DIFFERENTIAL EXPRESSIONS AND ACTIVITIES OF THE CYSTEINE PROTEINASES OF CELLULAR SLIME MOLDS

by

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ABSTRACT

Of the proteinases present in the slime molds, the cysteine proteinases (CP) are of great interest. There are multiple forms of these proteinases within the cell and their expression is developmentally regulated with the majority of the activity found in the amoeba stage and decreasing with progression of the life cycle. These lysosomal proteinases are stored inactive inside the lysosyme and the production of mature proteinases requires them to be modified. This modification can be carried out *in vitro* on Gelatin-SDS-PAGE by subjecting the gel to the action of 10% acetic acid. The change of activity can be visualized by an increase of gelatin hydrolysis in the polyacrylamide gel. However, the modification is reversible as subsequent treatment with ammonia solutions inactivates the enzyme but allows additional re-activation. This suggests that activation is brought about by conformational change rather than by an irreversible cleavage, or loss of an inhibitor.

The activities of CP's are not only under developmental control but also depend upon the type of food source. Previous studies demonstrated that there is differential expression of CPs of selected slime molds when fed on bacteria, as opposed to glucose containing media. Cysteine Proteinase Converting Factor (CPCF) partially purified from the bacteria, when added to the media caused the proteinases shifts.

In the present study the differential expression of the CPs has been examined in detail for two slime molds i.e., the AX3 strain of *Dictyostlium discoideum* and the WS320 strain of *Polysphondylium pallidum*. Lipopolysacharide (LPS) and peptidoglycan both components of the bacterial cell wall were tested for the CPCF activity. Additionally the effects of the presence bacterial components on the germination kinetics

were examined. This study shows that peptidoglycan is a very likely candidate for being cysteine proteinase converting factor. The peptidoglycan was able to induce changes in the types of proteinases expressed in the slime molds within the time expected time period. Secondly, it was able to override the dormancy of the wild type young spores of NC4, and it also affected the germination kinetics of SG1 spores by decreasing the lag period. Similar effects have been noted when whole bacterial cell extracts were used.

Further, this work also supports the view that the conformational change is responsible for the activation of cysteine proteinases in slime molds. It shows that it is possible to increase the proteolytic activity of the proteinase inside of the cell prior to the extraction of the protein and separation of the gel. The in situ activated proteinases show increased activities as opposed to the controls, and there is no detectable differences in the molecular weights of the enzymes.

DEDICATION

To my parents Mieczyslaw and Jadwiga Wolak
For constant encouragement and unconditional love, and quidance

To my wife Renata Wolak
For her companionship constant motivation, patience and
Love

Could not do it without YOU

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I. Introduction

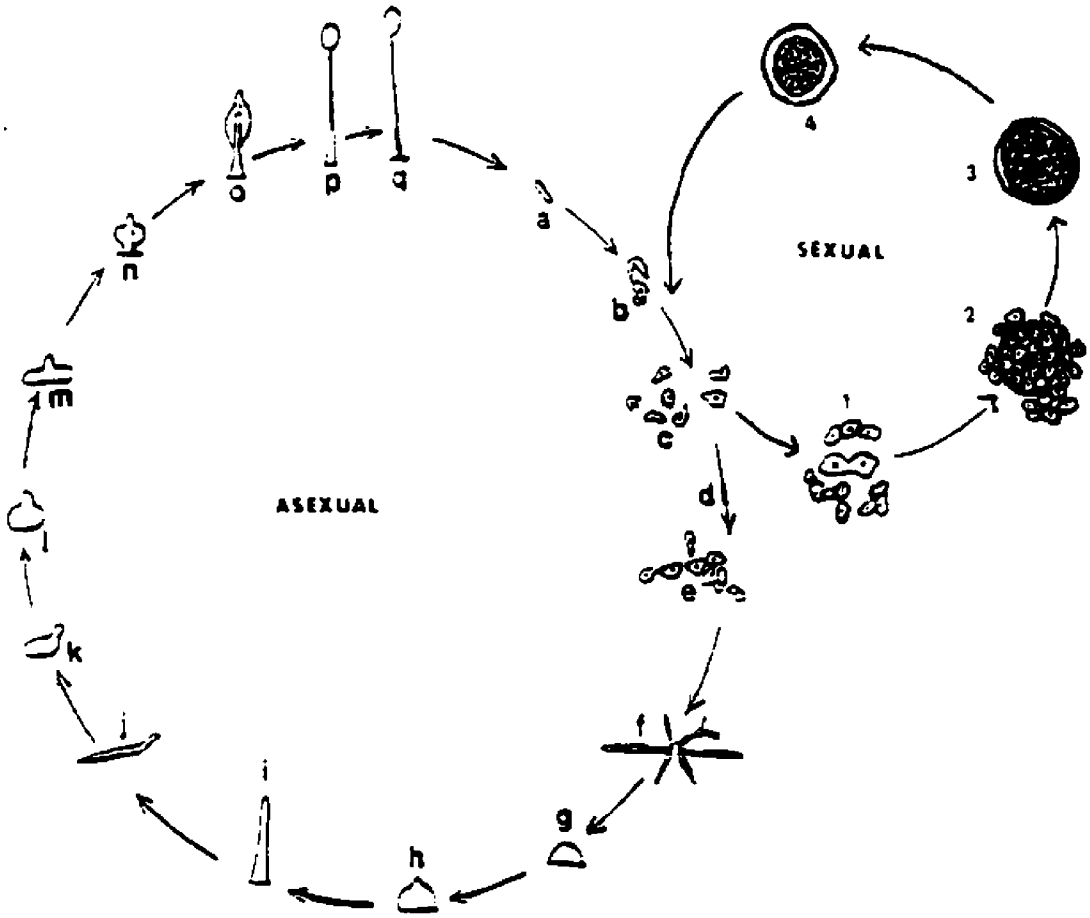
Cellular slime molds, members of the family Dictyosteliaceae, are valuable organisms for developmental studies. Although they are believed to have only 28,000 single copy genes, they are capable of entering three separate developmental cycles. *Dictyostelium discoideum*, first described by Raper (1935), is one of the most studied representatives of the family. It is capable of undergoing the asexual life cycle which is terminated by fruiting body formation. It can also enter an alternate sexual life cycle, where two cells of opposite mating types fuse together to form macrocysts. A third pathway is observed in the cellular slime mold *Polysphondylium pallidum*, the asexual cycle leading to the formation of microcysts.

I. 1.0 The Life Cycle of *Dictyostelium discoideum*.

The vegetative cells of *Dictyostelium discoideum* are phagocytic amoebae, feeding on common soil bacteria as well as other simple eukaryotes. Growth of the organism, by binary fission, continues until the food supplies are exhausted, followed by the onset of morphological differentiation six to eight hours later (Figure 1). The signal for the start of development involves secretion of cyclical pulses of adenosine-3', 5'-monophosphate (cAMP) by the founder cells (Barkley, 1969). The cAMP acts as a chemoattractant for the rest of the cell population. The surrounding cells are stimulated to similarly produce and secrete cAMP, in a pulsative manner, while at the same time migrating towards higher levels of the chemoattractant. Eventually a migratory multicellular pseudoplasmodium, also referred to as a slug or grex consisting of about 10^5

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

The events leading to the formation of macrocysts are outlined. Aggregation, cells aggregate together and two vegetative cells of opposite mating type fuse to form a zygote (**1**); developing macrocysts, the zygote engulf surrounding amoebae which become endocytes and primary wall is formed (**2**); maturing aggregates, most amoebae are engulfed and a secondary wall is formed (**3**); aged macrocysts, endocytes are degraded and the cytoplasm shrinks away from the outer wall (**4**) (Cotter *et al.*, 1992).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cells is formed. It migrates horizontally along the substratum in search of additional food sources (Loomis, 1982). However, it has to be noted that unlike the true slime molds the cells within the slugs of *Dictyostelium* retain their individual cellular identity as they are separated by plasma membranes. The cells secrete a mucopolysaccharide substance which forms a slimy sheath around the slug. The sheath is responsible for the slime trails left behind the migrating slug. In addition to being chemotactic the slug is also thermo- as well as phototactic (Bonner 1950). If the slug encounters suitable food sources it disintegrates into individual cells. Otherwise, the final stage of development is entered. At this point the aggregate forms a mound and cells begin to differentiate and by doing so they enter the final, developmental phase of the life cycle as a multicellular organism. Cells located at the tip of the mound begin to show pre-stalk characteristics, whereas the remaining cells exhibit characteristics of pre-spore cells (Jermyn *et al.*,1989). Culmination of the life cycle is dependent upon environmental factors; it will only take place in the presence of light, low humidity and high temperature (Raper, 1984). In the presence of these factors, the slug rounds up and stalk cells secrete cellulose, laying down rigid cell walls. Eventually the stalk cells expand and die. As this takes place the downward movement of the column's stalk cells lifts developing pre-spore cells vertically from the substratum (Bonner, 1967). At the top of the stalk these pre-spore cells mature and form spores. The spores of the mature fruiting bodies are surrounded by a viscous matrix which consists of complex carbohydrates, trehalose (Ceccarini & Fiosa, 1965), hydrolytic enzymes (Chan *et al.*, 1981), and discadenine, an autoinhibitor of spore germination (Abe *et al.*, 1976). The spores are capsule shaped, 6-9 μm long and 2.5-3.5μm in diameter (Raper 1935, Bonner 1967) and the spore wall is at least 200nm thick.

The spore is resistant to unfavourable conditions such as extremes of heat or cold, and desiccation (for review see Loomis, 1975, Cotter *et al.*, 1992).

I. 1.1 Spore Germination

There are a number of factors known to be capable of preventing spore germination. The principal component present in the sorocarp of the mature fruiting body is the autoinhibitor. This inhibitor has been suggested to be an adenine derivative called discadenine (Abe *et al.*, 1976). Although the mode of action for discadenine is still not fully known, recent work by Zinda and Singleton (1998) suggest that the autoinhibitor inhibits the phosphodiesterase activity of RegA. Other factors which prevent spore germination include osmotic pressure equivalent to 0.2 M sucrose (Cotter, 1977) which may result from high levels of ammonium phosphate in the matrix of the sorus (Cotter *et al.*, 1999).

Spore germination can be divided into the following stages: i) activation, ii) post activation lag, iii) swelling and iv) emergence (Cotter, 1975). Activation is defined as the period of time during which the spore is exposed to an activating agent. Post activation lag is the period of time after the termination of the activating stimulus and prior to spore swelling (Cotter *et al.*, 1992). The stages of spore swelling and amoeba emergence can be quite easily visualized with the help of phase-contrast microscopy. Over the years a number of physical and chemical treatments have been described which are capable of activating the dormant spore; these include incubation in 1% peptone (Cotter & Raper, 1966), 8M urea (Cotter & O'Connell 1976), 2M dimethyl urea, 2M tetramethyl urea, 6M guanidine HCl (Cotter *et al.*, 1979), 3M ethylene glycol (Cotter, 1977), 20% DMSO

(Cotter *et al.*, 1976), and controlled heat shock at 45°C (Cotter & Raper, 1966). Most of these treatments have destructive effects on some of the proteins and/or organelles within the cell. For example, DMSO is suggested to disrupt mitochondrial function, which is accompanied by a rapid uptake of oxygen following the removal of the protein denaturant (Cotter *et al.*, 1976). Interestingly, respiratory inhibitors such as azide and cyanide, cause spore deactivation. It should be noted that activation of the spore is reversible; however, deactivation has to take place during the lag phase prior to spore swelling (Cotter, 1977).

Wild type spores are capable of auto-germination after ageing for 10-14 days. The mutant strains SG1 and SG2 derived from wild types NC4 and V12, lack the ageing requirement (Dahlberg & Cotter, 1978). These spores germinate soon after the fruiting bodies mature. To date the genes required for spontaneous germination have not been identified.

I. 1.2 Sexual Life Cycle

An additional life cycle of *Dictyostelium discoideum* is the sexual or macrocyst cycle (Figure 1). Macrocysts are dormant structures formed by fusion of two amoebae. The two types of macrocysts are homothallic, created by fusion of identical cells of the same species and heterothallic, formed by fusion of cells of opposite mating types (Raper 1984).

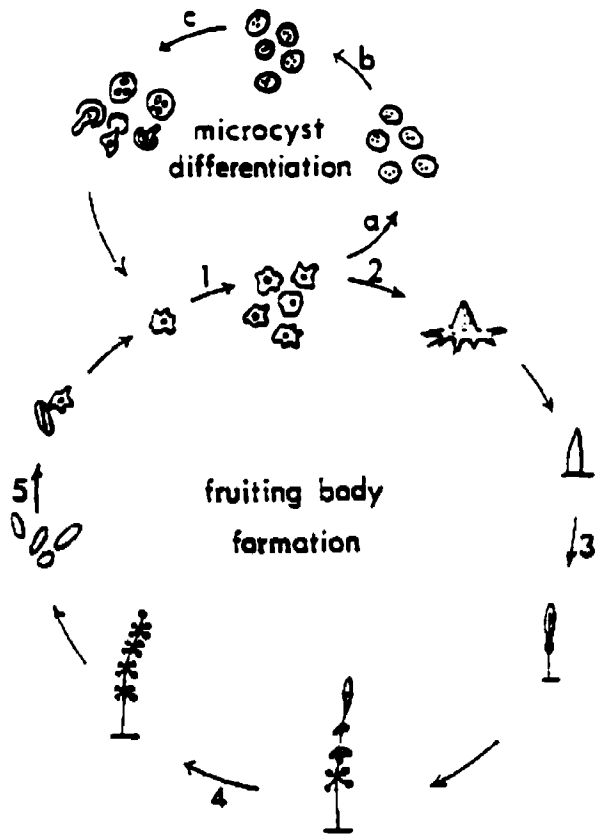
The sexual life cycle is heavily dependent on a number of environmental factors which include absence of light, and high humidity as well as the presence of very small amounts of magnesium, nitrate, sulphate, chlorine, and calcium (Erdors, *et al.*, 1976; Raper 1984). If these conditions are met, two cells fuse together forming an early

zygote or a giant cell. Once formed this cell begins to engulf surrounding amoebae of both mating types. The engulfed amoebae become endocytes (Raper, 1984). During this process a primary wall forms, surrounding a group of pre-endocytes (O'Day, 1979), before they are engulfed by the zygote. Upon complete ingestion of all amoebae, secondary and tertiary cellulose walls are constructed, forming a mature macrocyst. The final stage of macrocyst formation is characterized by the degradation of the endocytes resulting in a dark and granular cytoplasm (Raper, 1984). Termination of this stage is marked by entrance into dormancy by the macrocyst, where it will remain for weeks or months until conditions stimulate its germination. Whether an amoeba will enter the developmental life cycle or re-enter the sexual life cycle is also dependent on environmental factors.

I. 2.0 Asexual Life Cycle of *Polysphondylium pallidum*

The simplest of all the life cycles is the asexual, microcyst pathway as evidenced in *Polysphondylium pallidum* (Francis, 1982). Microcysts formation takes place after vegetative growth in the absence of light and moisture. When cells are subjected to these conditions they will round up and individually encyst to form dormant microcysts. Encystment involves production of a microcyst wall composed of an individually deposited loose fibrillar layer of cellulose around the cell (Hohl *et al.*, 1970). Germination of microcysts requires only rehydration, which can be achieved by placing the cells in a sterile-non nutrient media (Cotter & Raper 1968).

Figure 2. **Asexual life cycle of *Polysphondylium pallidum*.** After growth (**1**), amoebae either differentiate as microcysts or undergo multicellular development to form a fruiting body. The major events of fruiting body formation (**2-5**) follows the same as the asexual life cycle of *D. discoideum* (Figure 1). The microcysts differentiation pathway involves the rounding up of the amoebae (**a**), the formation of a definite cell wall (**b**), and germination (**c**) (O'Day and Francis, 1973).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fruiting body
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I. 3.0 Proteases

Proteases are very important and diversified enzymes found in organisms ranging from viruses and simple microbial cells to complex multicellular organisms (Mala *et al.*, 1998). The importance of proteases is highlighted by the wide array of functions which they perform within and outside of the cell. One of these functions is to maintain protein turnover (Bohely & Seglen, 1992), resulting in production of reusable amino acids for new protein synthesis as well as for energy production and hence, nutrition (Mala *et al.*, 1998). Additional physiological functions of proteases include activation and inactivation of other enzymes (Smeekens, 1993) as well as regulation of gene expression (Roberts *et al.*, 1977). The importance of properly functioning proteases is evident in diseases such as muscular dystrophy, diabetes, cancer and multiple sclerosis (Bond & Butler, 1987). Proteases are also a major component of commercially available enzymes where they are used in a wide variety of applications ranging from food to pharmaceutical industries (Mala *et al.*, 1998).

Proteases are generally designated as either peptidases (exo-peptidases) or proteinases (endo-proteinases). Exo-peptidases cleave their substrate at the carboxyl or amino end of the substrate, whereas endo-proteinases cleave proteins internally (Bond & Butler, 1987). It should be noted that the term proteases encompasses both the peptidases and proteinases however, for the purpose of this work the terms proteinase and protease will be used interchangeably. These groups are then further subdivided into four subgroups based on the essential catalytic residues at their active site: cysteine-, serine-, aspartyl-, and metallo-proteases. However, it is necessary to recognize that there are a

number of proteases which do not necessarily fit into any of these categories (Mala *et al.*, 1998).

In *Dictyostelium discoideum* the most studied proteases are the cysteine class due to their unusual characteristics to be described in latter chapters.

I. 3.1 Cysteine Proteases

Present mostly in the lysosomes and cytosol, cysteine proteinases (also referred to as thiol proteases), are considered to be "true intracellular proteases" (Bond and Butler, 1987), but secretion does occur in some circumstances. For example, in macrophages and fibroblasts the enzymes are secreted in an inactive form and only under proper conditions are they activated (Kirsche *et al.*, 1995). The enzymes have optimal activity at neutral pHs but lysosomal species often have an acidic optima.

Cysteine proteases are generally characterized by the presence of histidine and cysteine residues at their active sites, (Bond and Butler, 1987; Mala *et al.*, 1998). These residues are highly conserved among these proteinases, however, the order differs: His-Cys or Cys-His. Additionally, glutamine and asparagine are important to the formation and conformations of the active site in some members of this group (Rawlings & Barret, 1994). Four major groups of cysteine proteinases are recognized: (i) papain like (ii) trypsin like (iii) specific to glutamic acid, and (iv) other (Mala *et al.*, 1998).

A member of the cysteine proteinases, which has been intensively studied is papain, isolated from papaya fruits. These studies have helped in elucidating the mechanism of action of cysteine proteinases. Papain possesses a large groove where the thiol group of Cys-25 lies close to the imidazole ring of His-159 (Drenth *et al.*, 1976).

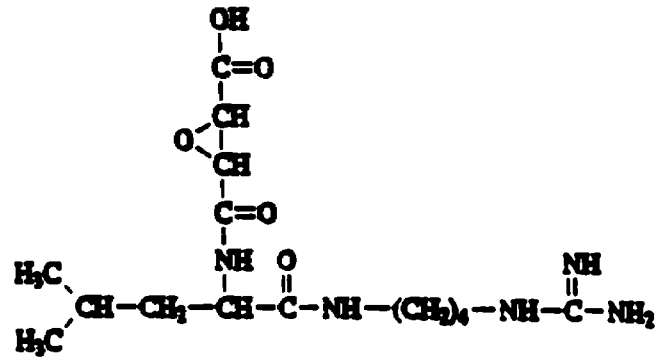
The SH- group of Cys-25 acts as a nucleophile, which attacks the carbonyl group of the substrate peptide bond. A charge relay assisted by the imidazole group of His-159, and possibly by ASP-158, results in the formation of a tetrahedral intermediate (Polgar & Halasz, 1982). An acyl-enzyme intermediate, a thioester, is produced as the substrate peptide bond is cleaved. The product of the catalysis is removed with the addition of water and creation of another tetrahedral intermediate (Darnell et al., 1990).

There are a number of commercially available cysteine proteinase specific inhibitors. An example is L-trans-epoxysuccinyl-leucylamido(4-guanidin)butane, more commonly known as E-64, which acts irreversibly to bind to the thiol group of the active site of a proteinase by forming a thioether bond with cysteine residues. An second example of a commercially available selectively reversible inhibitor is the 2,2'-Dithiodipyridine (Figure 3). Additional inhibitors include iodoacetamide, iodoacetate, n-ethylmaleimide, and p-chloromercuribenzoate (Bond and Butler 1987).

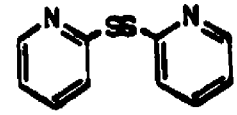
Numerous factors control the physiological activity of cysteine proteinases. Major players in the control of cysteine proteinases are naturally occurring proteinase inhibitor proteins. These include the cystatin family of inhibitors, specific for most cysteine proteinases (Bobek & Levine, 1992), the calpastatins, specific for the calpains (Pontremolli *et al.*, 1991), and α2-macroglobulin, which is a non specific inhibitor for all four classes of endoproteinases (Borth, 1992). Additional levels of control of these destructive enzymes include compartmentalization, localization, presence of metabolites, post-translational modifications and sequestration (Twining, 1994).

