The Multiple Proteolytic Enzymes of Two *Microsporum* Species

A Thesis Presented in Partial Fulfilment of the Requirements for the Degree of
Master of Science
in Microbiology at Massey University

Jon Stuart Palmer

1995

ABSTRACT

Dermatophyte infections can be contracted from animals, humans or from the soil. In the genus *Microsporum* some species commonly are associated with cats & dogs but also often cause infections in humans. Others are regarded as non-pathogenic & are commonly isolated from the soil. The present studies investigated the production of proteolytic enzymes by the zoophilic species *M.canis* & the geophilic species *M.cookei,* in various cultural conditions which might affect expression of such enzymes, in an attempt to detect differences between the two that could be associated with the ability of *M.canis* to invade skin *in vivo.*

Biochemical assays showed *M.canis* produced higher azocollytic & elastase activity in a keratin containing medium(BSW) than in Sabourauds Broth(SDB). In contrast, azocollytic & elastase activity of *M.cookei* in the two media was relatively similar. Azocollytic & elastase activity of both species peaked in the pH range 7-10 & azocollytic activity demonstrated highest activity around 45°C in both media. Both species produced some keratinolytic activity in BSW but not in SDB. Inhibition studies of azocollytic & elastase activity revealed the presence of an aspartic elastase with little or no azocollytic activity, which also was not detected using a substrate(gelatin) SDS-PAGE technique. Other proteinase types found were serine, cysteine & metalloproteinases.

Using the gelatin-SDS-PAGE technique, the mode of culture(shake & stationary) & the effect of substrate, time & temperature were analysed to compare the effects these factors may have on proteolytic enzyme expression between the two species.Substrate proved to be the most important factor in the expression of gelatinases. Mode of culture in SDB demonstrated that some proteinases were expressed in shake culture sooner than in stationary cultures. *M.canis* in both SDB & BSW produced 6 bands between 85,000 Da & 13,000 Da. *M.cookei* in SDB produced 7 bands between 64,000 Da to 19,000 Da but in BSW only 5 bands between 61,500 Da to 19,000 Da. Inhibition studies revealed that both species expressed several metalloproteinases & serine proteinases in BSW which were not expressed in SDB cultures. It is suggested that these proteinases may be important factors in the ability

of dermatophytes to colonise keratin & possibly, in the case of *M.canis*, to invade skin i*n vivo*.

ACKNOWLEDGMENTS

I would like to thank the Department of Microbiology & Genetics for providing the facilities & funding necessory for the project to be completed. To Dr. M. Baxter for his advice, extreme patience & grammatical artistry in the write up of this project.

I wish to thank Francis for the discussions on proteolytic enzymes & also for the philosophical discussions on the differences between NZ & Zambia, especially in the use of the English language. My thanks also to all my friends for all the help during this study.

I would like to thank Nigelhuw & Julie for the use of their printers & their patience & advice in demonstrating the modern complexities of a word processing program.

Finally, thanks must go to my family, especially my parents who have been wonderful, both financially & emotionally though good times & bad. Their help has been gratefully acknowledged.

TABLE OF CONTENTS

TABLE OF CONTENTS Contd.

TABLE OF CONTENTS Contd.

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES Contd.

LIST OF PLATES

LIST OF PLATES Condt.

Page

CHAPTER 1

INTRODUCTION

1.1 DERMATOPHYTES & DERMATOPHYTOSES

Dermatophytes are a group of filamentous fungi closely related in physiology & morphology. These organisms have the ability to invade cutaneous structures & break down keratin proteins of skin, nail & hair. In dermatophyte infections of the skin(dermatophytoses or ringworm) the fungus remains confined to the stratum corneum. Because of it's visibility, ringworm, the current & preferred terminology for the disease, was noted & described by many early civilisations. Growth of the fungus on the glabrous skin is more or less equal in all directions & tends to produce a ring, which the Greeks named herpes. The Romans associated the disease with insects & named it tinea, meaning small insect larva, & this term is still often used today to describe the clinical form of the disease. Because the same fungus may cause different lesion types depending upon the anatomic site of infection, the clinical disease can also be named according to site, for example,Tinea pedis, ringworm of the feet, Tinea barbae, ringworm of the beard & Tinea capitis, ringworm of the scalp. Different species of dermatophytes may predominate in these different clinical forms of ringworm.

1.2 CLASSIFICATION OF DERMATOPHYTES

At the turn of the century, Sabouraud classified dermatophytes into four genera, *Achorion*, *Epidermophyton*, *Microsporum* & *Trichophyton* However, the basis for the current taxonomy of these anamorphic conidial fungi was laid down by Emmons in 1934, who reduced the number of genera on morphological grounds to three, *Trichophyton*, *Microsporum* & *Epidermophyton*. In addition, there are known ascomycetous teleomorphic stages of a number of *Trichophyton* & *Microsporum* species(Nannizzia &/or Arthoderma)(Rippon 1985).

At least forty species of dermatophytes are recognised by fungal taxonomists in the three anamorphic genera of dermatophytes. These

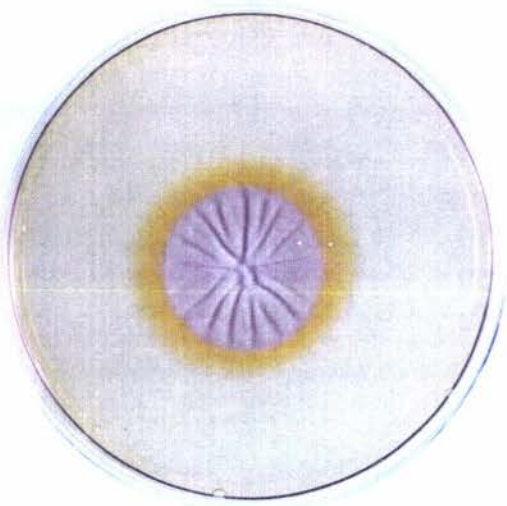
species can be differentiated on the basis of colony morphology, microscopic appearance, particularly the appearance of macroconidia & microconidia, & mating reactions(Ajello 1974).

The species selected for the present investigations are two of the most commonly encounted *Microsporum* species, *M.canis,* a very common dermatophyte causing infections in a wide range of animals as well as humans and *M.cookei,* a common soil saprophytic and non-pathogenic dermatophyte.

On Sabouraud Dextrose Agar(SDA) *M.canis* produces cottony, white fluffy mycelium(Plate 1.1) with yellow periphery & underside(Plate 1.2), which is very characteristic for *M.canis.* Macroconidia are large(10-20 x 50-60μm), thick walled & spindle shaped, terminating in a distinct knob. Microconidia are clavate(1-2 x 3-5μm) and borne on short pedicels laterally on the hyphae(Plate 1.3). *M.cookei* produces a powdery surface, which is dark tan(Plate 1.3), with a dark red-brown underside(Plate 1.4). Macroconidia are oval to ellipsoidal,(15 x 31-50μm) & thick walled. Microconidia are obovate & produced abundantly(Plate 1.5).

Plate 1.1:Colony of *M.canis* on Sabourauds Dextrose Agar(SDA) after two weeks of growth at 25°C.

Plate 1.2: Reverse with golden yellow pigmentation.



