Extracellular serine protease activity among selected members of the Saprolegniales: Potential role in pathogenicity.

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Extracellular Serine Protease Activity among Selected Members of the Saprolegniales: Potential Role in Pathogenicity

by

Stephen C. Kales

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

Windsor, Ontario, Canada

2001
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Abstract

Some of the world’s most notorious fungal pathogens belong to the phylum Oomycota. *Saprolegnia* and *Achlya*, both water-borne members of this phylum, have been implicated as pathogens of amphibians, fish and rice. Like all fungi, these organisms require nutrient uptake by external enzymatic degradation. It has long been hypothesized that such external enzymes may also play a crucial role in pathogenicity by degrading host structure and defenses, while providing nutrients for the growing pathogen as it penetrates deeper into host tissues. Proteases, enzymes that specifically degrade proteins, may serve a crucial role in animal host penetration through fibrous structural proteins. In this study, gelatin SDS-PAGE analysis has demonstrated multiple bands of inducible extracellular proteolytic activity among *Achlya ambisexualis*, and two species of *Saprolegnia*, including a species recently isolated as the cause of “winter kill” in Mississippi fish farms (Bly et al. 1992). This inducible proteolytic activity among the Saprolegniales may provide answers to their mechanism of potential pathogenicity. Proteolytic activity among these isolates was detectable over a wide range of pH, but optimized under alkaline conditions, conditions typical of protein supplemented cultures. Most proteolytic activity, detectable by gelatin SDS-PAGE analysis, was completely inhibited by the serine protease inhibitors PMSF, SBTI and leupeptin. Crude protease preparations demonstrated a wide range of substrate specificity to several synthetic serine protease substrates. Further characterization employing the trypsin substrate, BApNA, demonstrated susceptibility to TLCK, further supporting a serine protease mechanism and suggestive of a trypsin-like mechanism. Although not thermally optimized, this crude extracellular trypsin-like activity was highest per mycelial dry weight when grown in the presence of protein under low temperatures, similar to those observed during “winter kill”. These results suggest that this trypsin-like activity may play a role in low temperature induced fish diseases such as “winter saprolegniosis”.

iv
To my father, his analytical mind has always driven me to figure out how things work.
To my mother who always showed me to look things up when I couldn’t figure them out for myself.
Their qualities have inspired me and their love and support have carried me throughout my pursuits.
Acknowledgements

I would like to thank my advisors, Dr. David Cotter and Dr. Donovan Thomas, for their support and for providing me with the opportunity to test myself. To Dana Mahadeo and David Cervi, for their help, advice and friendship. To my wife Wendy for everything else.
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<th>Description</th>
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<tbody>
<tr>
<td>BApNA</td>
<td>N-Benzyol-arginine p-nitroanilide HCL</td>
</tr>
<tr>
<td>BM</td>
<td>Barksdale's Medium</td>
</tr>
<tr>
<td>DFP</td>
<td>di-isopropylfluorophosphate</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
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<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>E-64</td>
<td>L-3-carboxy-trans-2,3-epoxypropyleucylamido (4-guanidino) butane</td>
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<td>EDTA</td>
<td>ethylenediamine tetra-acetic acid</td>
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<td>MES</td>
<td>2-(N-Morpholino)ethanesulfonic acid</td>
</tr>
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<tr>
<td>NaOH</td>
<td>sodium hydroxide</td>
</tr>
<tr>
<td>p</td>
<td>para-</td>
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<tr>
<td>PIPES</td>
<td>1,4-Piperazinediethanesulfonic acid</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulphonyl fluoride</td>
</tr>
<tr>
<td>SAAPpNA</td>
<td>N-Succinyl-alanine-alanine-proline-phenylalanine p-nitroanilide</td>
</tr>
<tr>
<td>SBTI</td>
<td>soybean trypsin inhibitor</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
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<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
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<tr>
<td>SPPNA</td>
<td>N-Succinyl-phenylalanine p-nitroanilide</td>
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<tr>
<td>SPPApNA</td>
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<tr>
<td>Suc</td>
<td>succinyl</td>
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<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
</tr>
<tr>
<td>TLCK</td>
<td>L-1-chloro-3-[4-tosylamido]-7-amino-2-heptanone HCL</td>
</tr>
<tr>
<td>Tos-</td>
<td>tosyl, (4-toluenosulphonyl)</td>
</tr>
<tr>
<td>TPCK</td>
<td>L-1-chloro-3-[4-tosylamido]-4-phenyl-2-butanone</td>
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<tr>
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<td>tris-(hydroxymethyl)-aminomethane</td>
</tr>
<tr>
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<td>micromolar</td>
</tr>
<tr>
<td>μL</td>
<td>microlitre</td>
</tr>
<tr>
<td>Z</td>
<td>benzyloxy carbonyl, carbobenzoxy (=CBZ)</td>
</tr>
</tbody>
</table>
1. Introduction

1.1) *Fungi* and *fungi*

The term fungi encompasses a large, ubiquitous group of organisms. To many, this term immediately invokes an image of the famed speckled, red-capped mushroom of many fairytales. This, the *Amanita*, is but one of many thousands within the vast and diverse kingdom of fungi. A select number of fungi have become an intimate part of our daily lives, while most others remain completely foreign and some simply serve as a musty annoyance. Fungi can be microscopic or miles wide, as recently demonstrated by “fungus humongous” in Michigan (Alexopoulos et al. 1996). Traditionally, fungi have been defined as eukaryotic, spore-bearing, non-chlorophyllic organisms that reproduce both sexually and asexually, and having absorptive nutrition. As in many cases, such a definition always has room for exceptions, and such is the case with fungi. Many organisms, historically considered members of the kingdom Fungi, bear characteristics that make it difficult to place them in a single taxonomic group. It has, therefore, become common practice among authors to refer to this generally flexible group of organisms as “fungi”, and reserving the capitalized term, “Fungi”, for those that distinctly share characteristics and, therefore, seem to be truly related. One particular group of organisms, the Oomycota, has long been recognized as distinctly different from the typical *Fungi*. Although they are morphologically similar and have absorptive nutrition, it has become increasingly apparent that the Oomycota lack any close phylogenetic relationships with the true *Fungi*, as demonstrated by recent molecular comparisons (Leipe et al. 1994, Baldauf et al. 2000). Such comparisons have recently shown that they may be best described by their original term, Phycomycete, (*Algal fungus*). Now, commonly grouped with the brown algae, either within the kingdoms *Stramenopila* or *Chromista*, their place among taxonomists is still a topic of debate. The characteristics that make taxonomic placement of the Oomycota so difficult, also defines their phylum. Such features include biflagellate zoospores, like those of algae, cellulosic cell walls, like those of plants and most obvious, is their oogamous sexual reproduction, which gives this phylum its name. There are many other interesting characteristics, each of which could exhaust years of research, and pages of detail, but are not within the scope of this study.
1.2 The Saprolegniales

Oomycetes can be found around the world, from terrestrial habitats to marine and freshwater. The terrestrial forms comprise some of the world's most notorious phytopathogens, including *Plasmopara viticola*, which nearly destroyed the French wine industry and *Phytophthora infestans*, the potato late-blight fungus that led to great Irish famine of the 1850's. This single organism led to the emigration of millions and shaped social and economic history. Most of the aquatic members, commonly, yet improperly, referred to as "water molds", are restricted to fresh water. Most are saprophytic, living off the remains of decaying organic matter, and playing a key role in nutrient recycling. Some however, are parasites of algae, nematodes, mosquito larvae, many fish and crayfish. The Saprolegniales are one order of the fresh water Oomycetes, and as their name implies, have been historically generalized as saprophytic. Members of the Saprolegniales are however some of the most important fish pathogens (Jones, 1976). *Saprolegnia* and *Achlya*, both members of the Saprolegniales, are considered to be the major pathogenic fungi of the common carp, the most cultured fish in the world, topping one million metric tons in 1990 (Jeney & Jeney, 1995). *Achlya debaryana* was demonstrated for the first time as causing an epizootic mycosis of the Channel Catfish, *Mastacembelus armatus*, in Indian fish farms (Khulbe et al. 1992). A *Saprolegnia spp.* has also been isolated as the cause of "winter kill" in Mississippi fish farms (Bly et al. 1992), now termed "winter saprolegniosis". Such pathogenic outbreaks have caused significant economic damage and great attempts have been made to deal with this pathogen. *Saprolegnia spp.* and *Achlya spp.* can be found in almost any body of fresh water making the potential for infection difficult to avoid, and further highlighting the need for better understanding their biology. They are also among the easiest fungi to isolate and cultivate, allowing them to be readily studied under laboratory conditions, as they have for over 100 years. Baiting from fresh water can be performed using a variety of substrates, including snakeskin, sesame seed and hemp seed. They are characterized by profuse branching of aseptate, or coenocytic diploid hyphae, reserving septa, (hyphal cross-walls), strictly for delimiting reproductive organs. Unlike true *Fungi*, which have chitinous cell walls, the cell walls of oomycetes are composed primarily of β-glucans, small amounts of cellulose and the amino acid hydroxyproline (Alexopoulos et al. 1996).
The hyphae often give rise to terminal sporangia filled with asexual zoospores (Figure 1). Following their release through an exit papilla, these biflagellate zoospores, similar to those of the brown algae, can chemotactically "swim" towards a new substrate, or encyst under unfavourable conditions. The encysted zoospore can release a new secondary zoospore or, produce a germ tube and give rise to a new diploid thallus. Sexual reproduction also involves chemoattractants. Compatible mates, attracted by sexual hormones, can exchange genetic material following gametangial contact, giving rise to the production of thick-walled sexual spores, termed the oospores, (egg-seed), a feature unique to the Oomycota. As mentioned, there are several characteristics that separate the Oomycota from the true Fungi, however, one key feature that they do share with the true Fungi, is their absorptive nutrition. Nutrients must enter through the cell wall and plasma membrane. Instead of first ingesting, then digesting like animals, fungi must release digestive enzymes into the external environment. The smaller, more soluble end products of this extracellular digestion can then be absorbed (Alexopoulos et al. 1996). Many fungi can exploit nearly any substrate, while others are very specialized. Substrate range is largely determined by the battery of secreted enzymes at its disposal. The importance of secreted degradative enzymes not only to nutrition, but also to host invasion is quite clear. Numerous studies have investigated the roles of secreted degradative enzymes of fungi, including the Saprolegniales. Investigations into the role of secreted β-glucanases in fungal tip morphogenesis have been a topic of interest for a number of years. Secreted enzymes are considered by many to be of critical importance to successful invasion by wood-rot fungi. Wood decay by the brown rot fungi, such as the Aphyllophorales, involves extensive depolymerization of the cellulolytic wall components (Alexopoulos et al. 1996). Three classes of secreted enzymes have been identified in some white-rot fungi, including hydrolytic enzymes (Alexopoulos et al. 1996). It is understandable why secreted cellulolytic enzymes would be of critical importance to a phytopathogen such as the wood-rot fungi. Zoopathogens on the other hand, have an entirely different set of epidermal barriers to overcome. Due to the proteinaceous components found in animal host tissues, proteolytic enzymes are often the subject of interest.
Figure 1. Life cycle of *Saprolegnia* and *Achlyya*.

Somatic hyphae (A) give rise to terminal asexual sporangia (B). Release of a secondary zoospore (F) followed by encystment (G). Release of another secondary zoospore can occur or germination (H). Meiotic division occurs in gametangia, (antheridia and oogonia). Fusion can occur on the same thallus (homothallism), as shown, or from two compatible mates (heterothallism). Oospore germination (O) gives rise to a somatic thallus (A). Not to scale. (Drawing by R. W. Scheetz from Alexopoulos et al. 1996).

* In *Achlyya*, primary zoospores immediately encyst upon release giving rise to a cluster of encysted zoospores at the tip of the empty zoosporangium, a diagnostic characteristic of *Achlyya*.
Figure 1.
The secreted proteases and what role they may play in zoopathogenicity have been the subject of much study and speculation for many notorious zoopathogens, but very little is known regarding the secreted proteases of the *Saprolegniales*.

