature, autoclaved, distilled water. The spores were then washed by centrifuging them out of solution and resuspending them in 10mM KPi; this process was repeated three times to ensure that the spores were free of autoinhibitors. A high concentration of spores ($>1 \times 10^8$ cells/mL) was then inoculated at one side of a 2-3mm thick, 0.2% protein (gelatin) infused non-nutrient agar (1.5%) petri-plate in a single 4 cm line. The plates were then placed in the dark with a pinpoint light source illuminating the opposing side of the petri-plate. After approximately 24 hours the pseudoplasmodia phototropically migrated toward the light source

After the pseudoplasmodia had migrated across the plate, they were harvested and their slime trails were cleared with a stream of distilled water. The plate was then stained with Coomassie Brilliant Blue for 60 minutes, and then destained in a solution of 10% acetic acid (v/v) and 5% methanol (v/v).

In situ Analysis of Proteolytic Activity of *Achlya ambisexualis* colonies

An 8mm plug of a mature colony of *A. ambisexualis* was transferred to a petri plate of agar-stabilized Barksdale's media containing 0.2% of one of the following proteins: collagen, albumin, casein, peptone, or gelatin. After colony establishment, the medium was washed with the protein stain, Coomassie Brilliant Blue. Hydrolyzed protein is visible as a white clearing against a blue protein stained background.

Breakage of Cells

Approximately 50 μL of 10 mM KPi and 200 μL of small glass beads (212-300 μm diameter, Sigma, St. Louis, MO) were added to all spore samples after one freeze thaw cycle. In order to lyse larger cells, such as the ciliates and the myxamoebae, 200 μL of mixed glass beads were utilized (212-300 μm and 1000 μm diameters, Sigma, St. Louis, MO). All samples were vortexed with the glass beads for 5 minutes, and the homogenates were viewed under phase contrast microscopy for disrupted cell membranes. The homogenates were then collected using a micropipette and dispensed into microcentrifuge tubes. The samples were frozen and stored again in a 95% ethanol bath at -20°C .

The mycelia of *Achlya ambisexualis* were lysed by using a mortar and pestle with Ottawa sand and a small volume of 10 mM KPi buffer. The mycelial extract was centrifuged and the resulting supernatant sample was frozen and stored at -20°C .

Protein Assay on Samples

Protein Assays were performed in microtitre wells according to the Bradford method (Bradford, 1976) with bovine serum albumin as the standard.

Gelatin-SDS-Polyacrylamide-Gel-Electrophoresis

Gelatin-SDS-PAGE was performed using the Bio-Rad (Mississauga, ON) Mini Protean II Electrophoresis System to separate and identify cysteine proteinases. Gelatin, 0.2% (w/v), was used as a substrate for the proteinases on a 7.5 % or 10 % polyacrylamide separating gel (Appendix A, Table 1.0). Once the separating gel was polymerized (30 minutes), it was placed in the refrigerator at 4°C over night. A 4.0 % acrylamide solution (Appendix A, Table 2.0) containing a Teflon comb was allowed to polymerize and form a stacking gel above the separating gel. The thickness of both gels was 0.75 mm. The samples were prepared by adding 4 parts of sample to 1 part of 5 x sample buffer. Once the stacking gel polymerized (45 minutes), the combs were removed and the samples were loaded into the wells using a micropipette or Hamilton syringe. Electrophoresis of the proteinases was carried out at 4°C and at a constant voltage of 60 volts until the dye front reached the separating gel (30 minutes). Then the voltage was increased to 110 volts for the remainder of the electrophoresis (45 - 60 minutes). After electrophoresis, the gels were washed with 2.5% Triton x-100 (v/v) for 30 minutes to remove the SDS, and followed by an incubation in 0.033M sodium acetate pH 4.0 with 1 mM DTT for 18-20 hrs at room temperature. Gels were then stained with a solution of Commassie Brilliant Blue for 60 minutes, and then destained in a solution of 10% acetic acid (v/v) and 5% methanol (v/v). Gels were dried at 23°C for 24 hours between two BioGelWrap membrane sheets on a gel drying frame set. High molecular mass standard markers (Sigma, St. Louis, MO) were previously loaded onto one of the lanes of the gels and after the gels were dried these markers were used in determining the

relative molecular mass of the proteinases. Primarily, the R_f (relative to the dye front) values of the standard markers were calculated and plotted against the log of their respective molecular mass. Secondly, this graph and the calculated R_f values of the proteinases were used in determining their relative molecular mass.

Gelatin-Native-Polyacrylamide-Gel-Electrophoresis

Gelatin-Native-PAGE was performed using the Bio-Rad (Mississauga, ON) Mini Protean II Electrophoresis System to separate and identify cysteine proteinases. Gelatin, 0.2 % (w/v), was used as a substrate for the proteinases on a 7.5 % or 10 % polyacrylamide separating gel (Appendix A, Table 3.0). Once the separating gel was polymerized (30 minutes) it was placed in the refrigerator at 4°C over night. A 4.0 % acrylamide solution (Appendix A, Table 4.0) containing a Teflon comb was allowed to polymerize and form a stacking gel above the separating gel. The thickness of both gels was 0.75 mm. The samples were prepared by adding 4 parts of sample to 1 part of 5 x sample buffer. Once the stacking gel polymerized (45 minutes) the combs were removed and the samples were loaded into the wells using a micropipette or a Bradford syringe. Electrophoresis of the proteinases was carried out at 4°C and at a constant voltage of 60 volts until the dye front reached the separating gel (30 minutes). Then the voltage was increased to 110 volts for the remainder of the electrophoresis (45 - 60 minutes). After electrophoresis, the gels were incubated in 0.033M sodium acetate pH 4.0 for 18-20 hrs at room temperature. Gels were then stained with a solution of Commassie Brilliant Blue

for 60 minutes, and then destained in a solution of 10% acetic acid (v/v) and 5% methanol (v/v). Gels were dried at 23°C for 24 hours between two BioGelWrap membrane sheets on a gel drying frame set.

Gelatin-Nonreducing-SDS-PAGE

Experimental methodologies are the same as for the gelatin-SDS-PAGE protocols described above with the following variations. All lanes except the lane containing the molecular mass markers are loaded with identical sample. The sample buffer loaded into the outermost lanes of the gel (i.e. lanes 1,2,3,8,9,10) is identical to the standard gelatin-SDS-PAGE technique and contains the reducing agent β -mercaptoethanol. The sample buffer loaded into the innermost lanes of the gel (i.e. lanes 4,5,6,7) lacks the reducing agent β -mercaptoethanol. The mobility of polypeptides in the innermost lanes is then compared to that of the outermost lanes. If the mobility of the innermost lanes is greater relative to that of the outermost lanes intra-disulfide bonding is indicated. If the mobility of the innermost lanes is less compared to that of the outermost lanes inter-disulfide bonding is indicated. If the mobility of the innermost lanes is the same as that of the outermost lanes then an absence of inter and intra disulfide bonds is indicated (Allore and Barber, 1984).

Acid Treatments

Samples were electrophoresed via PAGE and prior to treatment with 2.5% Triton x-100, the gels were placed into a 1.7 M acetic acid bath at pH 2.1 bath for 60 seconds.

Base Treatments

Samples were electrophoresed via PAGE and prior to treatment with 2.5% Triton x-100, the gels were placed into a 1.7 M ammonium chloride bath at pH 9.5 for 60 seconds.

Acid and Base Treatments

Samples were electrophoresed via PAGE and prior to treatment with 2.5% Triton x-100. The gels were then treated with 1.7 M acetic acid at pH 2.1 for 60 seconds, then placed in H₂O for 3 minutes followed by treatment with 1.7 M ammonium chloride at pH values greater than 9.5 for 60 seconds. Some gels were then placed in H₂O for an additional 3 minutes and then retreated with 1.7 M acetic acid at pH 2.1 for 60 seconds.

Inhibitor Treatments

Inhibitor studies were performed to verify that the proteinases are cysteine proteinases. Inhibitor treatments were performed as described for experimental controls and prior to electrophoresis samples were incubated with 100 μ M E64 for 30 minutes at 23°C. The rest of the procedure followed the same as the control gels. An additional method used for inhibition of the proteinases required placing 50 μ M E64 into the incubation buffer which contained 0.033 M sodium acetate at pH 4.0 and 1 mM DTT after electrophoresis.

96-Well Microtitre Assay

Axenicly grown myxamoebae (strain AX3) were harvested, centrifuged, washed and resuspended in KPi at 1×10^7 cells/ml. An aliquot of 40 μ L of cells was then added to each well in a 96-well microtitre plate. An airtight plastic seal was then fastened to the top of the plate and development was allowed to proceed. After 6 hours the cells had adhered to the bottom of the well, had formed tipless mounds and development was suspended (Cotter *et al.*, 1992). It is believed that development is halted at this point because ammonia produced by the submerged mounds can not diffuse away through the 40 μ L of buffer (Cotter, unpublished results). The 40 μ L of buffer was then removed and the cells were washed with fresh KPi. An additional 20 μ L of buffer was then added to all wells, this lesser volume did not hinder development. All cells synchronously

continued with development and the 20 μ L of KPi, as well as whole cells, could then be harvested at various times in development for analysis by gelatin-SDS-PAGE.

Achlya Samples for SDS-PAGE

A 1 mL volume of *Achlya* spore suspension (Thomas, 1989) was added to 50 mL of liquid Barksdale's (Appendix B) medium plus the addition of 0.2% (w/v) of one of the following nitrogen sources: collagen, albumin, casein, peptone, or gelatin. The flasks were left on a rotary shaker for 120 hours at room temperature

Chironomid Samples

Dr. Jan Ciborowski graciously supplied the samples of 4th instar Chironomids larvae.

Tetrahymena Samples

Tetrahymena pyriformis strain T (ATCC, Manasses, VA) were grown in 1/20 TM media with shaking at 26°C. When the culture had reached stationary phase with a cell

density of approximately 3×10^5 cells/ml the medium was centrifuged at 1200 x g for 5 minutes to remove cells.

Euglena Samples

Samples of *Euglena gracilis* were grown photosynthetically in distilled tap water without shaking at 26°C. Alternatively, samples were grown in the absence of light with approximately 0.2% dissolved gelatin as a protein source. When the culture had reached stationary phase the medium was centrifuged at 1200 x g for 5 minutes to remove cells.

RESULTS

Proteinases Associated with Multicellular Development in

Dictyostelium discoideum.

During multicellular development *D. discoideum* ceases its existence as a single celled amoeba and begins to form the multi-celled pseudoplasmodium. The external proteinases secreted during the specific stages of aggregation and pseudoplasmodium migration were examined. The total observable proteolytic expression, internal and external, was also examined during the complete course of multicellular development in microtitre wells.

Gelatin-SDS-PAGE reveals that pseudoplasmodia as well as aggregating amoebae secreted three cysteine proteinases ddCP60, ddCP38, and ddCP24 (Figure 2 and Figure 5). All three proteinases were observed to be more active in the aggregating myxamoebae stage than the migrating pseudoplasmodia stage; these proteinases were also found to be acid activatable. *In situ* studies (Figure 4 and 6) show that these proteinases are externally active during the movement of both the myxamoebae and pseudoplasmodia. Their respective paths can be visualized as white trails that have been cleared of protein against a dark protein stained background.

Gelatin-SDS-PAGE, and corresponding densitometer analysis, illustrates that the total observable secreted proteinases associated with multicellular development increases in activity during culmination (Figure 9 and 10). However a decrease in proteolytic activity during multicellular development is observed internally.

Figure 2. Gelatin-SDS-PAGE analysis of proteinases secreted from the pseudoplasmodial stage of *D. discoideum*, AX3. (Gel A, control; Gel B, acid treatment). Samples were collected exclusively from the pseudoplasmodial stage during multicellular development in a microtitre well. These are 10% polyacrylamide gels with each lane containing 0.8 µg of protein.

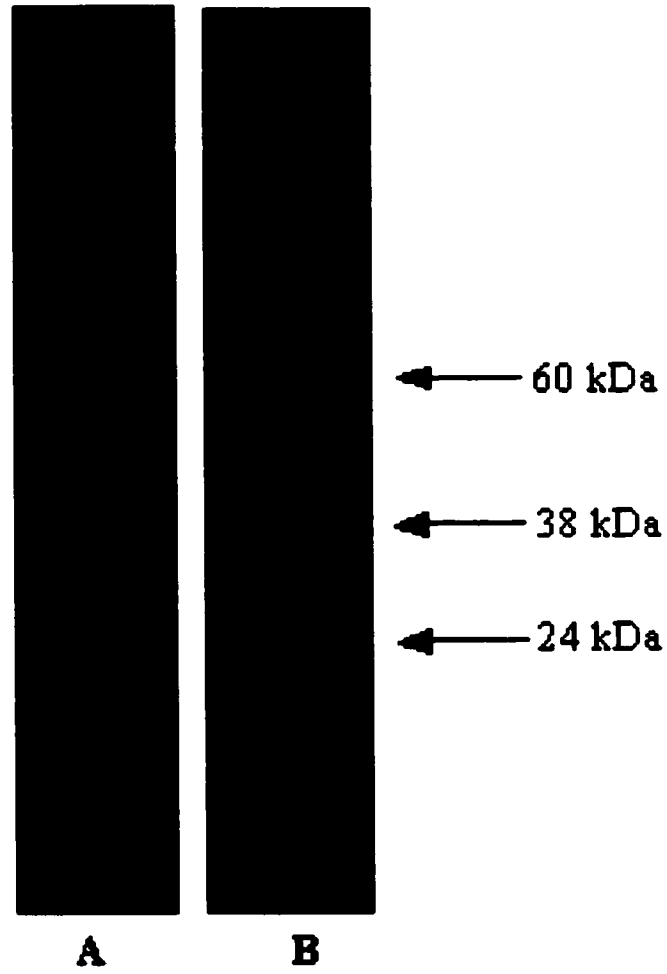


Figure 2.

Figure 3. *In situ* analysis of the proteinases secreted from the pseudoplasmodial stage of *D. discoideum*, SG1, 4 x magnification. The petri plate contains 3 mm thick non-nutrient agar infused with 0.2% gelatin. On the right most side of the petri plate washed spores of *D. discoideum* strain SG1 was inoculated in a 4 cm line. After inoculation the plate was placed in the dark save a pin point light source positioned on the rightmost side of the plate. After 24 hours multicellular development had completed and the cells, in the form of pseudoplasmodia, had phototropically moved towards the light source. The plates were then stained with Coomassie Brilliant Blue, the white trails left by the pseudoplasmodia indicate protein hydrolysis by the pseudoplasmodial proteinases.

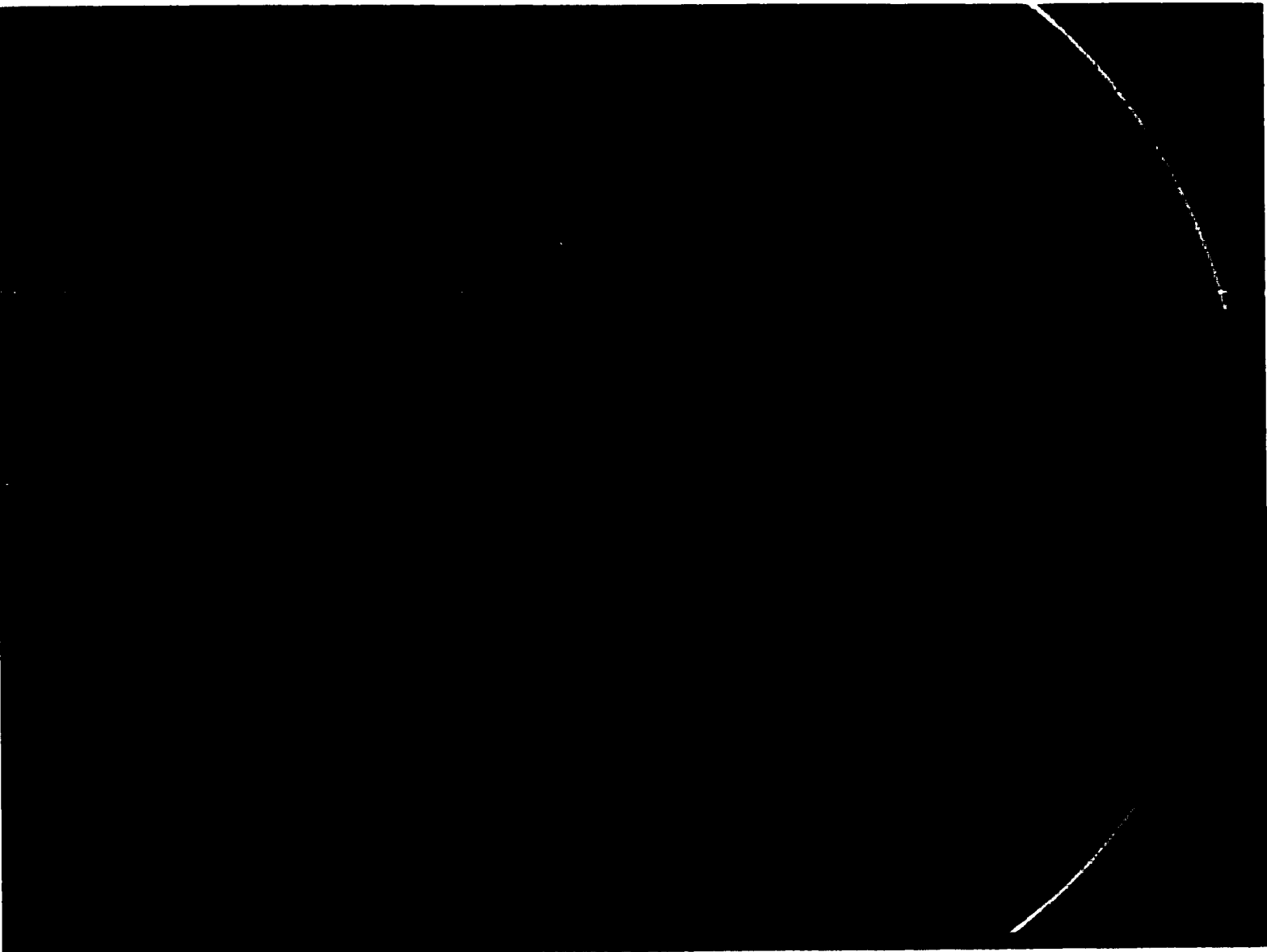


Figure 3.

Figure 4. *In situ* analysis of the proteinases secreted from the pseudoplasmodial stage of *D. discoideum*, SG1. (Figure 4A, 100 x magnification; Figure 4B, 40 x magnification). In Figure 4A the pseudoplasmodial slime trails have been washed off. In Figure 4B the slime trails were not washed off so that the fruiting body at the end of the slime trail could be visualized.

