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The Effects of Hydrogen Peroxide and the Involvement of the Cysteine Proteases in the Life Cycle of the Social Amoeba *Dictyostelium discoideum*, and the Acid Activatable Proteases of *Acanthamoeba castellanii*

by
Kashif Z. Kirmani

A Thesis
Submitted to the Faculty of Graduate Studies and Research
through Biological Sciences
in Partial Fulfillment of the Requirements for
the Degree of Masters of Science at the
University of Windsor

Windsor, Ontario, Canada
2004

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The Effects of Hydrogen Peroxide and the Involvement of the Cysteine Proteases in the Life Cycle of the Social Amoeba *Dictyostelium discoideum*, and the Acid Activatable Proteases of *Acanthamoeba castellanii*

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Abstract

It has been demonstrated previously that actin in the dormant spores of *Dictyostelium discoideum* can be dephosphorylated when incubated in glucose solutions. The work reported here supports and extends previous work demonstrating that actin as well as a 66 kilodalton (kDa) protein were dephosphorylated when dormant spores of *Dictyostelium* were incubated in a glucose of 100 mM for 1 hour. The dephosphorylation of both of these proteins was inhibited by hydrogen peroxide. Furthermore, the addition of the reducing agent, dithiothreitol (DTT), along with glucose and hydrogen peroxide (H_2O_2), allowed for the dephosphorylation of actin but not the 66 kDa protein. Recent reports have indicated that the spores of *Dictyostelium* are highly resistant to H_2O_2. Stress tests revealed that spores could survive in concentrations of 50 mM H_2O_2; however, this level of oxidant abolished the spore's ability to spontaneously germinate. It also was observed *in vitro* that the cysteine proteases of *Dictyostelium* could be inhibited by hydrogen peroxide and that enzyme activity could be restored by the addition of the reducing agent DTT. It was demonstrated here that the previously characterized non-acid activatable cysteine protease CP18 found in the spore matrix was acid activatable when sodium dodecyl sulfate (SDS) was absent from the polyacrylamide gel electrophoresis system (PAGE). However, the cysteine proteases of *Dictyostelium* were found to have no direct involvement anywhere in the life cycle. The spore matrices from *Dictyostelium* wild-type strains *NC4* and *V12* and spontaneous germinator mutant *SG2* were examined utilizing SDS-PAGE. It was shown that in the wild-type strains but not the mutant *SG2*, a 26 kDa protein accumulated from 1 day old spores and remained at a

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steady state level for up to 4 days in the matrix. A unique characteristic of *Dictyostelium* is the ability for its cysteine proteases to be acid activatable. In an effort to elucidate whether or not organisms phylogenetically related to *Dictyostelium* have acid activatable proteases, protein extracts from the microcysts of *Acanthamoeba castellanii* were separated on SDS-PAGE zymograms. It was observed that the proteases from the microcysts of *Acanthamoeba* were acid activatable, but not base deactivatable like the cysteine proteases of *Dictyostelium*. Incubation of protein extracts from microcysts with the cysteine protease inhibitor E-64 prior to separation revealed that a 40 kDa protease belonged to the cysteine class.

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Dedication

Although there have been plenty of people that have been extremely supportive of me during my time as a graduate student, there is one exceptional person who has been alongside me through many different climates of weather. As I look back now at my undergraduate years, I would describe myself as an individual whose skills needed a little more time to catch up with him. Had you asked me at the time, however, I had already written myself off for failure. This individual would tell you differently, though. For some reason, this person saw something exceptional of an individual who sometimes had lost a bit of his self confidence. As the years went by, this "cheerleader" of mine did manage to inspire a little bit of hope in me. Many years later, this person was so insistent in supporting me that she followed me to my new surroundings so I could pursue my master's degree. I must admit, I wouldn't know how to sustain had it not been for the constant love and support Shelly has given to me throughout this whole time. Not only has she stood by my side when things got a little hectic, but she arguably sacrificed as much as I've had to so I could be in the incredible situation that I find myself in today. With that in mind, I would like to dedicate this thesis to my better half, Shelly.

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Throughout the years, there have been many undergraduate students who have come and gone through the lab. I would like to acknowledge Yaa Forjour, Lindsay Beneteau, Maryam Al-Shaffei, Sotheary Kiev, Vivian Chow, Michelle Askew, Brett Bogucki, and Laura Sovran. The undergraduates have always done a good job of boosting my ego a bit by thanking me for my assistance. It is me who should be thanking them for their wisdom and knowledge. I have always found that I have taken more knowledge away from the undergraduates than I have been able to pass on

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to them. I would like to thank Marian and Farhad for being such good friends to me. I would also like to thank Ajay and Ed for being my friends and housemates for the last couple of years.

There have been quite a few professors who have been helpful to me. I would like to thank Dr. Michael Dufresne and Dr. Derick Jane. I would like to thank my thesis committee members Dr. Alden Warner and Dr. Siryam Pandey. I would also like to thank Dr. Adnan Ali, Dr. Michael Crawford, and Dr. Hugh Fackrell for allowing me to use some of the resources in their respective laboratories to conduct some necessary aspects of my research.

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List of abbreviations

Acanthamoebal keratitis; AK

Adenylyl cyclase A; ACA

Adenylyl cyclase B; ACB

Adenylyl cyclase G; ACG

Ammonium persulfate; APS

Bovine serum albumin; BSA

Cyclic adenosine monophosphate; cAMP

Dimethyl sulfoxide; DMSO

Dithiothreitol; DTT

(2S,3S)-trans-Epoxysuccinyl-L-leucylamido-3-methylbutane ethyl ester; E-64D

Ethylenediaminetetraacetic acid; EDTA

(2*S*,3*S*)-3-(*N*-{(*S*)-1-[*N*-(4-guanidinobutyl)carbamoyl]3-methylbutyl}carbamoyl)oxirane-2-carboxylic acid; E-64

Granulomatous amoebic encephalitis; GAE

Hydrogen peroxide; H_2O_2

Kilodaltons; kDa

Micrometer; µm

Micromolar; µM

Milliliter; ml

Millimolar; mM

Molar; M

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Nitric oxide; NO

Phenylmethylsulfonyl fluoride; PMSF

Polyacrylamide gel electrophoresis; PAGE

Potassium phosphate buffer; KPi

Reactive oxygen species; ROS

Sodium dodecyl sulfate; SDS

Standard media over 2; SM/2

Superoxide; O^-

N,N,N',N' Tetramethylethylenediamine; TEMED

Trypticase media; TM

Units; U

Water; H_2O

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Chapter 1. Introduction

The social amoeba, *Dictyostelium discoideum*, is a simple eukaryote that is part of the micro-flora of soil (Raper, 1935). Since there are no pathogenic diseases associated with the organism, and the biochemistry and molecular make-up of the organism are quite similar to higher eukaryotic cells, *D. discoideum* is an ideal laboratory model for biochemistry and molecular biology research (Cotter *et al*, 1992). The social amoeba *D. discoideum* is unique in that it can exist as a unicellular or multi-cellular organism (Poinar and Waggoner, 1992). *D. discoideum* is able to enter into one of two developmental pathways depending upon the environmental conditions (Firtel and Bonner, 1972; Loomis, 1982; Raper, 1984; Cotter *et al*, 1992). The most well established of these pathways is fruiting body formation, which occurs during the asexual life cycle (Loomis, 1982); (Figure 1). *D. discoideum* is also able to enter into a sexual life cycle in which cells of opposite mating types form heterothallic macrocysts (West and Erdos, 1990; Cotter *et al*, 1992).

The asexual life cycle and internal physiology of *D. discoideum*

D. discoideum is able to maintain itself in a dormant state encapsulated in a spore. In its dormant state there is a high level of cyclic adenosine monophosphate (cAMP) produced by adenylyl cyclase G (ACG). The level of ammonium phosphate in the sorocarp containing the spores is between 100 to 200 mM. It is believed that this level of ammonium phosphate supplies an osmotic stimulus that activates ACG (van Es *et al*, 1996; Cotter *et al*, 1999; Virdy *et al*, 1999). Discadenine, which is contained in the fluid spore matrix in the sorus, is also believed to act as an inhibitor

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towards spontaneous spore germination. *Dictyostelium* histidine kinase B (DhkB) is believed to be stimulated by the adenine derivative to inhibit the internal cAMP phosphodiesterase, RegA (Zinda and Singleton, 1998). The actin filaments in the dormant spore are phosphorylated on a tyrosine residue, and the cysteine protease activity within the spore is low (Kishi *et al*, 1998; Cavallo *et al*, 1999).

When the environmental conditions become favorable, the spore will germinate. Germination occurs in 3 steps: activation of the dormant spore, spore swelling, and finally emergence of a nascent myxamoeba (Cotter *et al*, 1992). The environmental factors that regulate spore germination are temperature, pH, osmotic pressure, and natural autoinhibitors and autoactivators (Cotter *et al*, 1979). If the spores of *D. discoideum* sense that there are bacteria in the area, germination occurs. This can be mimicked in the laboratory by incubating wild-type spores one to three days of age in peptone or glucose solutions in which bacteria have been growing for twenty four to forty eight hours. Germination can be induced by a heat shock at 45°C for thirty minutes (Cotter and Raper, 1966; Hashimoto *et al*, 1976; Dahlberg and Cotter, 1978; Ihara *et al*, 1990; Kishi *et al*, 1998). Germination of one to three day old wild-type spores can also be induced in either of the following reagents; 20% DMSO, 8 M urea, 6 M guanidine HCl, 2 M dimethylurea, 2 M tetramethyl urea, or 30% methanol followed by a rapid deplasmolysis (Cotter *et al*, 2000). In nature, if environmental conditions such as heat or bacterial food sources have not initiated spore germination, the natural autoinhibiters become diluted and the spores will spontaneously germinate when they are six to ten days of age (Cotter and Dahlberg, 1977; Dahlberg and Cotter, 1978). This spontaneous germination process in aged

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spores is induced by a low molecular weight "autoactivator". Once the autoinhibitors have been diluted or removed, the autoactivator is released by a few spores which induces swelling of neighboring spores, which in turn induces the release of more autoactivator (Cotter and Dahlberg, 1977; Dahlberg and Cotter, 1978). Two of the biochemical hallmarks of spore germination are the dephosphorylation of actin and an increase in the level of cysteine protease activity (Kishi *et al*, 1998 and 2000; Cavallo *et al*, 1999). Since the ammonium/ammonia and discadenine concentrations have become dilute in the sorus, the cAMP phosphodiesterase inhibitor DhkB and ACG are no longer stimulated; as a result, RegA phosphodiesterase is able to turnover the cAMP that has accumulated in the spore (van Es *et al*, 1996; Virdy *et al*, 1999; Loomis, 1998). Therefore, another biochemical marker of spore germination is the decrease in the level of cAMP in the germinating spore.

When the nascent amoeba is released from the spore, the growth phase of the life cycle begins. The internal physiology shows that the cysteine protease activity is highest during vegetative growth and actin has been completely depolymerized (Kishi *et al*, 1998; Cavallo *et al*, 1999). As long as there are numerous bacterial cells for the amoeba to ingest, the organism will grow and divide (Cotter *et al*, 1992). When the nutritional requirements have been depleted, especially free amino acids, the developmental life cycle is initiated. The vegetative cells of *D. discoideum* will secrete waves of cAMP that are produced by adenylyl cyclase A (ACA) and adenylyl cyclase B (ACB) (Cotter *et al*, 2000). The waves of cAMP serve as a chemoattractant to signal the vegetative cells to aggregate together (Bonner, 1967). Other factors secreted by the vegetative cells that aid in the aggregation process

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include the multi-subunit protein complex known as counting factor and superoxide (O^{-}) (Gomer *et al*, 2002; Bloomfield and Pears, 2003; Brock *et al*, 2003a and b). It has been reported that the cysteine proteases of *D. discoideum* are also involved in the aggregation process (Fong and Bonner, 1979). The internal cysteine protease activity is decreasing at this time (Cavallo *et al*, 1999). As the cells aggregate, they bind together in a linear fashion to form a mound containing approximately 10^5 cells (Loomis, 1982). The vegetative cells that are at the tip of the mound differentiate into pre-stalk cells, while those in the rest of the mound differentiate into pre-spore cells (Jermyn *et al*, 1989). The tip of the mound will then become erect to form a vertical slug, which will fall over and migrate (Raper, 1940). At this time, the majority of cAMP is produced primarily by ACB (Meima and Schaap, 1999; Soderbom *et al*, 1999; Cotter *et al*, 2000). The slug migrates in such a manner that the pre-stalk cells, which comprise 20% of the slug, will move to the anterior portion of the slug, the remaining pre-spore cells will move to the posterior portion of the slug (Cotter *et al*, 1992).

The final stage of the classical developmental life cycle is known as culmination. During the culmination stage of development, the pre-stalk cells form a vertical tube in response to avoid the increasing concentration of ammonia that is being produced, and terminally differentiate into stalk cells inside the tube (Schindler and Sussman, 1977; Bonner *et al*, 1989; Cotter *et al*, 2000). The cAMP phosphodiesterase inhibitor DhkA acts to initiate a rising level of cAMP in the pre-spore cells (Loomis, 1998). Monomeric actin starts to polymerize and becomes tyrosine phosphorylated (Kishi *et al*, 1998). While the stalk cells will eventually die,

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the pre-spore cells are lifted towards the top of the hollow vertical tube and terminally differentiate into spores. During encapsulation, the levels of ammonium/ammonia accumulate and stimulate ACG to produce cAMP. The level of discadenine has also increased, which may result in the RegA-phosphodiesterase being inhibited by DhkB (Bonner, 1967; Zinda and Singleton, 1998).

The events occurring during post-encapsulation over a six to ten day period which prepare spores to spontaneously germinate are under investigation in our laboratory.

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Figure 1. The life cycle of *Dictyostelium discoideum*. (A). The vertical hollow tube, or the sorocarp, holding a ball of dormant spores; (B-E). When conditions are favorable, the spores will undergo germination; (F). At the end of the germination process, a single myxamoeba will emerge from the spore; (G-M). When nutrients in the environment become depleted, the developmental life cycle begins. (Image A is from Loomis, 1976; Images B-F are from Raper, 1984; Images G-M are courtesy of Grimson and Blanton, 1995)

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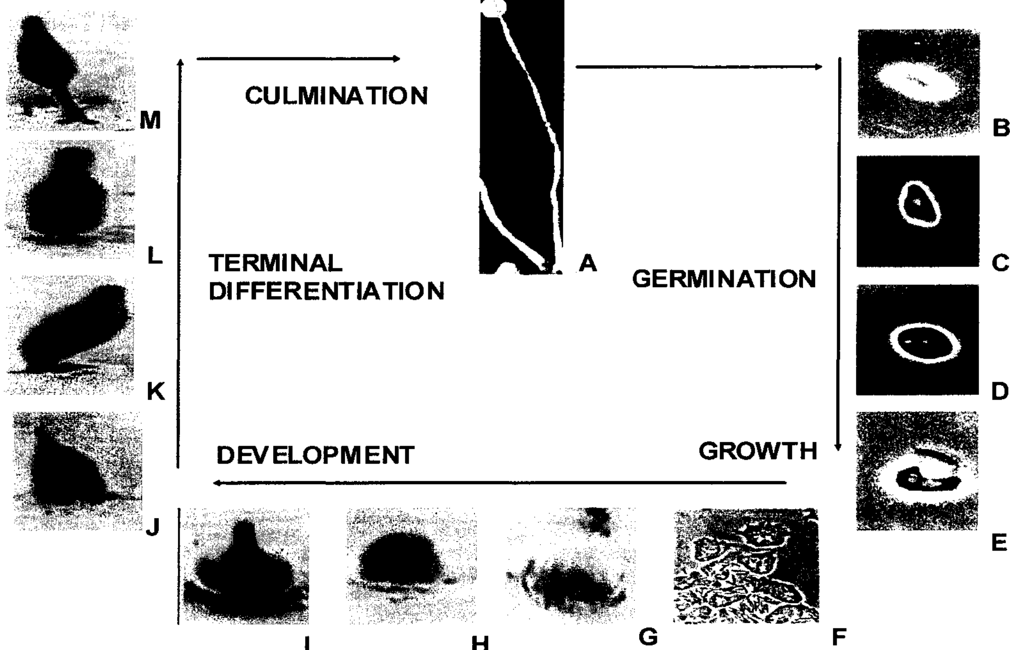

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The role of reactive oxygen species (ROS) in eukaryotic cells

An enigma for all aerobic cell systems is that oxygen, which is required for any aerobic organism to maintain its life cycle, can also become the source of toxic agents (Katoch and Begum, 2003). To ward off the effects of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), nitric oxide (NO), superoxide (O^-) and the hydroxyl radical (·OH), to name a few, cells maintain a level of anti-oxidants to counter-balance the toxic effects of ROS (Kamata *et al*, 1999; Katoch and Begum, 2003). ROS have been found to be involved directly in cell growth, cell-type differentiation and development, and in the up-regulation of gene expression (Schreck *et al*, 1991; Olsen *et al*, 1995; Joneson *et al*, 1998; Li *et al*, 1998; Garcia *et al*, 2000 and 2003; Shibakura *et al*, 2002; Nimnual *et al*, 2003).

ROS also has been shown to be a negative regulator by acting as a second messenger in signal transduction events (Bloomfield and Pears, 2003; Garcia *et al*, 2003; Nimnual *et al*, 2003). Protein tyrosine phosphatases (PTP), enzymes responsible for dephosphorylating tyrosine residues, are inhibited by ROS such as NO and H_2O_2. Inhibition of PTP's occurs by the oxidation of the active site cysteine of the phosphatases. This inhibition is reversible by treating the enzymes with anti-oxidant agents that act upon cysteine residues (Caselli *et al*, 1994; Caselli *et al*, 1998; Bloomfield and Pears, 2003; Nimnual *et al*, 2003).

In the cellular slime mold *Dictyostelium discoideum*, recent studies have shown that ROS contained in the fluid extra-cellular matrix plays an important role in multi-cellular development. Recent reports have also shown that *D. discoideum* is highly resistant to oxidataive stress (Garcia *et al*, 2000; Bloomfield and Pears, 2003;

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Katoch and Begum, 2003). With the exception of these studies, there has not been much research conducted on *D. discoideum* compared to higher eukaryotic cell system in regards to the role ROS may play in signaling events.

The cysteine proteases of *D. discoideum*

In eukaryotic organisms, lysosomal proteases are designated into four different classes; aspartic, metallo, serine, and cysteine proteases. The proteases of the cysteine class are the most abundant of the four classifications. A few of the enzymes that belong to the cysteine class are papain, lysosomal cathepsins, and caricain. Classically, cysteine proteases have been shown to be housekeeping enzymes that act to turn over proteins for various biochemical pathways (Turk *et al*, 2000). However, lysosomal cysteine protease cathepsin B has been shown to be directly involved in cell differentiation (Jane *et al*, 2002).