### 1.3 Proteases

To try to understand the organismic role of a protease, one must first try to understand its molecular function. Proteases are a class of enzymes that catalyze the cleavage of the peptide bonds of other proteins. They play a variety of physiological functions, ranging from protein processing to protein digestion. They are found in all eukaryotes, some prokaryotes and viruses where they occupy as crucial a role in commercial applications as they do in physiology. Their substrate and hydrolysis specificity allows them to conduct selective modifications of other proteins resulting in their diverse applications in industry and medicine. They are used in the preparation of a wide range of products ranging from leather production to dairy processing. It was estimated that worldwide sales of industrial enzymes, 60% of which were proteases, topped $1 billion in 1998 (Rao *et al.* 1998). The classification of proteases is currently based upon the following criteria: type of reaction, the chemical nature of the catalytic site, and structural relationships. There are two main types of proteases, those that cleave peptide bonds within a polypeptide chain, and those that cleave proximal to the termini. These are referred to as endo and exopeptidases, respectively. Within these two groups, proteases are further sub-divided into four prominent groups, based upon their catalytic sites. The four catalytic types are the cysteine, serine, aspartic and the metalloproteases. A fifth characteristic type is the threonine mechanism of the multiprotein, barrel-shaped complex called the proteosome.

#### 1.3.1 Serine Proteases

Serine proteases are characterized by the presence of a serine residue in their active site. They can be endo- or exopeptidases and are found in nearly all types of organisms. Structural similarities have led to divisions of the serine proteases into as many as 20
families and 6 clans, three of the more common clans being chymotrypsin, carboxypeptidase C and subtilisin. All three clans share a common mechanism involving a catalytic triad of three amino acids brought together by tertiary structure. The serine residue acts as the nucleophile, a histidine as the base and an aspartate residue as the electrophile (Rao et al. 1998). The catalytic site of a protease is flanked by specificity subsites. Each protease subsite is deemed $S_i$ through $n$ and $S_i'$ through $n$, from the catalytic site towards the N terminus and C terminus, respectively (Figure 2). Complementary subsites within the substrate are similarly deemed P and P’ flanking the scissile bond, the site of cleavage. The serine protease mechanism follows a two-step reaction in which a covalently linked enzyme-peptide intermediate is formed. Nucleophilic attack by water upon the intermediate results in hydrolysis of the peptide bond. Serine endopeptidases are further grouped according to their substrate P1 preference. As an example, trypsin-like and chymotrypsin-like proteases prefer positive and hydrophobic P1 residues respectively. Serine proteases are generally active at neutral and alkaline pH, and have mass ranges from 18 to 35 kDa, with some, however, reaching 126 kDa, as in the zygomycete, Blakeslea trispora (Rao et al. 1998). Alkaline serine proteases are the largest subgroup of serine proteases and are produced by several bacteria, molds, yeasts and fungi (Rao et al. 1998). They are typically inhibited by DFP or a potato protease inhibitor, but not by the trypsin inhibitor TLCK nor the chymotrypsin inhibitor, TPCK. They do, however, typically share similar substrate specificity to that of chymotrypsin (Rao et al. 1998). The serine proteases, as mentioned above, involve a mechanism of acylation/deacylation using a Ser-His-Asp catalytic triad. The remaining three main protease types involve somewhat different catalytic mechanisms. Cysteine proteases follow a general acid-base attack followed by the hydrolysis of an acyl-thiol intermediate, very similar to serine proteases. Aspartic proteases also follow an acid/base attack involving an Asp-Thr-Gly active site. Metalloproteases depend upon the presence of divalent cations, usually zinc, and bound in most cases, by a His-Glu-Xaa-Xaa-His motif.
Figure 2. The Schecter and Berger nomenclature for peptide-peptidase binding. The protease is represented as the shaded area. Amino acid side chains of the polypeptide substrate are represented by $P_1$ to $P_1'$. $S_1$ to $S_1'$ represent the corresponding side chains of the protease. Drawing from Beynon & Bond, 1989.
Figure 2.
1.4 Protease and Pathophysiology

As mentioned, the physiological roles of proteases are as numerous as their industrial applications. Proteases can activate required metabolic enzymes by limited hydrolysis or completely degrade other proteins. Proteases allow leukocytes the capacity to leave the blood vessels by penetrating the basement membrane, which lines the vascular lumen and to degrade collagen, thereby gaining access to microorganisms that may be infecting the surrounding connective tissues (Kohnert et al. 1988). They also play a pivotal role in many other complex processes involved in normal cellular metabolism. They can, unfortunately, also serve a role in abnormal physiology. The same capacity proteases play in degrading extracellular matrices for proper immune response, is also a prerequisite for the metastasis of malignant cancer cells (Kohnert et al. 1988). It is for their involvement in cancer and AIDS, that most people recognize the terms “protease” and “protease inhibitors”. Proteases have also been implicated in diseases caused by other viruses, bacteria and fungi. A group of protease genes have been clearly linked with virulence in Candida infection (Staib et al. 2000). Candida albicans is an opportunistic fungal pathogen of humans, commonly causing oral thrush. Existing primarily as a benign commensal organism in healthy individuals, it can quickly cause superficial, as well as life-threatening, systemic infections in those with compromised immunity, especially those suffering from AIDS. Although it is a general view that no single virulence factor is responsible for Candida infection, various characteristics have been implicated. Such characteristics include: colony morphology (switching from a yeast form to a hyphal form), the ability to adhere to a variety of substrates as well as the correlation between the ability to secrete acid proteases and virulence in laboratory animal models (Morschhäuser et al. 1997). Several roles of these proteases have been proposed including digestion of host proteins for nutrient supply, evasion of host defenses (by degradation of immunoglobulins), adherence and invasion (Morschhäuser et al. 1997). In the latter, invading microorganisms establishing a systemic infection would be required to penetrate the basement membrane underlying the host epithelium, comprised of a tight network of extracellular matrix proteins.
It has also been proposed that proteolytic degradation of keratin and collagen may facilitate the penetration of tissue barriers in cutaneous infections (Morschäuser et al. 1997). Potential for evasion of host defenses by a protease was demonstrated in a bacterial fish pathogen. Culture supernatant of *Aeromonas salmonicida* was shown to suppress the humoral response of the Atlantic salmon, *Salmo salar*, and the factor responsible was a serine protease (Hussain et al. 2000). A 200kDa metalloprotease has been associated with virulence in the fish pathogen *Cryptobia salmositica* (Zuo & Woo, 1998). The purified metalloprotease directly lysed fish erythrocytes by digesting membrane proteins and found to be secreted into the blood causing haemolysis (Zuo & Woo, 1998). Secretion of this protease was significantly enhanced by collagen type I or IV supplemented media, which it readily degraded (Zuo & Woo, 1998). The theory that protease may play a key role in zoopathogenicity is not a new one, and the supporting evidence is growing.

### 1.5 Saprolegniosis

As mentioned, several members of the Saprolegniales have been demonstrated as significant pathogens of freshwater fish (Jeney & Jeney, 1995, Bly et al. 1992, Alvarez et al. 1994 & Neish, 1977), exhibited by hyphal penetration of the skin and underlying muscle in low temperature-induced immunosuppressed fish (Bly et al. 1992). As proposed in many cutaneous and systemic infections, secreted proteases may facilitate saprolegniosis. Typical signs of saprolegniosis and related *Achyrea spp.* infections have been described as fungal associated skin lesions, mucous-depleted skin and sunken eyes (Bly et al. 1992). Lesions can occur anywhere on the body of the fish but are most common on the dorsal surface of the head, in front of the dorsal fin, other fins and any sites of mechanical trauma (Neish, 1977). Increased lethargy is associated with the progression of the disease, and infected fish tend to congregate in quiet areas of streams (Neish, 1977).
Histopathological analysis demonstrated that the radiating fungus first destroys the epidermis followed by penetration of the basement membrane and subsequent invasion into the dermis and in some cases, into the hypodermis and musculature with little or no inflammatory response (Neish, 1977, Hatai & Hoshiai, 1992). The damage done by the fungus is perceived to be directly related to the tissue destroyed in the immediate area of the hyphae, as there is no known toxin produced and no sign of systemic infection (Neish, 1977). Such mass tissue destruction clearly implicates proteolytic digestion of the collagen and other proteinaceous components of the dermal tissue, and underlying basement membrane and muscle.

1.6 Temperature and Saprolegniosis

Channel catfish, Ictalurus punctatus cultured in earthen ponds in the southern United States often suffer mass mortalities during the months of October through March, to a syndrome commonly referred to as “winter kill”. Such large-scale mortalities result in major financial losses, and are distinctly different than ice cover-induced anoxia, also termed “winter kill” (Bly et al. 1992). Observations by farmers immediately implicated temperature-dependent immunosuppression as a factor. “Winter kill” tends to follow the passage of severe cold weather fronts, where pond temperatures drop as much as 9°C in 12 hours and remain at low temperatures (6° to 12° C), for 1 to 4 weeks (Bly et al. 1992). Previous studies involving laboratory catfish have shown that a severe drop in tank temperature, (from 22° to 12° C over 24 hours), leads to temperature-induced immunosuppression. Other studies have demonstrated temperature-dependent regulation of immune related genes in various teleosteans (Quiniou et al. 1998, Rodrigues et al. 1998). Pure cultures of fungus were isolated from the commercially raised channel catfish exhibiting symptoms of “winter kill”. Challenge experiments, at both 22° and 10° C, were performed satisfying Koch’s postulates. Rapid fungal growth was seen at both temperatures. Histopathological analysis revealed fungal hyphae at, and deep below, the surface of degenerated dermal tissue (Bly et al. 1992). Lesions showed complete destruction of epidermal tissue, typically containing mucous secreting cells, generally considered the first line of defense against infectious diseases (Quiniou et al. 1998).
Severe water temperature decrease did not lead to fish mortality, unless in the presence of *Saprolegnia* *spp.* CF1 zoospores. Considering the apparent link between cold temperature and saprolegniosis, one must consider that in order for protease activity to be playing an active and crucial role in disease progression, the ability of the fungus to produce active proteases at such temperatures is imperative.

1.7 Protease and the Saprolegniales

Considering their pathogenic potential, little is known regarding the proteases of the Saprolegniales. A previous study demonstrated the ability of *Achlya ambisexualis* Raper E87 to produce extracellular protease under an induction+derepression regulation pattern, a pattern similar to those demonstrated in other fungi including *Neurospora crassa*, *Mucor miehei*, *Ustilago maydis* and *Beauveria bassiana* (Hill & Pott, 1997). Detectable levels of extracellular protease activity were only found in culture filtrates where growth occurred in the absence of readily available nitrogen or sulfur (derepression) plus the presence of an extracellular source of protein (induction). In this study, collagen seemed to induce the highest levels of detectable protease per dry weight of mycelium, as compared to other sources of protein (Hill & Pott, 1997). In a separate study, immunological analyses were performed on the mycelial extracts from four of the most frequently pathogenic species of the genus *Saprolegnia* (Peduzzi & Bizzozero, 1977). The objective of their study was to compare antigenic compositions among the pathogenic species and evaluate the degree of relationship between those species. The four species of study were the following: *S. parasitica*, *S. ferax*, *S. declina* and *S. delica*. Comparative immuneelectrophoretic analysis revealed that all four antisera contained at least one antibody capable of precipitating antigens exhibiting chymotrypsin-like activity. This activity was detected by the ability of these antigens to hydrolyze the chymotrypsin substrate, *N*-acetyl-L-phenyl alanine methyl ester (Peduzzi & Bizzozero, 1977). Similar experiments also demonstrated the presence of this enzyme activity in culture media. This extracellular chymotrypsin-like activity was found to be two to five times higher than that found in the mycelial extract, suggesting an extracellular destination of these proteolytic enzymes (Peduzzi & Bizzozero, 1977).
Furthermore, this exoenzyme reacted with antisera formed against the cytoplasmic constituents, suggesting that secretion did not change its antigenic properties (Peduzzi & Bizzozero, 1977). It was further suggested that such extracellular activity could be related to degradation of host tissues (Peduzzi & Bizzozero, 1977). A more recent study failed to demonstrate any convincing extracellular, or free, proteinase activity among selected members of the Saprolegniales (Smith et al. 1994). Their study was designed to test whether selected species of *Saprolegnia* demonstrated specific amino acid requirements by selective uptake. Utilization of protein under various conditions was tested upon: *S. diclina, S. ferax* and *S. parasitica.* Casein, as a sole source of carbon, (and potentially a nitrogen and sulfur source) supported growth in the three test species, suggestive of effective casein utilization. Increased alkalinity followed incubation with a pH change from an initial 6.83 to near and/or above 8.00, and that such alkalinity was associated with ammonia release. The addition of glucose to protein supplemented media led to increased biomass without suppressing proteolytic activity (Smith et al. 1994). Similar results of glucose addition were seen in *Achlya ambisexualis,* provided that at least one other essential element was lacking (Hill & Pott, 1997). Selective amino acid uptake was demonstrated by an increased biomass when methionine was used in combination with other amino acids, as the sole sources of nitrogen and sulfur (Smith et al. 1994). Plate halo diffusion tests, however, failed to demonstrate free proteinase activity, suggesting that proteinases were potentially immobilized (Smith et al. 1994). Based upon increased alkalinity and end-point culture pH, the authors suggested the probability that *Saprolegnia* produces alkaline serine proteases.