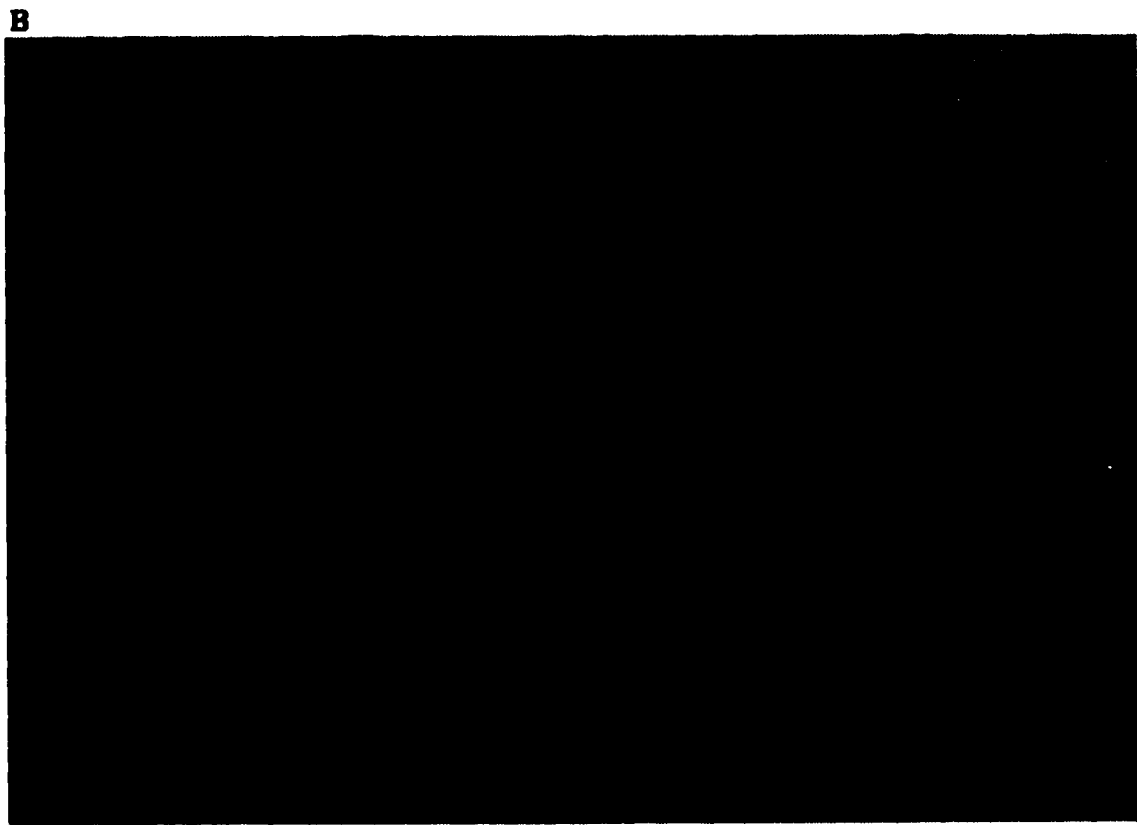
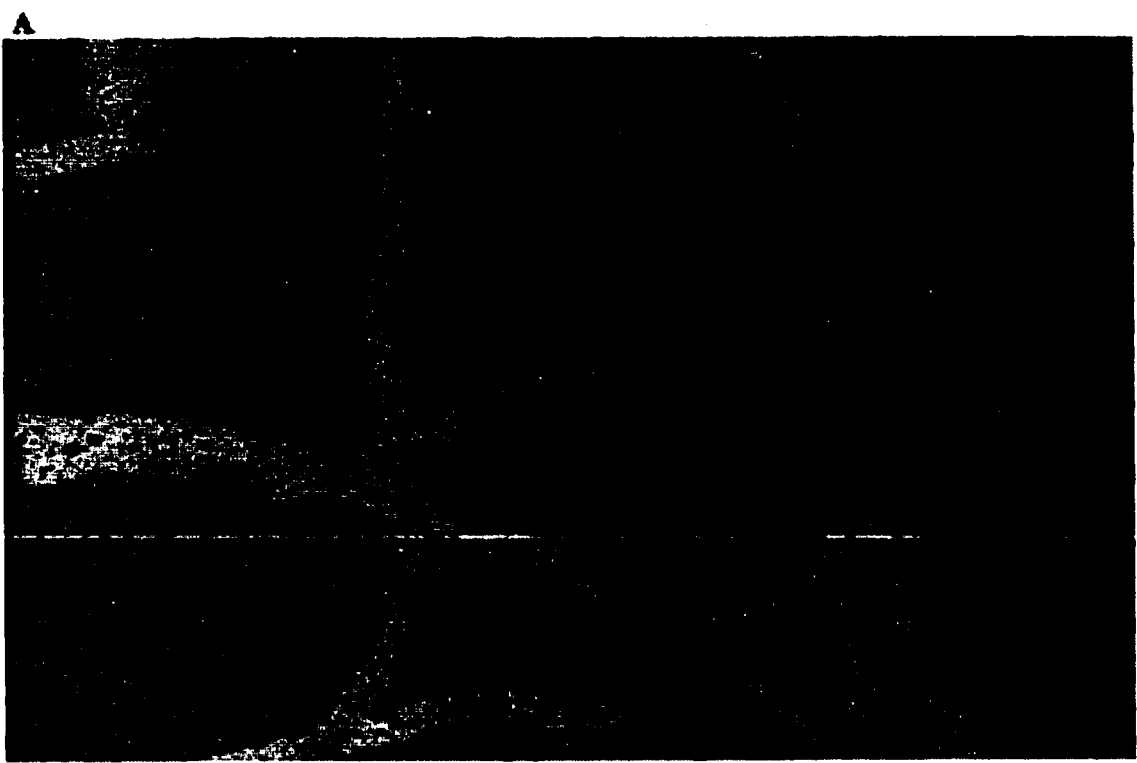


Figure 4.

Figure 5. Gelatin-SDS-PAGE analysis of proteinases secreted from aggregating myxamoebae of *D. discoideum*, AX3. (Gel A, control; Gel B, acid treatment). Samples were collected exclusively from the aggregating myxamoebae stage during multicellular development in a microtitre well. These are 10% polyacrylamide gels with each lane containing 0.8 μ g of protein.

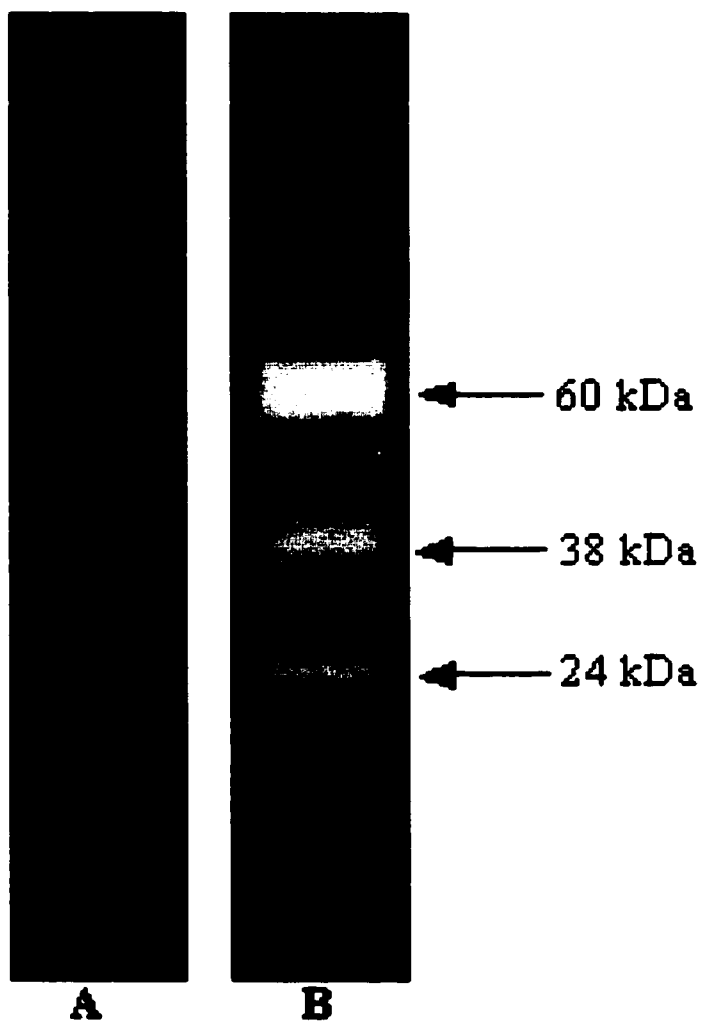
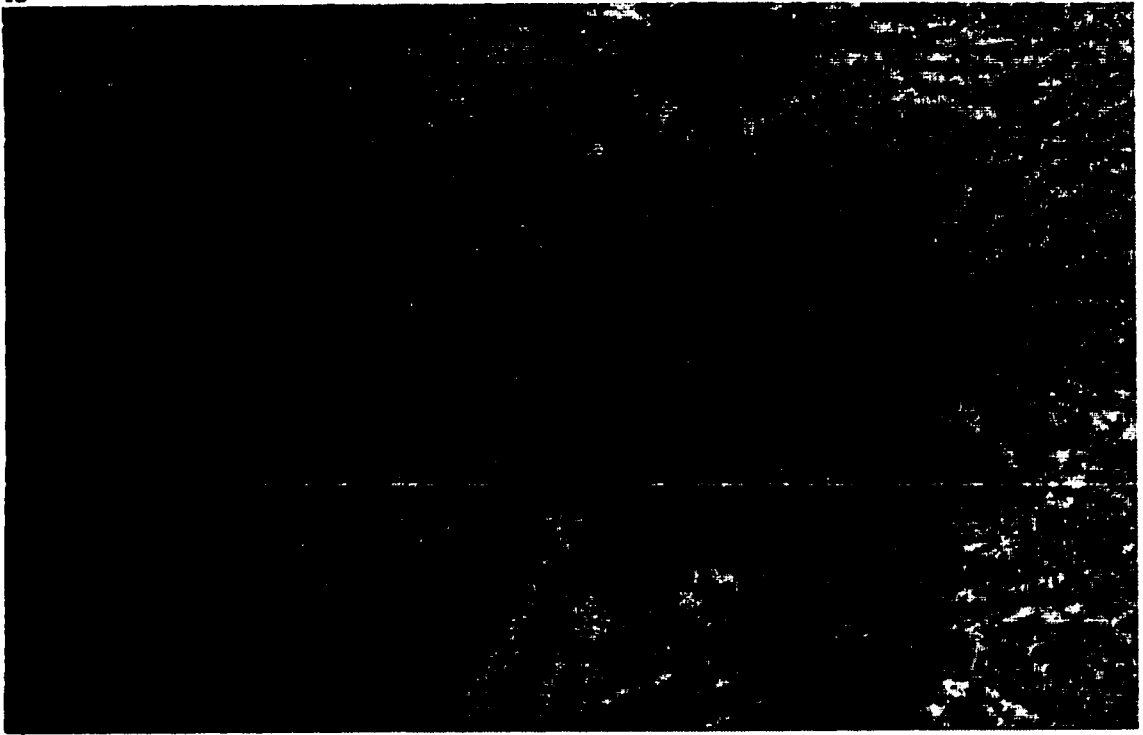


Figure 5.

Figure 6. *In situ* analysis of the proteinases secreted from the aggregating myxamoebae of *D. discoideum*, SG1, 40 x magnification. (Figure 6A, 40 x magnification; Figure 6B, 100 x magnification). This observation of aggregating myxamoebae is performed on a petri plate that contains 3 mm thick non-nutrient agar infused with 0.2% gelatin. Here washed spores of *D. discoideum* strain SG1 were inoculated. After inoculation the plate was placed in the dark and allowed to proceed through multicellular development. After 24 hours multicellular development had completed. The plates were then stained with Coomassie Brilliant Blue, the white radiating lines indicate protein hydrolysis by myxamoebal proteinases as they are aggregating to form a multicelled structure.

A



B

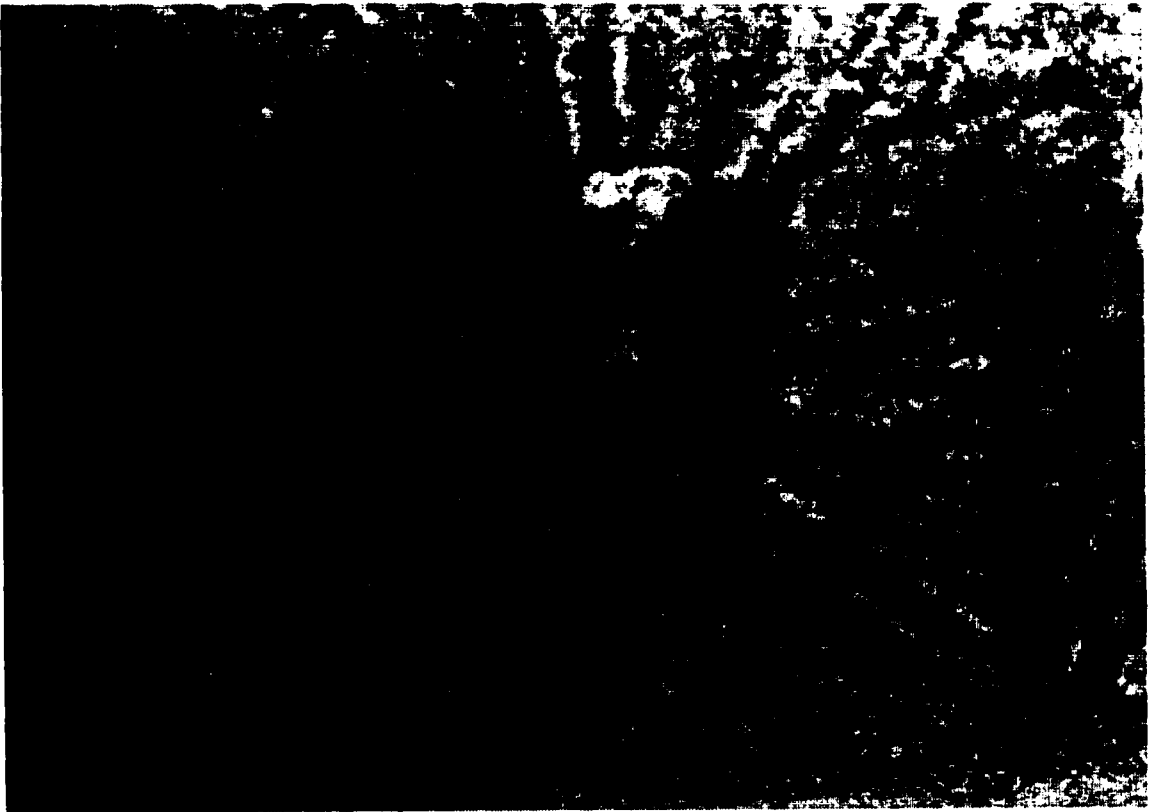


Figure 6.

Figure 7. Gelatin-SDS-PAGE analysis of the proteinases secreted during the multicellular development of *D. discoideum*, AX3. (Gel A, control; Gel B, acid treatment). Samples were collected at various times of multicellular development in a 96-well microtitre plate. During multicellular development the cells were covered with 20 μ l of KPi buffer. This buffer that was analyzed for secreted proteinases every four hours during multicellular development (*Lane 1*, 0 hr; *Lane 2*, 4 hr; *Lane 3*, 8 hr; *Lane 4*, 12 hr; *Lane 5*, 6 hr; *Lane 6*, 20 hr; *Lane 7*, 24 hr). These are 7.5% polyacrylamide gels with each lane containing 0.8 μ g of protein.

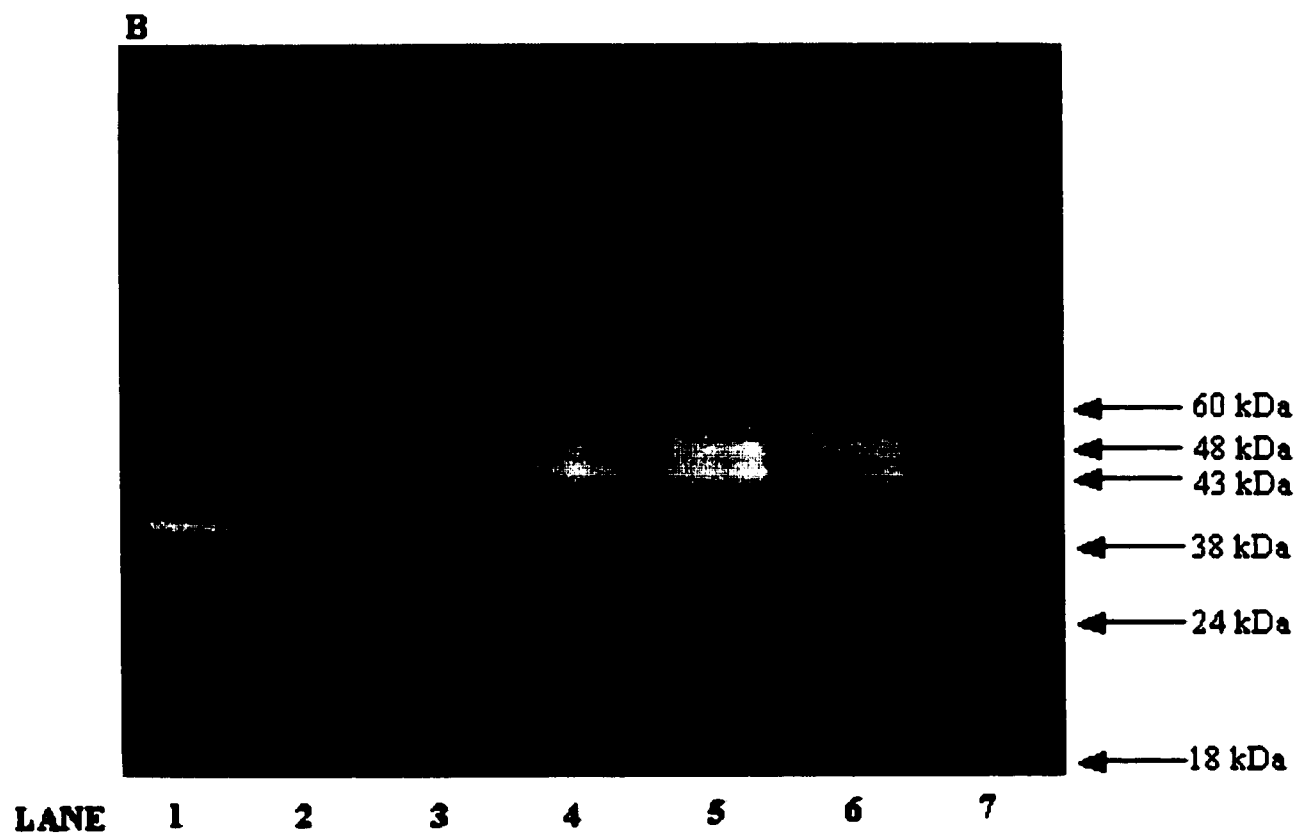
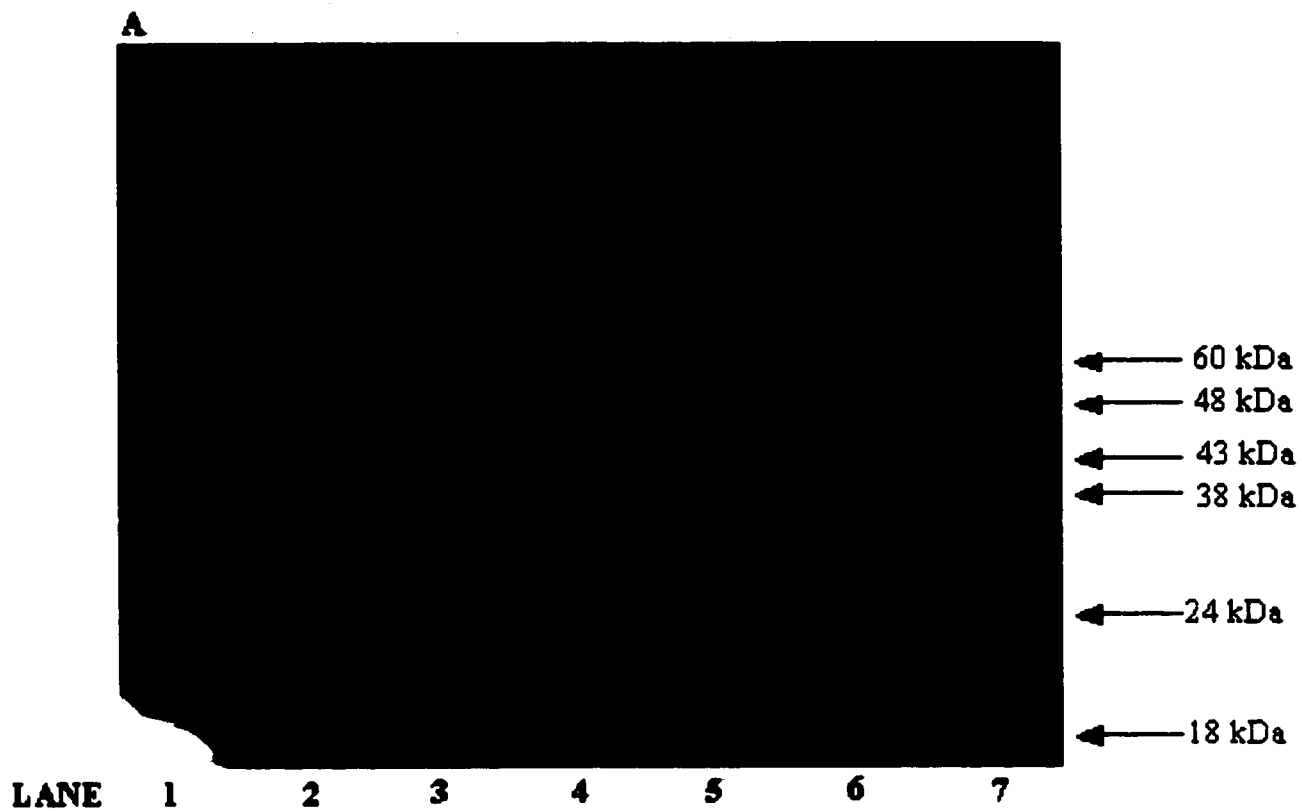


Figure 7.

