Differential activity and secretion of multiple proteinases in vegetative amoebae and in germinating spores of the cellular slime mold Dictyostelium discoideum.

Karl J. Franek

University of Windsor

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DIFFERENTIAL ACTIVITY AND SECRETION OF MULTIPLE PROTEINASES
IN VEGETATIVE AMOEBAE AND IN GERMINATING SPORES
OF THE CELLULAR SLIME MOLD DICTYOSTELIUM DISCOIDEUM

by

Karl J. Franek

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

Windsor, Ontario, Canada
1991
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ABSTRACT

by

Karl J. Franek

Lysosomal enzymes in the cellular slime mold *Dictyostelium discoideum* are differentially secreted during growth and starvation. Axenic amoebeae linearly secreted over 30% of the total acid phosphatase activity during starvation in phosphate buffer. Addition of sucrose (a nonmetabolizable disaccharide) stimulated secretion of acid phosphatase; over 70% of the total enzyme activity was rapidly released. Moreover, the secretion kinetics of the enzyme activity shifted to a sigmoidal pattern resembling the complex secretion kinetics of other lysosomal enzymes (β-glucosidase, N-acetylglucosaminidase, α-mannosidase). In an extension of these findings proteinase secretion was examined during starvation using three peptide-nitroanilide substrates N-benzyol-L-prolyl-L-phenylalanyl-L-arginine 4-nitroanilide (BzPFR), N-carbobenzoxy-L-arginyln-L-arginine 4-nitroanilide (ZRR), and N-carbobenzoxy-L-tyrosyl-L-lysyl-L-arginine 4-nitroanilide (ZYKR), the former two substrates being specific to cysteine proteinases. The hydrolytic activities toward BzPFR, ZRR, and ZYKR were designated BzPFRase, ZRRase, and ZYKRase, respectively. Starved amoebeae linearly secreted large amounts of BzPFRase activity. Sucrose stimulated and altered the secretion of BzPFRase activity; over 80% of the total activity was released with sigmoidal kinetics. Analysis using gelatin sodium dodecyl sulfate polyacrylamide electrophoresis (gelatin-SDS-PAGE) revealed a major 42 kilodalton (kDa) *Dictyostelium discoideum* cysteine proteinase, ddCP42, which corresponded to BzPFRase activity.
Proteinase activity was also examined in spores and during spore germination under various activation treatments. Dormant spores contained a major 58 kDa aspartic proteinase, designated ddAP58. Matrix material contained a novel 18 kDa cysteine proteinase, named ddCP18. During spore germination a decrease in intracellular ddAP58 activity heralded the appearance of two new cysteine proteinase activities, designated ddCP43 and ddCP48. Increases in BzPFRase and ZYKRase activities were also observed during emergence of myxamoebae. The use of different spore activation treatments, which altered the timing of events during germination, revealed that ZYKRase activity could be uncoupled from emergence; ZYKRase activity may be more closely associated with the late spore swelling stage. The differential proteinase activities and secretion characteristics observed in this study may reflect different lysosomal enzyme targeting mechanisms, and differences in physiological functions, among proteinases and other lysosomal enzymes.
DEDICATION

To my parents, Mildred and Sidney Franek.
ACKNOWLEDGEMENTS

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ABREVIATIONS

Arg  arginine
Asp  aspartic acid
APS ammonium persulfate
BzPFR N-benzoyl-L-prolyl-L-phenylalanyl-L-arginine
        4-nitroanilide
BzPFRase enzyme which hydrolyses BzPFR
cAMP cyclic adenosine monophosphate
Cys cysteine
ddCPXX Dictyostelium discoideum cysteine
         proteinase of XX kDa molecular weight
ddH₂O double distilled water
DMSO dimethyl sulfoxide
DTT dithiothreitol
E-64 trans-ε-(γ-succinyl-L-leucylamido)
        (4-guanidino)-butane
EDTA ethylenediaminetetraacetic acid
ER endoplasmic reticulum
gelatin-SDS-PAGE SDS-PAGE using gelatin copolymerized
         with acrylamide
His histidine
hsp heat shock protein
MeOH methanol
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</tr>
<tr>
<td>NAP</td>
<td>naphtylamide</td>
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<tr>
<td>NEM</td>
<td>N-ethylmaleimide</td>
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<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PB</td>
<td>10 mM phosphate buffer, pH 6.5</td>
</tr>
<tr>
<td>Phe</td>
<td>phenylalanine</td>
</tr>
<tr>
<td>PHMB</td>
<td>p-hydroximercuribenzoate</td>
</tr>
<tr>
<td>PNPP</td>
<td>p-nitrophenylphosphate</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
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<td>SDS</td>
<td>sodium dodecyl sulfate</td>
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<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
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<tr>
<td>TEMED</td>
<td>N,N,N',N'-tetramethylethylenediamine</td>
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<td>L-tosyl-amido-2-phenylethyl chloromethyl ketone</td>
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<td>UDP-GP</td>
<td>uridine diphosphate glucose phosphorylase</td>
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<td>enzyme which hydrolyses ZRR</td>
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<td>ZYKR</td>
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INTRODUCTION

The Dictyostelium Lifestyle

The cellular slime mold *Dictyostelium discoideum* (Order: Acrasiales, Family: Dictyosteliaceae) is a soil amoeba inhabiting decaying leaf matter in hardwood forests, and was first described by K.B. Raper (1935). *Dictyostelium* occupies a unique taxonomic niche: botanists and mycologists claim slime molds for the kingdom of Fungi while zoologists classify them as Protists. Mycologists first studied slime molds because they were found on the same substrates as fungi and produced aerial fruiting structures much like other fungi. The amoeboïd nature and the "animal-like" behaviour of the vegetative cells interested protozoologists. Regardless of its formal classification, *Dictyostelium*, and its close cousin *Polysphondylium*, have become popular in laboratories devoted to developmental biology, morphogenesis, genetics, cell to cell interactions and signalling, phagocytosis, and many other biochemical and molecular biological endeavors (see Loomis, 1975; Raper, 1984).

*Dictyostelium discoideum* has an interesting life cycle which includes a vegetative growth phase, and a developmental phase featuring sorocarp formation (Fig. 1). Vegetative myxamoebae of *D. discoideum* are phagocytic, feeding on common soil bacteria, and multiplying by binary fission. This growth continues until the food supply is depleted after which the organism commences morphological differentiation. The developmental journey begins with aggregation of individual myxamoebae to form migratory multicellular pseudoplasmodia. Culmination follows resulting in fruiting body and spore formation. Under favourable environmental conditions spores germinate releasing myxamoebae thus completing the life cycle. The behavioural change in the
Figure 1.
The life cycle of *Dictyostelium discoideum*.
Figure 1.

- Spores
- Stalk
- Basal disk
- Mature fruiting body
- Spore
- Germination
- Amoeba
- Multiplication
- Aggregation
- Prestalk cells
- Prespore cells
- Migrating slug

TImes:
- 0 h
- 6 h
- 10 h
- 12 h
- 14 h
- 16 h
- 17 h
- 19 h
- 20 h
- 24 h
lifestyle of *Dictyostelium* ensures survival of the organism during starvation and in harsh conditions such as extremes of heat or cold, and desiccation (see Loomis, 1975, for review).

In the laboratory vegetative amoebae of *D. discoideum* can be cultivated on solid, or on liquid media in association with live or heat-killed bacteria, typically *Escherichia coli* Bf or *Klebsiella aerogenes*. Mutant strains have been found which may be grown axenically in defined liquid medium. Upon depletion of the food source a group of centrally-located founder cells begins to secrete pulses of "acrasin", a chemoattractant identified as adenosine-3',5'-monophosphate (cAMP) (Konijn et al., 1968; Barkley, 1969). The surrounding myxamoebae are stimulated to similarly produce and secrete cAMP in a pulsatile manner. Chemotactic movement of the responding amoebae occurs in concentric waves with a periodicity of 5 minutes, and results in the streaming of cells toward the founder cells (Gerisch, 1968; Robertson et al., 1971). The aggregates of approximately $10^4$ cells form a pseudoplasmodium (slug or grex) which migrates horizontally along the substratum in search of an appropriate food source. However, unlike true slime molds (Myxomycetes), such as *Physarum* and *Didymium*, the myxamoebae of the cellular slime mold retain their plasma membranes and thus their individual cellular identity. The cells continually secrete a mucopolysaccharide substance which forms a slimy sheath around the slug; the trail of the slime remains in the wake of the migrating grex as the amoebae coordinately move through the sheath. In addition to chemotaxis, the pseudoplasmodium exhibits sensitivity to light and heat (Bonner, 1950). Once the migrating slug encounters bacteria the structure dissociates into individual cells.
If no food is found migration ceases and culmination, the subsequent stage in differentiation, begins. Culmination is induced by factors which inhibit migration; lower humidity, higher temperature, and overhead light (Raper, 1940; Newell et al., 1969). The amoebae at the anterior tip of the slug (about 15%) differentiate into pre-stalk cells while those at the posterior end are committed to become dormant spores. The slug rounds up and the anterior tip begins to flow downward through the culminating mass to the surface of the substratum. Stalk cells secrete cellulose, and lay down rigid cell walls; they then expand and vacuolate and, in effect, die. The downward movement of the column of stalk cells lifts developing pre-spore cells vertically off the substratum. Bonner (1967) likens this process to a fountain running backward. Cells located at the periphery of the rising cell mass which have not entered the stalk will encapsulate and differentiate into dormant spores (see Raper & Fennel, 1952). A small number of cells forms a basal "disk" which adjoins the stalk and the substratum (this structure lends itself to the species name discoideum). The sorus topples to the surface, releasing spores which may germinate giving rise to vegetative amoebae (for review see Loomis, 1975; Newell, 1971, 1978).

Fruiting Bodies of Dictyostelium

The sorus is comprised of spores surrounded by a viscous matrix material which consists of complex carbohydrates, trehalose (Ceccarini & Filosa, 1965), hydrolytic enzymes, such as carbohydrases and proteinases (Chan et al., 1981), and discadenine, an autoinhibitor of spore germination (Abe et al., 1976). Some extracellular matrix proteins may aid in formation of the spore and stalk cells (Hohl & Hamamoto, 1969). The spores of D. discoideum are capsule-shaped structures 6 to 9 μm long and 2.5 to
3.5 μm in diameter (Raper, 1935; Bonner, 1967). Dormant spores are typically resistant to unfavourable conditions such as desiccation, freezing and heat which may adversely affect vegetative cells (Raper & Cotter, 1968c). The mature spore wall is at least 200 nm thick and consists of three discrete layers. An outermost polysaccharide sheath (30 nm thick) surrounds a middle layer (150 nm) consisting of two sheets of cellulose fibrils arranged in perpendicular orientation. The innermost layer (30 nm) is composed of cellulose and protein (Cotter et al., 1969; Hemmes et al., 1972; Hohl & Hamamoto, 1969). The spore wall is in part formed by "prespore vesicles", heavy walled organelles which fuse with the plasma membrane and donate their contents to the developing wall (Maeda & Takeuchi, 1969). Prespore vesicles appear in late aggregation in the posterior (prespore) region of the pseudoplasmodium (Maeda & Takeuchi, 1969; Hohl & Hamamoto, 1969; Gregg & Badman, 1970) and persist through to encapsulation.

**Spore Germination**

The dormant wild type NC4 spore germinates to release a myxamoeba capable of vegetative growth. *Dictyostelium* spore germination may be divided into four morphologically distinct stages: i) activation, ii) post-activation lag, iii) swelling, and iv) emergence (Cotter, 1975). Spore dormancy is constitutive and thus physical or chemical treatments are required to activate spore germination (Cotter & Raper, 1966; Sussman, 1976). These are varied and may include incubation in 1% peptone (Cotter & Raper, 1966), 8 M urea (Cotter & O'Connell, 1976), 2 M dimethyl urea, 2 M tetramethyl urea, 6 M guanidine HCl (Cotter, 1979), 3 M ethylene glycol (Cotter, 1977), 20% dimethyl sulfoxide (DMSO) (Cotter et al., 1976), gamma irradiation (Hashimoto & Yanagisawa, 1970), and a controlled heat shock at 45°C (Cotter &
Raper, 1966, 1968a,b). Most of the agents which activate spores commonly have
denaturing effects upon proteins, or in the least may promote conformational shifts in
hydrophobic side chains of polypeptides (Cotter, 1975). Aged spores of the wild type
NC4 germinate spontaneously and release an autoactivator substance during the process
(Dahlberg & Cotter, 1978a). When partially-purified autoactivator is added to a
population of mature dormant NC4 spores, germination ensues (Glaves & Cotter, 1989).
A spontaneous germination mutant, SG1, derived from NC4, has been described by
Cotter & Dahlberg (1977). Young mature SG1 spores have the ability to autoactivate
and require no external activation treatments.

Vegetative Growth of Dictyostelium discoideum

Myxamoebae of the wild type Dictyostelium discoideum are grown in the
laboratory on a bacterial food source, typically Escherichia coli B/r or
Klebsiella aerogenes. Live or heat-killed suspensions of the bacteria sustain the growth
of myxamoebae in liquid culture (Raper, 1939) but it is often more convenient to grow
myxamoebae in association with bacteria on a solid medium such as "SM"
(Sussman, 1966). Rapid bacterial growth results in lawn formation upon which the
myxamoebae feed, and continue to grow until the lawn is cleared. The vegetative cells
may then be washed from the agar surface and isolated from the bacteria by differential
centrifugation (Loomis, 1975).

While wild type NC4 myxamoebae cannot be grown successfully on a defined
medium in the absence of bacteria, a number of mutant strains have been isolated which
may be cultured in very rich nutrient liquid medium. The axenic strains Ax1
A-3 (Loomis, 1971; Dimond et al., 1973; Firtel & Bonner, 1972a,b; Rossomando & Sussman, 1973) have been comprehensively characterized, and all thrive quite well in buffered media containing protease peptone, yeast extract, and glucose. No significant differences in morphological and growth characteristics among the strains of axenically grown myxamoebae are apparent, and all grow exponentially with a doubling time of 8 to 10 hours (Loomis, 1975). A mutant, strain M31, derived from Ax3, carries a recessive mutation, mod A, which results in a lack of α-1,3-glucosidase. The mutant is blocked in the addition of the two outermost glucose moieties to the putative mannose core which is N-linked to an asparagine residue of the nascent polypeptide. Subsequent processing steps, such as phosphorylation and sulfation are also blocked (Freeze et al., 1983; Free et al., 1978).