The proteases of the cysteine class are the most abundant in the social amoeba *D. discoideum* (Cavallo *et al*, 1999). The cysteine proteases of *D. discoideum* exist in multiple isoforms, and are thought to be required for nutritional functions during vegetative cell growth and to degrade endogenous proteins during the developmental life cycle (North and Cotter, 1991; Cavallo *et al*, 1999). Acidic conditions are usually required for the activation of cysteine proteases in higher eukaryotic cell systems. In most eukaryotic cell systems, the cysteine proteases are synthesized as preproenzymes and activation of the cysteine protease requires the cleavage of a pro-region. The mature form of the proteases exists in an acidic environment in its endosomal or lysosomal compartment (Turk *et al*, 2000). In the case of *D.*

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discoideum, it is known that a number of inactive cysteine proteases are acid activatable. *In vitro* analysis of the inactive cysteine proteases of *D. discoideum* has revealed that incubation of the proteolytic enzymes on polyacrylamide gels in 1.7 M acetic acid, pH 2.1 for sixty seconds results in greater activity (North *et al*, 1996; Cavallo *et al*, 1999). Another striking characteristic about the cysteine proteases of *D. discoideum* that makes these proteolytic enzymes different from the rest of the cysteine proteases in higher eukaryotic cells is that whereas most of the cysteine proteases in higher eukaryotes are *N*-linked glycosylated with methylated mannose 6-phosphate (Man6POCH$_3$), the cysteine proteases of *D. discoideum* are glycosylated with *N*-acetylglucosamine-1-phosphate (GlnNAc1*P*) linked onto serine residues (Mehta *et al*, 1996; Freeze, 1997)

In *D. discoideum*, the activity of the cysteine proteases is differentially regulated. In the dormant spore, the internal cysteine protease activity is barely detectable. As the germination process proceeds, the internal cysteine protease activity constantly increases. When the myxamoeba emerges from the spore, the internal cysteine protease activity is at its peak activity. During the developmental life cycle, the internal cysteine protease activity decreases constantly, with the activity become barely detectable in the dormant spore (Cavallo *et al*, 1999).

During the developmental life cycle, the acid activatable cysteine proteases are also differentially secreted by *D. discoideum* (North *et al*, 1990 and 1996). At twelve to twenty-four hours into the developmental life cycle, a 24 kDa weight acid activatable cysteine protease and a non-acid activatable cysteine protease of 18 kDa are secreted into the slime sheath and can also later be found in the spore matrix

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(North *et al*, 1996; Cotter *et al*, 1997). It is thought that secreted proteases may be required to orient the slug towards the soil surface in preparation for the culmination stage of development (Bonner, 1993). It has also been reported previously that the cysteine proteases of *D. discoideum* are required for the developmental life cycle to progress (Fong and Bonner, 1979).

The proteases of *Acanthamoeba castellanii*

A close phylogenetic relative of *Dictyostelium* is *Acanthamoeba*, which consists of a collection of free-living amoebae that are found in soil, heating and cooling ducts, fresh water, and marine environments (Khan *et al.*, 2001). These organisms grow and proliferate as phagotrophic trophozoites (vegetative cells) that feed on microorganisms and inert particles, which can survive inorganic levels of chlorine as high as 2 ppm (Khan *et al.*, 2001). When conditions become harsh, the trophozoites can encyst and resist highly unfavourable conditions, such as 50 ppm inorganic chlorine (Khan *et al.*, 2001). These organisms are relevant to this study for two reasons. Firstly, the trophozoites are natural hosts of a number of intracellular microbes that are pathogenic to humans (Kim *et al.*, 2003). Secondly, they are associated with granulomatous amoebic encephalitis (GAE), which only affects immunosuppressed individuals, and acanthamoebal keratitis (AK), which is found in healthy individuals, principally contact lens wearers (Alfieri *et al.*, 2000). Until recently it was thought that neutrophils were accountable for the pathology of the cornea in AK (Na *et al.*, 2002). However, recent evidence has shown that various proteases of *Acanthamoeba* spp. are implicated in the pathogenesis of AK and have

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the potential to be biochemical markers of AK (Alfieri *et al.,* 2000; Khan *et al.,* 2001; Mitro *et al.,* 1994; Kim *et al.,* 2003; Na *et al.,* 2002).

The cytopathic effects of AK are mediated mainly by proteases of the serine and cysteine classes. These proteases have been found to be differentially secreted, as serine proteases from *A. castellanii* are not found in the internal extracts of the *A. castellanii* microcysts (Na *et al.,* 2002). As well, certain species lack active proteases in their microcysts (Mitro *et al.,* 1994). Together, these data suggest different roles for the various proteases in *Acanthamoeba* spp., depending on the species type and whether they are in the trophozoite stage or the microcyst stage. Furthermore, it has been shown that these proteases have a wide range of sizes across various species from 12 – 130 kDa, with the larger sized enzymes being associated with the cysteine class (Alfieri *et al.,* 2000; Na *et al.*, 2002). In addition, they also exhibit activity over an extensive range of temperatures from 30 – 55˚C with the highest activity being seen at 50˚C (Na *et al.,* 2002). However they show less than 30% activity at 4 - 37˚C (Kim *et al.*, 2003). They also show a wide range of activity at pH 5.5 – 10 (Kim *et al.*, 2003). These broad temperature and pH optima are more than likely due to the wide range of environments these organisms inhabit. The proteases themselves interact with a wide range of protein substrates from endogenous protease inhibitors, several immmunoglobulins, and to various structural proteins such as types I and IV collagen and fibronectin (Kim *et al.*, 2003; Na *et al.*, 2002). All of these proteins are found in the corneal stroma and are broken down by the proteases during invasion of the host tissue and devasion of the host's immune system.

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As stated earlier, the social amoeba *Dictyostelium discoideum* is located very close to the entamoebae branch of organisms on the phylogenetic tree of life (Figure 2) (Woese, 1998). The cysteine proteases of *D. discoideum* have been shown to be acid activatable (North *et al*, 1996; Cavallo *et al*, 1999). To date, there have not been any reports addressing whether or not the cysteine proteases of organisms closely related to *D. discoideum* are acid activatable.

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Figure 2. An example of a rooted phylogenetic tree according to the three domain system. (Illustration taken from Woese, 1998).

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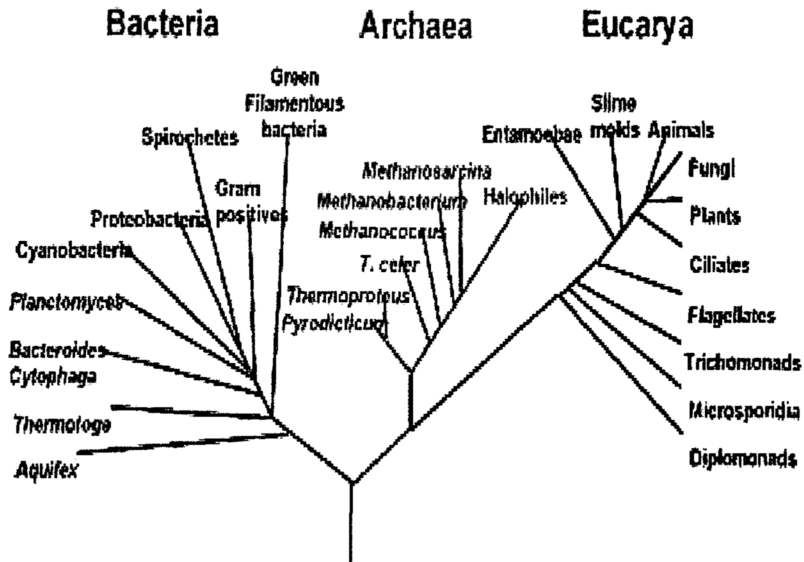
Phylogenetic Tree of Life



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Thesis objectives

The objectives of this thesis were to elucidate four things. The first was to elucidate what type of observable effects H_2O_2 had on spontaneous spore germination in *Dictyostelium discoideum*. The second was to shed more light on the peculiar non-acid activatability characteristic of the spore matrix cysteine protease CP18. The third was to assign a function to the cysteine proteases in the life cycle of *Dictyostelium*. The fourth was to elucidate whether the acid activatability characteristic of cysteine proteases extended to a phylogeneticaly related strain of *Dictyostelium*, *Acanthamoeba castellanii*.

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Chapter 2. Methods and Materials

Media

Half Standard media agar plates (SM/2) were prepared by combining 5.0 g D-(+)-glucose (Sigma), 5.0 g bacto-peptone (Difco), 0.5 g yeast extract (Becton Dickson), 1.1 g KH_2PO_4 (BDH Inc.), 0.5 g K_2HPO_4 (Becton Dickson), 0.5 g $MgSO_4$ (Anachemia) and 15.0 g bacto-agar (Difco) in 1.0 L dH_2O. Alternatively, SM/2 agar plates were prepared by combining 21.0 g SM agar (Formedium) with 8.5 g bacto-agar in 1.0 L of dH_2O. The pH of the SM/2 agar was adjusted to 6.5 and autoclaved. Trypticase media (TM) was prepared by combining 10.0 g trypticase peptone (Becton Dickson), 5.0 g yeast extract, 10.0 g D-(+)-glucose, 0.35 g $Na_2HPO_4 \cdot 7H_2O$ (Fisher) and 1.2 g KH_2PO_4 in 1.0 L dH_2O. The pH of the TM was adjusted to 6.5 and autoclaved. 1% bacto-peptone agar plates were prepared by combining 10.0 g bacto-peptone and 15.0 g bacto-agar in 1.0 L of dH_2O, and autoclaving. Non-nutrient agar plates were prepared by combining 15.0 g bacto-agar in 1.0 L of dH_2O and autoclaving.

Cell Culture maintenance of *Dictyostelium discoideum* strains and *Acanthamoeba castellanii*

Dictyostelium discoideum strains *V12, SG2, NC4, SG1, AX4,* and *NC4A2* fruiting bodies were grown on *E.coli* B/r on SM/2 agar plates. With the exception of strains *V12, NC4, SG1* and *SG2*, all remaining strains of *D. discoideum* and *Acanthamoeba castellanii* were also grown axenically in TM in shake flask culture.

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Vegetative cells of *A. castellanii* from TM were plated onto lawns of *E. coli* B/r on SM/2 agar plates to establish microcyst formation.

Collection of *D. discoideum* dormant spores and spore matrix

Stock cultures of *D. discoideum* strain *V12, NC4, SG1* and *SG2* growing on lawns of *E.coli* B/r on SM/2 agar plates were collected with a sterile loop and mixed with four loops of E.coli B/r in sterile water and vortexed. 1.0-2.0 ml of the spore/*E.coli* solution was dispensed onto SM/2 agar plates. The first day that fruiting bodies had emerged was recorded as day zero. The end of a microscope slide that was immersed in 5.0 ml of 10 mM potassium phosphate buffer (KH_2PO_4), pH 6.5, was used to shave the spores from the top of the fruiting bodies on the SM/2 agar plates. All of the spores were then transferred into a test tube and centrifuged at 4500 g in an IEC clinical centrifuge for three minutes. The supernatant was carefully transferred into another collection tube, passed through a 0.2 micron syringe filter, and retained as spore matrix. The spores were resuspended in 5.0 ml of 10 mM KH_2PO_4, pH 6.5, and centrifuged at 4500 g for three minutes two times. The spores were then resuspended in 5.0 ml of 10 mM KH_2PO_4, pH 6.5. A sample (10 μl) was then placed under a hemacytometer to calculate the spore density.

Collection of *D. discoideum* vegetative cells during growth

A sample (10 μl) of *D. discoideum* strains in TM was placed under a hemacytometer to determine the cell density. The cells were then transferred into a

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test tube and centrifuged at 4500 g for three minutes. The supernatant was discarded and the cell pellet was retained and stored at -20°C.

Collection of *D. discoideum* vegetative cells during development

A sample (10 μl) of *D. discoideum* stains in TM media was placed under a hemacytometer to determine the cell density. The cells were then transferred into a test tube and centrifuged at 4500 g for three minutes. The supernatant was discarded and the cells were resuspended in 10 mM KH_2PO_4, pH 6.5, at 1.0×10^7 cells/ml. Aliquots of this cell suspension (40 μl) were dispensed into the wells of a 96-well flat bottom plate (Nunc). The cells were then collected at specified time points from the 96-well plates into a test tube and centrifuged at 4500 g for three minutes. The supernatant and pellets were collected into separate test tubes and stored at -20°C.

Collection of *A. castellanii* microcysts

Aliquots of *A. castellanii* vegetative cells (100 μl) were placed in the middle of bacterial lawns of *E.coli* B/r on SM/2 plates. After two weeks, the microcysts that had formed were washed from the agar surface with 10 mM KH_2PO_4, pH 6.5. KH_2PO_4 containing microcysts were centrifuged at 4500 g for three minutes. The pellet was retained and stored at -20°C.

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Experimental conditions of *D. discoideum* vegetative cells used for the biochemical inhibition of the cysteine proteases

For growth experiments, vegetative cells of *D. discoideum* strain *AX4*, which are derived from the wild-type strain *NC4*, were shaken in TM containing 0.5% dimethyl sulfoxide (DMSO, BDH Inc.) with or without 50 μM (2S,3S)-trans-epoxysuccinyl-L-leucylamido-3-methylbutane ethyl ester (E-64D, Biomol and Peptide Institute). Cell samples were collected at indicated time points and centrifuged at 4500 g for three minutes. The cell pellets were retained and either prepared immediately for further analysis or stored at -20°C. The decision to utilize E-64D at a 50 μM concentration was determined by incubating vegetative cells of *D. discoideum* strain *AX4* in TM with 0.5% DMSO containing 0.5, 5, and 50 μM E-64D for one hour. After one hour, the cells were centrifuged at 4500 g for three minutes and the cell pellet was retained. Cell pellets were either prepared immediately for use or retained at -20°C.

For developmental assays, vegetative cells in TM were resuspended in 10 mM KH_2PO_4, pH 6.5, containing 0.5% DMSO with or without 50 μM E-64D and placed into microtiter wells as described above. The samples were collected at indicated time points and centrifuged at 4500 g for three minutes. The supernatants and pellets were separated and prepared immediately for further analysis or stored at -20°C.

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Experimental conditions of *D. discoideum* spores used for the biochemical inhibition of the cysteine proteases

Two day old spores from *D. discoideum* spontaneous germinator strain *SG2* were resuspended in 20% DMSO in 10 mM KH_2PO_4, pH 6.5, containing 50 µM E-64D for one hour. Spores were then centrifuged at 4500 g for three minutes and the DMSO solution was discarded. Spores were then resuspended in 5.0 ml 10 mM KH_2PO_4, pH 6.5, and centrifuged at 4500 g for three minutes to wash out any residual DMSO. Spores were then resuspended at 1.0 x 10^7 spores/ml in 10 mM KH_2PO_4, pH 6.5, and allowed to germinate for five hours. The germination kinetics of the spores was determined as described above. Samples were collected at indicated time points and centrifuged once again at 4500 g for three minutes. The pellets were retained and either prepared immediately for further analysis or stored at -20 °C.

It has been demonstrated previously that under inhibitory conditions for spore germination, the *D. discoideum V12* derived strain *SG2* yields more accurate kinetics than the *NC4* derived strain *SG1* (Cotter *et al*, 1990). For this reason, *D. discoideum* strain *SG2* was used as the model for spore germination in this set of experiments.

Experimental conditions for germination of *D. discoideum* spores under oxidative stress

Two-day old spores from *D. discoideum* wild-type strain *NC4* were resuspended in 10 mM KH_2PO_4, pH 6.5, containing either 100 mM dithiothreitol (DTT, ICN Biomedicals, Inc.), 50 mM hydrogen peroxide (H_2O_2, Sigma), or 100 mM D-(+)-glucose. After one hour incubation, the spore suspensions were centrifuged at

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4500 g for three minutes to wash out the solution, and the pelleted spores were resuspended in sterile H_2O. Spore survival was determined according to a previously published protocol (Cotter and Raper, 1966). Briefly, the spores were washed out of their incubating agents by centrifugation at 4500 g for three minutes and resuspended in sterile H_2O. The spores were resuspended at 2.0×10^5-10^6 spores/ml and a 0.5 ml volume was dispensed onto 1% bacto-peptone agar plates. Eight hours post plating, the agar plates were viewed under bright-field microscopy at 100X and the first three hundred objects were scored as dormant spores or emerged amoebae.

Two-day old spores from spontaneous germinator strain *SG2* were resuspended at 1.0×10^7 spores/ml in 10 mM KPO_4, pH 6.5, containing either 1 mM, 10 mM, or 50 mM H_2O_2, 278U of heat-treated catalase (Sigma), or 69U, 139U, or 278U of native catalase and allowed to germinate under shaking conditions. Every hour, aliquots (10 μl) were dispensed onto a microscope slide and viewed under a phase-contrast objective. The first three hundred objects were scored as either dormant spores or swollen spores. After four hours, the spores were either diluted to 2.0×10^5-10^6 spores/ml in their respective incubating agents, diluted in fifty fold lower concentrations of their respective incubating agents, or washed away completely from their respective incubating agents and resuspended in 10 mM KH_2PO_4, pH 6.5. Aliquots (0.5 ml) of the diluted spore suspension were dispensed onto 1% bacto-peptone agar plates to assess spore survival and onto non-nutrient agar plates to assess the ability of the spores to germinate (Cotter and Raper, 1966). Eight hours post-plating, the agar plates were viewed under bright-field microscopy and the first three hundred objects were scored as dormant spores or amoebae.

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Two-day old spores from *D. discoideum* spontaneous germinator strain *SG1* were resuspended at 1.0 x 10^7 spores/ml in 10 mM KH_2PO_4, pH 6.5, containing either 10 mM or 50 mM H_2O_2 and allowed to spontaneously germinate under shaking conditions. After four hours of shaking, the spores were diluted in a fifty fold lower concentration of their incubating agents and resuspended at 2.0 x 10^5-10^6 spores/ml. Aliquots of this spore suspension (0.5 ml) were dispensed onto 1% bacto-peptone to assess spore survival and on non-nutrient agar plates to assess the ability of the spores to germinate (Cotter and Raper, 1966). Eight hours post-plating, the agar plates were viewed under bright-field microscopy and the first 300 objects were scored as dormant spores or amoebae.

It has been demonstrated previously that under inhibitory conditions for spore germination, the *D. discoideum V12* derived strain *SG2* yields more accurate kinetics than the *NC4* derived strain *SG1* (Cotter *et al*, 1990). For this reason, *D. discoideum* strain *SG2* was used primarily in this set of experiments as a model for spore germination and strain *SG1* was used mainly to confirm the results observed for strain *SG2*.

Isolation of protein samples

300 µl of glass beads 212-300 µm in diameter (Sigma) and 200 µl of 10 mM KH_2PO_4, pH 6.5, or 200 µl of dH_2O were added to *D. discoideum* and *A. castellanii* pellets. Samples were vortexed for thirty seconds followed by a thirty second incubation on ice. Samples were vortexed for a total of three minutes. Samples were then centrifuged at 4500 g for three minutes. Supernatants were transferred to

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microcentrifuge tubes and centrifuged for three minutes to remove any glass beads and cellular debris. Supernatants were then transferred to clean microcentrifuge tubes and placed on ice.

Supernatant samples from *D. discoideum* vegetative cells were transferred to a centrifuge filter device (Millipore) with a 30 kDa molecular weight cut-off. Samples were centrifuged at 4500 g for three minutes or until the volume in the upper chamber of the centrifugal filter device had reached a volume of 500 μl. These samples were transferred to clean microcentrifuge tubes and kept on ice until ready for use.

Experimental conditions for internal protein extracts from *A. castellanii* microcysts

Protein extracts from *A. castellanii* microcysts were incubated for one hour in the following reagents; 0.9% ethanol and 0.1% acetic acid containing 1 mM phenylmethylsulfonyl fluoride (PMSF, Sigma), 1% methanol containing 10 μM Pepstatin A (Sigma), 10 mM ethylenediaminetetraacetic acid (EDTA, Baker), or 1 mM (2*S*,3*S*)-3-(*N*-{(*S*)-1-[*N*-(4-guanidinobutyl)carbamoyl]3-methylbutyl}carbamoyl)oxirane-2-carboxylic acid (E-64, Sigma). After one hour, the protein samples were prepared for further analysis.