Figure 8. Densitometer analysis of the proteinases secreted during the multicellular development of *D. discoideum*, AX3. The total observable proteolysis through secreted proteinases during multicellular development in Figure 7 is analyzed by a densitometer (Bio-Rad, Mississauga, ON). The optical density (O.D.) of every square mm of each lane in Figure 7 is read and compared to a blank lane not containing proteinases.

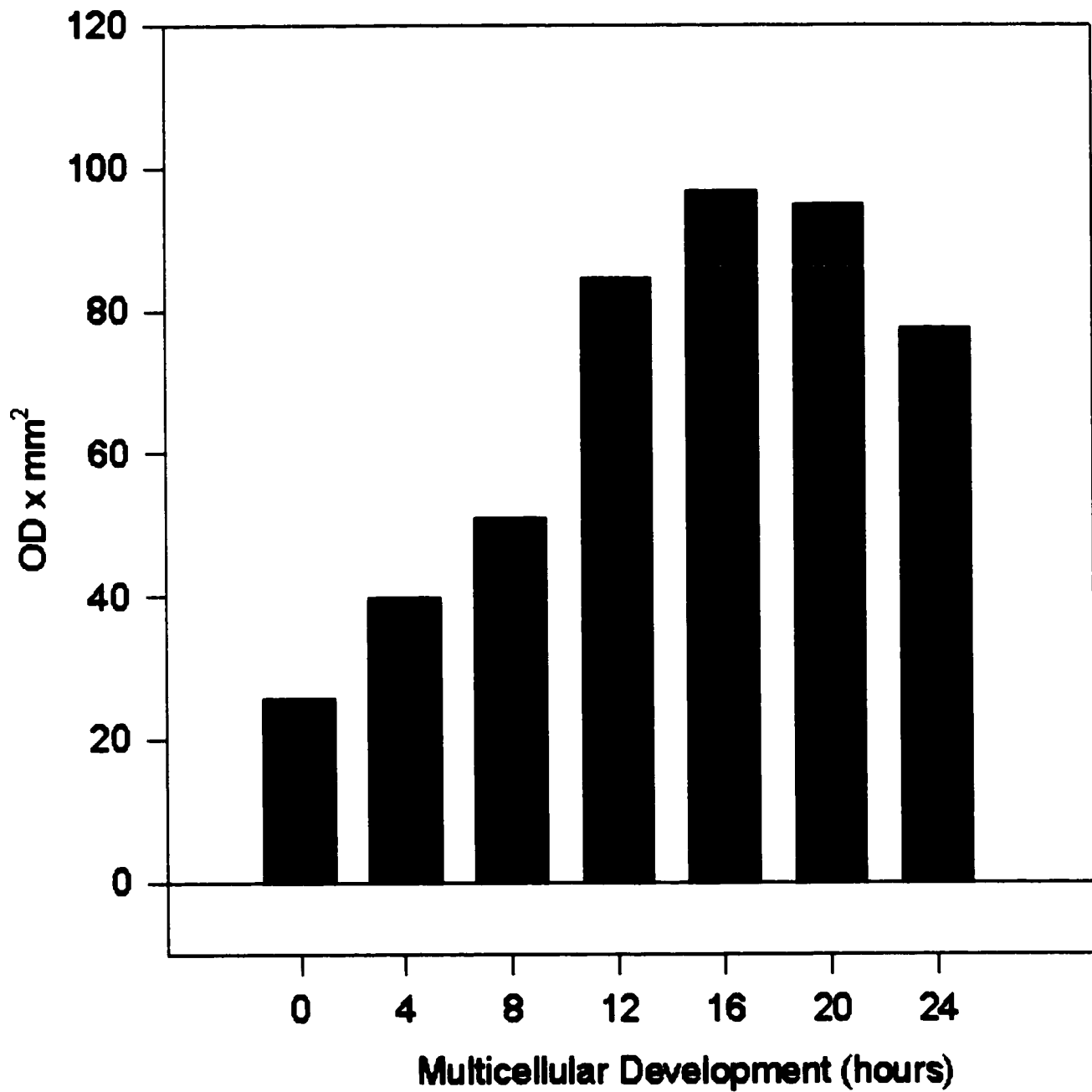


Figure 8.

Figure 9. Gelatin-SDS-PAGE analysis of the internal proteinases during the multicellular development of *D. discoideum*, AX3. (Gel A, control; Gel B, acid treatment). Samples were harvested at various times during multicellular development in a 96-well microtitre plate. (*Lane 1*, 0 hr; *Lane 2*, 4 hr; *Lane 3*, 8 hr; *Lane 4*, 12 hr; *Lane 5*, 16 hr; *Lane 6*, 20 hr; *Lane 7*, 24 hr). These are 7.5% polyacrylamide gels with each lane containing 0.8 μ g of protein.

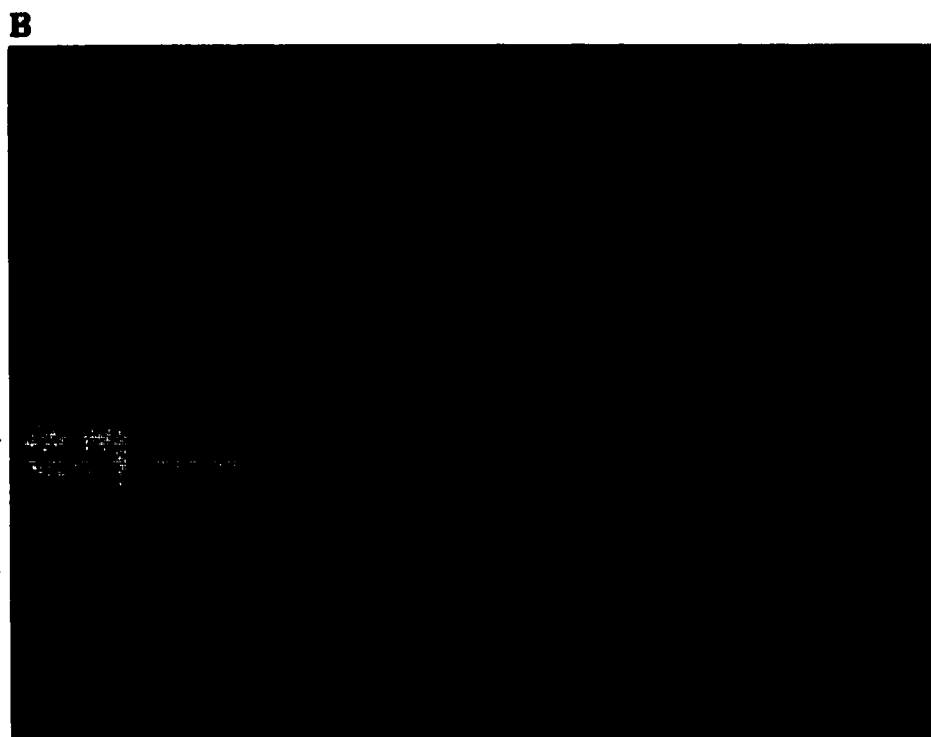


Figure 9.

Figure 10. Densitometer analysis of gelatin-SDS-PAGE of internal proteinases during the multicellular development of *D. discoideum*, AX3. The total observable proteolysis of internal proteinases during multicellular development in Figure 9 is analyzed by a densitometer (Bio-Rad, Mississauga, ON). The optical density (O.D.) of every square mm of each lane in Figure 9 is read and compared to a blank lane not containing proteinases.

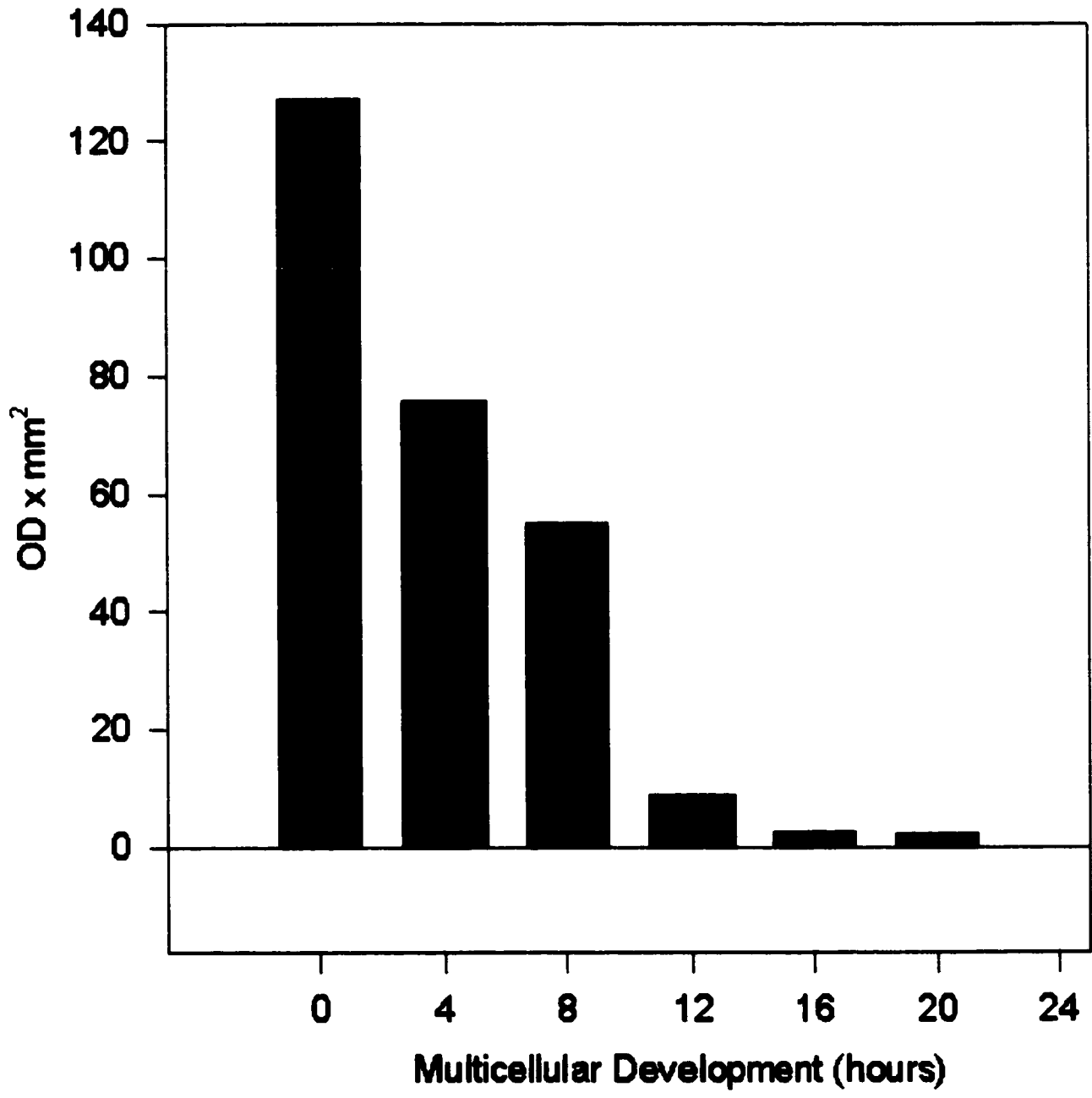


Figure 10.

Acid Activatable Cysteine Proteinases in the Dictyosteliaceae

Through gelatin-SDS-PAGE analysis of spores, the acid activatable nature of the cysteine proteinases was determined to be conserved outside of the previously studied strains of *D. discoideum*.

Within *D. discoideum* the strains JH10, NC4mL, M31, and *spiA*⁻ were assayed by gelatin-SDS-PAGE and found to have proteinases capable of acid activation (Figure 11, A-H). Other members of the Dictyosteliaceae, *Dictyostelium mucoroides* (strain DM7), and *Polysphondylium pallidum* (strain WS320) were similarly assayed and found to have proteinases capable of acid activation (Figure 12).

The apparent molecular masses of the proteinases in the *D. discoideum* strains were 43 and 48 kDa, however individual levels of expression were found to vary. The mutant *spiA*⁻ was an exception; however the *spiA*⁻ sori that were harvested contained a mixture of viable spores, germinating spores, and dead spores.

Figure 11. Gelatin-SDS-PAGE analysis of the proteinases of spores of *D. discoideum* strains NC4ml, JH10, M31, and *spiA*⁻. (Gels A, C, E and F are controls; Gels B, D, G and H are acid treated). Gels A and B, strain JH10; Gels C and D, strain M31; Gels E and F, strain NC4ml; Gels G and H, strain *spiA*⁻. These are 7.5% polyacrylamide gels with each lane containing 0.8 µg of protein.

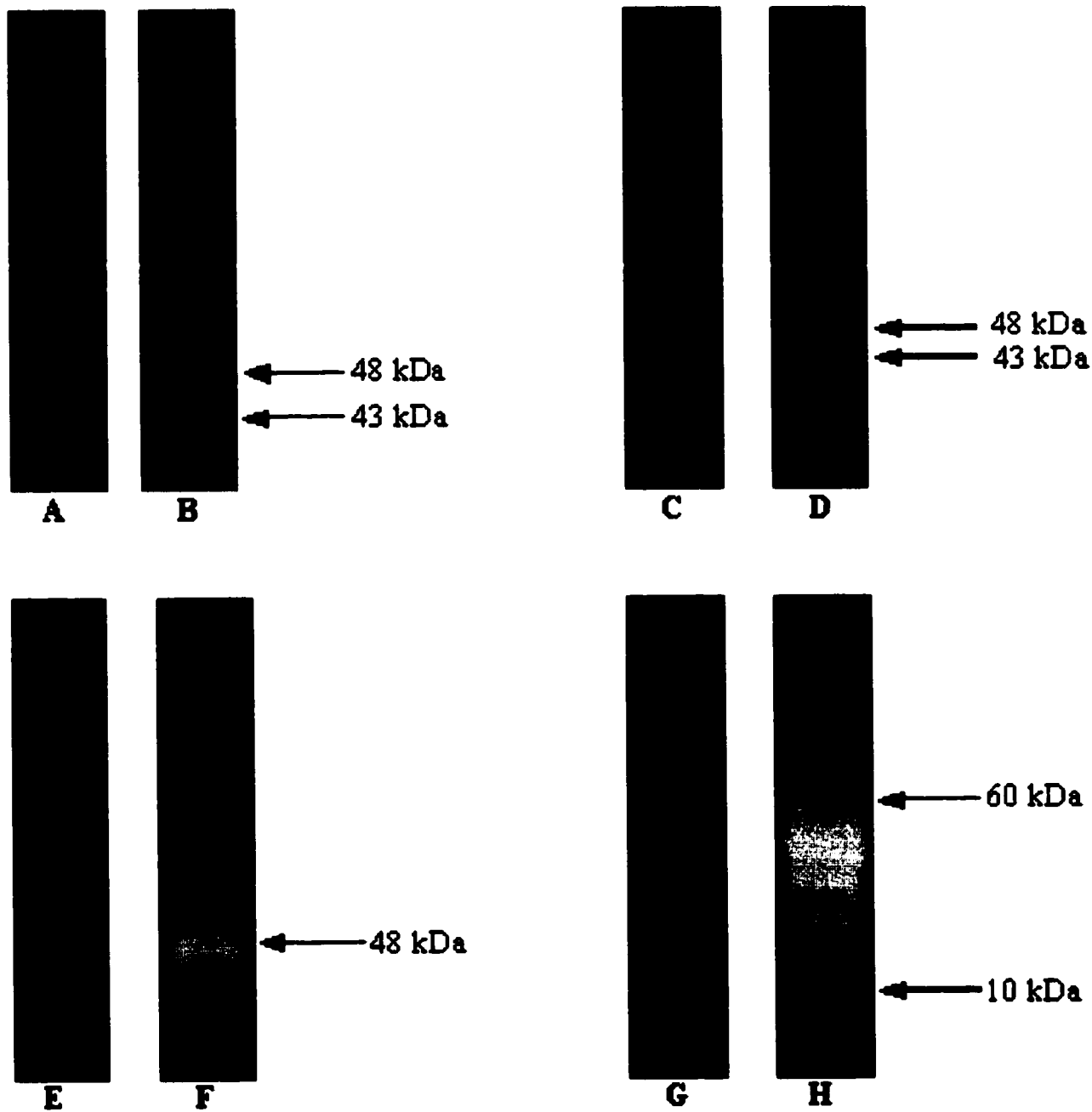


Figure 11.

Figure 12. Gelatin-SDS-PAGE analysis of proteinases of spores of

***Dictyostelium mucoroides*, strain DM7 and *Polysphondylium pallidum*, strain**

WS320. These are 7.5% polyacrylamide gels with each lane containing 0.8 µg of protein.