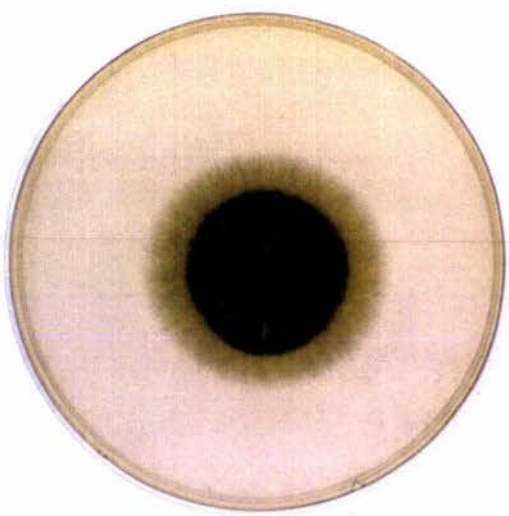
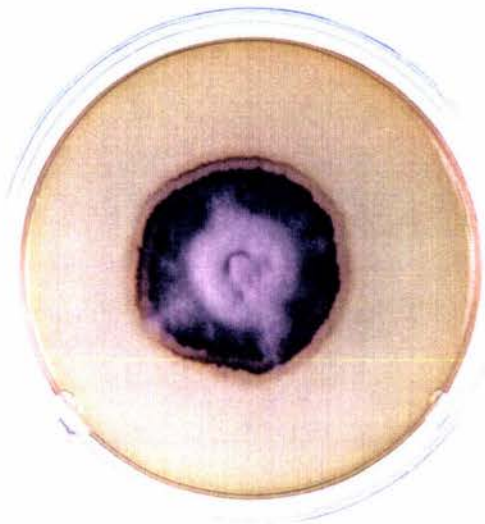


Plate1.3:*M.canis*:spindle shaped & pointed macroconidia & small microconidia(magnification X400).

Plate 1.4:Colony of *M.cookei* on SDA after two weeks of growth at 25°C.

Plate 1.5: Reverse with dark red-brown pigmentation.



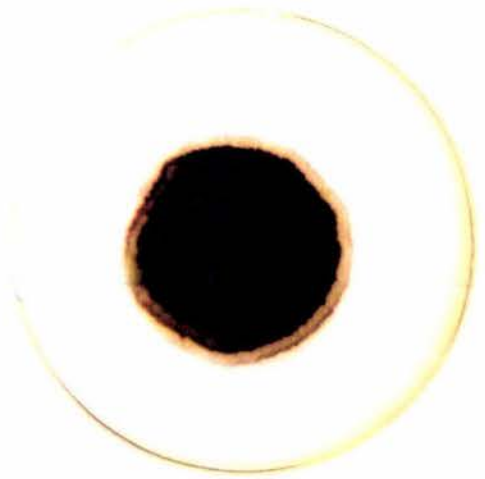
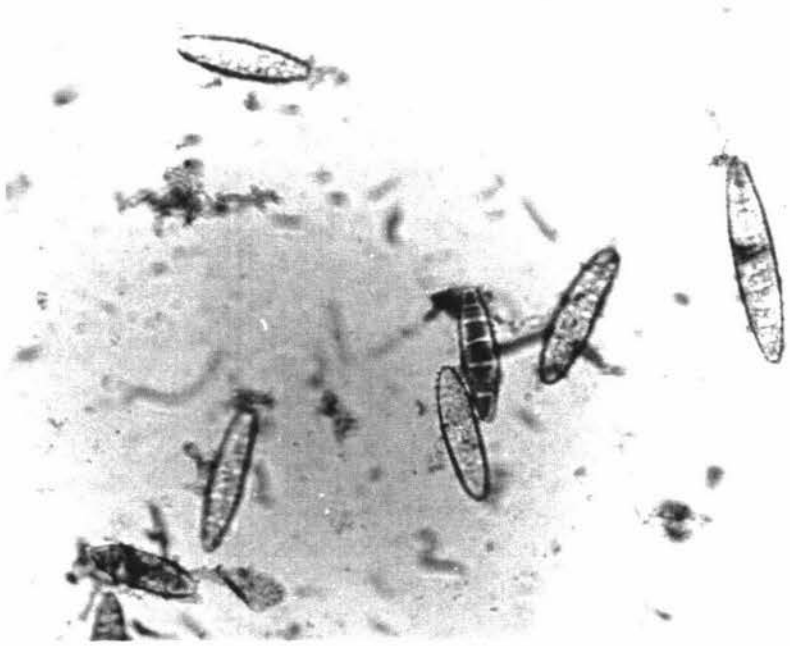


Plate 1.6:*M.cookei*:oval to ellipsoidal macroconidia(magnification X400).



1.3 ECOLOGICAL GROUPING.

The dermatophytes can also be classified into three ecological groups in relation to their "reservoirs" in the environment.

Anthropophilic - Those found almost exclusively on humans
Zoophilic - Those found mostly on animals
Geophilic - Those found mostly in soils

This grouping not only reflects major reservoirs for each species but to some degree the clinical characteristics of the infection caused by these organisms. The anthropophilic species tend to produce less inflammatory & more chronic infections in humans than do zoophilic or geophilic species.

1.3.1 Geophiles

These fungi exist as saprophytes in the soil, where they colonise keratin substrates present there. The distribution of geophilic dermatophytes is thought to be influenced by three important factors, availability of keratin, pH of the soil & temperature. Marples (1965) reported isolating *M.cookei* from areas closely associated with animal - contaminated soils, where skin & hair had been shed from the animals into the soil & then colonised by geophiles. Another common fungus in this group, *Trichophyton ajelloi,* was found to be closely associated with low pH soils in New Zealand(Marples 1965). *T.ajelloi* has been commonly isolated in cooler climates but its isolation in warmer climates is sporadic.

A few geophiles do have the capacity to cause ringworm infections on animals & man. Frey(1971) reported the isolation of *M.cookei* from a human case but *M.gypseum* is the most common geophilic dermatophyte to cause disease in animals & humans (Ajello 1974).

Tanaka et al (1992) list the geophilic dermatophytes as follows

Microsporum amazonicum
M.boullardii
M.cookei
M.fulvum
M.gypseum
M.nanum
M.persicolor
M.praecox
M.racemosum
M.riparae
M.vanbreuseghemii

Trichophyton ajelloi
T.flavescens
T.gloriae
T.longifusum
T.phaseoliforme
T.terrestre
T.vanbreuseghemii

Epidermophyton stockdaleae

1.3.2 Zoophilies

Zoophilic dermatophytes are mainly animal pathogens but several are also able to infect humans. Among the five *Microsporum* species, *M.canis* is the most common & is widely distributed throughout the world. *M.canis* infects a wide variety of animals, especially cats & dogs, from which humans readily acquire infections through direct contact with infected animals or by infected hairs etc shed by pets in domestic environments.There are five zoophilic *Trichophyton* species, of which *T.mentagrophytes* & *T.verrucosum* are the most common (De Vroey 1985).

Tanaka et al (1992) list the zoophilic dermatophytes as follows

Microsporum canis
M.equinium
M.gallinae
M.persicolor
M.distortum

Trichophyton equinum
T.mentagrophytes
(two sibling species & variants)
T.sarsikovii
T.simii
T.verrucosum

1.3.3 Anthropophiles

Anthropophilic species are primarly adapted for the parasitism of man, but have occasionally been reported to have caused infection in animals (Georg 1960). The spread of anthropophilic species is more common in communities such as schools, military establishments, prisons etc, where the communal use of showers etc can lead to the rapid spread of infection (Philpot 1977, Rippon 1982a). Anthropophilic species tend to produce less inflammatory & more chronic infections than do either zoophilic or geophilic species. It has been suggested that anthropophilic species are better adapted to human hosts than zoophilic or geophilic species (Sohnle 1989). Anthropophilic species tend to lack the capacity for sexual reproduction & some have lost the ability to produce macroconidia (Tanaka et al 1992).

The most common anthropophilic species, *T. rubrum*, is an extremely common cause of skin infections throughout the world (De Vroey 1985). Other anthropophilic dermatophytes are endemic in particular areas. *M.ferrugineum,* for example, is found only in certain parts of Asia & *T.concentricum* in certain parts of Central & South America. Other species may be sporadic in occurrence but distributed worldwide

Different populations can show a different response to the same dermatophyte species. An example of this occurred in Vietnam, where Vietnamese troops had little trouble with the endemic strain of *T.mentagrophytes* but American troops living under the same conditions developed severe inflammatory lesions, sometimes covering half of the body. On the other hand, Vietnamese troops were troubled by *T.rubrum*, which was uncommon among American troops (Rippon 1982a).