**Endomembrane System: Intracellular Protein Traffic**

The eukaryotic cell has a high degree of internal ultrastructural organization. The intracellular space is pervaded by an internal, or "endomembrane", system which includes the nuclear envelope, the endoplasmic reticulum (ER), and the Golgi complex. This membrane network of lumina, tubules and cisternae effectively partitions the cell into major "macro environments", chiefly the cytosol and the lumen of the endomembrane system. Different regions of the Golgi, as well as offshoots of these systems, such as peroxisomes, lysosomes, and secretory granules, represent "micro environments" where enzymes having associated functions and requiring similar physical conditions (pH, oxidation-reduction states) reside. The ER plays an essential role in protein biosynthesis and in the subsequent transport of proteins to various cellular destinations including the cytosol, the cell surface, lysosomes, secretory vesicles, and
other organelles (mitochondria, chloroplasts, nucleus). As well, some of the proteins managed by the ER remain there and in the Golgi stacks to perform maintenance and processing functions (Griffiths & Simons, 1986; Pfeffer & Rothman, 1987). Nascent polypeptides synthesized on ER membrane-bound ribosomes are directed into the lumen of the ER by signal peptides inherent to their structure. Generally proteins synthesized in the ER are glycoproteins; high mannose-containing polysaccharides (conjugated to dolichol phosphate carriers) are cotranslationally N-linked to asparagine residues of the growing polypeptide chain. Additional modifications to the oligosaccharides may follow in the ER and in the Golgi stacks (for review see Lennarz, 1987). These secondary modifications may be sorting signals which target certain proteins to their destinations. Bulk flow of proteins through the ER and Golgi is signal-independent whereas specific retention of proteins in the ER, cis, medial or trans Golgi, and lysosomal and secretory vesicles is signal-mediated (Pfeffer & Rothman, 1987). The signals which endorse this retention are in the form of "signal patches", as described by Pfeffer & Rothman (1987), formed by the juxtaposition of noncontiguous regions on the same polypeptide during protein folding. Unlike signal peptides whose functions are often maintained when transferred to different proteins through molecular manipulation, signal patches are surface characteristics dependent upon protein conformation. The study of these features is encumbered by their vulnerability to genetic variation which may affect conformation; a change in three dimensional structure may perturb signal patches.
Lysosomal Protein Targeting

The signal patches on the surface of glycoproteins appear to be responsible for directing sorting in the Golgi en route to lysosomes. Membrane-bound mannose-6-phosphate (M-6-P) receptors in the Golgi sacs and the shuttling vesicles (primary lysosomes) have been implicated in lysosomal hydrolase sorting in mammalian systems. Two species of M-6-P receptor, 215 kD and 46 kD molecular weight are present intracellularly and at the cell surface; the surface receptors are believed to play a role in recycling hydrolases which have been secreted. The 215 kD species is cation-independent while the 46 kD receptor is dependent upon a cation (Mg²⁺) species for binding ability. The significance of this feature, or the differential size of the two receptors is not known. The current provisional model of M-6-P receptor functioning is such: Lysosomal hydrolases bearing oligosaccharides decorated with M-6-P are transported by bulk flow to the Golgi where they encounter M-6-P receptors and become closely associated with them. The receptors bind the enzymes and sequester them in transport vesicles which bud from the trans face of the Golgi and fuse with lysosomal (or pre-lysosomal) compartments. The affinity of the 215 kD receptor for M-6-P is diminished by low pH and the acidic environment characteristic of the lysosomal compartment promotes the dissociation of the receptor-hydrolase complex. The enzyme is thus deposited in the lysosomal space where it may carry out its degradative function, while the M-6-P receptors are recycled back to the Golgi apparatus in vesicles or small tubules (for review see Hoiman, 1989).
**Dictyostelium Lysosomal Enzymes**

*Dictyostelium* shares with many other lower eukaryotes the ability to phagocytose complex exogenous food sources, and possesses a well-developed lysosomal system involved in phagocytosis and digestion (deChastellier & Riter, 1977). The *Dictyostelium* lysosomal system is of particular interest to those who study lysosomal protein targeting. The organism has no detectable mannose-6-phosphate receptors (Cardelli *et al.*, 1987) and, because it can be easily manipulated both genetically and biochemically, it presents a novel system to investigate alternate lysosomal enzyme processing and targeting pathways. Although a number of modifications may be involved in some way in targeting the retention of glucose residues, the absence of Man-6-PO₄, a reduction in phosphorylation, or the size of N-linked oligosaccharide sidechains did not affect the proteolytic processing and targeting of the lysosomal enzymes α-mannosidase, β-glucosidase, or acid phosphatase (Ebert *et al.*, 1989; Bush & Cardelli, 1990; Cardelli *et al.*, 1990; Freeze *et al.*, 1989). An inability to achieve proper folding may, however, affect the rate at which these enzymes are transported from the ER to the lysosomes (Woychik *et al.*, 1986). Alternate modifications, such as phosphorylation may reveal information about targeting (for review see Cardelli *et al.*, 1993b).

Lysosomal enzymes are known to be regulated during *Dictyostelium* development (Loomis, 1969; Coston & Loomis, 1969; Dimond *et al.* 1976; Dimond & Loomis, 1976) and this feature as well as the ability to construct various structural gene mutants (Free & Loomis, 1974; Dimond & Loomis, 1976) makes this organism ideal for the study of protein targeting and secretion. The known lysosomal enzymes which have received much attention include acid phosphatases (Parish, 1976), β-galactosidase
(Dimond et al., 1976); α-glucosidase (Every & Ashworth, 1973); β-glucosidase (Coston & Loomis, 1969); N-acetyl-β-D-glucosaminidase (Loomis, 1969; Dimond & Loomis, 1974), α-mannosidase (Loomis, 1971); proteases, RNases, DNases and amylase (Weiner & Ashworth, 1970).

Although these enzymes are typically sequestered in lysosomes where they perform degradative functions, lysosomal hydrolases may alternatively be secreted into the extracellular milieu. Myxamoebae in normal growth conditions, or during axenic growth, secrete significant levels of lysosomal enzymes (Ashworth & Quance, 1972; Every & Ashworth, 1973). The food source available to myxamoebae appears to regulate lysosomal enzyme release as cells grown axenically secrete enzymes more efficiently than those grown on bacteria (Dimond et al., 1983). Amoebae of Dictyostelium discoideum suspended in starvation buffer with a metabolizable sugar (glucose) fail to synthesize a number of components which serve as markers for early stages of development (Rahmsdorf et al., 1976); the presence of the nutrient appears to postpone development. Similarly, vegetative cells susupended in starvation buffer supplemented with yeast extract and proteose peptone secreted less glycosidase activity than cells incubated in starvation buffer alone. Earlier, Marin (1976, 1977) suggested that starvation, particularly for amino acids, induced aggregation and multicellular development. It may be that secretion of glycosidases, and lysosomal enzymes in general, is an initial response to nutrient depletion.
Secretion of Lysosomal Enzymes in *Dictyostelium*

In order to better study the secretion process in *Dictyostelium discoideum*, Dimond *et al.* (1981) developed standard secretion conditions which imposed starvation upon vegetative cells. Myxamoebae in late exponential phase of growth in nutrient broth were washed free of the medium and resuspended in dilute potassium phosphate buffer. These conditions appear to mimic the later vegetative stages prior to development. It was discovered that during starvation lysosomal enzymes were secreted at rates comparable to, or above, those observed in cells undergoing normal growth and development. Lysosomal enzyme synthesis and degradation appears to cease under starvation conditions and thus members of this class of enzymes are considered to be markers for the vesicles (lysosomal, secretory) in which they are contained. Secretion of lysosomal enzymes is an energy-requiring process; it is inhibited by both cyanide (Dimond *et al.*, 1981) and azide (Crean & Rossomando, 1979). The presence of supplemental amino acids in the external environment inhibits lysosomal enzyme secretion. This suggests that the process is regulated and is not merely a passive function. Equally significant is that lysosomal enzyme release is a separate event from the perfunctory egestion of phagolysosomal contents as distinguished by latex bead ingestion studies (Dimond *et al.*, 1981).

Lysosomal enzymes may be segregated into three functional classes according to their respective secretion kinetics under standard secretion conditions. The first class of enzymes is secreted very efficiently with more than 50% of the total cellular activities detected extracellularly after 6 hours of incubation. All the enzymes of this class exhibit similar or identical secretion kinetics which include a short initial lag, a quick release of the enzyme followed by retardation and the halt of secretion. The resulting pattern of
secretion can be described as sigmoidal. This class includes the glycosidases N-acetylglucosaminidase (NAG), α-mannosidase, β-glucosidase-1, β-galactosidase-1, and α-glucosidase-1. Secretion of these enzymes is not sensitive to the protein synthesis inhibitor cycloheximide.

The second class of enzymes, represented by acid phosphatase, is secreted with linear kinetics, with approximately 30% of the total cellular enzyme activity being released. Secretion of this class is further distinguished by sensitivity to cycloheximide and chloroquine diphosphate. Chloroquine is a lysosomotropic agent, commonly used as an antimalarial drug, which accumulates in lysosomes and raises the pH of the compartment toward neutrality (pH 6-6.5). This, in effect, promotes influx of water to lysosomes and other compartments and is believed to interfere with lysosome and lysosomal enzyme function (Holtzman, 1989).

The third class of lysosomal enzymes includes β-galactosidase-2 and is non-secretory, as very little enzyme is detected extracellularly.

Dimond et al. (1981) account for this differential secretion of lysosomal enzymes by hypothesizing the existence of different multiple lysosomal vesicles, both secretory and non-secretory. Different functional types of enzymes may be targeted to different lysosomes (viz. glycosidases and acid phosphatase).

**Alteration of Lysosomal Enzyme Secretion**

The secretion of lysosomal glycosidases observed in starving cells (Crean & Rosomando, 1977) is enhanced in the presence of non-metabolizable sugars in the starvation buffer (Crean & Rosomando, 1979). A variety of sugars were found to prompt secretion of at least 90% of the total cellular NAG activity including mono-, di-, 
tri-, and tetrasaccharides (galactose, sucrose, raffinose, and stachyose), and sugar alcohols such as mannitol and inositol. Non-metabolizable sugars were taken up poorly by the cell but readily stimulated secretion suggesting that glycosidase secretion was not correlated with sugar uptake. Addition of glucose to the starvation buffer had no stimulative effect upon secretion while maltose and glycerol increased secretion slightly (Crean & Rossomando, 1979).

Along with the stimulation of NAG activity by non-metabolizable sugars the pattern of secretion over time was altered compared to secretion in starvation buffer alone. Under control conditions NAG was secreted with near linear kinetics reaching maximum levels quite early. In the presence of 0.1 M sucrose rapid secretion of NAG occurred after a short initial lag; the resulting kinetics of secretion were "sigmoidal". Extracellular levels of the enzyme were six-fold higher than in the control situation.

The glycosidase trehalase is a lysosomal enzyme in Dictyostelium which is secreted with kinetics similar to those observed for the secretion of β-glucosidase under starvation conditions. There is a short initial lag in enzyme secretion after which release into the external medium becomes more rapid, and finally decreases to a halt. Trehalase secretion does not depend heavily upon protein synthesis but is an energy-dependent process as shown by inhibitor studies using cycloheximide and sodium azide (Seshadri et al., 1986). Non-metabolizable disaccharides such as 0.1 M sucrose and 0.1 M lactose stimulate trehalase secretion and elicit a shift in the kinetics of enzyme release to a sigmoidal pattern as classified by Dimond et al. (1981).
Characteristics of Eukaryotic Proteinases

Cellular proteolysis is highly regulated in eukaryotes and impacts upon essential cellular functions, including digestion of nutrient protein, limited proteolysis in the regulation of zymogens, and turnover of cellular protein. Proteases, or peptidases, the enzymes which degrade proteins by hydrolysing peptide bonds, are found virtually in all compartments of the cell. Internal peptide bonds are hydrolysed by endopeptidases (proteinases) while bonds situated near either N- or C-terminus of the polypeptide are recognized by exopeptidases (Barrett & McDonald, 1986).

Proteinases vary widely according to their function and mode of action. These enzymes can be generally grouped in one of four classes: i) aspartic proteinases (EC 3.4.23), ii) metalloproteinases (EC 3.4.24), iii) serine proteinases (EC 3.4.21) and iv) cysteine proteinases (EC 3.4.22). As their names imply aspartic, serine and cysteine proteinases require one or more aspartic, serine or cysteine residue respectively at or near the active site. Metalloproteinases function best in the presence of certain metals (Ca²⁺, Mg²⁺, Mn²⁺, Fe³⁺, Cu²⁺, Cd²⁺, Zn²⁺). The proteinases studied to date range in size from approximately 20,000 to 800,000 daltons. Although some lysosomal proteinases are smaller monomeric units, many larger proteinases are multimeric enzymes comprised of subunits of 50-100 kDa (for review see Bond & Butler, 1987).

Lysosomal Proteinases

Proteinases may be compartmentalized in subcellular locations to optimize enzyme activity and to facilitate enzyme-substrate association, and to quarantine rogue hydrolytic enzymes with the potential to indiscriminately degrade proteins. Lysosomes
sequester proteinases in a membrane-bound acidic environment where the enzymes
degrade nutrient proteins and proteins from other subcellular locations.

The lysosomal proteinases, also known as cathepsins, are typically of the aspartic
and cysteine class and have been well characterized in mammals. They are generally
small enzymes (20 - 40 kDa), optimally active at acidic pH values and unstable at neutral
and alkaline pH values. Like other lysosomal enzymes, cathepsins are commonly
glycoproteins (see Bond & Butler, 1987). Cathepsins B, H, L, S, and D have been the
most widely studied. Cathepsin B exhibits trypsin-like substrate specificity and is
generally active against synthetic substrate containing arginine in the P1 position
(Bz-Arg-Nap, Bz-Arg-Arg-NMec). A relatively small molecule, approximately
25,000 Da, it has an optimum pH value of 5. Cathepsin B is believed to perform a
processing function against proinsulin in mammals based upon its specificity toward
synthetic substrates.

Cysteine Proteinases

Cysteine proteinases, or "thiol" proteinases, demonstrate general proteolytic
activity and are involved in initial and terminal stages of extensive protein degradation.
They are found in the cytosol and in lysosomes. There are several related families of
cysteine proteinases, the "papain" superfamily being the most notable in eukaryotes.
Papain, an enzyme isolated from papaya, is a single protein 23,400 Da in molecular
weight. Extensive amino acid sequence homology between papain and cathepsins B, H,
L and S, and the conservation of crucial sequences surrounding the active site Cys-25 and
His-159 suggests that members of the superfamily have evolved from a common ancestor
(Barrett, 1986).
Mechanism of Cysteine Proteinase Action

A number of X-ray crystallographic and chemical modification studies of serine proteinases have helped clarify the mode of action of cysteine proteinases. Exploration of the active site structures and the substrate binding pockets of chymotrypsin, trypsin and elastase has revealed the involvement of acyl-enzyme and tetrahedral intermediates (Hess, 1971; see also Price & Stevens, 1989; Darnell et al., 1990). The cysteine proteinase papain possesses a large groove wherein lies the thiol group of Cys-25 close to the imidazole ring of His-159 (Drenth et al., 1976). The SH-group of Cys-25 acts as a nucleophile which attacks the carbonyl group of the substrate peptide bond. A charge relay assisted by the imidazole group of His-159, and possibly by Asp-158, results in the formation of a tetrahedral intermediate (Polgar & Halasz, 1982). As the substrate peptide bond is cleaved and the modified substrate removed an acyl-enzyme intermediate, a thiolester, is produced. By the addition of water and the creation of another tetrahedral intermediate involving Cys-25 and the product, the latter is released and the catalysis is complete (Darnell et al., 1990; Price & Stevens, 1989).

Proteinases are often distinguished according to their sensitivity to inhibitors and activating agents. Cysteine proteinases are inhibited by p-hydroxymercuribenzoate (pHMB) and alkylating agents such as iodoacetate, iodoacetamide and N-ethylmaleimide (NEM) which interfere in the formation of the acyl-enzyme intermediate (Bond & Butler, 1987; Polgar & Halasz, 1982). The site-specific cysteine reagent E-64 reacts with the active site cysteine residues to form thioethers (see Katunuma et al., 1983). Peptidyl diazomethyl ketones (Z-Phe-Phe-CHN₂ for eg.) are quite effective in blocking cysteine proteinases when designed to interact specifically (Green & Shaw, 1981). Some microbial peptides such as leupeptin,
antipain, chymostatin and elastinal form hemiacetal and hemithioacetals with the active-
site cysteine interfering in association with the substrate. These inhibitory compounds
may be active against other types of proteases and sensitivity to them is not necessarily
indicative of cysteine proteinases. Cells may also produce inhibitors to regulate
proteinase activity. The cytosolic polypeptide inhibitors cystatins (reviewed in
Barrett, 1987), stefins, kininogens and calpastatin are believed to block proteinases which
may have escaped the lysosome (Barrett et al., 1986; Murachi, 1983).

Lysosomal cysteine proteinases reside in acidic and highly reducing
environments. Reducing agents such as dithiothreitol, cysteine and 2-mercaptoethanol
have been commonly used to stimulate the activity of cysteine proteinases. In addition,
certain metals (Ca\(^{2+}\), Mg\(^{2+}\)) may repress activity and the presence of EDTA generally
encourages cysteine proteinase activity (Bond & Butler, 1987).

**Cysteine Proteinases in Dictyostelium discoideum**

Proteinase activity in *Dictyostelium discoideum* was first reported by Sussman
and Sussman (1969). A number of acid proteinases, with optimal activities at pH 2, were
found to be lysosomal (Weiner & Ashworth, 1970) as well as enzymes displaying
activities similar to the mammalian proteinases, cathepsin D and cathepsin B
(Fong & Bonner, 1979; Fong & Rutherford, 1978). The cryptic nature of the latter two
enzymes in *Dictyostelium* suggested that they too were lysosomal (North, 1982b).

Upon isolation and purification of two of the acid proteinases direct relationships
between proteinases of *Dictyostelium* and other known forms were established.
Proteinase E, an enzyme of approximately 53,000 Da, performs optimally at a pH of 5
(North & Harwood, 1979). Inhibitor studies indicated that proteinase E is an aspartic
proteinase and probably contributes to the cathepsin D-like activity in the cells (North, 1982a).