### 1.8 Objective

The implications that proteases may play a key role in pathogenicity and host tissue penetration is broadly accepted, and supporting evidence is building throughout various fields of study, from human illnesses such as cancer and *Candida,* to other non-human pathogens, including many fish diseases. Considering the ubiquity of the Saprolegniales and increased rise in densely populated fish farming, a better understanding of their ability to produce extracellular protease is imperative.
As mentioned, little is known regarding the regulation or the mechanism of extracellular proteases among these organisms. The objective of this study is to further investigate and compare the presence of extracellular protease activity among selected members of the Saprolegniales, and discuss their potential role in fish pathogenicity. This study will first attempt to replicate, then supplement, the work of Hill & Pott, 1997, by including Achlya ambisexualis Raper E87. Attempts will then be made to determine the potential for, and regulation of extracellular proteolytic activity in two other test species, Saprolegnia ferax and Saprolegnia spp. CF1 Bly. S. ferax was a test species of previous studies and is considered one of several species potentially parasitic to fish. The later species, S. spp. CF1 Bly, has been isolated as the causative agent of “winter saprolegniosis” in Mississippi fish farms (Bly et al. 1992), and to this author’s knowledge, is yet to be included as a test species for extracellular protease activity. The onset of winter saprolegniosis has accompanied low water temperature and, therefore, an investigation into the ability of this test species to produce extracellular proteolytic activity at such temperatures will serve as a secondary objective.
2. Materials & Methods

2.1. Preparative Methods:

2.1.1. Fungal Isolates

The three isolates under study were *Achlya ambisexualis* Raper E87 (ATCC 11399), *Saprolegnia ferax* Gruithuisen (ATCC 36051) provided by Dr. B. Heath, York University and *Saprolegnia spp.* CF1 Bly (ATCC 200048). Any other cultures were isolated from local fresh-water systems.

2.1.2. Maintenance of Stock Cultures

All fungal isolates were maintained on corn meal agar (Difco). Fresh plates of maintenance cultures were made every 8 weeks by transferring a 6 mm cork bore plug from the colony periphery to a fresh 100 mm diameter petri plate of corn meal agar. Colonies were then allowed to grow to the plate edge, a diameter of 80 mm, and then subsequently stored at 4°C. Colonies were periodically observed for any noticeable changes in colony morphology, signifying significant mutations or contamination.

All solid media contained 1.5 % w/v agar.

2.1.3. Culture and Growth Conditions

All isolates under study were grown in defined liquid media with slight modifications from Hill & Pott (Hill & Pott, 1997). Originally developed by Mullins and Barksdale, 1965, Barksdale mating medium is an autoclaved liquid, or 1.5 % agar-infused, medium containing monosodium glutamate as a source of organic nitrogen,
D-glucose as a carbon source and L-methionine as a source of sulfur and buffered to pH=6.9 (See Appendix B). Control growth experiments involved standard Barksdale Medium (BM). In most protease induction experiments, one or more of the C, N and/or S sources were replaced with 0.2 % w/v protein prior to autoclaving, as described in Hill & Pott, 1997. In most cases, gelatin served as the protein source, exceptions included collagen, casein or albumin.

2.1.3.1 Shaken Cultures

Initial growth experiments were performed, with slight modifications, as described by Hill & Pott, 1997, involving 100 mL volumes (in 250 mL Erlenmeyer flasks) of the BM media, with or without protein supplementation, inoculated with six cork bore plugs (6mm) from an actively growing colony periphery. Incubations were performed at room temperature, (22° C) with reciprocal shaking (100 rpm; 3.5 cm excursion) on a rotary shaker.

2.1.3.2 Unshaken Cultures

In order to mimic natural growth conditions, later growth experiments were performed without reciprocal shaking, but in smaller volumes to increase replicate numbers. One 6 mm plug of colony periphery was used to inoculate a 25 mL volume of the various media types in 100 mm petri plates and incubated without shaking for the entire growth period. The absence of rotary shaking allowed increased radial growth, similar to that seen in natural pond conditions (personal observation during separate field studies) and avoided the dependence on limited shaker space.
2.1.3.3 Unshaken Cultures & Protein Utilization

Later, preliminary growth experiments were also performed as above, using protein as the sole source of all added nutrients. These involved inoculations as described above except plates contained dH₂O instead of liquid Barksdale media. Sets of 60 mm petri plates contained 10 mL of ddH₂O with various proteins, (0.2 % w/v), as the sole source of all nutrients. Under these growth conditions, the protein would serve as the sole source of nutrients for all growth requirements. All protein types, gelatin, collagen, casein and albumin were supplied by Sigma and added prior to autoclaving.

2.1.3.4 Hair Plates

Human hair served as a source of keratin and was prepared according to Safranek & Goos, 1981. Hair was acquired from a local barber (Holland Distributing & Barber Shop), rinsed in ddH₂O, dried and cut into small fragments. Lipids were removed by immersing in chloroform for 24 h. with rotary shaking followed by equal washing in ddH₂O to remove chloroform. Dried hair was then used as a source of keratin, (6g/L), prior to autoclaving, and subsequently included in the preliminary growth study, as above. Positive controls contained Barksdale medium to support growth. This also served to allow for the detection of any inhibitory effects of colony growth by residual chloroform, in plates containing hair. Results of this preliminary growth study were then simply recorded as growth observed or not observed.
2.1.4. Crude Protease Sample Harvesting

Cultures were harvested by vacuum filtration through 2.5 cm Whatman no. 2 filter paper with sacrifice, or by micropipette, without sacrifice, at timed intervals throughout the growth period and subsequently filtered to remove any hyphal filaments. Crude protease samples were then dispensed into pre-autoclaved 1.5 mL Eppendorf tubes and stored at −20°C for later analysis.

2.1.5. Dry Weights

Dry weights were determined by vacuum filtration, as described above, upon dried and tared 2.5 cm Whatman no. 2 filter paper and re-weighed following drying for 24 h. at 80°C, as described by Hill & Pott, 1997.

2.2. Analytical Methods:

2.2.1. Substrate SDS-PAGE Analysis

2.2.1.1. Experimental Controls

All electrophoretic separations were performed using Bio-Rad Mini Protean II Electrophoresis Cells. A 2% w/v gelatin stock was used as protease substrate in all cases unless otherwise stated (Appendix A). Unless specified, all gelatin was co-polymerized within a 10% acrylamide separating gel (Appendix A). Following polymerization of the separating gel, (approximately 30 minutes), a 4% acrylamide stacking gel, lacking the protein substrate, but containing a teflon lane comb, was then polymerized atop the separating gel (Appendix A). All acrylamide gels were 0.75 mm in thickness unless otherwise stated. Samples were prepared, without boiling, by adding 4 parts of sample to 1 part of 5X Sample Buffer.
The 5X sample buffer contained 2.5 mL β-mercaptoethanol, 5.0 mL glycerol, 2.5 mL 0.5 M Tris-HCl; pH 6.8, 1.0 g SDS and 1.0 mg Bromophenol Blue, all acquired from Sigma. Following polymerization of the stacking gel, (approximately 30 minutes), the teflon lane combs were removed and lanes rinsed with 1X Running Buffer prior to sample loading. The 1X running buffer, pH 8.3, included 3.0 g Tris Base (Sigma), 14.4 g glycine (Sigma), 1.0 g SDS (Sigma) in 1.0 L nanopure water. Electrophoretic separation was performed at 4 °C at a constant voltage of 200 volts until the dye front reached the bottom of the separating gel, (approximately 55 minutes). Following electrophoresis, the gels were then washed in 2.5 % Triton X-100 (v/v) for 30 minutes to remove SDS. Gels were then rinsed in distilled water and placed in the appropriate incubation buffer for 18 hours at room temperature. Gels were then stained for protein with a Coomassie Blue solution for 1 h. and subsequently destained in a 10% v/v acetic acid/ 5% v/v methanol solution until bands of protease activity were detectable. Coomassie Blue solution contained 2.5 % w/v Coomassie Brilliant Blue R-250, 50 % v/v methanol and 10 % v/v acetic acid. Gels were then dried at room temperature between two BioGelWrap membrane sheets within a drying frame set. Relative molecular weights of protease banding were determined by comparison with SDS-6H molecular weight markers (Sigma) loaded in a separate lane of each gel. Molecular weight markers were boiled for 60 seconds prior to loading as directed.
2.2.1.2. Native Substrate SDS-PAGE Analysis

Electrophoretic separations were performed as above but using a 5X Native Sample Buffer, lacking the dissociating reagent, SDS and the reducing agent, β-mercaptoethanol. This was done to allow separation under more native conditions with the possibility of retaining any intact disulphide bridges and allowing separation to be a product of charge, size and shape. These gels also lacked the molecular weight markers mentioned above due to their indeterminant mobilities under such conditions. Following electrophoresis, gels were treated as described above.

2.2.1.3. Substrate SDS-PAGE Analysis in the Presence of Inhibitors

Inhibitor studies were performed to test the mechanistic type of protease activity. Inhibitor treatments were performed as described in experimental controls with the following modifications: crude protease samples were pre-incubated for 15 minutes on ice, with the appropriate protease concentration prior to sample buffer loading and subsequent electrophoresis. Following electrophoresis, gels were incubated in the control buffer containing the appropriate protease concentration, and then stained as described above.

2.2.1.4. pH optima Substrate SDS-PAGE analysis

Early attempts to determine optimal pH for crude exoenzyme of *A. ambisexualis* were performed by incubating gels in a variety of buffer systems. Experiments were performed as described in experimental controls except that gels with replicate lanes were sliced and separately incubated in the following buffers: 0.05 M Sodium Acetate (pH 4-6),
0.05 M Tris-HCl (pH 7-9) & 0.05 M Glycine-NaOH (pH 10-11). Following the appropriate 18 h. incubation, gels were then stained for protease detection as described in experimental controls.

2.2.1.5. Protease Inhibitors

All inhibitors were purchased from Sigma and used at concentrations according to Beynon & Salveson 1989. SBTI at 0.005 μM in ddH₂O, PMSF at 1mM in 2-propanol, E-64 at 10 μM in ddH₂O, leupeptin at 100 μM in ddH₂O, TLCK and TPCK at 100 μM in 1mM HCL, pH=3.0 and 95 % ethanol respectively.

2.2.2. Protein Assay

Protein assays were performed in microtitre wells with bovine serum albumin as the standard according to Bradford, 1976.