Tanaka et al (1992) list the anthropophilic dermatophytes as follows

Microsporum audouinii
M.ferrugineum

Trichophyton concentricum
T.gourvillii
T.kanei
T.kuryangei

	T.megninii
Epidermophyton floccosum	*T.mentagrophytes*
	(two sibling species & variants)
	T.rubrum
	T.schoenleinii
	T.soudanense
	T.tonsurans
	T.violaceum
	T.yaoundei

1.4 THE HOST & SKIN INVASION BY DERMATOPHYTES

1.4.1. Predisposing factors to infection

Predisposing factors to infection can be broken down into two groups, Cutaneous & Systemic factors.

Cutaneous factors

In general, experimental infections with dermatophytes are difficult to reproduce unless some measure is taken to alter the skin of the inoculation area. Mild abrasion of the skin has been found to enhance infection (Sohnle 1989) & is often used on experimental animals to enhance infection rates. However, abrasion to the point of bleeding decreases the chance of infection due to the inhibitory effects of serum on dermatophytes. Warmth & moisture are important for infection & can be responsible for epidemics of dermatophytoses in some parts of the world(Blank et al 1969). Other factors such as fungistatic lipids in sebum & disorders of keratinisation have also been found to be important(Sohnle 1989).

Systemic factors

Factors increasing susceptibility to dermatophytosis include collagen vascular disease, Diabetes mellitus, Cushing disease & atopy. Most of the systemic factors have been associated with decreased effectiveness of certain immunological functions, many of which are inheritable(Sohnle 1989).

1.4.2 Immunity to Dermatophytes

During the colonisation process the dermatophyte soon meets the host defence mechanisms, which usually are well capable of fighting off a continuing infection.

Natural Resistance

The skin surface is a highly hostile environment for microorganisms because of exposure to drying, UV light & competition from the skin's natural flora. The continual shedding of the upper stratum corneum will eventually eliminate any organism that does not firmly establish itself on the skin surface. Suggestions have been made that the main reason dermatophytes produce annular rings when causing an infection is that the organism advances outward in a expanding ring, so allowing the centre to eventually heal due to the shedding of the stratum corneum(Sohnle 1989). Inflammation of the epidermis increases epidermal activity, thus increasing the rate at which the upper stratum corneum is shed, which could be an important pathological defence against dermatophytes(Sohnle 1989).

The Inflammatory Response

Dermatologists have long been aware of the relationship in dermatophyte infections between the length of infection & inflammation. Anthropophilic species such as *T.rubrum* & *M.audouinii* produce chronic infections with little inflammation & these fungi have developed the ability to avoid eliciting a marked inflammatory response. Zoophilic species such as *M.canis* & *T.verrucosum,* on the other hand, produce highly inflammatory infections which are self limiting in duration. This relationship between duration of infection & inflammatory response suggests the inflammatory response is an important mechanism by which the host clears the infection.

Acquired Resistance

For many years the skin was regarded as a non-specific structural barrier against infection but today it is clear the skin plays an important role in developing specific immunity against infections, with most immunocompetent cells being able to inflitrate the epidermis. Sohnle (1993) reported experimental dermatophyte infections in animals do result in cell-mediated immunity to the antigens of the infecting dermatophyte. Delayed type hypersensitivity reactions on infected skin appeared to clear the infection by increasing epidermal cell proliferation(Lepper 1976). Injection of soluble antigen during infection caused lesions to heal faster, indicating a possible lack of antigenic stimulus in chronic infections(Grappel 1974). Also, common sites of infection are the palms & soles, suggesting the thickened epidermis may provide protection against host defences (Smith 1982). *In vitro* lymphocyte transformation assays have been used to demonstrate cell-mediated immunity to dermatophyte antigens & partial immunity to reinfection has been demonstrated. Humoral immunity to dermatophytes is still not fully understood but antibodies to *T.rubrum* have been found in infected humans using enzyme-linked immunosorbent assay(ELISA) (Sohne 1993). Antibody titres are usually higher in infected patients than controls but humoral response appears to be variable(Sohne 1993). Smith (1982) noted that serum antibodies could be demonstrated in infected patients, particularly in patients with a strong inflammatory response. Antibodies were produced by immunizaton against keratinases of *T.mentagrophytes* (Grappel 1972) & *M.canis* (Takiuchi et al 1982). These antibodies were capable of inhibiting enzyme action & if they were produced during normal infection could aid the clearing of the infection. Another possible function of antibody production suggested by Smith (1982) is that of activating complement, leading to increased capillary permeability & phagocytosis.

Thus the host is quite capable of fighting dermatophyte infections with a whole battery of defence mechanisms. However, the type of lesion, if any, which eventuates, in many cases would seem to be related to the efficient production & secretion of proteolytic enzymes by the fungus.

1.5 PROTEOLYTIC ENZYMES & THEIR ROLE IN PATHOGENISIS

1.5.1 Proteinases of Dermatophytes

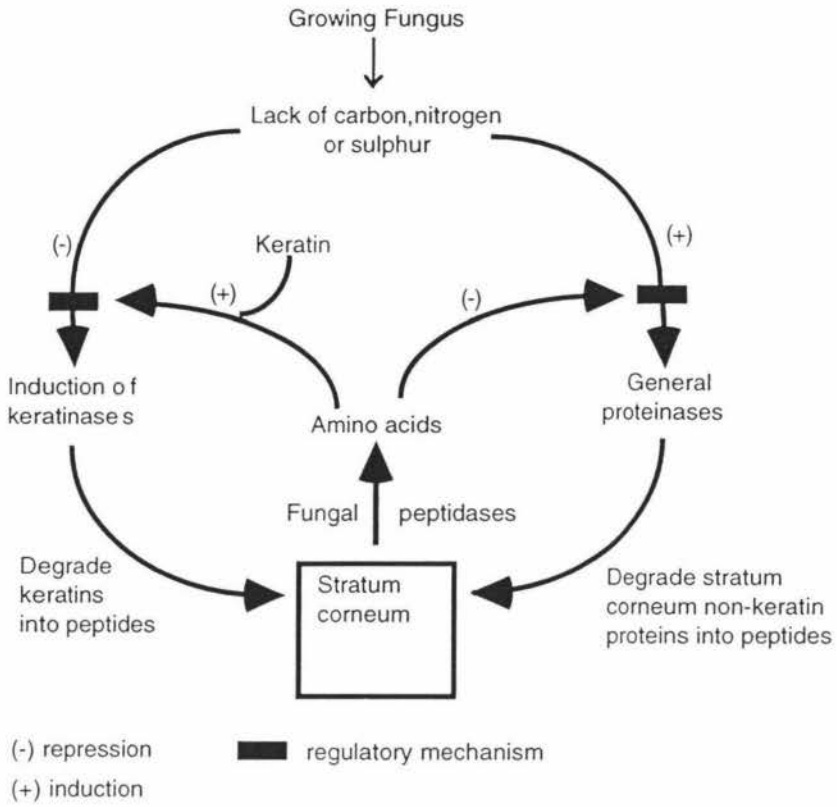
Following the initial invasion of the skin by dermatophytes, pathological changes occur in the deeper layers of the epidermis, probably caused by the diffusible products produced by the fungus or by the interaction of the fungus with the stratum corneum (Minocha et al 1972). Dermatophytes produce a number of secreted enzymes & their role as virulence factors has been inferred by many authors (Rippon & Varadi 1968, O'Sullivan & Mathison 1971, Tanaka et al 1992, Hellgren & Vicent 1980, Apodaca & McKerrow 1989a, 1989b, 1990, Takiuchi et al 1982, 1984, Tsuboi et al 1989, Brasch et al 1991).

Early work on dermatophytes demonstrated that this group of organisms possessed the ability to degrade wool & hair & release sulphydryl containing compounds into the medium (Stahl et al 1950, Raubitschek 1961). Degraded wool fibres examined microscopically showed that cuticles remain intact but the central medullary region of the wool(Stahl et al 1950) or guinea pig hair(Yu et al 1969) is dissolved, with randomly fragmented shafts remaining.