Figure 3. **Stuctures of two commercially available cysteine proteinases inhibitors L-trans-epoxysuccinyl-leucylamido(4-guanidin)butane (E64) (A) and 2,2'-dithiodipyridine (B).**

A)



B)



It is not the purpose of this work to extensively review all of the groups of proteases. Bond and Butler (1987) and Mala *et al.* 1998 provide more detailed treatments of the subject.

I. 4.0 Cysteine Proteinases in *Dictyostelium discoideum*

Among the hydrolytic enzymes present in *Dictyostelium discoideum* cysteine proteinases appear to have the most interesting and unusual characteristics. Activity of these enzymes appears to be under strict control by the lysosomal pH. Their activity also appears to be developmentally regulated and different types of proteinases are utilized during development. Additionally, the proteinases expressed are dependent upon the type of food sources available to the slime mold. Finally the carbohydrate modifications of the enzymes are not typical of other lysosomal enzymes.

I. 4.1 Properties of Cysteine Proteinases in *Dictyostelium discoideum*

The most active proteinases in *Dictyostelium discoideum* are of the aspartic and cysteine types. However, while the activity of the aspartic forms remain relatively unchanged during the life cycle of the organism that of cysteine proteinases differs significantly throughout both the vegetative and developmental phases. Additionally CPs are also subject to nutritional regulation (North, 1985; North et al., 1988). Dormant spores possess little cysteine proteinase activity compared to the cysteine proteinase activity found in the extracts of vegetative cells. It is during the germination process that the cysteine proteinase activity becomes apparent. The change is readily visualised using

one-dimensional gelatin containing sodium dodecyl sulfate-polyacrylamide gels (also referred to as zymograms or gelatin-SDS-PAGE). The major cysteine proteinase activity detected in this fashion is attributed to an enzyme with apparent molecular weight of 48 kDa (ddCP48). Additional minor proteinase activity is also detected and is attributed to a proteinase identified as 43kDa (ddCP43) (North *et al.*, 1990). Treatments of the zymograms with 10% (v/v) glacial acetic acid, after electrophoresis, but before incubation in acetate buffer at pH 4.0, reveals that the ddCP48 is present in the protein extracts of dormant spores. This result suggests that the activation of cysteine proteinases may be due to a conformational change. This view is supported by two-dimensional gel electrophoresis which does not detect any changes in apparent molecular weight of the enzymes before and after acid activation (Cotter *et al.*, 1992; North *et al.*, 1996). Moreover, the acid activation appears to be reversible since secondary treatments of the gel slab with ammonium chloride at pH 9.5-10.5 for one minute greatly diminishes the activities of the activated proteinases. This deactivation is again reversible; additional treatment with acetic acid results in reappearance of proteinase activity (Cotter *et al.*, 1997). Cavallo *et al.* (submitted) presents further evidence for the hypothesis of reversible conformational change as the mechanism of cysteine proteinase activation. Using the irreversible inhibitor E-64, she showed that only active proteinases are affected by this suicide inhibitor; extracts of dormant spores do not react with E-64, until after they are acid activated. It is therefore possible that the control of activity of cysteine proteinases *in vivo* may be achieved by shifts of lysosomal pH. This process in turn may be controlled in part by vacuolar H^+-ATPase. The acidification process is greatly reduced in a mutant strain HGR8 partially defective in vacuolar H+-ATPase as well as in

the parent strain in the presence of bafilomycin A1, a selective inhibitor of this enzyme (Aubry *et al.*, 1993). The ability of the lysosomal cysteine proteinases to be acid activated is not unique to *Dictyostelium discoideum*. Others organisms in which the phenomenon is detectable include *D. mucoroides*, *D. purpereum* and *Polysphondylium pallidum* (K.E. Gale, *unpublished results*).

During spore germination the net cysteine proteinase activity increases and remains at high levels in the cells during vegetative growth. The enzymes detected at these stages with the help of zymograms also demonstrate reversible activation/deactivation through pH shifts (North & Cotter 1991).

Myxamoebae may be grown in association with bacteria such as *E.coli*, and *K. aerogenes*; some strains are capable of growth in a liquid partially defined medium. Ashworth and co-workers (Watts and Ashworth, 1970; Ashworth and Quance, 1972) have characterized differences in the activities of acid hydrolases between axenically and bacterially grown amoebae. Axenic cells produce higher levels of certain enzymes such as β-N-acetylglucosaminidase and α-mannosidase. Generally, cells grown in the presence of bacteria have higher levels of proteolytic activity than cells grown in their absence.

The expression of proteinases within vegetative cells depends upon the growth medium. Axenically grown myxamoebae of the strain Ax2 were found by electrophoretic analysis to produce a number of cysteine proteinases (North, 1988). In the growth medium containing glucose as major carbohydrate source, two major intracellular forms of 51 kDa and 45 kDa, as well as ddCP30, ddCP38 and ddCP42 have been discovered using gelatin-SDS-PAGE. However, addition of bacteria (gram-

negative *E.coli, K. aerogenes*, gram-positive *Micrococcus lysodeikticus*, and *Bacillus subtilis*) to the growth medium has resulted in a dramatic change in the observable pattern of cysteine proteinase expression. Zymograms have revealed three prominent bands, ddCP48, ddCP43, and ddCP38B, as well as low levels of ddCP30. However, the total proteinase activity remains similar in the two treatments. An acid-precipitable macromolecule named the cysteine proteinase converting factor (CPCF), originating from the bacterial cell wall was found to have the ability to induce the switch from A-pattern (axenically grown) to B-form (grown in presence of bacteria) cysteine proteinase expression. North (1988) hypothesized that the possible candidate for the CPCF is peptidoglycan. This phenomenon has also been observed in yet another member of the slime molds *Polysphondylium pallidum*, strain PPHU8 (North *et al.*, 1984). In this case however, there was an increase in the net proteinase activities in the protein extracts derived from bacterially treated cells.

Developmental regulation of cysteine proteinase activity is evident from molecular biological studies of *Dictyostelium* in the later stages of the life cycle. Genes encoding cysteine proteinases CP1 (*cprA*), and CP2 (*cprB*), have a high degree of similarity to members of the papain superfamily, while CP3 may be a truncated cysteine proteinase (Pears *et al.*, 1985; Presse *et al.*, 1986a,b; Williams *et al.*, 1985). These genes are expressed late in development, when overall cysteine proteinase activity appears to decrease (North and Cotter, 1991). The proteins encoded by these genes have not yet been identified and characterized in detail.

I. 4.2 Cysteine Proteinases in *Dictyostelium discoideum* – Differential Modifications

Vegetative cells of *Dictyostelium discoideum* release numerous proteinase activities when starved in shaken suspensions (Rossomando, *et al.*, 1978). In 1982 North presented evidence for the possible distinct localization of cysteine proteinases as opposed to other acidic hydrolases. After re-suspension of Ax2 myxamoebae in phosphate buffer, activity of various enzymes was detected including one of the β-N-acetylglucoseaminidases and acid phosphatases. However, incubation of the cells in the presence of cyanide inhibited the release of these typical lysosomal hydrolases, but not the thiol proteases. This indicated a differential type of storage of the cysteine proteinases compared to other hydrolytic lysosomal enzymes. Further studies of lysosomal enzymes such as α-mannosidase and β-glucosidase detected the presence of N-linked oligosaccharides, containing Man-6-P in a stable phosphomethyldiester linkage (Freeze 1997; Freeze and Wolgast, 1986). The cysteine proteinases in *Dictyostelium*, however, have been found to contain a novel serine-rich domain which was determined to be the site of phosphoglycosylation with GlcNAc-1-P (Souza *et al.*, 1995) and it has been determined that the two kinds of sugar modifications are not overlapping (Metha *et al.*, 1996). Most importantly Souza and co-workers (1997) have shown that the proteins with different sugar modification are sorted into functionally distinct compartments. Vesicles with the cysteine proteinases containing GlcNAc-1-P fuse with bacteria loaded phagosomes in less than three minutes after ingestion of the bacteria, but vesicles with other lysosomal enzymes fuse with the phagosomes later. After the degradation of bacteria is completed the two kinds of enzymes are again segregated.

I. 4.3 Functions of Cysteine Proteinases in the Development *of Dictyostelium discoideum*

By raising the average pH of lysosomal/endosomal compartments to pH 6.4, Cardelli *et al.* (1989) showed that the complete processing of lysosomal enzymes depends on an acidic environment, as the higher pH resulted in accumulation of newly synthesized intermediate forms of α-mannosidase and β-glucosidase. A similar effect has been achieved with the use of the cysteine proteinase inhibitor, Z-Phe-AlaCHN$_2$, as well as with leupeptin and antipain. These results implicate modifications to be carried out by two classes of cysteine proteinases, which depend on acidic environments for their proper function. Activity of proteinases has been shown to be pH dependent in other systems including yeast (Monaro and Klionsky 1994).

Properly functioning cysteine proteinases are required for aggregation, and for proper orientation of slugs. Aggregation begins only after starvation, and it has been noted that during the time when no exogenous sources of energy are found, proteins are degraded (Gregg *et al.*, 1954). The work by Fong and Bonner (1979) concluded that proteolysis was necessary in cells preparing for aggregation, based on their experiments involving cysteine proteinase inhibitors. It was thus hypothesized that proteolysis is not just a means of producing available elements necessary for generation of energy (glucose did not rescue inhibited cells) and amino acids available to the starving cells, but it is an essential component in normal cellular development. Moreover, the secretion of proteinases into the slime sheath of a slug can be important for proper orientation towards the soil surface in preparation for culmination. Bonner (1993) has shown that the ammonia avoidance reaction of the pre-stalk region of a slug may be simulated by the

addition of papain in a polyacrylamide bead to one side of a slug causing the slug to turn away from the enzyme diffusing from the bead.

Cysteine proteinases could play yet an additional function during the development of *Dictyostelium*. A small 18 kDa cysteine proteinase can be found in the matrix of a fruiting body, it has been hypothesized that this enzyme could play a possible role in the aging phenomenon by which wild type spores acquire ability for spontaneous germination. It is possible that the ddCP18 could cleave and hence, proteolytically inactivate the ACG osmotic sensor and/or proteolytically activate the PLC pathway receptor resulting in spores poised to germinate (Cotter *et al.*, 1997).

I. 5.0 Objectives

The work of North (1988) has indicated that the switch between the expression of the A and B patterns of cysteine proteinases was controlled by the Cysteine Proteinases Converting Factor (CPCF). The molecular weight of this factor has been suggested to be in excess of 50,000 Da. The active factor was neither affected by treatment with digestive enzymes nor by boiling. In addition it was not precipitated by 80%(v/v) ethanol, and 1M-KOH had no effect on overall activity. However, it was precipitated by HCl and by trichloroacetic acid, and the activity recovered by dissolving the precipitates in 20mM-sodium phosphate buffer pH 6.0, and dialysing against the same buffer. All of these characteristics combined indicated that the CPCF could not be a protein or nucleic acid. Having a large molecular weight this factor most likely was a component of the bacterial cell wall.

The differentiation of proteinase expression was examined with the purpose of identifying the CPCF in a more specific manner. The alterations of the proteinase pattern from A to B will be examined in two different species of slime molds, *Dictyostelium discoideum* axenic strain Ax3 and *Polysphondylium pallidum* axenic strain WS320. Additionally, the specific conditions required for the shift, as well as the effects of the CPCF were tested for its ability to cause a change in the pattern of germination of dormant spores and/or the possibility of being an activating agent in the germination process.

A secondary and minor purpose of this work was to further test the hypothesis that a conformational change in the proteinase, due to pH shifts, may be the main mode of controlling the activity of cysteine proteinases. Thus, the question which will be attempted to be answered here was: Is the phenomenon of *"in vitro"* acid activation a simulation of *"in vivo"* activation of cysteine proteinases by vesicular ATPases? Or is activation of the proteinase an artifact of *in vitro* acid treatments of the zymograms?

II. Materials and Methods

II. 1.0 Media used

II. 1.1 Glucose Salts (GS)

The GS medium contained an autoclaved solution of 1.0g of NH_4Cl (Sigma), 3.0g KH_2PO_4 (Sigma), 6.0g Na_2HPO_4 f (Sigma), 0.13g $MgSO_4$ (Sigma), 4g of glucose (Sigma) and 15g agar (Difco) in 1L of distilled water. The solution's pH was adjusted to 6.4-6.6. After autoclaving approximately 15 mL of the solution was poured into each petri plate.

II. 1.2 Standard Media (SM)

SM/2 medium contained a mixture of three separately autoclaved solutions. The first solution contained 5g of bactopeptone (Sigma), 0.5g of yeast extract (Difco), 1.1g of KH_2PO_4 (Sigma), 0.5g of K_2HPO_2 (Sigma) into 500mL of distilled water. The second solution contained 0.5g $MgSO_4$ (Sigma) and 15g of agar (Difco) in 450mL of distilled water. Finally the third solution contained 5.0g of Glucose in 50mL of distilled water. All of the solutions pHs were adjusted to 6.4-6.6. After autoclaving all of the solutions were mixed and poured into petri plates.

II. 1.3 LP

The 0.1% LP medium contained an autoclaved solution of 1.0g β-Lactose, 1.0g Bacto-peptone and 15.0g of granulated agar dissolved in 1.0L of nanopure water. The solution's pH was adjusted to 6.4-6.6. Plates were poured to contain 10mL of agar when cooled.

II. 1.4 Non Nutrient Agar (N/N)

Non nutrient agar contained 15% (w/v) agar in ddH_2O, with a pH of 6.4 to 6.6.

II. 1.5 Transfer Medium (TM)

The TM contained 10g of trypticase peptone (Difco), 5g of yeast extract (Difco), 10g of glucose 0.4g of NaH_2PO_4 (Sigma) and 1.2g of K_2HPO_4 (Sigma) in 1L of distilled water. The solution's pH was adjusted to 6.4-6.6. The solution was dispensed into 150mL flasks with 50 mL in each flask. The flasks with the media were autoclaved.

II. 2.0 Organism Used

Two types of slime molds have been used in this study: *Dictyostelium discoideum* strains SG1 and SG2, NC4mL, and axenic strains Ax3 and *Polysphondylium pallidum*, strain WS320.

II. 2.1 Cultivation and Production of Spores

Spores from fruiting bodies were transferred by sterile loop into 10 to 20mL of autoclaved 10mM potassium phosphate buffer at pH 6.5 (10mM KPi). Two loops of *Escherichia coli* B/r (*E. coli* B/r) or *Klebsiella aerogenes* were added to the spores and the suspension was mixed on a vortex mixer. A 1.5mL aliquot of the mixture was transferred to glucose salts (GS) agar plates or standard medium (SM/2) plates. The plates were incubated at 23°C in the presence of light and allowed to develop for 3-4 days until fruiting bodies formed. To collect the

spores a microscope slide was held 1mm above the agar plate at an angle of 45° and the petri plate was rotated. This resulted in deposition of spores onto the slide. The slide was rinsed into 2mL of ddH_20. The spores were washed twice in either KPi or ddH_20 by centrifugation at 1400x g.

II. 2.2 Axenic Strains

The axenic strains of the social amoebae were grown in transfer media (TM). To inoculate the axenic cultures, 1mL of cells (at $1.0x10^7$ cells/mL) were transferred to the medium. The flasks with the culture were placed on a low speed shaker for the duration of the growth. The cells usually required 4-5 days to reach maximum concentration at room temperature. Once this occurred they were harvested by centrifugation at 1400x g and washed twice in KPi buffer.

II. 2.3 Formation of Heterothallic Macrocysts Using Strains SG1 and SG2

Strains SG1 and SG2 of *D. discoideum* were employed in the formation of heterothallic macrocysts. The cultures were initially grown asexually as described above on 0.1% LP agar plates. Twenty four hours after fruiting bodies formed, spores from both SG1 and SG2 cultures were harvested into a 250mL flask of cool boiled distilled water. Two loops of *E.coli* B/r were mixed into the suspension and 5 mL of this solution was placed onto fresh 0.1% LP agar plates and swirled to ensure even distribution of spores. The plates were then placed in the dark and after 24 hours, vegetative cells were collected. An additional 5mL of boiled water was added to the plates. After another 24 hours vegetative cells began to form aggregates and cells of opposite mating types began to fuse and form zygotes. After an additional 3 days, the remaining plates contained

developing macrocysts. Cells from this stage were collected and centrifuged at 1400x g.

II. 2.4 Formation of Microcysts Using *Polysphondylium pallidum* WS320

P. pallidum was originally grown asexually as described for *D. discoideum* (see section II. 2.1). After the formation of fruiting bodies, the spores were harvested into a test tube containing 15mL of ddH_2O and one loop of *E. coli* B/r. The suspension was mixed and a 0.2mL of the aliquot was dispensed onto GS plates. This dispensation was spread evenly across the surface of the plates with a sterile rod. The plates were then stored in the absence of light, and they were allowed to dry for one day before being placed upside down. After approximately 10 days microcysts formed. The microcysts were washed from the plates into a test tube, using ddH_2O and a sterile bent glass rod. The microcysts were washed by centrifugation at 1400x g in KPi buffer, and stored at –20°C.

II. 2.5 Formation of Pseudoplasmodia Using *Dictyostelium discoideum* NC4

Petri dishes containing approximately 10 mL of non-nutrient agar were inoculated with a single streak of *E. coli* B/r. Following this procedure, 5-10 sorocarps of NC4 strain of *D. discoideum* were collected with a sterile loop and smeared into the inoculate line of *E. coli*. The plates were then placed under a dark box containing a small aperture in such a way that the inoculate line was perpendicular to the hole. A lamp was then placed directly in front of the aperture outside of the box. This subsequently allowed directional movement of the pesudoplasmodium towards the point source of light. Following a three day period a large number of migrating slugs were formed. To collect the slugs a

small amount of KPi buffer was placed on the plates, and the slugs were washed off with the help of a sterile glass rod and washed twice in KPi.

II. 3.0 Auto-Induced Activation of Spore Germination of Strain SG1 and SG2

Twenty-four hours after the formation of fruiting bodies, the spores were harvested into 4mL of distilled water, vortexed and washed twice by centrifugation at 1400x g. The spores were re-suspended in KPi buffer at a final cell density of 2.0×10^7 to 3.0×10^7 cells/mL. The suspension was placed on a high speed shaker to keep the spores evenly dispersed and to allow oxygen to enter during the germination process. Spores and nascent amoebae were collected at each hour and counted on a microscope slide. The collected sample was centrifuged at 1400x g and the supernatants were either stored at – 20°C or discarded. The pellets were quickly frozen and stored in a 95% ethanol bath at – 20°C.

II. 4.0 Germination in the Presence of Bacteria or CPCF

The germination protocol in the presence of heat killed bacteria was similar to the control with one modification which included the presence of *K. aerogenes* at the same concentration as was required to obtain a B-pattern of cysteine proteinases (otherwise indicated). Similarly for germination in the presence of any other factors which were tested for their ability to mimic the effects of CPCF, the germination procedure was identical to the controls only with the addition of the tested factor in KPi buffer.

II. 5.0 Germination of Immature Wild Type Spores

The germination of young wild type spores cannot occur spontaneously, however the incubation of young spores on a solid surface in the presence of bacteria will promote germination. To achieve this germination, young wild type spores were placed in 12 well culture plates at a concentration of $1x10^5$ cells/mL. The bottom of the well was covered with 0.5mL of autoclaved 10% (w/v) agar which was a sufficient amount to cover the bottom of each well. The spores were collected one day after formation and washed twice in KPi buffer, and re-suspended in KPi at a desired concentration, together with the component which was tested at the concentration indicated. Finally, 300µL of the suspension was placed in individual wells and the plates were covered to prevent contamination. The culture plates were placed on a low speed shaker and checked periodically for evidence of germination. The suspensions were checked periodically for evidence of germination. Since, the spores on the agar can only be viewed at low power magnification (100x) a precise quantification of the results was difficult. Therefore, the numbers of germinating spores only yielded a rough estimate and should be considered semi-quantitative

II. 6.0 Breakage of Spores and Cells

Approximately 50µL of 10 mM KPi and 200µL of small glass beads (213-300 µm diameter, Sigma) were added to all samples after one freeze thaw cycle. The samples were vortexed with the glass beads in 20-sec intervals for a total of 2 minutes. Between intervals the samples were placed on ice for at least 30 sec. The homogenates were then centrifuged at 2000x g, and supernatants were collected using a micropipette and

dispensed into microcentrifuge tubes. The centrifugation at 2000x g was repeated to ensure that no glass beads and unbroken cell/spores remained in the protein preparation. Samples were frozen and stored again in –20°C until further use.