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Protein Quantification

Protein concentrations of experimental samples were elucidated using the Bradford assay (Bradford, 1976). Bovine serum albumin (BSA) was used as the protein standard.

Electrophoretic separation

All protein samples were separated electrophoretically by sodium dodecyl sulfate polyacrylamide gel electrophoesis (7.5-10% polyacrylamide) (SDS-PAGE) gels or SDS-PAGE gels containing 2% gelatin (zymograms). Separating gels were prepared by combining 2.5 ml of 1.5 M Tris-HCl (Biorad), pH 8.8, 100 μl of 10% SDS, 50 μl of 10% ammonium persulfate (APS, Biorad), and 10 μl of N,N,N',N' tetramethylethylenediamine (TEMED, Sigma). Acrylamide/bis (30%T, Biorad) was added in appropriate amounts to give 7.5-10% polyacrylamide separating gels. Gelatin (Sigma) was added to acrylamide gel solutions to give 0.2% gelatin for 7.5-10% polyacrylamide zymograms (Cavallo *et al.*, 1999). After thorough mixing, approximately 3.5 ml of the solution was dispensed into a 0.75 mm space created between a large and small glass plate on a Mini-Protean II or Mini-Protean III casting stand (Biorad), and allowed to polymerize. Stacking gels containing 4% polyacrylamide for SDS-PAGE and SDS-PAGE zymograms were prepared by combining 5.9 ml of dH_2O, 2.5 ml of 0.5 M Tris, pH 6.8, 1.5 ml of 30% acrylamide/bis (30%T), 100 μl of 10% SDS, 50 μl of 10% APS, and 10 μl of TEMED. The solution was mixed thoroughly and pipetted on top of the polymerized separating gel until the stacking gel solution had over-flowed. A 0.75 mm 10-well

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comb was inserted into the stacking gel solution and the gel was allowed to polymerize.

Samples were prepared by mixing 4 parts of protein sample with 1 part 5X sample buffer. 5X sample buffer was prepared by combining 5.0 ml of glycerol (EM Science), 2.5 ml of β-mercaptoethanol (Sigma), 2.5 ml of 0.5 M Tris, pH 6.8, 1.0 g of SDS, and 1.0 mg of bromophenol blue (Baker). Samples were incubated at 95°C for three minutes. Samples that were to be separated on zymograms were not incubated at 95°C. Samples were then dispensed into the wells of the stacking gel. SDS-PAGE and zymogram gels were then placed into a Mini-Protean II or Mini-Protean III electrophoresis gasket (Biorad), which was then placed into a buffer dam. The buffer dam holding the gasket and SDS-PAGE or zymogram gels was filled to the top with SDS running buffer. SDS running buffer was prepared by combining 3.0 g of Tris, 14.0 g of glycine (Biorad), and 1.0 g of SDS in 1.0 L of dH_2O with the pH adjusted to 8.0. The apparatus was connected to a power supply and a voltage of 60 mV was applied. When it was observed that the dye front had migrated from the stacking gel to the separating gel, the voltage was increased to 100 mV. The voltage was discontinued when it was observed that the dye front had reached the bottom of the separating gel.

For samples that were to be separated electrophoretically in the absence of SDS, SDS was replaced with dH_2O in the separating and stacking gels, and removed from the 5X sample buffer and running buffer. For samples that were to be separated electrophoretically in the absence of β-mercaptoethanol, β-mercaptoethanol was replaced with dH_2O in the 5X sample buffer.

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Treatments and staining of PAGE gels

When electrophoresis was complete, SDS-PAGE gels were immersed in 50% methanol, 10% acetic acid (fix solution) for 30 minutes with gentle agitation. SDS-PAGE gels were then incubated in dH_2O for ten minutes two times. After the two ten minute washes in dH_2O, gels were incubated in phosphoprotein stain (Molecular Probes) for two hours. SDS-PAGE gels were then destained in 20% acetonitrile containing 50 mM sodium acetate (Sigma), pH 4.0 for one hour.

PAGE zymograms were incubated in 2.5% Triton X-100 (Sigma) for thirty minutes. Prior to the Triton X-100 incubation, PAGE zymograms were incubated in either 1.7M acetic acid, pH 2.1, 0.3-2 mM H_2O_2, 1.7 M ammonium phosphate, pH 9.0, or 100 mM DTT for sixty seconds, then incubated in Triton X-100. Zymograms were then incubated in 99 mM sodium acetate, pH 4.0 containing 1 mM DTT for sixteen hours. For some experiments, the following solutions were included in the overnight incubation buffer; 0.9% ethanol and 0.1% acetic acid containing 1 mM PMSF, 1% methanol containing 10 μM Pepstatin A, 100 mM EDTA, or 100 μM E-64. For PAGE zymograms that required the absence of DTT, DTT was replaced with dH_2O. PAGE zymograms were then stained with coomassie blue for one hour and destained in 40% methanol, 10% acetic acid for one hour.

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Chapter 3. Results

The effects of reactive oxygen species (ROS) on the cysteine proteases of *D. discoideum*

It has been shown that reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) are capable of inhibiting protein tyrosine phosphatases by oxidizing the active site cysteine contained in the protein phosphatase (Caselli *et al*, 1994 and 1998; Denu *et al*, 1998; Nimnual *et al*, 2003). This type of inhibition is reversible by treating with anti-oxidants capable of reducing sulfur groups that are singly bound (Denu *et al*, 1998). Given that the cysteine protease of any organism contains a thiol bond in which the sulfur group is donated from a cysteine residue, the first part of this study focused on whether or not ROS is capable of reversibly inhibiting the cysteine proteases of *D. discoideum*. Internal protein extracts from *D. discoideum* strain *NC4A2* in growth phase were subjected to electrophoresis on an SDS-PAGE zymogram in the absence of reducing agents such as DTT and β-mercaptoethanol. After electrophoresis was complete, the lanes from the zymograms were carefully cut out and incubated for 60 seconds in various concentrations of H_2O_2 up to 2 mM. This data in Figure 3 revealed that 0.6 mM H_2O_2 was capable of partially inhibiting the cysteine proteases of *D. discoideum*. Concentrations of H_2O_2 0.7 mM and above up to 2 mM were capable of totally inhibiting the enzymes, as they were not visible on SDS-PAGE zymograms (Figure 3A). The cysteine proteases from strain *NC4A2* were run under native conditions to rule out that the SDS was not affecting the data set (Figure 3B). Furthermore, under SDS and non-SDS conditions, when zymograms

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were incubated for sixty seconds in 2 mM H_2O_2 followed by sixty second incubation in 100 mM dithiothreitol (DTT), cysteine protease activity was recovered (Figure 3A and 3B).

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Figure 3. The *in vitro* effects of H_2O_2 on the cysteine proteases of *D. discoideum*. Vegetative cells from *D. discoideum* strain *NC4A2* in growth phase were harvested for internal protein and subjected to electrophoresis on SDS-PAGE zymograms (A) or non-SDS-PAGE zymograms (B) (7.5% polyacrylamide in the separating gel). Both conditions were run in the absence of reducing agents in the PAGE system. The zymograms were incubated in 2.5% Triton-X 100 for thirty minutes. After the Triton-X incubation, the zymograms were incubated in the indicated concentrations of H_2O_2 for sixty seconds, or incubated in 2 mM H_2O_2 for sixty seconds, followed by sixty second incubation in 100 mM DTT. The zymograms were then incubated in 99 mM sodium acetate, pH 4.0 for sixteen hours. The zymograms were then stained in coomassie blue for at least one hour and destained in 40% methanol, 10% acetic acid for at least one hour. All lanes contain 10-15 μg of total protein.

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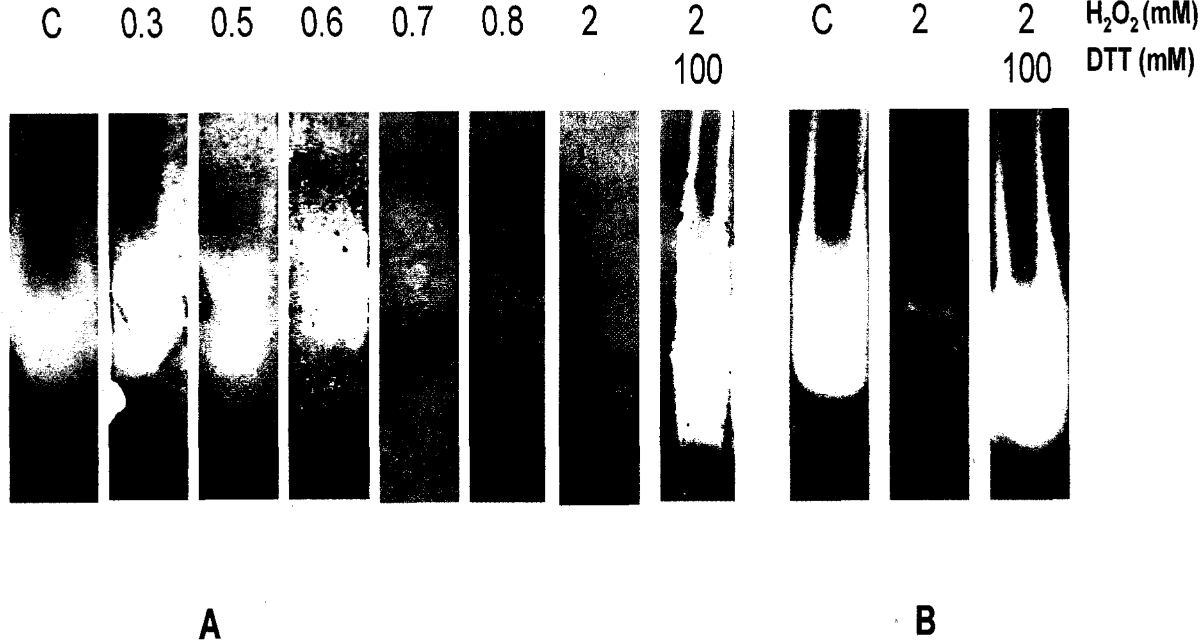

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The effects of ROS on actin phosphorylation in the dormant spores of *D. discoideum*

It was mentioned earlier that one of the biochemical hallmarks of dormancy in *D. discoideum* is tyrosine phosphorylation on actin. Conversely, as the spore is in the process of germinating, actin will become dephosphorylated and depolymerize. It has been reported previously that actin can be dephosphorylated independent of the spore germination process by incubating dormant spores in 30-300 mM glucose for one hour. It is thought that glucose mediates a signal transduction mediated event, resulting in the dephosphorylation of actin (Kishi *et al*, 2000). In an effort to reconfirm the results that have been previously generated, two-day old dormant spores from *D. discoideum* strain *NC4* were incubated in 100 mM glucose for one hour. The spores were then washed away from the solution and harvested for their internal protein. The protein was separated on SDS-PAGE. The SDS-PAGE gel was stained with a proprietary phosphoprotein stain. This proprietary stain was utilized for its sensitivity towards phosphorylated proteins. It has been shown previously that this proprietary stain can detect as little as 8 ng of mono-phosphorylated proteins in SDS-PAGE gels (Steinberg *et al*, 2003). The data in Figure 4A revealed that when the dormant spores were incubated in glucose, a 43 kDa protein was dephosphorylated when compared to the control lanes. Ovalbumin was included in the molecular weight markers and was phosphorylated according to the phosphoprotein stain at 45 kDa (Figure 4A). Reports that have utilized these same incubation conditions followed by phosphoprotein western blot analysis have shown

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that the protein depicted in Figure 4A at 43 kDa is actin (Kishi *et al*, 2000; Gauthier *et al*, 1997).

It has been demonstrated in higher eukaryotic cells that the small GTPase Rac down-regulates the small GTPase Rho through an ROS mediated inhibition of the low molecular weight protein tyrosine phosphatase required to dephosphorylate Rho (Nimnual *et al*, 2003). Based on this set of knowledge, the dormant spores were incubated in glucose and H_2O_2 simultaneously for one hour. The solution was washed away from the spores and the protein extract was separated on the same SDS-PAGE gel in Figure 4. It can be seen clearly in Figure 4A that simultaneous incubation of the dormant spores in glucose and H_2O_2 acted to inhibit the actin dephosphorylation event normally observed when the dormant spores are incubated in glucose alone. Furthermore, this type of inhibition was reversible by incubating the spores simultaneously in glucose, H_2O_2, and the reducing agent DTT (Figure 4A). After the gel had been stained with the proprietary phosphoprotein stain, the gel was stained in coomassie blue for total protein composition (figure 4B). A small portion of the spores from every condition was plated onto 1% bacto-peptone agar plates to assess viability. Viability was calculated by the spore's ability to release an amoeba on the agar plates. The data in Table 1 illustrate that none of the conditions utilized had an effect on the spore viability.

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Table 1. Survival of two-day old spores from *D. discoideum* strain *NC4* after one hour incubation in glucose, H_2O_2, and DTT. Two-day old spores of *D. discoideum* strain *NC4* were incubated in 10 mM KH_2PO_4, pH 6.5, containing 100 mM DTT, 50 mM H_2O_2, or 100 mM glucose. After one hour, the spores were washed free from their incubating agents and resuspended in sterile H_2O at 2.0 x 10^5-10^6/ml. 1.0 x 10^5-10^6 total spores were plated onto 1% bacto-peptone agar plates and viewed under bright-field microscopy eight hours post-plating. The first three hundred objects were scored as dormant or emerged (n=3).

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Treatment	**% emergence**
Control	98.1 ± 0.7
100 mM DTT	95.4 ± 0.3
50 mM H_2O_2	96.5 ± 0.6
100 mM Glucose	92.8 ± 0.3
100 mM Glucose and 50 mM H_2O_2	91.9 ± 0.1
100 mM Glucose, 100 mM DTT and 50 mM H_2O_2	97.1 ± 0.5

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Figure 4. The phosphorylation state of actin in the presence of 50 mM H_2O_2. (A). Two-day old spores from *D. discoideum* wild-type strain *NC4* were incubated in 10 mM KH_2PO_4, pH 6.5, containing either 100 mM DTT, 50 mM H_2O_2, or 100 mM glucose (solid line) for one hour. The spores were then washed free of the KH_2PO_4, followed by a second wash in dH_2O to remove any residual KH_2PO_4. Spores were harvested for their internal spore extracts and subjected to electrophoresis on SDS-PAGE (10% polyacrylamide in the separating gel). The gel was then incubated for thirty minutes in 50% methanol, 10% acetic acid followed by two ten minute washes in dH_2O. The gel was then incubated in phosphoprotein stain for two hours and then destained in 20% acetonitrile, 50 mM sodium acetate, pH 4.0. (B). The SDS-PAGE gel was then incubated in coomassie blue for one hour and then destained in 40% methanol, 10% acetic acid. Molecular weight markers were run alongside the samples (Fermentas). All experimental lanes contain 3.2 μg of protein.

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The effects of ROS on the spores of *D. discoideum*

Since the data in Table 1 and Figure 4A revealed that there was a possibility that the germination process in the spores of *D. discoideum* might be inhibited by H_2O_2, the next part of this study was to observe the germination process in the spores of *D. discoideum* which had been exposed to different levels of oxidative stress. This was accomplished by incubating two-day old spores of *D. discoideum* strain *SG2* in 1, 10, and 50 mM H_2O_2 and allowing germination to occur. As can be viewed in Figure 5B, the spores of *D. discoideum* strain *SG2* displayed a decreased rate of spore swelling in the presence of H_2O_2 in a dose dependent manner. The *SG2* spores were able to reach approximately 60%, 40%, and 25% swelling in the presence of 1, 10, and 50 mM H_2O_2, respectively, after being allowed to germinate for four hours. Furthermore, the decreased swelling kinetics observed for strain *SG2* in the presence of 50 mM H_2O_2 was recovered in the presence of exogenously added catalase (Figure 5C). The recovery was dose dependent. After four hours of germination in the presence of 50 mM H_2O_2 and 69U of catalase, the swelling kinetics looked nearly identical to the swelling kinetics after four hours when the spores were incubated in 50 mM H_2O_2 by itself. However, when the spores were allowed to germinate in the presence of 50 mM H_2O_2 and 139U of catalase, the swelling kinetics after four hours was at approximately 40% after four hours (Figure 5C), which was comparable to the swelling kinetics of the *SG2* spores when they were incubated in 10 mM H_2O_2 for four hours. When the spores were incubated simultaneously in 50 mM H_2O_2 and 278U of catalase, the swelling kinetics after four hours was at approximately 60%

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(Figure 5C), which was comparable to the swelling kinetics observed for the *SG2* spores when allowed to germinate in the presence of 1 mM H_2O_2 (Figure 5C).

To ensure that this type of oxidative stress had no effect in the spore viability of *D. discoideum* strain *SG2*, the spores were washed free of their respective incubating agents after four hours and resuspended in KH_2PO_4. A small population of these spores was then plated onto 1% bacto-peptone agar plates to assess spore viability. The data in Table 2 show that there was no effect on the viability of the *SG2* spores after having been incubated for four hours under any of the conditions tested, as the spores were able to release amoebae in the presence of bacto-peptone. In the same manner, these spores were washed, resuspended in KH_2PO_4, and plated onto non-nutrient agar to assess the effect this type of oxidative stress may have had on the spores ability to germinate. As the data indicate in Table 3, the *SG2* spores were inhibited in their ability to germinate even when they were washed free of the oxidative environment. To get some insight on how resistant the spores of *D. discoideum* were to these levels of oxidative stress, this ecological spore dispersal simulation was modified slightly. After the spores of strain *SG2* had been given the opportunity to germinate for four hours, the spores were then resuspended in the same oxidizing conditions, or were resuspended in a fifty-fold lower concentration of their oxidative agents. As expected, the spores were inhibited in their ability to germinate when the spores were exposed to concentrations of H_2O_2 that were fifty-fold lower than the oxidative environments that they were exposed to for four hours. The data in Table 5 show that this type of treatment had no effect in the spore viability.

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However, when the spores were continuously exposed to these oxidative environments, spore viability was decreased in a concentration dependent manner.

To ensure that the results produced in these experiments with *D. discoideum* strain *SG2* could be reproduced with other strains of *D. discoideum*, two-day old spores of *D. discoideum* strain *SG1* were allowed to spontaneously germinate for four hours in 1, 10, and 50 mM H_2O_2. After four hours, a small portion of the spores was diluted in fifty fold lower concentrations of H_2O_2, and then plated onto non-nutrient agar and 1% bacto-peptone agar plates. The data in Tables 8 and 9 show that similar results could be achieved with *D. discoideum* strains *SG1* and *SG2*.

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Figure 5. Spore swelling kinetics of *D. discoideum* strain *SG2* allowed to germinate in oxidized environments. (A). Two-day old spores of *D. discoideum* strain *SG2* were resuspended at 1.0×10^7 spores/ml in 10 mM KH_2PO_4, pH 6.5 (black), containing 278U of heat treated (white) or non neat treated catalase (polka dots). (B). Two-day old spores of *D. discoideum* strain *SG2* were resuspended at 1.0×10^7 spores/ml in 10 mM KH_2PO_4, pH 6.5, containing 1 (black), 10 (white), or 50 mM H_2O_2 (polka dots). (C). Two-day old spores of *D. discoideum* strain *SG2* were resuspended at 1.0×10^7 spores/ml in 10 mM KH_2PO_4, pH 6.5, containing 50 mM H_2O_2 and either 278U of heat treated catalase (black), 69U of catalase (white), 139U of catalase (polka dots), or 278U of catalase (checkers). Samples (10 μl) were placed onto microscope slides and viewed under phase-contrast microscopy at the indicated time points. The first three hundred objects were scored as dormant spores or swollen spores (n=3).