Raubitschek (1961) & others discussed whether it was mechanical action or chemical factors which were the most important in natural keratin breakdown. But the importance of chemical degradation of keratin was demonstrated by Weary & Canby (1969), when strains of *T.rubrum* & *T.schoenleinii* were separated from radio-labelled wool by a permeable membrane & diffusible products produced by the dermatophytes degraded the radio-labelled wool. Since then the proteolytic enzymes produced by dermatophytes have been studied by many people, including important contributions by Roberts & Doetsch (1967), Yu et al (1968,1969,1971), Weary & Canby(1969), Day et al(1968), Grappel & Blank(1972), Minocha et al (1972), O'Sullivan & Mathison(1971), Kunert (1992), Apodaca & McKerrow(1989,1990), Tsubio et al(1989), Sanyal et al(1985), Takiuchi et al(1982,1984) & Quin et al (1992).

Nevertheless, little is know about how dermatophytes actually control proteinase production, O'Sullivan & Mathison(1971) noted that extracellular proteinases produced by *M.canis* were inducible by protein(casein) & repressible by amino acids. Meevootisom(1979) studied the control of extracellular proteinases in *T.rubrum* & reported that glucose & other carbohydrates suppressed proteinase production.

More recently, Apodaca & McKerrow (1989b) also investigated the control of proteinases produced by *T.rubrum* & noted that when carbon, nitrogen or sulphur were deleted from the medium, increased azocollytic activity occurred. *T.rubrum* was grown on nitrogen, carbon or sulphur depleted media & the culture filtrate examined by Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis(SDS-PAGE) with gelatin as incorporated substrate. Apodaca & McKerrow observed a 71,000 Da molecular weight(Mr) proteinase band in nitrogen-depleted cultures & in sulphur-depleted cultures, 124,000 Da & 27,000 Da proteinase bands were observed. Keratinolytic activity, on the other hand, was not repressed by carbon, nitrogen or sulphur but induced when a protein source was added to the medium. Apodaca & McKerrow(1989b) proposed the figure below as a model for the regulation of *T.rubrum* proteolytic activity in log cultures.



The proposed model suggested whenever the fungus lacks carbon, nitrogen or sulphur a group of general proteinases is expressed which act upon the non-keratinous proteins contained in the stratum corneum. The amino acids produced provide the fungus with a source of carbon, nitrogen & sulphur, which in turn has a feed back affect & represses the general proteinases. On the other hand, keratinases are induced by the presence of insoluble proteins such as keratin. The black boxes represent regulatory elements that sense the concentration of carbon, nitrogen & sulphur & control the expression of proteolytic activity.

Apodaca & Mckerrow (1989b) proposed that there were differences between proteolytic enzyme expression of log & stationary phase cultures. In the initial stages of infection *T.rubrum* grows logarithmically & whenever carbon, nitrogen or sulphur is lacking the general proteinases are induced acting upon the non-keratinous proteins in the stratum corneum. Once the stationary phase of growth occurs proteinases are expressed constitutively, coinciding with disease. These proteinases may directly or indirectly incite a host response, resulting in inflammation & dermatophytosis.

Grappel & Blank (1971) were able to demonstrate with guinea pigs that "keratinases" produced by *T.mentagrophytes var. granulosum* elicited a delayed-type cutaneous hypersensitivity reaction & circulating antibody in both previousely infected guinea pigs & those immunised with what were described as active keratinases were present indicating that keratinases are produced during infection.

Minocha et al (1972) noted that zoophilic *T.mentagrophytes* uniformly produced much higher levels of proteolytic enzymes than the anthropophilic *T.rubrum*. They related this to the findings of Pillsbury et al (1956) & Riddell (1958) that zoophilic species induce a more severe inflammatory response than anthropophilic species. Rippon(1982) noted that strains of *T.mentagrophytes* isolated from cattle produced few proteinases when growing on keratin from cattle, but when grown on human keratin proteolytic activity increased over three-fold but keratinase activity remained constant over the two sources of keratin. A human strain of *T.mentagrophytes* when grown on keratin from cattle produced over a four -fold increase in proteinase activity compared to when grown on human keratin ,with keratinase activity again remaining constant.This species specialisation shown by dermatophytes for a particular host can also be seen within human ethnic groups. In the United States, for example, white children infected with *T.tonsurans* get a mild chronic infection but the same species infecting black children produces a severe inflammatory disease. Rippon (1982) considered that as the molecular structure of keratin varies from species to species, the dermatophytes have evolved proteinases with high specificity to the keratin of their host.

Several groups have isolated proteinases from dermatophytes & attempted to characterise them, Asahi et al(1985), Sanyal et al(1985) & Apodaca & McKerrow(1989a) isolated proteinases from *T.rubrum* & Takiuchi et al (1982,1984) isolated a proteinase from *M.canis*.

In 1985 Asahi et al purified two proteinases of molecular weights 90,000 Da & 71,000 Da. In the presence of reducing agents both proteinases broke down into two subunits of 44,000 Da for the 90,000 Da proteinase & 36,000 Da for the 71,000 Da proteinase. Both were inhibited by phenylmethanesulphonyl fluoride (PMSF), suggesting they

were serine proteinases with alkaline pH optima. That same year Sanyal et al (1985) isolated a 34,700 Da proteinase from *T.rubrum* which was also inhibited by PMSF & had an alkaline pH optimum. It appears Asahi et al (1985) & Sanyal et al (1985), both of whom used the same substrate, a glucose-peptone broth, may have purified the same proteinase.

In 1989 Apodaca & McKerrow purified a 27,000 Da serine-like proteinase from *T.rubrum* with an alkaline pH optimum but a very low elastase activity compared to the 71,000 Da & 90,000 Da proteinases purified from *T.rubrum* in 1985. Apodaca & McKerrow concluded each proteinase has a different substrate specificity & when certain elements such as nitrogen, carbon or sulphur are lacking the fungus responds by secreting different proteinases.

Simpanya (1994), using the substrate SDS-PAGE technique noted that *M.canis* expressed six gelatinases(122,000 - 28,000 Da) of which three were more highly expressed in shake cultures. *M.cookei* expressed seven(67,000 - 42,000 Da) of which two were found to be more highly expressed in stationary cultures. Biochemical assays showed higher azocollytic & keratinase-activity in shake cultures of both *M.canis* & *M.cookei*. Both species produced serine proteinases but only *M.canis* produced cysteine & possibility aspartic & metalloproteinaes. The serine & cysteine proteinases were considered to be of particular significance in the pathogenesis of *M.canis* infections. Thus Simpanya concluded that the differing enzyme expression may reflect the differing ecological roles of the two *Microsporum* species.

Many of the proteinases purified from dermatophytes have neutral or alkaline pH optima but the human skin surface has a weakly acidic pH, so questions were raised as to whether these proteinases are important virulence factors(Tanaka et al 1992, Tsuboi et al 1989). Tsuboi et al (1989) isolated a serine-like proteinase from *T.mentagrophytes*, with a pH optimum of 4.5 for keratin as a substrate. Quin et al (1992) isolated a 38,000 Da serine-like keratinase from *T.schoenleinii* , with an optimal pH of 5.5, so providing some evidence dermatophytes do produce proteinases with slightly acidic pH optima.

Takiuchi et al (1982) demonstrated proteolytic activity of *M.canis* was much higher in cultures with human hair as substrate than in cultures without hair. An extracellular proteinase was purified by chromatography & later calculated to be 45,000 Da (Takiuchi et al 1983). IgG antibody was raised against this enzyme & was used to neutralise the proteinase activity.In 1984 Takiuchi et al reported the 45,000 Da *M.canis* proteinase purified in 1982 released peptides from alpha-type fibrous proteins purified from human skin & concluded that proteinases from *M.canis* are able to degrade insoluble proteins present in human skin. Tsuboi et al (1994) reported what were described as three keratinases from *M.canis* with molecular weights of 48,000 Da, 34,000 Da & 31,500 Da. Anti-keratinase IgG, produced by immunization of rabbits & purified by affinity chromatography, reacted with keratinases from different species of dermatophytes, suggesting structurally similar proteinases may be expressed by many dermatophytes.