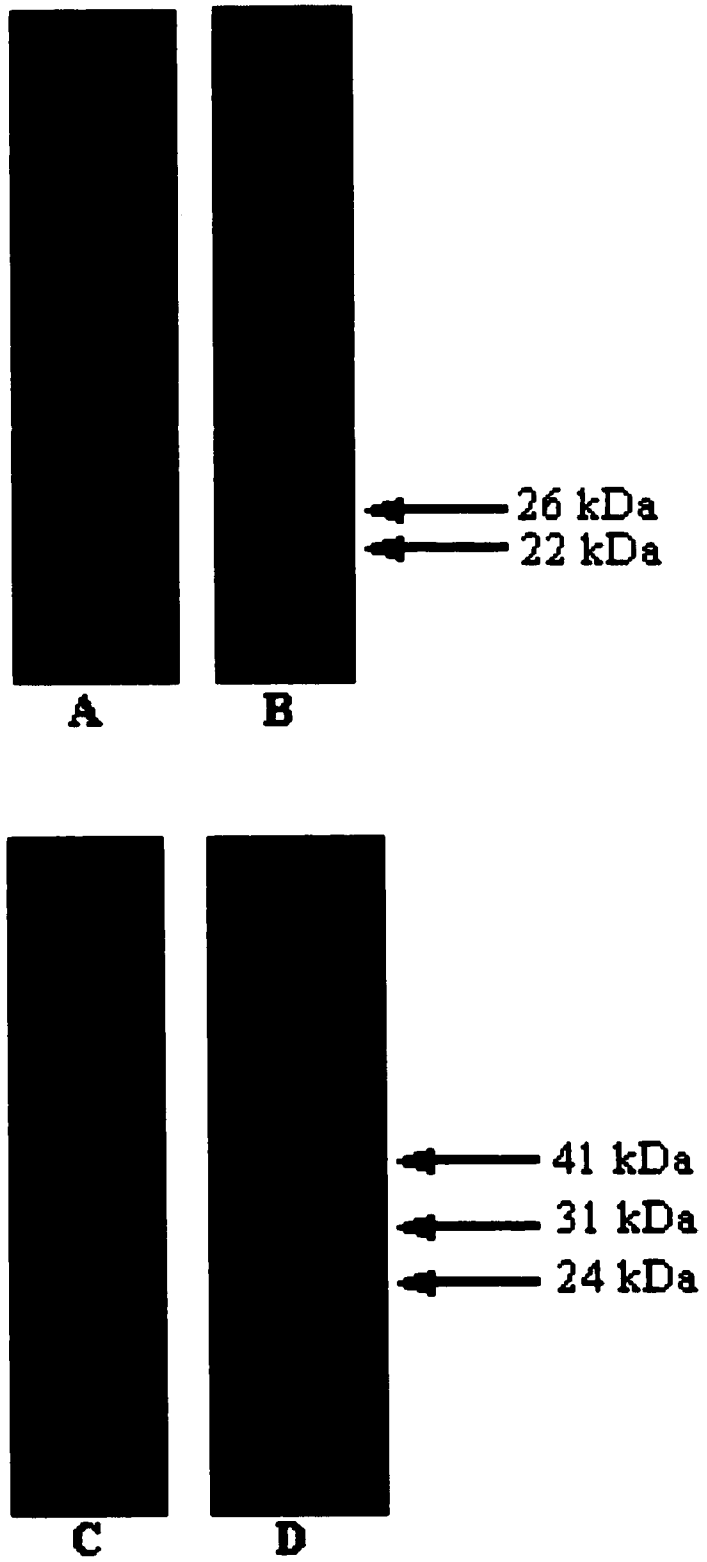


Figure 12.

The Proteinases of *Achlya ambisexualis*

The external proteinases of *A. ambisexualis* were not found to be acid activatable. The majority of proteinases in *A. ambisexualis* showed decreased activity after acid treatment when assayed by gelatin-SDS-PAGE. The external proteinase with an apparent molecular mass of 98 kDa and the internal proteinase with an apparent molecular mass of 48 kDa were observed to be resistant to acid treatment (Figure 13).

In support of the work of Hill and Pott (1997), the assayed proteinases were found to be induced when *A. ambisexualis* was grown in the presence of protein. Different proteins were found to elicit different levels of proteinase activity with collagen inducing the greatest response. The internal and external proteinases revealed by gelatin-SDS-PAGE were found to independently vary in activity with the protein present during growth (Figure 13 and Figure 14). Inhibitor studies with E64 indicate that the internal and external enzymes were cysteine proteinases (Figure 15).

In situ studies of external proteinase activity supported the *in vitro* studies by illustrating the protein requirement for external proteinase production (Figure 16).

Figure 13. Gelatin-SDS-PAGE analysis of secreted proteinases of the mycelium of *Achlya ambisexualis*. (Gel A, control; Gel B, acid treatment). Samples were collected during mycelial growth in the presence of various proteins. (*Lane 1*, no protein; *Lane 2*, collagen; *Lane 3*, albumin; *Lane 4*, casein; *Lane 5*, peptone; *Lane 6*, gelatin). These are 10% polyacrylamide gels with each lane containing 2.0 μ g of protein.

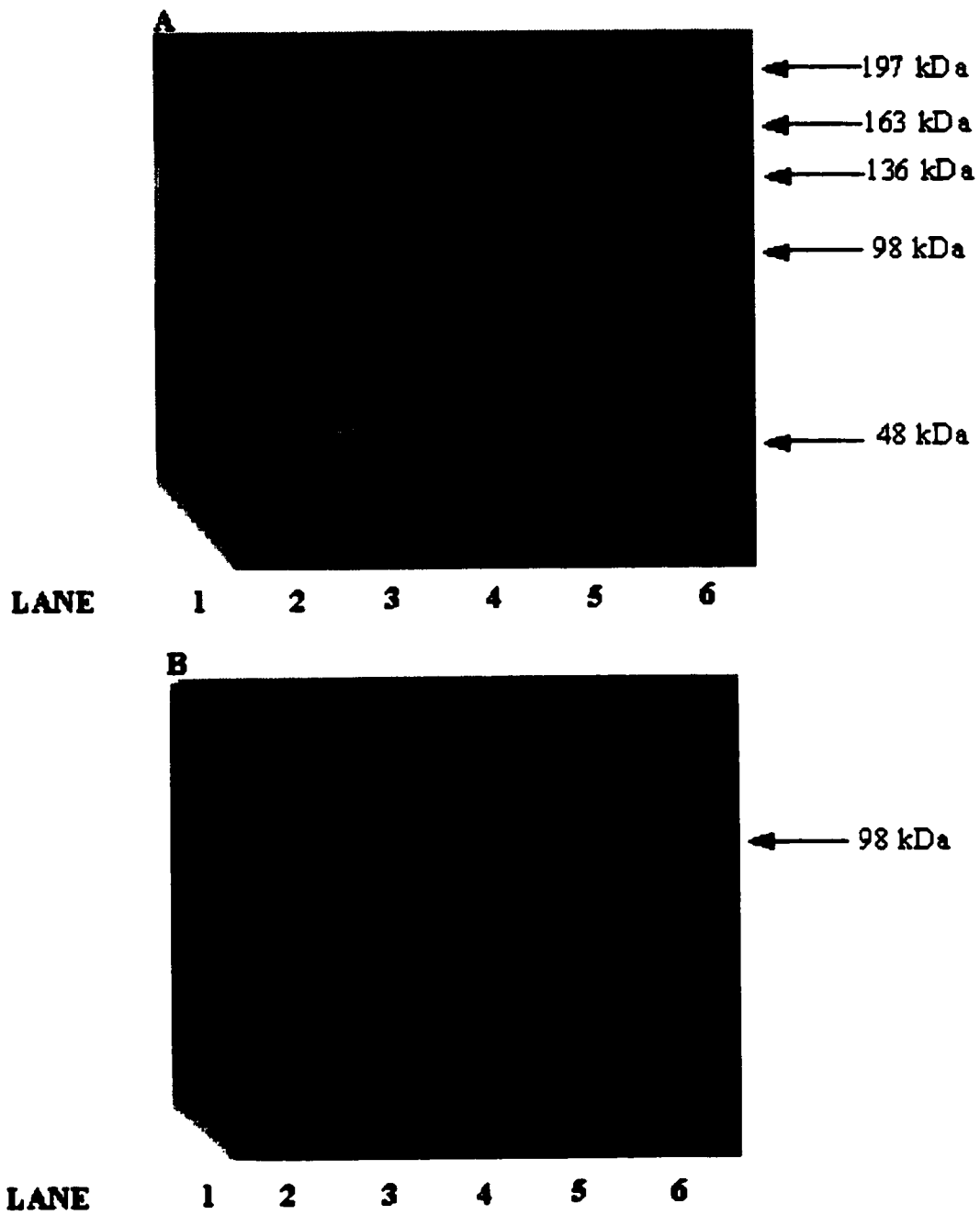


Figure 13.

Figure 14. Gelatin-SDS-PAGE analysis of internal proteinases of the mycelium of *A. ambisexualis*. (Gel A, control; Gel B, acid treatment). Samples were collected during mycelium growth in the presence of various proteins. (*Lane 1*, no protein; *Lane 2*, collagen; *Lane 3*, albumin; *Lane 4*, casein; *Lane 5*, peptone; *Lane 6*, gelatin). These are 10% polyacrylamide gels with each lane containing 2.0 µg of protein.

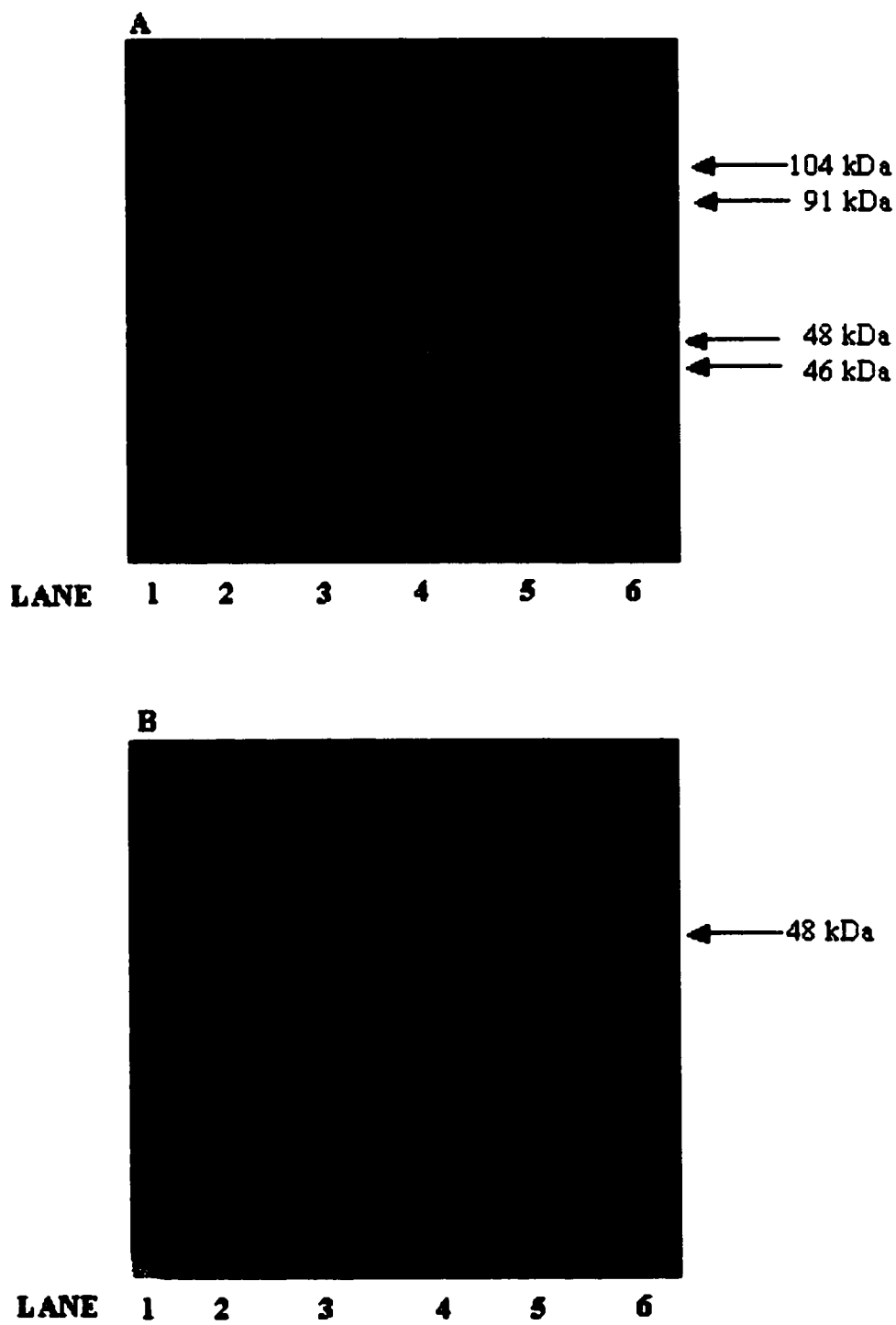


Figure 14.

Figure 15. Gelatin-SDS-PAGE inhibitor studies of the proteinases of the mycelium of *A. ambisexualis*. Gels A and C, control; Gels B and D, E64 treatment. Gels A and B represent internal proteinases when grown in the presence of collagen. Gels C and D represent external proteinases when grown in the presence of peptone. These are 10% polyacrylamide gels with each lane containing 2.0 μ g of protein.

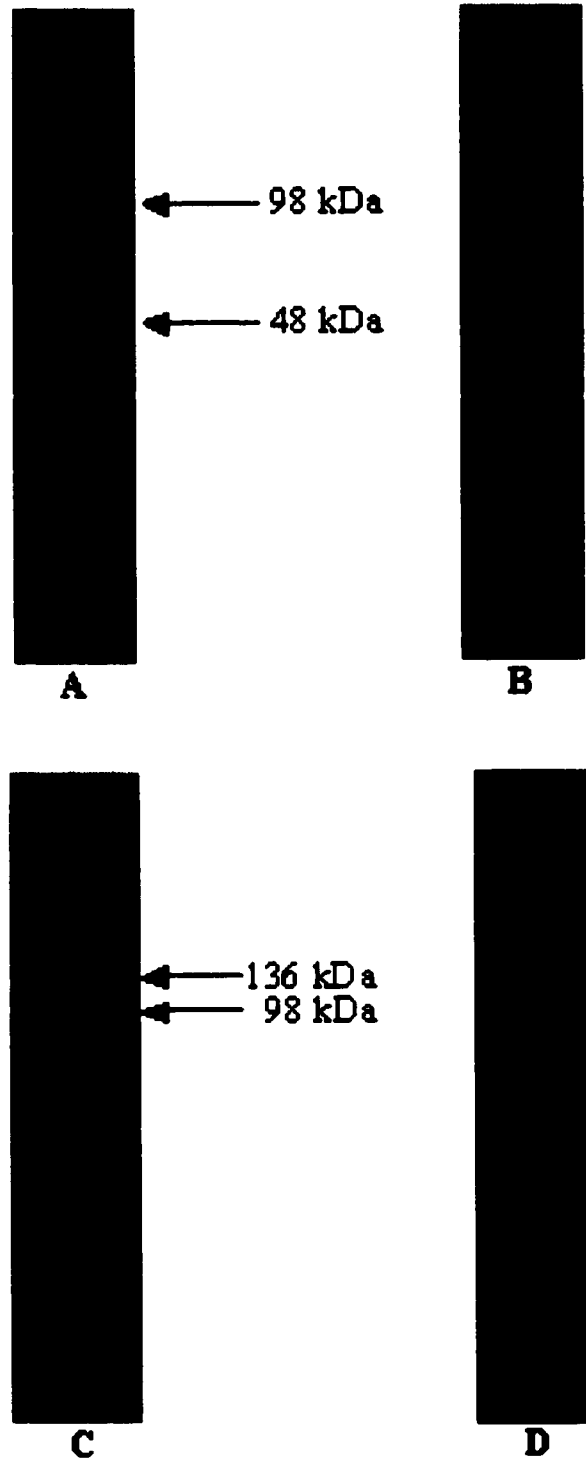
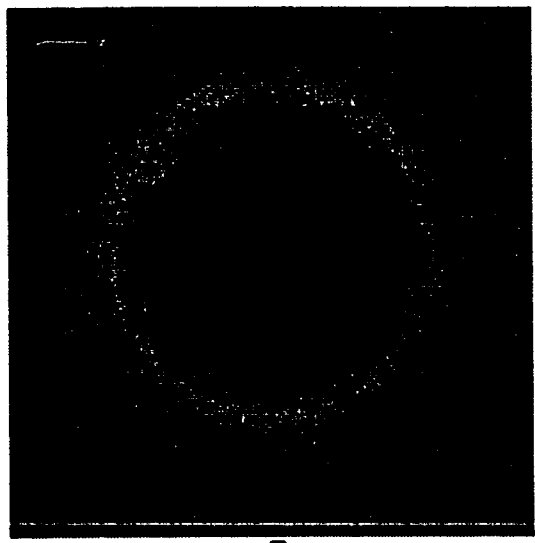


Figure 15.

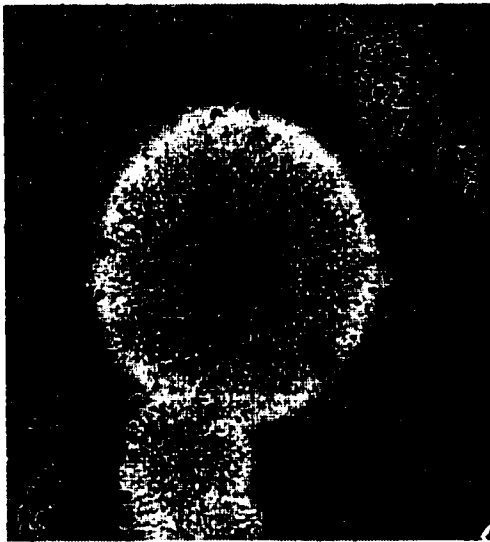
Figure 16. *In situ* localization of the proteinases secreted from *A. ambisexualis*, 2 x magnification. Mycelium growth is observed in the presence of various proteins added to the agar-stabilized growth medium. After colony establishment the agar was stained with Coomassie Brilliant Blue. Protein clearing, shown as a white halo, indicates the location of proteinase activity. (Figure 16A, no protein; Figure 16B, gelatin; Figure 16C, albumin; Figure 16D, casein; Figure 16E, collagen; Figure 16F, peptone).