2.2.3. Azocoll Protease Assay

2.2.3.1. Control Experiments

General protease activity was assayed according to Zuo & Woo 1998, with modifications, using Azocoll, a dye-impregnated collagen. Crude enzyme (100 μL) was incubated in 300 μL 0.1 M Hepes buffer + 4mM CaCl₂, pH=7.0 and 300 μL of Azocoll (Calbiochem), (10mg/mL buffer) and incubated with rocking (American tube rocker) at 37° C for 2h. The azocoll was insoluble and, therefore, required constant rocking of reaction vessels in an attempt to maintain equal mixing of substrate and enzyme.
Reactions were stopped with the addition of 300 μL of ice cold 10 % v/v TCA and placed on ice for 30 minutes and subsequently centrifuged for 10 min. at 13,000 g. A volume of 200 μL of the supernatant was then loaded onto a microtitre plate and absorbance at 510 nm recorded against its blank (ice cold TCA loaded prior to incubation).

2.2.3.2. Azocoll Protease Assay in the Presence of Inhibitors

Assays in the presence of selected protease inhibitors were performed as above except for the following: A volume of 20 μL of inhibitor or its control solvent were diluted into the assay buffer prior to incubation.

2.2.4. p-nitroanilide Protease Assays

All p-nitroanilide-based protease substrates were acquired from Sigma Chemical and prepared as 10 mM stock solutions in the appropriate solvent according to the manufacturer and subsequently stored at 4°C. N-Benzyl Arg p-nitroanilide, (BAPNA), N-Succinyl Phe p-nitroanilide, (SPpNA) and N-Succinyl Pro-Phe-Arg p-nitroanilide, (SPPApNA) in DMSO, N-Succinyl Ala-Ala-Pro-Phe p-nitroanilide, (SAAPPpNA) in 95 % ethanol. A standard curve of p-nitroaniline absorbance at 405 nm was generated by serial dilution of a 100 mM p-nitroaniline stock solution (Sigma) in DMSO. Concentrations ranged from 10 mM to 10⁻⁴ mM.

2.2.4.1. Control Experiments

Assays were performed, with slight modifications, as described in Larcher et al. 1996, on polystyrene 96 well microtitre plates.
Each well contained 20 μL of stock substrate in 180 μL of crude exoenzyme. Unless otherwise stated, plates were incubated for 45 minutes at 37° C. Reactions were stopped by the addition of 50 μL of 4 % v/v acetic acid and the amount of p-nitroaniline released was measured against its blank and standard curve, at 405 nm. Blanks contained the stopping agent, acetic acid, prior to substrate addition and incubated as above.

2.2.4.2. p-nitroanilide Protease Assays in the Presence of Inhibitors

All inhibitor-treated assays were performed as described in Larcher et al. 1996. According to inhibitor concentrations described by Beynon & Salveson 1989, 20 μL of solvent diluted inhibitor or its control solvent were pre-incubated at room temperature in 160 μL of crude exoenzyme. Reactions were then performed as above following the addition of substrate. Effects of inhibitors were determined by measuring p-nitroaniline release as compared with their control solvents.

2.2.4.3. pH Optima for BApNA Hydrolysis

Optimal pH conditions for BApNA hydrolysis by crude exoenzyme were determined at 37° C with the following buffer systems: 0.05 M Citric Acid-HCL (pH 3.0), 0.05 M Sodium Acetate (pH 4.0 & 5.0), 0.05 M MES (pH 6.0), 0.05 M PIPES (pH 7.0), 0.05 M Tris-HCL (pH 8.0), 0.05 M Borate (pH 9.0) & 0.05 M glycine-NaOH (pH 10.0 & 11.0). To avoid competition between buffering systems, crude exoenzyme was harvested as described above except from cultures incubated in non-buffered media.
Assays were performed in the appropriate pH buffering system as described by Larcher et al. 1996, with the following modifications: 20 μL of substrate was added to 100 μL of crude exoenzyme in 80 μL of buffer. Reactions were stopped after 45 minutes and recorded against their blanks (stopped prior to substrate addition). Buffer systems giving peak average absorbance were then recorded as optimal pH for BApNA hydrolysis. To test the effects of pH upon p-nitroaniline absorbance, 100 μL of a 1mM stock solution of p-nitroaniline (Sigma) was diluted in equal volumes of each buffer, giving proportions as described above. Absorbance was recorded following subsequent incubation at 37°C for 45 minutes.

2.2.4.4. Optimal Temperature for BApNA Hydrolysis

For determination of optimal temperature, the reaction was carried out as described by p-nitroanilide assay controls with the following modifications. Separate reaction stock mixtures and microtitre plates were pre-incubated for 30 minutes at 4, 10, 22, 30, 37, 45 & 60 °C. Assay reactions were then carried out at pre-incubation temperatures and stopped after 45 minutes. Assays were recorded, against their appropriate blanks, for absorbance at 405 nm. Temperature incubations giving peak average absorbancies were recorded as optimal temperature for BApNA hydrolysis.
3. Results


In support of Hill & Pott, 1997, protease plate halo assays revealed evidence of gelatin hydrolysis by *A. ambisexualis* E87 (Figure 3). Inocula were acquired from the colony periphery of an actively growing culture on Peptone Yeast Glucose Agar (PYG). To ensure that no hydrolysis occurred due to diffusion of media ingredients, a control plug of agar medium was included, which revealed no evidence of gelatin hydrolysis. To further investigate extracellular proteolytic activity, cultures were grown in various liquid media as described in Materials and Methods. Gelatin SDS-PAGE analysis of 72 h. culture filtrates, grown with rotary shaking in BM nutrient media alone, revealed no evidence of proteolytic banding (Figure 4, lane N). Colonies grown in media containing 0.2 % w/v collagen, but lacking glucose, MSG & L-methionine as sources of C, N & S, respectively, revealed proteolytic banding at pH 4 (Figure 4, lane P). Detectable levels of protease activity by gelatin SDS-PAGE analysis therefore seems to require growth in the presence of an extracellular source of protein. As demonstrated by Hill & Pott, high activity was demonstrated at the 66kDa range, however banding within the 97kDa range was not previously reported. The addition of nutrients to protein supplemented media did not inhibit detectable activity after 72 h. of growth (Figure 4, lane P+N). The addition of nutrients revealed a similar banding pattern as well as a new band of activity within the 44kDa range, similar to that described by Hill & Pott, 1997.
Both culture filtrate types, described above, were performed in duplicate with one gel incubated at pH 4, the other at pH 10 (Figure 4, lanes P' & P+N*). Alkaline incubated gels revealed higher activity within the 66kDa range, however limited activity was detected within the 97 and no activity within the 44kDa ranges. Such conditions did however reveal activity within the 29kDa range, as reported by Hill & Pott. Such results suggest an alkaline optimum for this activity.
Figure 3. **Protease Plate Halo Assay of *A. ambisexualis***

Triplicate colonies of *A. ambisexualis*, (A-C) grown on a gelatin-agar plate for 48 h. at room temperature, (22°C), followed by staining with Coomassie Blue solution.

A plug of agar medium serves as a control, (D).

Clear areas indicate absence of protein.
Figure 3.
Figure 4. Gelatin SDS-PAGE analysis of *A. ambisexualis* culture filtrate. Cultures were grown for 72 h. in three media types; Barksdale's media containing sources of C, N & S (N), media lacking C, N & S and replaced with collagen (P) and media with all nutrients and protein (P+N). Protein supplemented culture filtrates were run in duplicate, one gel set incubated at pH 4, (P, P+N), the other set at pH 10, (P', P+N').
Figure 4.
Similar banding of proteolytic activity was also demonstrated in cultures grown in the presence of various other proteins (Figure 5). As before, no activity was detected in cultures grown in nutrient media alone. (Figure 5, lane BM+). No growth was observed, nor activity was detected in cultures incubated in media lacking all sources of C, N or S. (Figure 5, lane BM). Cultures grown for 72 h. in the presence of gelatin and nutrients (Figure 5, lane Gel.+ ) or gelatin, collagen, albumin or an equal mix of all protein types, without nutrients, (Figure 5, lanes Gel., Col., Alb. & Mix), revealed varying levels of a similar banding pattern. Activity within the 44kDa range, however, seemed to be due to multiple bands of activity rather than one discreet band of activity. Various proteins seemed to induce a varying level of activity among a similar set of banding and that multiple banding of activity occurs within the 44kDa range. A time series of culture filtrates, throughout a 3 day growth period, revealed evidence of a nutrient delay of proteolytic activity detectable by gelatin SDS-PAGE analysis (Figure 6). A. ambisexualis cultures were grown in protein supplemented media with and without sources of C, N or S, (Figure 6, lanes P+N and P), respectively. Duplicate lanes were incubated in pH 4 or 10 buffers for detection of the various banding of proteolytic activity.
Culture filtrates after 52 h. in protein alone (Figure 6, lanes 52 P) revealed high activity, while those in protein and nutrients (Figure 6, lanes 52 P+N) demonstrated little or no detectable levels of activity. These results suggest a delay in protease activity by the presence of glucose, MSG and L-methionine. Activity detected in previous gels using 72 h. cultures, grown in P+N, failed to indicate such repression of protease induction.
Figure 5. Gelatin SDS-PAGE analysis of *A. ambisexualis* grown in several protein sources.

Culture filtrates of *A. ambisexualis* were incubated in nutrient media alone (BM+), or nutrient media lacking glucose, MSG and L-methionine (BM) served as controls. Cultures were also incubated in 0.2 % w/v gelatin supplemented nutrient media (Gel+), or in media lacking glucose, MSG and L-methionine, but supplemented with 0.2 % w/v gelatin, collagen, albumin or an equal mix of all protein types. Electrophoresis was performed in duplicate. Each replicate gel was then incubated at pH 4 or 10.
Figure 5.
Figure 6. Repression: Nutrient delay of proteolytic activity in
*A. ambisexualis*. Cultures were incubated in two protein
supplemented BM media types: with nutrients (P+N),
or without nutrients, (P). Lanes refer to time of growth
prior to culture filtrate harvesting, 24, 52 & 72 h.
respectively. Gels were performed in duplicate, one
incubated at pH 4, the other at pH 10.
Figure 6.
Gelatin SDS-PAGE analysis was also performed upon eight replicate lanes of a pool of prepared culture filtrate of *A. ambisexualis* (Figure 7). Each replicate lane was cut away and incubated in a different pH buffer system, as described in Materials and Methods. Activity of *A. ambisexualis* demonstrated a wide range of pH tolerance, from pH 4 to 11. The multiple banding of activity associated with 44kDa range seemed to be more acid optimized, as compared to the other banding of activity detected. Equal staining time and conditions revealed that overall extracellular activity, detectable by this method, seemed to be optimized at pH 8, suggesting alkaline protease activity. Subsequent gel incubations employed the use of this buffer system, 0.05 M Tris-HCL, pH 8, rather than previous pH 4 and 10 incubations.
Figure 7.  **Optimal pH of *A. ambisexualis* extracellular protease.**

Gelatin SDS-PAGE analysis was performed upon eight lane replicates of the same preparation. Lanes were cut apart following electrophoresis and subsequently incubated in a variety of buffer systems, as described in Materials and Methods. Gel slices were stained for equal time in the same Coomassie Blue solution staining vessel.
Figure 7.
3.2. Extracellular proteolytic activity among selected Saprolegniales.

Protease plate halo assays were once again performed upon *A. ambisexualis* (E), and also *Saprolegnia ferax* (S) and *Saprolegnia spp.* CF1. (C) (Figure 8). As demonstrated in *A. ambisexualis*, both species of *Saprolegnia* also demonstrated the ability to hydrolyze the extracellular source of gelatin. Such results also suggest that gelatin may be serving as the sole source of nutrients. One day old (24h.) cultures also revealed higher rates of radial growth among the two *Saprolegnia* species as compared to the *Achlya* culture. Such increased growth in Saprolegnia species may not be due to increased ability to degrade and or utilize protein, as increased radial growth was repeatedly observed under all nutrient conditions (observation). Upon further magnification of halos, (Figure 9) proteolytic activity was clearly demonstrated well beyond the colony periphery of *A. ambisexualis* (Figure 9A). The two *Saprolegnia* species, *S. spp.* CF1 and *S. ferax* (Figure 9B) demonstrated reduced extension of proteolytic clearing as compared to *A. ambisexualis* (Figure 9A). It should be noted that these magnifications are from two separately stained plates, evident by absence of Coomassie blue powder clumps in 9A, as compared to those seen in 9B. These figures, although only representative, are typical of the reduced diffusion of proteolytic clearing demonstrated by both *Saprolegnia* species. Such differences in halo extension were repeatedly demonstrated throughout all plate halo assays. Reduced diffusion of protease halo in *S. ferax* was also previously reported by Smith et al. 1994.
Figure 8. Protease Plate Halo Assay of three *Saprolegniales*.