However, the actual mechanism by which dermatophytes break down keratin is not well understood. Kunert (1972) first suggested the cysteine disulpide bonds present in keratin, which form the basis of keratin resistance, are not enzymatically cleaved as suggested by Chattaway et al (1963) & Weary et al (1965 , 1969), but are degraded by sulphitolysis in which the fungus excretes sulphite into the medium which then reacts with cysteine disulphide bonds according to the equation,

$$Cys\text{-}SS\text{-}Cys + HSO_3^- \rightarrow Cys-SH + Cys-SSO_3^-$$

This renders the reduced keratin more susceptible to enzymatic attack. Work done by Kunert (1992) confirmed that proteolytic enzymes of *M.gypseum* are more active in the presence of reducing agents such as sulphite but this effect depended upon the content of cysteine (disulphide bonds) in the substrate. Ruffin et al (1976) found the expected products of sulphitolysis (peptides containing S-sulphocysteine) in *T.ajelloi* culture medium. This evidence supports the theory of Kunert that dermatophytes denature keratin by excretion of sulphite to attack cysteine (disulphide bonds) prior to attack by fungal proteinases.

1.5.2 Proteinases of Other Pathogenic Fungi

The demonstration of secreted proteinases produced by pathogens does not prove their association with virulence, as most proteinases of microorganisms have been studied *in vitro,* so that the role of these enzymes remains hypothetical. Two pathogenic fungi which have recieved most attention in terms of proteinase production & virulence are *Aspergillus fumigatus* & *Candida albicans*. Proteinases from *A.fumigatus* have been purified & the gene cloned by Tang et al (1992, 1993) & Moser et al (1994). Gene disruption of the proteinase & virulence determinations of both proteinase producing & gene disrupted proteinase deficient mutants has produced confusing results. Some proteinase deficient mutants caused reduced mortality in mice (Kolattukudy et al 1993) but others have found no differences in mortality rates between proteinase producing & proteinase deficient mutants (Tang et al 1993, Monod et al 1993).

The evidence for the role of aspartic proteinases in the virulence of *Candida albicans* is slightly more compelling. Early observations of the low virulence proteinase deficient *C.albicans*, induced chemically, compared to wild-type *C.albicans* gave initial evidence of the importance of aspartic proteinases in the virulence of *C.albicans* (McDonald et al 1983 , Kwon-Chung et al 1985, Ross et al 1990). The specific aspartic proteinase inhibitor, Pepstatin A, interfered with the adherence of *C.albicans* & its invasion of human oral epithelium (Borg & Rüchel 1988).

Tsuobi et al (1985) reported Pepstatin A caused inhibition of *C.albicans* at very low concentrations (0.01μg/ml) & this inhibitor was suggested as a possible antifungal agent. However, Rüchel et al (1992) found that mice immunised with purified aspartic proteinase, when later challenged with the *C.albicans* strain from which the proteinase was derived, showed only some protection against infection

1.6 LABORATORY PRODUCTION OF *M.CANIS* & *M.COOKEI* PROTEINASES

Among the factors that could influence production of proteinases are substrate, period of incubation, temperature & method of culture(ie shake or stationary).

1.6.1 Media

Media used for the production of proteolytic enzymes by dermatophytes are in no way standardised, with different research groups using their own recipes to induce proteolytic enzyme expression. In general, the most common substrate is Sabouraud's Broth, containing glucose as a carbohydrate source & peptone as a protein source. Another commonly used medium is Basal Salts Medium containing vital elements such as Mg , K , PO_4 etc in addition to keratin sources such as hair or wool.

Meevootisom et al(1979) reported exogenous glucose & various amino acids caused suppression of elastase & proteinase activity in *M.gypseum*, *T.mentagrophytes* & *T.rubrum*. Brasch et al(1991) studied the effects of nutritional conditions on enzyme release by *T.rubrum*. By using an api-zym® test they demonstrated seven different extracellular enzymes released when keratin was supplied as a substrate but only five when lipids & two when peptone was available. Among the enzymes released, N-acetyl-ß-glucosaminidase & alkaline phosphatase were detected in all cultures lacking glucose. Enzyme release was inhibited completely when glucose was added to the media, except for N-acetyl-ß-glucosaminidase in peptone cultures. From these results, Brasch et al considered alkaline phosphatase & N-acetyl-ß-glucosaminidase important virulence factors for dermatophytes.

Takiuchi (1982) cultured *M.canis* in a medium containing

0.5g glucose
0.6g $MgSO_4.12H_2O$
0.05g inositol
0.01g pyridoxine

0.01g thiamine
2.6g human hair

& compared proteinase production when the same basic medium was used but human hair substituted by 2.6g of peptone. Proteinase expression appeared not to be supressed by exogenous glucose present, but induced by the presence of keratin. Apodaca & McKerrow (1989) gave a more detailed review of the regulation of proteolytic enzymes produced by *T.rubrum*. They concluded different proteinases are expressed when in log phase of growth, in response to vital elements the fungus is lacking, but once stationary phase is achieved proteolytic enzymes are expressed constitutively. Keratinolytic activity was not repressed by the presence of glucose but induced by the presence of an insoluble protein source. In 1990, Apodaca & McKerrow reported glucose caused partial repression of general proteolytic activity in *T.rubrum*.

1.6.2 Period of Incubation & Temperature

Other factors which could affect proteinase expression are period of incubation & temperature. The period of incubation used varies from report to report, some authers using as little as five days(Tsuboi,1989), Takiuchi (1982) incubated their cultures for eleven days. There is little information on the effect temperature may play on proteolytic enzyme expression. Most workers use the temperature range 25°C to 35°C.

1.6.3 Stationary & Agitated(Shake) Cultures

Raubitschek in 1955 noted shake cultures of dermatophytes produce hyphae, arthrospores & chlamydospores resembling the parasitic phase of growth found when infecting skin, nails or hair. Stationary cultures, on the other hand, produce hyphae & macro- & microconidia, resembling the saprophytic phase of growth. Thus proteinases produced in shake culture may reflect more closely those involved in skin invasion *in vitro*. However, most workers have used either stationary or shake cultures only, without any reason given for their choice of culture technique.

1.7 SUBSTRATE CO-POLYMERISED SDS-PAGE ELECTROPHORESIS

In Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) proteins are mixed with buffer at a pH where proteins remain stable, generally in the pH range 7.0 - 9.0. SDS is added which binds to the protein, giving the protein a negative net charge per unit. Once electrical current is connected the proteins migrate towards the anode at a rate dependent upon their size & subsequently can be detected by suitable staining techniques.

$$Rf = \frac{\text{distance migrated by protein}}{\text{distance migrated by dye*}}$$

*The dye bromophenol blue(BPB) allows the progress of the electrophoresis to be monitored.

A plot, $\log_{10}$ of polypeptide molecular weight versus relative mobility(Rf), reveals a straight line relationship (Hames 1991). Therefore by adding to the gel a set of known molecular weight proteins & using the distance migrated by each protein to construct a standard curve, the molecular weight of proteins from samples can be calculated. The pore size of acrylamide gels can be varied, according to the size of molecules to be separated, by changing the concentration of total acrylamide or by changing the concentration of the cross-linker, bis-acrylamide in the gel. It should be noted that acrylamide concentration also has an effect on the relationship between $\log_{10}$ molecular weight & relative mobility(Rf), which is linear over a limited range of molecular weights according to the concentration of acrylamide in the gel.