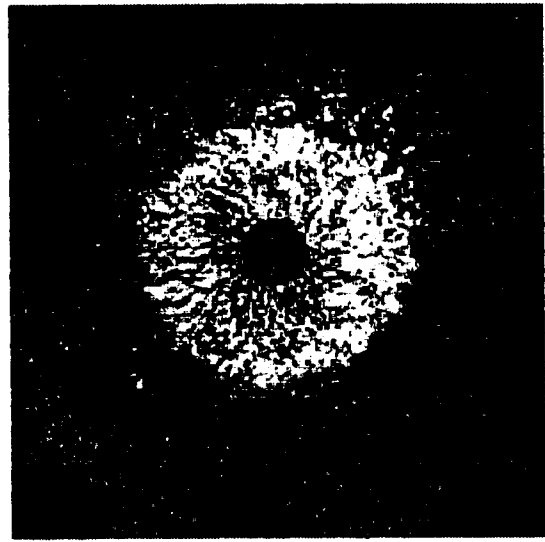
A



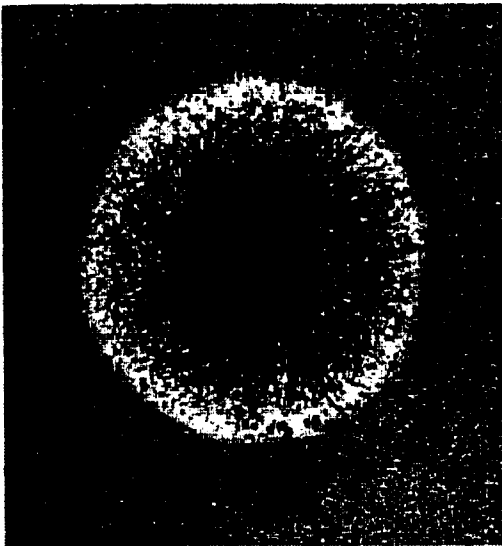
B



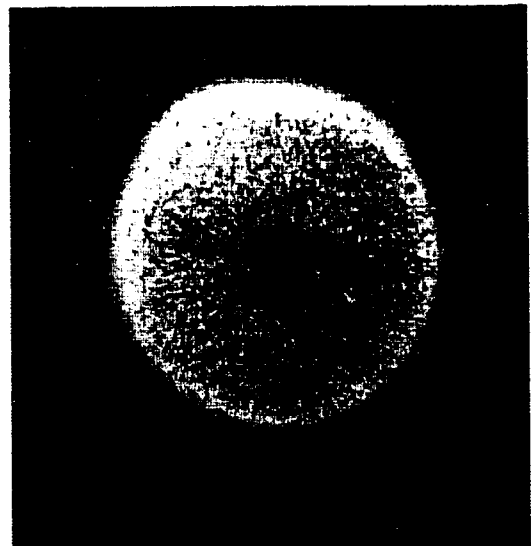
C



D



E



F

Figure 16.

The Proteinases of *Chironomus*, *Euglena gracilis*, and *Tetrahymena pyriformis*

Gelatin-SDS-PAGE revealed that the proteinases of *Chironomus* and *Euglena gracilis* were neither acid activatable nor cysteine proteinases as indicated by E64. Chironomid was found to have three proteinases with apparent molecular masses of 195, 185, and 116 kDa (Figure 17). *Euglena gracilis* was found to have a single proteinase of apparent molecular mass of 140 kDa. This proteinase was only observable when *E. gracilis* was grown in the presence of protein (gelatin) and not when the organism was grown photosynthetically (Figure 18).

Gelatin-SDS-PAGE revealed that *Tetrahymena pyriformis* possessed three proteinases of apparent molecular masses of 132, 43, and 28 kDa. E64 inhibition assays revealed that all proteinases were cysteine proteinases. In addition the proteinases were acid activatable, base deactivatable, and acid reactivatable (Figure 19).

Figure 17. Gelatin-SDS-PAGE analysis of the proteinases of 4th instar Chironomid larvae. (GelA, control; GelB, acid treatment; Gel C, E64 treatment). In this analysis whole larvae homogenate is assayed. These are 10% polyacrylamide gels with each lane containing 5 µg of protein.

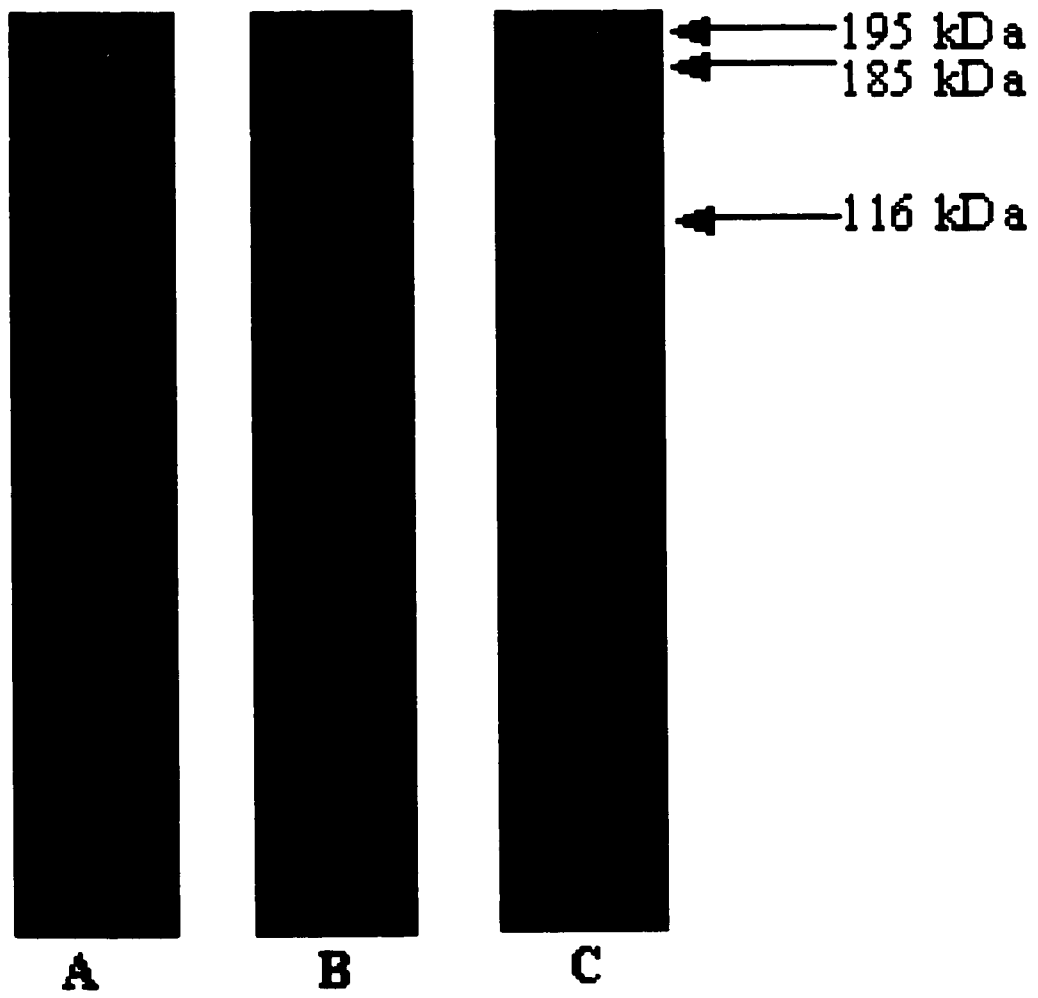


Figure 17.

Figure 18. Gelatin-SDS-PAGE analysis of the proteinases of *Euglena gracilis*.

Samples from Gels A and B were grown photosynthetically. (Gel A, control; Gel B, acid treatment). Samples from Gels C, D, and E were grown with 0.2% gelatin. (Gel C, control; Gel D, acid treatment, Gel E, E64 treatment). These are 10% polyacrylamide gels with each lane containing 25 μ g of protein.

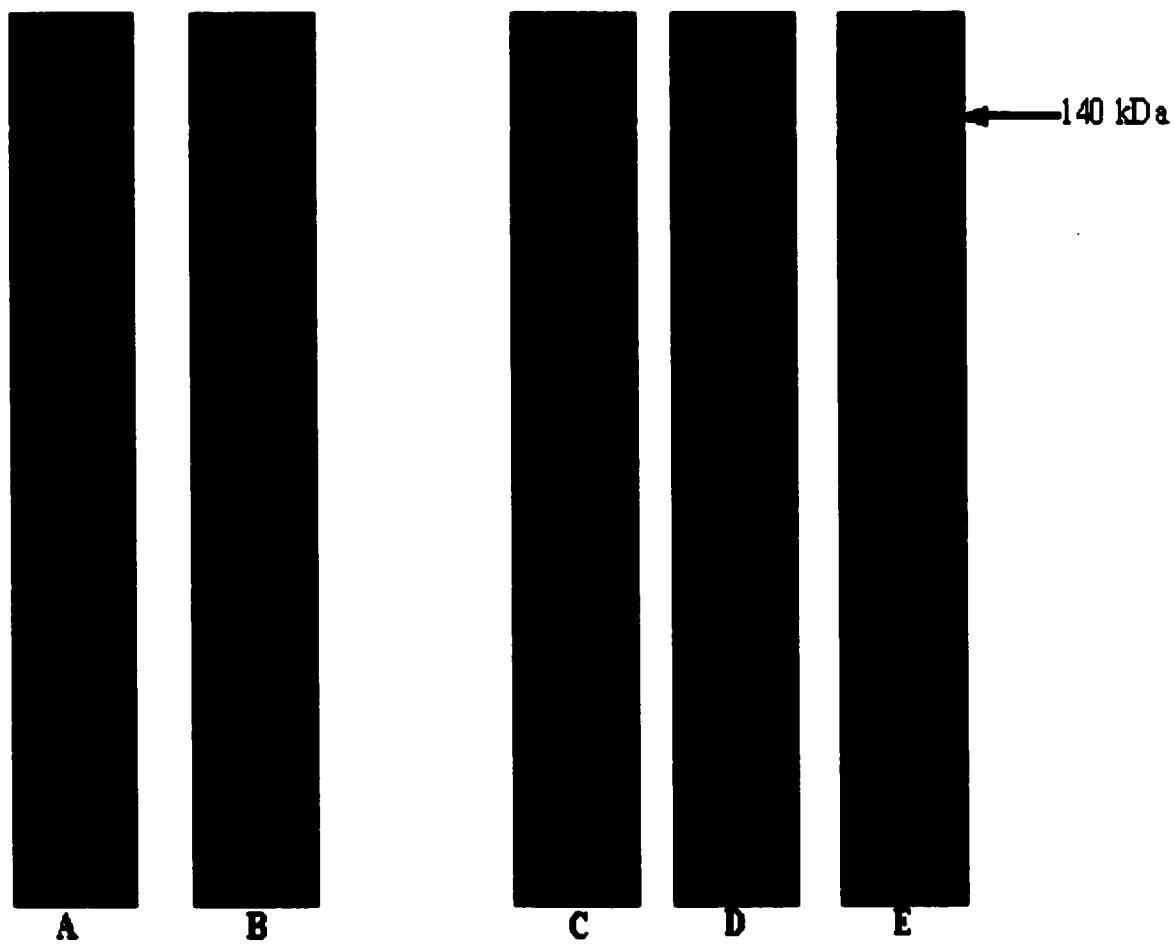


Figure 18.

Figure 19. Gelatin-SDS-PAGE analysis of proteinases of *Tetrahymena pyriformis*.

(Gel A, control; Gel B, acid treatment; Gel C, acid and base treatment; Gel D, acid reactivation; Gel E, E64-inhibitor treatment). These are 10% polyacrylamide gels with each lane containing 2.0 μg of protein. The blanched appearance of Gel E is due to extensive destaining which failed to illustrate any bands.

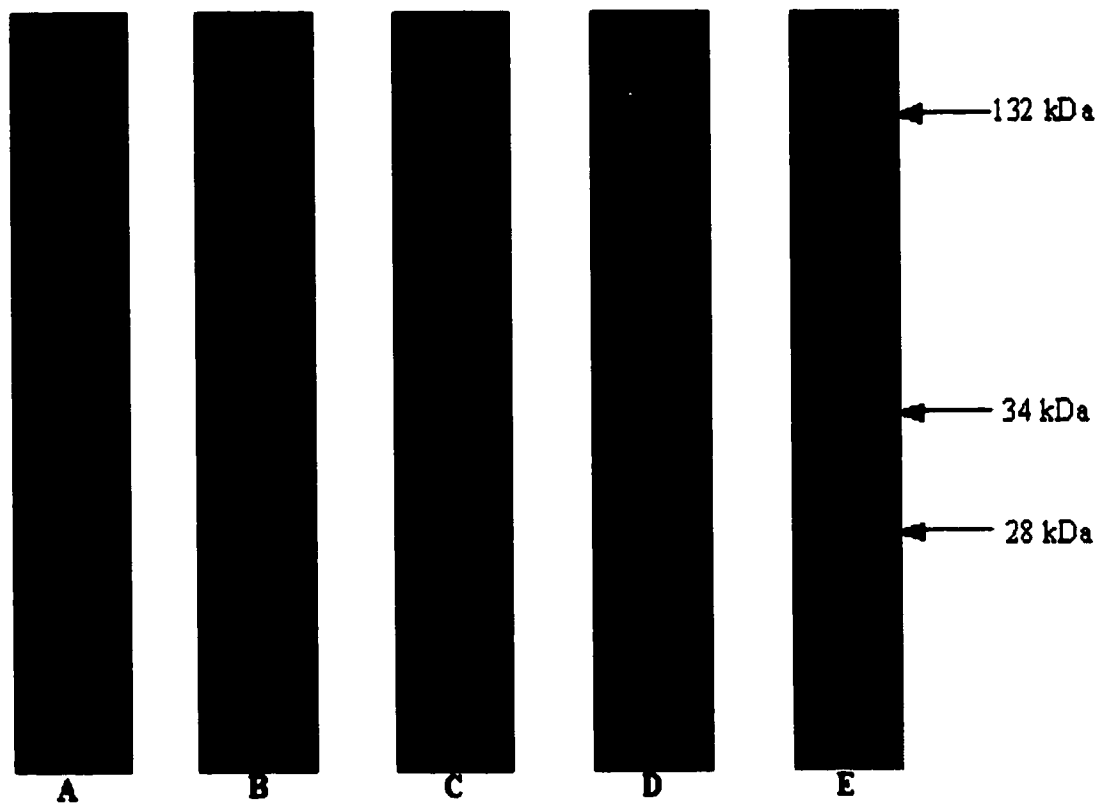


Figure 19.

Bacterially Induced Proteolytic Responses in *Polysphondylium pallidum*

When the myxamoebae from *P. pallidum* are grown axenically only the aspartyl proteinase is observed, ppAP65 (Figure 20A, lanes 1-3). If the myxamoebae are instead grown with bacteria (*E. coli*) a cysteine proteinase band becomes evident, ppCP31 (Figure 20A, lanes 4-6).

After acid treatment the cysteine proteinase ppCP47, becomes evident from the axenic cells (Figure 20B, lanes 1-3). In the case of the bacterially grown myxamoebae, two additional cysteine proteinases become evident ppCP47, and ppCP24 (Figure 20B, lanes 4-6).

Figure 20. Gelatin-SDS-PAGE analysis of the proteinases of *P. pallidum*

myxamoebae grown axenically or with bacteria. (Gel A, control; Gel B, acid treatment. In this analysis all lanes were ran in triplicate. *Lanes 1-3* represent myxamoebae grown axenically. *Lanes 4-6* represent myxamoebae grown with bacteria). These are 10% polyacrylamide gels with each lane containing 0.8 μg of protein.

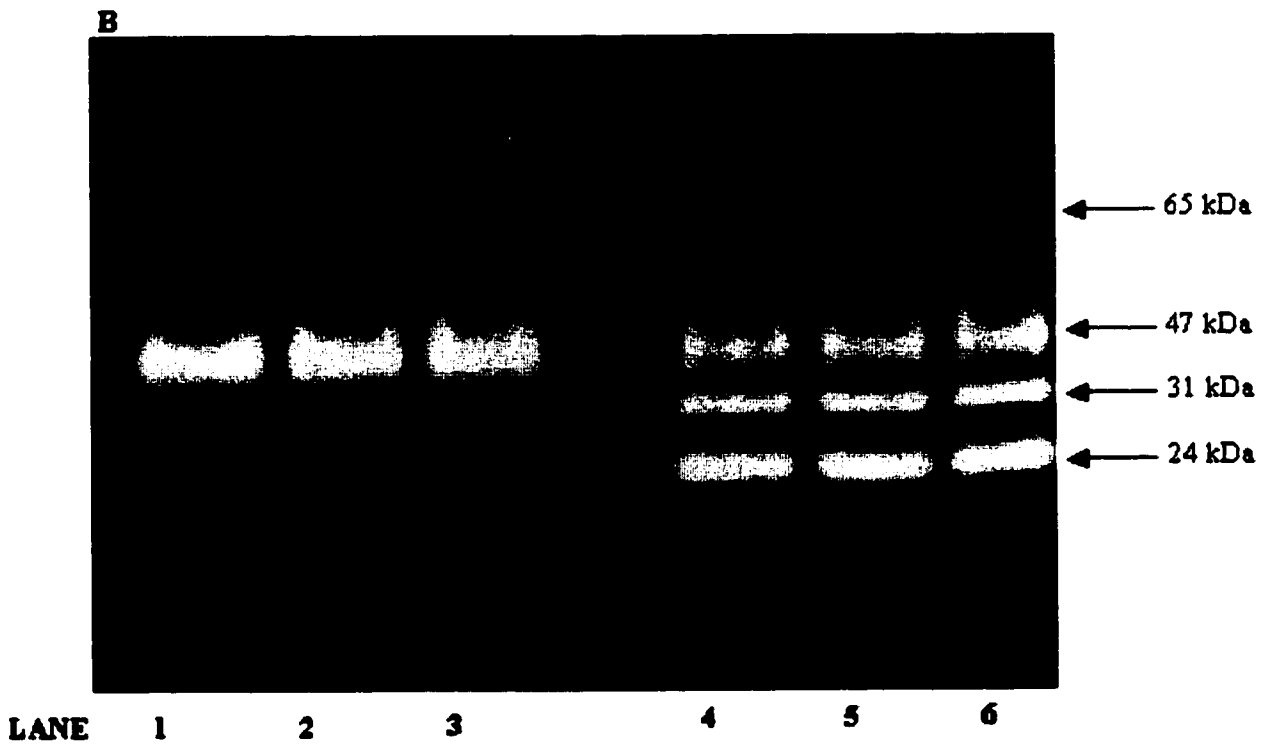
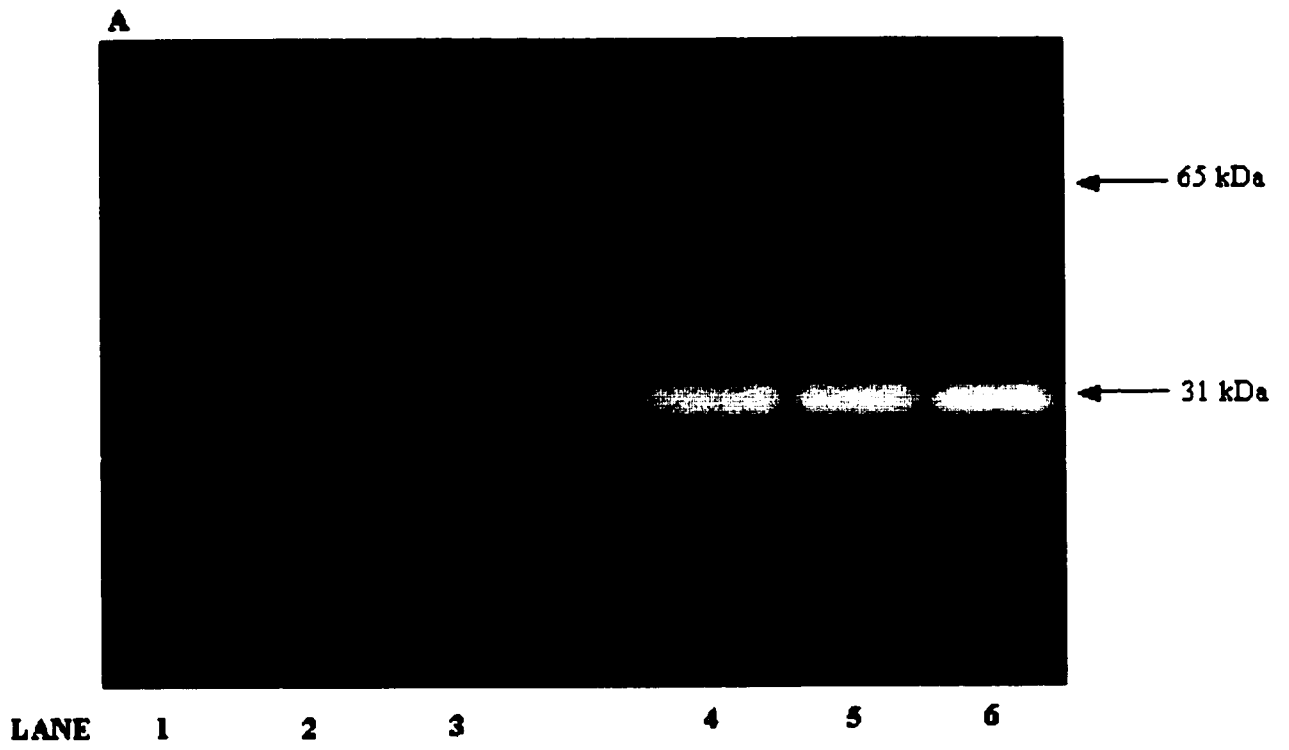


Figure 20.