A very prominent enzyme, proteinase I, was isolated and characterized by Gustafson and coworkers (Gustafson & Thon, 1979; Gustafson & Milner, 1980b); it had a pH optimum value of 4 and was comprised of three heterogeneous subunits. The sensitivity of proteinase I to iodoacetimide, cystamine and TLCK, as well as its activation by DTT was representative of cysteine proteinases with cathepsin B-like activity (Fong & Rutherford, 1978). Moreover, N-acetylglucosamine-1-phosphate residues have been found in proteinase I; these are antigenic moieties common to lysosomal enzymes such as β-N-acetylglucosaminidase (Gustafson & Milner, 1980a; see also Knecht & Dimond, 1981). An additional enzyme, proteinase B, has been purified and extensively characterized (North & Whyte, 1984) and may be identical to proteinase I.

Role of Cysteine Proteinases in Dictyostelium

Of the acid proteinases studied in Dictyostelium the cysteine proteinases provide the most interesting information. Unlike aspartic proteinases which are present at relatively constant levels throughout the Dictyostelium life cycle, net cysteine proteinase activity is highest during the growth phase and decreases prior to development. In view of this, vegetative amoebae likely employ cysteine proteinases in the degradation of nutrient protein, coactively with aspartic proteinases (North, 1985). During starvation, and at the onset of development when cells depend upon endogenous nutrients for metabolic precursors and energy, complete intracellular protein degradation results (Hames & Ashworth, 1974). Studies on the inhibition of protein breakdown in
*Dictyostelium* provide evidence that not only are general proteolysis leading to free amino acid pools and the continuation of development linked, but that cysteine proteinases are most certainly involved in this process (Fong & Bonner, 1979; North, 1985). In addition, cysteine proteinase I-like enzymes have been implicated in the activation of UDP-glucose phosphorylase *in vitro* (Gustafson & Thon, 1979) and in the regulation of intracellular UDP-GP levels *in vivo* (DeToma *et al.*, 1979). In spite of this evidence cysteine proteinases are not renowned as major regulatory enzymes in *Dictyostelium*.

The multiple cysteine proteinases in *Dictyostelium discoideum* are subject to nutritional and developmental regulation. *Myxamoebae* may be grown in association with bacteria such as *Escherichia coli* and *Klebsiella aerogenes*, while some laboratory contrived strains thrive axenically in a partially defined medium. Differences in the activities of acid hydrolases between axenically and bacterially grown amoebae have been characterized by Ashworth and coworkers (Watts & Ashworth, 1970; Weiner & Ashworth, 1970; Ashworth & Quance, 1972). Axenic cells produce higher levels of certain enzymes such as β-N-acetylglucosaminidase and α-mannosidase. Generally, bacterially grown amoebae exhibit high levels of hydrolase activity primarily during starvation early in development (Quance & Ashworth, 1972).

Axenically grown vegetative myxamoebae of the strain Ax2 were found by electrophoretic analysis to produce a number of cysteine proteinases (North *et al.*, 1988). In the presence of glucose two major intracellular forms of 51 kDa and 45 kDa, named ddCP51 and ddCP45 respectively, as well as ddCP30, ddCP38A and ddCP42 were discovered using gelatin-SDS-PAGE. The previously purified enzyme proteinase B (North & Whyte, 1984) was found to be identical to ddCP30. When grown in the
absence of glucose cells produced similar proteinase forms but ddCP42 and ddCP38A were more abundant.

Bacterially grown Ax2 amoebae exhibit a different pattern of cysteine proteinase expression from those grown axenically. Gelatin-SDS-PAGE revealed three prominent bands, ddCP48, ddCP43 and ddCP38B, as well as low levels of ddCP30 (North et al., 1988). Excepting ddCP30, the bacterially induced, or "B-form" enzymes, were different forms of cysteine proteinases from the axenic forms (A-form) of the enzymes; despite similar inhibition by E-64, leupeptin and TLCK, the two forms differed in sensitivity to chymostatin, TPCK and antipain. Interestingly, axenically grown cells could be induced to "switch" from the A-form to B-form cysteine proteinase expression within 4 hours after the addition of K. aerogenes. North (1988) found that Gram-negative (E. coli, K. aerogenes) as well as Gram-positive bacteria (Micrococcus lysodeikticus, Bacillus subtilis) induced the switch even though the latter group of organisms are generally a less suitable nutrient source for Dictyostelium myxamoebae. An acid-precipitable macromolecule termed the cysteine proteinase converting factor (CPCF) originating in the bacterial cell wall demonstrated the ability to induce the switch from A-form to B-form cysteine proteinase expression in Ax2 amoebae. Its susceptibility to lysozyme suggests that the CPCF may be peptidoglycan (North, 1988).

Cysteine Proteinase Activity During Starvation and Development

During starvation amoebae behave as they would in the early stages of development when they become aggregation competent (Lee, 1972). Generally, there is a marked release of some lysosomal enzymes including proteinases. Starvation of
axenically grown cells results in the gradual disappearance of A-form proteinases (North et al., 1988) and the appearance of a cysteine proteinase of an apparent molecular weight of 48,000 Da. North (1982c) showed that cathepsin B-like activity was released 30 minutes after the onset of starvation, while other lysosomal enzymes, acid phosphatase and β-N-acetylgalactosaminidase, were secreted maximally after 60 to 90 minutes. The major proteinase, ddCP42, was present in the extracellular milieu as were lower levels of ddCP41, ddCP51 and ddCP30 (North et al., 1988).

Developmental regulation of cysteine proteinases is evident from molecular biological studies of Dictyostelium in later stages of the life cycle. Genes encoding cysteine proteinases, CP1, CP2 and CP3 are expressed late in development, ironically when cysteine proteinase activity appears to decrease (Pears et al., 1985; Presse et al., 1986a,b; Williams et al., 1985). The proteins encoded by these genes have not yet been identified.

Cysteine Proteinase Activity During Spore Germination

Very low levels of proteolytic activity are present in spores and matrix material. Jackson and Cotter (1984) examined the levels of three types of proteinases in dormant wild-type NC4 spores. Leucine aminopeptidase was maintained at constant levels in spores aged 1 to 13 days, while aspartic proteinase (cathepsin D-like) activity decreased during aging reaching 50% of the original levels (1-day old) after 13 days. Cysteine proteinase (cathepsin B-like) activity remains constant during spore aging. Interestingly, cathepsin B-like activity in spontaneous germination mutants, SG1 and SG2, was much lower in 2-day old spores than in NC4 spores of the same age. Activity levels peaked at 7 days of age and decreased thereafter. In spite of these data there is no evident
correlation between cathepsin B-like activity and the ability of spores to be autoactivated or heat-activated (Jackson & Cotter, 1984).

During spore germination, levels of cysteine proteinases, specifically cathepsin B-like activity, increases during early swelling, approximately 2 hours postactivation (Jackson & Cotter, 1984; North & Cotter, 1984). Enzyme levels increase as myxamoebae begin to emerge. Increases in other lysosomal hydrolase activities at this stage in germination have been documented (Jackson et al., 1982; Tisa & Cotter, 1979, 1980; Chan & Cotter, 1982). Late in spore swelling the activity is detected externally; secretion continues after emergence of myxamoebae. Cysteine proteinase activity generally increases throughout spore germination, and in newly emerged myxamoebae this activity may achieve levels 11-fold higher than those observed in dormant spores (Jackson & Cotter, 1984). Nonetheless, cathepsin B-like activity in spores is much less than in vegetative amoebae (North & Cotter, 1984).

Role of Cysteine Proteinases During Spore Germination

These studies were intended to clarify the role of proteinases in spore germination. In light of previous findings that levels of protein and amino acid pools decreased during multicellular development (Wright & Anderson, 1960), and that high amino acid levels in spores decreased during germination (Ennis, 1981), it seemed plausible that proteins or amino acids may be involved in energy production during spore germination. Jackson and Cotter (1984), however, showed that acid proteinases, including cysteine proteinases, did not increase significantly during early swelling when energy reserves are needed (Jackson et al., 1982); it is unlikely that proteinases play a critical role in providing energy for germination. Alternatively, they do suggest that
cathepsin B-like activity may be involved in the breakdown of the cell wall of the germinating spore. Just as the cell wall is composed of a variety of components, cellulose, protein and carbohydrate, different types of enzymes such as cellulases, proteinases and carbohydrates work in concert to digest the wall (Chan & Cotter, 1982; Jackson & Cotter, 1984). Indeed, spores blocked at the swelling stage by cycloheximide release myxamoebae when treated with Pronase in association with cellulase and β-glucosidase (Chan & Cotter, 1982).

As well as serving as metabolites in energy production free amino acids are diverted into protein synthesis. Since spore germination leads to the emergence of amoebae which are primed to pursue vegetative growth, proteolytic activity may provide building blocks for de novo protein synthesis. In effect, the proteinases expressed during spore germination prepare the cell for subsequent vegetative growth functions (North & Cotter, 1984).

Purpose of This Study

A variety of lysosomal hydrolases exhibit differential secretion characteristics suggesting that differential targeting to lysosomes, or subpopulations of lysosomes, exists in Dictyostelium (Dimond et al., 1981). There is also evidence that the characteristic secretion patterns for a particular enzyme may be modified by the addition of a nonmetabolizable sugar, sucrose (Crean & Rossomando, 1979). In view of these data, expression of cysteine proteinases and their secretion characteristics were examined in vegetative cells to observe whether differential secretion occurs among these proteinases. A preliminary study of acid phosphatase secretion sheds light upon this portion of the work.
Spore germination in *Dictyostelium* brings about changes in the regulation and expression in a number of lysosomal enzymes (Tisa & Cotter, 1980; Chan & Cotter, 1982a,b). In light of previous studies of cysteine proteinases during spore germination (Jackson & Cotter, 1984; North & Cotter, 1984), the current work investigates further the expression, regulation and secretion of cysteine proteinases during this process under altered secretion conditions, and under alternate activation treatments.
MATERIALS AND METHODS

Growth of Dictyostelium discoideum

A number of haploid strains of Dictyostelium discoideum (Raper, 1935) were employed to study the expression of lysosomal enzymes during development. Spore germination was studied in the strains NC4H and SG1. The wild type NC4H (ATCC 28245) is a haploid strain from which the spontaneous germination mutant SG1 (ATCC 44840) was derived (Cotter & Dahlberg, 1977). Both of these strains produce healthy fruiting bodies when grown in association with Escherichia coli B/r on glucose-salts agar (Adams, 1959). This medium contained: 1.0 g NH₄Cl, 3.0 g KH₂PO₄, 6.0 g Na₂HPO₄, 0.13 g MgSO₄, and 15.0 g agar (Gibco) in 1.0 litre of double distilled water (deionized) (ddH₂O) (see Cotter & Dahlberg, 1977). Sterile glucose (40.0 g/100 mL) was added to the autoclaved medium to give a final concentration of 0.4%. The medium was cooled to approximately 50°C and dispensed into sterile plastic Petri plates (100 X 15 mm).

The axenic mutants of D. discoideum, Ax3 and Ax2, were used to study lysosomal enzyme secretion during vegetative growth and starvation. The strain Ax3 is derived from the wild type NC4 and was obtained from Dr. R.L. Dimond. The strain Ax2 (ATCC 24397), also derived from NC4, was a gift from Dr. M.J. North.

Vegetative amoebae of D. discoideum were grown in TM medium (Free & Loomis, 1974) which was composed of the following: 10.0 g trypsinase peptone (BRL), 5.0 g yeast extract (Difco), 10.0 g glucose, 0.35 g Na₂HPO₄, 1.2 g KH₂PO₄ in 1.0 L of ddH₂O. The pH of the medium was adjusted to 6.5 and the medium was
autoclaved (121°C, 15 minutes). Cooled sterile TM medium was inoculated using
0.2 - 0.5 mL of inoculum from previously grown cultures.

Formation of Dictyostelium discoideum Sorocarps

Fruiting bodies of Dictyostelium were prepared according to the methods of
Cotter and Raper (1968a). Spores of D. discoideum strains NC4 and SG1 were
aseptically transferred by loop into a flask with 10 - 20 mL of sterile
ddH₂O to obtain a concentration of approximately 10⁷ spores/ mL. One loopful of the
bacterium E. coli B/r was transferred to the flask, and the mixture agitated on a
Vortex mixer to disperse clumped spores and bacteria.

Approximately 1.5 to 2.0 mL of the spore/bacterium suspension was pipeted onto
glucose-salts agar plates which were then incubated at room temperature (23 - 25°C).
The plates were shaken 24 to 48 hours after plating to ensure uniform distribution and
development of Dictyostelium sorocarps. Aggregation centres appeared 3 days after
plating, and sorocarps were formed by the fourth day of development. Spores were
allowed to age 1 to 2 days before they were used in germination studies.

Germination of Spores

Mature dormant spores of D. discoideum are suspended in a sorus above the
substratum by a thin stalk consisting of dead vacuolated cells. The spores were harvested
by passing a moistened microscope slide 2 to 3 mm above the agar surface; this allowed
the capture of the sorus leaving behind the dead stalk cells. The spores and matrix were
deposited in 5 to 10 mL of ddH₂O. This material was centrifuged at room temperature in
an IEC clinical centrifuge at a setting of 6 (500 X g) for 5 minutes separating the dormant
spores and matrix. The supernatant (matrix) was decanted and reserved on ice or frozen at -20°C until it could be assayed for extracellular enzyme activity. The pelleted spores were washed twice in PB before they were finally resuspended in 5 mL of buffer. Spore density was determined by counting a representative sample using a hemacytometer. The final spore concentration was adjusted to 1.0 - 1.2 X 10^7 spores/ml with PB.

Dormant wild type (NC4) spores were suspended in test tubes and heat activated by incubation at 45°C±0.1°C, for 30 minutes in a temperature-controlled water bath (Braun) (Cotter & Raper, 1968a). The test tube was suspended in the water bath such that the meniscus was at least 2 cm below the surface of the bath. Following heat activation the spores were quickly cooled to 23°C by immersing the test tube in cool water. Germination was carried out at 23°C±0.1°C by placing the spore suspension in a circulating water bath (Thermomix). Spores were kept in suspension by placing micro stir bar magnets in the test tubes and propelling them by a submersible stirring unit (Troemner, model 700). The stirring action was just enough to circulate the spores to prevent clumping and to provide adequate aeration required for the germination process, but not harsh enough to damage spores and emerging myxamoebae. Zero time was designated as the point that heat-induced activation was terminated.

The volume of the mixture of germinating spores in test tubes was kept between 1.0 to 5.0 mL to ensure all spores received adequate surface aeration. Where large volumes of germinating spores were required spore suspensions were incubated in 125 mL or 250 mL Erlenmeyer flasks and agitated in an oscillating temperature-controlled water bath (83 oscillations per minute) (Precision Instruments). The volumes of the suspension in 125 mL and 250 mL flasks were typically below 50 mL and 100 mL, respectively; this permitted sufficient surface area for aeration.
Flasks were loosely covered with aluminum foil to prevent airborne contamination. No substantial difference in the germination kinetics or in enzyme activities were observed between spore suspensions incubated in small or larger volumes.

**Autoactivation and Germination of SG1 Spores**

Germinating spores of *Dictyostelium discoideum* produce an autoactivator factor which stimulates germination (Cotter & Dahlberg, 1977). Spores of the spontaneous germination mutant SG1 have the ability to autoactivate and require no external activation; in effect SG1 spores begin to germinate immediately upon removal of the matrix material.

Spores of the mutant SG1 were harvested, washed 3 times in PB and incubated directly at 23° C (without heat-shocking) as described previously. Zero time was taken to be when spores were transferred to the incubation bath.

Chemical activation involved incubation of harvested SG1 spores in 20% (v/v) dimethylsulfoxide (DMSO) for 30 minutes at 23.5°C (Cotter et al., 1979). The DMSO was removed from spores by filtration through a 1.2 μm filter (Sartorius) and spores were washed twice in PB. The spores were diluted to a final concentration of 10⁷ spores/mL. The instant spores were diluted to the germination concentration was designated as zero time.