The substrate co-polymerised gel technique is a modified version of the above Laemmli's (1970) procedure, using a non-substrate stacking gel. Several differences exist between the two techniques, namely

a) Substrate gels are made by incorporating the protein substrate of interest into the gel during mixing of acrylamide solutions, buffers & initiators of polymerisation, namely freshly made up ammonium

persulphate & a catalyst for polymerisation N,N,N',N'-tetramethyethylenediamine.

b) In the sample buffer no reducing agents, such as ß-mercaptoethanol are added & the sample is not boiled. However, to standard molecular weight markers ß-mercaptoethanol is added & the mixture boiled.

c) After electrophoresis the gel is incubated in TritonX-100 for 1hr to inactivate the SDS. Incubation buffer is added & the gel incubated overnight at 37°C to allow digestion of substrate & thus formation of proteolytic bands.

The most common substrate used by other workers is gelatin (Heussen & Dowdle 1980, Apodaca & McKerrow 1989,1990 & North et al 1988). However, some workers have used elastin (Apodaca & McKerrow 1990).

The staining process for substrate co-polymerised gels is performed as for normal denatured protein gels, except that the gels are only destained until visualisation of the clear bands against the Coomassie blue stained background is achieved.

There are several limitations with substrate co-polymerised gels which need to be noted & taken into consideration when using this technique to study proteolytic enzymes. Some proteinases in the presence of SDS may be irreversibly denatured. With crude samples several different proteinases may be present, each with different pH optima compared to the pH of the buffers present. Different proteinases of identical size may not be differentiated on a one dimensional gel & only proteinases of high activity can be investigated.

1.8 PROTEINASES & PROTEINASE INHIBITORS

1.8.1 Proteinases

Proteolytic enzymes or proteinases that catalyze the degradation of peptide bonds in proteins are found throughout the plant & animal kingdoms. Proteinases that catalyze the cleavage of an internal peptide bond are sometimes referred to as endoproteinases. Proteinases that catalyse the cleavage of one or more amino acids from the extreme amino terminus or carboxy terminus are referred to as exopeptidases or more specifically aminopeptidases & carboxypeptidases, depending upon which end of the protein the amino acids are liberated. Proteinases are classified today on comparison of active sites, mechanism of action & 3-D structure. Four different mechanistic classes are recognised by the International Union of Biochemistry & are named according to the mechanism of action,

Serine proteinases
Cysteine proteinases
Aspartic proteinases
Metalloproteinases.

Serine Proteinases

These are the most thoroughly studied class of enzymes in the proteinase field (Dunn 1989). The serine class of proteolytic enzymes includes trypsin & many elastases from mammals & subtilisin from bacteria. Serine proteinases form covalent complexes between enzyme & substrate by the hydroxyl(OH) group from the serine amino acid making a nucleophilic attack on the acyl portion of the substrate, with the formation of an ester bond between enzyme & substrate. Via the hydrolysis of water the ester bond is broken resulting in the breaking of the peptide bond present in the protein.

Cysteine Proteinases

These include cathepsins found in mammalian lysosomes & the plant proteinase papain. Cysteine proteinases also form a covalent bond between enzyme & substrate but with cysteine proteinases the

attacking nucleophile is a sulphur atom from the cysteine side chain. From this point on the reaction is the same as for serine proteinases.

Aspartic proteinases

The asparatic proteinases do not cleave peptide bonds by nucleophilic attack. The catalytic apparatus consists of two aspartic acid side chain residues and at low pH(3-5), the carboxyl groups are thought to cause cleavage of the peptide bond.

Metalloproteinases

This group of proteinases, like aspartic proteinases, do not form covalent intermediates. A metal ion such as zinc provides a strong electrophilic pull which assists an attack by a water molecule causing cleavage of the peptide bond.

1.8.2 Proteinase Inhibitors

Today many different types of proteinase inhibitor can be employed in an effort to classify a newly discovered proteinase. Dunn (1989) suggested that each inhibitor should be individally applied, with the effect of inhibitor on activity of the enzyme carefully measured. Each inhibitor should be incubated with the unknown proteinase for up to 1 hr at 20°C & controls treated the same way without inhibitor added. The following inhibitors were used in the present study.

Phenylmethanesulphonyl fluoride(PMSF)

This is a very effective irreversible inhibitor of serine proteinases, & reacts with the amino acid serine at the active site of the enzyme. PMSF decays rapidly in aqueous solutions ($t_{1/2}$ 25°C pH 7.5, 55 min; James 1978) & this has to be taken into account when using this inhibitor.

p-Chloromercuric acid(p-CMB)

This is a cysteine proteinase inhibitor that reacts with -SH groups found in the amino acid cysteine, which is a vital component of the active site in cysteine proteinases.

Pepstatin A(PEP)

This is a well known inhibitor of aspartic proteinases, orginally isolated from a *Streptomyces* species but today it is often modified to increase/decrease selectivity for different proteinases.

Ethylenediaminetetraacetic acid(EDTA)

This is a commonly used inhibitor of metalloproteinases. The EDTA acts as a metal chelator of the active site metal such as Zn, Ca, or Fe, acting as a reversible inhibitor. Many other types of proteinases can be inhibited by EDTA as some serine or cysteine proteinases are stabilised by a metal ion such as Zn or Ca. With this in mind it is important to classify metalloproteinases on the insensitivity to the other three types of inhibitor, plus the observation of inhibition by EDTA or another chelating agent.

THE AIMS OF THIS PROJECT

(a)To seek possible differences in proteolytic enzyme expression between the pathogenic species *M.canis* & the saprophytic species *M.cookei*, using various biochemical assays over varying pH ranges & temperatures & to determine the effects proteinase inhibitors have on activity.

(b)To use the substrate SDS-PAGE technique to demonstrate the effect environmental factors such as substrate, mode of culture, temperature & time have on the expression of proteolytic(gelatinase) enzymes.

(c)To attempt to characterise, using proteinase inhibitors, the gelatinase enzymes expressed by both species, as detected by the substrate SDS-PAGE technique, so allowing an estimation of the similarity or dissimilarity of the various gelatinases in the proteinase mixes.

CHAPTER 2

MATERIALS & METHODS

2.1 MYCOLOGICAL TECHNIQUES

2.1.1 Basic Media

(a)Sabouraud's Dextrose Agar(SDA)

Glucose(Serva D-Glucose)	40g
Neopeptone(Difco)	10g
Agar	15g
Distilled Water (DW)	1000ml

Mixed & boiled before autoclaving for 15 min at 120°C.

(b)Sabouraud's Dextrose Agar with Antibiotics(SDAXX)

To make 1L 0f SDAXX the ingredients were mixed as above & the following added prior to autoclaving

Chloramphenicol(Sigma)	0.05g/5ml ethanol(95%)
Cycloheximide(Sigma)	0.5g/5ml acetone

(c)Dilute Sabouraud's Dextrose Agar(Dil SDA)

Neopeptone(Difco)	1.0g
Glucose(Serva D-Glucose)	2.0g
Agar	15g
DW	1000ml

Prepared & autoclaved as above

(d)Sabouraud's Dextrose Broth (SDB)

Neopeptone	10g
Glucose	40g
DW	1000ml

(e)Basal Salts Medium(BS)

$MgSO_4.7H_20$	0.025g
$CaCl_2$	0.025g
KH_2P04	0.05g
$FeSO_4$	0.005g

The components were added to 1L of DW, mixed until dissolved & stored at 4°C until required.

2.1.2 Sterile Wool

Clean wool was rinsed & then soaked in soap & water for several days. The wool was washed with distilled water, squeezed to remove excess water & allowed to dry overnight at 37°C. Once dried the wool was then soaked in ether for 48 hours to remove fatty material, the ether was then decanted off & the wool allowed to dry inside a fume cupboard overnight. Samples of 0.5g were packed into small paper bags & sterilised with ethylene oxide(88% CO_2, 12% ethylene oxide) for 2 hours at 35 kPa by L.J.Eccles Ltd, Auckland.

2.1.3 Source of Cultures

The four *M.canis* isolates(Departmental culture collection codes MB6, MB21, MB42 & MB47) had been isolated from cases of ringworm in the Palmerston North region & were maintained on slopes of SDA & SDAXX.