Preliminary Modeling of the Disulfide Bonding of ddCP43 and ddCP48

When the proteinases of the spores of *D. discoideum*, strain SG1, are analyzed by gelatin-SDS-PAGE the cysteine proteinase ddCP48 is weakly present before acid treatment (Figure 21A). After acid treatment ddCP48 is strongly activated and ddCP43 is now weakly observed (Figure 21B). These results confirm the work done by North *et al.* (1996).

When the proteinases of the spores of *D. discoideum*, strain SG1 were analyzed by gelatin-Native-PAGE no proteinase bands were observed in the control (Figure 22A). However, after acid treatment two bands of similar molecular mass are observed (Figure 22B).

When the proteinases of the spores of *D. discoideum*, strain, SG1, were analyzed by gelatin-nonreducing-SDS-PAGE the proteinase ddCP43 shows a higher relative mobility (M_r) in lanes lacking the reducing agent β -mercaptoethanol (Figure 23, lanes 3-4) than in the lanes containing the reducing agent (Figure 23, lanes 1-2 and 5-6). This indicates the presence of intradisulfide bonding within the proteinase ddCP43. The proteinase ddCP48 elicited no changes in the M_r when run on a gel with or without reducing agent.

No indication of intradisulfide bonding was observed as this would have resulted in a lower M_r in the lanes lacking reducing agent.

Figure 21. Gelatin-SDS-PAGE analysis of the proteinases of *D. discoideum* spores,

SG1. (Gel A, control; Gel B, acid activation). In this analysis the sample was run in the centre most lanes in triplicate. This gel is to be used in reference to Figure 22 and Figure 23. These are 10% polyacrylamide gels with each lane containing 0.8 μg of protein.

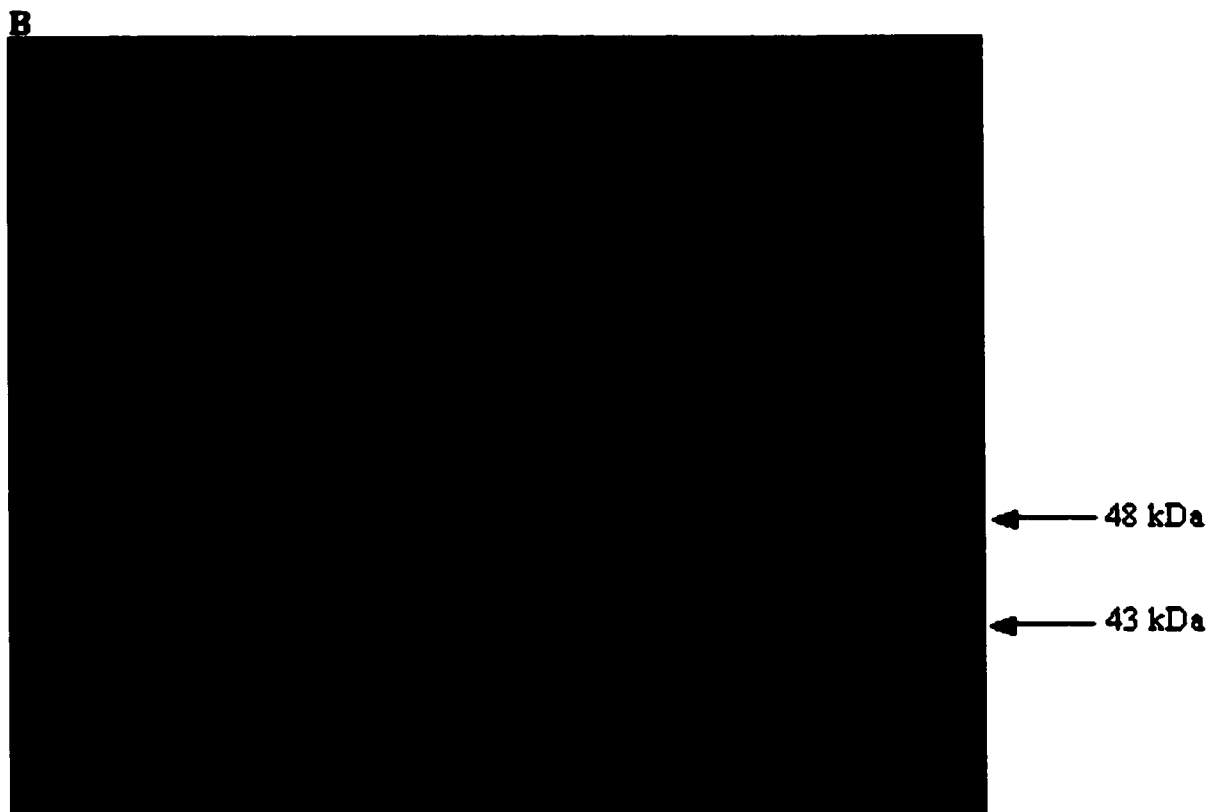
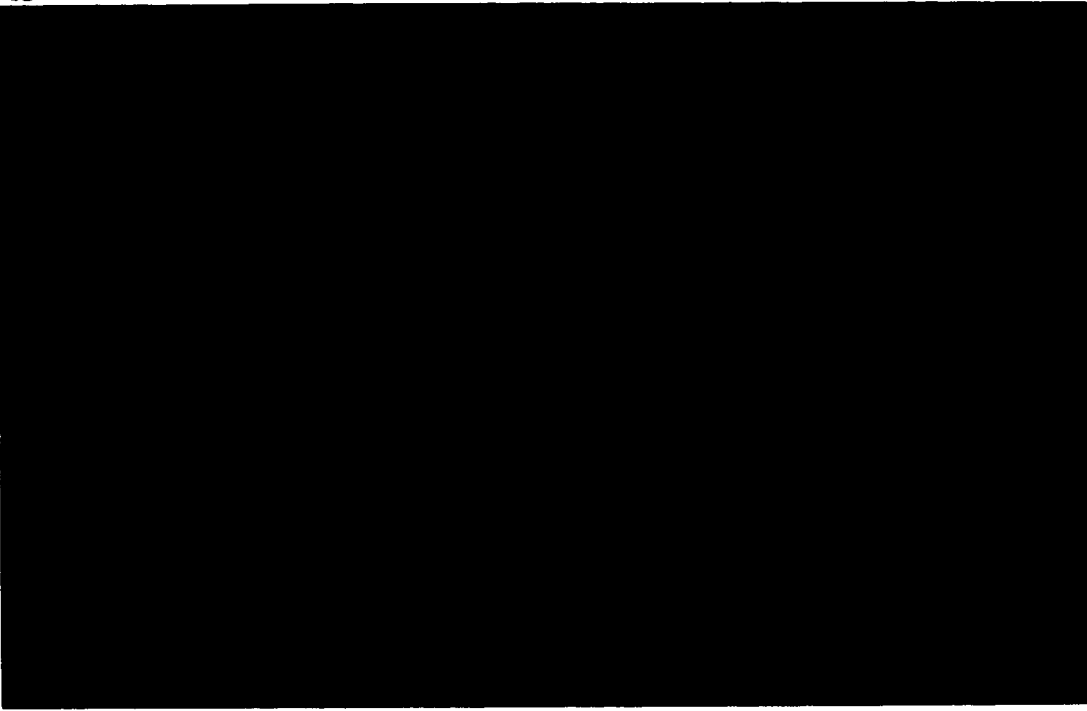


Figure 21.

Figure 22. Gelatin-Native-PAGE analysis of the proteinases of *D. discoideum* spores, strain SG1. (Gel A, control; Gel B, acid activation). These are 5% polyacrylamide gels with each lane containing 0.8 μ g of protein. The two arrows indicate the two separate proteinases assayed, molecular masses of these enzymes could not be determined under native conditions.

A



B

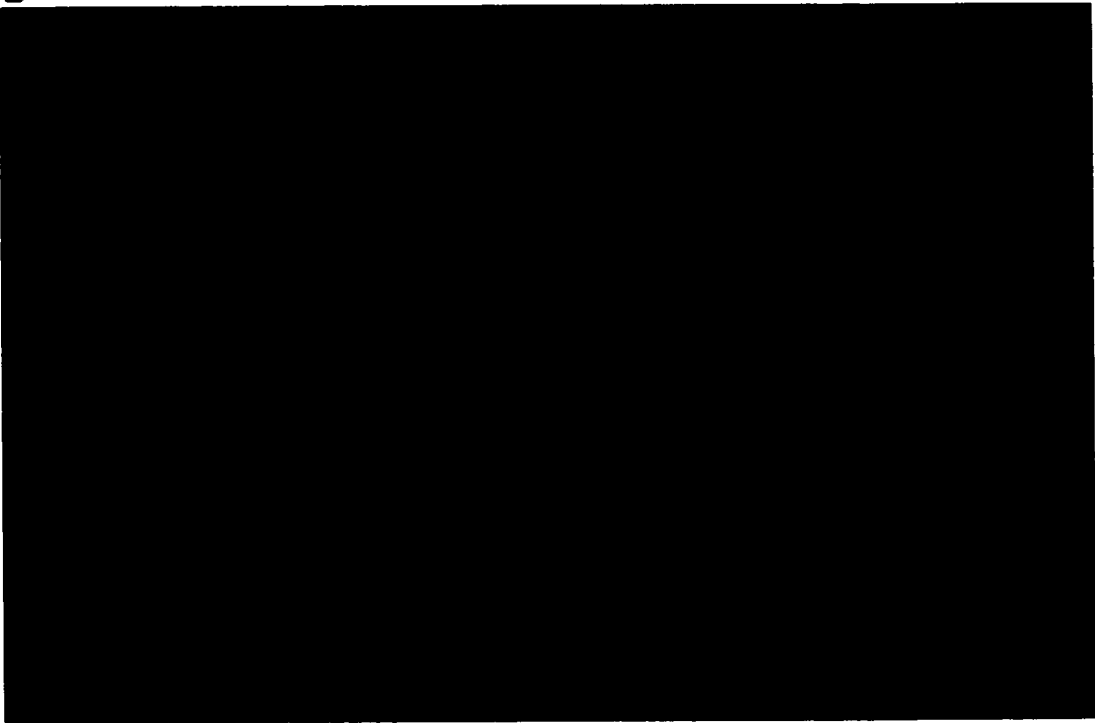


Figure 22.

Figure 23. Semi-Reducing gelatin-SDS-PAGE analysis of the proteinases of

***D. discoideum* spores, strain SG1.** This gel has been acid treated. *Lanes 3 and 4* contain a reducing agent in the sample buffer; *Lanes 1, 2, 5 and 6* do not contain a reducing agent in the sample buffer. These are 10% polyacrylamide gels with each lane containing 0.8 μg of protein. A higher *Mr* of the lanes lacking a reducing agent in the sample buffer of lanes 3 and 4 indicate the presence of intradisulfide bonding in ddCP48. The *Mr* of both ddCP43 and ddCP48 lacking reducing agent is equal to or greater to the *Mr* of samples containing reducing agent. This infers a lack of interdisulfide bonding between the two proteinases.

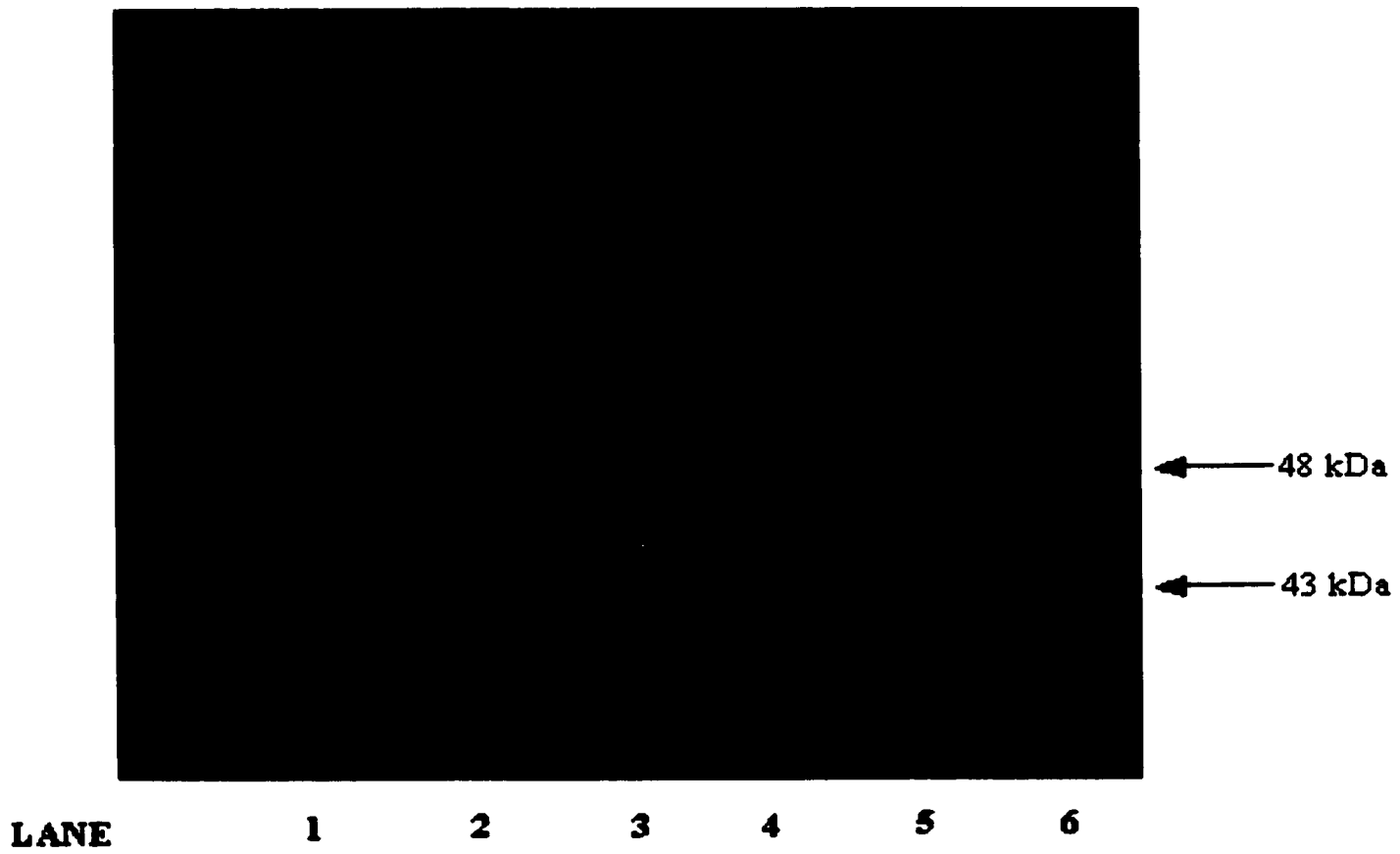


Figure 23.

DISCUSSION

In 1996 North *et al.* reported that the majority of the cysteine proteinases in *Dictyostelium discoideum* strain SG1 were acid activatable. This work was substantiated in 1997 by Cotter *et al.* who determined that this acid activatable characteristic of the cysteine proteinases was prevalent in all stages of development of *D. discoideum*. Furthermore Cotter *et al.* (1997) determined that this process of acid activation was reversible, a base treatment was found to deactivate the cysteine proteinases yet leave them able to be reactivated. These results are substantiated by this study.

External Proteinases of the Pseudoplasmodia and Aggregating

Myxamoebae

As postulated by Bonner in 1993 it has been found that the pseudoplasmodial slime trail contains cysteine proteinases. In experiments where axenically grown *D. discoideum* (AX3) myxamoebae were allowed to develop in 96-well micro-titre plates three proteinases were found to be secreted, namely ddCP60, ddCP38, and ddCP24 (Figure 2). These secreted proteinases were recovered from the buffer in an inactive state but readily displayed proteolytic activity after an acid treatment. These proteinases were previously associated with the pseudoplasmodial stage by North *et al.* (1996) who performed whole cell analysis at the slug stage. North also found that the

pseudoplasmodial proteinases were initially relatively inactive but responded with increased proteolysis after an acid treatment. A preliminary report from North *et al.* (1996) indicated that the ddCP60 was believed to be internal, as it could not be removed from the cells by washing. The current results show that the ddCP60 was secreted into the KPi buffer during the 96-well experiment.

The proteolytic nature of the pseudoplasmodium was also observed *in situ*. As phototrophically directed slugs migrated along a protein infused agar surface, a slime trail was observed to slough off. After mechanical removal of the slime trail the agar was stained with Coomassie Brilliant Blue, and the white trail left by the slug on a blue background indicates pseudoplasmodium associated proteolysis (Figure 4). Similar experiments were performed to show mycelium associated proteolysis in *Achyla ambisexualis* (Figure 16). Similar methodologies have been employed to qualitatively determine extracellular proteolysis in various *Saprolegnia* species (Smith *et al.*, 1994).

Other indications of CP activity in the ECM of the pseudoplasmodium may be found in the independent work of Champion and West. A group of glycoproteins extracted from *D. discoideum* pseudoplasmodia are recognized by the monoclonal antibody MUD62 and are mobile within the ECM, and hence are non-structural molecules. When examined by immunofluorescence microscopy, they are found within the tube defined by the structural molecules of the ECM, accumulating along folds of the trail. The identity of the proteins is unknown but MUD62 has been shown to recognize the cysteine proteinases secreted from vegetative amoebae (Champion *et al.*, 1991, West *et al.*, 1988).