II. 7.0 Protein Assay on Samples

Protein assays, using the supernatant (as per section II. 8.0), were performed in microtitre wells according to the Bradford method (Bradford, 1976) with bovine serum albumin as the standard.

II. 8.0 Gelatin-SDS-Polyacrylamide-Gel-Electrophoresis

Gelatin-SDS-PAGE was performed using the Bio-Rad Mini Protean II Electrophoresis System to separate and identify cysteine proteinases. 2% gelatin (w/v) was used as a substrate for proteinases. The final concentration of gelatin in the gels was 0.2% (v/v). The separating polyacrylamide gel concentrations used in this work were 7.5% 10% and 12% (refer to Appendix A). Once the separating gel was polymerized (30 minutes) a 4.0% acrylamide solution was placed on top of the separating gel together with a teflon comb and the solution was allowed to polymerize and form a stacking gel. The thickness of gels was 0.75 mm. Once the gel polymerized (40 min), the comb was removed and the gel was washed with 1x running buffer (14.4g of glycine (Sigma), 1.0g of SDS (Sigma), and 3.0g of Tris (Sigma) in 1L of distilled water). The samples were prepared by adding 4 parts of sample to one part of 5x sample buffer. The 5x sample buffer contained 2.5mL β-mercaptoethanol (Sigma), 5.0mL of glycerol (Sigma), 2.5mL of 0.5M Tris (Sigma) at pH 6.8, and 1.0mg of Bromophenol Blue (Sigma). The samples

were loaded into the wells of the stacking gel using micropipettes followed by careful overlying of the wells with more running buffer. Electrophoresis was carried out in the running buffer at 4°C and at a constant voltage. The voltage was set at 70 V until the dye front was observed to reach the separating gel, following which the voltage was increased up to 150 V for the remainder of the electrophoresis run (45-60 minutes). After electrophoresis the stacking gel was removed and discarded and the separating gel placed in a 2.5% Triton-X 100 (v/v) wash for about 30 minutes to remove the SDS. The gels were then incubated in 0.033M sodium acetate buffer at pH 4.0 with 1mM DTT for 18-24 hours at room temperature. Gels were stained with a solution of Commassie Brilliant Blue (2.5% Commassie Brilliant Blue R-250 (w/v), 50% methanol (v/v) and 10% acetic acid (v/v)) for 60 minutes, and then destained in a solution of 10% acetic acid (v/v) and 5% methanol (v/v) for 60 minutes. Gels were dried at room temperature for 24 hours in between two BioGelWrap membrane sheets on a gel drying frame.

Wide range molecular weight standard markers (prepared according to Sigma), were boiled for 1 minute and loaded onto the lanes of the gels. Once the gels were dried the apparent molecular weights of the proteinases were calculated. The calculation utilized the R_f values (Relative to the dye front), of the standard markers plotted against the log of their respective molecular weights.

II. 9.0 Acid Treatments

Samples were electrophoresed as described above. For acid activation prior to the treatment with 2.5 Triton X-100, the gels were placed into a bath of 1.7 M acetic acid at pH 2.1 for 60 seconds

II. 10. Inhibitor Treatment

Two inhibitors were utilized in this study to verify that the proteinases were cysteine proteinases. The inhibitors used were E64 (Sigma) and 2,2'-Dithiodipyridine (Sigma). In the case of the second inhibitor, incubation of gels in acetate buffer was performed without DDT. The samples, prior to electrophoresis were incubated with 100 μM of the inhibitor for 30 minutes at 23°C. The rest of the procedure followed the same as the control gels. To reverse the effects of the 2,2'-Dithiodipyridine, the samples were incubated with 1 mM DTT after electrophoresis. The compound 2,2'-Dithiodipyridine is insoluble in water; to overcome this problem the inhibitor was at first dissolved in small amounts of methanol and the solution was further diluted to achieve the desired concentrations.

II. 11.0 Induction of the B-Pattern of Cysteine Proteinases

The bacterium *K. aerogenes* was grown on SM/2 plates until a uniform lawn was formed. The cells were harvested by placing 1mL of KPi onto the plates, and gently scraping the surface with a bent glass rod. The bacteria were centrifuged and washed twice in KPi, and resuspended in the same buffer to give an absorbency of 2.5 at λ=650 nm (MSE-Spectro-Plus). The bacterial suspension was added to axenic cultures of myxamoebae in a ratio of 1:10 (v/v). To heat kill, the bacteria were heat-treated by incubating the suspension above 80°C for 15 minutes. After various times of incubation at room temperature with the bacteria, the amoebae were collected, centrifuged, washed twice, pelleted, and stored at –20°C until further use.

II. 12.0 *In situ* Activation of Cysteine Proteinases

Cells at different stages in the life cycle were grown and collected as described (sections II. 2.1-II. 2.5). For activation of spore enzymes, Triton X-100 was used as a detergent (0.5% (v/v)) and 20 %(v/v) DMSO as a solvent, along with 10 % acetic acid. For all of the other stages there was no need to use the detergents as acetic acid was capable of penetrating the plasma membrane.

The spores/cells were placed in test tubes and treated with the solution for various time periods as indicated. For the duration of the treatments the tubes were placed in ice. This was followed by a double wash in KPi buffer. The samples were homogenized as described (see II. 6.0)and the protein extracts were analysed for proteolytic activity using zymography (i.e. gelatin SDS-PAGE).

III. Results

III. 1.0 Patterns of Proteinases in *Dictyostelium discoideum* and *Polysphondylium pallidum*

Two types of cellular slime molds were examined for their ability to shift protease patterns from A to B: *Dictyostelium discoideum* (Ax3) *and Polysphondylium pallidum* (WS320). Both strains were grown in TM medium for a period of 24 hrs in the presence of the heat killed bacterium *Klebsiella aerogenes*. As can be seen in figures 4 and 5, the zymogram method was useful in detecting the shifts. The proteinases present at time 0h range from 57 kDa to 29 kDa in the Ax3 cells. Upon acetic acid treatment the activities of all other regions were enhanced. Within 24 hours of incubation of the cells in the presence of heat killed *K. aerogenes* the proteolytic pattern was changed. The B pattern of the Ax3 strain includes proteinases not present at time 0h: 46kDa and 35kDa with the majority of activity attributed to the high molecular weight proteinase. In addition, the 38kDa band as well as the 29kDa band present at time 0 are also active at this point. Acid activation revealed an additional major band at molecular weight of 53kDa and activities of all others were enhanced.

In WS320 cells the major protease present in the cells grown in the absence of bacterial factors is the 48kDa, with an additional band present with an apparent molecular weight of 63kDa. Gelatin clearing is also evident at a region corresponding to a molecular weight of about 29kDa. This activity is very weak and can only be visible at high protein concentrations as in Figure 11 (to be discussed later). However, acid treatment increased the activities of the 48kDa protease and it also revealed an additional

Figure 4. **Gelatin SDS-PAGE analysis of bacterially induced cysteine proteinase pattern changes in *Dictyostelium discoideum*, Ax3 cells.** (Gel A, control; Gel B, acid treatment). Samples were collected after addition of heat killed *K. aerogenes* at the times indicated. Each lane contains 0.8 μg of protein. Unless otherwise indicated, 10% polyacrylamide gels were used for SDS-PAGE experiments.

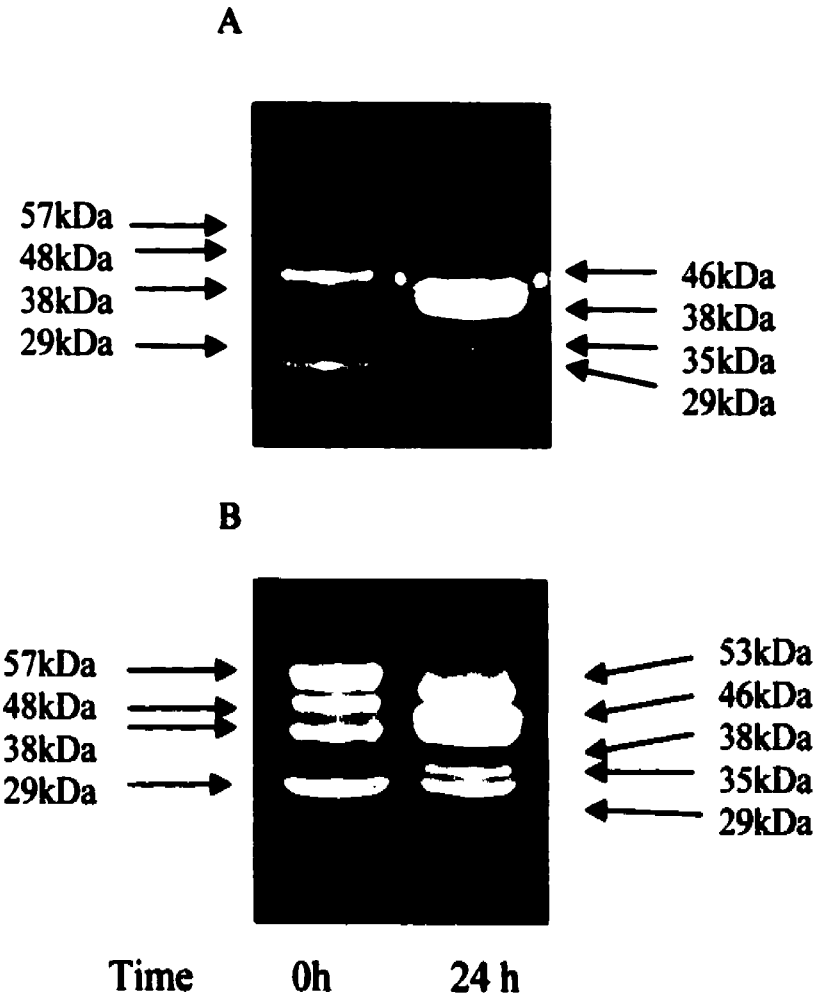

A
57kDa
48kDa
38kDa
29kDa
46kDa
38kDa
35kDa
29kDa
B
57kDa
48kDa
38kDa
29kDa
53kDa
46kDa
38kDa
35kDa
29kDa
Time
0h
24 h

Figure 4.

Figure 5. **Gelatin SDS-PAGE analysis of bacterially induced cysteine proteinase pattern changes in *Polysphondylium pallidum*, WS320 cells.** (Gel A, control; Gel B, acid treatment). Samples were collected after addition of *K. aerogenes* at the times indicated. Each lane contains 0.8μg of protein.

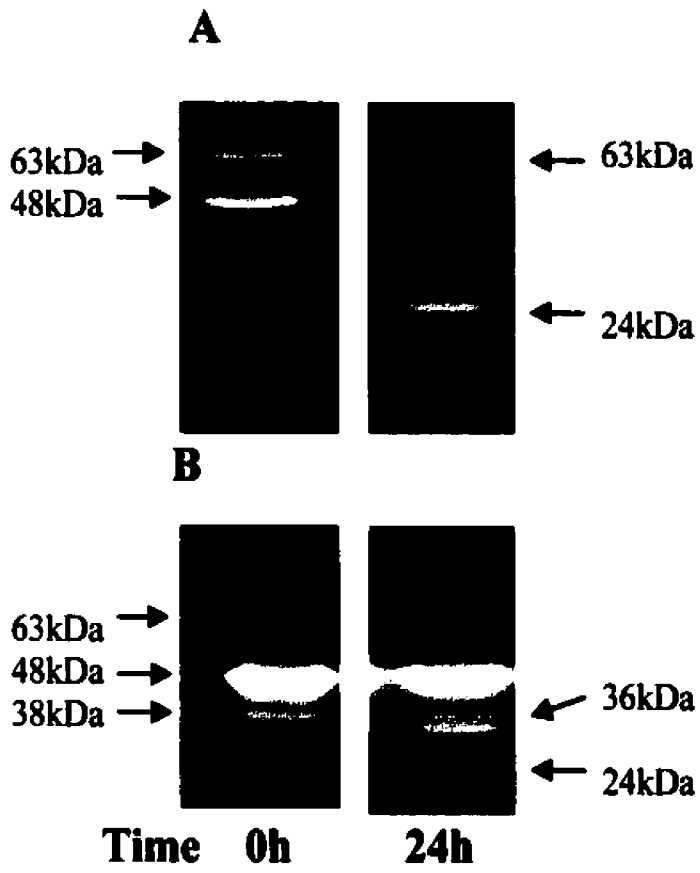

A
63kDa
48kDa
63kDa
24kDa
B
63kDa
48kDa
38kDa
36kDa
24kDa
Time 0h 24h

Figure 5.

band of activity present at 38kDa. Growth of the cells in the presence of bacteria induced a dramatic change in the proteinase patterns. The 48kDa band disappeared with the appearance of an additional band with a molecular weight of 24kDa. However, upon treatment of the gel slab with acetic acid, the band with the molecular weight of 48kDa reappeared, as well as the one with the molecular weight of 38kDa and minor 36kDa activity was detected as well. The activity of the 24 kDa protease appeared to be decreased by the treatment.

III. 2.0 Changes of the Proteinase Patterns in the Slime Molds Under Different Growth Conditions or Factors.

The proteinase patterns of *Dictyostelium discoideum* and *Polysphondylium pallidum*, were examined in detail. The Ax3 cells were grown in TM medium in the presence of heat killed bacteria. Samples were removed at each hour and analyzed for the protease pattern with the aid of zymographs (Figure 6). Similarly, the patterns were checked for cells grown in phosphate buffer in the presence of heat killed bacteria (Figure 7), as well as in the presence of live bacteria (Figure 8). These conditions were also tested for cells of *Polysphondylium pallidum* (Figures 9, 10, 11).

The proteinase pattern shift in the Ax3 cells, grown in TM in the presence of heat killed bacteria, began at 3h after the introduction of the bacteria to the culture. The change was seen with the appearance of the 53 kDa and 35 kDa bands and with gradual disappearance of the 57 kDa band commencing at two hours. The third clearing, typical of the B pattern, at the apparent weight of 46 kDa became visible at 5 hours. This change took place at the same time as the 48 kDa clearing became less detectable. Under this

condition the change in the proteinase pattern was complete at 8 hours. Incubation of the gel in acetic acid for 1 minute resulted in increased activities of all of the proteinases.

Ax3 cells grown in potassium phosphate buffer in the presence of heat killed bacteria experienced different timing in the shift of the proteinase pattern (Figure 7). In this case the change was slower, in comparison to that of the previously described experiment. However, the acid treated gel revealed that the change was starting to take place at 2-3 hours with the appearance of the 53-kDa band. Incubation of Ax3 cells in phosphate buffer in the presence of live bacteria resulted in an even slower change in the proteolytic patterns as indicated in Figure 8. Nevertheless, all of these experiments succeeded in changing the patterns from A to B within 24 hours of the introduction of any bacteria to the cultures under all of the conditions tested (results not shown).

Similar experiments with WS320 cells revealed different timing in the switch of the patterns. In this case the switch was more rapid when compared to the Ax3 cells; the switch as completed at 5 hours as opposed to 8 hours for the Ax3. It was characterized by a gradual disappearance of activity at the apparent weight of 48kDa, and the appearance of activity at 24kDa. However, acid activation revealed that the 48kDa protease was present in the protein extracts, but in an inactive state. Secondly, acid treatment revealed yet another bacterially induced proteolytic band at 36kDa, this activity was first detected at the same time as the 24kDa. The proteolytic clearance around 38kDa not present in untreated gels was revealed by the acid activation, and was present throughout the experiment. As previously indicated there was a minimal activity at the region of 29 kDa in the A pattern. To visualize this band the protein extracts from the WS320 cells grown in association with live bacteria were prepared at a high

Figure 6. **Changes of proteinase patterns in Ax3 cells grown in TM medium in the presence of heat killed bacteria.** (Gel A, control; Gel B, acid treatment) Samples were collected after the addition of *K. aerogenes*, at the indicated times. Each lane contains 0.8 µg of protein.

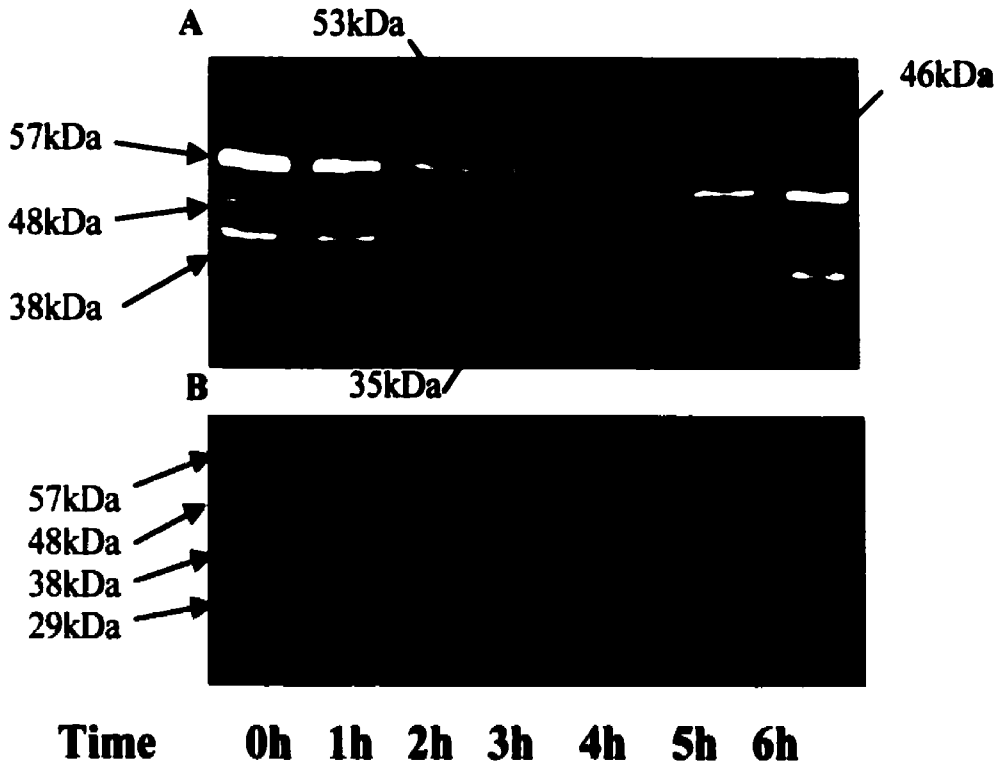

A
53kDa
46kDa
57kDa
48kDa
38kDa
B
35kDa
57kDa
48kDa
38kDa
29kDa
Time
0h
1h
2h
3h
4h
5h
6h

Figure 6.

Figure 7. **Changes of proteinase patterns in Ax3 cells over time grown in KPi buffer in the presence of heat killed bacteria.** (Gel A, control; Gel B, acid treatment) Samples were collected after the addition of *K. aerogenes*, at the indicated times. Each lane contains 0.8 μg of protein.

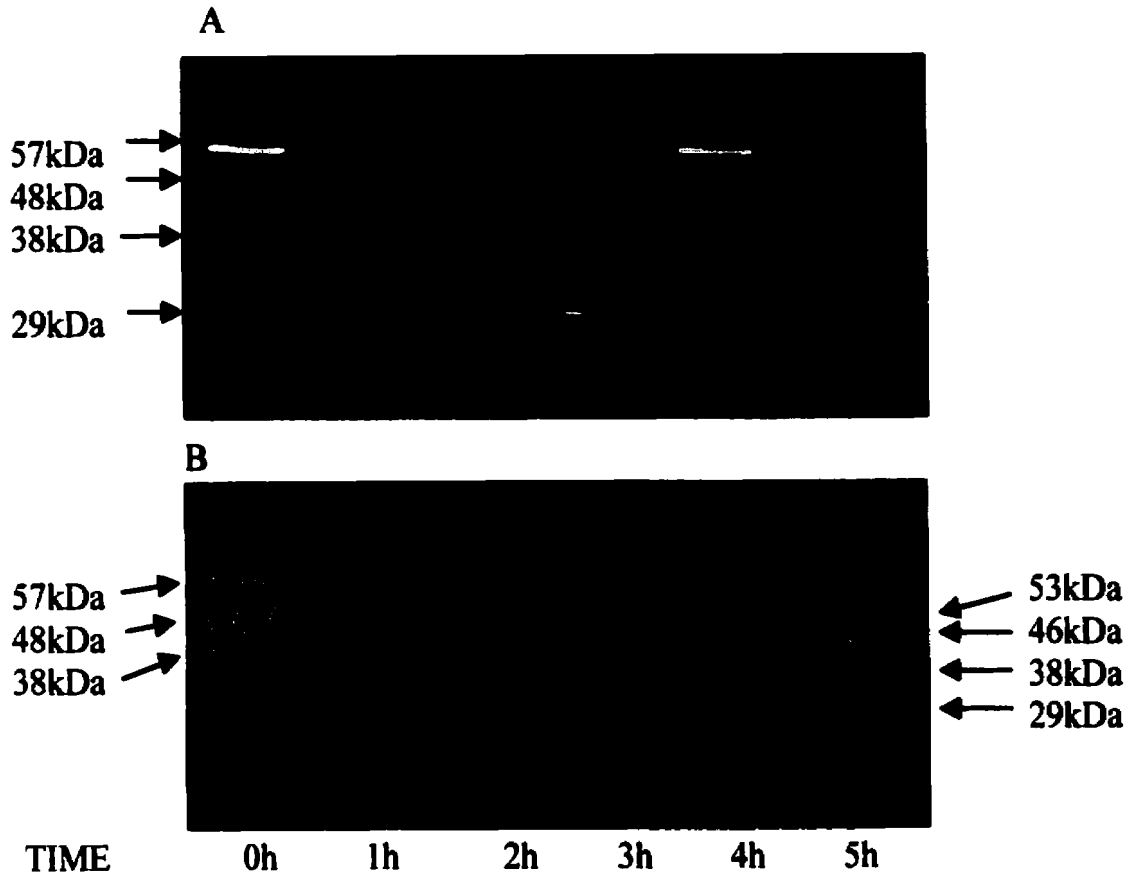

A
57kDa
48kDa
38kDa
29kDa
B
57kDa
48kDa
38kDa
53kDa
46kDa
38kDa
29kDa
TIME
0h
1h
2h
3h
4h
5h

Figure 7.