The four *M.cookei* isolates(24H, 125H, 152H & 192H) had been isolated from soil samples taken from various sites in Palmerston North using the keratin-baiting technique & were maintained on slopes of SDA & SDAXX.

2.1.4. Preparation of Cultures

2.1.4.1 Spore Suspension

Spore suspensions were used for inoculating SDB cultures & BS + wool cultures. Cultures were established on Dil SDA slopes to induce spore production. After 2-3 weeks growth at 25°C, 5ml of sterile distilled water was washed over the surface & sucked up & down with a 5ml autopipette to dislodge conidia. 4ml was then removed & autopipetted into a bijou bottle. The lids were tightened & the suspension stored at 4°C.

2.1.4.2 SDB Cultures

(a)Shake Cultures

SDB in 50ml aliquots was added to 125ml conical flasks, plugged with cotton wool & autoclaved at 120°C for 15 min.

Spore suspension(0.1ml) was aseptically added & the cultures placed on a rotatory shaker(100rpm) in the dark for incubation.

(b)Stationary Cultures

Cultures were established as above but in Kimax tissue culture bottles & placed flat on a shelf for incubation in the dark.

2.1.4.3 Basal Salts + Wool (BSW) Cultures

(a)Shake Cultures

BS medium in 50ml aliquots was added to 125ml conical flasks, plugged with cotton wool & autoclaved for 15 min at 120°C. Sterile wool(0.5g) was aseptically added & gently mixed with the medium.

Spore suspension(0.1ml) was aseptically added & the cultures placed on a rotatory shaker(100rpm) for incubation.

(b)Stationary Cultures

Cultures were established as above but using Kimax bottles & placed flat for incubation.

2.1.5 Preparation of Culture Filtrates

After 14 days incubation of cultures, samples of culture broth were removed & spun in a Eppendorf tube for 15 min at 13,000rpm at 4°C in a Micro-Centaur to remove cellular material from the sample. The supernatant was then used in assays for proteolytic activity.

2.2 PROTEOLYTIC ASSAYS.

2.2.1 Materials

Azocoll (Sigma) - stored at room temperature
Elastin- congo red (Sigma) - stored at 4°C
Keratin azure (Sigma)- stored at room temperature.

(a)Substrate buffers: Sodium Acetate/HCl (pH 3, 4 & 5)

Sodium acetate	1.64g
$CaCl_2$	0.022g
DW	200ml

The sodium acetate was added to 150ml of DW & mixed until dissolved. The pH was adjusted with HCl to the desired level & the mixture brought up to volume with DW & stored at room temperature.

(b)Substrate buffers: Tris-HCl (pH 6,7 & 8)

Tris	2.42g
$CaCl_2$	0.022g
DW	200ml

The Tris buffer mixtures were prepared as above except Tris replaced sodium acetate.

(c)Substrate buffers: Glycine-NaOH (pH 9,10, 11 & 12)

Glycine	1.50g
$CaCl_2$	0.022g
DW	200ml

The glycine buffer mixtures were prepared as above except glycine replaced sodium acetate & NaOH was used to adjust the pH.

2.2.2 Methods

2.2.2.1 Azocollytic, Elastase & Keratinase Activity

(a) Azocollytic Activity

Estimation of proteolytic activity(proteinase) using azocoll was a modification of the method of Walter (1984).

Suspensions of azocoll were made by mixing the appropriate buffer with azocoll to give a concentration of 4mg/1.35ml.

The azocoll suspension was dispensed in 1.35ml aliquots into Eppendorf tubes, 0.15ml of culture filtrate was added & the mixture incubated for 24hr at the appropriate temperature & pH in a controlled temperature waterbath. Each assay was performed at least in duplicate.

Control samples of culture filtrate were boiled for 45 min & 0.15ml of this boiled sample was added to 1.35ml of azocoll suspension & incubated for 24 hr with the experimental assays.

After incubation the tubes were spun for 15 min at 13,000rpm at 4°C. The supernatant was autopipetted off into another Eppendorf tube for ease of handling.

The azocoll degradation was measured by determining the absorbance of the supernatant at 520nm wavelength using a Schimadzu UV-160A spectrophotometer

A change of 0.1 A_{520} units per hour was defined as one unit of azocollytic activity(μ/ml hr^{-1}).

(b)Elastinolytic Activity

Estimation of Elastinolytic activity using elastin congo-red was a modification of the method of Monod (1993).

The procedures for elastinolytic activity were the same as for azocollytic activity, except elastin congo-red replaced azocoll & the wavelength 495nm was used to read absorbances.

A change of 0.01 A_{495} units per hour was defined as one unit of elastinolytic activity(μ/ml hr^{-1}).

(c)Keratinase Activity

Estimation of Keratinase activity using keratin azure was a modification of the method of Apodaca & McKerrow (1989a, 1989b & 1990).

Keratin azure(0.5mg aliquots) was added to Eppendorf tubes & 0.8ml of the appropriate buffer added.

The procedures for keratinolytic activity were the same as for azocollytic activity except 0.2ml of culture filtrate was added to the buffer-keratin mixture & the wavelength 595nm was used to read absorbances.

The keratinase activity of the supernatant was measured by determining absorbance at 595nm. One unit of keratinase activity was defined as a change of 0.01 A_{595} per hour(μ/ml hr^{-1}).

2.2.2.2 pH Profiles

Proteolytic activities measured as described in section 2..2.1 were estimated in the following buffers:

0.1M Sodium Acetate-HCl + 1mM $CaCl_2$	pH range 3 - 4
0.1M Tris-HCl + 1mM $CaCl_2$	pH range 6 - 8
0.1M Glycine-NaOH + 1mM $CaCl_2$	pH range 9 - 12

The assays were incubated for 24 hr & results plotted as activity against pH.

2.2.2.3 Temperature Optima

Azocollytic activity was measured as before except the assays were incubated for 6 hr & not 24 hr to avoid excessive loss of water in the water baths at the higher temperatures. The temperatures used were

25°C
30°C
37°C
45°C
50°C
60°C

All assays were in 0.1M Tris-HCl + 1mM $CaCl_2$, pH 8 Substrate buffer & the results were plotted as azocollytic activity against temperature.

2.2.3 Characterisation of Proteolytic Enzymes

The inhibitors used are listed in Table 2.1:

Table 2.1: Inhibitors & Inhibitor Concentrations

Inhibitor	Abbreviation	Solvent	Concentration*
Phenylmethanesulphonyl fluoride	PMSF	DMSO	2mM
Ethylenediaminetetraacetic acid	EDTA	DW	5mM
p-Chloromercuric acid	p-CMB	0.2M NaOH/HCl	2mM
Pepstatin A	PEP	DMSO	50µg/ml

*Working concentration in both sample & incubation buffers
The PMSF & p-CMB were prepared fresh just before each experiment.

Filtrates from 14 day shake cultures grown at 25°C were prepared as described in section 2.1.5. Appropriate inhibitor was added to 1ml aliquots of filtrates & the mixture incubated at room temperature for 10 min.

Suspensions of azocoll or elastin congo-red were made by mixing substrate buffer (0.1M Tris-HCl + 1mM $CaCl_2$, pH 8) with the substrate to give a concentration of 4mg/1.35ml of buffer. Aliquots of 1.35ml of these suspensions were autopipetted into Eppendorf tubes.

After 10 min incubation of the inhibitor-culture filtrate mixture, 0.15ml was added to the substrate-buffer suspension & the appropriate inhibitor added to give the correct working concentration required(Table 2.1) & the assay performed as in 2.2.2.

At the same time, postive control assays without inhibitor added were included to calculate total azocollytic & elastase activity. For the two inhibitors PEP & PMSF, the solvent DMSO is required to completely dissolve the inhibitors, so the positive control assays for the two above inhibitors contained the solvent DMSO to allow for any affects DMSO may have had on enzyme activity.