To summarize, the *in situ* proteinase assay demonstrated that the slug or slug trail has proteolytic capabilities. The gelatin SDS-PAGE assay showed no naturally active proteolytic activity from the proteinases secreted from the slug. However, after an acid treatment the cysteine proteinases ddCP60, ddCP38, and ddCP24 were activated. It is suspected that active proteinases were not originally observed via gelatin-SDS-PAGE since they were all deactivated by the alkaline nature of the relatively large prespore section of the pseudoplasmodium.

These results indicate that the ECM of the pseudoplasmodium is capable of proteolysis. Experiments done with the pH indicator neutral red indicate that the prestalk region of the slug contains vesicles with a pH <6.5, compared to the prespore region which has a pH >8.0 (Dormann *et al.*, 1996). If this is a true indication of the pH levels of the two regions then the prespore region is too alkaline for proteolytic activity therefore the prestalk region must contain the active proteinases of the slug.

This conclusion is substantiated by the work done by Bonner (1993) who illustrated that a slug will move away from a point source of the cysteine proteinase papain but towards a point source of a cysteine proteinase inhibitor. Since all slug movement is directed by the slug tip, therefore it must be the cells in slug tip that are interacting with the proteinases.

It is hypothesized that either the whole pseudoplasmodium is secreting proteinases, or that the prestalk/tip cells are secreting proteinases and that the proteinases are moving to the rear of the slug in the ECM. This hypothesis is again supported by Bonner (1993) who reported that when slugs move over agar of different pH values, the more acid the agar the faster they will move. This may be a result of the dormant

cysteine proteinases in the extracellular matrix becoming active at the lower pH. The now active proteinase from the large prestalk region would create ammonia through proteolysis which has been found to increase slug speed. In other words, the speed of the pseudoplasmodium is believed to be directly proportional to the number of active cysteine proteinases in the pseudoplasmodium.

The mechanism of an acidic prestalk zone may exist in a theory adapted from Inouye (1989). Inouye invokes a lateral-inhibition model (Cox, 1992; Gierer and Meinhardt, 1972) involving a local proteinase activator and a long range inhibitor displaying autocatalytic and cross-inhibitory dynamics. According to this proposal, prestalk cells produce, in addition to large amounts of ammonia that diffuses rapidly, a high local concentration of a slowly diffusing activator, possibly an organic acid. This local activator partially neutralizes ammonia, and a reduction in the concentration of ammonia leads to a sustained increase in proteolytic activity due to the lower pH environment.

Inouye's proposal gains some support from the intriguing observation that the pH of the front of the slugs drops by some 0.5 pH units within minutes from the onset of culmination (Rand, 1985), as well as from the increased cellular content of several organic acids during development (Kelley *et al.*, 1979). In addition, there is an indication of a weak acid liberated by culminating stalk cells (Inouye, 1990).

Gelatin-SDS-PAGE and the previously mentioned *in situ* technique were also employed to illustrate the presence of external cysteine proteinases in aggregating myxamoebae of *D. discoideum*. North previously stated that proteinases were found to be secreted when myxamoebae were subjected to starvation conditions (North *et al.*,

1990a). The starvation conditions utilized by North are utilized by *D. discoideum* as a signal to begin multicellular development (Raper, 1984). From these observations and from the identification of secretion of cysteine proteinases through gelatin-SDS-PAGE (Figure 5) it is evident the aggregating (streaming) myxamoebae secreted cysteine proteinase which can be visualized *in situ* (Figure 6).

Proteinases Associated with Multicellular Development

When individual cells of *D. discoideum* strain AX3 were allowed to synchronously proceed through multicellular development, several trends in the observed cysteine proteinase patterning were noted.

Internally, as previously reported (North *et al.* 1996), the highest level of proteolytic activity was found in the myxamoebae and was found to decrease as multicellular development proceeded. This observation was made before the discovery that the cysteine proteinases of the Dictyosteliaceae could be acid activated. These results demonstrate that after acid activation a decrease in the total protease activity was measured by densitometer analysis on the acid activated gel (Figure 9, Figure 10). This work illustrates that all naturally active and inactive proteinases levels decrease with multicellular development.

The reverse was true of the proteinases observed externally as an increase in total proteolytic activity could be observed during multicellular development with a peak observed at 16 hrs, the pseudoplasmodial stage. Overall, proteinases were externally

weakly active during all stages of multicellular development, after acid treatment the majority of proteinases expressed a heightened activity illustrated by increased proteolysis (Figure 7 and 8). The cysteine proteinase ddCP18 was found to be inhibited by the acid treatment while ddCP24 was not observed to be affected by the acid treatment. This difference in activity between ddCP18, ddCP24 and the other acid activatable should prove to be of significance as all the proteinases in *Dictyostelium* are modeled. Structural differences between ddCP18 and ddCP24 and the other proteinases capable of acid activation may reveal the mechanism of the conformational method of activation.

Dictyosteliaceae Proteinases

Within the species *Dictyostelium discoideum*, acid activatable proteinases have been previously observed (North *et al.*, 1996; Cotter *et al.*, 1997). The major acid activatable proteinases previously reported were the SG1 dormant spore proteinase ddCP48, and the SG2 dormant spore proteinase ddCP43. Acid activatable proteinases have also been determined to be present in the spores of *D. discoideum*, the microcysts of *P. pallidum*, and the macrocysts of *D. mucoroides*.

In this study the *D. discoideum* strains NC4ml, JH10, M31, *spiA*⁻ were assayed for acid activatable spore associated cysteine proteinases (Figure 11). The strain NC4mL is a wild-type strain that does not spontaneously germinate as the SG1 strain does. The strain JH10 has a mutation in pyrimidine biosynthesis which is not associated with proteinase

activation. The strain M31 has a α -glucosidase mutation interfering with n-glycosylation, this is inconsequential in proteinase analysis, as the cysteine proteinases in the Dictyosteliaceae are known to be o-glycosylated. The strain *spiA*⁻ lacks a spore coat protein, which causes premature germination resulting in the cell death of a large number of nascent cells in the sorus. The strains NC4ml, JH10, M31, and *spiA*⁻ were all found to have spore associated, acid activatable, cysteine proteinase with apparent molecular masses of 43 and/or 48 kDa. These results further solidify the idea that the acid activatable nature of the cysteine proteinases is prevalent in all members of *D. discoideum*.

Interestingly strain *spiA*⁻ was found to have a wide range of observable proteinases ranging from an apparent molecular mass of 10 to 60 kDa. To reiterate, this is explained since the *spiA*⁻ strain is found to produce spores of which a certain percentage germinate and die while still residing in the sorus. The proteinases observed here are likely a mixture of spore and myxamoebae associated proteinases as well as proteinase associated with cell death.

Outside of *D. discoideum* two other members of the Dictyosteliaceae were found to have spore associated, acid activatable cysteine proteinases when analyzed by gelatin-SDS-PAGE (Figure 12). *Dictyostelium mucoroides*, strain DM7, was found to have two with apparent molecular masses of 22 and 26 kDa. *Polysphondylium pallidum* was found to have three with apparent molecular masses of 24, 31, and 41 kDa.

The observable proteolytic pattern observed from gelatin-SDS-PAGE has been found to vary from between species as well as within strains of the Dictyosteliaceae. These differences range from the minor to the major but could be used to help identify an

unknown strain or species. A good example of this is the unmistakable pattern of the *D. discoideum* strain *spiA*⁻.

Achyla ambisexualis Proteinases

Recently, *A. ambisexualis* was found to produce extracellular proteinases only when a protein or related substrate was present as an inducer (Hill and Pott, 1997). These observations are consistent with an “inducer + derepression” control mechanism like those of *Neurospora crassa* (Cohen *et. al*, 1975), *Ustilago maydis*, (Hellmich and Schauz 1988) and *Beauveria bassiana* (Bidochka and Khachatouris, 1988). This is a versatile mechanism, which would be of nutritional advantage under the widest possible range of conditions of availability of exogenous protein (Cohen, 1980).

The work of Hill and Pott (1997) further illustrated that when different proteins are utilized to induce proteolytic responses, the total proteolytic response varies with the protein inducer. Through gelatin-SDS-PAGE the individual proteinases secreted from *A. ambisexualis* can be visualized and separate proteinases are seen to vary independently from each other. It appears that there is an optimum proteinase combination for each protein substrate that *A. ambisexualis* utilizes (Figure 13).

Some discrepancy is seen to exist between the apparent molecular masses observed in the current study and that of Hill and Pott (1997). These results illustrate external proteinases of apparent molecular mass of 197, 163, 136, 98, and 48 kDa. The work of Hill and Pott (1997) found active proteinases of apparent molecular masses of 26, 48, and

56 kDa. The most active *A. ambisexualis* proteinase of 48 kDa was found to be present by both researchers, interestingly this was the only proteinase to be resistant to acid treatment.

Internally, the proteinase pattern is also seen to express a proteolytic pattern that appears to depend on the protein included in the culture medium (Figure 14).

Interestingly the apparent molecular mass of the proteinases found externally are greater than those found internally. This may possibly represent a complex that the proteinase forms with a protein used to anchor them to the cell wall. This occurrence of slow running high apparent molecular mass proteinases has also been detected in other systems (North, 1994).

With collagen used as an inducer, inhibitor assays with E-64 illustrate both external and internal proteinases to be cysteine proteinases (Figure 15b).

As previously mentioned, the proteolytic activity of the cysteine proteinases is also observable *in situ* (Figure 16). External proteolysis is represented as a halo around the mycelium, however it is only observed when *Achlya* is grown on a solid substrate infused with protein. These results support the observations of Hill and Pott that *A. ambisexualis* produces extracellular proteinases only when a protein is present as an inducer.

The proteinases in *A. ambisexualis* are not thought to be sequestered into endo/lysosomes as in *D. discoideum* but are thought to be cell wall bound as in *Neurospora* (Mahadevan and Makadkar, 1970). Naturally immobilized proteinases would offer advantages to *A. ambisexualis*. The natural habitats of the mycelium are solid substrates, which are also inhabited by a large number of other micro-organisms.

Competition for nutrients is likely to be intense. Surface located proteinases would only degrade proteinaceous material in the close proximity of the producing cell, ensuring efficient utilization of the substrate protein. Secreted enzymes would catalyze the hydrolysis of proteins both close and far from the mycelium which would be wasteful to the economy of the producing organism. On depletion of the protein substrate the wall-bound proteinases may be released by an as yet undefined mechanism to scavenge in the substratum. (Adapted from Kalisz *et al.*, 1987).

In the basidiomycetes (Kalisz *et al.*, 1987) and in *Saprolegnia* (Smith *et al.*, 1994) proteinase activity is believed to be localized in the mycelium and its immediate environments. The reasoning for this is that proteinases are either trapped by a membrane or bound to the outside of the cell wall resulting in greater enzyme control and degradation of proteinaceous material in close proximity to fungal hyphae. In nature this control of proteinases close to cell walls will reduce the loss of amino acids and dipeptides to competitors.

Chironomus Proteinases

Gelatin-SDS-PAGE revealed that the proteinases of 4th instar chironomid larvae do not contain acid activatable proteinases. Inhibitor analysis with E-64 showed no reduction in proteolytic activity thus indicating that the proteinases assayed were not cysteine proteinases (Figure 17).

In *Chironomus* as well as *Achlya*, high molecular mass (slow running) proteinases were detected, these have also been found in other species (North, 1994). These proteinases have yet to be analyzed in detail and the possibility that they represent aggregates or complexes of proteinases with other proteinases can not be eliminated. However, it is known that this multiplicity of proteinase forms can be explained by the fact that many species have multiple genes.

Euglena gracilis Proteinases

When grown photosynthetically *Euglena gracilis* produced no proteinases that could be detected by gelatin-SDS-PAGE (Figure 18). When grown with bacteria as an energy source, a single proteolytic band could be detected having an apparent molecular mass of approximately 140 kDa. The discovery of this band, as intuitively expected, illustrates that proteinases are utilized for the degradation of bacterial protein; however their lack of expression indicates that they are not playing a role in the energy production derived from photosynthesis. After acid treatment this band of proteolytic activity significantly diminished, it is believed that the acid treatment denatured the enzyme. Inhibitor treatments with E-64 characterized the enzyme from *E. gracilis* as not being a cysteine proteinase.

Tetrahymena pyriformis Proteinases

The proteinases of the ciliated protozoan *Tetrahymena pyriformis* were analyzed by gelatin-SDS-PAGE in hopes of finding a pH regulated cysteine proteinase system similar to that found in the Dictyosteliaceae. The results were very pleasing as gelatin-SDS-PAGE illustrated that *T. pyriformis* has three cysteine proteinases that could be acid activated, base deactivated, and acid reactivated (Figure 19). Also E-64 inhibitor analysis defines all the proteinases as cysteine proteinases. The apparent method of pH regulation appears to be similar to , if not the same, as that found in the Dictyosteliaceae.

This organism was not selected as one of convenience, as the others were, rather it was sought out and ordered from the American Type Culture Collection (ATCC, Manasses, VA) due to published similarities between the cellular slime moulds and the ciliates. Cysteine proteinases of *Tetrahymena thermophila* and *Dictyostelium discoideum* have been shown to share a highly conserved amino acid motif in the propeptide region of the protein, the ERFNIN motif (Cohen, *et al.*, 1986; Feng *et al.*, 1985). This motif has been shown to be present in virtually all studied cysteine proteinases with the exception of the cathepsin-B like proteins. Differences in the propeptides and in the conserved amino acids of the mature proteins suggest that the ERFNIN proteinases and the cathepsin B-like proteinases constitute two distinct subfamilies within the cysteine proteinases (Karrer *et al.*, 1993). These results therefore place the cysteine proteinases from *Tetrahymena* and *Dictyostelium* in the same family.

Dr. Hud Freeze made an observation that *Dictyostelium* and *Tetrahymena* share a physiological trait that correlates with the existence of acid activatable proteinases. In *Dictyostelium*, endocytosed material enters the cell at ambient pH (>6.0), and over the

next 15-20 minutes the environment surrounding the material decreases to pH 4.5-5.0. However, in the following 20-40 minutes the pH rises again to >6.0 (Aubry *et al.*, 1993). Aggressive competitors such as *Acanthamoeba*, *Paramecium*, and *Tetrahymena* also do similar vesicular pH shuffles, but mammalian cells do not (Bowers and Olszewski, 1983; Fok and Allen, 1990; Nilsson, 1979).

In 1977 Carl Woese was able to order biological diversity based on the concept that nucleic acid sequences could be used to relate organisms (Woese, 1987). His universal phylogenetic tree based on small subunit-ribosomal-RNA sequences places the evolution of the ciliates shortly after that of the Dictyosteliaceae (Figure 25). Therefore it is conceivable that the ciliates and the Dictyosteliaceae share similarities in their enzyme systems.

The results of the studies with *Tetrahymena* show that the proteinases are activated by an acid treatment, deactivated by a treatment with base, and reactivated by a secondary acid treatment. Inhibitor studies performed with E-64 characterize all assayed proteinases as cysteine proteinases. Five proteolytic bands were evident in *T. pyriformis* when a crude homogenate was assayed by gelatin-SDS-PAGE; all bands responded similarly to acid/base and inhibitor treatments. The proteolytic patterning as a result of the acid/base and inhibitor treatments is very similar to that found in the Dictyosteliaceae (Cotter *et al.*, 1997) and was the only other species tested which exhibited this phenomenon. I intuitively believe that the cysteine proteinases in *Tetrahymena* are pH regulated by conformational change as they are believed to be in the Dictyosteliaceae. Further structure modeling experiments with both species are required to prove this hypothesis.

Interestingly, a group in Japan has recently purified a *Tetrahymena* proteinase with an apparent molecular mass of 28 kDa which they designated 'tetrain' (Suzuki *et al.*, 1997). They found tetrain to have different activities at different pH ranges but the mechanism of tetrain activation was found to be elusive. As previously mentioned, we believe the method of activation to be pH regulated, this mode of activation would not be obvious as classical literature states that the cysteine proteinase are activated by a cleavage in the pro-region. In the current study, the cysteine proteinase with the apparent molecular mass of tetrain showed the greatest amount of proteolytic activity of all proteinases observed by gelatin-SDS-PAGE.

Figure 25. Universal phylogenetic tree based on small-subunit ribosomal RNA

Sequences (Kessin, 1997). The general overview of phylogeny and the position of the cellular slime molds, as derived from ribosomal sequence.

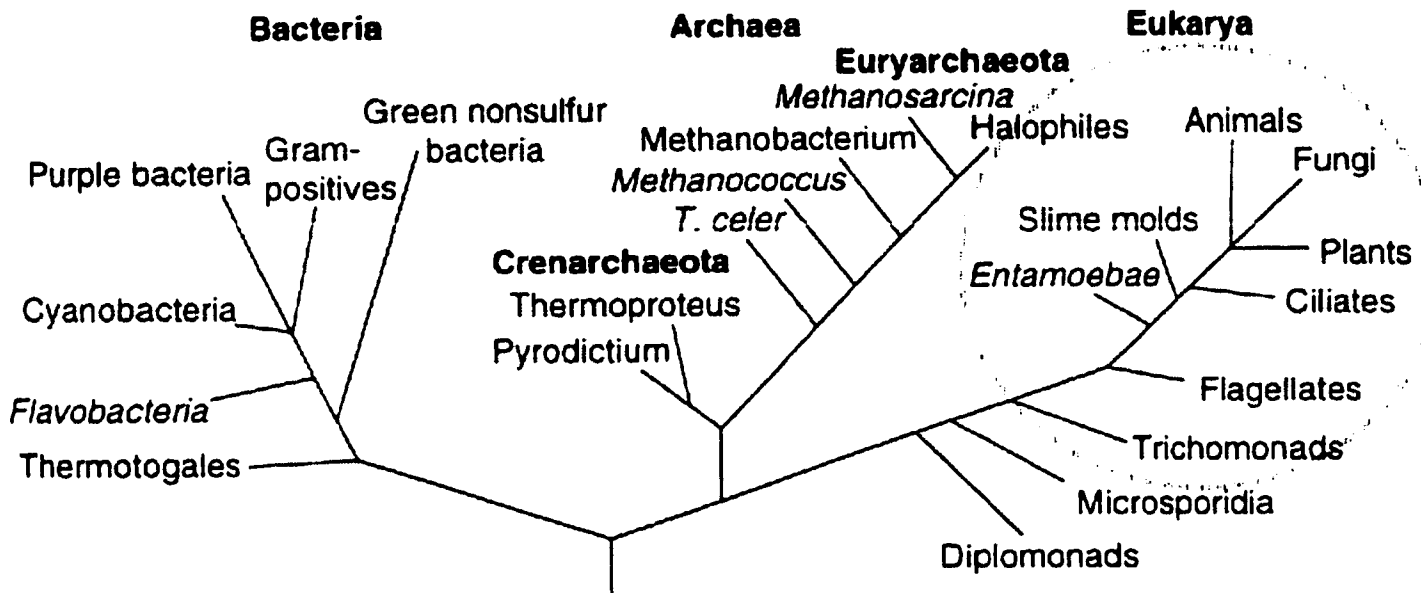


Figure 25.