**Scoring of Germination**

At designated time intervals about 0.1 mL of spore suspension was deposited on a microscope slide and examined using a Zeiss phase contrast microscope under a magnification of 320X. The first 200 objects observed were scored as either i) unswollen
spores, ii) swollen spores, or iii) emerged myxamoebae, representing the three primary stages in *Dictyostelium* spore germination (Cotter & Raper, 1968). Unswollen spores appeared capsule-shaped and bright under phase contrast, whereas swollen spores had lateral protuberances of the spore wall, were slightly rounded, and appeared phase dark. Emerged amoebae were irregularly shaped and phase dark.

**Extraction of Intracellular Enzymes**

Germinating spores of *D. discoideum* were monitored for expression of lysosomal enzymes. Samples of spore suspension (2 mL) were centrifuged at low speed (500 X g) at room temperature. Supernatants and pelleted spores were separated, frozen immediately and stored at -20°C until needed.

Spores were thawed and disrupted by grinding with glass beads (0.25 - 0.32 mm in diameter) (Sigma) using a method modified from North and Cotter (1984). Extracts were prepared by adding 500 μL of 0.1% (v/v) Triton X-100 in PB and 1.0 mL of glass beads to the spores, such that the solution was 1 - 1.5 mm above the level of the beads. The mixture was subjected to vigorous agitation on a Vortex mixer at high speed for 6 seconds, followed by a 3 sec. rest period on ice for a total of 5 minutes. An additional 500 μL of detergent mixture was added to the tube, the solution was mixed 3 X after which it was removed with a Pasteur pipet. The extract was centrifuged for 2 minutes (12000 X g) in a Fisher benchtop microcentrifuge to remove particulate cellular debris. The supernatant was stored on ice, or frozen at -20°C until enzyme assays and electrophoresis could be performed. Storage of cell extracts and supernatants at -20°C had no apparent effect on enzyme activity.
Vegetative myxamoebae of *D. discoideum* were also examined for the presence of intracellular lysosomal enzymes. Aliquots of 2 mL of cell suspension were centrifuged and the supernatant containing the spent medium was frozen at -20°C as before. The pelleted cells were washed once more in PB to remove residual TM medium, and finally resuspended in 0.1% Triton X-100 in PB and shaken vigorously on a Vortex mixer to cause lysis of the plasma membrane as well as the lysosomal membrane. This lysate was frozen at -20°C, thawed and centrifuged as previously described for spore lysates.

**Protein Determination**

Protein was measured according to the method of Bradford (1976) using the Bio Rad Protein Assay Kit. The assay was standardized using bovine serum albumin (Sigma).

**Lysosomal Enzyme Secretion in Vegetative Cells**

Secretion of acid phosphatase and cysteine proteinases was observed under standard secretion conditions as described by Dimond *et al.* (1981). Cells grown axenically in TM medium were harvested in late log phase as previously described. After washing, vegetative cells were resuspended in PB to a titre of 1.0 to 1.5 X 10⁷ cells/ mL in sterile 125 mL or 250 mL Erlenmeyer flasks. Alternatively, cells were resuspended in PB containing 0.1 M sucrose. The sucrose was dissolved in autoclaved buffer and filter sterilized using a disposable 0.2 µm membrane filtration unit (Nalgene). Experimental flasks were shaken on an oscillating shaker bath (83 oscillations per minute) at 23°C. Flask openings were stoppered with loose-fitting foam plugs which
prevented airborne contaminants from entering, but allowed gas exchange with the atmosphere.

**Acid Phosphatase Activity Assay**

Extracts were examined for intracellular and secreted acid phosphatase activity by methods modified from Tisa and Cotter (1979). The compound p-nitrophenylphosphate (PNPP) (Sigma) was used as a substrate for the enzyme. The reaction mixture consisted of 100 μL of 10 mM PNPP in 50 mM sodium acetate buffer, pH 5.5, and 100 μL of extract containing the enzyme. This mixture was incubated in test tubes at 23°C on a Precision shaker bath for 30 minutes. Termination of the reaction was achieved by the addition of 200 μL of 1.0 M Na₂CO₃ to the mixture. Colour development was monitored using a Chromoscan microtitre plate reader (BioTek) at 405 nm. One unit of enzyme activity was defined as the number of nmol p-nitrophenol released per minute; specific activity was expressed as units of enzyme activity per mg of protein.

**Cysteine Proteinase Activity: Nitroanilide Hydrolysis**

Extracellular (secreted) and intracellular extracts were examined for their ability to hydrolyse three different synthetic substrates (see Achstetter et al., 1981): N-carbobenzoxy-L-arginyl-arginine 4-nitroanilide (ZRR) (Novabiochem, Switzerland), N-carbobenzoxy-L-tyrosyl-L-lysyl-L-arginine 4-nitroanilide (ZYKR) (Bachem, Switzerland), and N-benzoyl-L-prolyl-L-phenylalanyl-L-arginine 4-nitroanilide (BzPFR) (Sigma). The nitroanilide substrates were dissolved in ddH₂O and used as 10 mM stock solutions.
Two assay systems were employed to measure specific cysteine proteinase activity. High enzyme activity was measured using a substrate mixture consisting of 1.0 mL 0.1 M phosphate buffer, pH 6.0, 10 μL stock substrate, and 10 μL 0.1 M dithiothreitol (DTT) (Sigma). The reaction was carried out in microtitre plate wells, and was initiated by adding 150 μL of substrate mixture to 15 μL of sample and incubated at 23°C. The release of 4-nitroaniline was monitored spectrophotometrically at 405 nm using a Chromoscan microtitre plate reader. Readings were taken after 15 minutes of incubation.

The measurement of low proteinase activity required a substrate mixture of 1.0 mL 0.1 M phosphate buffer, pH 6.0, and 36 μL 0.1 M DTT. The reaction mixture consisted of 45 μL of substrate mixture and 120 μL of sample. Readings were recorded as before. Values were corrected using both enzyme and substrate controls to account for non-enzymatic hydrolysis. Units of 4-nitroaniline release were calculated from these values using the molar extinction coefficient of 9500 cm² mol⁻¹ (North & Cotter, 1984). A unit of enzyme activity was defined as the amount of enzyme which released 1 nmol of 4-nitroaniline per minute. Specific activity was taken to be the unit of enzyme activity per mg of released protein.

**Proteinase Inhibition Studies**

The three specific cysteine proteinase substrates BzPFR, ZRR and ZYKR were examined for their sensitivity to the specific cysteine proteinase inhibitors E-64 (trans-epoxysuccinyl-L-leucylamido(4-guanidino)-butane) and two peptidyl diazomethanes Z-Phe-Phe-CHN₂ and Z-Phe-Ala-CHN₂. Stock solutions of each inhibitor compound were dissolved (E-64 in water and the peptidyl diazomethanes in acetonitrile) and diluted
appropriately to give final effective concentrations ranging from $10^4$ to $10^3$ M. To test inhibition of cysteine proteinase activity samples containing proteinase were preincubated with each compound for 5 minutes before the addition of substrate mixtures; the assay was carried out as above.

**Gelatin-SDS-PAGE Analysis of Proteinases**

Cysteine proteinases from *D. discoideum* were separated electrophoretically under partial denaturing conditions, allowed to renature, and were subsequently visualized using a "reverse staining" technique. Cell extracts and protein preparations were electrophoresed on sodium dodecyl sulfate-polyacrylamide electrophoretic gels impregnated with heat-denatured gelatin (gelatin-SDS-PAGE) using a conventional discontinuous buffer system as first described by Laemmli (1970). The gelatin formed an immobile proteinaceous network within the polyacrylamide matrix which had no apparent effect on either the polymerization process or the electrophoretic migration of proteins. The denaturing conditions of the sample buffer and running buffer systems in this procedure allowed proteins to migrate according to molecular weight, while preventing proteinases from digesting the gelatin substrate. Following electrophoresis SDS and the running buffer were removed from the gel using Triton X-100; this allowed proteins to regain their native conformation. Selective activation of cysteine proteinases was achieved by incubating the washed gel in an activation buffer (0.1 M sodium acetate, pH 4.0) including dithiothreitol (DTT), a known activator of cysteine proteinases.

Protein staining of the gelatin-SDS-PAGE gel with Coomassie Brilliant Blue gave a very dramatic image; unhydrolyzed gelatin stained blue (background) while clear bands appeared where cysteine proteinases hydrolysed the gelatin. The resulting gel resembled
a "negative" of a conventional Coomassie Blue-stained gel. Where certain proteins were abundant, deeply stained electrophoretic bands were visible above the background stain.

**Preparation of Stock Solutions**

Gelatin-SDS-PAGE gels were prepared according to North, Scott & Lockwood (1988), and the methods were modified to accommodate the Bio Rad Mini Protean system. A stock acrylamide monomer solution (30% T) was prepared by first dissolving 29.2 g acrylamide (Bio Rad) in 50 mL ddH₂O, and 0.8 g N,N'-methylene bis-acrylamide (Bio Rad) in 25 mL ddH₂O. The two solutions were mixed, filtered through a Whatman No. 1 filter, the volume was adjusted to 100 mL, and the solution was stored refrigerated in a light-proof glass bottle. Stock buffer solutions of 1.5 M Tris-Cl, pH 8.8 and 0.5 M Tris-Cl, pH 6.8, were made using Tris (hydroxymethyl) aminomethane (Sigma) and stored refrigerated. The pH of Tris-based buffers was adjusted using 6 M HCl. A 2% (w/v) gelatin stock solution was prepared by dissolving swine gelatin (0.4 g) (Sigma) in ddH₂O (20 mL) and heating at 80°C for 10 minutes. This solution was refrigerated and gently melted using a heating pad just prior to use. The above stock solutions were replaced after 30 days.

The sample buffer, in which protein preparations were solubilized prior to electrophoresis, was concentrated 5 times to avoid extensive dilution of sample protein.
A 10 mL mixture of the 5X sample buffer consisted of:

(i) 2.5 mL β-mercaptoethanol (as supplied) (Sigma)

(ii) 5.0 mL glycerol (Sigma)

(iii) 2.5 mL 0.5 M Tris-Cl, pH 6.8

(iv) 1.0 g SDS (Bio Rad)

(v) 0.4 mg Bromophenol Blue (J.T. Baker)

This solution was dispensed into 1.0 mL microcentrifuge tubes in aliquots of 100 μL and stored at 4°C for up to 30 days. Protein samples were dissolved 4:1 in 5X sample buffer before loading onto the gel.

Electrophoresis running buffer, Tris-glycine, consisted of 3.0 g Tris, 14.4 g glycine (Sigma) and 1.0 g SDS dissolved in 1000 mL of ddH₂O. The pH was adjusted to 8.3 using 6 M HCl.

Gelatin-SDS-PAGE processing solutions were prepared as follows. A stock solution of 10% (v/v) Triton X-100 was prepared using ddH₂O. A stock supply of 0.1 M acetate buffer, pH 4.0 was prepared using acetic acid/ sodium acetate (2:1).

It was convenient to maintain a stock solution of 0.1 M DDT to be used in cysteine proteinase band development on gels. Aliquots of 1.0 mL were stored in 1.5 mL microcentrifuge tubes at -20°C until required.

Other mixtures required for gelatin gel development included Coomassie Blue Protein staining solution (0.25% (w/v) Coomassie Brilliant Blue R-250 (Bio Rad) in 50% (v/v) methanol and 10% (v/v) glacial acetic acid), concentrated destaining solution (40% MeOH, 10% acetic acid), and mild destaining solution (5% MeOH, 10% acetic acid).
Preparation of Gelatin-SDS-PAGE Gels

Mini gels were produced using the Bio Rad Protean electrophoresis system. The polyacrylamide gel matrix consisted of 7.5%, 10% or 12% acrylamide polymer, with dimensions of 8 cm X 5.0 cm, and was 0.75 mm or 1.0 mm in thickness. The 7.5% separation gel consisted of:

(i) 3.85 mL ddH₂O
(ii) 1.0 mL 2% gelatin
(iii) 2.5 mL 1.5 M Tris-Cl (pH 8.8)
(iv) 2.5 mL acrylamide monomer solution (30% T)
(v) 100 µL 10% SDS
(vi) 50 µL 10% (w/v) ammonium persulfate (APS)
(vii) 5 µL N,N,N',N'- tetramethylethylenediamine
(TEMED) (as supplied by Sigma)

The first six components were mixed in a vacuum flask and degassed for 5 minutes before TEMED was added. The solution was quickly poured into the plate glass sandwich and overlayed carefully with 0.1% (w/v) SDS in 1.5 M Tris-Cl. The mixture was allowed to polymerize for 1.0 hour.

The 4% polyacrylamide stacking gel consisted of the following:

(i) 6.1 mL ddH₂O
(ii) 2.5 mL 0.5 M Tris-Cl (pH 6.8)
(iii) 1.3 mL acrylamide (30% T)
(iv) 100 µL 10% SDS
(v) 50 µL 10% APS
(iv) 10 µL TEMED
As before, the first six components were mixed in a vacuum flask and degassed for 15 minutes. TEMED was added to the mixture and the solution was overlayed onto the separation gel using a ten or fifteen-well comb.

Protein preparations were solubilized in sample buffer (dilution of 4:1) and loaded directly onto the gel. Typically, 10 - 30 μg of protein was loaded into each well. Overloading caused the appearance of stained protein electrophoretic bands which interfered with proteinase band development. Conventional SDS-PAGE protein molecular weight standards were used: α-lactalbumin (14,200 Da), trypsin inhibitor (20,100 Da), trypsinogen (24,000 Da), carbonic anhydrase (29,000 Da), glyceraldehyde-3-phosphate (36,000 Da), egg albumin (45,000 Da), bovine serum albumin (66,000 Da), phosphorylase B (97,400 Da) and β-galactosidase (E. coli) (116,000 Da) (Sigma). The standards were used at concentrations twice those for non-gelatin gels, approximately 2 μg/μL, to facilitate observation of marker bands against the stained background.

Electrophoresis was carried out using SDS-Tris-glycine running buffer (pH 8.3) system at approximately 10°C (in a cold room) to prevent overheating of the polyacrylamide gels and the subsequent damage to proteins. Current was applied at 5 mA per gel. Electrophoretic runs were continued until the marker dye (Bromphenol Blue) had reached the bottom of the separation gel, about 2 to 4 hours.
Development of Gelatin-SDS-PAGE Gels

Gels were washed in 2.5% Triton X-100 for 60 minutes to remove SDS and running buffer. Development of cysteine proteinase bands was carried out by incubating gels at room temperature in 0.1 M sodium acetate buffer, pH 4.0, supplemented with 1.0 mM DTT. Development times varied with the nature of the source of the protein preparation: 2 - 4 hours (intracellular samples) or 16 - 24 hours (extracellular samples). Visualization of cysteine proteinase bands was accomplished by staining gels in Coomassie Blue stain (1.0 hour) and destaining in concentrated destain (1 - 3 hours) and mild destain solutions (overnight).

Gelatin-SDS-PAGE gels were photographed on a light box using Panatomic-X black and white film, ASA 32 (Kodak), at aperture settings bracketed from f8 to f11 and a shutter speed of 1/4 second. Contrast between the hydrolytic proteinase bands and the background was enhanced with a red filter.
RESULTS

Acid Phosphatase Secretion

The secretion of lysosomal enzymes in *Dictyostelium discoideum* can be classified according to the kinetics of enzyme release: (i) nonsecretory, (ii) sigmoidal or (iii) linear (Dimond *et al*., 1981). The addition of nonmetabolizable disaccharides has been shown to alter secretion of glycosidases (Crean & Rossomando, 1979) including trehalase (Seshadri *et al*., 1986). The alteration of the external environment of vegetative amoebae may have an effect on the secretion pattern of various lysosomal enzymes and would therefore be an appropriate topic of study.

It is evident from Figure 2 that sucrose had a dramatic effect on the secretion of acid phosphatase in starving amoebae. The amount of secreted acid phosphatase activity was expressed as the percentage of the total acid phosphatase (i.e. the extracellular activity divided by the sum of the intracellular and extracellular activities). Under standard secretion conditions vegetative amoebae of *D. discoideum* secreted acid phosphatase in a linear fashion with extracellular levels of the enzyme representing approximately 40% of the total acid phosphatase activity over a 6-hour period. In the presence of 0.1 M sucrose (sucrose-PB) there was an increase in secretion. Following a short lag of 1 hour acid phosphatase was rapidly released; the kinetics of this release were sigmoidal. Furthermore, at least 85% of the total acid phosphatase activity was extracellular by 6 hours of incubation.
Figure 2.