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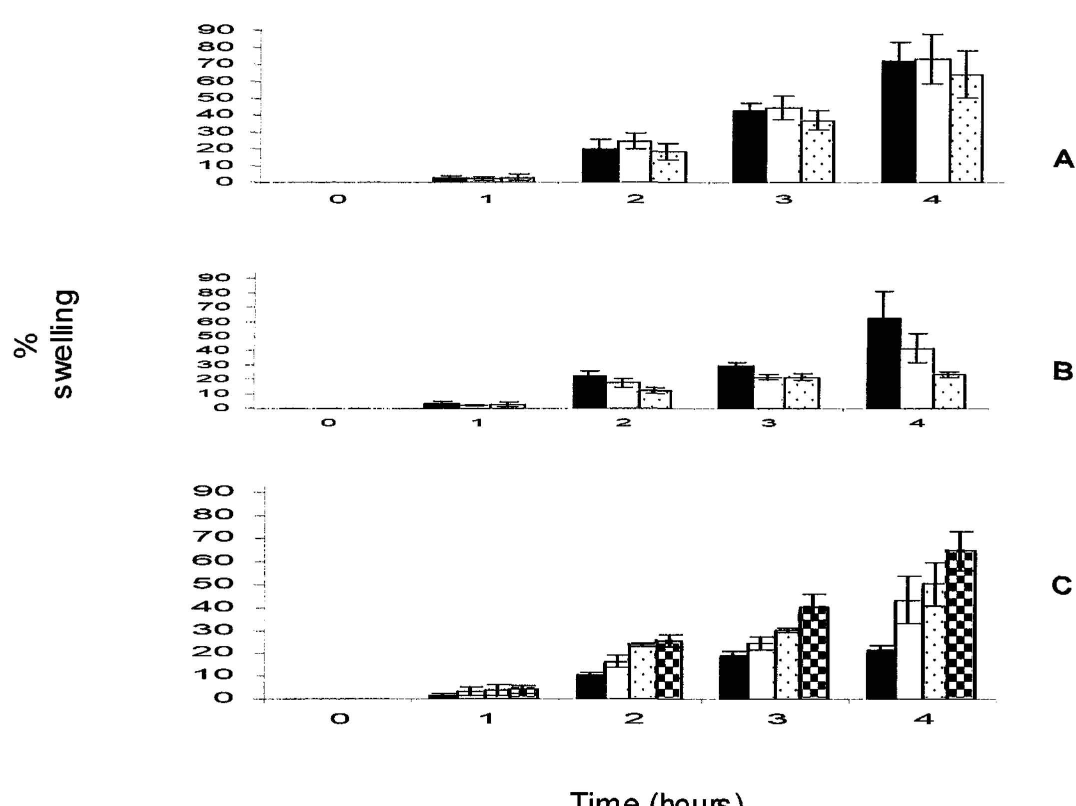

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Table 2. Spore viability of *D. discoideum* strain *SG2* when allowed to germinate under oxidative stress conditions for four hours. Two-day old spores of *D. discoideum* strain *SG2* from the experiments in Figures 5-7 were washed free of their respective incubating agents after four hours of spontaneous germination and resuspended at 2.0 x 10^5-10^6 spores/ml in 10 mM KH_2PO_4, pH 6.5. 0.5 ml of this spore suspension was plated onto 1% bacto-peptone agar plates. Eight hours post plating, the plates were viewed under bright-field microscopy and the first three hundred objects were scored as dormant spores or emerged amoebae (n=3).

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Treatment	average % emergence
Control	97.1 ± 0.4
278U heat treated catalase	95.4 ± 1.1
278U catalase	96.3 ± 0.2
1 mM H_2O_2	96.0 ± 0.6
10 mM H_2O_2	97.0 ± 0.5
50 mM H_2O_2	97.0 ± 1.0
50 mM H_2O_2/278U heat treated catalase	96.7 ± 1.1
50 mM H_2O_2/69U catalase	96.6 ± 1.5
50 mM H_2O_2/139U catalase	96.0 ± 0.8
50 mM H_2O_2/278U catalase	97.2 ± 0.6

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Table 3. The ability of spores from *D. discoideum* strain *SG2* to germinate after being exposed to oxidative stress conditions for four hours. Two-day old spores of *D. discoideum* strain *SG2* from the experiments in Figures 5-7 were washed free of their respective incubating agents after four hours of spontaneous germination and resuspended at 2.0 x 10^5-10^6 spores/ml in 10 mM KH_2PO_4, pH 6.5. 0.5 ml of this spore suspension was plated onto non-nutrient agar plates. Eight hours post plating, the plates were viewed under bright-field microscopy and the first three hundred objects were scored as dormant spores or emerged amoebae (n=3).

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Treatment	average % emergence
Control	75.0 ± 9.8
278U heat treated catalase	71.6 ± 12.3
278U catalase	66.8 ± 11.2
1 mM H_2O_2	64.2 ± 19.8
10 mM H_2O_2	41.3 ± 8.5
50 mM H_2O_2	26.3 ± 2.3
50 mM H_2O_2/278U heat treated catalase	22.9 ± 1.1
50 mM H_2O_2/69U catalase	40.2 ± 9.4
50 mM H_2O_2/139U catalase	51.6 ± 5.2
50 mM H_2O_2/278U catalase	66.8 ± 6.3

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Table 4. The ability of spores from *D. discoideum* strain *SG2* to germinate for four hours and then having been diluted fifty fold from their oxidative environments. Two-day old spores from the experiment in Figure 6 were allowed to spontaneously germinate for four hours. At four hours, the spores were diluted to 2.0 x 10^5-10^6 spores/ml in 10 mM KH_2PO_4, pH 6.5, containing fifty fold lower of the H_2O_2 concentrations indicated in the table (at four hours, 1, 10, and 50 mM H_2O_2 were diluted down to 0.02, 0.2, and 1 mM H_2O_2, respectively). 1.0 x 10^5-10^6 spores were then plated onto non-nutrient agar plates. Eight hours post plating, the plates were viewed under bright-field microscopy and the first three hundred objects were scored as dormant spores or emerged amoebae (n=3).

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Treatment	**average % emergence**
Control	73.5 ± 9.8
1 mM H_2O_2	63.1 ± 9.7
10 mM H_2O_2	43.6 ± 10.2
50 mM H_2O_2	24.9 ± 3.2

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Table 5. Spore viability of *D. discoideum* strain *SG2* when allowed to germinate under oxidative stress conditions for four hours, and then having been diluted fifty fold lower from their oxidative environments. Two-day old spores of *D. discoideum* strain *SG2* from the experiment in Figure 6 were allowed to spontaneously germinate for four hours. At four hours, the spores were diluted to 2.0 x 10^5-10^6 spores/ml in 10 mM KH_2PO_4, pH 6.5, containing fifty fold lower of the H_2O_2 concentrations indicated in the table above (1, 10, and 50 mM H_2O_2 were diluted down to 0.02, 0.2, and 1 mM H_2O_2, respectively). 1.0 x 10^5-10^6 spores were plated onto 1% bacto-peptone agar plates. Eight hours post plating, the plates were viewed under bright-field microscopy and the first three hundred objects were scored as dormant spores or emerged amoebae (n=3).

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Treatment	**average % emergence**
Control	94.4 ± 1.1
1 mM H_2O_2	94.3 ± 0.6
10 mM H_2O_2	94.4 ± 0.3
50 mM H_2O_2	94.8 ± 0.9

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Table 6. The ability of spores from *D. discoideum* strain *SG2* to germinate after having been continuously exposed to oxidative stress. Two-day old spores of *D. discoideum* strain *SG2* from the experiment in Figure 6 were allowed to spontaneously germinate for four hours. At four hours, the spores were diluted to 2.0 x 10^5-10^6 spores/ml in 10 mM KH_2PO_4, pH 6.5, containing concentrations of H_2O_2 listed in the table above. 1.0 x 10^5-10^6 spores were plated onto non-nutrient agar plates. Eight hours post plating, the plates were viewed under bright-field microscopy and the first three hundred objects were scored as dormant spores or emerged amoebae (n=3).

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Treatment	average % emergence
Control	75.0 ± 11.2
1 mM H_2O_2	60.3 ± 19.2
10 mM H_2O_2	43.5 ± 10.4
50 mM H_2O_2	26.2 ± 2.8

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Table 7. Spore viability of *D. discoideum* strain *SG2* when allowed to germinate after having been continuously exposed to oxidative stress. Two-day old spores of *D. discoideum* strain *SG2* from the experiment in Figure 6 were allowed to spontaneously germinate for four hours. At four hours, the spores were diluted to 2.0 x 10^5-10^6 spores/ml in 10 mM KH_2PO_4, pH 6.5, containing concentrations of H_2O_2 listed in the table above. 1.0 x 10^5-10^6 spores were plated onto 1% bacto-peptone agar plates. Eight hours post plating, the plates were viewed under bright-field microscopy and the first three hundred objects were scored as dormant spores or emerged amoebae (n=3).

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Treatment	average % emergence
Control	76.7 ± 9.7
1 mM H_2O_2	66.3 ± 15.4
10 mM H_2O_2	43.1 ± 6.8
50 mM H_2O_2	19.6 ± 3.1

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Table 8. The ability of spores from *D. discoideum* strain *SG1* to germinate for four hours under oxidative stress conditions, then having been diluted fifty fold lower from the oxidative environments. Two-day old spores from *D. discoideum* strain *SG1* were allowed to spontaneously germinate for four hours in 10 mM KH_2PO_4, pH 6.5, containing concentrations of H_2O_2 listed in the table above at 1.0 x 10^7 spores/ml. At four hours, the spores were diluted to 2.0 x 10^5-10^6 spores/ml in 10 mM KH_2PO_4, pH 6.5, containing fifty fold lower concentrations of H_2O_2 listed in the table above (10 and 50 mM H_2O_2 were diluted to 0.2 and 1 mM H_2O_2, respectively). 1.0 x 10^5-10^6 spores were plated onto non-nutrient agar. Eight hours post plating, the plates were viewed under bright-field microscopy and the first three hundred objects were scored as dormant spores or emerged amoebae (n=3).

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Treatment	average % emergence
Control	77.4 ± 7.5
10 mM H_2O_2	37.6 ± 8.3
50 mM H_2O_2	28.1 ± 1.1

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Table 9. Spore viability of *D. discoideum* strain *SG1* when allowed to germinate under oxidative stress conditions for four hours, and then having been diluted fifty fold lower from their oxidative environments. Two-day old spores from *D. discoideum* strain *SG1* were allowed to spontaneously germinate for four hours in 10 mM KH_2PO_4, pH 6.5, containing the concentrations of H_2O_2 listed in the table above at 1.0 x 10^7 spores/ml. At four hours, the spores were diluted to 2.0 x 10^5-10^6 spores/ml in 10 mM KH_2PO_4, pH 6.5, containing fifty fold lower concentrations of H_2O_2 in the table listed above (10 and 50 mM H_2O_2 were diluted to 0.2 and 1 mM H_2O_2, respectively). 1.0 x 10^5-10^6 spores were plated onto 1% bacto-peptone agar plates. Eight hours post plating, the plates were viewed under bright-field microscopy and the first three hundred objects were scored as dormant spores or emerged amoebae (n=3).

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Treatment	average % emergence
Control	94.4 ± 0.9
10 mM H_2O_2	94.4 ± 0.1
50 mM H_2O_2	94.8 ± 0.8

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Analysis of the spore matrix proteins of *Dictyostelium discoideum*

The objective in this set of experiments was to elucidate if there were any visible differences in the spore matrix proteins as the spores of *D. discoideum* approach the age of spontaneous spore germination. This was done by collecting spore matrix from *D. discoideum* wild-type strains *NC4* and *V12* and from spontaneous germinator strain *SG2* when the spores were one, two, three, four, six, eight, ten, and twenty days of age. When the spore matrix from the different strains was collected and separated electrophoretically on SDS-PAGE, it was revealed that there was a difference in the spore matrix between the wild-type strains and the spontaneous germinator strain. Figures 6 and 7 show that a protein at 26 kDa accumulated over time. The abundance of this protein was highest when the spore matrix was four days old. However, Figure 8 shows that this protein was always visible in the spore matrix from day one in the spontaneous germinator strain *SG2*.

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Figure 6. Total protein analysis of the spore matrix from *D. discoideum* strain *NC4*. The spore matrix from the spores of *D. discoideum* strain *NC4* at one, two, three, four, six, eight, ten, and twenty days of age was collected and separated electrophoretically by SDS-PAGE (7.5% polyacrylamide). When electrophoresis was complete, the PAGE gel was incubated in 2.5% Triton-X 100 for thirty minutes, followed by incubation in coomassie blue staining solution over night. The PAGE gel was then destained in 40% methanol, 10% acetic acid until the protein bands were clearly visible. Molecular weight markers (M) were alongside the samples (Fermentas). The spore matrix was collected in the same manner as described in the Methods and Materials. Equal volumes were utilized for analysis on SDS PAGE.

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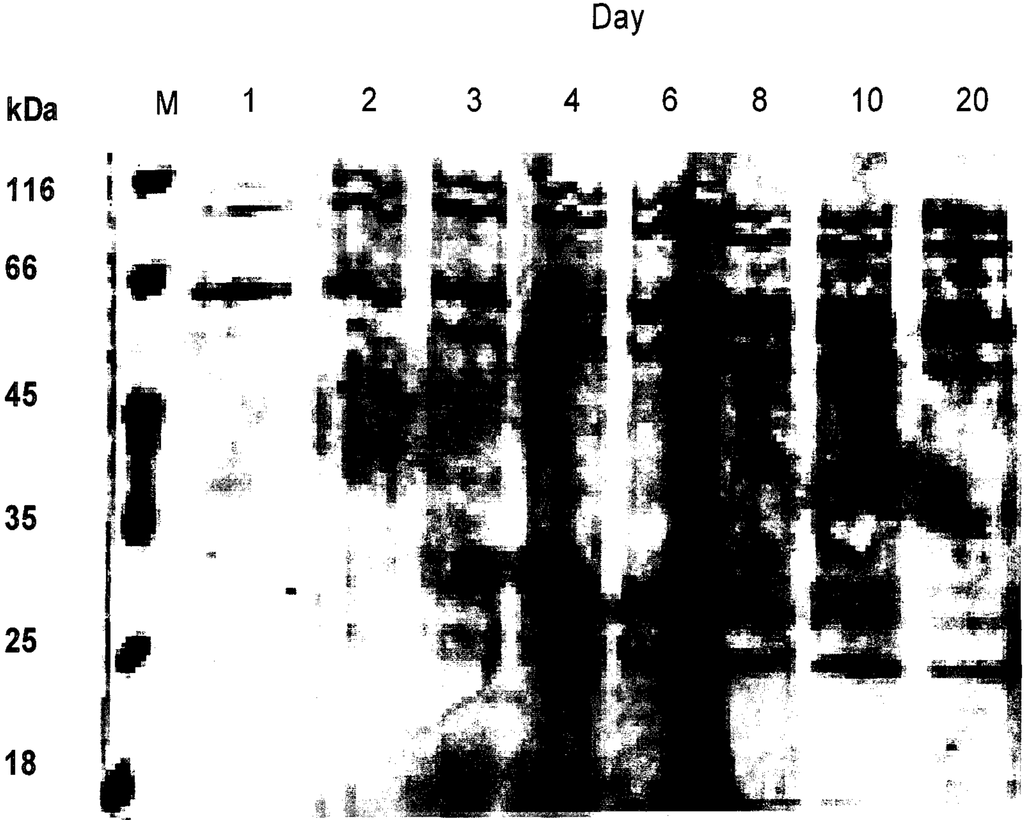

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Figure 7. Total protein analysis of the spore matrix from *D. discoideum* strain *V12*. The spore matrix from the spores of *D. discoideum* strain *V12* at one, two, three, four, six, eight, ten, and twenty days of age was collected and separated electrophoretically by SDS-PAGE (7.5% polyacrylamide). When electrophoresis was complete, the PAGE gel was incubated in 2.5% Triton-X 100 for thirty minutes, followed by incubation in coomassie blue staining solution over night. The PAGE gel was destained in 40% methanol, 10% acetic acid until the protein bands were clearly visible. Molecular weight markers (M) were alongside the samples (Fermentas). The spore matrix was collected in the same manner as described in the Methods and Materials. Equal volumes were utilized for analysis on SDS PAGE.

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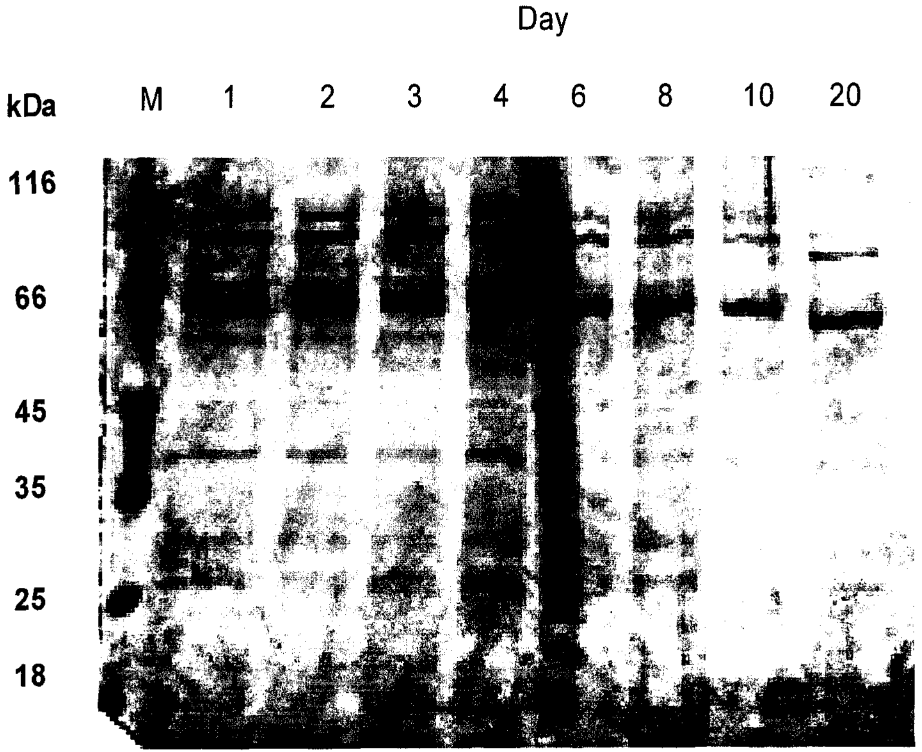

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Figure 8. Total protein analysis of the spore matrix from *D. discoideum* strain *SG2*. The spore matrix from the spores of *D. discoideum* strain *SG2* at one, two, three, four, six, eight, and ten days of age was collected and separated electrophoretically by SDS-PAGE techniques (7.5% polyacrylamide). When electrophoresis was complete, the PAGE gel was incubated in 2.5% Triton-X 100 for thirty minutes, followed by incubation in coomassie blue staining solution over night. The PAGE gel was then destained in 40% methanol, 10% acetic acid until the protein bands were clearly visible. Molecular weight markers (M) were alongside the samples (Fermentas). The spore matrix was collected in the same manner as described in the Methods and Materials. Equal volumes were utilized for analysis on SDS PAGE.