Figure 8. **Gelatin-SDS-PAGE analysis of changes of proteinase patterns in Ax3 cells over time grown in KPi buffer in the presence of live bacteria.** (Gel A, control; Gel B, acid treatment) Samples were collected after the addition of *K. aerogenes*, at the indicated times. Each lane contains 0.8 μg of protein.

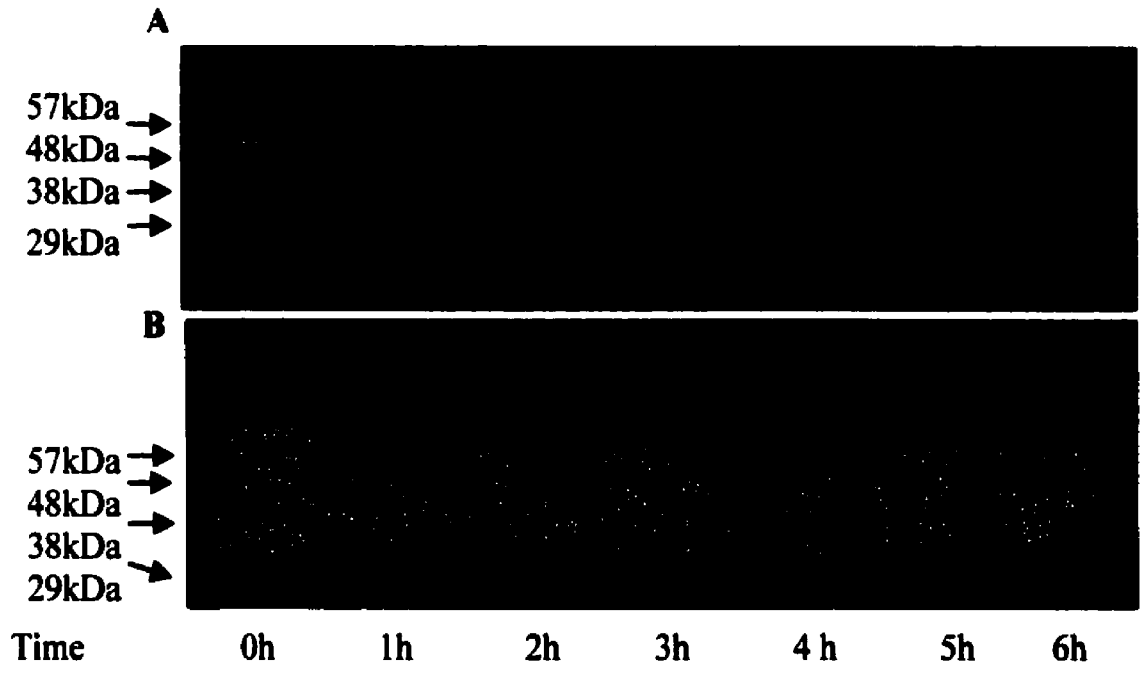

A
57kDa
48kDa
38kDa
29kDa
B
57kDa
48kDa
38kDa
29kDa
Time
0h
1h
2h
3h
4 h
5h
6h

Figure 8.

Figure 9. **Gelatin SDS-PAGE analysis of heat killed bacteria-induced proteinase pattern change in WS320 cells grown in KPi buffer**. (Gel A, control: Gel B, acid treatment) Samples were collected after the addition of *K. aerogenes*, at the indicated times. Each lane contains 0.8 μg of protein.

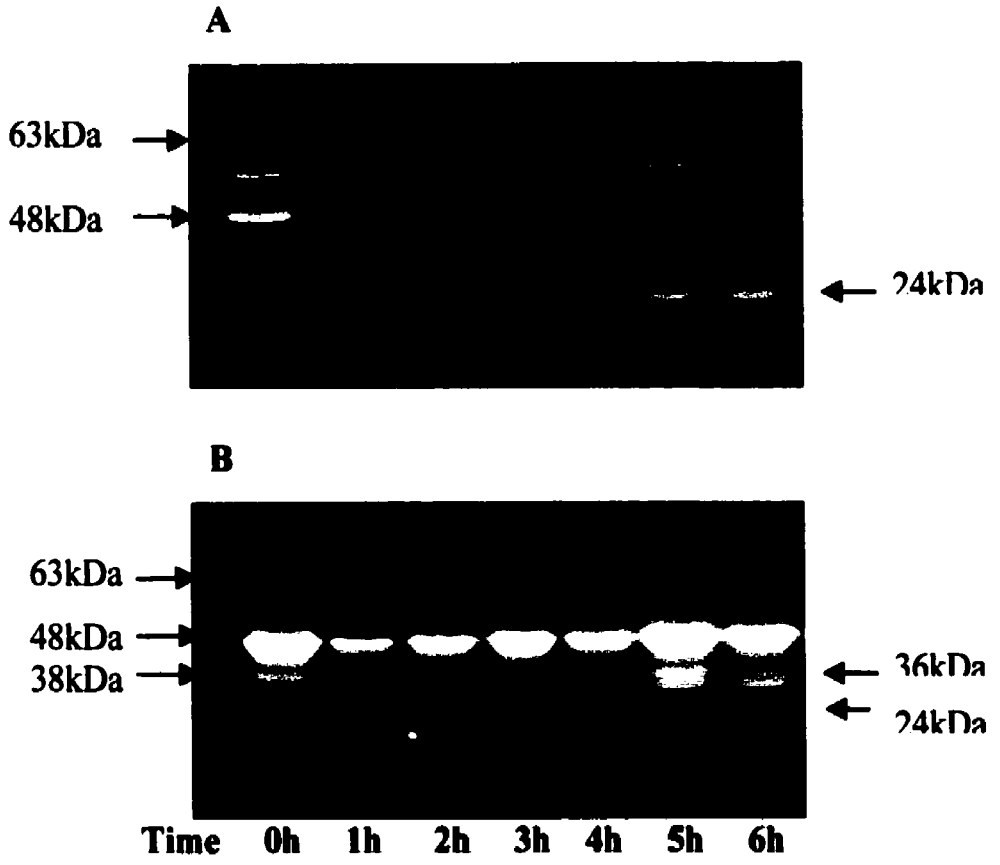

A
63kDa
48kDa
24kDa
B
63kDa
48kDa
38kDa
36kDa
24kDa
Time 0h 1h 2h 3h 4h 5h 6h

Figure 9.

Figure 10. Gelatin-SDS-PAGE analysis of heat killed bacteria-induced proteinase pattern change in WS320 cells grown in TM medium. Samples were collected after the addition of *K. aerogens*, at times indicated. Each lane contains 0.8 μg of protein.

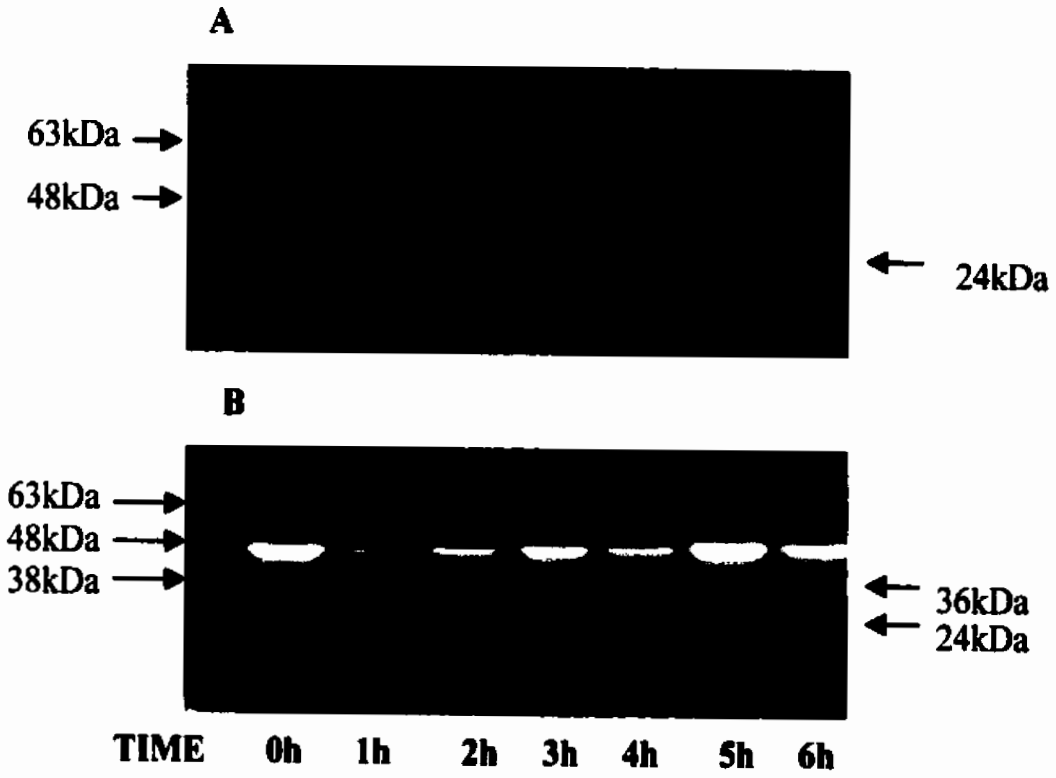

A
63kDa
48kDa
24kDa
B
63kDa
48kDa
38kDa
36kDa
24kDa
TIME
0h
1h
2h
3h
4h
5h
6h

Figure 10.

Figure 11. Gelatin-SDS-PAGE analysis of Gelatin SDS-PAGE analyses of live bacteria-induced proteinase pattern change in WS320 cells grown in KPi buffer. Samples were collected and analyzed at the indicated times. Each lane contains 1.5 μg of protein.

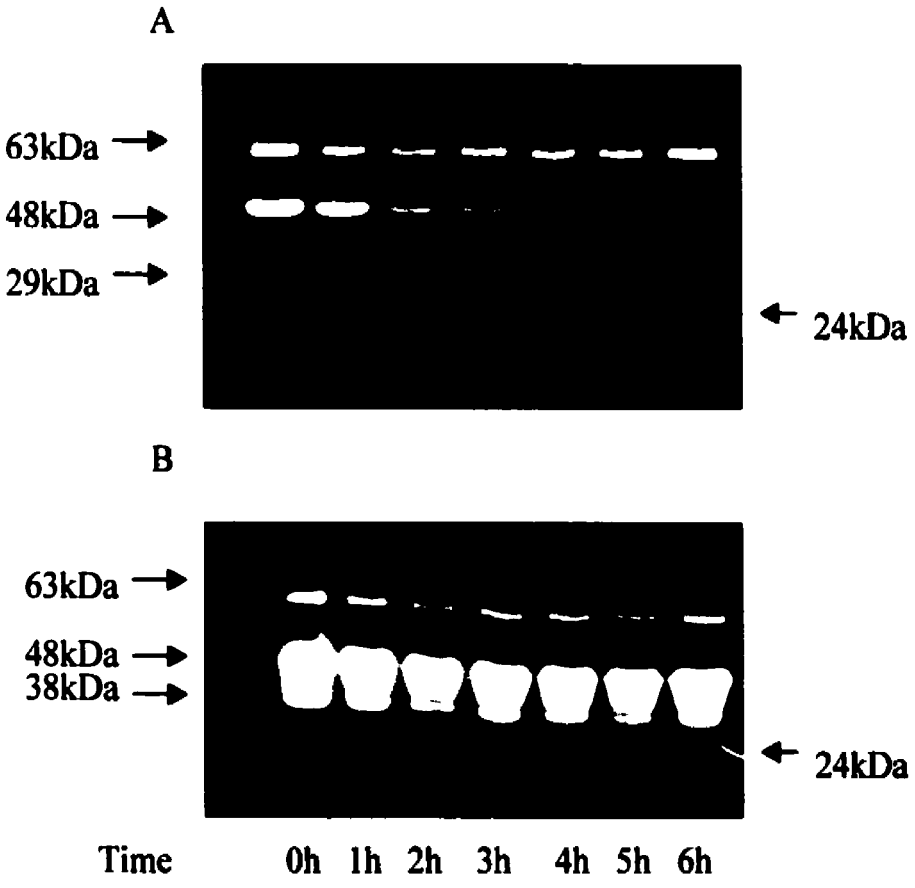

A
63kDa
48kDa
29kDa
24kDa
B
63kDa
48kDa
38kDa
24kDa
Time
0h 1h 2h 3h 4h 5h 6h

Figure 11.

concentration of 1.5 µg (Figure 11). This proteinase decreases in activity as the bacteria is introduced into the culture and is replaced with the 24kDa activity. The remaining banding patterns exhibited similar timing in shifts as the previous experiments.

Since the growth of the slime molds in TM buffer with heat killed *K. aerogenes* yielded the fastest switch of patterns, these conditions were the ones used for the remaining experiments, whenever the induction of the B pattern became necessary.

III. 3.0 Protein Synthesis and Proteinase Shifts in Ax3 and WS320 Cells

In order to determine if protein synthesis is necessary for the switch of proteinase patterns, the cells were grown in the presence of heat killed *K. aerogenes* and 400 µg\mL cycloheximide, a potent inhibitor of protein synthesis. The samples were removed at each hour for the time period required for the switch to take place, and analyzed using zymograms.

The inspection of the results of these experiments (Figures 12 and 13) indicated that protein synthesis is an important component of the shifts in the proteinase pattern. There was no detectable shifts from the A to B pattern in Ax3. In the WS320 cells there was no proteinase shifts as well however, the 48kDa activity was decreased substantially in both the control and acid treated gels within one hour of introduction of cycloheximide.

III. 4.0 Inhibitor Studies

Cysteine proteinase inhibitors were utilized to determine whether the activities of the proteinases of both the Ax3 (Figure 14) cells and WS320 (Figure 15) cells of both patterns can be attributed to cysteine proteinases. Two types of inhibitors were used: the irreversible E64 and the reversible inhibitor 2,2'-Dithiodipyridine.

The proteinases extracted from cells grown in axenic medium of the strain Ax3 were inhibited with E64, and acid treatment only slightly increased any activities. Protein extracts from bacterially grown Ax3 cells when treated with E64 resulted in decreased activities of all but one proteinase with apparent molecular weight of 53kDa. Moreover, the proteolytic activity in this region increased with acid treatment (Figure 14, Gel D, *Lane 2*). Treatment of WS320 cells with E64 inhibited all activities accept for one with apparent molecular weight of 63kDa. *In vitro* activation with acetic acid was unsuccessful in causing reappearance of any additional activities (Figure 15, Gels C and D).

Use of 2,2`-Dithiodipyridine diminished activities of all proteinases in the axenically grown cells. However, this inhibitor similar to E64, failed to inactivate the 53kDa present in the extract prepared from the Ax3 strain grown in the presence of bacteria (Figure 14, Gel E, *Lane 2*). Acid treatment resulted in a much higher reappearance of activities, as opposed to the E64 treatments (Figure 14, Gel F), and all of the proteolytic activities of both patterns were accounted for. The only activity in the presence of the inhibitor in the WS320 extracts was due to the 63kDa proteinase (Figure 15 Gels E and F). Only the 48kDa proteinase reappeared after the gel was treated with acid for 1 minute (Figure 15, Gel F).

Figure 12. Gelatin SDS-PAGE analysis of proteinase activities of Ax3 cells when grown in the presence of cycloheximide and heat killed *K. aerogenes*. (Gel A. control; Gel B, acid treatment). Samples were collected after the addition of the tested component, at the indicated times. Each lane contains 1.0 μg of protein.

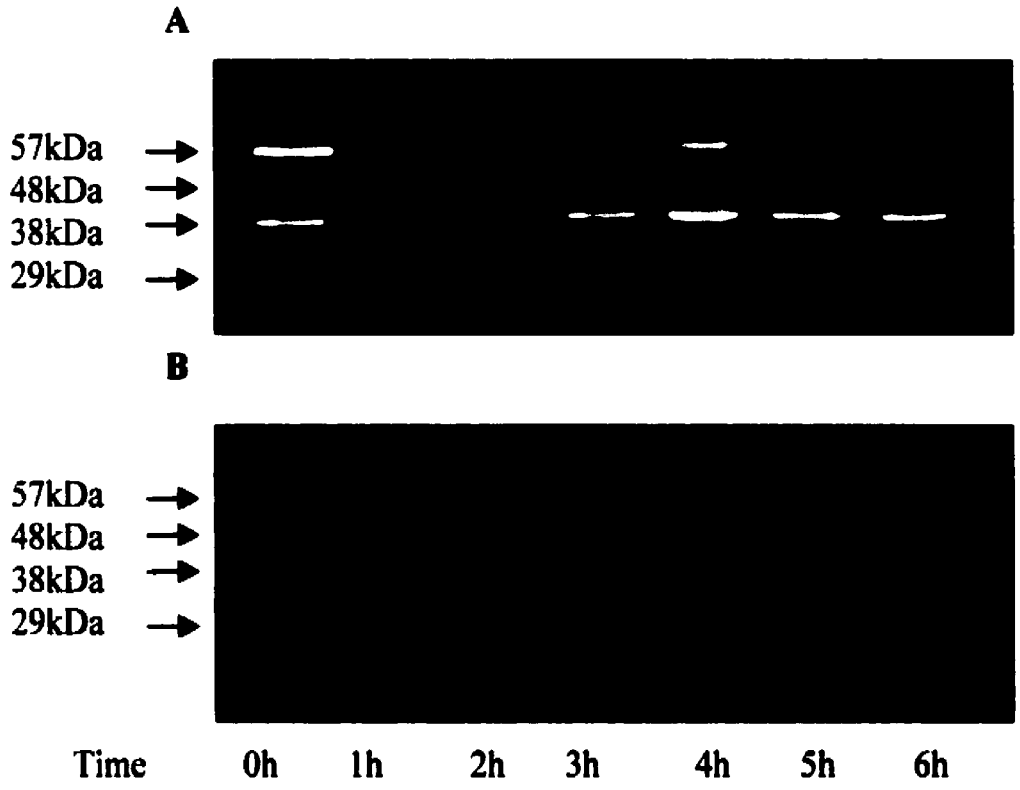

A
57kDa
48kDa
38kDa
29kDa
B
57kDa
48kDa
38kDa
29kDa
Time
0h
1h
2h
3h
4h
5h
6h

Figure 12.

Figure 13. **Gelatin SDS-PAGE analysis of proteinase activities of WS320 cells, grown in the presence of cycloheximide and heat killed *K. aerogenes*.** (Gel A, control; Gel B, acid treatment). Samples were collected after the addition of the tested component, at the indicated times. Each lane contains 1.0 μg of protein.

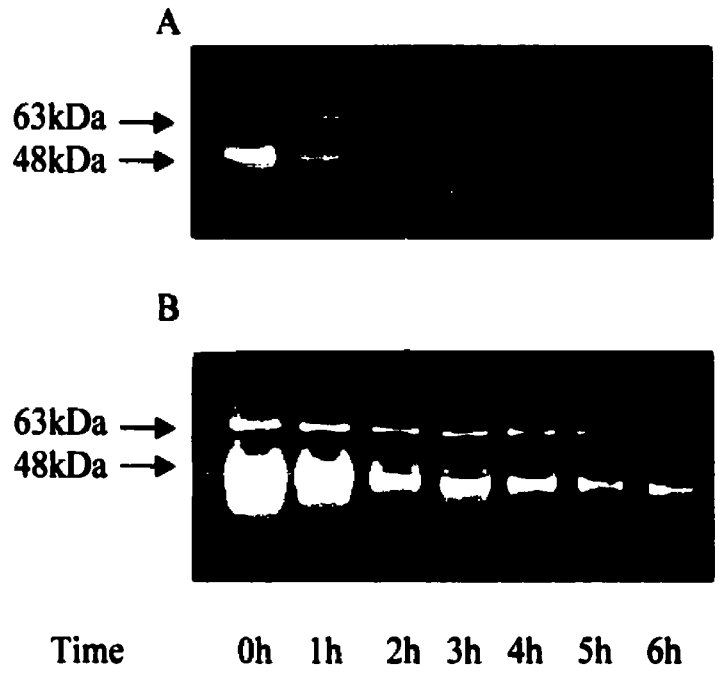


Figure 13.