Secretion of acid phosphatase in response to starvation and sucrose. Growing Ax3 amoebae were washed free of TM medium and resuspended in PB with or without 0.1 M sucrose (see Materials & Methods). Acid phosphatase secretion from cells incubated with (●) or without (○) 0.1 M sucrose is shown.
Figure 2.
Excessive heat normally has adverse effects on vegetatively growing cells. However, some thermoprotection may be gained through a universal cellular response, termed the "heat shock response", whereby cells synthesize specific heat shock proteins (hsp) which appear to confer limited heat resistance upon the stressed cell. Essentially all organisms (bacteria to humans) exhibit a similar heat stress response and major heat shock proteins are conserved throughout evolution. Vegetative amoebae of *Dictyostelium discoideum* characteristically exhibit hsp synthesis when exposed to temperatures of 30°C or higher (Loomis & Wheeler, 1980).

Heat appeared to have little effect on the secretion of acid phosphatase (Fig. 3). Starving amoebae in either the absence or presence of 0.1 M sucrose exhibited characteristic acid phosphatase secretion patterns depicted in Figure 2. Both control cells (30°C) and heat shocked cells were viable in starvation buffer although the latter population of cells gradually lost viability over the 6-hour incubation period (Fig. 4). Interestingly, the cells heat shocked at 30°C in the presence of sucrose appeared to exhibit greater viability than those cells lacking the disaccharide. It could be that 0.1 M sucrose imparted some degree of heat resistance to stressed amoebae.

Though global protein synthesis is suppressed under starvation conditions, the addition of the protein synthesis inhibitor cycloheximide partially inhibits secretion of acid phosphatase without affecting the secretion of lysosomal carbohydrates (Dimond et al., 1981). Differential secretion among lysosomal enzymes (acid phosphatase versus glycosidases) implies that different secretion mechanisms may be responsible; differential requirements for protein synthesis for each mechanism may
Figure 3.
Effect of heat stress on acid phosphatase secretion. Vegetatively grown Ax3 amoebae were washed free of TM medium and resuspended in PB with or without 0.1 M sucrose. Cell suspensions were incubated at 23°C or 30°C for 6 hours. Secreted acid phosphatase activity was examined in buffer medium (see Materials & Methods). Secreted acid phosphatase activity from amoebae incubated at 23°C with (●) or without (○) 0.1 M sucrose; at 30°C with (■) or without (□) 0.1 M sucrose.
Figure 4.
Viability of amoebae heat stressed at 30°C. Growing Ax3 amoebae were washed free of TM medium and resuspended in PB with or without 0.1 M sucrose. Cell suspensions were incubated at 23°C or 30°C for 6 hours. Aliquots (1 mL) of cell suspension were removed at intervals, diluted, and plated on nutrient medium in association with an E. coli bacterial lawn. Plaque formation on the bacterial lawn indicated viable cells. Depicted is the log of survivorship (viability) (S). Viable cells incubated at 23°C in PB with (▲) or without (△) 0.1 M sucrose or incubated at 30°C in PB with (■) or without (□) 0.1 M sucrose.
reflect this also. The effect of cycloheximide on secretion of acid phosphatase is depicted in Figure 5. The presence of cycloheximide in cultures starved in PB depressed acid phosphatase secretion slightly (to approx. 15%) compared to the control (PB alone). Cells treated with cycloheximide in the presence of sucrose showed a marked depression in acid phosphatase secretion to a level approximately 5% of the total enzyme activity. The decrease in secretion by cells starved in PB alone suggests that the mechanism responsible for acid phosphatase secretion requires protein synthesis. It is interesting that cycloheximide severely depressed secretion of acid phosphatase in cells starved in sucrose-PB; a differential requirement for protein synthesis in cells starved in the absence or presence of the disaccharide supports the existence of differential mechanisms for secretion.

Changes in Cellular Morphology of Starving Amoebae

Dramatic changes in morphology are apparent in cells starved in PB in the presence and absence of sucrose. Figure 6 depicts amoebae incubated in (A) nutrient medium and after one hour in (B) PB and (C) sucrose-PB. Cells grown in nutrient medium were amorphously shaped and contained few large intracellular granules. Starvation in PB caused cells to secrete lysosomal enzymes and vacuoles to appear in the cell. Some evaginations in the cell membrane associated with vesicles were apparent and the cell shape was more rounded.

The addition of 0.1 M sucrose-PB caused amoebae to actively secrete enzymes. Concomitant with the increase in secretion the cell shape became more rounded and robust. Many vesicles were observed to be associated with large spherical extrusions of the cell membrane. In some preparations Neutral Red-stained spherical
Figure 5.

Effect of cycloheximide on acid phosphatase secretion. Growing Ax3 amoebae were washed free of TM medium and were resuspended in PB with or without 0.1 M sucrose (see Materials & Methods). Cycloheximide was added to the cell suspension to give a final concentration of 500 µg/mL. Acid phosphatase activity in cells incubated in PB (○); PB with cycloheximide (○); PB with sucrose (●); and PB with sucrose and cycloheximide (●).
Figure 6.

Changes in morphology of Ax2 amoebae in response to starvation. Amoebae of the strain Ax2 grown in TM medium were washed free of medium and starved in PB with or without 0.1 M sucrose. The photographs depict Ax2 amoebae during vegetative growth (A), and approximately 1 hour after the onset of starvation in PB (B) and in sucrose-PB (C).
Figure 6.

A  
**Nutrient Medium**

B  
**PB**

C  
**PB + Sucrose**
extracellular vesicles were observed. These observations lend support to the hypothesis that secretion of enzymes is a vesicle-mediated event.

**Proteinase Activity in Starved Amoebae of *Dictyostelium***

Intracellular and secreted proteinase activity was examined in vegetative amoebae of *D. discoideum* using peptide-nitroanilide substrates and gelatin-SDS-PAGE. Specific proteinase activities were correlated with the appearance of proteinase activity electrophoretic bands.

**Inhibition of Peptide-nitroanilide-hydrolysing Activities**

Proteinases produced by *Dictyostelium discoideum* hydrolyse a number of synthetic peptide substrates. The arginine-containing peptide-nitroanilide compounds used in this study were suitable for examining cysteine proteinase expression in this organism (North, 1982a, 1985) because they were rapidly hydrolysed by specific cysteine proteinases in cell extracts. Extracts from vegetative amoebae hydrolysed Bz-Pro-Phe-Arg-Nan, Z-Arg-Arg-Nan and Z-Tyr-Lys-Arg-Nan with specific activities of 7.1±2.1, 2.9±1.0 and 2.8±1.9 nmol/min mg protein respectively (values represent average of eight trials ±SD). The cysteine proteinase activator, DTT, stimulates both BzPFRase and ZRRase activities 3-fold at concentrations above 0.1 mM but 1.0 mM DTT did not enhance ZYKR-hydrolysing activity (data not shown).

Peptide-nitroanilide-hydrolysing activity may be further defined by sensitivity to specific inhibitors of cysteine proteinases. The specific inhibitor E-64 (Kirschke & Barrett, 1987) depressed Bz-Pro-Phe-Arg-Nan and Z-Arg-Arg-Nan hydrolysing activity (referred to as BzPFRase and ZRRase respectively), ZRRase
being the more sensitive. E-64 blocked Z-Tyr-Lys-Arg-Nan hydrolysing activity (ZYKRase) to a much lesser degree than either BzpFRase or ZRRase. The two peptidyl Diazomethanes, Z-Phe-Ala-CHN$_2$ and Z-Phe-Phe-CHN$_2$, were similarly more effective in impeding BzpFRase and ZRRase than ZYKRase. While BzpFRase and ZRRase were equally sensitive to Z-Phe-Ala-CHN$_2$ the latter was less sensitive to Z-Phe-Phe-CHN$_2$; this characteristic may differentiate the two cysteine proteinase activities. Peptidyl Diazomethane inhibition of ZYKRase was substantial compared to the inhibition by E-64.

The three peptide-nitroanilide substrates may be specific for different populations of proteinases. Enhancement of activity by DTT, and sensitivity of both BzpFRase and ZRRase to E-64 and the peptidyl Diazomethanes, is indicative of cysteine proteinases and differential Z-Phe-Phe-CHN$_2$ sensitivity distinguishes the two activities. ZYKRase responses to DTT and E-64 were less characteristic of cysteine proteinases and it is unlikely that the enzyme possesses cysteine proteinase-like activity.

**Intracellular Proteinase Activity**

As the inhibitor studies had previously shown, two of the synthetic substrates, BzpFR and ZRR, are hydrolysed selectively by cysteine proteinases. Expression of BzpFRase, ZRRase and ZYKRase activities in starving amoebae, as well as the secretion of these enzymes, is differential. BzpFRase activity exhibited the most interesting pattern of expression (Fig. 7). During starvation in PB BzpFRase activity increased almost two-fold within 2 hours, peaking at this time. A sharp decline in BzpFRase activity followed this peak and continued through 6
Figure 7.

Effects of sucrose on intracellular BzPFRase activity in starved axenic amoebae.

Growing Ax2 myxamoebae were washed free of TM medium and incubated in PB under standard secretion conditions with or without 0.1 M sucrose for 6 hours. Cellular extracts were examined for proteinase activity (see Materials & Methods). BzPFRase activity in the presence (●) or absence of (○) 0.1 M sucrose.
Figure 7.
Figure 8.

Effects of sucrose on intracellular ZRRase activity in starved axenic amoebae.

Growing Ax2 myxamoebae were washed free of TM medium and incubated in PB under standard secretion conditions with or without 0.1 M sucrose for 6 hours. Cellular extracts were examined for proteinase activity (see Materials & Methods). ZRRase activity in the presence (●) or absence of (○) 0.1 M sucrose.
Figure 8.
hours of starvation. Intracellular ZRRase and ZYKRase activities are more stable during starvation in PB. Figure 8 shows that ZRRase activity decreases very slightly during the 6-hour starvation period. ZYKRase activity (Fig. 9) changes very little, increasing over 6 hours.

Initial observations suggest that in sucrose-treated cells the disaccharide caused a depression in intracellular cysteine proteinase activity levels. Intracellular BzPFRase activity (Fig. 7) decreased linearly by more than 70% over the 6-hour incubation. Similarly, decreases in ZRRase activity (Fig. 8) by 38% and ZYKRase activity (Fig. 9) by 32% were also evident. To determine whether these decreases were due to a "suppression" in cysteine proteinase activity or release of the enzymes the extracellular buffer was examined for activity.

**Extracellular Cysteine Proteinase Activity**

Ax2 myxamoebae secreted cysteine proteinase activity during starvation in PB. Extracellular peptide-nitroanilide-hydrolysing activity was expressed relative to total specific activity. The amount of secreted proteinase activity was expressed as the percentage of the total proteinase activity (i.e. the extracellular activity divided by the sum of the intracellular and extracellular activities). Approximately 35% of the BzPFRase (Fig. 10) and 25% of the ZRRase (Fig. 11) activities were secreted in a linear fashion over the 6-hour starvation period. The appearance of the enzyme in the buffer corresponded to the disappearance of activity in the cell. Little or no extracellular ZYKRase activity was detected (Fig. 12).
Figure 9.

Effects of sucrose on intracellular ZYKRase activity in starved axenic amoebae.

Growing Ax2 myxamoebae were washed free of TM medium and incubated in PB under standard secretion conditions with or without 0.1 M sucrose for 6 hours.

Cellular extracts were examined for proteinase activity (see Materials & Methods). ZYKRase activity in the presence (●) or absence of (○) 0.1 M sucrose.
Figure 9.
Figure 10.
Secretion of BzPFRase activity in starved axenic amoebae. Growing Ax2 myxamoebae were washed free of TM medium and incubated in PB under standard secretion conditions with or without 0.1 M sucrose for 6 hours. The extracellular buffer was examined for proteinase activity (see Materials & Methods). BzPFRase activity in the presence (●) or absence of (○) 0.1 M sucrose.
Figure 10.
Figure 11.

Secretion of ZRRase activity in starved axenic amoebae. Growing Ax2 myxamoebae were washed free of TM medium and incubated in PB under standard secretion conditions with or without 0.1 M sucrose for 6 hours. The extracellular buffer was examined for proteinase activity (see Materials & Methods). ZRRase activity in the presence (●) or absence of (○) 0.1 M sucrose.
Figure 11.
Figure 12.

Secretion of ZYKase activity in starved axenic amoebae. Growing Ax2
myxamoebae were washed free of TM medium and incubated in PB under standard
secretion conditions with or without 0.1 M sucrose for 6 hours. The extracellular
buffer was examined for proteinase activity (see Materials & Methods). ZYKase
activity in the presence (●) or absence of (○) 0.1 M sucrose.
Figure 12.
Effects of Sucrose Upon Proteinase Secretion

Starvation in the presence of 0.1 M sucrose enhanced secretion of cysteine proteinases into the surrounding buffer by amoebeae. The most dramatic effect of sucrose upon starving vegetative cells was observed as a change in BzPFRase secretion (Fig. 10). Sucrose stimulated the secretion of BzPFRase such that over 80% of the total activity was extracellular after 6 hours of starvation. As before, BzPFRase activity release corresponded to decreases in intracellular activity. Furthermore, the kinetics of enzyme release were altered in the presence of sucrose. Cells incubated in PB alone secreted BzPFRase activity linearly, whereas those incubated in the presence of 0.1 M sucrose consistently secreted the enzyme activity in a sigmoidal pattern. That is, a short one hour lag in secretion was followed by a rapid release of BzPFRase activity and a subsequent "plateau" of activity.

Amoebeae starved in sucrose-PB released higher levels of ZRRase activity (Fig. 11) than amoebeae starved in PB alone. Approximately 35% of the total ZRRase activity was found outside the cell after 6 hours of starvation in the presence of sucrose. The kinetics of this release were nearly linear. Appearance of ZRRase in the buffer accompanied the disappearance of the enzyme from the cell.

Sucrose had an interesting effect upon extracellular ZYKRase activity. Under standard secretion conditions ZYKRase behaves as a nonsecretory enzyme (Dimond et al., 1981). In the presence of 0.1 M sucrose approximately 10 to 15% of the total ZYKRase activity was detected extracellularly (Fig. 12). While it is possible that sucrose stimulated some secretion of ZYKRase, the extracellular levels do not correspond to the amount of ZYKRase which had disappeared from the
cell (32%) during this time. Alternatively, it is possible that cell lysis contributed to extracellular activity.

**Gelatin-SDS-PAGE Analysis of Secreted Proteinases**

While specific types of cysteine proteinase activities can be defined using peptide-nitroanilide substrates, gelatin-SDS-PAGE detects general proteinase activity. Electrophoresis allowed the separation of individual species of cysteine proteinases and the relation of these to specific activities investigated above. The gelatin-SDS-PAGE technique used in this study is one adapted to *Dictyostelium* by North *et al.* (1988) and was used to examine cysteine proteinases during the *Dictyostelium* life cycle. A specific inhibitor and specific activator were used to determine whether activity bands were a result of gelatin hydrolysis by cysteine proteinases. Duplicate sample lanes were incubated in development buffer containing E-64 (10 μg/mL) in the presence and absence of DTT, and compared to the original gels (incubated normally). Suspect activity bands were taken to be cysteine proteinases if they exhibited sensitivity to E-64 inhibition (disappearance of the band) and DTT activation (greater intensity of the band).

Figure 13 depicts the proteinase electrophoretic pattern in starving vegetative amoebae using gelatin-SDS-PAGE. The pattern of expression of proteinase activity did not differ significantly between cells starved in phosphate buffer (PB) and those starved in the presence of 0.1 M sucrose (Fig. 14a). One major proteinase band of apparent molecular weight 42 kDa, designated ddCP42 (North *et al.*, 1988), and a minor band, ddCP30, decreased in intensity over the 6-hour incubation period; this
Figure 13.

Cysteine proteinase activity in starved Ax2 amoebae. Vegetative amoebae were washed free of growth medium and resuspended in PB and starved for 6 hours. Proteinases from intracellular extracts (A) and extracellular extracts (B) were separated using gelatin-SDS-Page (see Materials & Methods). The apparent molecular weights of the proteinases in kDa are shown.

(see also North et al., 1990c.)
Figure 13.