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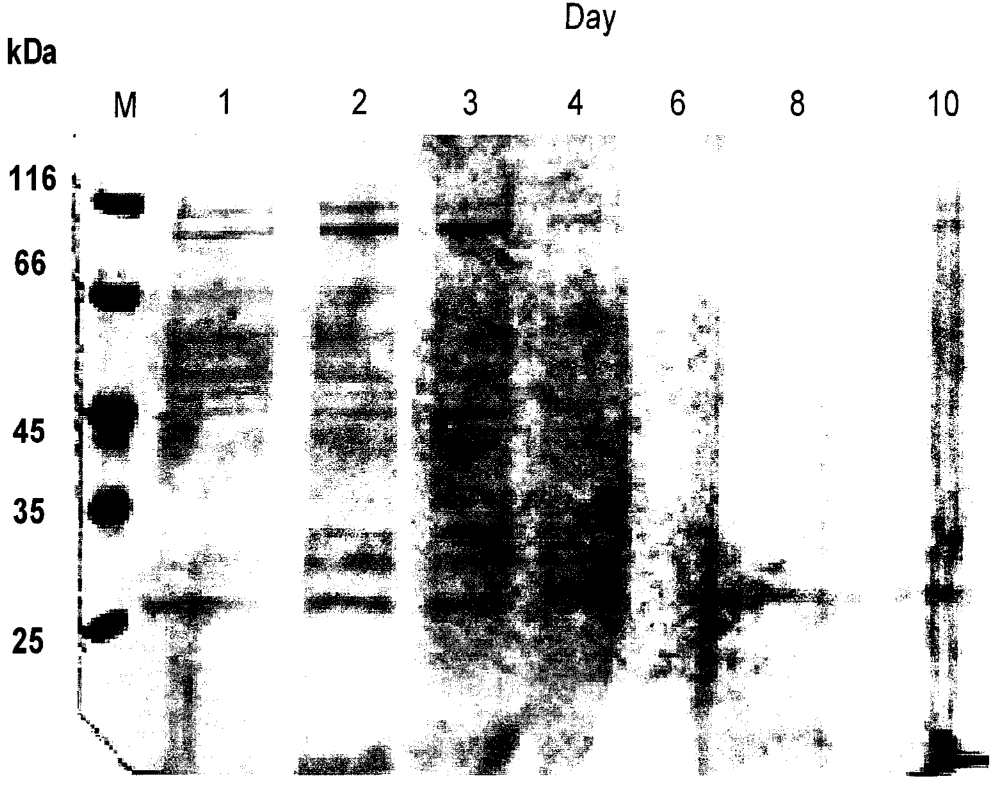

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Characterization of *Dictyostelium discoideum* spore matrix cysteine protease 18 kDa (CP18)

The objective of this set of experiments was to gain more information on the *Dictyostelium discoideum* spore matrix cysteine protease CP18. There is not very much information on the spore matrix protease. The most striking characteristic of this enzyme is that unlike the rest of the cysteine proteases in *D. discoideum*, CP18 has been reported to be non-acid activatable (North *et al*, 1996; Cotter *et al*, 1997). The initial experiments conducted with the spore matrix of *D. discoideum* involved characterizing the native state of CP18. This was done by isolating the spore matrix from two-day old spores of the *D. discoideum* spontaneous germinator strain *SG2*, and subjecting it to electrophoresis on native-PAGE zymograms. Native-PAGE analysis did not show any band at the 18 kDa range (Figure 9). The data in Figure 10 revealed that under native zymographic conditions, there was a lone protease that migrated relative to the position of 18 kDa that was acid activatable.

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Figure 9. Native -PAGE analysis of the spore matrix from *D. discoideum* strain *SG2*. Spore matrix from *D. discoideum* strain *SG2* at two days of age was collected and separated electrophoretically native PAGE techniques (7.5% polyacrylamide). When electrophoresis was complete, the PAGE gel was incubated in 2.5% Triton-X 100 for thirty minutes, followed by overnight incubation in coomassie blue staining solution. The PAGE gel was then destained in 40% methanol, 10% acetic acid until the protein bands were clearly visible. The lane contains 1.5 μg of protein.

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Figure 10. Native-PAGE zymogram analysis of the spore matrix from *D. discoideum* strain *SG2*. Two-day old spore matrix from *D. discoideum* spontaneous germinator strain *SG2* was subjected to electrophoresis on native-PAGE zymograms (7.5% polyacrylamide). When electrophoresis was complete, the zymograms were incubated in 2.5% Triton-X 100 for thirty minutes (A), or incubated in 1.7 M acetic acid, pH 2.1 for sixty seconds prior to the Triton-X incubation (B). The zymograms were then incubated in 99 mM sodium acetate, pH 4.0 for sixteen hours. The zymograms were then stained in coomassie blue for at least one hour and then destained in 40% methanol, 10% acetic acid for at least one hour. All lanes contain 2 μg of total protein.

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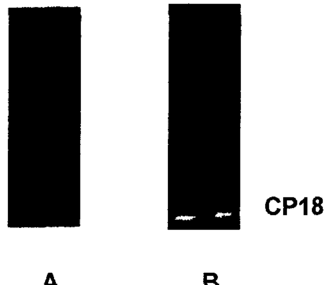

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The data obtained in Figure 10 seemed a bit peculiar given the fact that under the classical, partially reduced conditions prescribed for zymographic analysis of cysteine proteases in *D. discoideum*, the 18 kDa spore matrix protease seemed to be non-acid activatable (North *et al*, 1996; Cotter *et al*, 1997). Therefore, this part of the study dealt with the acid-activatability of CP18.

The acid-activatibility of CP18

To elucidate what could be contributing to the acid-activatability of the 18 kDa weight protease, various components of the PAGE system were excluded in an effort to determine the cause of these characteristics. Two-day old spore matrix from *D. discoideum* strain *V12* was subjected to electrophoresis on SDS-PAGE zymograms (Figure 11) and native-PAGE zymograms (Figure 12). When electrophoresis was complete, the zymograms were incubated overnight in 99 mM sodium acetate, pH 4.0 with or without 1 mM DTT. The data shown in Figure 11 are similar to the data previously described for CP18 (North *et al*, 1996). Under non-acid treated conditions (Figure 11A), there was a significant amount of enzymatic activity from CP18. Incubation of the same zymogram in acetic acid resulted in little to no enzymatic activity (Figure 11B). When the two-day old spore matrix from *D. discoideum* strain *V12* was subjected to electrophoresis under native conditions, the results were consistent with the data produced for two-day old spore matrix from spontaneous germinator strain *SG2*. Under native-PAGE conditions, there was very little enzymatic activity from CP18 under non-acid treated conditions (Figure 12A) and an increase in the level of enzymatic activity when the same zymogram was incubated in

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acetic acid (Figure 12B). The cysteine protease inhibitor, E-64, was included in the overnight incubation buffer to confirm the fact that this enzyme belongs to the cysteine class. The removal of DTT from the overnight incubation buffer had no effect on the acid activatable characteristic of CP18.

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Figure 11. SDS-PAGE zymography on *D. discoideum* wild-type strain *V12* spore matrix cysteine protease CP18 while DTT is excluded from the PAGE system. Two-day old spore matrix from *D. discoideum* wild-type strain *V12* was subjected to electrophoresis on SDS-PAGE zymograms (7.5% polyacrylamide). When electrophoresis was complete, the zymograms were incubated in 2.5% Triton-X 100 for thirty minutes (A), or incubated in 1.7 M acetic acid, pH 2.1 for sixty seconds prior to the Triton-X incubation (B). When electrophoresis was complete, the zymograms were then incubated in 99 mM sodium acetate, pH 4.0 with or without 1 mM DTT and with or without 1 mM E-64 for 16 hours. The zymograms were then stained in coomassie blue for at least one hour and then destained in 40% methanol, 10 % acetic acid for at least one hour. All lanes contain 2 µg of protein.

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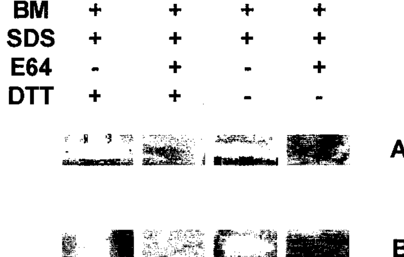

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Figure 12. Native-PAGE zymography on the spore matrix cysteine protease CP18 of *D. discoideum* wild-type strain *V12* while DTT is excluded from the PAGE system. Two-day old spore matrix from *D. discoideum* wild-type strain *V12* was subjected to electrophoresis on native-PAGE zymograms (7.5% polyacrylamide). When electrophoresis was complete, the zymograms were incubated in 2.5% Triton-X 100 for thirty minutes (A), or incubated in 1.7 M acetic acid, pH 2.1 for sixty seconds prior to the Triton-X incubation (B). After the Triton-X incubation, the zymograms were incubated in 99 mM sodium acetate, pH 4.0 with or without 1 mM DTT and with or without 1 mM E-64 for sixteen hours. The zymograms were then stained in coomassie blue for at least one hour and destained in 40% methanol, 10% acetic acid for at least one hour. All lanes contain 2μg of total protein.

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BM	-	-	-	-
SDS	-	-	-	-
E64	-	+	-	+
DTT	+	+	-	-

A

B

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Next, the two-day old spore matrix from *D. discoideum* wild-type strain *V12* was subjected to electrophoresis on PAGE zymograms in which either β-mercaptoethanol (BM) or SDS was excluded from the PAGE system. The data in Figure 13 revealed that when BM was excluded, CP18 was non-acid activatable. However, when SDS was removed from the PAGE system, CP18 regained the acid activatable characteristic. The experiment in Figure 13 was done in the presence of DTT in the overnight incubation buffer. Experiments that were conducted in the absence of DTT yielded similar results (results not shown).

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Figure 13. PAGE zymography on the spore matrix cysteine protease CP18 of *D. discoideum* wild-type strain *V12* while β-mercaptoethanol and SDS is excluded from the PAGE system. Two-day old spore matrix from *D. discoideum* wild-type strain *V12* was subjected to electrophoresis on PAGE zymograms in which either β-mercaptoethanol (BM) or SDS was removed from the system (7.5% polyacrylamide). When electrophoresis was complete, the zymograms were incubated in 2.5% Triton-X 100 for thirty minutes (A), or incubated in 1.7 M acetic acid, pH 2.1 for sixty seconds prior to the Triton-X incubation (B). After the Triton-X incubation, the zymograms were incubated in 99 mM sodium acetate, pH 4.0 containing 1 mM DTT for sixteen hours. The zymograms were then stained in coomassie blue for at least one hour and destained in 40% methanol, 10% acetic acid for at least one hour. All lanes contain 2 μg of total protein.

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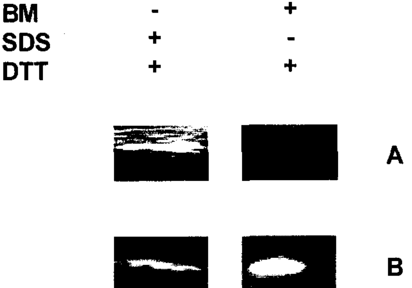

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To confirm the fact that the results observed for the two-day old spore matrix of *D. discoideum* wild-type strain *V12* was universal to the spore matrix of the social amoeba, similar experiments on the two-day old spore matrix of *D. discoideum* spontaneous germinator strain *SG2* was subjected to the same electrophoresis techniques as was done for the two-day old spore matrix for *D. discoideum* wild-type strain *V12*. The results in Figures 14 and 15 showed that when the two-day old spore matrix of *D. discoideum* strain *SG2* was subjected to electrophoresis in which BM was excluded from the sample buffer and DTT was excluded from the overnight incubation buffer, it had no effect on the acid activatability on CP18 in the *SG2* spore matrix. However, when SDS was excluded from the PAGE system, CP18 from the two-day old spore matrix of *D. discoideum* strain *SG2* was acid activatable (Figure 16).

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Figure 14. SDS-PAGE zymography on the spore matrix cysteine protease CP18 of *D. discoideum* spontaneous germinator strain *SG2* while DTT is excluded from the PAGE system. Two-day old spore matrix from *D. discoideum* spontaneous germinator strain *SG2* was subjected to electrophoresis on SDS-PAGE zymograms (7.5% polyacrylamide). When electrophoresis was complete, the zymograms were incubated in 2.5% Triton-X 100 for thirty minutes (A), or incubated in 1.7 M acetic acid for sixty seconds prior to the Triton-X incubation (B). After the Triton-X incubation, the zymograms were incubated in 99 mM sodium acetate, pH 4.0 with or without 1 mM DTT for sixteen hours. The zymograms were then stained in coomassie blue for at least one hour and then destained in 40% methanol, 10% acetic acid for at least one hour. Each lane contains 2 µg of total protein.

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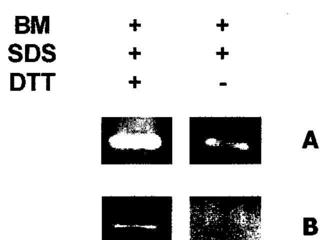

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Figure 15. Native-PAGE zymography on the spore matrix cysteine protease CP18 of *D. discoideum* spontaneous germinator strain *SG2* while DTT is excluded from the PAGE system. Two-day old spore matrix from *D. discoideum* spontaneous germinator strain *SG2* was subjected to electrophoresis on native-PAGE zymograms (7.5% polyacrylamide). When electrophoresis was complete, the zymograms were incubated in 2.5% Triton-X 100 for thirty minutes (A), or incubated in 1.7 M acetic acid, pH 2.1 for sixty seconds prior to the Triton-X incubation (B). After the Triton-X incubation, the zymograms were incubated in 99 mM sodium acetate, pH 4.0 with or without DTT for sixteen hours. The zymograms were then stained in coomassie blue for at least one hour and then destained in 40% methanol, 10% acetic acid for at least one hour. All lanes contain 2 μg of total protein.

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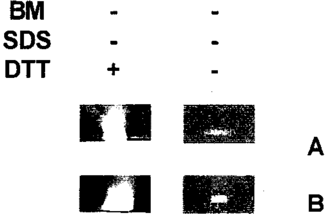

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Figure 16. PAGE zymography on the spore matrix cysteine protease CP18 of *D. discoideum* spontaneous germinator strain *SG2* while β-mercaptoethanol and SDS are excluded from the PAGE system. Two-day old spore matrix from *D. discoideum* wild-type strain *SG2* was subjected to electrophoresis on PAGE zymograms in which either β-mercaptoethanol (BM) or SDS was removed from the system (7.5% polyacrylamide). When electrophoresis was complete, the zymograms were incubated in 2.5% Triton-X 100 for thirty minutes (A), or incubated in 1.7 M acetic acid, pH 2.1 for sixty seconds prior to the Triton-X incubation (B). After the Triton-X incubation, the zymograms were incubated in 99 mM sodium acetate, pH 4.0 containing 1 mM DTT for sixteen hours. The zymograms were then stained in coomassie blue for at least one hour and destained in 40% methanol, 10% acetic acid for at least one hour. All lanes contain 2 μg of total protein.

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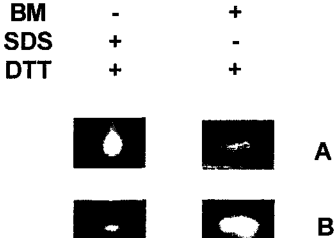

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Inhibition of the internal cysteine proteases during growth of *Dictyostelium discoideum*

To investigate whether the cysteine proteases of *Dictyostelium discoideum* are involved in the growth phase of the social amoeba's life cycle, the rationale of this set of experiments was to inhibit the cysteine proteases during logarithmic growth. To accomplish this, the cell permeable cysteine protease suicide inhibitor (2S,3S)-trans-epoxysuccinyl-L-leucylamido-3-methylbutane ethyl ester (E-64D) was used to biochemically inhibit the active cysteine proteases in the vegetative cells of *D. discoideum.* E-64D inhibits active cysteine proteases by attaching to the active site thiol of the enzyme, creating a thiosulfate group. The formation of a thiosulfate group is irreversible (Tamai *et al*, 1986).

Prior to this experiment, however, it was necessary to elucidate which concentration of the drug would result in total inhibition of the cysteine proteases. Therefore, vegetative cells of *D. discoideum* strain *AX4* were resuspended at 1.0×10^7 cells/ml in TM containing 0.5, 5, or 50 μM E-64D in 0.5% DMSO. After one hour, the cells were washed free of the TM, harvested for their internal protein extracts, and subjected to electrophoresis on SDS-PAGE zymograms. The zymogram in Figure 17 revealed that 5 μM E-64D was sufficient to illicit total inhibition of the endogenously active cysteine proteases in *D. discoideum* strain *AX4.*

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Figure 17. The effect of E-64D on the cysteine proteases from the vegetative cells of *D. discoideum*. Vegetative cells from *D. discoideum* strain *AX4* in growth were resuspended at 1.0 x 10^7 cells/ml in TM with increasing concentrations of E-64D in 0.5% DMSO. After one hour, the cells were washed free of the TM, harvested for their internal protein extract, and subjected to electrophoresis on SDS-PAGE zymograms as described in the Methods and Materials (7.5% polyacrylamide). When electrophoresis was complete, the zymograms were incubated in 2.5% Triton X-100 for thirty minutes (A), or were incubated for sixty seconds in 1.7 M acetic acid, pH 2.1 and then incubated in Triton X-100 (B). The zymograms were then incubated in 99 mM sodium acetate, pH 4.0 containing 1 mM DTT for sixteen hours. The zymograms were then stained in coomassie blue for at least one hour and destained in 40% methanol, 10% acetic acid for at least one hour. All lanes were loaded with 0.8 μg of total protein.

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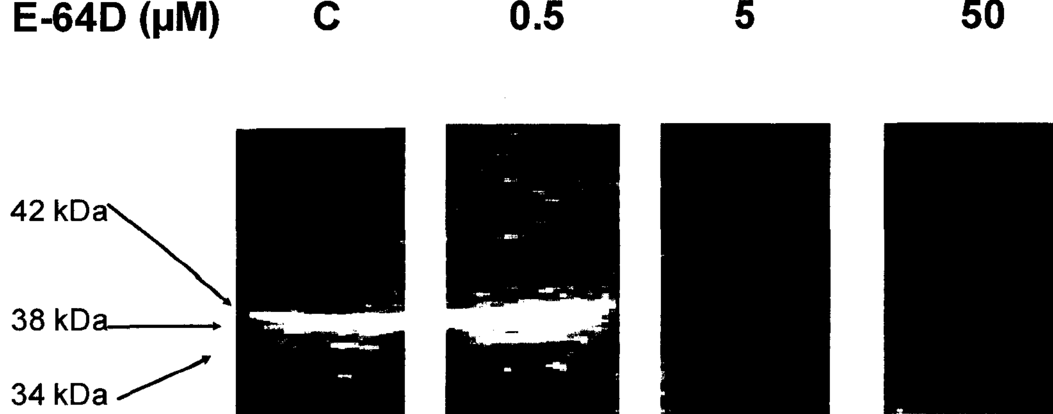

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5.0×10^6 cells/ml from *D. discoideum* strain *AX4* were incubated with 50 µM E-64D. Cell samples from times zero, two, and twenty-four hours were harvested for their internal protein extracts and analyzed by electrophoresis on SDS-PAGE zymograms. The zymograms in Figure 18 revealed that the internally active cysteine proteases were inhibited by 50 µM E-64D (Figure 18A). The only instance when enzyme activity from the drug treated samples could be observed was when the zymograms were incubated in acetic acid for sixty seconds, revealing the acid activatability characteristics of the cysteine proteases in the cellular slime mold.