Figure 14. **Gelatin-SDS-PAGE analysis of proteinase patterns in Ax3 cells incubated with 100 μM 2,2`-Dithiodipyridine or 100 μM E64, prior to electrophoresis.** (Gel A, control; Gel B, acid treatment of gel A; Gel C, E64 treatment; Gel D, acid treatment of Gel C; Gel E, 2,2`-Dithiodipyridine treatment; Gel F, acid treatment of Gel E). *Lane 1* Ax3 cells; *Lane 2* Ax3 strain after 24 hour growth with heat killed bacteria. Each lane contains 0.5 μg of protein.

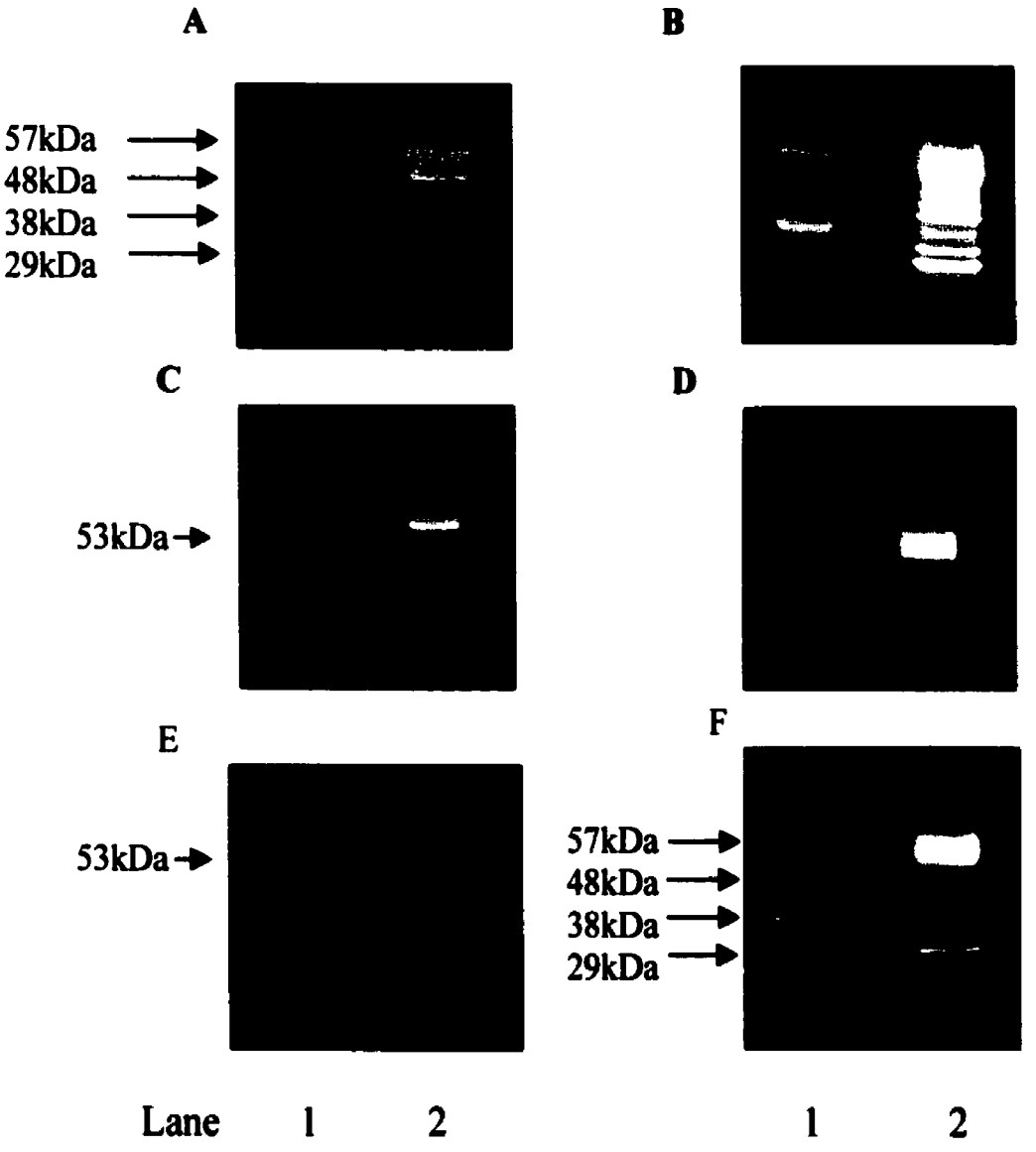

A
B
57kDa
48kDa
38kDa
29kDa
C
D
53kDa
E
F
53kDa
57kDa
48kDa
38kDa
29kDa
Lane 1 2 1 2

Figure 14.

Figure 15. **Gelatin-SDS-PAGE analysis of proteinase patterns in WS320 cells incubated with 100 μM 2,2`-Dithiodipyridine or 100 μM E64, prior to electrophoresis.** (Gel A, control; Gel B, acid treatment of gel A; Gel C, E64 treatment; Gel D, acid treatment of Gel C; Gel E, 2,2`-Dithiodipyridine treatment; Gel F, acid treatment of Gel E). *Lane 1* WS320 cells; *Lane 2* WS320 strain after 24 hour growth with heat killed bacteria. Each lane contains 0.5 μg of protein.

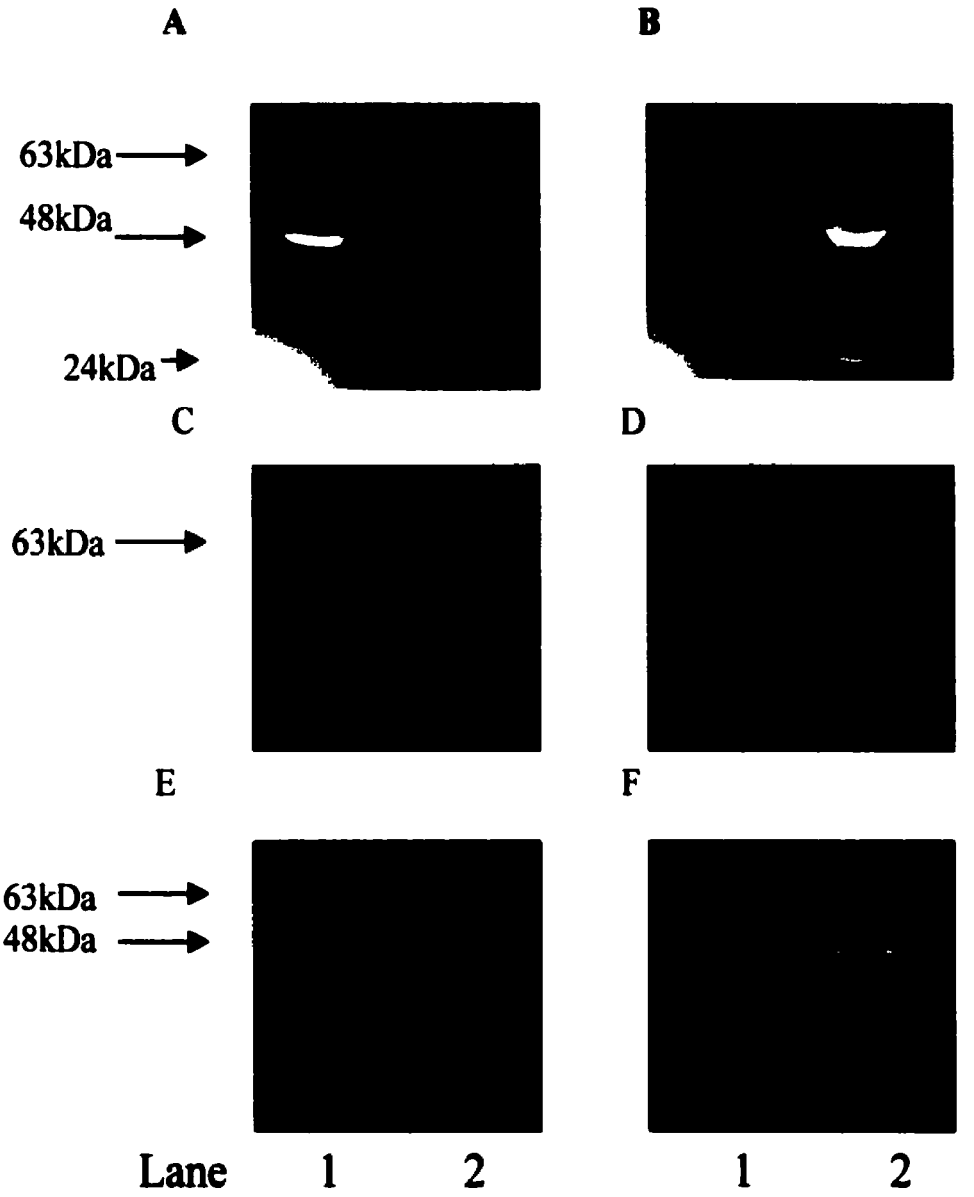


Figure 15.

III. 5.0 Cysteine Proteinase Converting Factor-Exclusive Property of Prokaryotic Cells?

To determine whether the CPCF is the exclusive property of prokaryotic cells, *Dictyostelium discoideum* Ax3 cells and *Polysphondylium pallidum* WS320 cells were grown in the presence of heat killed yeast cells. Additionally, to rule out the possibility of CPCF being an endogenous part of slime molds, these cells were also grown in the presence of heat killed "self". The samples were collected at 24 hours and 48 hours after introduction of the tested component, and the proteinase patterns were analyzed with the aid of zymograms.

The analysis using gelatin SDS-PAGE of Ax3 cells grown in the presence of heat killed yeast cells as well as heat killed Ax3 cells did not detect any changes in the proteinase pattern (Figure 16).

III. 6.0 Effects of Heat Killed *K. aerogenes* on the Germination Kinetics and Proteinase Patterns of Germinating Spores.

Germination of 3-day old spores of SG1 was performed to determine if heat killed *K. aerogenes* is capable of inducing germination of dormant spores. Additionally, the proteinase patterns of spores undergoing germination were examined using gelatin-SDS-PAGE.

The main difference in spores germinating in the presence of heat killed bacteria was the shorter lag time. As can be seen in Figure 17A, control spores (without bacteria) were 40% swollen at 1.5 hours compared to over 90% in the treated batch. The proteinase activities of the germinating spores were also examined and no major

differences were observed in the proteolytic activities of the control germination (Figure 18) as opposed to that of germination in the presence of heat killed bacteria (Figure 19).

Figure 16. Gelatin SDS-PAGE analysis of proteinase pattern in Ax3 cells grown in TM medium in the presence of heat killed yeast and heat killed Ax3. A) Cells have been incubated in the presence of heat killed yeast cells. B) Acid treatment of A. C) Cells have been incubated in the presence of heat killed Ax3 cells. D) Acid treatment of C. Samples were collected after the addition of tested component at the indicated times. Each lane contains 0.8 μg of protein.

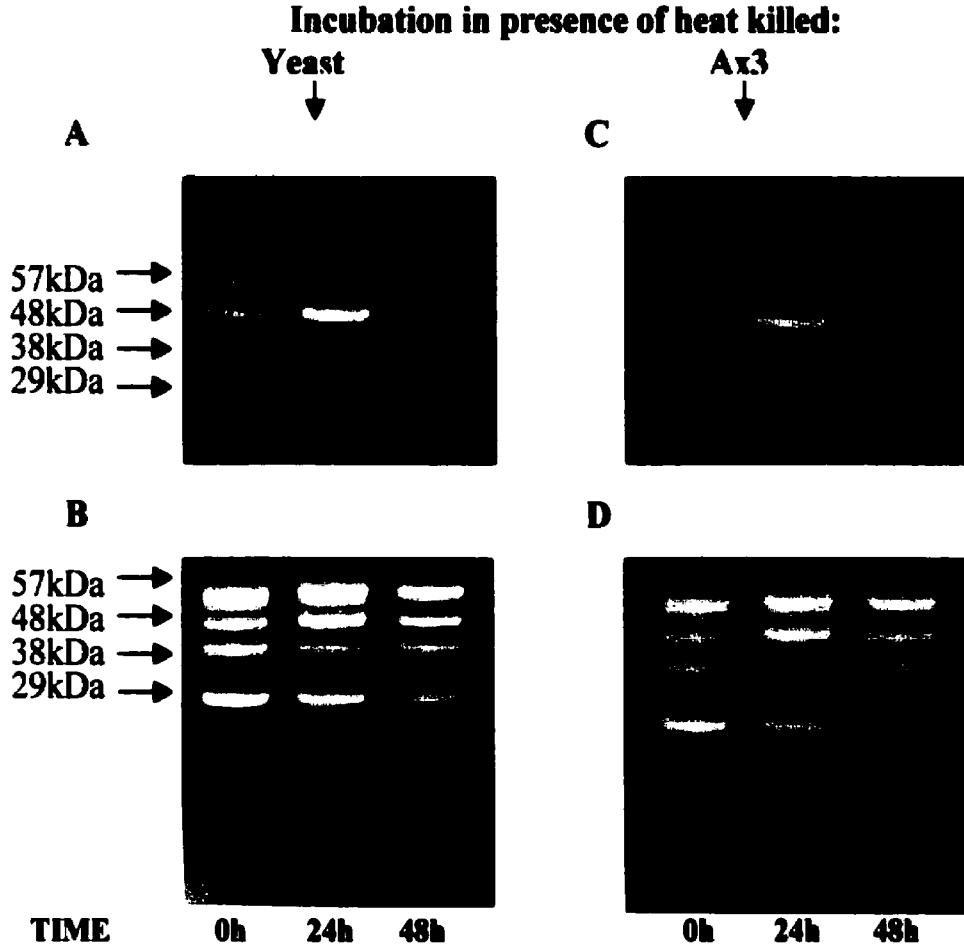

Incubation in presence of heat killed:
Yeast
Ax3
A
C
57kDa
48kDa
38kDa
29kDa
B
D
57kDa
48kDa
38kDa
29kDa
TIME
0h
24h
48h
0h
24h
48h

Figure 16.

Figure 17. Germination kinetics of autoactivation of 3-day old spores of SG1. A) Control germination. B) Germination in presence of heat killed *K. aerogenes*. Starting spore concentration was $2x10^7$ cells/mL.

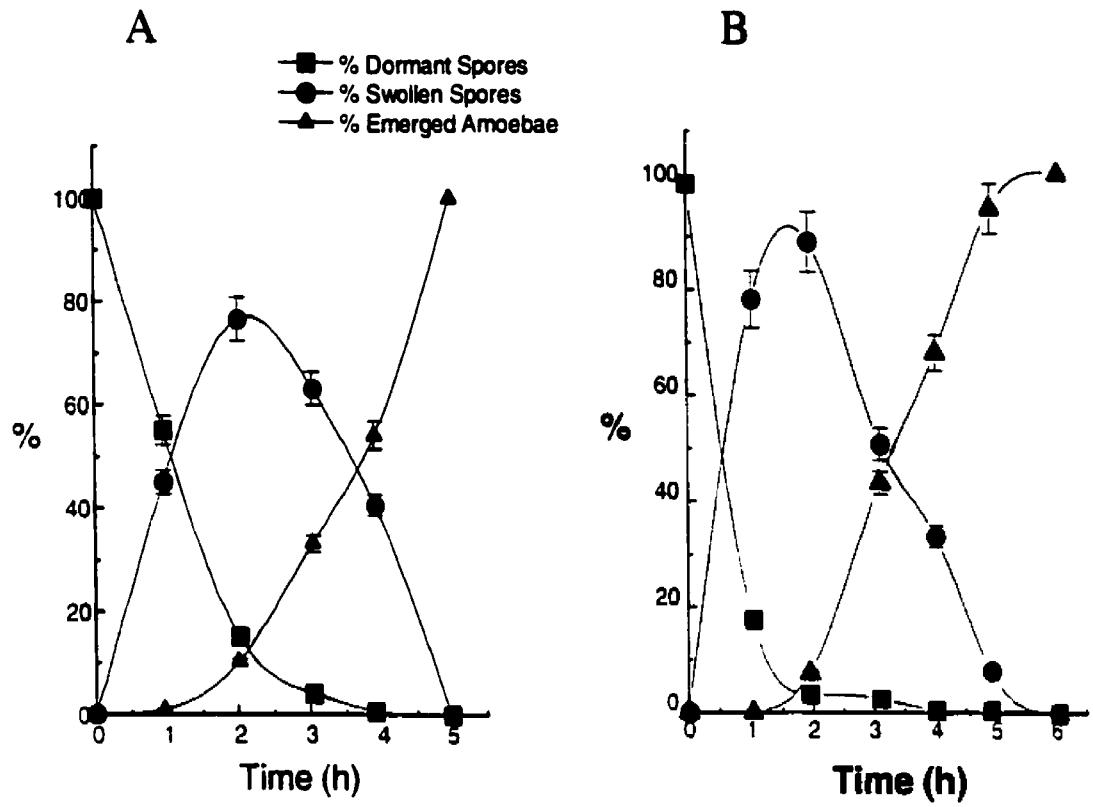

A
B
% Dormant Spores
% Swollen Spores
% Emerged Amoebae
%
Time (h)
Time (h)

Figure 17.

Figure 18. Gelatin-SDS-PAGE analysis of proteinases throughout auto-induced activation of spores of SG1. (Gel A, control; Gel B, acid treatments). Samples were collected after the addition of the tested component, at the indicated times. Each lane contains 0.7 μg of protein.

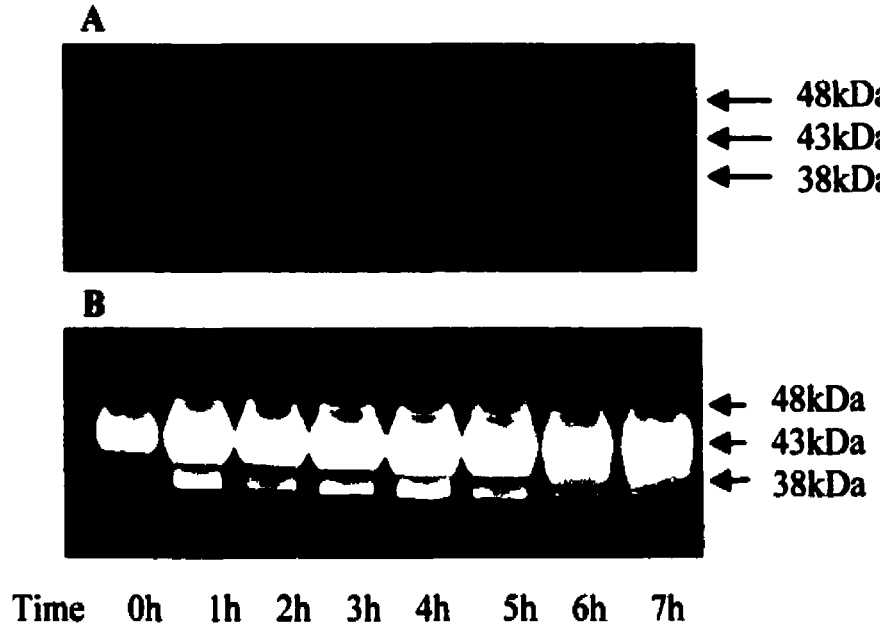

A
48kDa
43kDa
38kDa
B
48kDa
43kDa
38kDa
Time 0h 1h 2h 3h 4h 5h 6h 7h

Figure 18.

Figure 19. **Gelatin-SDS-PAGE analysis of proteinases throughout auto-induced activation of spores of strain SG1 in the presence of heat killed *K. aerogenes*.** (Gel A, control; Gel B, acid treatments). Samples were collected after the addition of the tested component, at the indicated times. Each lane contains 0.7 μg of protein.