A

B

<table>
<thead>
<tr>
<th>54</th>
<th>51</th>
<th>45</th>
<th>42</th>
</tr>
</thead>
</table>

| 54 | 51 | 42 |

| 30 |

Time (h)
Figure 14.
Cysteine proteinase activity in Ax2 amoebae starved in the presence of sucrose. Vegetative amoebae were washed free of growth medium and resuspended in PB containing 0.1 M sucrose and starved for 6 hours. Proteinases from intracellular extracts (A) and extracellular samples (B) were separated using gelatin-SDS-PAGE (see Materials & Methods). The apparent molecular weights of the proteinases in kDa are shown.

(see also North et al., 1990c.)
Figure 14.

A

B
correlated with the gradual decrease in intracellular enzyme activities shown previously.

Secreted Proteinase Activity During Starvation

It is evident from Figure 13b that a major cysteine proteinase was secreted by cells starved in phosphate buffer. The proteinase band, which represents ddCP42, appeared about one hour after the onset of starvation, and gradually increased in intensity. The secretion kinetics of BzPFRase complemented the extracellular appearance of ddCP42, and it is possible that the protein was responsible for a majority of the BzPFRase activity (Fig. 10). Sucrose clearly has a stimulatory effect on the secretion of ddCP42. Appearance of ddCP42 is concomitant with the intracellular disappearance of ddCP42 activity. Concurrent with ddCP42 secretion is the detection of ZYKRase activity in the extracellular fraction, though at levels somewhat lower than those of the BzPFRase enzyme(s).

These results indicate that under standard secretion conditions vegetative cells of D. discoideum secrete proteinases differentially. This is reflected by the differential secretion of peptide nitroanilide-hydrolysing activity as well as by the appearance of proteinase activity on gelatin-SDS-PAGE gels. The enzymes also behave differently with respect to their secretion kinetics. It is also clear that the presence of 0.1 M sucrose, a nonmetabolisable disaccharide, enhances secretion of certain cysteine proteinases and not others. Sucrose may also influence the synthesis of these enzymes.
Proteinase Secretion During Recovery From Starvation

After 6 hours of starvation in PB (with or without 0.1 M sucrose) cells were centrifuged, and washed twice with equal volumes of fresh PB. The cells were finally resuspended in the original volume of sterile nutrient medium and allowed to recover from starvation.

Intracellular BzPFRase, ZRRase and ZYKrase activities did not accumulate during recovery (Figs. 15a, 16a, 17a, respectively). In contrast, cells recovering from starvation in sucrose-PB continued to accumulate proteinases to varying degrees. Intracellular ZRRase and ZYKrase activities were greater in sucrose-treated cells than in control cells (Figs. 16a, 17a, respectively). The most notable change was the exponential increase in the level of BzPFRase activity (more than 3-fold) during recovery over the 6-hour incubation period (Fig. 15a).

Standard secretion conditions impose a state of starvation upon vegetative cells which retards cell growth and major macromolecular synthesis. Secretion of lysosomal enzymes (Dimond et al., 1981) may deplete intracellular pools. One would expect that if starved cells were returned to a nutrient-rich medium macromolecular synthesis would resume, secretion of lysosomal enzymes would cease, and intracellular levels of the enzymes would increase. A gradual increase in ZRRase (Fig. 16a) and ZYKrase (Fig. 17a) activities was evident in recovering cells. Intracellular BzPFRase activity (Fig. 15a), however, increased at a greater rate, and achieved higher levels in cells pre-starved in sucrose-PB than in control cells. It is unlikely that this was a passive accumulation, and may represent the cells' attempt to overproduce the enzyme in order to attain some internal "threshold" level quickly.
Figure 15.

Recovery of BzPFRase activity in amoebae starved in phosphate buffer.

Ax2 amoebae were starved in PB with or without 0.1 M sucrose under standard secretion conditions for 6 hours. Cells were washed free of starvation buffer, resuspended in fresh TM medium and allowed to recover for up to 6 hours. Cell extracts and the extracellular medium were examined for BzPFRase activity during recovery (see Materials & Methods). Intracellular (A) and secreted (B) BzPFRase activity from cells starved in the presence (●) or absence (○) of 0.1 M sucrose.
Figure 15.

A

Specific Activity (nmol min^{-1})^2 vs Time (h)

0 1 2 3 4 5 6
Figure 15.

![Graph showing specific activity vs time (h) with two lines: one for black circles and another for white circles. The y-axis represents specific activity in nmol min^{-1} mg protein^{-1} ranging from 0 to 2, and the x-axis represents time in hours from 0 to 6.](image)
Figure 16.

Recovery of ZRRase activity in amoebae starved in phosphate buffer. Ax2 amoebae were starved in PB with or without 0.1 M sucrose under standard secretion conditions for 6 hours. Cells were washed free of starvation buffer, resuspended in fresh TM medium and allowed to recover for up to 6 hours. Cell extracts and the extracellular medium were examined for ZRRase activity during recovery (see Materials & Methods). Intracellular (A) and secreted (B) ZRRase activity from cells starved in the presence (●) or absence (○) of 0.1 M sucrose.
Figure 16.
Figure 17.
Recovery of ZYKase activity in amoebae starved in phosphate buffer.
Ax2 amoebae were starved in PB with or without 0.1 M sucrose under standard secretion conditions for 6 hours. Cells were washed free of starvation buffer, resuspended in fresh TM medium and allowed to recover for up to 6 hours. Cell extracts and the extracellular medium were examined for ZYKase activity during recovery (see Materials & Methods). Intracellular (A) and secreted (B) ZYKase activity from cells starved in the presence (●) or absence (○) of 0.1 M sucrose.
Figure 17.
As predicted, little secretion of ZRRase and ZYKRase activity was indicated in each of the cultures pre-starved in either phosphate or sucrose-Pb (Figs. 16b, 17b, respectively). Interestingly there was a sustained secretion of BzPFRase activity by cells pre-starved in sucrose (Fig. 15b); the enzyme(s) was secreted at somewhat lower levels in control cells.

**Gelatin-SDS-PAGE Analysis of Recovering Amoebae**

Gelatin-SDS-PAGE analysis of intracellular extracts from recovering cells revealed the accumulation of ddCP42 activity, and lower levels of ddCP30 activity, accumulated in cells pre-starved in PB (not shown). The appearance of ddCP42 was concomitant with an increase in BzPFRase activity over the same time period. Cells recovering from starvation in sucrose-PB exhibited a dramatic increase in the activity of all the major proteinases: ddCP42, ddCP45, ddCP51, and ddCP54 (Fig. 18a). The accumulation of ddCP42 and ddCP45 was readily detected at zero time in recovering cells and the activity levels of ddCP51 and ddCP54 increased after the first hour of recovery. Although it was present with the other proteinase activity bands, ddCP42 activity again correlated with the increase in BzPFRase activity (Fig. 15a).

Cells pre-starved in PB did not secrete detectable levels of ddCP42 or other cysteine proteinases during recovery. Sucrose-induced secretion of ddCP42 continued into the regrowth period after the removal of sucrose (Fig. 18a) and coincided with the increased secretion of BzPFRase activity (Fig. 15b).
The observations indicate that the stimulatory effect of sucrose upon lysosomal proteinase secretion persists even though cells are recovering from starvation, and the nonmetabolizable sugar is no longer an influence.

**Proteinase Activity During Spore Germination**

Spores of the strain NC4 and SG1 were examined for the presence of proteinase activity. In order to extend the observations from previous studies (Jackson & Cotter, 1984; North & Cotter, 1984) the three specific proteinase activities, as well as the cysteine proteinase activities on gelatin gels, were examined during spore germination.

**Proteinase Activity in Aged Fruiting Bodies**

Fruiting bodies were allowed to develop and intracellular NC4 spore extracts and matrix material were examined for hydrolytic activity against the synthetic peptide substrates (Figs. 19, 20).

Two-day-old NC4 spores contained predominant ZYKRase and BzPFRase activity with the latter being less abundant (Fig. 19). ZRRase activity was very low and represented less than 14% of the total proteinase activity measured. As spores age (7-29 days) ZYKRase and BzPFRase activities are very high, representing over 90% of the total proteinase activity measured. In spores aged 49 days ZRRase levels suddenly increased accounting for approximately 30% of the total proteinase activity. Cysteine proteinase-like activity (BzPFRase and ZRRase) was responsible for 50-60% of the total proteinase activity measured.
Figure 18.

Recovery of cysteine proteinase activity in cells after starvation in the presence of sucrose. Growing Ax2 amoebae were washed free of growth medium and starved in PB buffer containing 0.1 M sucrose for 6 hours. Cells were washed free of the starvation buffer, resuspended in fresh TM medium and allowed to recover for up to 6 hours. Cell extracts, and spent buffer and medium were electrophoresed using gelatin-SDS-PAGE, and the gels activated with DTT and stained. Intracellular (A) and extracellular (B) proteinases in cultures restored to growth medium (see Materials & Methods). The apparent molecular weights of the proteinases in kDa are shown.

(see also North et al., 1990c.)
Figure 18.
Figure 19.
Proteinase activation in spores and matrix of the strain NC4. Fruiting bodies were
allowed to develop on solid glucose-salts medium and spores were allowed to age
up to 49 days. Sori were collected and cysteine proteinase activity in spores was
determined (see Materials & Methods).

- ZRRase
- ZYKRase
- BzPFRase
Figure 20.
Proteinase activation in spores and matrix of the strain NC4. Fruiting bodies were allowed to develop on solid glucose-salts medium and spores were allowed to age up to 49 days. Sori were collected and cysteine proteinase activity in matrix material was determined (see Material & Methods).

\[
\begin{array}{c}
\text{ZRRase} \\
\text{ZYKRase} \\
\text{BzPFRase}
\end{array}
\]
Matrix material of NC4 fruiting bodies of all ages contained peptide-nitroanilide-hydrolysing activities at levels 10-fold those found in spores (Fig. 20). The high specific activities of the enzymes may be due to lower levels of total protein in the matrix material than in spores. As was the case in spores, matrix contains very little ZRTase activity, typically less than 10% of the total proteinase activity measured. ZYKRTase accounts for the majority of proteinase activity and BzPFRase is present at slightly lower levels. Overall proteinase activity increases from 2 days of age to 16 days after which it declines.

The cysteine proteinase activity examined appeared to be stable inside the spore for at least 28 days, and as long as 49 days. Spore dormancy imposes a metabolic quiescence, which extends to RNA and protein synthesis. Thus, it is unlikely that the increase in ZRTase activity was due to an increase in the level of the enzyme. It is plausible that the specificity of one or more other cysteine proteinases is changed; proteolytic cleavage of the enzyme, or a change in the internal environment of the spore may affect substrate specificity. The reduction in proteinase activity had little correlation with the viability of old spores (data not shown).

Diminished proteinase activity in the matrix may be due to inactivation of the enzymes, either by proteolytic degradation, or denaturation possibly mediated by oxidation. Aged sorocarsps have been exposed to the atmosphere for longer periods and therefore are subjected to oxidation effects; spores provide a comparatively less harsh internal milieu. Humidity has an effect on sorocarp development and supposedly upon the matrix material itself. Normal evaporation of water from the sorocarp would concentrate the matrix material, which may include any number of
other proteinases and glycosidases, and may impose osmotic effects upon constituent proteins.

**Gelatin-SDS-PAGE Analysis of Proteinases in Spores and Matrix**

Dormant NC4 spores which were washed to remove matrix material, contained a major proteinase band having a relative mobility of 58 kDa as identified using gelatin-SDS-PAGE (Fig. 21). This proteinase, though present after DTT treatment, was not considered to be a cysteine proteinase because its activity did not show sensitivity to E-64. Instead, the 58 kDa proteinase band was believed to be an aspartic proteinase observed previously (North & Whyte, 1984). Proteinase E, or ddAP58 as it will be referred to herein, (see nomenclature scheme, North et al., 1988) is present in both vegetative cells and spores as detected by hemoglobin-PAGE (North & Cotter, 1984). The vegetative form of the enzyme may be slightly inhibited by SDS since little activity can be detected using gelatin-SDS-PAGE (North et al., 1988). A minor 35 kDa proteinase band, which did not exhibit cysteine proteinase activity (E-64 sensitivity), is also present in some preparations.

The matrix material surrounding NC4 spores revealed a number of lower molecular weight proteinases, the most prominent being a novel proteinase band of approximately 18 kDa (Fig. 21). The enzyme which was sensitive to E-64 was considered to be a cysteine proteinase and was named ddCP18. Other minor proteinase bands having molecular weights of 22 kDa, 36 kDa and 38 kDa were
Figure 21.

Proteinase activity in NC4 spores and matrix as detected by gelatin-SDS-PAGE. Spores were harvested and washed free of matrix material. Spore extracts and matrix material were electrophoresed using gelatin-SDS-PAGE (see Materials & Methods). Depicted are major proteinase activity bands. Apparent molecular weights of the proteinases in kDa are shown.
also detected. Spores of the spontaneous germinating stain SG1 revealed spore and matrix proteinase banding patterns similar to NC4 spores (not shown).

**Effect of Sucrose on Spore Germination**

Heat-activated NC4 spores of *Dictyostelium discoideum* underwent synchronous germination when incubated in PB (Fig. 22). Spores began to swell after a postactivation lag of 1.0 hour. Spore swelling peaked at about 2.5 hours when at least 95% of the spores were swollen. Emergence of myxamoebae began at 2.5 hours and was complete by 5-6 hours postactivation after which about 95% of the spores had fully germinated.

Filter-sterilized sucrose was added to cells suspended in PB at 2.5 hours to give a final concentration of 0.1 M in the germination mixture. Spore germination was inhibited by sucrose as previously shown by Cotter (1977); emergence advanced to only 50% (Fig. 22a).

Figure 22b shows the effect of 0.25 M sucrose on NC4 spore germination. Inhibition of emergence appeared to be dependent upon sucrose concentration as only 35% of the myxamoebae had emerged 6 hours after activation.

**Cysteine Proteinase Activity During Germination**

Germinating NC4 spores exhibited differential proteinase activity when they were incubated in PB with or without sucrose. BzPFRase activity in heat-activated spores incubated in PB alone remained low during early spore swelling but increased during emergence peaking at 4.5 to 5 hours into the germination program (Fig. 23). Approximately 90% of the myxamoebae had emerged by this time.
Figure 22.
NC4 spore germination in the presence of sucrose. Spores were harvested, washed free of matrix material and heat-activated at 45°C for 30 minutes. Sucrose was added 2.5 hours postactivation to germinating spores to give final concentrations of 0.1 (A) or 0.25 M (B) (see Materials & Methods). Percentage swollen spores incubated in PB (○), percentage emerged myxamoebae (●); percentage swollen spores incubated in sucrose (□), percentage emerged myxamoebae (■).
Figure 22.
Figure 23.

Effect of sucrose on intracellular BzPFRase activity during NC4 spore germination. NC4 spores were harvested, washed free of matrix material and heat-activated at 45°C for 30 minutes. Activated spores were incubated at 23°C. Sucrose was added 2.5 hours postactivation to germinating spores to give final concentrations of 0.1 M (A) or 0.25 M (B) (see Materials & Methods). Specific BzPFRase activity in the presence (●) or absence (○) of sucrose.
Figure 23.
Emergence was delayed and BzPFRase activity was reduced in activated spores treated with sucrose. The BzPFRase activity peak, however, does not coincide with emergence and occurs 0.5 hour sooner than in spores which were left untreated. The new "sucrose-modified" BzPFRase peak occurred during early emergence.

ZRRase activity does not increase appreciably during NC4 spore germination (Fig. 24). Sucrose treatment merely diminishes whatever intracellular ZRRase activity is present.

The ZYKRRase activity in germinating spores is significantly affected by the sucrose-induced delay in emergence (Fig. 25). In spores germinated in PB alone the increase in ZYKRRase activity appears to coincide with emergence and peaks at maximum emergence, approximately 5 hours postactivation. When added during germination 0.1 M sucrose decreased ZYKRRase activity slightly whereas 0.25 M sucrose diminished the activity. In spite of the delay in emergence in sucrose-treated spores ZYKRRase activity still peaks at 5 hours postactivation, and enzyme activity no longer coincides with emergence.

The changes in BzPFRase and ZYKRRase activities may reflect an "uncoupling" of the activation of these two enzymes from the emergence process.

Effect of Sucrose on SG1 Spore Germination.

Sucrose affects spore germination similarly in NC4 and SG1 spores. A high concentration of sucrose (0.25 M) added to spores 2.5 hours after activation effectively delayed emergence of myxamoebae and severely blocked cysteine proteinase accumulation (not shown). When 0.1 M sucrose was added to swollen spores emergence was delayed (Fig. 26) but proteinase activity did accumulate.
Figure 24.