*A. ambisexualis* (E), *S. ferax* (S) and *S. spp. CF1* (C)

were grown for 24 h. on gelatin-agar. Proteolytic activity
is revealed by the areas absent of staining with Coomassie
blue solution.
Figure 8.
Figure 9. Magnified view of Protease Plate Halo.

A 10X magnified view of protease haloes extending beyond the colony peripheries of *A. ambisexualis* (A) and *S. ferax* (B). Scale bar equals 1 mm.
Figure 9.
3.3. Characterization of crude culture filtrate activity.

All three test species were harvested for crude extracellular protease following growth in protein supplemented media, as described in Materials and Methods. Gelatin SDS-PAGE analysis was performed upon all three culture filtrates, *A. ambisexualis*, (E), *S. ferax*, (S) and *S. spp.* CF1, (C). (Figure 10 Control). Proteolytic clearing is evident throughout the entire high molecular weight range. Similar multiple low molecular weight banding is visible within the 29kDa range (Figure 10, lanes S & C). These results clearly demonstrate extracellular protease from culture filtrates. Based upon previous reports suggesting a serine protease mechanism, the use of the serine protease inhibitor, PMSF, was included in both the preparation and gel incubation buffer. Results demonstrate that PMSF effectively inhibited nearly all activity detected in the control gel (Figure 10). These results further support the suggestion of a serine protease mechanism, not only in the two *Saprolegnia* species, but also in *A. ambisexualis*. The cysteine protease inhibitor, E-64, failed to demonstrate any inhibitory effects detected by gelatin SDS-PAGE analysis (Figure 11). To test whether this method allows proper function of this inhibitor, two control lanes were also included. Trypsin, a serine protease and papain, a cysteine protease, were loaded in separate lanes and served as a negative and positive control respectively.
The cysteine protease inhibitor, E-64, failed to inhibit trypsin, but did inhibit all papain activity evident in the control solvent gel (Figure 12). It was, therefore, assumed that this method allows proper function of the cysteine protease inhibitor, E-64, and that these results support a serine protease mechanism.
Figure 10. **Gelatin SDS-PAGE analysis of all three culture filtrates in the presence of PMSF.** All three culture filtrates were pre-incubated with the serine protease inhibitor, PMSF. Subsequent electrophoresis was followed by gel incubation in the standard Tris-HCL; (pH=8) buffer with either the control solvent, (2-propanol), or PMSF. *A. ambisexualis* (E), *S. férax*, (S) and *S. spp.* CF1, (C).
Figure 10.
Figure 11. Gelatin SDS-PAGE analysis of all three culture filtrates in the presence of E-64. All three culture filtrates were pre-incubated with the cysteine protease inhibitor, E-64. Subsequent electrophoresis was followed by gel incubation in the standard Tris-HCl: (pH=8) buffer with either the control solvent, ddH$_2$O, or E-64. *A. ambisexualis* (E), *S. ferax* (S) and *S. spp.* CF1, (C).
Figure 11.
Figure 12. Gelatin SDS-PAGE of positive and negative controls in the presence of E-64. Lanes were loaded with either trypsin, (T), or papain, (P) in duplicate. Sets were then incubated with the cysteine protease inhibitor, E-64, or its solvent, ddH$_2$O, as a control.
Figure 12.
Due to the proteolytic smearing demonstrated by gelatin SDS-PAGE analysis of culture filtrates from the two *Saprolegnia* species, a timed gel incubation was performed to detect whether gelatin hydrolysis occurs during electrophoresis. As demonstrated, high molecular weight range activity is evident immediately following electrophoresis (*Figure 13, lane 0 h.*), suggesting that some protease activity is occurring during electrophoretic separation in the presence of 0.1% w/v SDS. Continued incubation of replicates demonstrates further clearing may be due to multiple faint bands of activity leading to a smeared appearance of proteolytic activity. Gelatin PAGE analysis performed under native conditions revealed the absence of the low molecular banding (*Figure 14A*) demonstrated by gelatin SDS-PAGE analysis (*Figure 14C*). The low molecular weight activity associated within the 29kDa range under gelatin SDS-PAGE may, therefore, be due to the dissociating agent SDS or the reducing agent β-mercaptoethanol. The serine protease inhibitor, PMSF, also effectively inhibits much of the activity detected by this method (*Figure 14B*). Less activity, however, is detected when used under non-native conditions (*Figure 14D*).
Figure 13. **Timed incubation of gelatin SDS-PAGE analysis.**

Equal volumes of protein supplemented culture filtrates of *S. ferax*, (S) and *S. spp. CF1*, (C), were loaded in replicates. Sets of replicates were sliced apart following electrophoresis and Triton X washing, then incubated in the same incubation vessel for various times. Activity was stopped by staining with Coomassie blue solution. Time in hours refers to gel incubations of replicate lanes.
Figure 13.
Figure 14. Native & Non-Native Substrate PAGE Analysis of the Culture Filtrates in the Presence of PMSF.

Culture filtrates from all three test species, *A. ambisexualis* (E), *S. ferax* (S) and *S. spp. CF1* (C), were analyzed under native conditions with the control solvent, (2-propanol) or the serine protease inhibitor PMSF, gels A & B respectively.

For comparison, substrate SDS-PAGE analysis is shown for the same three culture filtrates, control and inhibited, gels C & D, respectively.
Figure 14.
3.4. Quantitative analysis of extracellular protease activity among the Saprolegniales.

Attempts to quantify crude protease activity of all three culture filtrates in the presence of inhibitors and their control solvents, using the general protease substrate, Azocoll (Calbiochem), proved unreliable. *A. ambisexualis* was the only culture filtrate demonstrating assay results with reasonable deviations between replicates (Figure 15). Hydrolysis of the substrate by culture filtrates of the two Saprolegnia species were highly variable and therefore considered unreliable. Based upon previous results suggesting a serine protease mechanism, further tests were attempted using synthetic *p*-nitroanilide-based substrates designed for specific protease mechanisms. A standard curve was first generated to determine absorbance of *p*-nitroaniline at 405 nm (Figure 16). A linear relationship was demonstrated between *p*-nitroaniline concentration and absorbance at 405 nm. Due to its cost effectiveness and immediate availability, assays were first performed using the trypsin substrate, BApNA. Culture filtrates of the three test species, following growth in protein supplemented media, demonstrated hydrolysis of the trypsin substrate in a linear fashion with respect to time of incubation at 37°C (Figure 17).
Figure 15. **Azocoll Protease Assay in the Presence of Inhibitors**

Crude enzyme was analyzed for its ability to hydrolyze the general protease substrate Azocoll (Calbiochem). Assays were performed on crude protease of the three test species in the presence of soybean trypsin inhibitor, leupeptin and the control solvent, ddH₂O. Values are absorbance means at 510 nm with standard error, N=2.
Figure 15.
Figure 16. Standard curve of \textit{p}-nitroaniline absorbance.

Serial dilution of a 100 mM stock solution was performed to generate a standard curve of absorbance at 405 nm.

Values represent raw data with no replicates.

Concentrations higher than 0.20 mM exceeded the limit of absorbance and are, therefore, not shown.
Figure 16.
Figure 17. **Timed hydrolysis of the trypsin substrate, BApNA.**

Based upon inhibitor results suggesting a serine protease mechanism, a timed absorbance of nitroanilide hydrolysis by crude enzyme of the three test species was performed as described in Materials and Methods, using the trypsin substrate BApNA. Values are means with standard error, N=3.
Figure 17.
The addition of the serine protease inhibitors, SBTI and leupeptin, to the BApNA assays of the three test species, and the positive control trypsin, further supported a serine protease mechanism (Figure 18). Values are means of raw absorbance without subtraction of zeroed blanks. Based upon the absorbance of blanks, SBTI and leupeptin effectively inhibited nearly all activity. It should be noted that leupeptin is specific for trypsin-like proteases and some cysteine proteases (Beynon & Bond, 1989). Effects of this inhibitor should, therefore, be considered less specific. Failure of the cysteine protease inhibitor, E-64, to inhibit hydrolysis of the substrate, however, does further support a trypsin-like mechanism. Similar assays employing other p-nitroanilide-based protease substrates were performed to determine substrate specificity of culture filtrates. Culture filtrates of the two Saprolegnia test species demonstrated high preference for the chymotrypsin substrate, SAAPPpNA (Figure 19). Release of p-nitroaniline was so high that it exceeded measurable absorbance and was, therefore, arbitrarily recorded as 2.0 (using broken columns) (Figure 19). Such a value exceeds 0.2 mM p-nitroaniline, the limit of absorbance according to the standard curve. Culture filtrate of A. ambisexualis showed preference for the kallikrein substrate, BPPApNA. No hydrolysis was detected by the culture filtrates towards the substrate, SPpNA, reported to be a chymotrypsin substrate (Sigma). It should be noted that a later assay using preparations of chymotrypsin (Sigma) did demonstrate hydrolysis of this substrate (data not shown).
**Figure 18. BApNA Assay of Culture Filtrates in the Presence of Inhibitors.**

To further test the results of inhibitor-treated substrate SDS-PAGE analysis, inhibitor-treated BApNA assays were performed upon culture filtrates of the three test species. Inhibitor concentrations were as described by Beynon & Salveson, 1989. Diluted trypsin (36 μg total protein) served as a positive control for trypsin activity. Values represent mean absorbance with standard errors, N=3. Mean absorbance are raw figures without subtraction of blanks. Zeroed blanks showed a mean absorbance of 0.196 +/- 0.00096 for water, leupeptin and E-64. Zeroed blanks for SBTI were 0.331 +/- 0.0143.
Figure 18.
Figure 19. **Specificity towards various p-nitroanilide substrates.**

Assays using different p-nitroanilide substrates demonstrated substrate preference of crude protease from the three test species. 10mM stock solutions of each substrate were used in the appropriate solvent. Mean absorbance of p-nitroaniline release by the culture filtrates of each test species are recorded for each substrate, listed according to its residue(s): SPPNA (Phe), SPPApNA ProPheArg, SAAPPpNA (AlaAlaProPhe) and BApNA (Arg). N=3.
Figure 19.
Assays were also performed employing the trypsin substrate, BApNA, with the addition of the chymotrypsin inhibitor, TPCK (Sigma), and the trypsin inhibitor, TLCK (Sigma). Table 1.0 demonstrates the effect of these inhibitors upon $p$-nitroaniline release by culture filtrates of the three test species (Table 1.0). The chymotrypsin inhibitor, TPCK, had little or no effect upon BApNA hydrolysis. In support of a trypsin-like protease mechanism, the trypsin inhibitor, TLCK, did show some inhibitory effects. TLCK was most effective at inhibiting BApNA hydrolysis by culture filtrate of *A. ambisexualis*. Recall that culture filtrates of *A. ambisexualis* also demonstrated the highest preference for hydrolysis of BApNA as compared to the two *Saprolegnia* species. These results suggest that the mechanism for extracellular protease activity of *A. ambisexualis* may be more trypsin-like than that of the two *Saprolegnia* species.
Table 1. **Effects of specific protease inhibitors upon culture filtrates of the three test species.**

Employing the use of the trypsin substrate BApNA, assays were performed upon culture filtrates of the three test species in the presence of 100 μM TPCK and TLCK, specific for chymotrypsin and trypsin-like proteases respectively. Controls included the inhibitor solvent in equal volumes to treatments, 95 % ethanol and 0.1 M HCl; pH 3.0, respectively.
Table 1.1. Effects of specific protease inhibitors upon BApNA hydrolysis by culture filtrate of *A. ambisexualis*.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Mean Absorbance of Control</th>
<th>Mean Absorbance of Treatment</th>
<th>% of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPCK</td>
<td>0.720 (.026)</td>
<td>0.743 (.022)</td>
<td>103</td>
</tr>
<tr>
<td>TLCK</td>
<td>0.697 (.009)</td>
<td>0.169 (.006)</td>
<td>24</td>
</tr>
</tbody>
</table>