Bacterial Induced Proteolytic Responses in *Polysphondylium pallidum*

In *D. discoideum* differences between the levels of hydrolytic enzymes in axenically and bacterially grown cells have been reported and in most cases the activities were higher in the latter (Ashworth and Wiener, 1972; Burns *et al.*, 1981). Relevant gelatin-SDS-PAGE experiments performed on axenically and bacterially grown myxamoebae of *Polysphondylium pallidum* support this observation (Figure 20).

The results shows little proteolysis occurs when *P. pallidum* is grown axenically, only the aspartyl proteinase ppAP65 is active. As seen in *D. discoideum*, the aspartyl proteinase (ddAP58) activity does not change greatly throughout the entire lifecycle of *D. discoideum* and is unaffected by acid and base treatments (Cotter *et al.*, 1997). It appears that the aspartyl proteinase of *P. pallidum* is similar to that of *D. discoideum* from the commonalties of an ambivalence to acid/base treatments, a relatively similar apparent molecular mass, and an apparent lack of regulation by axenic or bacterial growth. In the Dictyosteliaceae the developmental roles of the aspartyl proteinases remain unclear at this time.

When *P. pallidum* is grown in the presence of bacteria (*E. coli*) a single proteinase of apparent molecular mass of 31 kDa is active. This is supportive of the work of North *et al.* (1984) with *P. pallidum* who found the contribution of cysteine proteinases to be much lower when grown axenically than when grown with bacteria. It has been suggested that cells growing in an axenic medium are in a transition state between true vegetative growth and the complete developmental cycle so that axenic growth leads to premature developmental changes (Burns *et al.*, 1981). This hypothesis is supported by the striking effects of acid treatment on the cysteine proteinases separated by gelatin-

SDS-PAGE. After acid treatment of the proteinases extracted from axenically grown cells the cysteine proteinase, ppCP47 becomes active. If one conforms to Burns' hypothesis, it follows that the ppCP47 was produced in an inactive, or immature form, which later can be activated by acid activation. If Burns' hypothesis is true then these results elucidate the maturation pathways of the pH regulated cysteine proteinases in the Dictyosteliaceae. The cysteine proteinases are produced in an inactive form which can be activated by acidic environments.

In bacterially grown cells ppCP31 is naturally active in the cells growing with bacteria, after acid treatment two new proteinases are visible, ppCP47, and ppCP24. These results indicate that the proteinase regulation is further complicated. It is seen that when *P. pallidum* is grown with bacteria there is not simply the activation of proteinases but also the production of new proteinases. These proteinases are presumably involved in the digestion of the bacterial nutrients whose production is regulated by some bacterial factor. The results of these experiments are summarized in Figure 24. In this figure CP 1,2, and 3 represent the proteinases ppCP24, ppCP31, and ppCP47. When these numbers are circled an active enzyme is indicated (as assayed by gelatin-SDS-PAGE in Figure 23).

In *D. discoideum* the observed differential proteolytic responses from cells grown axenically and with bacteria have respectively been termed the A (axenic) and B (bacterial) patterns (North, 1988). This nomenclature appears well suited to describe the comparable phenomenon found in *P. pallidum*.

In order to elucidate whether the bands of acid activatable cysteine proteinase activity found within the spores of *D. discoideum* (ddCP43, and ddCP48) were individual proteinases or whether they were merely the active subunits of one large protein, gelatin-Native-PAGE was performed. The results from these experiments show two distinct

Figure 24. Model of proteinase regulation in *P. pallidum* in axenic or bacteria supplemented conditions. In this figure CP 1,2, and 3 represent the cysteine proteinases ppCP24, ppCP31, and ppCP47 from homogenized myxamoebae of *P. pallidum*. When these numbers are circled an active enzyme is indicated (as assayed by gelatin-SDS-PAGE in Figure 20).

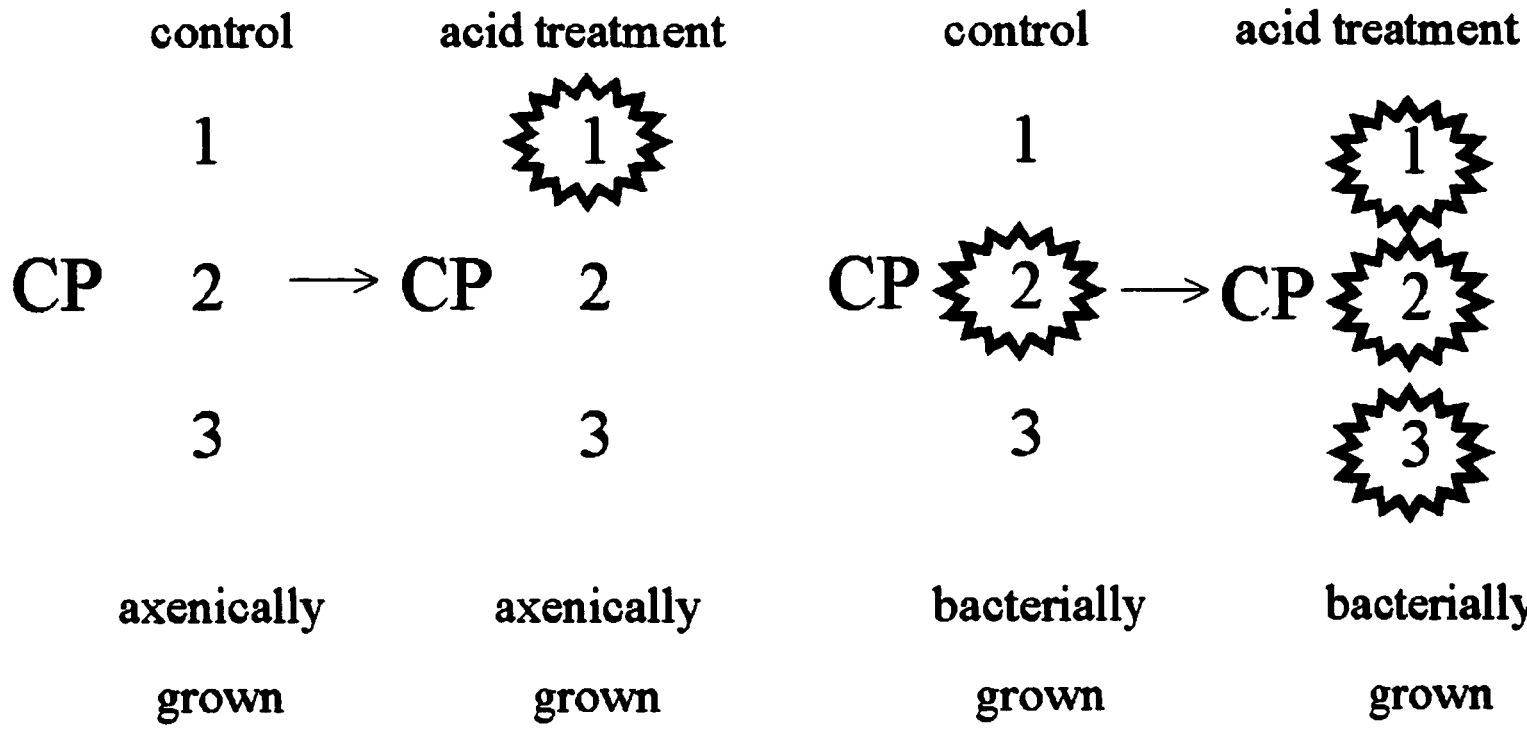


Figure 24.

bands of proteolytic activity having similar molecular masses (Figure 22). These results suggest that the two cysteine proteinases ddCP43 and ddCP48 are not the active subunits of a large enzyme but that they are two separate enzymes. For reference the cysteine proteinase gelatin-SDS-PAGE patterning of the acid activated proteinases of the spores of SG1 has been included (Figure 21).

A novel method of visualizing disulfide bonding in proteins by one-dimensional SDS-PAGE was constructed by Allore and Barber in 1984. Their insight allowed researchers to display the extent of inter- and intramolecular disulfide bonding within a protein. Proteins and protein complexes which contain disulfide bonds can be specifically identified by the alterations in apparent mobility (M_r) which occurs when samples are electrophoresed in the presence or absence of reducing agents. When the M_r of an electrophoresed sample lacking reducing agents is greater than the M_r of the same sample containing reducing agents, then intradisulfide bonding is inferred. When the M_r of an electrophoresed sample lacking reducing agents is less than the M_r of a the same sample containing reducing agents, then interdisulfide bonding is inferred. When the relative mobility of the bands is unchanged an absence of inter- and intramolecular disulfide bonding is inferred.

Allore and Barber's protocols were adapted for use with gelatin-SDS-PAGE zymograms (Figure 23). The results from these experiments demonstrate that the M_r of the ddCP48 in reducing and nonreducing conditions was indistinguishable, thus suggesting that ddCP48 has no inter- or intramolecular disulfide bonds. The M_r of the ddCP38 in nonreducing conditions showed an increased mobility when compared to

reducing conditions, suggesting the presence of intramolecular disulfide binding within the proteinase.

The lack of intermolecular disulfide bonds detected by this technique, as well as the presence of two bands of proteolytic activity in gelatin-Native-PAGE strongly suggests that the cysteine proteinases ddCP38 and ddCP48 are independent enzymes rather than active subunits of a larger enzyme that is dissociated by the semi-denaturing conditions of SDS-PAGE.

In *D. discoideum* other more defined cysteine proteinases, CP1 and CP2, have been found to be not only independent enzymes but that the gene duplication that gives rise to the two separate cysteine proteinase genes of *Dictyostelium* is very ancient. It is believed to have taken place early in the history of the eukaryotes before the divergence of *Dictyostelium*, plants, and animals (Hughes, 1994).

The *in vitro* activation of the cysteine proteinases is believed to simulate the *in vivo* activation of these enzymes. It is believed that the cysteine proteinases are sequestered into vacuoles that are sites for the digestion of nutrients absorbed by phagocytosis. The pH of the vacuoles is thought to be regulated by vacuolar H⁺-ATPases (V-H⁺-ATPases); these enzymes are known to exist in the membranes of *D. discoideum* (Heuser *et al.*, 1993; Bush *et al.*, 1994; Fok and Allen, 1994; Nolte and Steck, 1994) as well as in mammals (Holtzman, 1989). The vacuolar proton pump is a multimeric enzyme that, through the use of the energy from ATP hydrolysis, transports protons across membranes (reviewed by Glock, 1993). This active influx of hydronium ions

serves to lower the pH of the vesicles, and is also thought to play a role in water accumulation (Clarke and Heuser, 1997).

Further evidence of this phenomenon is supported by the recent findings in the yeast *Saccharomyces cerevisiae*. Mutants lacking the VMA genes which encode the subunits of the vacuolar H⁺-ATPase were found to accumulate autotrophic bodies in their vacuoles. This accumulation was shown to result from a defect in the protein degradation mechanism, as proteinase concentrations in the *vma*⁻ cells were remarkably lower than the wild-type (Nakamura *et al.*, 1997).

The role of V H⁺-ATPases in *D. discoideum* is not completely understood however. It is unknown why the contractile vacuole, an organelle used to maintain osmotic regularity, is not found to be acidic despite the presence of V H⁺-ATPases within the vacuolar membrane (Zhu and Clarke, 1992). The work of Dr. J. Gross has allowed him to theorize an explanation for this phenomenon (Gigliione and Gross, 1995). He believes that bicarbonate is utilized as an expendable osmolyte by the Dictyosteliaceae and other fresh water amoebae instead of salt, which while available in mammals can not be readily replaced by the environment in freshwater. This anion can be produced by CO₂ by the action of carbonic anhydrase, which is present in the cytoplasm of *Dictyostelium* cells. Bicarbonate is cotransported with protons into the lumen of the contractile vacuole, where it would act to draw in water (Heuser *et al.*, 1993). In support of this hypothesis, Gross has found that vesicles derived from a proton pump-rich membrane of *Dictyostelium* possess a bicarbonate channel or transporter in addition to an electrogenic proton pump. In the presence of bicarbonate, H⁺ and HCO₃⁻ are cotransported into the vesicles, thereby preventing the formation of a substantial pH

gradient across the vesicle membrane (Clarke and Heuser, 1997). Therefore the observation that endocytic compartments in myxamoebae are highly acidic while contractile vacuoles are not is unlikely to be due to a difference in the vacuolar H⁺-ATPase of these organelles. It suggests instead that the contractile vacuoles possesses a bicarbonate channel or transporter absent from the endolysosomal system (Giglione and Gross, 1995).

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APPENDIX A

Table 1.0 Preparation of 7.5% and 10% acrylamide separating/resolving gels for gelatin-SDS-PAGE.

Constituents	7.5 % Acrylamide	10 % Acrylamide
Nanopure H ₂ O	3.85 mL	3.00 mL
2 % Gelatin	1.00 mL	1.00 mL
1.5 M Tris-HCL, pH 8.8	2.50 mL	2.50 mL
30 % T Acrylamide	2.50 mL	3.35 mL
10 % SDS	100 µL	100 µL
10 % Ammonium Persulfate	50 µL	50 µL
TEMED	10 µL	10 µL

Table 2.0 Preparation of 4.0% acrylamide stacking gels for gelatin-SDS-PAGE.

Constituents	
Nanopure H ₂ O	5.90 mL
0.5 M Tris-HCl, pH 6.8	2.50 mL
30 % T Acrylamide	1.50 mL
10 % SDS	100 µL
10 % Ammonium Persulfate	50 µL
TEMED	10 µL

Table 3.0. Preparation of 7.5 % and 10 % acrylamide separating gel for gelatin-Native-PAGE

Constituents	7.5 % Acrylamide	10 % Acrylamide
Nanopure H ₂ O	3.85 mL	3.00 mL
2 % Gelatin	1.00 mL	1.00 mL
1.5 M Tris-HCL, pH 8.8	2.50 mL	2.50 mL
30 % T Acrylamide	2.50 mL	3.35 mL
TEMED	10 μ L	10 μ L

Table 4.0. Preparation of 4.0% Acrylamide Stacking Gel for Gelatin-Native-PAGE.

Constituents	
Nanopure H ₂ O	5.90 mL
0.5 M Tris-HCl, pH 6.8	2.50 mL
30 % T Acrylamide	1.50 mL
TEMED	10 μ L

APPENDIX B

Glucose Salts Agar

1.0 g	NH ₄ Cl (Sigma, St. Louis, MO, St. Louis, MO)
0.13g	MgSO ₄ (Sigma, St. Louis, MO, St. Louis, MO)
3.0 g	KH ₂ PO ₄ (Sigma, St. Louis, MO, St. Louis, MO)
6.0 g	Na ₂ HPO ₄ (Sigma, St. Louis, MO)
4.0 g	glucose (Difco, Mauston, WI, Mauston, WI)
15.0 g	agar (Difco, Mauston, WI)

Dissolved in 1 litre of distilled water, pH 6.5.

Standard Medium/2 (SM/2) Agar

5.0 g	glucose (Difco, Mauston, WI)
5.0 g	bactopeptone (Difco, Mauston, WI)
0.5 g	yeast extract (Difco, Mauston, WI)
1.1 g	KH ₂ PO ₄ (Sigma, St. Louis, MO)
0.5 g	K ₂ HPO ₄ (Sigma, St. Louis, MO)
0.5 g	MgSO ₄ (Sigma, St. Louis, MO)
15.0 g	agar

Dissolved in 1 litre of distilled water, pH 6.5.

Non-Nutrient Agar

20 g	agar (Difco, Mauston, WI)
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Dissolved in 500 mL of distilled water plus 500 mL of sterile 10 mM KPi, pH 6.5.

TM Liquid Medium

10.0 g	trypticase peptone (Difco, Mauston, WI)
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5.0 g	yeast extract (Difco, Mauston, WI)
10.0 g	glucose (Difco, Mauston, WI)
0.35 g	NaH ₂ PO ₄ (Sigma, St. Louis, MO)
1.2 g	K ₂ HPO ₄ (Sigma, St. Louis, MO)

Dissolved in 1 litre of distilled water, pH 6.5.

Barksdale's Medium

D-glutamate (Monosodium glutamate)	0.4 g
Glucose	2.8 g
Tris	1.2g
L-methionine	1.0ml (stock 15 mg/mL in 1 N HCl)
KCl	1.0ml (stock 14.9 g/100mL in distilled water)
MgSO ₄ ·7H ₂ O	1.0 mL (stock 12.32 g/ 100mL in distilled water)
CaCl ₂	1.0 mL (stock 5.55 g/ 100mL in distilled water)
EDTA	2.0 mL (stock 1.0 g/ 100mL in distilled water)
KH ₂ PO ₄	2.0 mL (stock 10.2 g/ 100mL in distilled water)
Metal mix	10 mL (stock 0.2 g/ 100 mL in distilled water)
Distilled water	1 L
pH	6.9 (adjust with a small volume of acid)
For Agar Stabilization	1.5 % Agar

Metal mix

Fe(NH ₄) ₂ (SO ₄) ₄ ·6H ₂ O	1.44 g
ZnSO ₄ ·7H ₂ O	0.44 g
MnSO ₄ ·H ₂ O	0.15 g
Grind to a fine powder using a mortar and pestle.	

APPENDIX C

5x Sample Buffer for Gelatin SDS PAGE

2.5 mL	β -mercaptoethanol (Sigma, St. Louis, MO)
5.0 mL	glycerol (Sigma, St. Louis, MO)
2.5mL	0.5 M Tris pH 6.8 (Sigma, St. Louis, MO)
1.0 g	SDS (Sigma, St. Louis, MO)
1.0 mg	Bromophenol Blue (Sigma, St. Louis, MO)

Running Buffer (pH 8.3) for Gelatin SDS PAGE

3.0 g	Tris Base (Sigma, St. Louis, MO)
14.4 g	glycine (Sigma, St. Louis, MO)
1.0 g	SDS (Sigma, St. Louis, MO)

Dissolve in 1.0 L nanopure water.

Coomassie Brilliant Blue Stain

2.5 %	Coomassie Brilliant Blue R-250 (w/v)
50 %	methanol (v/v)
10 %	glacial acetic acid (v/v)

5 x Sample Buffer for Gelatin Native PAGE

2.5 mL	water
5.0 mL	glycerol (Sigma, St. Louis, MO)
2.5 mL	0.5 M Tris pH 6.8 (Sigma, St. Louis, MO)
1.0 mg	Bromophenol Blue (Sigma, St. Louis, MO)

Running Buffer (pH 8.3) for Gelatin Native PAGE

3.0 g Tris Base (Sigma, St. Louis, MO)

14.4 g glycine (Sigma, St. Louis, MO)

Dissolve in 1.0 L nanopure water.

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Cotter, D.A., Gale, K.E., Dunbar, A.J., Cervi, D., Wolak, T., Sands, T.W. and J.F. Wheldrake. 1998. Ammonia is both a positive and a negative upstream regulator of PKA activity in the life cycle of *Dictyostelium discoideum*. International Conference on *Dictyostelium discoideum*. Munich, Germany.

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Cavallo, D., Gale, K.E., Wolak, T., Sands, T.W. and D.A. Cotter. 1997. Detection of cryptic cysteine proteinases in the cellular slime moulds. American Society of Cell Biology. Washington, D.C.

Cavallo, D., Gale, K.E., Sands, T.W. and D.A. Cotter. 1997. Regulation of cysteine proteinases from alternative life cycles of the Dictyosteliaceae. American Society of Microbiology. Miami, FL.

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