Effect of sucrose on intracellular ZRRase activity during NC4 spore germination. NC4 spores were harvested, washed free of matrix material and heat-activated at 45°C for 30 minutes. Activated spores were incubated at 23°C. Sucrose was added 2.5 hours postactivation to germinating spores to give final concentrations of 0.1 M (A) or 0.25 M (B) (see Materials & Methods). Specific ZRRase activity in the presence (●) or absence (○) of sucrose.
Figure 24.
Figure 25.

Effect of sucrose on intracellular ZYKRase activity during NC4 spore germination. NC4 spores were harvested, washed free of matrix material and heat-activated at 45°C for 30 minutes. Activated spores were incubated at 23°C. Sucrose was added 2.5 hours postactivation to germinating spores to give final concentrations of 0.1 M (A) or 0.25 M (B) (see Materials & Methods). Specific ZYKRase activity in the presence (●) or absence (〇) of sucrose.
Figure 25.
Effect of sucrose on SG1 spore germination. SG1 spores were heat-activated and allowed to germinate. After 2.5 hours the suspension was divided into two portions, one receiving sucrose to a final concentration of 0.1 M while the remaining portion was untreated (see Materials & Methods). Percentage spores swollen in PB (○) and in 0.1 M sucrose (□); percentage emerged myxamoebae in PB (●) and in 0.1 M sucrose (■).
Figure 27 depicts an increase in ZYKRase activity in the presence of 0.1 M sucrose; maximum activity occurs earlier when approximately 35% of the myxamoebae had emerged. No changes were observed in BzPFRase and ZRRase activities. Little or no cysteine proteinase activity was detected in the extracellular fraction from germinating spores.

**Gelatin-SDS-PAGE Analysis of Germinating Spores**

Figure 28 shows the electrophoretic separation of cysteine proteinases from spore extracts during germination of SG1 spores. Dormant spores possessed significant levels of ddAP58, which began to diminish shortly after heat activation. During early spore swelling, approximately 2-3 hours postactivation, a lower molecular weight cysteine proteinase, ddCP48 became apparent. This proteinase was also very active in vegetative amoebae (North et al., 1988). ddCP48 was accompanied by a proteinase having a slightly lower apparent molecular weight, ddCP43.

**Alternate Activation Treatments and Proteinase Activity**

Spores of *Dictyostelium discoideum* can be activated to commence germination using any one of a number of treatments. Some of these may alter the timing of the events in germination. Two of the proteinase activities examined in the current work, BzPFRase (North & Cotter, 1984) and ZYKRase accumulate concurrently with the emergence of myxamoebae. The activation treatments used in this study (heat, autoactivation, DMSO) may help uncouple emergence and proteinase accumulation.
Figure 27.

Changes in cysteine proteinase activity during SG1 spore germination in the presence of sucrose. Heat-activated SG1 spores were allowed to germinate. After 2.5 hours the suspension was divided into two portions, one receiving sucrose to give a final concentration of 0.1 M and the other portion untreated (see Materials & Methods). ZYKase activity in the presence (□) and (○) of 0.1 M sucrose.
Figure 27.
Figure 28.

Gelatin-SDS-PAGE analysis of proteinase activity during SG1 spore germination.

Spore extracts were electrophoresed on a 7.5% gelatin-SDS-PAGE gel. The gel was incubated at pH 4 with 0.1 mM DTT and stained with Coomassie Blue (see Materials & Methods). D, dormant spores; 0-7, time (in hours) after heat activation. The apparent molecular weights of the proteinases in kDa are shown.

(see also North et al., 1990a.)
Figure 28.

Grid 01 2 3 4 5 6 7

D 3 4 5 6 7

Time (h)

AP58 48 43
Heat activated SG1 spore germination proceeded very much like the germination of heat activated NC4 spores (Fig. 29). Spore swelling began at 1.0 hour postactivation and advanced rapidly peaking at 2.5 hours. Emergence of myxamoebae began at this time and continued logarithmically to 5 hours postactivation reaching a level of 93%.

Accumulation of proteinase activity in germinating SG1 spores was similar to that in germinating NC4 spores (Fig. 30). ZYKRase activity began to increase 2 hours postactivation and coincided with emergence. BzPFRase activity also increased peaking one hour sooner than ZYKRase.

Spores of the mutant SG1 were able to germinate spontaneously in the absence of heat activation (Fig. 31). Both swelling and emergence were approximately 0.5 to 1 hour later than in heat-activated SG1 spore germination. Figure 32 depicts the intracellular levels of hydrolytic activity against ZRR, ZYKR, and BzPFR during autoactivated germination. Enzymatic activity levels against all three synthetic substrates remained low during a lag period of 3 hours. After 4 hours of germination proteinase activities rapidly increased reaching maximum levels at 6 hours, concomitant with maximal emergence. The accumulation of proteinases was delayed by approximately 0.5 hour.

Spores activated with DMSO swelled sooner than heat or autoactivated SG1 spores (Fig. 33). Emergence of myxamoebae from DMSO-activated spores, however, was delayed by 0.5 to 1.0 hour but the accumulation of proteinase activity occurred sooner than in heat-activated spores (Fig. 34). This accumulation commenced before most of the myxamoebae had emerged; at 50% emergence the maximum ZYKRase and BzPFRase activities had been reached. This is in sharp
Figure 29.
Heat-activated SG1 spore germination. SG1 spores were harvested, washed free of matrix material and heat-activated at 45°C for 30 minutes. Activated spores were incubated at 23°C (see Materials & Methods). Percentage swollen spores (○) and percentage emerged myxamoebae (●) were recorded.
Figure 30.
Intracellular cysteine proteinase activity during heat-activated SG1 spore germination. SG1 spores were harvested, washed free of matrix material and heat-activated at 45°C for 30 minutes. Activated spores were incubated at 23°C. Cell extracts were assayed for activity against synthetic peptide substrates (see Materials & Methods). ZYKase activity (□); BzPFRase activity (∆); ZRRase activity (○).
Figure 31.
Auto-activated SG1 spore germination. SG1 spores were harvested, washed free of matrix material and were incubated at 23°C (see Materials & Methods). Percentage swollen spore (⊙) and percentage emerged myxamoebae (●) were recorded.
Figure 31.

% Swollen Spores
or Emerged Myxamoebae

Time (h)
Figure 32.

Intracellular cysteine proteinase activity during auto-activated SG1 spore germination. SG1 spores were harvested, washed free of matrix material and were incubated at 23°C. Cell extracts were assayed for activity against synthetic peptide substrates (see Materials & Methods).

ZYKRase activity (□); BzPFRase activity (△); ZRRase activity (○).
Figure 32.
Figure 33.

DMSO-activated SG1 spore germination. SG1 spores were harvested, washed free of matrix material and incubated in 20% DMSO at 23°C for 30 minutes. Activated spores were incubated at 23°C (see Materials & Methods). Percentage swollen spores (○) and percentage emerged myxamoebae (●) were recorded.
Figure 34.
Intracellular cysteine proteinase activity during DMSO-activated SG1 spore germination. SG1 spores were harvested, washed free of matrix material and incubated in 20% DMSO at 23°C for 30 minutes. Activated spores were incubated at 23°C. Cell extracts were assayed for activity against synthetic substrates (see Materials & Methods). ZYKase activity (□); BzPFase activity (△); ZRase activity (○).
Figure 34.
contrast to heat-activated and autoactivated spores in which maximum ZYKRase
and BzPFRase activities are reached when 70% of myxamoebae had emerged.

Both NC4 and SG1 spores exhibited an increase in intracellular ZYKRase
activity during germination. The final level of the enzyme in NC4 spores, however,
is 4-fold less than in SG1 spores.

Extracellular levels of cysteine proteinase activity were too low to be
efficiently detected in germinating spore suspensions and secretion kinetics for the
enzymes are not shown.
DISCUSSION

Secretion of Acid Phosphatase

*Dictyostelium* offers an interesting model in which to study the lysosomal system. Many microorganisms obtain nutrients through endocytosis, and *Dictyostelium* especially possesses well-developed phagocytic machinery. In nature, myxamoebae engulf soil bacteria, whereas axenically grown laboratory strains of *Dictyostelium* thrive well on nutrient medium, feeding by fluid-phase pinocytosis. Endosomes containing engulfed material fuse with primary lysosomes permitting hydrolytic enzymes to degrade the contents of the endocytic vesicle. Permeases in the secondary lysosome membrane allow release of nutrients into the cytosol after which the waste byproducts and lysosomal enzymes are released into the extracellular surroundings. Endocytosis of certain nutrients impacts upon intracellular vesicular traffic, and may affect the release of lysosomal enzymes (Klein *et al.*, 1988).

Release of lysosomal enzymes by *Dictyostelium discoideum* is nutritionally and developmentally regulated. Lysosomal carbohydrases, and acid phosphatase are secreted during starvation (Dimond *et al.*, 1981). Addition of non-metabolizable disaccharides such as sucrose promotes release and alters the secretion kinetics of lysosomal enzymes (Crean & Rossomando, 1979; Seshadri *et al.*, 1986).

The pattern of acid phosphatase secretion from starving myxamoebae is linear and the extracellular fraction represents more than a third of the total cellular activity. The addition of 0.1 M sucrose to starving cells induces secretion of acid phosphatase such that over two thirds of the total activity is detected extracellularly.
Moreover, the kinetics of secretion resemble the sigmoidal pattern observed for the secretion of glycosidases during starvation in phosphate buffer (Dimond et al., 1981). Thus, not only is there differential secretion among lysosomal enzymes, but the secretion patterns of one species of enzyme may differ under different conditions.

Under heat stress vegetative myxamoebae of *Dictyostelium* produce heat shock proteins which impart thermotolerance upon cells (Loomis & Wheeler, 1980). Heat shock protein synthesis occurs at the expense of putative cellular protein synthesis. The linear and sigmoidal kinetics of acid phosphatase secretion were unaffected by incubation at 30°C. Starving cells gradually lost viability during the six hour incubation period when incubated in phosphate buffer at 30°C. It is possible that the ability of cells to maintain an adequate heat shock response is diminished during starvation.

The gradual loss in viability did not alter the secretion kinetics. Sucrose in fact appeared to enhance the viability of cells at 30°C. The reason for this is not clear, however, sucrose may act as an osmotic agent in this situation. Increased intracellular osmotic pressure in the presence of sucrose may decrease the number of water molecules available to interact with cellular proteins. Under heat stress intracellular protein is prone to heat denaturation by the increased vibration of the water molecules interacting with hydrophillic amino acid residues. Thus, the occupation of water molecules by sucrose may enhance thermotolerance in stressed cells.
Protein synthesis is required for the efficient secretion of acid phosphatase. Cycloheximide inhibited secretion of the enzyme during starvation, and severely blocked the inductive effect of 0.1 M sucrose. The requirement for protein synthesis is similar whether the signal for secretion is generated internally (starvation) or externally (sucrose).

The mechanism by which sucrose exerts an inductive effect upon lysosomal enzyme secretion is yet unknown. Sucrose receptors or permeases do not exist on the plasma membrane of Dictyostelium and sucrose must be internalized by pinocytosis. Since it cannot cross the endosomal membrane to the cytosol sucrose remains in the secondary lysosome where it is osmotically active; water retention in the vesicles results in vacuolated cells (Cohn & Ehrenreich, 1969; Swanson et al., 1986). Starving Dictyostelium myxamoebae exhibit some vacuolation whereas those cells starved in sucrose become highly vacuolated to the extent that large vesicles distend the plasma membrane (Fig. 6).

The secretion of lysosomal enzymes appears to be a constitutive phenomenon. Eukaryotic microorganisms such as Acanthamoeba castellanis (Hohman & Bowers, 1984,1985), Dictyostelium discoideum (Dimond et al., 1981) and the protozoa Tetrahymena pyriformis and T. thermophila (Banno et al., 1987; Hunseker et al., 1987) release large amounts of lysosomal enzymes. Mammalian fibroblasts and macrophages have very active lysosomal systems (Schnyder & Baggioni, 1980; Leoni et al., 1985; Hermelin et al., 1988). While the mechanisms or the reasons for their release are still unclear two pathways for the secretion of enzymes have been observed. Errors in intracellular targeting of proteins to primary
lysosomes due to a lack of phosphomannosyl residues may result in the premature secretion of lysosomal enzymes (Creek & Sly, 1984; Von Figura & Hasilik, 1986).

Another mechanism by which lysosomal enzyme secretion occurs involves the membrane recycling process when secondary lysosomal vesicles fuse with the plasma membrane during eggestion (Hohman & Bower, 1984). Klein and coworkers (1988) used this phenomenon to study lysosomal enzyme secretion.

**Proteinase Secretion in Vegetative Amoebae**

The expression (North, 1985; North *et al.*, 1988) and secretion of proteinases (North, 1982b) in *Dictyostelium discoideum* had been studied earlier. The current findings elaborate upon the secretion of proteinases through the coordinate use of synthetic peptide substrates and gelatin-SDS-PAGE.

Of the three enzyme activities examined in vegetative amoebae BzPFRase was the most abundant. The enzyme(s) was secreted into the nutrient medium by growing cells, and into the buffer medium during starvation. As mentioned previously, BzPFRase exhibits sensitivity to E-64 and enhanced activity in the presence of DTT and is therefore considered to be a cysteine proteinase. The presence of another putative cysteine proteinase activity, ZRRase, was also detected. ZYKRase activity was not inhibited by E-64 and not activated significantly by DTT, and so was not considered to possess typical cysteine proteinase activity.

Gelatin-SDS-PAGE of intracellular samples revealed a number of proteinase bands ranging in apparent molecular weights from 30 kDa to 54 kDa. The activity of one in particular, a cysteine proteinase ddCP42, coincided with intracellular and secreted BzPFRase activity. Although ddCP42 likely contributes to much of the
BzPFRase activity the participation of ddCP41 and ddCP30 cannot be overlooked.

ddCP30 (previously named proteinase B) which has been purified and has been
found to possess BzPFRase activity (North & Whyte, 1984) likely contributes, at
least partly, to the cellular enzyme activity. Despite the weak gelatin hydrolytic
activity shown in this and previous studies (North et al., 1988) ddCP30 hydrolyses
the substrate on hemoglobin-PAGE gels (North & Harwood, 1979).

The secretion of proteinase activity from starved amoebae of *D. discoideum*
showed very interesting characteristics. Both BzPFRase and ZRRase activities were
secreted with linear kinetics by amoebae starved in phosphate buffer. ZYKKrase
activity was very low in extracellular fractions and was probably not actively
secreted. The proteinase bands ddCP51, ddCP42, ddCP41 and ddCP30 were also
released into the surrounding buffer. As was shown previously for other lysosomal
hydrolases (Seshadri et al., 1986) sucrose greatly enhanced the secretion of
proteolytic activity. Most dramatic was the stimulation of BzPFRase secretion; a
short lag followed by a rapid release of enzyme(s) resembled the shift of acid
phosphatase secretion kinetics to a sigmoidal pattern during sucrose treatment. This
change in BzPFRase coincided with the release of significant quantities of ddCP48
and ddCP41. The appearance of enzyme activity extracellularly accompanied a
decrease in intracellular enzyme levels. Sucrose promotes secretion of enzymes and
appears to depress the further synthesis of the enzymes.

Starved cells which are allowed to recover in growth medium cease to secrete
enzymes and resynthesize the released enzymes. Sucrose-treated cells, however,
continue to secrete enzymes, primarily BzPFRase activity and ddCP42. The
stimulatory effect of sucrose upon the cells persists for six hours after restoration to
growth medium. Intracellular levels of the proteinases also increase during recovery.

The current data support the Dimond et al. (1981) hypothesis of differential secretion among lysosomal enzymes. There is differential secretion among the proteinases studied; BzPFRase, a cysteine proteinase, is secreted while ZYKrase is not. The two cysteine proteinases BzPFRase and ZRRase are differentially regulated as revealed by sucrose stimulation. Furthermore, there are differences in the secretion of the cysteine proteinases and other lysosomal hydrolases such as trehalase and β-glucosidase (Seshadri et al., 1986), NAG, and α-mannosidase (Dimond et al., 1981). On the basis of starvation-induced and sucrose-enhanced secretion kinetics BzPFRase and acid phosphatase may share a similar release mechanism.