Once inhibition of the internally active cysteine proteases during growth was confirmed, the growth kinetics of the vegetative cells in the presence of the inhibitor was analyzed. Figure 19 showed that there was no significant difference in the doubling times between the control and drug treated groups. The doubling time of the vegetative cells was about fourteen hours.

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Figure 18. Inhibition of the internally active cysteine proteases in *D. discoideum* during growth with E-64D. Vegetative cells from *D. discoideum* strain *AX4* were resuspended at 5.0 x 10^6 cells/ml in TM containing 50 μM E-64D in 0.5% DMSO. At the indicated time points, cell samples were collected, harvested for their internal protein extracts, and subjected to electrophoresis on SDS-PAGE zymograms. When electrophoresis was complete, the zymograms were incubated in 2.5% Triton-X 100 for thirty minutes (A), or incubated in 1.7 M acetic acid, pH 2.1 for sixty seconds prior to the Triton-X incubation (B). Following the Triton-X incubation, the zymograms were incubated in 99 mM sodium acetate, pH 4.0 containing 1 mM DTT for sixteen hours. The zymograms were then stained in coomassie blue for at least one hour, and then destained in 40% methanol, 10% acetic acid for at least one hour. All lanes contain 0.8 μg of total protein.

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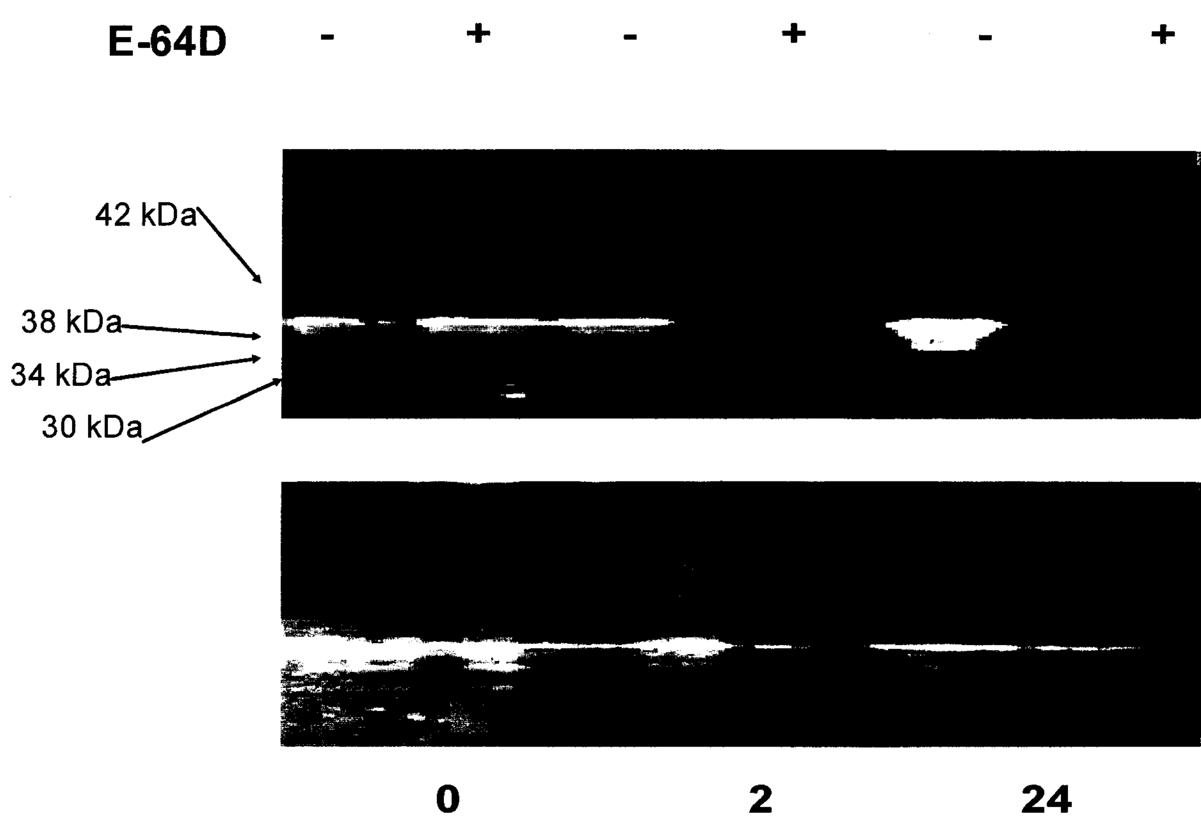

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Figure 19. Growth kinetics of *D. discoideum* in the presence or absence of E-64D. Vegetative cells from the experiment in Figure 18 were sampled at the indicated time points and viewed on a hemacytometer under bright field microscopy to determine the cell density (Control cells are represented by diamonds and cells incubated with 50 μM E-64D are represented by squares). (n=3)

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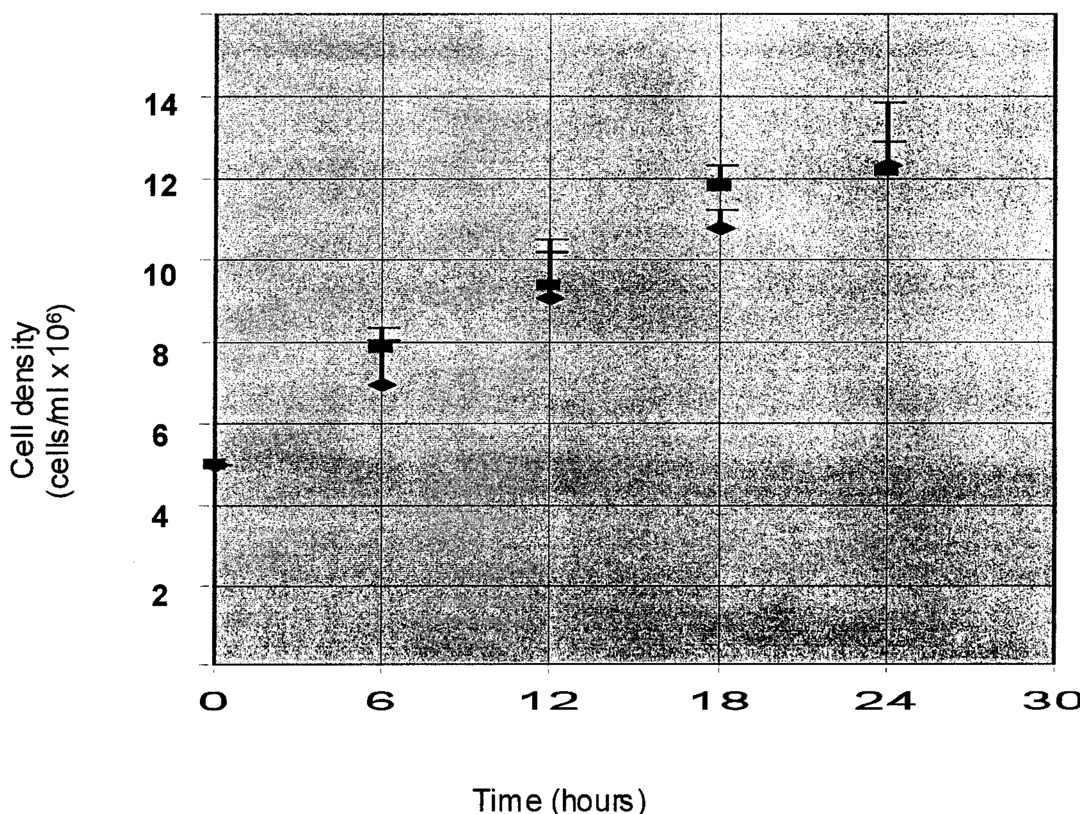

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Inhibition of the internal and secreted cysteine proteases of *D. discoideum* during development

To investigate whether or not the internal cysteine proteases of *D. discoideum* are directly involved in cellular developmental, vegetative cells of *D. discoideum* strain *AX4* were used as a model for the developmental life cycle. Vegetative cells were washed free from TM and resuspended at 1.0 x 10^7 cells/ml in 10 mM KH_2PO_4, pH 6.5 containing 0.5% DMSO containing 50 µM E-64D. Aliquots (40 µl) of the cell suspension were dispensed into wells of a 96-well microtitre plate. At the indicated time points, the cells were collected from the microtitre wells, harvested for their internal protein extracts, and subjected to electrophoresis on SDS-PAGE zymograms. As expected, the endogenously active cysteine proteases were inhibited with 50 µM E-64D, (Figure 20A). Zymogram analysis of the internal cysteine proteases during development also revealed that there was a fraction of the enzyme class that was inactive. Acetic acid treatment of the zymogram in Figure 20B revealed that there was acid activatable cysteine proteases in samples that were treated with E-64D.

When the cell samples from the microtitre wells were collected, the supernatant fractions were retained and subjected to electrophoresis on SDS-PAGE zymograms. Zymogram analysis in Figure 21A on the supernatant fractions revealed that *D. discoideum* strain *AX4* secreted cysteine proteases into the medium. Furthermore, supernatant fractions from the E-64D treated samples revealed that the secreted cysteine proteases were inhibited. It was also apparent that vegetative cells secreted a fraction of the cysteine proteases in an inactive form. Figure 21B shows

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that there were acid activatable cysteine proteases in the secreted portion of the control and E-64D treated samples.

Once enzyme inhibition was confirmed, digital images were captured of the cells to view their phenotypes. At approximately six hours, images were taken of the cells in the microtitre wells to view the aggregation process of *D. discoideum* strain *AX4*. Figure 24 revealed that there was virtually no observable difference in the aggregation process between the control (Figure 22A) and E-64D treated samples (Figure 22C). Furthermore, images were captured twenty-four hours later to view the vegetative cells proceeding into fruiting body formation. Once again, the control (Figure 22B) and E-64D treated samples (Figure 22D) showed no observable difference in the ability to enter into fruiting body formation.

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Figure 20. Inhibition of the internally active cysteine proteases during development with E-64D. Vegetative cells of *D. discoideum* strain *AX4* were washed out of TM and incubated in 10 mM KH_2PO_4, pH 6.5, containing 50 μM E-64D in 0.5% DMSO at 1.0 x 10^7 cells/ml (time 0 hours). Aliquots (40 μl) of the cell suspension were placed in microtitre wells of a 96-well plate. Cells were collected from the wells at the indicated time points, harvested for their internal protein extracts, and subjected to electrophoresis on SDS-PAGE zymograms (7.5% polyacrylamide). When electrophoresis was complete, the zymograms were incubated in 2.5% Triton X-100 for thirty minutes (A), or incubated in 1.7 M acetic acid, pH 2.1 for sixty seconds prior to the Triton X-100 incubation (B). Zymograms were then incubated in 99 mM sodium acetate, pH 4.0 containing 1 mM DTT for sixteen hours. The zymograms were then stained in coomassie blue for at least one hour and then destained in 40% methanol, 10% acetic acid for at least 1 hour. All lanes contain 0.8 μg of total protein.

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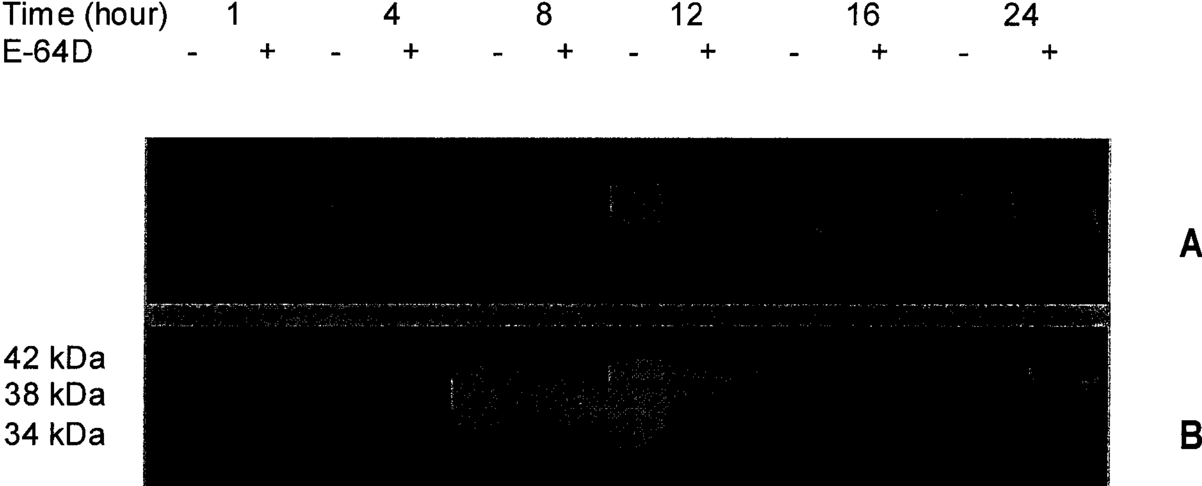

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Figure 21. Inhibition of the active cysteine proteases secreted by *D. discoideum* during development with E-64D. The supernatants from the experiment in Figure 22 were retained from the indicated time points, concentrated, and subjected to electrophoresis on SDS-PAGE zymograms (7.5% polyacrylamide). When electrophoresis was complete, the zymograms were incubated in 2.5% Triton-X 100 for 30 minutes (A), or incubated in 1.7 M acetic acid, pH 2.1 for 60 seconds prior to the Triton-X incubation (B). Following the incubation in Triton-X, the zymograms were incubated in 99 mM sodium acetate, pH 4.0 containing 1 mM DTT for 16 hours. The zymograms were then stained in coomassie blue for at least one hour and destained in 40% methanol, 10% acetic acid for at least one hour. All lanes contain 0.8 μg of total protein.

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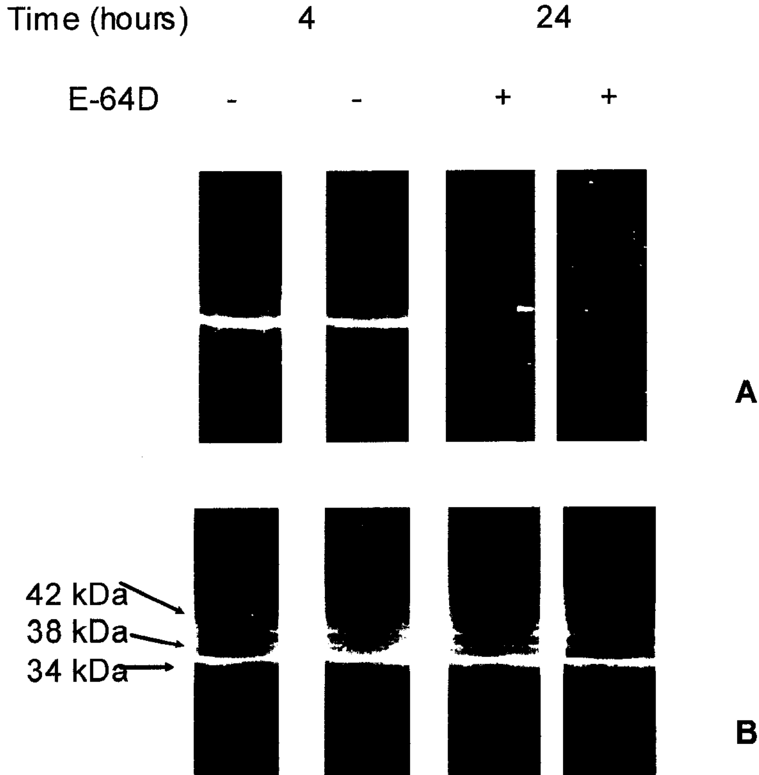

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Figure 22, cell aggregation and fruiting body formation in the presence of E-64D. Digital images from the experiment in Figures 22 and 23 were captured at six hours (A and C) and at twenty-four hours (B and D). The cells in panels A and B were incubated in 0.5% DMSO. The cells in panels C and D were incubated in 50 μM E-64D in 0.5% DMSO.

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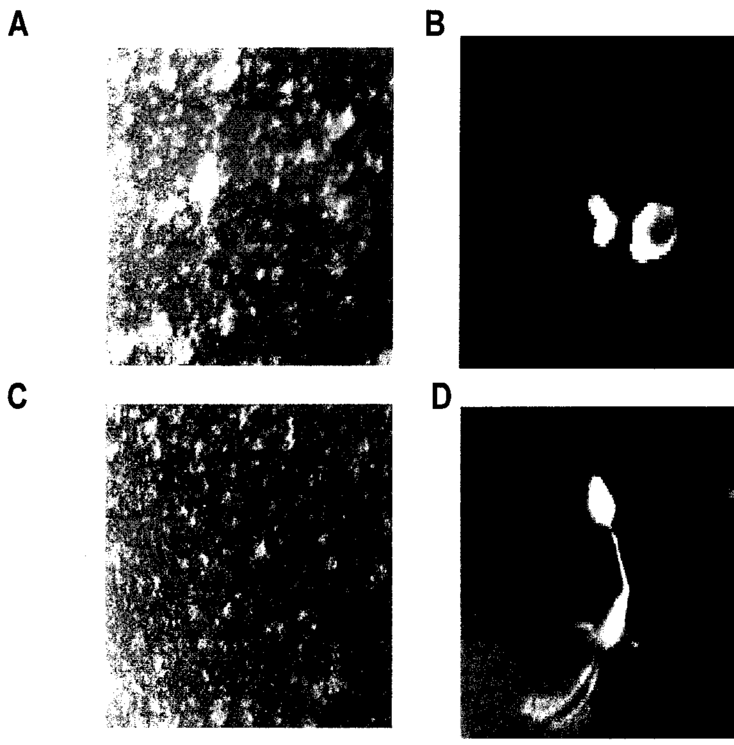

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Inhibition of the internal cysteine proteases in *D. discoideum* during spontaneous spore germination

The last part of this study was to elucidate whether or not the internal cysteine proteases of *D. discoideum* were required for the spore germination process. *D. discoideum* spontaneous germinator strain *SG2* was used as a model for this part of the study. E-64D (50 μM) was utilized once again to inhibit the internally active cysteine proteases during the germination process. It has been reported previously that dormant spores can be activated to germinate when incubated in 20% DMSO (Cotter *et al*, 1976). Using this rationale, the spores were incubated in 20% DMSO containing 50 μM E-64D. After one hour, the spores were washed free of DMSO and resuspended at 1.0 x 10^7 spores/ml in 10 mM KH_2PO_4, pH 6.5, and allowed to germinate over five hours. SDS-PAGE zymogram analysis in Figure 25C revealed that the endogenously active cysteine proteases were inhibited when the spores were allowed to germinate. Once again, the only time enzymatic activity from the E-654D treated samples could be observed was when the zymogram was treated with acetic acid (Figure 23D), confirming the acid activatability of the cysteine proteases in the spores of *D. discoideum*.

Once inhibition of the internally active cysteine proteases during spore germination was confirmed, the kinetics of the spore germination process was assessed. Figure 24 revealed that there was no significant difference in the percentage of spore swelling between the control and drug treated groups.