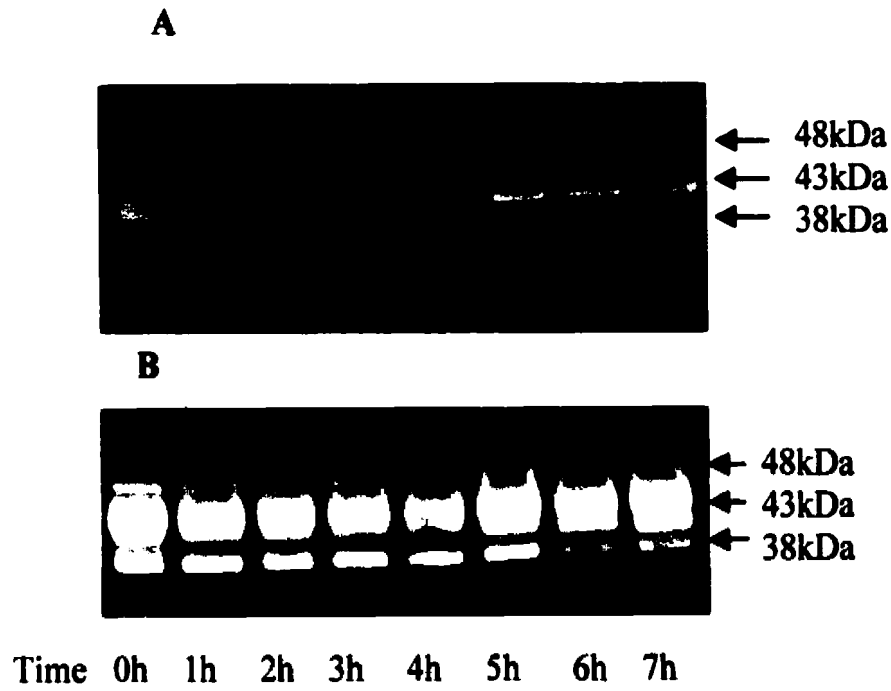

A
48kDa
43kDa
38kDa
B
48kDa
43kDa
38kDa
Time 0h 1h 2h 3h 4h 5h 6h 7h

Figure 19.

Both gram positive and gram negative bacteria are known to be capable of inducing changes of the protease patterns. Therefore, it was rather unlikely that the CPCF could belong to only one group of bacteria. However, a possibility existed that CPCF could be a combination of two or more substances normally present in bacteria. Furthermore, previous reports indicated a decrease of β-N-acetylglucosaminidase activity of *D. discoideum* when the cells were incubated in the presence of LPS (Longmore and Watts, 1980). To determine if LPS could cause effects similar to those of whole bacteria, Ax3 cells were grown in phosphate buffer (pH 6.5) in the presence of LPS purified from *H. influenzae* (Figure 20). No detectable changes took place in these cells. There were no shifts detected in either the intracellular or extracellular activity (Figure 21). It should be noted however that cells with LPS did manage to maintain cell concentrations higher than the controls (Figure 22); hence, LPS must have provided some energy for the population. Secondly, the control cells did reveal more extracellular activity than treated cells (Figure 21).

Additionally, LPS was tested for its ability to induce or affect germination kinetics of 3 day old SG1 spores. The amoeba appeared a little faster in the presence of LPS, other than this there was no noticeable difference in germination kinetics of the spores in the presence of LPS (Figure 23). Furthermore, there were no detectable differences of proteinase activities of the germinating SG1 spores in the presence of LPS, the pattern reassembled the one of control spores showed in Figure 16.

Figure 20. Gelatin SDS-PAGE analysis of changes of the proteolytic activities of Ax3 cells grown in the presence of 1mg/mL LPS. (Gel A, acid treatment; Gel B control). Lanes contain protein extracts from cells grown at various times with either LPS or heat killed *K. aerogenes* as indicated. Each lane contains 0.8 μg of protein.

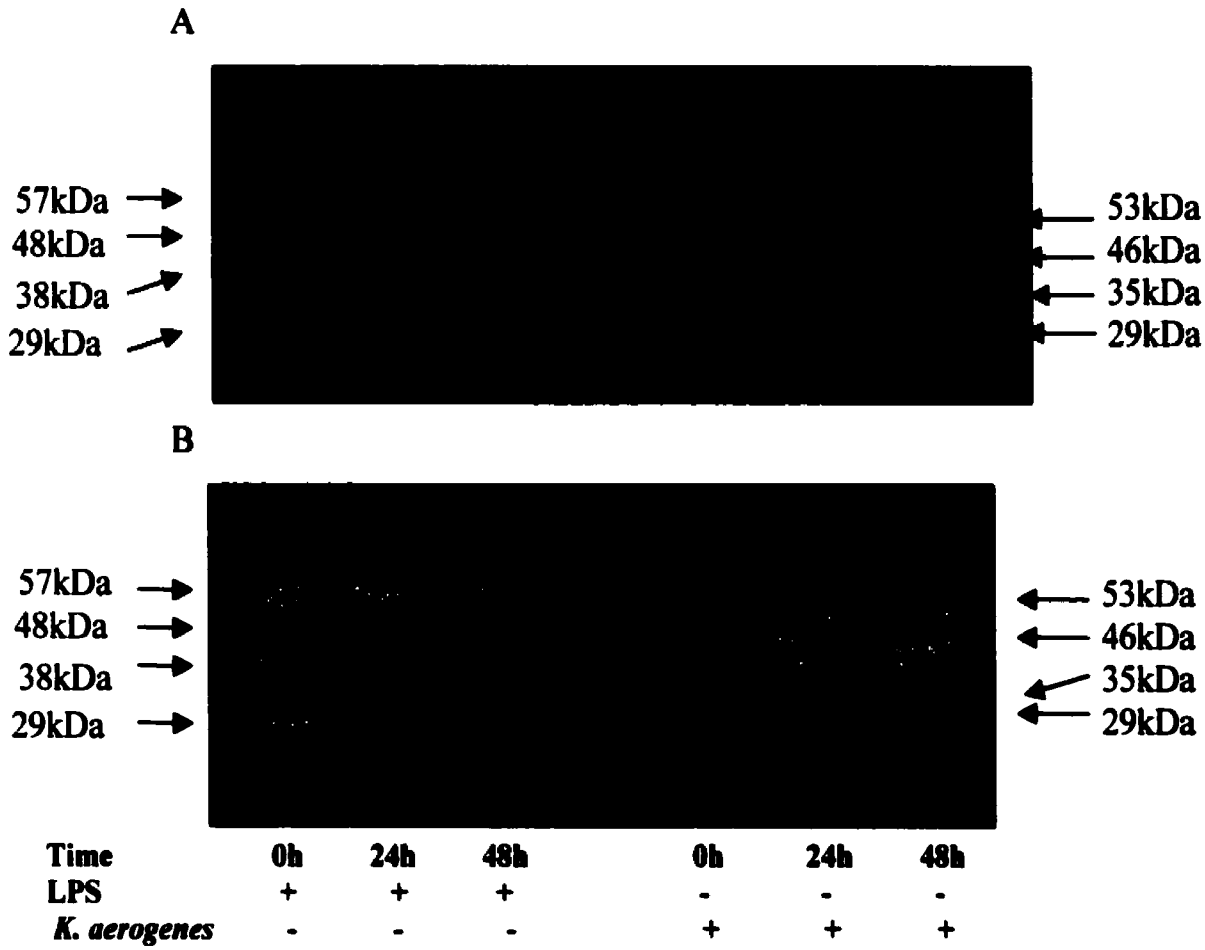

A
57kDa
48kDa
38kDa
29kDa
53kDa
46kDa
35kDa
29kDa
B
57kDa
48kDa
38kDa
29kDa
53kDa
46kDa
35kDa
29kDa
Time
0h
24h
48h
0h
24h
48h
LPS
+
+
+
-
-
-
K. aerogenes
-
-
-
+
+
+

Figure 20.

Figure 21. Gelatin SDS-PAGE analysis of secreted proteinase activities of Ax3 cells grown in the presence of 1mg/mL LPS. (Gel A, acid treatment; Gel B control). *Lane 1*, Control at time 0 hours, *Lane 2*, control at 24 hours, *Lane 3*, Growth with LPS at 24 hours, *Lane 4*, Control at 48 hours, *Lane 5*, Growth with LPS at 48, *Lane 6*, Control at 72 hours, *Lane 7*, Growth with LPS at 72 hours.

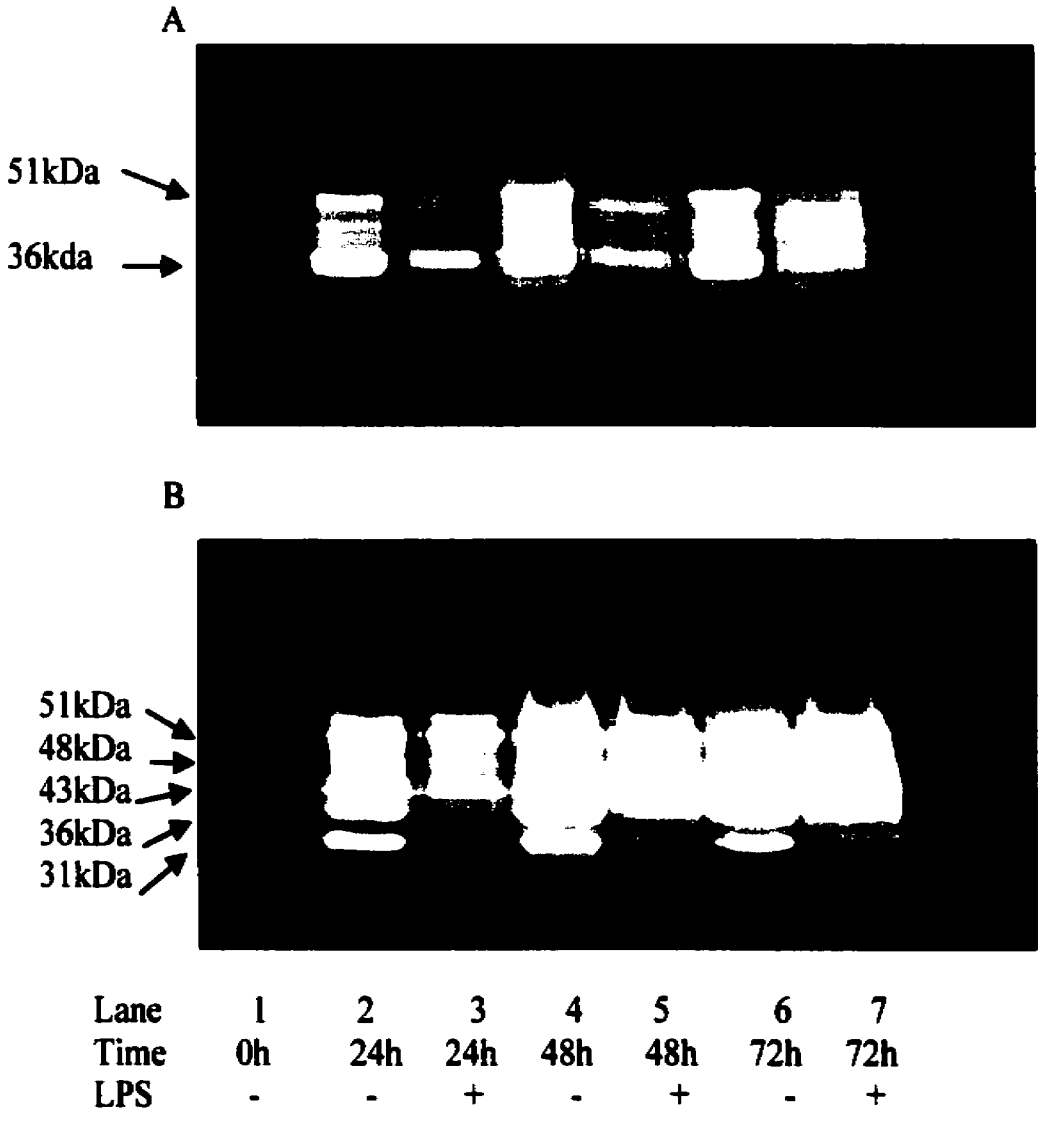

A
51kDa
36kda
B
51kDa
48kDa
43kDa
36kDa
31kDa
Lane 1 2 3 4 5 6 7
Time 0h 24h 24h 48h 48h 72h 72h
LPS - - + - + - +

Figure 21.

Figure 22. **Changes in amoeba concentrations of Ax3 when grown in potassium phosphate buffer with LPS.**

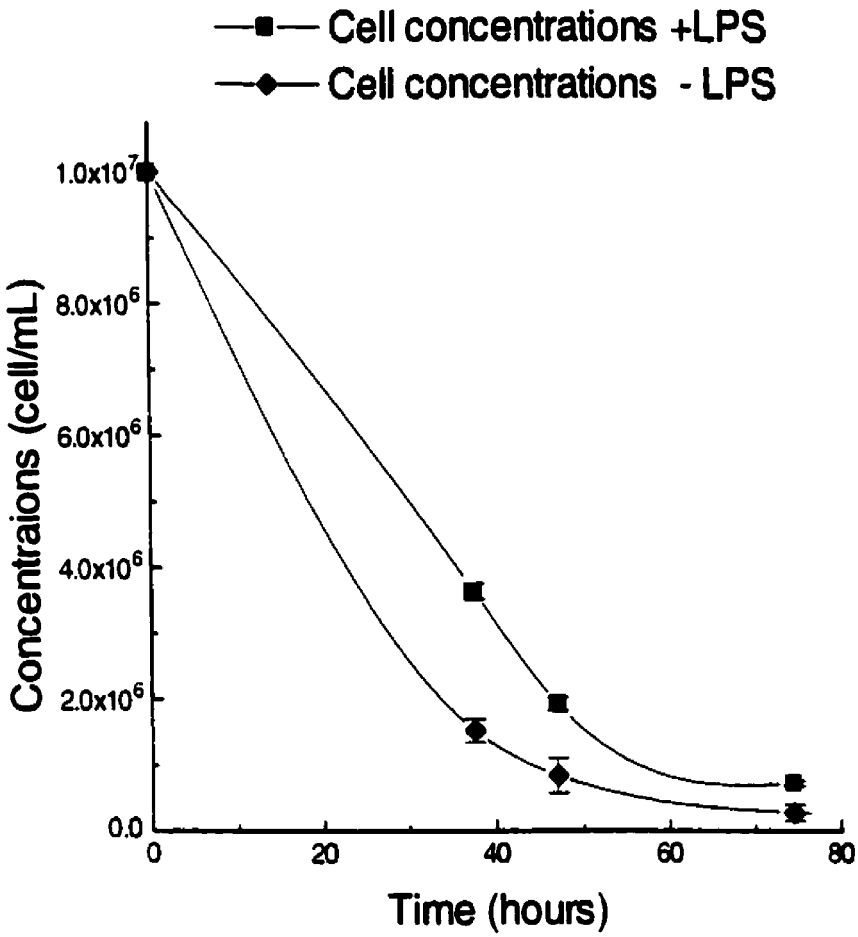

Cell concentrations +LPS
Cell concentrations - LPS
1.0x10^7
8.0x10^6
6.0x10^6
4.0x10^6
2.0x10^6
0.0
Concentraions (cell/mL)
0
20
40
60
80
Time (hours)

Figure 22.

Figure 23. Germination kinetics of auto-activated spores of strain SG1 in presence of LPS. (A, control; B, germination in presence of 1 mg/mL LPS). Starting spore concentration was $2x10^7$ cells/mL.

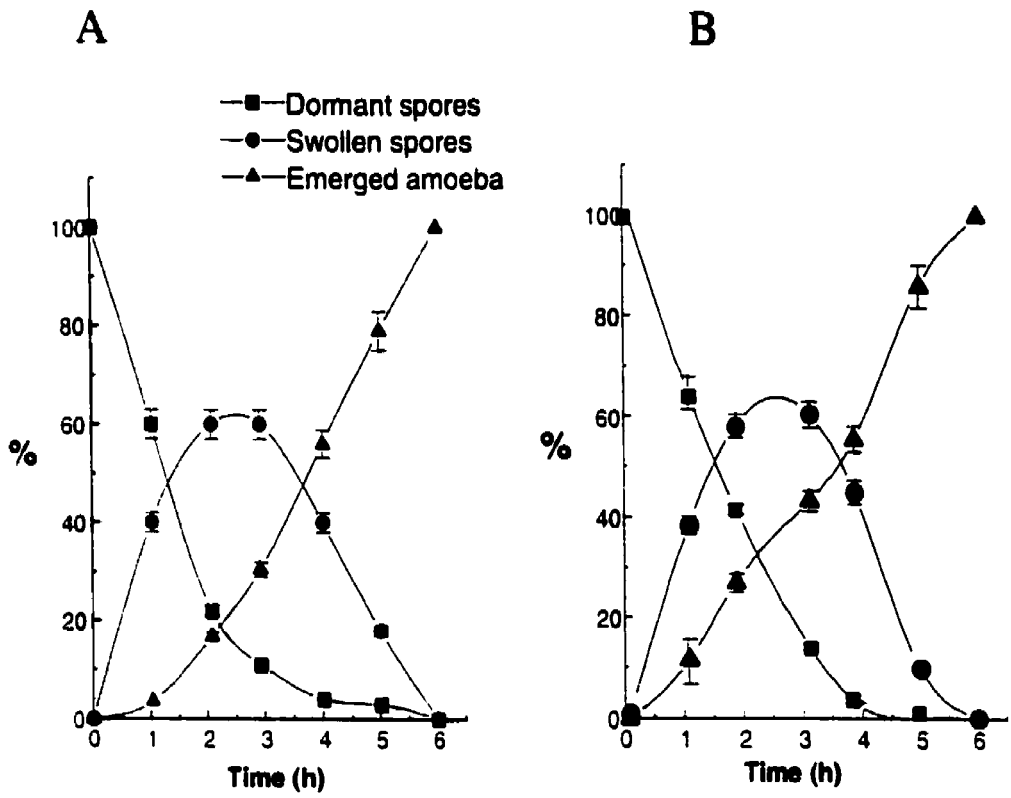

A
B
Dormant spores
Swollen spores
Emerged amoeba
%
Time (h)
0
20
40
60
80
100
0 1 2 3 4 5 6

Figure 23.

III. 8.0 Cysteine Proteinase Converting Factor: Peptidoglycan

Due to the characteristics previously described by North (1988), the CPCF was speculated to be a component of the bacterial cell wall, common to both gram positive and negative bacteria. The very likely candidate for CPCF was peptidoglycan. Therefore, to test peptidoglycan's ability to act as CPCF, Ax3 cells were incubated in the presence of 0.1mg/mL of *Staphyloccocus aureus* derived peptidoglycan. Samples were removed at 7 and 24 hours after addition, and tested with the use of zymograms for the shifts in the proteolytic patterns (Figure 24). The results revealed that the proteolytic patterns differ in the samples isolated at 7 and 24 hours. Furthermore, the proteolytic pattern detected on zymograms in the samples incubated with peptidoglycan correlated with the B pattern.

Germination of SG1 spores showed slightly altered germination kinetics in the presence of peptidoglycan. In contrast to the control germination there was about 15% more swollen spores when germination occurred in buffer plus 0.1 mg/mL of peptidoglycan; the overall germination time was comparable to the control (Figure 25). However, there was no detectable difference in the proteolytic pattern of germinating SG1 spores in the presence of peptidoglycan (results not shown).

III. 9.0 Germination of Wild Type Immature Spores in Presence of Peptidoglycan

Wild type spores are not capable of spontaneous germination unless, they are aged for 12-16 days. However, these spores can germinate at an early age (one day), if germination is performed on agar plates in the presence of bacteria. To determine if the peptidoglycan can stimulate similar effects to that of whole bacteria, NC4 one-day-old

spores were placed on non-nutrient, agar plates. The cell concentration was at $1.0x10^5$ cells/mL and peptidoglycan concentration ranged from 0.0125 mg/mL to 1 mg/mL. The controls used were spores with no additions and spores with live and heat killed *K. aerogenes*, at concentrations of 0.5, 0.25, 0.125, 0.06 and 0.03 units.

The results of this experiment are summarized in Table 1. Since the germination of wild type spores has to take place on solid medium a difficulty arose in quantifying the germinating spores. However, most of the spores in the presence of either live or heat killed bacteria have germinated within 72 hours. Peptidoglycan although at a much lower rate, succeeded in overriding the dormancy of the spores and caused germination. There is no reliable quantification for the high concentration of peptidoglycan treatments. This problem arose due to low solubility of peptidoglycan in KPi buffer, and hence low visibility of the spores under the microscope because of the floating debris. Nevertheless, the spores germinated because after 96 hours slug formation, and later fruiting body formation was noticed. There was no germination noticed for the control spores with no addition of any substances over the time tested.

Figure 24. Gelatin SDS-PAGE analysis of proteinase activities of strain Ax3 when grown in the presence of 0.1 mg/mL S. aureus' derived peptidoglycan. (Gel A, Control: Gel B, Acid treatment) Samples were collected and analyzed at the indicated times, starting at the addition of peptidoglycan. Each lane contains 0.8 µg of protein.