Table 1.2. Effects of specific protease inhibitors upon BApNA hydrolysis by culture filtrate of *S. ferax*.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Mean Absorbance of Control</th>
<th>Mean Absorbance of Treatment</th>
<th>% of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPCK</td>
<td>0.282 (0)</td>
<td>0.274 (.007)</td>
<td>97</td>
</tr>
<tr>
<td>TLCK</td>
<td>0.267 (.006)</td>
<td>0.166 (.002)</td>
<td>62</td>
</tr>
</tbody>
</table>

Table 1.3. Effects of specific protease inhibitors upon BApNA hydrolysis by culture filtrate of *S. spp.CFI*.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Mean Absorbance of Control</th>
<th>Mean Absorbance of Treatment</th>
<th>% of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPCK</td>
<td>0.278 (.008)</td>
<td>0.254 (.011)</td>
<td>91</td>
</tr>
<tr>
<td>TLCK</td>
<td>0.265 (0)</td>
<td>0.159 (.001)</td>
<td>60</td>
</tr>
</tbody>
</table>

Table 1
3.5. Optimal conditions for trypsin-like activity among selected Saprolegniales

Assays employing the use of the trypsin substrate, BApNA, were performed under a variety of conditions in an attempt to determine optimal conditions for the trypsin-like activity demonstrated among the three test species. Recall that slight tip high alkalinity has been previously demonstrated in Achlya cultures (Gow et al. 1984). Previous studies have reported increasing alkalinity associated with growth in protein supplemented media, up to pH 8.02 for S. ferax (Smith et al. 1994, Hill & Pott, 1997), Based upon this rise in alkalinity, attempts were made to determine optimal pH conditions for BApNA hydrolysis. In order to determine effects of pH upon enzyme action, it was first necessary to determine if changes in pH had any significant effect upon p-nitroaniline absorbance. Therefore, a stock solution of p-nitroaniline, was diluted to 0.05 mM, a concentration providing an absorbance within the typical range of assay absorbance, and was added, in triplicate, to equal volumes of buffer and incubated for 45 minutes at 37° C. Absorbance of each replicate was recorded and plotted to determine effects of pH upon absorbance. All variations of absorbance fell within the standard error, N=3 (Figure 20). Based upon these results, it was concluded that pH has no significant effect upon p-nitroaniline absorbance, and that any differences noted in subsequent assays would be due to effects other than absorbance of the substrate alone.
Figure 20. Effects of pH upon absorbance of stock p-nitroaniline

A 100 μL volume of 0.1 mM stock solution of p-nitroaniline was diluted in an equal volume of DMSO and incubated at 37°C for 45 minutes prior to recording of absorbance. Values represent mean absorbance at 405 nm with standard error, N=3.
Figure 20.
Assays, employing the trypsin substrate, BApNA, were performed upon culture filtrates of the three test species grown in non-buffered protein supplemented media. Recall that BM medium is PIPES buffered to pH 6.9. Although adjusted to pH 6.9, all media used for production of this crude protease was devoid of buffer. Assays were performed as described in Materials and Methods and absorbance recorded for each pH replicate. The culture filtrates of all three test species demonstrated a slight alkaline optimum (Figure 21). Due to a slight increase in absorbance of the alkaline controls, each recording was adjusted by subtraction of the appropriate control value for each pH replicate. This correction, however, did not significantly alter the peak pH for each culture filtrate (Figure 22). A. ambisexualis demonstrated two peaks of absorbance corresponding to pH 8 & 10. The slight drop in absorbance at pH 9 was not considered significant, as it did not exceed the standard error demonstrated in figure 21. It was therefore considered that A. ambisexualis culture filtrate demonstrated peak BApNA hydrolysis at pH 8 through 10. Culture filtrates of S. ferax and S. spp. CF1 demonstrated single peaks of BApNA hydrolysis corresponding to pH 9 and 8 respectively (Figure 22). Assays, employing the trypsin substrate, BApNA, were also performed upon culture filtrates of the three test species under various assay incubation temperatures. Results indicated that optimal BApNA hydrolysis by culture filtrates occurred at temperatures much higher than those expected to occur in natural ponds or fish farms (Figure 23). A. ambisexualis and S. ferax demonstrated peak hydrolysis at 37°C, while S. spp. CF1, reported as the cause of winter saprolegniosis, demonstrated peak hydrolysis at 45°C (Figure 23).
Figure 21. **Optimal pH for BApNA hydrolysis by all three test species.**

Culture filtrates from all three test species were incubated in a variety of pH buffers ranging from pH=3 to 11, as described in Materials and Methods. Values represent mean absorbance with standard error, N=3. Blanks were performed by the addition of 4% v/v acetic acid prior to the addition of substrate.
Figure 21.
Figure 22. Corrected optimal pH for BApNA hydrolysis for test species.

Absorbance of controls were subtracted from test means in order to correct for the slight increase in alkaline control absorbance demonstrated in figure 21.

Values represent mean absorbance, N=3.
Figure 22.
Figure 23.  **Optimal temperature for BApNA hydrolysis.**

Culture filtrates from all three test species were assayed for 45 minutes at various temperatures following a 15 min. pre-incubation. Absorbance means are given with standard errors. N=3.
Figure 23.
3.6. Optimal growth conditions for trypsin-like activity among selected Saprolegniales

Assays employing the trypsin substrate, BApNA, were also performed upon culture filtrates of the three test species following growth under a variety of conditions. Test species were grown in two media types at two different incubation temperatures. Media types were BM media containing all essential nutrients serving as a control, and media lacking MSG and L-methionine, as sources of nitrogen and sulfur respectively, but replaced with 0.2% w/v gelatin, serving as protein-induced media. Each type of medium was inoculated, as described in Materials and Methods, and subsequently incubated, in triplicate, at either 22 °C or 10 °C. Culture filtrates were harvested throughout the six day growth period and analyzed for BApNA hydrolysis at 37 °C. When analyzed for BApNA hydrolysis at 37 °C, culture filtrates of *A. ambisexualis* demonstrated detectable activity only in those cultures containing protein (Figure 24). Highest activity demonstrated by *A. ambisexualis* occurred in cultures grown with protein at the warmer temperature. Interestingly, both *Saprolegnia* species demonstrated highest activity in culture filtrates grown with protein at the colder temperature (Figures 25 & 26). Although warm temperature incubation initially demonstrated higher activity, all warm temperature culture filtrates following 4 days of growth of the two *Saprolegnia* species showed a significant decrease in their overall ability to hydrolyze the BApNA substrate (Figures 25 & 26).
Harvesting of cultures for the above growth condition experiments also involved determining mycelial dry weights of cultures, as described in the Materials and Methods. Activity specific for BApNA hydrolysis, was corrected for mycelial dry weight to provide units of trypsin activity per gram of mycelial dry weight. All three test species demonstrated the highest trypsin-like activity per mycelial dry weight, (TU/mg), when grown in the presence of protein under cold temperature conditions (Table 2.0).
Figure 24. Growth conditions upon crude BApNA specific activity in *A. ambisexualis*.

Growth experiments were performed to test the effects of temperature and protein upon the regulation of BApNA specific activity. Single 6 mm cork bore plugs of inocula were added to 25 mL volumes of media in 60 mm petri plates. Plates contained either BM (control) or BM with 0.2 % w/v gelatin instead of MSG and L-methionine, as a source of N and S respectively. Plates were then incubated at either 10°C (cold) or 22°C (warm) and harvested for crude protease in triplicate at days 1, 2, 4 & 6.
Figure 24.
Figure 25. Growth conditions upon crude BApNA specific activity in *S. ferax*.

Growth experiments were performed as described for *A. ambisexualis*. Plates contained either BM (control) or BM with 0.2 % w/v gelatin instead of MSG and L-methionine, as a source of N and S respectively. Plates were then incubated at either 10° C (cold) or 22° C (warm) and harvested for crude protease in triplicate at days 1, 2, 4 & 6. Values represent mean absorbance of equal triplicate volumes of culture filtrates with standard error.
Figure 25.
Figure 26. Growth conditions upon crude BApNA specific activity in *S. spp.* CF1.

Growth experiments were performed as described for *A. ambisexualis*. Plates contained either BM (control) or BM with 0.2 % w/v gelatin instead of MSG and L-methionine, as a source of N and S respectively. Plates were then incubated at either 10°C (cold) or 22°C (warm) and harvested for crude protease in triplicate at days 1, 2, 4 & 6. Values represent mean absorbance with standard errors, N=3.
Figure 26.
Table 2. **Effects of growth conditions upon mycelial dry weight and trypsin-like activity (BApNA hydrolysis).**

In the same growth experiment, involving single inoculation of 25 mL volumes of media in 60 mm petri plates. Plates contained either BM (control) or BM with 0.2 % w/v gelatin instead of MSG and L-methionine, as a source of N and S respectively. Plates were then incubated at either 10° C (cold) or 22 °C (warm) and harvested in triplicate for mycelial dry weight and culture filtrate. BApNA hydrolysis by crude culture filtrate was used to determine trypsin activity. One trypsin unit (TU) was defined arbitrarily as an increase in absorbance of 0.01 at 405 nm in 1 hr., at 37 °C. Trypsin-like activity was then recorded per gram of mycelial dry weight (TU/mg).
Table 2.1. Effects of growth conditions on mycelial dry weight and crude protease activity specific for BApNA hydrolysis in *A. ambisexualis* at day 6 of growth.

<table>
<thead>
<tr>
<th><em>A. ambisexualis</em> Growth Conditions</th>
<th>Mean Dry Weight (mg)</th>
<th>Trypsin Activity (TU)</th>
<th>Activity per Dry Weight (TU/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cold (control)</td>
<td>9.83 (1.44)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cold + Protein</td>
<td>7.0 (0.5)</td>
<td>40.6</td>
<td>5.8</td>
</tr>
<tr>
<td>Warm (control)</td>
<td>19.83 (0.58)</td>
<td>0.3</td>
<td>0</td>
</tr>
<tr>
<td>Warm + Protein</td>
<td>34.5 (0.5)</td>
<td>63.7</td>
<td>1.8</td>
</tr>
</tbody>
</table>

Table 2.2. Effects of growth conditions on mycelial dry weight and crude protease activity specific for BApNA hydrolysis in *S. ferax* at day 6 of growth.

<table>
<thead>
<tr>
<th><em>S. ferax</em> Growth Conditions</th>
<th>Mean Dry Weight (mg)</th>
<th>Trypsin Activity (TU)</th>
<th>Activity Per Dry Weight (TU/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cold (control)</td>
<td>26.0 (0.87)</td>
<td>1.9</td>
<td>0.1</td>
</tr>
<tr>
<td>Cold + Protein</td>
<td>32.33 (0.76)</td>
<td>122.2</td>
<td>3.8</td>
</tr>
<tr>
<td>Warm (control)</td>
<td>19.5 (0.5)</td>
<td>3.8</td>
<td>0.2</td>
</tr>
<tr>
<td>Warm + Protein</td>
<td>27.0 (0)</td>
<td>30.3</td>
<td>1.1</td>
</tr>
</tbody>
</table>

Table 2.3. Effects of growth conditions on mycelial dry weight and crude protease activity specific for BApNA hydrolysis in *S. spp. CF1* at day 6 of growth.