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Figure 23. Inhibition of the internally active cysteine proteases during spontaneous spore germination with E-64D. Two-day old spores from *D. discoideum* spontaneous germinator strain *SG2* were incubated in 20% DMSO containing 50 µM E-64D. After one hour, the DMSO was washed from the spores and the spores were resuspended at 1.0 x 10^7 spores/ml in 10 mM KH_2PO_4, pH 6.5, and allowed to germinate in shake flask culture over a period of five hours. Every hour, spore samples were collected, harvested for their internal protein extracts, and subjected to electrophoresis on SDS-PAGE zymograms (7.5% polyacrylamide). When electrophoresis was complete, the zymograms were incubated in 2.5% Triton-X 100 for thirty minutes (A and C), or incubated in 1.7 M acetic acid, pH 2.1 for sixty seconds prior to the Triton-X incubation (B and D). The zymograms in panels A and B contain extracts from spores that were incubated in 20% DMSO for one hour and allowed to germinate. The zymograms in panels C and D contain extracts from spores that were incubated in 20% DMSO containing 50 µM E-64D for one hour and allowed to germinate. All lanes contain 2 µg of total protein.

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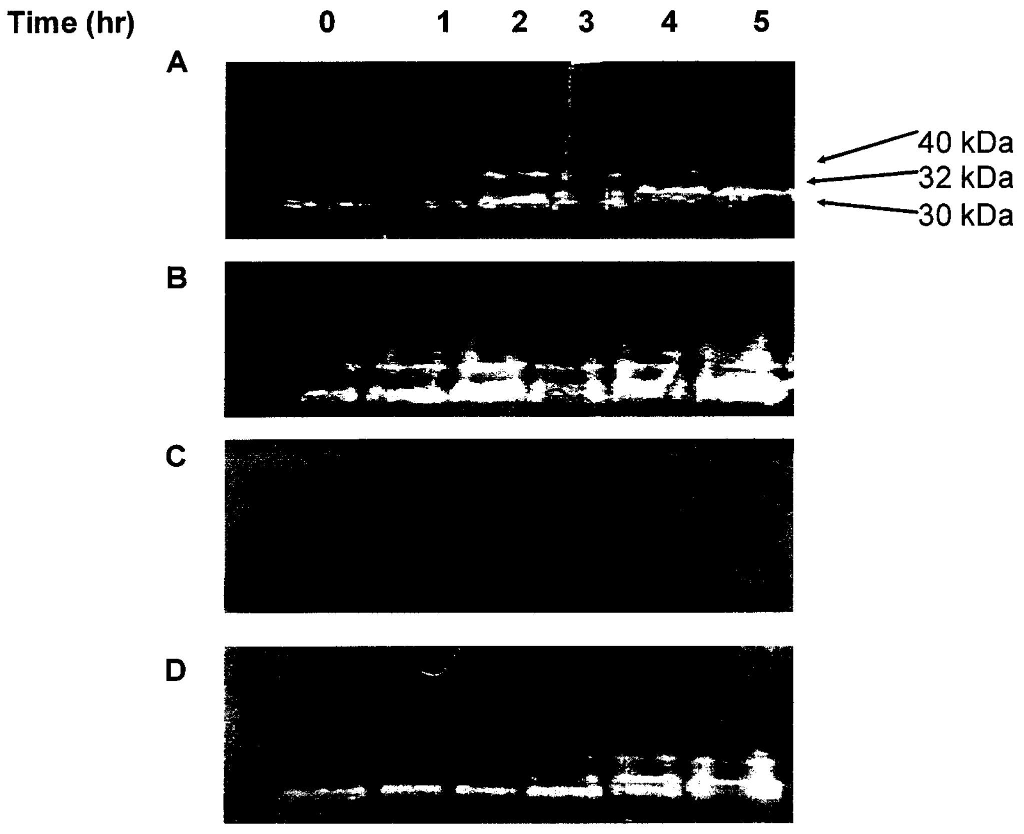

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Figure 24. Spore germination kinetics of *D. discoideum* in the presence of E-64D. Samples from the experiment in Figure 23 were viewed under phase contrast microscopy at the indicated time points (Control is represented by diamonds and spores that were incubated with 50 μM E-64D are represented by squares). The first two hundred objects were scored as dormant spore or swollen spore (n=9).

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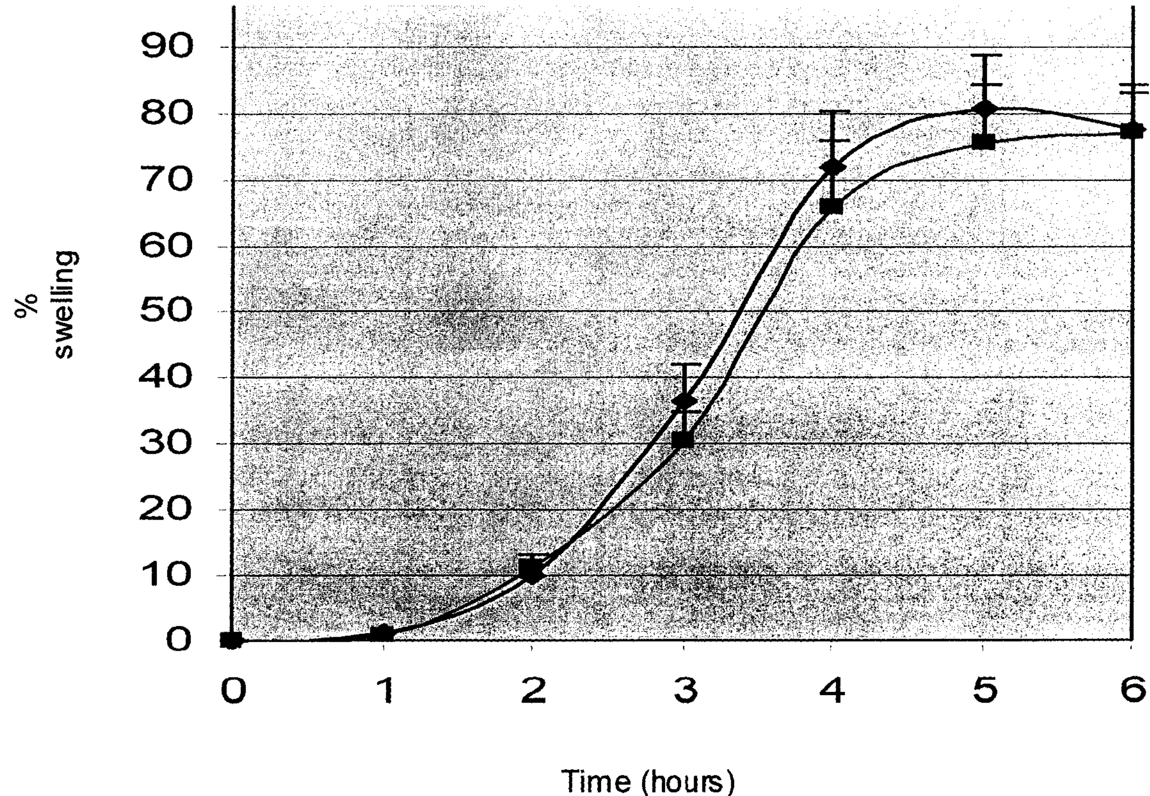

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The proteolytic enzymes in the microcysts of *Acanthamoeba castellanii*

It has been previously reported that the proteolytic enzymes of the cysteine class in *D. discoideum* are unique in that their activity can be switched on and off by the induction of conformational changes when exposed to acidic and/or alkaline environments (North *et al*, 1996; Cotter *et al*, 2000). *Acanthamoebae castellanii* is a free living amoeba closely related to *D. discoideum* on a phylogenetic basis (Woese, 1998). The purpose of this study was to investigate what similarities, if any, existed in the proteolytic enzymes in the microcyst stage of *A. castellanii* compared to *D. discoideum*.

Microcysts on lawns of *E.coli* B/r were collected as described in Methods and Materials. The microcysts were harvested for their internal protein extracts and separated on SDS-PAGE zymograms. A range of protein concentrations from 0.02-7.2 μg was run on the gel and separated. It was observed that there were enzymes with an approximate molecular weight of 60 and 40 kDa (Figure 25A and B). A block of unresolvable enzymes ranging in molecular weight of approximately 116-160 kDa was also visible. It was noted that at least 7.2 μg of protein was needed to obtain clear visibility of the 40 kDa weight enzyme. No proteolytic activity from *E. coli* B/r contributed to the results seen on the SDS-PAGE zymograms (results not shown). To view whether or not the proteolytic enzymes of *A. castellanii* were acid activatable, a duplicate gel was exposed to 1.7 M acetic acid, pH 2.1. It was observed that there was an acid activatable component of the 40 and 60 kDa weight enzyme (Figure 25B). It could be seen that there was also acid-activatibility of the 116-160 kDa weight block of unresolvable enzymes. Comparison of the non-acid and acid

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treated lanes loaded with 7.2 μg of protein in Figure 25 showed slightly more intensity in the 40 kDa weight enzyme. Comparison of the non-acid and acid treated lanes loaded with 1.2 and 2.4 μg of protein revealed slightly more intensity in the sixty kDa weight enzyme. After acid treatment, the zymograms were further incubated in 1.7 M ammonium phosphate, pH 9.0, or incubated in 1.7 M ammonium phosphate, pH 9.0 followed by an incubation in 1.7 M acetic acid, pH 2.1 to view whether or not the proteolytic enzymes of *A. castellanii* were capable of being inactivated and reactivated by alkaline and acidic environments as is observed with *Dictyostelium discoideum* cysteine proteases. As can be seen in Figure 25C, the 40 kDa weight enzyme in the lane loaded with 2.4 μg of protein was barely detectable. Furthermore, the 60 kDa weight enzyme and the 116-160 kDa weight block of proteolytic enzymes had lost a significant amount of intensity when treated with the alkaline solution. Figure 25D revealed that all of the proteolytic enzymes lost even more of their intensity on the SDS-PAGE zymograms when the acid-base treated gel was subsequently treated with acid.

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Figure 25. Acidic and alkaline tolerance of the proteolytic enzymes in *A. castellanii* microcysts. Microcysts of *A. castellanii* were harvested for their internal protein extracts and separated on SDS-PAGE zymograms (10% polyacrylamide) Gels were incubated for thirty minutes in 2.5% Triton-X 100 (A). Before the Triton-X incubation, gels were incubated in 1.7 M acetic acid, pH 2.1 for sixty seconds (B), then incubated in 1.7 M ammonium phosphate, pH 9.0 for sixty seconds (C), followed by an incubation in 1.7 M acetic acid, pH 2.1 for sixty seconds (D). Gels were then incubated overnight in 99 mM sodium acetate, pH 4.0 containing 1 mM DTT. Gels were then stained with coomassie blue for at least one hour and destained in 40% methanol, 10 % acetic acid for at least one hour. Molecular weight markers (Fermentas) were loaded in gels A and B. Lanes 1-8 contain 7.2, 2.4, 1.2, 0.6, 0.3, 0.15, 0.075, 0.0375 and 0.02 μg of protein, respectively.

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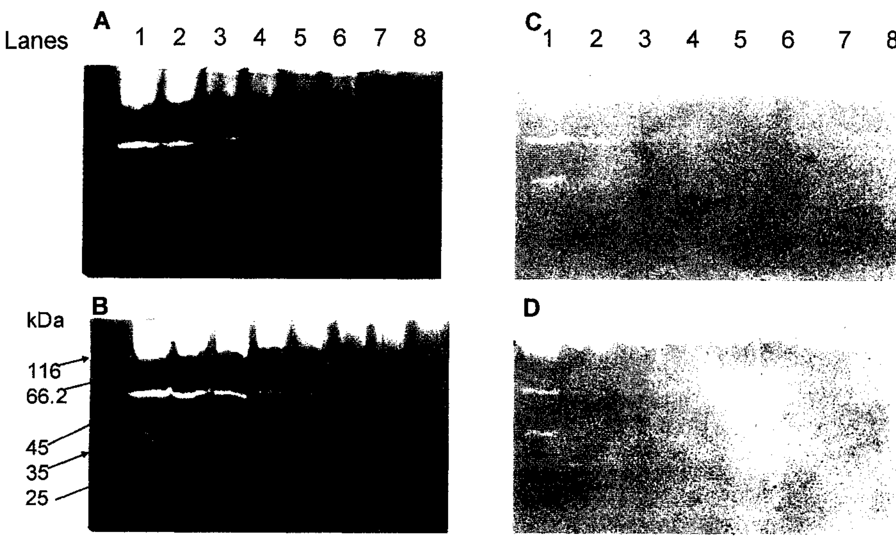

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Class characterization of the proteolytic enzymes in the microcysts of *A. castellanii*

It has been well established that the acid-activatability of the proteolytic enzymes in *D. discoideum* are only restricted to the cysteine class (North *et al*, 1996). Since all of the proteolytic enzymes separated on SDS-PAGE zymograms from the microcysts of *A. castellanii* in Figure 25 were shown to be acid activatable, the next stage of this study dealt with assigning these proteolytic enzymes to their proper class. This was accomplished by treating the proteolytic enzymes with EDTA, a metallo-protease inhibitor, PMSF, a serine protease inhibitor, pepstatin A, an aspartyl protease inhibitor, and E-64, a cysteine protease inhibitor.

As can be seen in Figure 26, 7.2 μg of internal protein extracts from the microcysts of *A. castellanii* were separated on SDS-PAGE zymograms. After the electrophoresis was complete, strips of the gels containing the proteolytic enzymes were incubated in sodium acetate, pH 4.0 containing 1 mM DTT. The inhibitors just mentioned were incorporated into the overnight incubation buffer. Figure 26 shows that the SDS-PAGE zymogram strip incubated in the overnight buffer with 100 μM E-64 abolished the activity of the 40 kDa weight enzyme. Incubation of the zymogram strips in 1 mM EDTA, 10 μM pepstatin A, or 1 mM PMSF did not abolish activity of the 60 kDa weight enzyme or the 116-160 kDa weight block of enzymes. As a control, the zymogram strips were incubated in 0.9% ethanol/0.1% acetic acid and in 1% methanol. The ethanol/acetic acid and methanol were the reagents used to solubilize pepstatin A and PMSF, respectively.

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Figure 26. Overnight incubation of zymogram strips containing the proteolytic enzymes of *A. castellanii* in the presence of various enzyme inhibitors. 7.2 μg of internal protein extracts harvested from *A. castellanii* microcysts were separated on SDS-PAGE zymograms (10% polyacrylamide). The zymograms were incubated in 2.5% Triton-X 100 for thirty minutes, followed by an overnight incubation in 99 mM sodium acetate, pH 4.0 containing 1 mM DTT. The reagents listed in the picture were incorporated in the overnight incubation. Gels were then stained in coomassie blue for at least one hour and destained in 40% methanol, 10% acetic acid for at least one hour.

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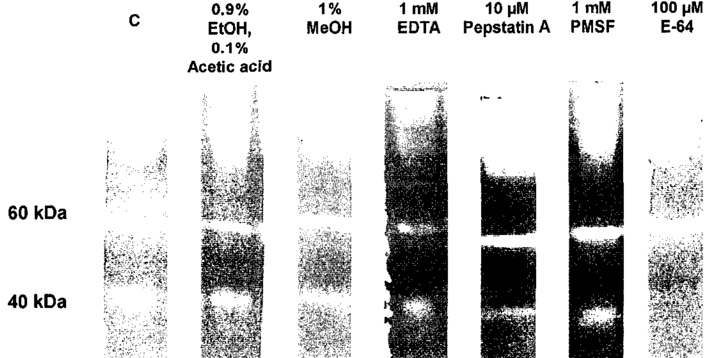

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To further elucidate the class designation of the 60 kDa weight enzyme and the 116-160 kDa weight block of enzymes in the microcysts of *A. castellanii*, internal protein extracts were incubated in the various inhibitors for one hour. After the one hour incubation, 1.2 µg of protein was separated on an SDS-PAGE zymogram. It was mentioned earlier that at least 7.2 µg of protein from the microcysts of *A. castellanii* was needed to clearly view the 40 kDa weight enzyme. However, since it had been recently established from Figure 26 that the 40 kDa weight enzyme was of the cysteine class, it was agreed that 1.2 µg of protein was sufficient to clearly view the 60 kDa weight enzyme and 116-160 kDa weight enzyme block on the SDS-PAGE zymogram. Figure 27 shows that the activity of the 60 kDa weight enzyme was abolished when the protein extract was incubated in 10 µM pepstatin A. However, it was determined that the absence of the 60 kDa weight enzyme in the presence of 10 µM pepstatin A did not confirm that this enzyme was designated to the aspartyl class of proteases. Incubation of the protein extract in 0.9% ethanol and 0.1% acetic acid, the reagents that pepstatin A was solubilized in, also resulted in the absence of the sixty kDa weight enzyme. The ethanol/acetic acid more than likely denatured the enzyme. Furthermore, when the protein extracts were incubated in 10 mM EDTA, 1 mM PMSF and 1 mM E-64, there was no abolishment of the 60 kDa weight enzyme or the 116-160 kDa weight enzyme block.

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Figure 27. SDS-PAGE zymogram analysis of internal protein extracts from the microcysts of *A. castellanii* incubated in various protease inhibitors. Internal protein extracts from the microcysts of *A. castellanii* were incubated in the above concentrations of protease inhibitors for one hour. After the one hour incubation, 1.2 μg of protein was separated on a SDS-PAGE zymogram containing 10% polyacrylamide in the separating gel. The gels were incubated in 2.5% Triton-X 100 for thirty minutes (A) or incubated in 1.7 M acetic acid, pH 2.1 for sixty seconds prior to the Triton-X incubation (B). The gels were then incubated overnight in 99 mM sodium acetate, pH 4.0 containing 1 mM DTT. The gels were then stained in coomassie blue for at least one hour and destained in 40% methanol, 10% acetic acid for at least one hour.

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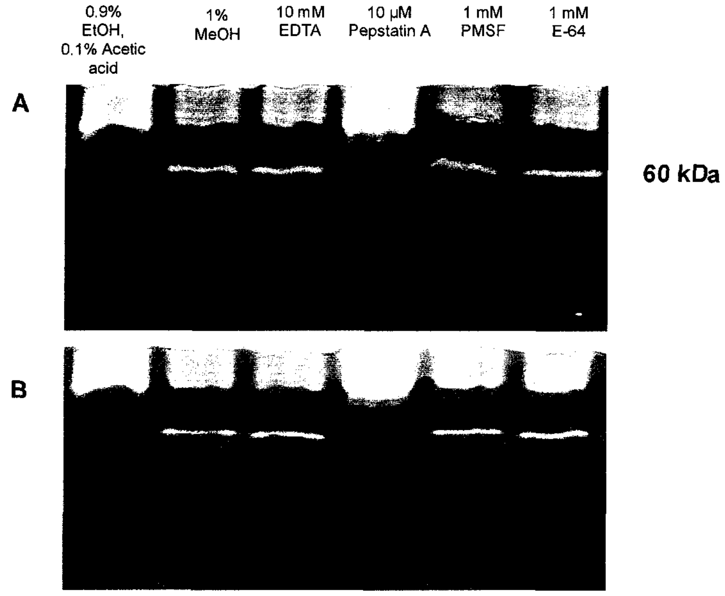

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Chapter 4, Discussion

Protein tyrosine phosphatases and cysteine proteases as targets of hydrogen peroxide in the oxidized inhibition of spontaneous spore germination in *Dictyostelium discoideum*

The involvement of reactive oxygen species (ROS) as a second messenger in signal transduction has gained a wealth of appreciation over the last few years. The best example of this is the involvement of ROS in the antagonistic relationship between the small GTPases Rac and Rho. When mammalian cells commit to growth and proliferation, there is an up-regulation in the small GTPase Rac, and when the cells commit to becoming stationary, there is an up-regulation in the small GTPase Rho (Nimnual *et al*, 2003). It has now been established that when there is an up-regulation in the small GTPase Rac, there is also an increased intracellular production of ROS. The increased production of ROS results from a signal peptide sequence on the small GTPase Rac that alerts the cell in an unknown way to produce ROS (Freeman *et al*, 1996). The small GTPase Rho requires a dephosphorylated state to become up-regulated. However, ROS has been shown to be an inhibitor of protein tyrosine phosphatases (Denu *et al*, 1998). Therefore, when there is an upregulation of Rac, Rho is down-regulated because of the increased ROS concentration in the cell that acts as an inhibitor towards Rho.