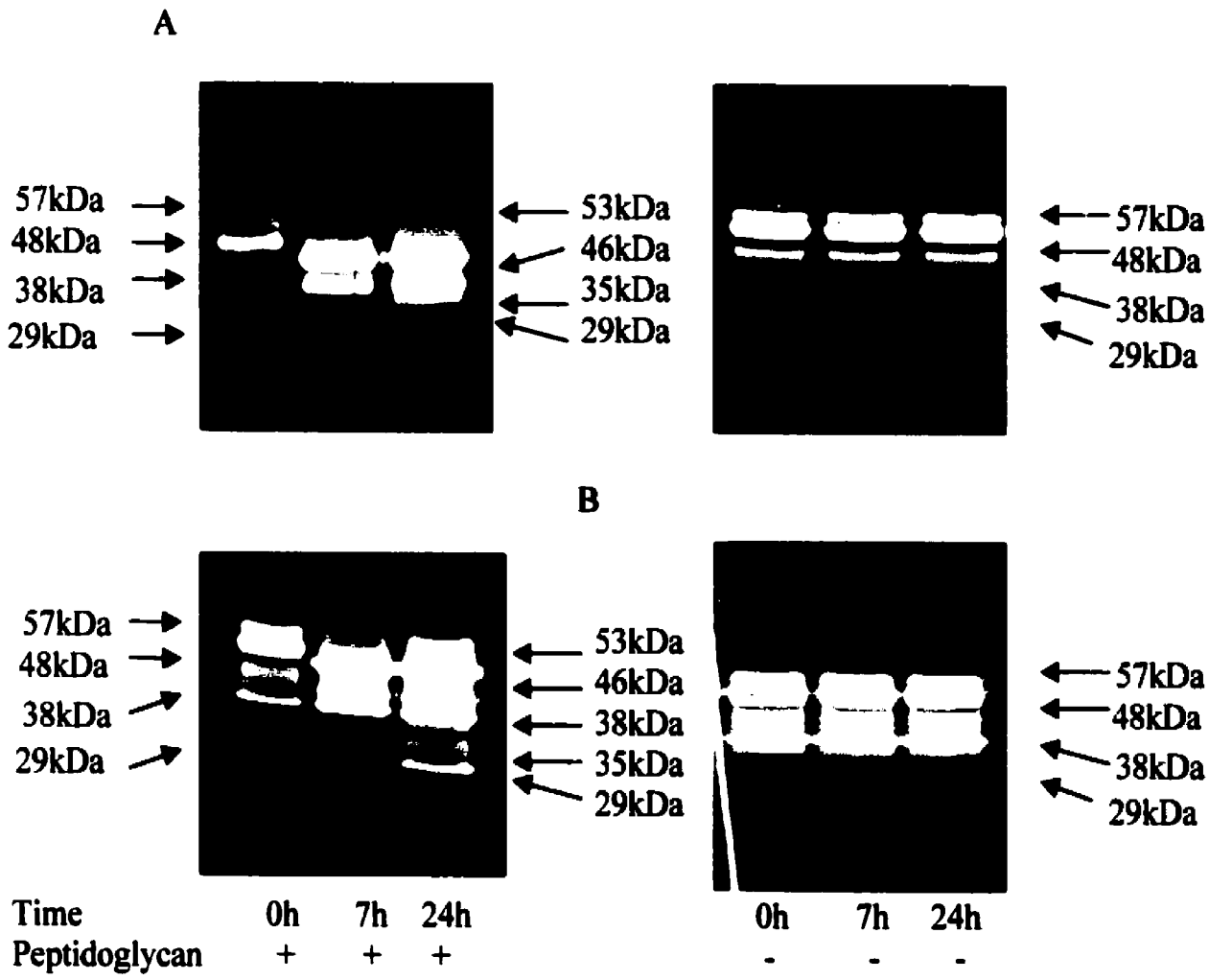

A
57kDa
48kDa
38kDa
29kDa
53kDa
46kDa
35kDa
29kDa
57kDa
48kDa
38kDa
29kDa
B
57kDa
48kDa
38kDa
29kDa
53kDa
46kDa
38kDa
35kDa
29kDa
57kDa
48kDa
38kDa
29kDa
Time
0h
7h
24h
0h
7h
24h
Peptidoglycan
+
+
+
-
-
-

Figure 24.

Figure 25. Germination kinetics auto-activated spores of SG1 strain in presence of peptidoglycan. (A, control; B, in the presence of 0.1 mg/mL peptidoglycan). Staring spore concentration was $2x10^7$ cells/mL.

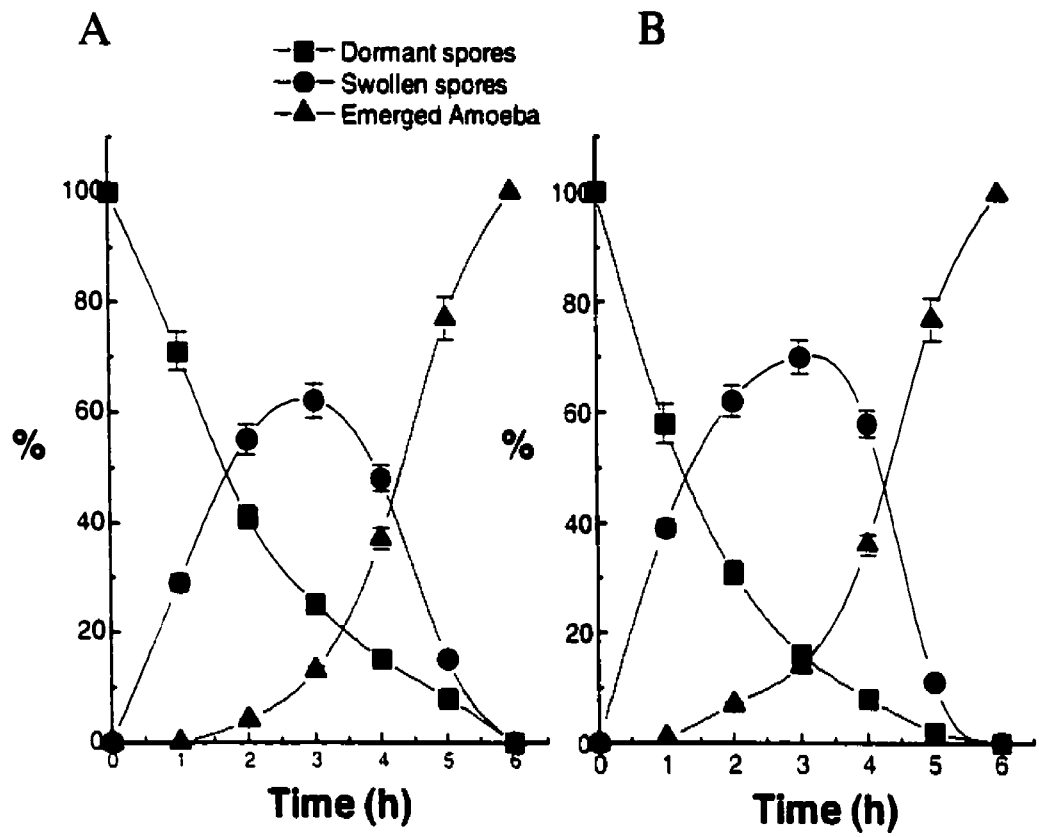

A
B
Dormant spores
Swollen spores
Emerged Amoeba
%
%
100
80
60
40
20
0
0
1
2
3
4
5
6
Time (h)
Time (h)

Figure 25.

Table 1. **Summary of results of germination of wild type NC4 one-day-old spores.** (A, germination in the presence of live bacteria; B, germination in the presence of heat killed bacteria; C, germination in the presence of peptidoglycan). * is roughly equivalent to 25% germination, N/A = not available.

A

	Treatment					
	Live *K. aerogenes* (units @ A_{650})					Control
Time	0.5	0.25	0.125	0.06	0.03	0%
24 hours	**	***	**	*	0	0%
48 hours	***	***	***	**	*	0%
72 hours	***	****	***	**	**	0%

B

	Treatment					
	Heat killed *K. aerogenes* (units @ A_{650})					Control
Time	0.5	0.25	0.125	0.06	0.03	0
24 hours	*	**	*	0	0	0
48 hours	**	***	**	*	*	0
72 hours	***	****	***	**	**	0

C

	Treatment						
	Peptidoglycan mg/ml						
Time	1	0.5	0.25	0.1	0.05	0.025	0.0125
24 hours	N/A	N/A	N/A	0	0	0	0
48 hours	N/A	N/A	N/A	*	*	0	0
72 hours	N/A	N/A	N/A	**	*	*	0
96 hours	N/A	N/A	N/A	***	**	*	*

Figure 26. Gelatin-SDS-PAGE analysis of *in situ* activation of cysteine proteinases of one day old spores of NC4. (A, control; B, acid treatment of gel A). Spores were treated with 10% acetic acid and either 0.5% Triton X-100 or 20% DMSO as indicated. Each lane contains 0.5 µg of protein.

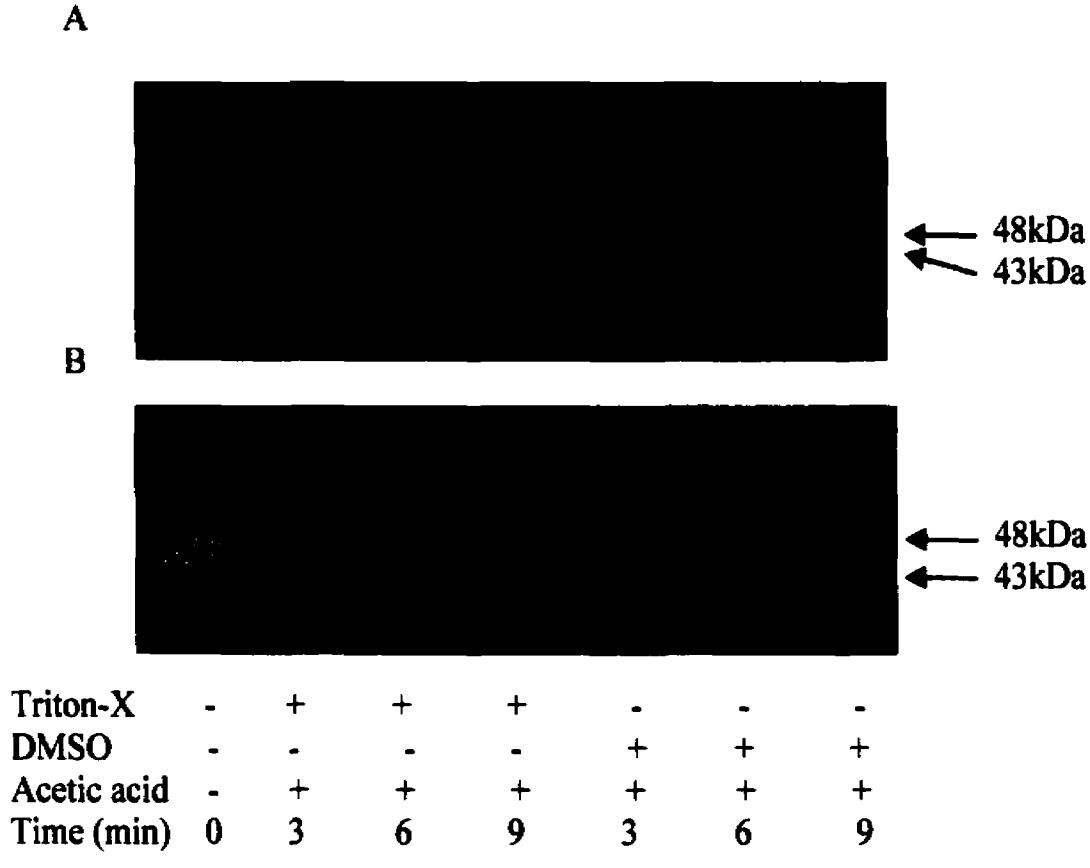

A
48kDa
43kDa
B
48kDa
43kDa
Triton-X - + + + - - -
DMSO - - - - + + +
Acetic acid - + + + + + +
Time (min) 0 3 6 9 3 6 9

Figure 26.

III. 10.0 *In Situ* Activation of Cysteine Proteinases

If acid-activation is truly a simulation of an *in vivo* process and not simply an artifact of SDS-PAGE, then cysteine proteinases should be activatable prior to extraction from the organism and electrophoresis. Therefore, an attempt has been made to activate the proteinases throughout the different life stages of the organism.

Treating dormant spores with acetic acid for extended times did not result in any changes of activities of the proteinases (results not shown). This is very likely a result of inability of acid to completely penetrate the spore coat and saturate the components of the spores. To overcome this difficulty two different penetrating agents were used: 0.5 % Triton X-100 or 20% DMSO, along with acetic acid. Treatment of NC4 1 day old spores with any of these two agents along with 10% glacial acetic acid for up to 18 minutes resulted in an increase in activities of the proteinases when compared to the control, as seen by gelatin-SDS-PAGE (Figure 26). Additionally, the proteinases were shown to be quite capable of surviving these harsh treatments for an extended period of time (up to one hour). NC4 spores treated for 30 and 60 minutes continued to show increased activity of the proteinases (Figure 27). However the spores did not survive the treatments of 10% acetic acid with and without detergents, even for a short 3-minute exposure.

Gelatin-SDS analysis of proteinases from vegetative amoebae of axenically grown *D. discoideum* Ax3 cells when exposed to 10 % glacial acetic acid for 3 to 9 minutes, revealed changes of proteinase activities (Figure 28). Only mid-range proteinases appeared to have elevated levels of activities, where all other activities were lower compared to the control. Furthermore, *in vitro* acid treatment only significantly

increased activity within the control lanes (Figure 28 *Lanes 1* and *2*), and there was no observable *in vitro* activation of the treated samples (*Lanes 3,4* and *5*).

Other life stages have been examined as well. However due to very rigorous treatments with acetic acid the protein concentrations recovered from these experiments were very low, nevertheless zymogram analysis were performed using pseudoplasmodium cells of wild type NC4 (Figure 29) and SG1 X SG2 macrocysts (Figure 30).

There was a partial activation of proteinases within the pseudoplasmodium cells. In the control lane there was no initial activity detected using the gelatin-SDS-PAGE, but in the samples obtained from 10% acetic acid treatment there were detectable levels of activity in the 38kDa region after 6 and 9 minutes of treatment. Further exposure to acid resulted in undetectable levels of activities. *In vitro* acid treatment resulted in detectable activities of the control sample in the 38kDa as well as 43kDa region. this is also evident in all remaining samples.

The original levels of proteinase activities within macrocysts are relatively high and this is probably the reason for which no further activation was possible. The amount of gelatin hydrolysis in the control lane (Figure 30), were a lot higher than in all of the others, and further acetic acid treatment failed to significantly increase the activities in any of the lanes. Similar findings have been determined for *Polysphondylium pallidum* WS320 microcysts (results not shown).

Figure 27. Gelatin SDS-PAGE analysis of *in situ* activation of cysteine proteinases of one-day old of NC4 spores. (A, control; B, acid treatment of gel A). Spores were treated with 0.5% Triton-X 100 and 10% acetic acid for times indicated. Each lane contains 0.5 µg of protein.

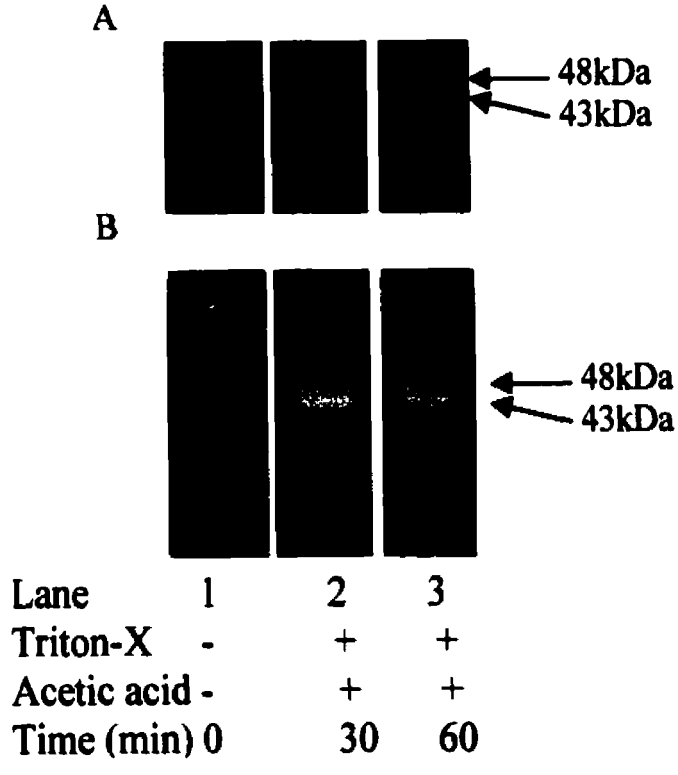

A
48kDa
43kDa
B
48kDa
43kDa
Lane 1 2 3
Triton-X - + +
Acetic acid - + +
Time (min) 0 30 60

Figure 27.

Figure 28. Gelatin SDS-PAGE analysis of *in situ* activation of cysteine proteinases of Ax3 amoebae. (A, control; B, acid treatment). Amoebae were treated with 10% acetic acid for time as indicated. Each lane contains 0.5 μg of protein.

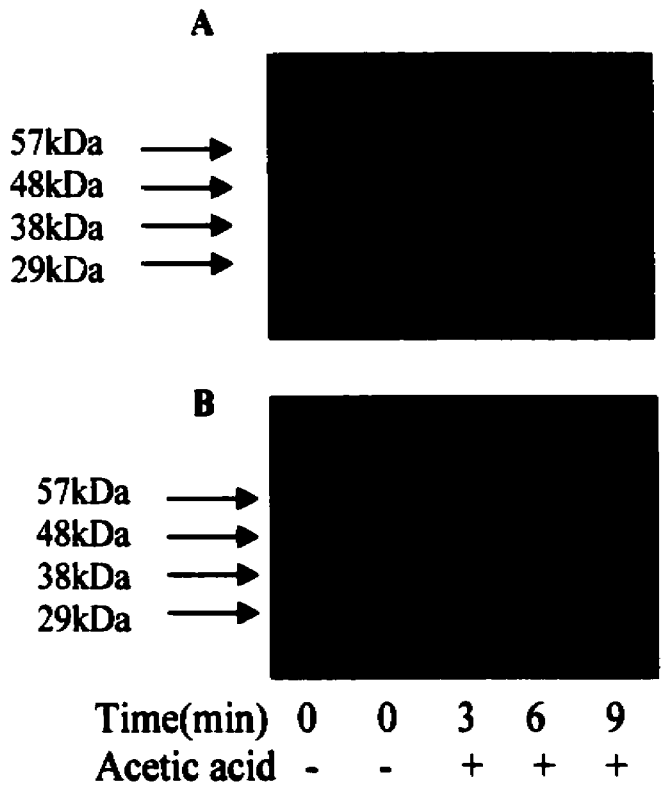

A
57kDa
48kDa
38kDa
29kDa
B
57kDa
48kDa
38kDa
29kDa
Time(min) 0 0 3 6 9
Acetic acid - - + + +

Figure 28.

Figure 29. Gelatin SDS-PAGE analysis of *in situ* activation of cysteine proteinases of the NC4 pseudoplasmodium. (A, control; B, acid treatment). Each lane contains protein extracts from slugs treated with 10% acetic acid for various amounts of time. Each lane contains 0.4 µg of protein.

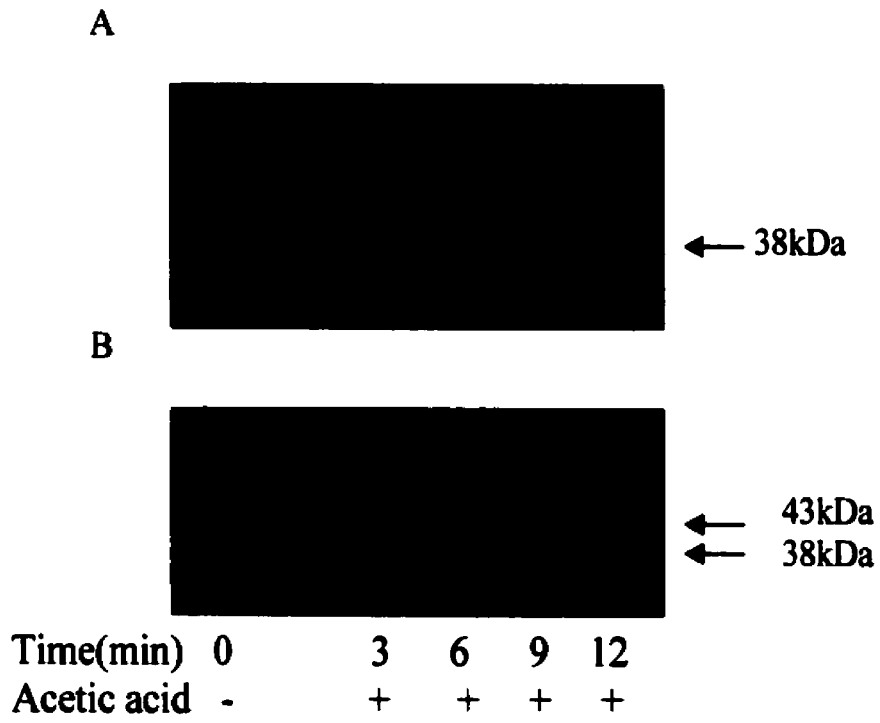


Figure 29.