**Lysosomal Enzyme Secretion**

Lysosomal enzymes differ in the kinetics of secretion and in the extent to which they are secreted. Dimond and coworkers (1981) postulated three classes of secreted enzymes in *D. discoideum*. Generally, lysosomal hydrolase secretion can be observed as occurring rapidly as exhibited by NAG and β-glucosidase, or more slowly as in the release of acid phosphatase. Acid phosphatase secretion is further distinguished from the secretion characteristics of the other group of enzymes by sensitivity to cycloheximide and chloroquine diphosphate. A simple explanation of this heterogeneity in secretion kinetics is that lysosomal enzymes are packaged in different types of lysosomal vesicles; each type of vesicle permits mechanistically different enzyme secretion (Dimond et al., 1981). This model, however, does not
account for the differential secretion of acid phosphatase during starvation in the absence and presence of sucrose.

Alternatively, lysosomal enzymes may be packaged in homogeneous vesicles but possess different binding affinities for the inner surface of the lysosomal membrane. Enzymes having a greater binding affinity for the membrane (and possibly for a receptor) would exhibit a slower rate of release into the extracellular medium. If this is so the lysosomal membrane would have to accommodate alterations in binding affinities to account for differential secretion among enzymes during starvation. Moreover, altered secretion of one type of enzyme in the presence of a nonmetabolizable disaccharide (viz. acid phosphatase) would be indicative of a change in membrane binding affinity of the enzyme. This would be efficiently accomplished through an enzyme receptor mechanism associated with the lysosomal membrane.

Whichever mechanism of lysosomal enzyme secretion one prefers, it is evident that enzymes are not passively released as a consequence of egestion. Lysosomal enzymes are involved in intracellular degradation and may be egested along with waste materials via terminal phagosomes. Dimond and coworkers (1981) used latex beads to monitor the release of phagosomal material by *D. discoideum* amoebae. After they were ingested by the cells, the beads were found in secondary lysosomes and were egested along with other indigestible material. The timing of the release of the beads was different from the secretion of the lysosomal enzymes. Moreover, latex bead egestion was stimulated by cycloheximide and thus is distinguished from acid phosphatase secretion which is inhibited by the drug.
The exact mechanism of differential lysosomal enzyme secretion is unclear, but the involvement of a secretion signal is very likely. The early speculation that differential secretion is due to segregation of lysosomal enzymes to subpopulations of lysosomal vesicles, each one capable of releasing the enzymes in response to different stimuli (Dimond et al., 1981), has given way to other mechanisms. Sorting of proteins to different types of lysosomes would require a sorting signal, much like the signals required to sort proteins to different cellular locations. Thus, an alternative model describing the release of enzymes from secondary lysosomes would be contingent upon the segregation of enzymes within the lysosome itself. Differential secretion kinetics could be regulated by the stringency of the enzyme release-signal relationship. Bush and Cardelli (1989) showed that glycosidases and acid phosphatase were secreted with different kinetics but were colocalized in the same population of lysosomes. This suggests that some yet undefined mechanism recognizes different classes of enzymes at a stage after targeting to lysosomes but prior to secretion. It is plausible that an enzyme membrane receptor complex may regulate release from the lysosome, and the shift in secretion kinetics in the presence of sucrose, could be attributable to a shift in the binding affinity of the enzymes to the receptor.

**Proteinase Detection Using Gelatin-SDS-Page**

The variety of proteolytic enzymes in the different stages of the *Dictyostelium discoideum* life cycle complicates the study of cysteine proteinases. Previous electrophoretic studies of *Dictyostelium* proteinases employing copolymerized haemoglobin inadequately resolved many of the enzymes, especially
cysteine proteinases (North & Harwood, 1979). The gelatin-SDS-PAGE technique used here effectively separates cysteine proteinases and permits their detection. The procedure of North et al. (1988) was adapted to accommodate a mini-gel electrophoretic system; economy of both time and materials makes the technique particularly attractive. The first documented use of copolymerized gelatin and acrylamide was by Heussen and Dowdle (1980) who studied the activity of plasminogen activators and other proteinases, and the technique has since been used to detect trypsin-like and chymotrypsin-like proteinases in picogram quantities (DiStefano et al., 1988). The success of gelatin-SDS-PAGE depends upon a number of fortuitous features of gelatin and the electrophoretic system. Firstly, gelatin is a very suitable substrate because it is readily hydrolysed by most proteinases. Gelatin forms an immobile copolymerized proteinaceous network within the polyacrylamide matrix having no affect on the polymerization of acrylamide or the mobility of sample proteins and protein molecular weight markers (see Heussen & Dowdle, 1980; North et al., 1988). Secondly, the inhibition of proteins by SDS appears to be reversible: proteinases can be reactivated by removing SDS following electrophoresis. As in conventional SDS-PAGE (Laemmli, 1971) association of SDS with charged groups on amino acid residues results in uniformly-charged proteins and molecules are electrophoretically separated on the basis of molecular weight. Although β-mercaptoethanol was omitted in the sample buffer in the initial work by Heussen and Dowdle (1980) proteins incubated with the compound (without heating) migrate consistently (see also North et al., 1988). Removal of SDS and β-mercaptoethanol allows proteins to refold to their native
conformation. This manipulation apparently does not affect most proteinase activity.

Additional advantages of gelatin-SDS-PAGE make it a technique favoured over other enzyme detection techniques. The gelatin polymer has a structure having a small pore size which limits lateral diffusion of proteins. This feature is important during gel incubation over long intervals at room temperature prior to gel staining and "fixation" of proteins in the matrix. Small molecules such as proteinase activators and inhibitors move freely into the matrix and allow specific proteinase detection. Moreover, the incorporation of the substrate into the polyacrylamide gel exempts the requirement for a second indicator matrix (zymogen) to detect specific proteinases. Electrophoretic detection of the lysosomal enzymes acid phosphatase (Franek, unpublished results) and trehalase (Temesvari & Cotter, 1990) suffers from band diffusion over extended incubation with nonsupported chromogenic substrates.

Like other molecular biological techniques gelatin-SDS-PAGE bears some minor limitations. Electrophoresis at room temperature may result in trailing and smearing of proteolytic bands. This could be due to incomplete inactivation of proteinases by SDS. Alternatively, high salt concentration in any of the electrophoretic buffers may cause ionic discontinuities leading to local heating in the gel; gelatin melting may appear as smearing within a lane. Either of these problems can be relieved by performing electrophoresis in the cold 4 - 15°C (Heussen & Dowdle, 1980). The information obtained with this technique is qualitative and only the relative amounts of cysteine proteinases can be inferred. A biochemical assay gives specific cysteine proteinase activity and can be coupled to
the appearance or disappearance of proteinase bands. In addition, differential activity of particular proteinases against gelatin gives misleading information regarding the relative amounts of cysteine proteinase in representative bands.

Proteinase Activity in Spores and During Spore Germination

Spores and matrix material contained significant levels of ZYKRase activity which persisted in aged fruiting bodies. Gelatin-SDS-PAGE analysis revealed a novel major cysteine proteinase in the matrix of NC4 and SG1 spores, which has been named ddCP18. A number of other low molecular weight proteinases were also detected, one of which was ddCP22. Spores contained a very prominent aspartic proteinase, ddAP58.

During heat-activated spore germination ddAP58 disappears from the spore and two cysteine proteinases identified as ddCP48 and ddCP43 appear 2 to 3 hours postactivation.

The study of different spore activation treatment sheds new light on the involvement of proteinases during germination. Regardless of the activation treatment ZYKRase and BzPFRase contribute to much of the spore proteinase activity during germination. In autoactivated SG1 spores ZYKRase and BzPFRase begins to accumulate after emergence has commenced. This is in contrast to DMSO-activated and sucrose-treated spores in which the enzymes accumulate slightly before emergence. The increase in both enzymes in heat-activated SG1 spores coincides with emergence. In response to different activation treatments the pattern of enzyme activity shifts regardless of the extent of emergence; emergence proceeds whether the proteinases appear before, during or after the process. The
onset of accumulation of ZYKRase and BzPFRase activities appears to be linked more closely with events during the late swelling stage prior to emergence.

The enzymes responsible for ZYKRase and BzPFRase activity appear to be coordinately regulated even though they differ in their respective substrate specificities. BzPFRase activity is sensitive to E-64 and is enhanced by DTT; ZYKRase activity is slightly hindered by high concentrations of E-64 and is unaffected by DTT. The most prominent cysteine proteinase in germinating spores is ddCP48; the time of ddCP48 activation most resembled that of BzPFRase activity. Although ddCP48 is not detected in dormant spores it appears during germination at approximately 2 hours postactivation and accumulates throughout the remainder of the process. The enzyme(s) is also detected in vegetative cells.

The most prominent proteinase activity during spore germination was that of ZYKRase. Despite the lack of correlation between increased ZYKRase activity during germination and the appearance of proteinase bands on gelatin-SDS-PAGE gels, ZYKRase may yet play an important role in spore germination. Indeed, ZYKRase levels in newly emerged NC4 myxamoebae were higher than in vegetative cells of the same strain. In vegetative amoebae ZYKRase is not secreted in significant amounts, or even in the presence of sucrose. It is unlikely that the enzyme(s) responsible for ZYKRase is lysosomal and in fact may be active in the cytosol (M.J. North, unpublished observations). Curiously, ZYKRase activity in the spore matrix is substantial. Previous studies showed that matrix material contained a high proportion of lysosomal enzymes such as trehalase and β-glucosidase (Seshadri et al., 1986).
What is the Nature of ddCP18?

The abundance of lysosomal enzymes in the matrix material suggests that ddCP18 may also be a lysosomal enzyme. Unlike other lysosomal enzymes ddCP18 has not been detected in the spores or the starving amoebae examined in the current work. In an independent study, however, Ax2 growing logarithmically in nutrient medium were found to have low ddCP18 activity and high proteinase activity corresponding to ddCP22 as detected by gelatin-SDS-PAGE. Amoebae in late log or early stationary phase of growth were found to have high levels of ddCP18 and diminished levels ddCP22 (K.J. Franek, unpublished observations). The significance of these preliminary observations is unclear but the appearance of ddCP18 and the disappearance of ddCP22 activities may be triggered by nutrient depletion or a critical cell density. One may envision that these two proteinases may be activated in preparation for development (i.e. fruiting body formation) and participate in certain developmental events.

The molecular nature of ddCP18 is even more curious. Is it possible that ddCP18 is actually a proteolytically active subunit of a larger multimeric proteinase? Or even a proteolytic fragment? The nature of gelatin-SDS-PAGE itself may produce artifacts (see earlier section). The partially-denaturing system containing SDS and β-mercaptoethanol allows separation of polypeptides according to molecular size and not charge. The β-mercaptoethanol reduces disulfide bridges between linked subunits and portions of same strands. Heating of the sample before electrophoresis facilitates this reaction, and even though in the gelatin-SDS-PAGE technique used samples were not heated some reduction may have occurred. This
could have resulted in limited polypeptide fragmentation. When ddCP18 is eluted from gels and applied to a Sephadex gel filtration column much of the activity is detected in the fractions immediately following the void volume. In effect, ddCP18 coelutes with molecules four to six times its apparent molecular weight (K.J. Franek, unpublished observations; similar independent results corroborated by M.J. North). It is possible that under electrophoretic conditions a larger multimeric proteinase is separated into homologous or heterologous fragments some of which migrate to the ddCP18 position in gelatin-SDS-PAGE gels. Upon removal of the SDS and β-mercaptoethanol the ddCP18 subunits renature, re-form into functional proteolytic multimers, and hydrolyse the gelatin substrate. Alternatively each ddCP18 subunit may retain its own proteolytic activity.

Proteinases and Other Dictyostelium Lysosomal Hydrolases

One of the objectives of this study was to relate the activity and secretion of lysosomal and other proteinases to what is already known about lysosomal hydrolase activity in D. discoideum. Spore germination induced by different spore activating agents provides an excellent stage for comparison. Specifically, one can consider two other lysosomal enzymes, trehalase and β-glucosidase (Chan & Cotter, 1982 a,b) for opposition. Autoactivated spores produce trehalase and β-glucosidase early in germination whereas BzPFRase and ZRRase activities appear after emergence begins. Trehalase and β-glucosidase in DMSO-activated spores are synthesized before emergence as are BzPFRase and ZYKRase. It is evident that the lysosomal hydrolases and BzPFRase and ZYKRase are not coordinately regulated and that the enzymes may follow different agenda during spore germination. Despite
the lysosomal and secretory behaviour of BzPFRase the enzyme is not coordinately regulated with trehalase and β-glucosidase, but with ZYKRase, a non-secretory, non-lysosomal enzyme.

Dictyostelium Cysteine Proteinases and Mammalian Cathepsins

The sophisticated developmental life cycle as well as the phagocytic nature of the vegetative stage of *Dictyostelium* have been exploited as a model system for human cells. This relationship may be extended to include a comparison between *Dictyostelium* cysteine proteinases and mammalian cathepsins. The substrates specifically hydrolysed by cysteine proteinases, Bz-Pro-Phe-Arg-Nan and Z-Arg-Arg-Nan, contain the amino acid residues Phe-Arg and Arg-Arg in the P2-P1 positions respectively (P1 is the first residue adjacent to the peptide bond cleavage site, toward the amino terminal). The mammalian cysteine proteinase cathepsin L preferably hydrolyses substrates having hydrophobic P2 residues and is active against BzPFR. Cathepsin B prefers similar substrates having hydrophobic P2 residues and is very active against Z-Arg-Arg-Nan as well as Bz-Pro-Phe-Arg-Nan. Thus BzPFRase and ZRRase emulate cathepsin L and cathepsin B, respectively (see Kirschke & Barret, 1987; Barrett *et al*., 1988).

Cysteine Proteinases in Alternate Developmental Structures

The cellular slime molds are fortunate to participate in a number of different developmental pathways, and the researcher is fortunate to have such a "multiuse" tool at his or her disposal. *Dictyostelium* species exhibit vegetative growth, fruiting body formation, and macrocyst formation as well as spore germination and
pseudoplasmodium formation. Each stage may require proteinases to aid in
digestion of nutrients, degradation of proteins to replenish amino acid pools, and
regulation in the form of processing and modification of proteins. Generally,
cellular proteinases are secreted in preparation for, and during development.
Amoebae of *D. discoideum* secrete lysosomal enzymes including cysteine proteinases
during starvation, at the onset of development, and in response to different nutrients.
Recent studies have revealed interesting observations regarding cysteine proteinase
activity in two alternate developmental pathways; macrocyst and microcyst
formation.

One developmental pathway, involving parasexual cell fusion, leads to
macro cyst formation. Two cells of the homothallic organism
*Dictyostelium mucoroides* DM7 may fuse forming a "giant cell" which then engulfs
surrounding myxamoebae and encysts them. These "endocysts" are subsequently
degraded by lysosomal enzymes, of which cysteine proteinases are believed to play
a major role. Developing myxamoebae deplete two cysteine proteinases in initial
stages of macrocyst formation and two others throughout development. The
secretion of vegetative proteinases coincides with the appearance of three macrocyst-
specific cysteine proteinases. The two species of proteinases are thought to be
encoded by the same genes but differ in modifications (North *et al.*, 1991).

In response to starvation, and in the absence of light, vegetative myxamoebae
of *Polyspondylium pallidum* may forgo fruiting body formation to produce
individual microcysts which later germinate releasing amoebae. Development in the
cellular slime mold *P. pallidum* has been recently found to elicit an interesting
pattern of cysteine proteinase expression. Vegetative cells undergoing microcyst
formation secrete large amounts of ZRRase (North et al., 1991). This is unlike starved myxamoebae of *D. discoideum* (North, *et al.*, 1990c) and *D. mucoroides* (North *et al.*, 1990b), which initially contain amounts of BzPFRase and ZRRase similar to *P. pallidum*, but secrete much less of the latter enzyme.

Proteinase content of vegetative amoebae is dependent upon the available nutrient source (North *et al.*, 1984) and this aspect affects proteinase patterns in subsequent developmental stages (North *et al.*, 1991). Amoebae of *P. pallidum* grown axenically or in the presence of *E. coli* differ in predominant cysteine proteinase content as detected by gelatin-SDS-PAGE during microcyst formation. Microcysts having either nutrient history germinated normally, therefore the specific proteinases observed apparently have little bearing upon encystment or germination. More importantly, the work reveals the prudence with which one must interpret results of proteinase studies during stages in development; proteinases observed in current stages in the life cycle may relate more closely to a previous stage.
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