The effect of the hydrogen peroxide (H_2O_2) on the dormant spores of *Dictyostelium discoideum* has recently been reported. The dormant spores of *D. discoideum* have been shown to exhibit fifty percent survival after one hour exposure

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to 100 mM H_2O_2 (Garcia *et al*, 2003). The regulation of the catalase enzymes is quite unique in *Dictyostelium*. Two catalase enzymes exist within the organism. The vegetative cell specific catalase, catalase A, is contained within a peroxisomal compartment. The second catalase is spore specific; catalase B. What is intriguing about catalase B is that it does not localize to any peroxisomal compartment, rather, it exists free floating in the cytoplasm (Garcia *et al*, 2003).

In the dormant spores of *Dictyostelium*, polymerized actin is phosphorylated on Tyr53. A hallmark of the germination process is the dephosphorylation of actin (Gauthier *et al*, 1997; Kishi *et al*, 2000). To explain the high resistance which dormant spores have to H_2O_2, it was hypothesized that H_2O_2 might act as a natural inhibitor towards spore germination since it has been shown that phosphatase inhibitors block spore germination (Kishi *et al*, 1998). Previously, an *in vivo* manipulation involving polymerized actin in the dormant spores of *Dictyostelium* has been reported (Kishi *et al*, 2000). Briefly, if dormant spores are incubated in 30-300 mM glucose for 1 hour and then the glucose is washed from the spores, actin will become dephosphorylated under these conditions without promoting germination. This was observed in Figure 4A when the internal protein of dormant spores incubated in 100 mM glucose was denatured, separated under standard SDS-PAGE techniques, and stained with a proprietary phosphoprotein stain. Figure 4A also showed that actin from spores incubated in glucose simultaneously with 50 mM H_2O_2 was inhibited from dephosphorylation. This is strong evidence that the protein tyrosine phosphatase (PTP) required to dephosphorylate actin was inhibited by H_2O_2. It has been reported previously that PTP1 is the phosphatase that interacts with the

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actin phosphorylation site in vegetative cells (Howard *et al*, 1993). However, data in recent years have shown that actin dephosphorylation in spores occurs normally in a *ptp1* null strain (Cotter and Mahadeo, unpublished results). Furthermore, the inhibition of the dephosphorylation event was reversible upon simultaneous treatment of the spores with a thiol reductant as has been reported previously (Denu *et al*, 1996). An unexpected result in these experiments was the observation of an irreversible inhibition of a 66 kDa protein from dormant spores that were incubated simultaneously in glucose, H_2O_2, and dithiothreitol (DTT) (Figure 4A).

Since this preliminary experiment gave good evidence that H_2O_2 could serve as an inhibitor towards spore germination, laboratory simulations were conducted wherein spores that were at the age of spontaneous germination were allowed to germinate under oxidative conditions. Spores at the age of spontaneous germination were incubated in concentrations of 1, 10, and 50 mM H_2O_2 in shake flask culture. The data in Figure 5B revealed that over a 4 hour time course, the swelling kinetics of the spores decreased in a dose dependent manner with H_2O_2. In another set of experiments, spores that were at the age of spontaneous germination were allowed to germinate in shake flask culture in 50 mM H_2O_2 simultaneously with increasing concentrations of exogenously added catalase. The data in Figure 5C showed that the decreased spore swelling kinetics observed with 50 mM H_2O_2 was recovered in a dose dependent manner with exogenously added catalase. After the 4 hour time course, the spores were washed free from the various incubation conditions and assayed for their continued ability to spontaneously germinate on non-nutrient agar plates. The data in Table 3 revealed that amoebal emergence decreased in a dose

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dependent manner when the spores had been treated with H_2O_2, and the emergence kinetics was recovered in a dose dependent manner when the spores had been treated with 50 mM H_2O_2 and exogenously added catalase. The spore germination experiments and the emergence experiments on non-mutrient agar plates do not shed any light on the viability of the spores. Therefore, the spores that had been washed after the various conditions were also plated onto 1% bacto-peptone agar plates and then analyzed for their ability to emerge. Amoebal emergence in the presence of bacterial or bacterial-like components is also an indication of spore viability (Cotter and Raper, 1966). The data in Table 2 showed that under all conditions, the emergence kinetics was almost 100 %, indicating that the conditions used had essentially no effect on the viability of the spores.

Inhibition of PTP's by ROS involves oxidation of the active site thiol. Once oxidized, the sulfer atom will be forced to form a disulfide bond or sulfenic acid with an amino acid residue in the near vicinity (Nimnual *et al*, 2003; Denu *et al*, 1996). A close inspection at the active site of lysosomal cysteine proteases reveals that they share some similarity with PTP's in that they also contain an active site thiol bond (Turk *et al*, 2000). It was hypothesized that during the inhibition of spontaneous germination by H_2O_2 described in this data set, the cysteine proteases of *Dictyostelium* also might be inhibited. It did not seem reasonable, however, to examine the cysteine protease activity during the oxidative inhibition of spontaneous germination since it has been reported previously that cysteine protease activity only increases during the latter portion of the spore germination process; i.e., spore swelling (North *et al*, 1996; Cavallo *et al*, 1999). Therefore, it was felt that

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attributing the low cysteine protease activity that would be observed during the oxidative inhibition of spontaneous spore germination would be an inaccurate observation. However, to test the hypothesis that H_2O_2 could reversibly inhibit cysteine proteases, the enzymes from vegetative cells in growth phase were isolated on PAGE zymograms. These zymograms were incubated in increasing concentrations of H_2O_2 and then analyzed for their observable proteolytic activity. The data in figure 3A revealed that when zymograms were exposed to 0.6 mM H_2O_2, there was partially diminished activity of the cysteine proteases. Incubation of the zymograms in 0.7 mM H_2O_2 resulted in total loss of cysteine protease activity. Furthermore, incubation of the zymograms in 2 mM H_2O_2 followed by incubation in 100 mM DTT resulted in recovery of the cysteine protease activity. To elucidate whether the cysteine proteases of *Dictyostelium* were directly required by the spores to spontaneously germinate, dormant spores at the age of spontaneous germination were incubated with the cell permeable cysteine protease inhibitor E-64D and allowed to germinate in shake flask culture over a five hour time course. The data in Figure 23C and Figure 24 show that spores treated with the cysteine protease inhibitor produced spore swelling kinetics similar to germinating spores in the control set while their active cysteine proteases were inhibited. This set of results confirms that the endogenously active cysteine proteases of *Dictyostelium* are not required for the spontaneous spore germination process.

In nature, it would seem highly unlikely that the dormant spores of *Dictyostelium* would be exposed to concentrations of H_2O_2 at the levels tested in these experiments. It is plausible, however, that the dormant spores would be

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exposed to lower levels of H_2O_2 emitted from lysed aerobic bacteria contained within the forest soil over a continuous period of time. As shown in Tables 4 and 5, continuous exposure to lower levels of H_2O_2 can also result in the inhibition of spore germination. If the spores were exposed to these levels of H_2O_2 for long enough, it could result in spore death as shown in Table 6. As shown in this data set, targets of H_2O_2 for the oxidative inhibition of spontaneous spore germination are PTP's and cysteine proteases. It is most likely that the reason why these oxidative conditions have no effect on the bacterial recognition mode of spore germination is because this pathway for spore germination provides the spores with enough energy and reducing power to repair the damage caused by the oxidative stress.

In summary, spontaneous spore germination in *Dictyostelium discoideum* is inhibited by the reactive oxygen species, hydrogen peroxide. Targets of this oxidative inhibition are protein tyrosine phosphatases and, although they are not required by the spores to spontaneously germinate, cysteine proteases.

Analysis of the fluid spore matrix in *Dictyostelium discoideum*

The spore matrix is the fluid environment contained between the individual dormant spores at the top of the fruiting body. In an effort to elucidate whether there were any differences in protein expression in the spore matrix of amongst different strains of *Dictyostelium*, the spore matrix from wild-type strains *NC4*, *V12* and spontaneous germinator strain *SG2* was isolated from different days and separated on SDS-PAGE. This type of differential analysis showed that a 26 kDa protein accumulates in the spore matrix and displays its maximum activity at four days in

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wild-type strains *NC4* and *V12*. In spontaneous germinator strain *SG2*, this 26 kDa protein displays constant activity from the first day on (Figures 6-8).

In nature, spontaneous germination of spores is dependent on age. Wild type spores spontaneously germinate when they are six to ten days of age, and the spontaneous germinating mutants when they are one day of age (Cotter and Dahlberg, 1977; Dahlberg and Cotter, 1978). It was interesting to see that as the wild-type spores came closer to the age of spontaneous germination, the accumulation of the 26 kDa protein became greater in the spore matrix. If this protein in the spore matrix were to be used as a marker to detect how close wild-type spores are to spontaneously germinating, then the data collected from the spontaneous germinating strain *SG2* remains consistent with this observation. Since these spores are ready to spontaneously germinate at one day of age, this means that this 26 kDa protein should already be present in the spore matrix at day one. This can be seen in Figure 8.

It has been reported previously that a cysteine protease that is 18 kDa in weight, CP18, is found exclusively in the spore matrix of *Dictyostelium*. What is unique about this cysteine protease is that it was also reported to behave in a manner opposite to that of the rest of the cysteine proteases of *Dictyostelium*; i.e., CP18 is non-acid activatable (North *et al*, 1996; Cotter *et al*, 1997). To investigate the peculiar nature of CP18, spore matrix from *Dictyostelium* was isolated and subjected to electrophoresis on PAGE zymograms under native conditions. The zymograms is Figure 10 show that under these conditions, CP18 was now acid activatable. When it was reported that CP18 is non-acid activatable, these experiments were conducted on SDS containing PAGE zymograms. It was thought that there were one or more

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components of the PAGE zymogram system that was accountable for the observations seen previously. To investigate this possibility, dithiothreitol (DTT) was removed from the overnight incubation buffer to determine if it was responsible for the acid activatability of CP18. The data in Figure 11 showed that the exclusion of DTT from the overnight incubation buffer had no effect in the acid activatablity of CP18. β-mercaptoethanol (BM) was targeted next and it was removed from the sample buffer. The data in Figure 13 revealed that BM was not responsible for the acid activatability of CP18. Exclusion of sodium dodecyl sulfate (SDS), however, resulted in the acid activatability of CP18.

The classically defined protocols for viewing the cysteine proteases of *Dictyostelium* on PAGE zymograms include the use of SDS and under these conditions, the rest of the cysteine proteases are acid activatable (Cavallo *et al*, 1999). Why then is this one cysteine protease, CP18, non-acid activatable in the presence of SDS? It is possible that since CP18 is smaller in molecular weight than the rest of the cysteine proteases, the overall negative charge that is applied to this protein by SDS might cause it unfold in such a manner that it would change its conformation into the "on" state, resulting in a high amount of proteolytic activity. Further acid activation of this highly active enzyme would then result in denaturation of the proteolytic enzyme, and would be viewed on an SDS-PAGE zymogram as a now low activity enzyme as was shown previously (North *et al*, 1996) and in Figure 12. The rest of the cysteine proteases of *Dicyostelium* are most likely resistant to the unfolding effects SDS would have on CP18, and therefore would still remain in their low activity state until acid activated.

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Another question that comes to mind in light of these results is why an enzyme with relatively low activity in its native state would be found exclusively in the spore matrix of *Dictyostelium*. It is possible that this proteolytic enzyme is found in a relatively low activity state because of the level of oxidation that may be present in the spore matrix. It has been recently reported that when the vegetative cells are in their developmental life cycle, superoxide (O^-) that is emitted by the cells is needed by the cells to aggregate properly. It is quite probable that the amount of reactive oxygen in the extracellular matrix of developing cells may carry over to the spore matrix and act as an inhibitor towards the low molecular weight cysteine protease. Although the data in Figure 3 show that the reactive oxygen species H_2O_2 is responsible for the oxidized inhibition of the cysteine proteases in *Dictyostelium*, it should be kept in mind that this type of oxidized inhibition is achievable amongst all reactive oxygen species, including O^- (Barrett *et al*, 1999).

In an effort to assign a role for CP18, it has been hypothesized that the proteolytic enzyme may serve as a timer towards spore germination. The spore specific adenylyl cyclase G (ACG) is an osmosensor that is stimulated by the 200 mM ammonium ion concentration in the fruiting body to produce cAMP, which in turn acts to maintain spore dormancy. ACG has an N-terminal loop that is believed to protrude from the plasma membrane, which may be slowly hydrolyzed by CP18. Once hydrolyzed, this would abruptly stop the production of cAMP, and thus predispose the spore to germinate.

In conclusion, the fluid spore matrix of *Dictyostelium* contains a 26 kDa protein that accumulates as the dormant spores move closer to the age of spontaneous

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spore germination. Also, the previously characterized non-acid activatable cysteine protease CP18, is in fact acid activatable under conditions where SDS is not present in the in vitro PAGE zymogram system.

The endogenously active cysteine proteases of *Dictyostelium discoideum* have no direct involvement in axenic growth or development

In mammalian cell systems, the cysteine proteases have been shown to have a direct involvement in cellular differentiation. For example, knockout of lysosomal cysteine protease cathepsin B at the molecular or biochemical level results in developmental defects in rat skeletal muscle cells (Jane *et al*, 2002). Lysosomal cysteine protease cathepsin L has been shown recently to have an important role in the G1-S transition of cell cycle progression (Goulet *et al*, 2004). In the social amoeba *Dictyostelium discoideum*, the cathepsin L-like cysteine proteases have been shown to have low activity in the dormant spore, a slowly increasing activity with progression of spore germination, highest activity during growth phase, and a decreasing activity with progression of the developmental life cycle (North *et al*, 1996; Cotter *et al*, 1997; Cavallo *et al*, 1999).

To elucidate whether the cysteine proteases had a direct involvement when the endogenous activity was at its peak (growth phase), the cysteine protease inhibitor E-64D was utilized to inhibit the endogenously active cysteine proteases. The data presented in Figures 18 and 19 indicated that the cells in growth phase in axenic media had a doubling time of approximately 14 hours in the presence or absence of

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the cysteine protease inhibitor, suggesting that the endogenously active cysteine proteases have no direct involvement in axenic growth.

It was hypothesized some time ago that the cysteine proteases of *Dictyostelium* existed exclusively for the vegetative cells to progress properly through the developmental life cycle (Fong and Bonner, 1979). The data presented in Figures 20-22, however, show that when the cells of *Dictyostelium* that were in the developmental life cycle were treated with E-64D, the cells were capable of forming fruiting bodies. This data set is in conflict with the previously reported observation. It is felt that this data set is more convincing, however, and that the conflicting results that had been previously reported were due to the fact that the research group had used two broad range anti-malarial drugs, chloroquine and quinone, as their cysteine protease inhibitors (Fong and Bonner, 1979; MacFarlane and Manzel, 1998; Hahn, 1974; Chou et al, 1974). It is quite reasonable to assume then that arrests in the developmental life cycle of *Dictyostelium* would be anticipated if broad spectrum drugs such as the ones used previously were administered.

Not only does this data set confirm that the endogenously active cysteine proteases of *Dictyostelium* have no direct involvement in axenic growth or development, but it was mentioned earlier that they also have no direct involvement in spontaneous spore germination. It is quite possible that the aspartyl proteases of *Dictyostelium* may have compensated for the loss of enzymatic activity from the cysteine proteases. It has been reported previously that molecular knockout of the aspartyl protease cathepsin D in *Dictyostelium* has no effect in the organisms ability to form fruiting bodies and grow in axenic media (Journet *et al*, 1999). It was

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hypothesized in this previous report that the cysteine proteases of *Dictyostelium* may have compensated for the loss of aspartyl cathepsin D (Journet *et al*, 1999). The data presented here may be the complement to the results generated previously with aspartyl cathepsin D.

The acid activatable proteases of *Acanthamoeba castellanii*

It was mentioned earlier that in most eukaryotic cells, the proteases of the cysteine class are activated upon cleavage of a pro-region (North *et al*, 1996; Mason *et al*, 1987 and 1989; McDonald *et al*, 1988; Mach *et al*, 1994). In *D. discoideum*, it has been shown that cleavage of a pro-region is not sufficient for the activation of the cathepsin L like cysteine proteases (North *et al*, 1996). Rather, conformational change, which is induced by an acidic environment, is needed to activate the cysteine proteases in *Dictyostelium* (North *et al*, 1996). This can be observed in the laboratory by treating an SDS-PAGE zymogram containing the cysteine proteases of *D. discoideum* with acetic acid (North *et al*, 1996; Cotter *et al*, 1997; Cavallo *et al*, 1999).

The cysteine proteases from the microcysts of *Acanthamoeba castellanii* were investigated to determine how similar they were to those of *D. discoideum*. When the protein extracts of the microcysts from *A. castellani* were separated on SDS-PAGE zymograms, it was observed that there were proteases of approximately 40 and 60 kDa. There was also a block of unresolvable enzymes in the range of 116-160 kDa. (Figure 27A). The 40 kDa protease was only clearly visible when at least 7.2 μg of protein was electrophoretically separated on the SDS-PAGE zymogram. All the

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for enzymatic activity. As a result of this less than ideal environment, certain proteases may have been present on the gels, but were just not active, and so could not be visualized. Further studies would need to be conducted in which the microcysts of *A. castellanii* would be incubated at temperatures of 30-55°C to observe the appearance of other proteolytic enzymes on an SDS-PAGE zymogram. More than likely, there would be a greater amount of activity on these zymograms than the ones observed in this data set. In addition, it is thought that there would be more inhibition with the various inhibitors used, especially E-64 and PMSF (serine protease inhibitor), since the most abundant proteases found in *Acanthamoeba* species are of the serine and cysteine class (Mitro *et al.*, 1994). However, the results that were observed may again be explained by the fact that the microcysts of *A. castellanii* were not incubated at their optimal temperature.

These results suggest that the acid activatability of cysteine proteases is not restricted to the slime molds on the phylogenetic tree of life in Figure 2 (Woese, 1998). Acid activatable characteristics in the proteases of *A. castellanii* would seem to make sense in light of the fact of how close the Entamoeba lineage and the slime mold lineage are to each other. Furthermore, it has been observed that there are acid activatable cysteine proteases in *Tetrahymena pyriformis*, which belongs to the Ciliate lineage on the phylogenetic tree of life (Gale and Cotter, unpublished results; Woese, 1998).

In conclusion, the acid activatable characteristics of proteolytic enzymes is a common trait not only of the cellular slime mold, *D. discoideum*, but of the free-living amoeba, *A. castellanii*.

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