Figure 30. Gelatin SDS-PAGE analysis of *in situ* activation of cysteine proteinases of SG1 and SG2 macrocysts. (A, control; B, acid treatment). Each lane contains protein extracts from macrocysts treated with 10% acetic acid for varying lengths of time. Each lane contains 0.4 μg of protein.

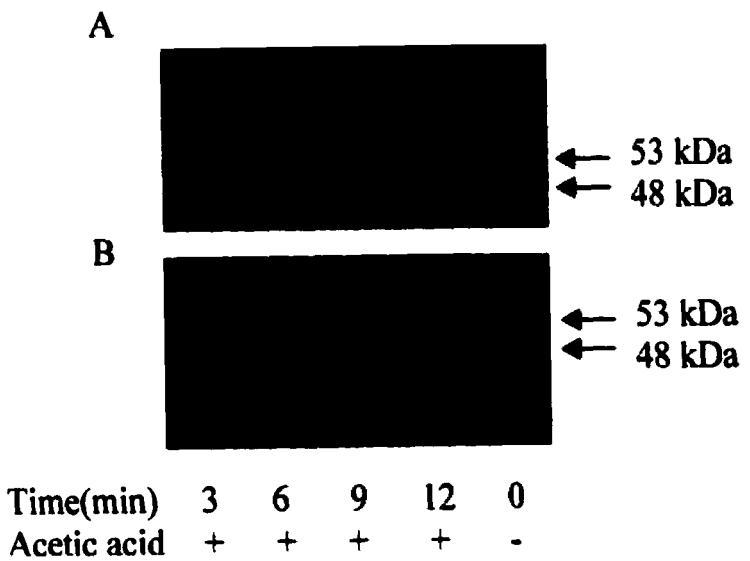


Figure 30.

IV. Discussion

"In cellular slime molds proteinases are likely to play a number of key roles during the life cycle. These include participation in the digestion of nutrients during the vegetative phase, degradation of endogenous proteins to release amino acids for developmental protein synthesis and energy metabolism, modification of pre-existing proteins for new developmental roles, selective inactivation of proteins during development, and hydrolysis of wall materials during germination processes. Given this potential diversity of roles and the possibility that some of the functions may be cell-type specific the control of proteinase activity is clearly of relevance to many other processes considered to be important to these organisms. (North and Cotter 1991)."

IV. 1.0 Differential Expression of Proteinases

A survey of the Sequence Database of the *Dictyostelium discoideum* cDNA project in Japan has revealed dozen of proteinase homologs. This would suggest that over millions of years the organism has developed quite an arsenal of proteinases used to degrade/digest different types of substrates.

North reported in 1988, that the nutrients on which *D. discoideum* feeds have a pronounced effect on the nature of the physical forms of the enzymes present in the vegetative cells. Gelatin-SDS-PAGE analysis of protein extracts of axenically grown Ax2 cells revealed major proteinases with apparent molecular weights of 38kDa, 42kDa, 51kDa and 54kDa. These comprised the A pattern. Bacterially grown myxamoebae had a distinct pattern or the B forms of proteinases, consisting of 38kDa, 43kDa and 48kDa forms. In conjunction to this finding his previous work in 1984 with *Polysphondylium pallidum* revealed that cysteine proteinase activity levels were elevated in the presence of bacteria in comparison to the control.

IV. 1.1 *Dictyostelium discoideum*, Ax3

Proteinases detected by gelatine-SDS-PAGE in this work correlated well with North's previous findings. The major A pattern proteinases found in Ax3 cells have apparent molecular weights of 57kDa, 48kDa, 38kDa, and 29kDa. Growth of cells in the presence of bacteria resulted in a change of the pattern of hydrolysis with the appearance of new activities in the region of 46kDa, and 35kDa with an additional 57kDa proteolytic band detected upon acetic acid treatment (Figure 4).

The most rapid changes induced by *K. aerogenes* in Ax3 cells occurred when myxamoebae were grown in TM media in the presence of heat killed bacteria. In this case the changes were initiated within 3 h with the appearance of the 53 kDa and 35 kDa bands and gradual disappearance of a 57kDa band (Figure 5). Loss of the A-form proteinases coincided exactly with the acquisition of the B-form proteinases, an observation consistent with the proteinase pattern changes occurring at a post-translational level. Under these conditions the changes were completed within 7 hours. In contrast, the previously investigated changes (under the same conditions), in Ax2 cells were initiated within 2h and completed within approximately 4h (North, 1988). Similar experiments were performed where KPi buffer was substituted in place of TM medium and in the presence of live or dead bacteria. In both these cases the changes were neither as rapid nor readily detectable. The possibility exists that the rapidly growing population of Ax3 cells experiences a shock by being placed in a nutrient depleted medium in the presence of a less favourable food source such as *K. aerogenes*. The cells might not possess ideal proteinases to adequately utilize bacteria and require additional time to

synthesize and activate different forms using internally stored resources first. This in turn slows their growth and response to different food sources.

IV. 1. 2. *Polysphondylium pallidum*, WS320

Since there are clear differences in proteolytic patterns between different strains of the same species it therefore follows that differences between different species are even more profound. The *P. pallidum* WS320 A pattern proteinases include 63kDa as well as 48kDa bands. Additionally, acetic acid treatment reveals an activity around 38kDa. The B-pattern activities includes a 24 kDa, and a 67 kDa proteinases. However acid treatment reveals the previously seen 48kDa and 38kDa activities along with a new 36kDa band (Figure 5). The 24kDa proteinase of the B-pattern reveals decreased level of activity following acid treatment. However this is consistent with previously reported results in which the low molecular weight proteinases with high initial activity are turned off with acid treatment or possibly denatured by the treatment (North *et al.*, 1996).

The timing of the proteolytic changes, in response to bacteria, is also different (Figure 9). As before, the loss of the A pattern proteinase (48kDa) coincides with acquiring the B-pattern activity at the 24kDa region (3h after the addition of the bacteria). However, unlike the Ax3 cells the A pattern 48kDa proteinase was not lost as the activity is brought back by acid treatment. In this case, the changes taking place are not consistent with the proteinase pattern changes taking place at a post-translational level of the A-form enzymes

Furthermore, in contrast to *D. discoideum*, there were no major differences

observed in the timing of the changes of cells grown under different growth conditions (Figures 10 and 11). *P. pallidum* WS320 cells grew at a slower rate compared to that of *D. discoideum* and hence they might not

experience as profound a shock to the removal of a more favourable food source. Additionally, the A-form enzymes might be more efficient at the task of utilizing bacteria present in their environment than Ax3 cells.

IV. 2.0 Characteristics of the Proteinases of the A and B Forms

Protein synthesis is a necessary component for the switch between the A and B forms of proteolytic activities. Incubation of the cells in the presence of bacteria and 400µg/mL of cycloheximide resulted in no appearance of the B-form enzymes within the time frame where the change is usually evident. In the case of Ax3 cells there was no major changes observed in the intensity of the individual bands (Figure 13). On the other hand, *P. pallidum* proteinase activities in the presence of the protein synthesis inhibitor decreased throughout the experiment (Figure 14). This observation can very likely be accredited to auto-degradation of pre-existing proteinases.

These results are indicative of the importance of protein synthesis in the switch between the patterns. However, it is impossible to know at this point, whether the proteinases of the B-form are synthesized *de-novo*, a process which would require an energy commitment on the part of the organisms. The changes might involve conversion of the A-form proteinases into the B-form, and these are dependent on some alternation to the machinery involved in post translational modification. This would be the most cost efficient way of producing the new species, since *de-novo* synthesis would require loss of

A-form enzymes by secretion, inactivation or degradation in *D. discoideum*. Secretion of A-pattern proteinases was not detected in the medium in response to the CPCF. Inactivation cannot involve lysosomal pH shifts since the acid treated gels would reveal the presence of these enzymes. There are no known endogenous inhibitors in *Dictyostelium discoideum*, and there is no experimental evidence thus far that they might be involved in any of the controls over cysteine proteinases. Therefore, the only alternative which remains is for the A-form enzymes to be degraded, a process that is very cost inefficient for the cell. There is evidence of changes in other hydrolytic enzymes in *D. discoideum*, but these occur during the developmental phase and not in response to an environmental factor (Bennet and Dimond, 1986; Moore et al., 1987).

In *P. pallidum* the changes in the forms of proteinases might be a result of a number of different mechanisms. The major A-form proteinase detected in the control gel is one with a MW of 48kDa. In the protein extracts from bacterially grown cells this enzyme clearly looses activity to a point of non detection in the control gels. However, acid treatment reveals that this proteinase is present in the extracts with similar concentrations to the controls as the activity levels are comparable. Secondly, the B form includes a new 24kDa and an acid activatable 36kDa proteinase not seen before in the A-form; the appearance of these are not coupled with the loss of the other activities. It is therefore very likely that these enzymes might be newly synthesized in response to the CPCF.

IV. 2.1 Cysteine Proteinase Inhibitor Studies

E64 and 2,2'-Dithiodipyridine were the two cysteine proteinase inhibitors used in this study. Use of these inhibitors on protein extracts from Ax3 cells grown in the presence and absence of *K. aerogenes* yielded interesting results. First, the majority of activities of both A and B pattern proteinases were diminished. However, the 53kDa proteinase activity present in the B form was not inhibited as adequately. Furthermore, acetic acid treatment increased proteolytic hydrolysis in this region of the gel. If the 53kDa proteinase were produced as a result of post-translational modification of the 57kDa (A-form) then one would expect similar reactivities of these two enzymes. It is however possible that the hydrolysis in this region might be due to two different types of proteinases with similar MW, and two dimensional gel electrophoresis might answer this question. Nevertheless it appears that most of the species of proteinases detected by gelatin-SDS-PAGE are of the cysteine type.

In *P. pallidum* the activities were greatly diminished in the presence of the inhibitors. The only band, which shows no effect, is the one with a MW of 63kDa. Additionally, this activity is also unaffected by acid treatments. Therefore, the gelatin hydrolysis in this region of the gel can be attributed to aspartic proteinases found previously in slime molds with a reported MW of approximately 60kDa (North and Whyte, 1984). The 48kDa proteinase shows a little less reactivity to 2,2'-Dithiodipyridine. Thus, activities of both the A and B form can be attributed to proteinases of the cysteine type.

IV 3.0. Cysteine Proteinase Converting Factor

The CPCF reported by North in 1988 is very likely unique to prokaryotic cells. Heat killed yeast cells and heat killed Ax3 cells failed to produce similar results as bacterial cells (Figure 16). On the other hand both gram positive and gram negative bacteria can stimulate changes in the activities of cysteine proteinases (North, 1984; North, 1988).

Germination kinetics of spores can be affected by a variety of treatments, some of which include; heat shock (Cotter and Raper, 1986) , 1% peptone (Cotter and Raper, 1966), and 8M urea (cotter and O'Connel 1976). However, most of these treatments are rather disruptive for the cell and normally not found in nature. A common signal for germination in the wild would be, as on can assume, return of favourable growth conditions. One of these can be the abundance of food source (Cotter 1975). Given that slime molds are capable of feeding on a number of different food sources (mostly on soil bacteria) it might be possible that the same bacterial factor can act as both the CPCF and an activator or at least an effector of germination.

The germination kinetics in the presence of heat killed *K. aerogenes* of SG1 spores yields very interesting and promising results (Figure 17). The lag period of cells in the presence of the bacterial factor is greatly reduced. Overall timing of germination is the same in both cases. However, there are no detectable changes in the proteolytic patterns found with gelatin-SDS-PAGE of the protein extracts, both for the control and bacterially treated spores. This is not surprising however, since the proteinases of the B-form resemble those found in slime molds grown on bacteria (North, 1988; North & Cotter, 1991).

IV.3.2. LPS and Peptidoglycan

The factor responsible for changes of cysteine proteinase forms was present in both gram positive and gram negative bacterial cultures (North 1988). Some of the characteristics of the cell free factor derived from *K. aerogenes*, clearly established that it was macromolecular bacterial cell wall component. The effect of lysozyme provided strong evidence in support of the view that its activity was dependant on peptidoglycan. However, previous investigations of Longmore and Watts (1980) has shown that another component unique to gram negative cell walls, LPS, affected the activities of lysosomal β-N-acetylglucosaminidase activity in *D. discoideum*. Secondly, one could not rule out the possibilty of CPCF being more than one compound.

Growth of Ax3 cells along with 1 mg/mL and 2 mg/mL of purified LPS from *H. influenzae* failed in inducing detectable changes in cysteine proteinase activities (Figure 20). However, the cells grown on LPS succeeded in maintaining higher cell concentrations compared to those without LPS in the media (Figure 22). Therefore, *D. discoideum* is capable of utilizing LPS as an energy resource especially since the control cells did secrete larger amounts of gelatin-SDS-PAGE detectable proteolytic activity (Figure 21). This finding correlates with previously published work showing that starved cells do release higher amounts of hydrolytic enzymes into the media (Seshardi *et al.*, 1986).

Additionally, LPS failed to induce major changes in the germination pattern of SG1 spores. The appearance of amoebae occurred slightly faster compared to the control, however, the timing in most other cases was similar to the control (Figure 23).

As expected, the proteolytic activities detected in germinating spores in the presence of LPS was not any different than the controls (results not shown).

Peptidoglycan appeared to be the ideal component acting as the CPCF due to its characteristics (North, 1988). To test this hypothesis Ax3 cells were grown in the presence of 0.1 mg/mL of purified peptidoglycan from *S. aureus* (Sigma). The fact that the peptidoglycan was isolated from gram positive cells gave assurance to its purity and to the absence of other factors found in some gram negative bacteria (i.e. LPS).

Figure 24 summarizes the results from this experiment. Peptidoglycan did manage to cause a shift of the proteinase forms in the time period expected. In addition, peptidoglycan also managed to affect the kinetics of spore germination in a similar fashion seen in germination experiments with whole bacterial cells (i.e. a decreased lag time) (Figure 25). More importantly, young wild type spores unable to germinate spontaneously, were stimulated to germinate by the peptidoglycan alone (Table 1). Thus peptidoglycan has all of the characteristics to safely consider it to bge CPCF.

It will now be interesting to examine the relationship in the conversion of the cysteine proteinase forms and the feeding of the slime molds. The question posed in this study would involve the mechanism of phagocytosis and digestion of bacteria, and also would point to whether an external receptor is involved in the detection of the CPCF. Answers to these questions would allow a further elucidation of the pathways involved in the conversion of these proteinases. In addition, the mechanism by which dormant spores are signalled in the presence of peptidoglycan and stimulated to germinate should be examined as well.

IV.4.0 Controls of Activities of Cysteine Proteinases in Cellular Slime Molds

Acid activation of the cysteine proteinases *in vitro* is believed to simulate the events of acidification of lysosomes by V-ATPases. This event is believed to create an acidic environment inside the lysosomal compartments. The lowest concentration of acetic acid required to activate these proteinases *in vitro* is 0.425M. This may appear to be quite drastic with respect to the entire cell, but many mammalian cells contain micro acidic environments, including the resorption chamber of osteoclasts for proteinase activation (Holtzman, 1989). Further evidence that these enzymes are present in either an active or inactive state is seen with E64 inhibitor treatments. Acetic acid treatments usually reveal some additional activities even in the presence of this inhibitor. Since E64 binds to the active site of the proteinase (Barret *et al.*, 1982) this would suggest that some of the enzymes' active sites are not exposed to the inhibitor. Furthermore, it is believed that the activation of these enzymes is due to conformational changes of the protein structure and neither to a cleavage of a pro-peptide region nor a dissociation of a proteinase inhibitor. North et. al., (1996) revealed that after acid activation two dimensional gel electrophoresis did not show a decrease in relative molecular weight of the cysteine proteinases. In addition, mercuric chloride ($HgCl_2$), which previously has been shown to inhibit autocatalytic cleavage of the pro-region of latent Cathepsin L for activation (Maciewicz *et al.*, 1988), had no affect on the activation of ddCP48 when treated prior to acid activation (North *et al.*, 1996). Additionally not only are the proteinases activated by the acid treatment but also can be de-activated with base treatment and again re-activated with an additional acid treatment. This is the strongest

argument so far that the conformational changes are responsible for the activities of these enzymes.

IV.4.1 *In situ* Activation of Cysteine Proteinases

If the hypothesis of a conformational change being produced through acidification is true, then cysteine proteinases should be activatable prior to extraction from the organisms and electrophoresis. The method used to experimentally activate the proteinases attempted to simulate the *in vivo* acidification that takes place in the lysosome. It should be kept in mind that this is simply a rudimentary simulation of a complex process, complete activation of all modifiable cysteine proteinase was not expected.

In situ activation of the dormant NC4 spores of *D. discoideum* was readily accomplished using 10% glacial acetic acid along with 0.5% Triton X-100 or 20% DMSO. Close examination of the lanes in figure 26 indicates that the 48kDa and 43kDa cysteine proteinases showed significantly increased activation as compared to the control lane. Examination of the *in vitro* acid activated version of the duplicate gel shows even higher activity levels, compared to its *in situ* activated counterpart. This indicates that not all the proteinases *in situ* are activated by the treatment. This was to be expected since the experimental approach was only a rough simulation of an *in vivo* process.

The resiliency of the spores to the above treatment solution is illustrated in figure 27. North (1996) pointed out that extended acid activation resulted in progressive decrease in proteinase activity. Therefore, spore resistance to acid/Triton treatments may be because not all lysosomes are affected at the same time. The thick wall of the spores may prevent some lysosomes from being subjected to treatment, while others may be

affected right away. Those that are activated early may experience denaturation over time, but those activated later may still be at their peak level. This also may explain why *in situ* activation never achieves the activity levels of an *in vitro* acid treatment. There are no noticeable differences in the molecular weights of the proteins, which would be indicative of a cleavage of pro-region or release of an inhibitor.

Vegetative myxamoebae (*D. discoideum* Ax3) treated with 10% glacial acetic acid yielded some interesting results when a zymogram was performed on the extracted proteins. First of all, only two bands correlating to molecular weights of 48kDa and 38kDa showed an increase in activity (Figure 29). Minimal increase in activity of the proteins was expected since the base level in the vegetative state is so high (North et al., 1996). The highest 57kDa and lowest 29kDa bands showed a decrease in activity with treatment. This decline was most likely due to the proteins innate susceptibility to acid denaturation. Another phenomenon of interest can be seen in the *in vitro* acid treated duplicate. While the control bands increase significantly with the treatment, lanes containing extracts from cells treated *in situ* prior to acid treatment showed no increase in activity. The reason for this may very well be that all the enzymes present are at their highest activity level, and are incapable of further activation.

Analysis of protein extracts from the pseudoplasmodium of NC4 treated with acetic acid for an extended period of time show increased activities of the 38kDa proteinase (Figure 29). Given that there are no apparent activities within the control lane, the treatment was minimally successful. Further treatment of the gel with acetic acid for 60 seconds revealed activity of an additional proteinase with a MW of 43kDa and an

increase in hydrolysis in the 38kDa region. Both of these are comparable to the control lane.

Macrocysts of SG1xSG2 when subjected to 10% glacial acetic acid showed no increased activity with the treatment (Figure 30). However, in this case little change was expected, due to the fact that the base activity level of the two proteinases present in the control lanes (53kDa and 48kDa) are already extremely high. *In vitro* acid treatment does not have a profound effect on the activities of any regions of the gel.

In conclusion these *in situ* studies found proteinases present at various stages of slime molds' life cycles to be activatable prior to extraction from the organism and electrophoresis. Enzymes were activated as a results of a pH shift within the organism; therefore, this comprises yet additional proof that increased activity is not an artifact of SDS-PAGE. Molecular weights of proteinases with increases activities were comparable to the controls and hence rule out the possibility of pro-region modifications or loss of inhibitor proteins. Purification, sequencing and 3-D analysis of these proteinases will greatly contribute to the discovery of the conformational structures under both active and inactive conditions.

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APPENDIX A

Preparation of 7.5% and 10% Acrylamide Separating Gels.

	7.5% Acrylamide	10% Acrylamide (mL)
DdH2O	3.85 mL	3.00 mL
2% gelatin	1.00 mL	1.00 mL
1.5M Tris-HCl, pH8.8	2.5 mL	2.5 mL
30% T Acrylamide	2.5 mL	3.35 mL
10% SDS	100 µL	100 µL
10% Ammonium Persulfate	50µL	50 µL
TEMED	10µL	10 µL

Preparation of 4.0% Acrylamide Stacking Gels.

	4.0% Acrylamide
DdH2O	5.90mL
0.5M Tris-HCl, pH 6.8	2.5 mL
30% T Acrylamide	1.5 mL
10% SDS	100 µL
10% Ammonium Persulfate	50µL
TEMED	10µL

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