<table>
<thead>
<tr>
<th><em>S. spp. CF1</em> Growth Conditions</th>
<th>Mean Dry Weight (mg)</th>
<th>Trypsin Activity (TU)</th>
<th>Activity Per Dry Weight (TU/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cold (control)</td>
<td>16.0 (0.5)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cold + Protein</td>
<td>30.67 (0.76)</td>
<td>69.8</td>
<td>2.3</td>
</tr>
<tr>
<td>Warm (control)</td>
<td>20.5 (0.5)</td>
<td>5.8</td>
<td>0.3</td>
</tr>
<tr>
<td>Warm + Protein</td>
<td>31.83 (1.25)</td>
<td>43.7</td>
<td>1.4</td>
</tr>
</tbody>
</table>

Table 2
In the same growth study, culture filtrate harvesting included recording of culture pH. The highest alkalinity was seen in culture filtrates of all three test species when grown at room temperature, (22° C) in the presence of protein. Results however varied, among the other growth conditions for each test species. *A. ambisexualis* also demonstrated increasing alkalinity in warm cultures without protein. The cold temperature controls showed more alkalinity than cold with protein (Figure 27). *S. ferax* initially demonstrated a similar pattern, but by day 6, the highest alkalinity was associated with protein supplemented growth (Figure 28). *S. spp.* CFI demonstrated increasing acidity with cold temperature protein supplemented growth, however, as with all cultures, alkalinity was associated with warm growth with protein (Figure 29). An association between protein supplemented growth and increasing alkalinity was, therefore, only demonstrated when incubated at room temperature. Cold temperature incubation failed to demonstrate a similar pattern in all three test species. It should be noted that all media in this growth experiment were PIPES buffered, yet still reached pH 8.0, as reported in other studies. Growth conditions resulting in culture pH similar to that found to be optimal for activity did not match the growth conditions attaining peak activity.
Figure 27. Growth conditions and culture pH of *A. ambisexualis.*

Growth experiments were performed to test the effects of temperature and protein upon the regulation of BApNA specific activity. Single 6mm cork bore plugs of inocula were added to 25 mL volumes of media in 60 mm petri plates. Plates contained either BM (control) or BM with 0.2 % w/v gelatin instead of MSG and L-methionine, as a source of N and S respectively. Plates were then incubated at either 10° C (cold) or 22 ° C (warm) and harvested for culture filtrate in triplicate at days 1, 2, 4 & 6. Culture filtrate pH was recorded following equipment calibration. Values represent mean pH with standard error, N=3.
Figure 27.
Figure 28. **Growth conditions and culture pH of S. ferax.**

Experiments were performed as described for *A. ambisexualis.*

Values represent mean pH with standard error, N=3.
Figure 28.
Figure 29. **Growth conditions and culture pH of S. spp. CF1**

Experiments were performed as described for *A. ambisexualis* and *S. ferax*. Values represent mean pH with standard error, N=3.
Figure 29.
A separate preliminary growth experiment was also performed upon the three test species using several protein sources as the sole source of added nutrients. These protein sources, 0.2 % w/v, were added to ddH₂O, in triplicate, as described in Materials and Methods and inoculated with the three test species. Observations were then recorded as growth or no growth. Positive growth was observed in gelatin, collagen, casein & albumin for all three test species, suggesting the ability to utilize these extracellular proteins as the sole source of added nutrients. Human hair, serving as a source of keratin failed to support any visible growth in either of the three test species. These results, (not shown), may indicate the inability of these species to degrade and/or utilize keratin.
4. Discussion

"Saprolegnia is the most important fungal parasite of fish" — Jones 1976.

The presence of considerable (protease) activity in the culture medium... deserves further attention" — Peduzzi & Bizzozero, 1977.

Although often difficult to demonstrate, the link between proteases and pathophysiology is commonly accepted. They have been linked to tissue invasion in Candida, gas gangrene and cancer, as well as virulence factors in many other non-human diseases including many fish. A correlation between activity of a secreted metalloprotease and virulence has been demonstrated in the fish pathogen, Vibrio anguillarum (Norqvist et al. 1990). Isolation of an invasiveness-defective mutant showed decreased proteolytic activity as the only detectable difference as compared to wild-type (Norqvist et al. 1990). Considering the increasing links between protease and pathogen virulence, it is surprising that little is known regarding the proteases of what many consider to be the most important fungal parasite of commonly cultured fish. In one of the few reports to investigate this, Hill & Pott demonstrated an induction-derepression regulation pattern of extracellular protease in A. ambisexualis E87 Raper (Hill & Pott, 1997). Although generally considered, at the time, to be strictly saprophytic, recent evidence has shown that several species of Achlya can act as opportunistic invaders (Khulbe et al. 1994, Jeney & Jeney, 1995).
In light of this new evidence, it was the objective of this report to further investigate the extracellular proteases of *A. ambisexualis* and those of two other selected Saprolegniales. Along with *A. ambisexualis* and *S. ferax*, this study included an isolate yet to be analyzed for its proteolytic abilities. Found by Bly *et al.* in 1992, and fulfilling Koch's postulates, *S. spp.* CF1 Bly, was isolated as the cause of "winter kill", now termed "winter saprolegniosis", in Mississippi fish farms. Based upon previous studies suggesting a link between pathogenicity and cold-temperature induced host immunosuppression, these three species were tested for their ability for induction of proteolytic activity under a variety of growth conditions, including the temperature at which winter saprolegniosis is observed (Bly *et al.* 1992).

In support of Hill & Pott, 1997, protease activity was only detected in *A. ambisexualis* when grown in the presence of a proteinaceous inducer, such as collagen, gelatin, casein or albumin. Growth in protein supplemented media in the presence of nutrients resulted in a delay of free protease, detectable by gelatin SDS-PAGE. In accordance with the model, this late detection of protease activity may be due to exhaustion of readily available nutrients, acting as repressors. Exhaustion of at least one of these readily available nutrients, (repressors), may then leave gelatin as the sole source of the limiting nutrients, leading to subsequent induction of free protease activity.

It has been proposed that nitrogen uptake from amino acids occurs by way of a general amino acid permease, GAP (Smith *et al.* 1994). The mechanism of protease induction remains unclear.
Protein clearly elicits protease activity in all three test species, definitive explanation of the mechanism for induction, however, is beyond the scope of this study. Preliminary data using various inducers showed no growth using the amino acid, glycine, as the sole source of nitrogen (observation). Similar results were shown in a previous report in which single amino acids failed to support growth in several species of *Saprolegnia* (Smith *et al.* 1994). It should also be noted that the control medium, Barksdale medium, contains L-methionine, an amino acid demonstrated by previous reports as inducing chemotaxis in active zoospores (Thomas & Peterson, 1990), and encouraged the greatest increase in biomass, when in combination with other amino acids (Smith *et al.* 1994). Considering their strong affinity for this essential amino acid, control media did not elicit detectable levels of protease in all test species. It is, therefore, unclear whether or not amino acids, alone or in combination, will also act as protease inducers. Casamino acids, by acid hydrolysis of casein (Difco), when used as the sole source of N & S did, however, support growth and elicited detectable levels of protease activity in culture filtrates (data not shown). It is unclear, however, whether any contaminating peptides are contained within such an undefined preparation and, therefore, fail to thoroughly test the properties of induction by combinations of individual amino acids.

To further support Hill & Pott, 1997, similar banding patterns of protease activity were detected by gelatin SDS-PAGE analysis using a variety of incubation buffer systems. Optimal activity was found under slight alkaline conditions. In their study, Hill & Pott demonstrated that collagen induced the highest level of protease activity per dry weight in *A. ambisexualis*. 
It is interesting to note that collagen is the most predominant protein found in the skin of fish and other animals. Collagen is also disproportionately high in the rare amino acid hydroxyproline, an amino acid also found in high amounts within the cell wall of all Oomycetes (Alexopoulos et al. 1996). Enzymatic degradation of collagens from host skin tissue could, therefore, serve not only an invasive role but also a nutritional role in an opportunistic Oomycete.

Following growth in gelatin, (or collagen, casein, or albumin), protease activity was detectable in culture filtrates and suggestive of truly extracellular protease activity. Based upon diffusion rates of protease halos in a previous study, it was suggested that protease activity among several Saprolegnia species, including S. ferax of this study, may be immobilized, and fails to demonstrate extracellular enzyme activity. Such a study however, only employed the use of gelatin-agar plates and monitoring of amino acid release during growth in casein supplemented media (Smith et al. 1992). Gelatin SDS-PAGE analysis, in this current study, clearly demonstrates bands of activity from culture filtrates, strongly suggesting truly free protease activity. Substrate SDS-PAGE analysis, under native and non-native conditions, further suggests that such activity has limited mobility due to a large molecular weight and may represent a large complex of multiple active sub-units. Such a large protease, or protease complex, may also have reduced diffusion through gelatin-infused agar and may explain such reduced diffusion demonstrated in the previous study. It is unclear how such a protease complex would explain the differences in protease diffusion between A. ambisexualis and the two Saprolegnia species.
A. ambisexualis did demonstrate a lower rate of growth (both radial and by dry weight). If one could assume equal diffusion of protease or protease complex occurs among all three test species, such a reduced radial growth seen in A. ambisexualis, may produce an extended halo, as demonstrated by protease plate halo assay. In other words, a difference between radial growth rate and protease diffusion might produce the differences in plate halo extensions seen in this study. The previous study, by Smith et al. 1994, however, involved incubation of 48 h. cultures for 24 h. at 5°C and subsequent return to initial growth temperatures. Their results failed to demonstrate a difference between clearing and colony extension rate (Smith et al. 1994). It should be noted that a large protease or protease complex as demonstrated by substrate SDS-PAGE, may also demonstrate reduced diffusion rates at such low temperatures. Future tests should investigate protease activity and the potential for association with the cell wall or plasma membrane. Preliminary attempts to address this by analyzing culture filtrates for alkaline phosphatase activity proved unreliable. Although low-level activity was detected in culture filtrates, it was not included due to its inconsistencies. Further reliable tests are needed to address whether a correlation exists between protease and alkaline phosphatase activity. Attempts to address hyphal lysis, resulting in extracellular presence of internal protease, also proved unsuccessful. Western blot analysis of culture filtrates for the internal proteins, actin and heat shock protein, hsp 70, proved unsuccessful. Internal controls failed to be detected; the tests were considered unreliable and are, therefore, not included in this report. This report, therefore, fails to thoroughly address the potential for membrane-bound protease activity or the possibility for lysis and subsequent release of an internal protease.
Considering only the presented data, substrate SDS-PAGE banding from vacuum-filtered culture media is suggestive of free extracellular protease activity. Absence of detectable levels of protease activity in experimental controls is suggestive of true secretion of protease as opposed to cell lysis, leakage or membrane fragmentation. Furthermore, a previous study demonstrated that the proteolytic activity found in the medium of several Saprolegnia species including S. ferax was two to five times higher than that found in the mycelial extract, indicating an extracellular destination of these proteolytic enzymes (Peduzzi & Bizzozero, 1977). The authors of this study further suggested that these enzymes could be related to the physiological function of degrading the host tissues (Peduzzi & Bizzozero, 1977). In the same study, the authors also demonstrated that such activity was specific for the chymotrypsin substrate, Acetyl-DL-phenylalanine β-naphthylester at pH 7.4 (Peduzzi & Bizzozero, 1977). This current report, through the use of several synthetic protease substrates and inhibitors, supports their findings. Crude culture filtrates of S. ferax and S. spp. CF1 were inhibited by several serine protease inhibitors supporting the possibility of a chymotrypsin-like mechanism. Tests using various p-nitroanilide substrates further supported these findings by demonstrating a high preference for the chymotrypsin substrate, SAAPPpNA. Tests employing the trypsin substrate BApNA, however, failed to demonstrate any effects on activity by the chymotrypsin inhibitor, TPCK. In a review by Rao et al. 1998, serine alkaline proteases, produced by several bacteria, molds, yeasts and fungi are listed as typically not affected by TPCK or TLCK, yet do demonstrate substrate specificity similar to, but less stringent than that of chymotrypsin (Rao et al. 1998).
Therefore, based upon the results of this study, together with the typical characteristics stated by Rao et al. 1998, this report further supports a chymotrypsin-like substrate specificity. The reduced stringency of substrate potential demonstrated by other alkaline serine proteases, therefore, allows the potential that activity present in the culture filtrates of this study could equally be due to several unrelated enzymes or a single large protease. In addition, and similar to characteristics demonstrated here, Rao et al. further state that serine proteases are typically active at neutral to alkaline pH, with optima between pH 7 to 11 and having a broad range of substrate specificity (Rao et al. 1998). Culture filtrates did demonstrate susceptibility to the trypsin inhibitor, TLCK. Aside from this, it can be considered then, that the characteristics demonstrated by crude culture filtrates of the three test species, A. ambisexualis, S. ferax and S. spp. CF1, are typical of the serine proteases produced by several bacteria, molds, yeasts and fungi. Considering its typically saprophytic nature, the wide range of potential growth substrates for these water molds, be it a decaying insect, a rotting seed or a weakened fish, would require the ability to secrete a protease or proteases with a broad range of substrate specificity.

Whether this protease activity, (be it a single or a group of enzymes), plays a role in the virulence of fish disease remains unclear. It has been reported that suppressed immunity in fish, due to sudden reduction of water temperatures, may lead to susceptibility to organisms that would otherwise be harmless. Bly et al. 1992, demonstrated that fish subjected to abrupt temperature decreases, from 22° to 10°C, were rapidly infected with Saprolegnia.
In this report, protease assays, using the trypsin substrate under various incubation temperatures, suggest an optimal temperature of 37°C for proteases of *A. ambisexualis* and *S. ferax* culture filtrates. Interestingly, the species isolated as the cause of “winter saprolegniosis”, *S. spp.* CF1 Bly, demonstrated optimal BApNA hydrolysis at 45°C. Such temperatures exceed the temperature range of the host fish, and are, therefore, not likely to occur during the disease outbreaks. Such results would exclude such protease activity as a virulence factor. This, however, is further complicated by the fact that low temperature growth conditions, similar to that of the Bly *et al.* (1992) study, although apparently not optimal for activity, did seem to be optimal for protease production. Higher BApNA hydrolysis activity per mycelial dry weight was associated with protein supplemented cultures grown at 10°C when compared to those grown at 22°C. Such temperatures were used by Bly *et al.* (1992) as treatment and controls, respectively. Both *Saprolegnia* culture filtrates demonstrated a decrease in the ability for BApNA hydrolysis following four days of growth at 22°C, but continued to demonstrate high activity from cold temperature cultures. This decrease of activity in warm temperatures, it should be noted, was also marked by a slight decrease in mycelial dry weight (data not shown). Such a decrease was accounted for by determining activity per mycelial dry weight. Based upon these results, it seems that such temperatures may optimize production of protease, at least specific for BApNA hydrolysis, but may not optimize for its activity, as such growth conditions did not provide optimal temperature or pH. It should be noted that although not optimal, low level activity was detected at such temperatures and culture pH was within the active range.
Whether such an increase in protease production (per mycelial dry weight) at this low
temperature can account for the associated decrease in activity, and thereby still serve as
a virulence factor, is unknown. The characteristics found in this report are based upon in
vitro studies of culture filtrates from pre-autoclaved media suspended in distilled water.
The conditions that would exist within or upon the epithelial surface of a fish would be
considerably different, and therefore so to would the proteolytic activity. The high
presence of protease activity per mycelial dry weight, under cold temperature growth
conditions, does raise suspicion in its role at such temperatures. Other previous studies
have clearly demonstrated associations between proteases and fish diseases. Culture
supernatant of *Aeromonas salmonicida* has been demonstrated to suppress the humoral
responsible was later identified as a 64kDa serine protease. An alkaline serine protease,
isolated from *Vibrio alginolyticus*, was demonstrated to be a lethal exotoxin to the
kuruma prawn, *Penaeus japonicus* (Kuo-Kau *et al.* 1997). Lethal effects of both the crude
extracellular products and the partially purified protease were completely inhibited by the
serine protease inhibitor, PMSF (Kuo-Kau *et al.* 1997). Subsequent casein SDS-PAGE
analysis later showed similar inhibition of a 33 kDa protease band in the presence of that
inhibitor. Whether the production of protease, by the three selected Saprolegniales of this
study, plays a role in immunosuppression or serves as an exotoxin is unknown. Previous
observations by Neish, in 1977, listed no data suggesting evidence of toxin production
(Neish, 1977).
This same report did, however, observe destruction of the epidermis, penetration of the basement membrane and subsequent extension into the dermal tissue and underlying musculature with little or no signs of an inflammatory response (Neish, 1977). The absence of any noticeable inflammatory response may implicate the potential for a suppression of immunity. The fact that these molds can actively grow in distilled water containing 0.2 % w/v collagen or gelatin suggests a more nutritional role. It could be expected that any extracellular protease activity, with the ability to degrade collagen, perhaps in the provision of nutrients to the growing fungus, could also serve a secondary function in tissue destruction. Proteolytic degradation of the epidermis and underlying tissue, similar to that described by Neish, could provide an excellent source of nutrients to support growth, as demonstrated by proteins alone. Collagen breakdown could provide the rare amino acid hydroxyproline, commonly found in the cell walls of Oomycetes. To further support the potential role in nutrition and tissue destruction, a recent study by Powell and Chambers, (2000), demonstrated reduced growth of Saprolegniales colonies upon the keratinized tissue of minnows. Similar results were demonstrated in preliminary tests using human hair as a source of keratin. All three Saprolegniales species failed to show any signs of growth in distilled water supplemented with human hair as the sole source of nutrients. If protease activity plays a role in the breakdown of extracellular sources of protein, one might expect that failure to grow on keratin might be due to the inability of this protease activity to hydrolyze keratin. It should be noted that keratins are typically resistant to the action of pepsin, trypsin and other non-substrate-specific proteases (Safranek & Goos, 1981).
Considering the less stringent trypsin and chymotrypsin-like nature of the Saprolegniales culture filtrates, failure to grow on keratin may be due to the inability of protease action. Furthermore, the characteristics of this proteolytic activity may not necessarily be restricted to zoopathogenicity. Trypsin-like activity has been isolated in the fungal plant pathogen *Verticillium dahliae*. Hydrolysis of the trypsin substrate BApNA by a 30kDa protease was inhibited by leupeptin (Dobinson *et al.* 1997). Hydroxyproline-rich cell wall glycoproteins, HPRGs, such as elicinins, are known to be synthesized by plants in response to wounding or infection (Rauscher *et al.* 1995 & Turner, 1994). It was suggested that extracellular proteases specifically degrade these HPRGs during the infection formation (Dobinson *et al.* 1997). These proteases may, therefore, facilitate localized penetration of the plant cell wall (Rauscher *et al.* 1995). Extracellular protease activity has been recently demonstrated among several species of *Pythium*. Activity among this Oomycete was found not only in the zoopathogen, *P. insidiosum*, but also among several plant pathogens (Davis *et al.* 2000). Proteolytic activity demonstrated among these three species of Saprolegniales may play a similar dual function for nutrition and invasiveness by degrading hydroxyproline-rich proteins, such as collagens of animal tissue or extensins of plant cell walls.

It is clear, from the results of this study, that these test species have the ability for induction of extracellular protease activity under a variety of growth conditions and that such activity is suggestive of truly free extracellular protease. Results also demonstrate that activity is alkaline optimized and follows a serine protease mechanism, typical of those produced by many other bacteria, molds and fungi.
Oomycetes have been clearly linked to diseases in various economically important plants, such as rice, and fish, such as carp, Atlantic salmon and rainbow trout. The potential roles such proteolytic activity plays in their virulence, either through nutrition, tissue penetration, toxicity or immune suppression, (although implicated), is still unclear. Our continued reliance upon hydroponic greenhousing and concentrated fish farming will undoubtedly lead to increasing potential for these opportunistic invaders and may support further interest in addressing these questions.
References


### Table 1.0. Preparation of 7.5 % and 10 % Acrylamide Separating Gels.

<table>
<thead>
<tr>
<th></th>
<th>7.5 % Acrylamide</th>
<th>10 % Acrylamide</th>
</tr>
</thead>
<tbody>
<tr>
<td>ddH2O</td>
<td>3.85 mL</td>
<td>3.00 mL</td>
</tr>
<tr>
<td>2 % w/v Gelatin</td>
<td>1.00 mL</td>
<td>1.00 mL</td>
</tr>
<tr>
<td>1.5 M Tris-HCl, pH 8.8</td>
<td>2.50 mL</td>
<td>2.50 mL</td>
</tr>
<tr>
<td>30 % T Acrylamide</td>
<td>2.50 mL</td>
<td>3.35 mL</td>
</tr>
<tr>
<td>10 % w/v SDS</td>
<td>100 μL</td>
<td>100 μL</td>
</tr>
<tr>
<td>10 % w/v Ammonium Persulfate</td>
<td>50 μL</td>
<td>50 μL</td>
</tr>
<tr>
<td>TEMED</td>
<td>10 μL</td>
<td>10 μL</td>
</tr>
</tbody>
</table>

### Table 2.0. Preparation of 4.0 % Acrylamide Stacking Gel.

<table>
<thead>
<tr>
<th></th>
<th>4.0 % Acrylamide</th>
</tr>
</thead>
<tbody>
<tr>
<td>ddH2O</td>
<td>5.90 mL</td>
</tr>
<tr>
<td>0.5 M Tris-HCl, pH 6.8</td>
<td>2.50 mL</td>
</tr>
<tr>
<td>30 % T Acrylamide</td>
<td>1.50 mL</td>
</tr>
<tr>
<td>10 % w/v SDS</td>
<td>100 μL</td>
</tr>
<tr>
<td>10 % w/v Ammonium Persulfate</td>
<td>50 μL</td>
</tr>
<tr>
<td>TEMED</td>
<td>10 μL</td>
</tr>
</tbody>
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Appendix B

Table 1.0. Barksdale Defined Liquid & Solid Medium.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-Glucose</td>
<td>2.8 g</td>
</tr>
<tr>
<td>Pipes</td>
<td>3.0 g</td>
</tr>
<tr>
<td>Monosodium Glutamate</td>
<td>0.4 g</td>
</tr>
<tr>
<td>L-Methionine (15 mg/mL 1 M HCl)</td>
<td>1.0 mL</td>
</tr>
<tr>
<td>KCL (14.9 g/100 mL)</td>
<td>1.0 mL</td>
</tr>
<tr>
<td>MgSO₄·7H₂O (12.32 g/100 mL)</td>
<td>1.0 mL</td>
</tr>
<tr>
<td>CaCl₂ (5.55 g/100 mL)</td>
<td>1.0 mL</td>
</tr>
<tr>
<td>EDTA (1 g/100 mL)</td>
<td>2.0 mL</td>
</tr>
<tr>
<td>KH₂PO₄ (10.2 g/100 mL)</td>
<td>2.0 mL</td>
</tr>
<tr>
<td>Metal Mix (2.0 g/100 mL) *</td>
<td>1.0 mL</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>to 1.0 L</td>
</tr>
<tr>
<td>Agar (for solid media only)</td>
<td>15.0 g</td>
</tr>
</tbody>
</table>

Adjust pH to 6.9 prior to autoclaving.

* Metal Mix: Fe(NH₄)₂(SO₄)₄·6H₂O 1.44g
ZnSO₄·7H₂O 0.44g
MnSO₄ 0.15g
Grind powder with mortar and pestle
**Vita Auctoris**

**Name**

Stephen C. Kales

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July 21, 1970

**Place of Birth**

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**Education**

University of Windsor  
Windsor, Ontario, Canada  
Master of Science – Biological Sciences  
1998-2001

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Honours Bachelor of Science - Biological Sciences  
Minor: Biochemistry  
1998

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General Physiology – Cell & Molecular Biology  
Laboratory Techniques - Winter 2000 & Winter 1999

Introductory Mycology  
Laboratory Techniques – Fall 2000  
Laboratory Techniques & Guest Lecturer - Fall 1999

Microbiology – Concepts & Techniques  
Laboratory Techniques - Fall 1998

**Honours and Awards**

Student Conference Travel Grant  1999 & 2000

**Professional Service & Voluntary Work**

Biology Department Graduate Committee Member - Fundraising  98-99 & 99-00

Voluntary Research Assistant – Summer 1997
Conference Presentations