To calculate the effect of each inhibitor on azocollytic or elastase activity results were expressed as percentage of inhibition by an individual inhibitor against a positive control.

$$\% \text{ inhibition} = \frac{Ai}{A} \times 100$$

Ai = absorbance value with inhibitor present

A = absorbance value without inhibitor present

2.3 SUBSTRATE CO-POLYMERISED SDS-PAGE GELS

The techniques used for electrophoresis were a modification of those used by Simpanya (1994).

2.3.1 Reagents & Materials

(a)Running Acrylamide

Acrylamide(BDH)	30.0g
Bis-Acrylamide(Sigma)	1.0g
DW	100ml

The acrylamide was added to approx 70ml of DW & stirred until dissolved. The bis-acrylamide was added & the preparation made up to volume with DW & stored at 4°C.

(b)Stacking Acrylamide

Acrylamide	30.0g
Bis-Acrylamide	1.6g
DW	100ml

Prepared as above

(c)Lower Tris-HCl Buffer(pH 8.8)

Tris(BDH)	18.10g
SDS*(BDH)	0.04g
DW	100ml

*SDS:Sodium dodecyl sulphate

Tris was added to 90ml of DW & mixed until dissolved. The pH was adjusted with HCl, the mixture brought up to volume & stored at 4°C.

(d)Upper Tris-HCl Buffer(pH 6.8)

Tris	6.05g
SDS	0.04g
DW	100ml

Prepared as for Lower Tris Buffer

(e)Tris -Glycine Reservoir Buffer(pH 8.3)

Tris	6.07g
Glycine(BDH)	28.8g
SDS	2.0g
DW	2.0L

The components were mixed & stored at 4°C.

(f)Sample Buffer(pH 6.8)

Tris	7.57g
Sucrose	2.5g
SDS	0.5g
Bromophenol Blue	0.0125g

The mixture was prepared as for Lower Tris buffer.

(g)Temed:N.N.'N'N'-Tetramethylethylenediamine(Sigma)(99%)

(h)Incubation buffer (pH 8.0)

Tris	1.51g
$CaCl_2$	0.027g
DW	250ml

The mixture was prepared as for Lower Tris buffer & stored at room temperature.

(i)Ammonium Sulphate Solution

Ammonium Sulphate	0.1g
DW	1ml

Once mixed, the solution was kept for no longer than 2 hours.

(j)Gelatin Solution

Gelatin(Type B)(Sigma)	0.05g
DW	5.0ml

The gelatin & DW were mixed by placing under a hot tap until the gelatin dissolved & the solution allowed to cool.

(k)TritonX-100 Washing Solution

TritonX-100(BDH)	20.0ml
DW	980.0ml

The components were stirred until dissolved

(l)Molecular Weight Markers

Broad range molecular weight markers were supplied by Bio-Rad & contained the following molecular weights protein.

Myosin	200,000 Da
β–Galactosidase	116,250 Da
Phosphorylase a	94,400 Da
Serum Album	66,200 Da
Carbonic Anhydrase	45,000 Da
Trypsin inhibitor	31,000 Da
Lysozyme	21,500 Da
Aprotinin	6,500 Da

(m)Bromophenol Blue Solution(0.1%)

Bromophenol blue	0.05g
DW	50ml

The Bromophenol blue was added to the DW, mixed & the solution stored at room temperature.

(n)SDS Solution (10%)

A 10% w/v solution was prepared in DW & stored at room temperature.

(o)Staining Solution

A 1% Coomassie blue solution was made by adding 2.0g of Coomassie blue to 200ml of DW & mixing well. It was filtered through Whatman N°1 paper & stored at room temperature.

The working staining solution was a (5:5:1 volume ratio of water:methanol:acetic acid)

110ml of 1% Coomassie blue
390ml of DW
500ml of methanol
100ml of acetic acid

to give 1100ml of staining solution.

(p)Destaining Solution

520ml DW
450ml methanol
30ml acetic acid

The components were mixed & stored at room temperature.

2.3.2 Methods

2.3.2.1 Preparation of Gels

(a) Moulds

A Bio-Rad Protean II X1 cell assembly was used with glass plates of sizes 200mm x 225mm(outer plate) & 200mm x 200mm(inner plate) with 1mm spaces. Petroleum jelly was used to help seal the joints.

(b) Running of Gel

Running gel of 12% Acrylamide content was prepared as follows.

Lower Tris buffer (pH 8.8)	10.0ml
Running Acrylamide	16.0ml
DW	9.9ml
Gelatin solution	4.0ml
Ammonium persulphate solution	0.2ml
Temed	0.02ml

The buffer, acrylamide,DW & gelatin solution were mixed together in a 250ml conical flask by swirling, trying not to create bubbles.The ammonium persulphate & Temed were added quickly, swirled & the suspension pipetted with a 5ml pipette into the prepared mould. A small volume of DW (≈2ml) was then distributed over the surface of the running gel & the gel left until polymerisation had taken place(≈45 min).

(c)Stacking Gel

A 30% acrylamide/1.6% bis-acrylamide stacking gel was prepared as follows:

Upper Tris buffer(pH6.8)	2.5ml
Stacking acrylamide	1.3ml
DW	6.1ml
Ammonium persulphate solution	0.05ml
Temed	0.01ml

The buffer,acrylamide & DW were mixed together by swirling trying not to create bubbles.The ammonium persulphate & Temed were added to the mixture & approximately 1ml was used to wash the surface of the polymerised running gel after the surface water had been decanted off. The mould was then filled with the stacking gel mixture to 5mm from the top of the mould. A 15 tooth comb was inserted to a depth of 25mm & the gel left until polymerised. After the stacking gel had polymerised the comb was carefully removed & the wells washed out with DW.The mould(s) were then clipped onto the water jacket & placed in the buffer chamber. Tris Glycine buffer was added to the upper buffer tank & a check made for leaks before the lower buffer tank was filled.

NB:note that stacking gel does not contain gelatin substrate.

2.3.2.2 Preparation of Samples

(a)A 1ml sample of culture filtrate was aseptically autopipetted into an Eppendorf tube & kept on ice while other samples from other strains were taken. The 1ml samples were spun in a Micro-Centaur Centrifuge for 10 min at 4°C.

(b)The top 0.5ml of the centrifuged culture filtrate was autopipetted into 0.5ml of sample buffer in a fresh Eppendorf tube, mixed &, using a 50µl micropipette, 50µl of this sample was added to the acrylamide stacking gel wells.

2.3.2.3 Preparation of Molecular Weight Standards

Buffer for the molecular weight standards was:

375µl	DW
125µl	Upper Tris buffer
200µl	Glycerol
200µl	SDS 10% sol
50µl	β-methcaptoethanol
50µl	Bromophenol blue 0.1% sol

The components were mixed in an Eppendorf tube & 19µl was autopipetted into a fresh Eppendorf tube.To this 1µl of the molecular weight marker solution was added, the mixture boiled for 5 min & a sample of 10µl was micropipetted into the acrylamide stacking gel well.

2.3.2.4 Gel Electrophoresis, Staining & Destaining

The samples were loaded into the acrylamide wells at 4°C. Once loaded, the electrophoresis unit was connected to a power unit (Pharmacia, ECDS 3000/150). The gel was electrophoresed at a constant current of 28mA until the tracking dye front was within ≈2cm of the bottom of the running gel.

At the end of the run the power source was switched off and the gel removed from the mould & soaked in 150ml of 2% Triton X-100 for 1 hr to neutralise the SDS.

The Triton X-100 was removed & 200ml of incubation buffer was added. The gel was left overnight at 37°C with gentle shaking.

The gel was then stained at room temperature with Coomassie blue stain for 1 hr with gentle shaking.

Once stained, the gel was destained until visualisation of proteolytic bands was reached & the gel then immersed in water & photographed as soon as possible.

2.3.2.5 Size Estimation of Proteolytic bands.

The size of the components in each proteolytic band was estimated by comparing their mobility in the polyacrylamide gels with that of the molecular weight standards run in the same gel.

$$\text{Relative mobility (Rf)} = \frac{\text{distance migrated by unknown protein}}{\text{distance migrated by dye}}$$

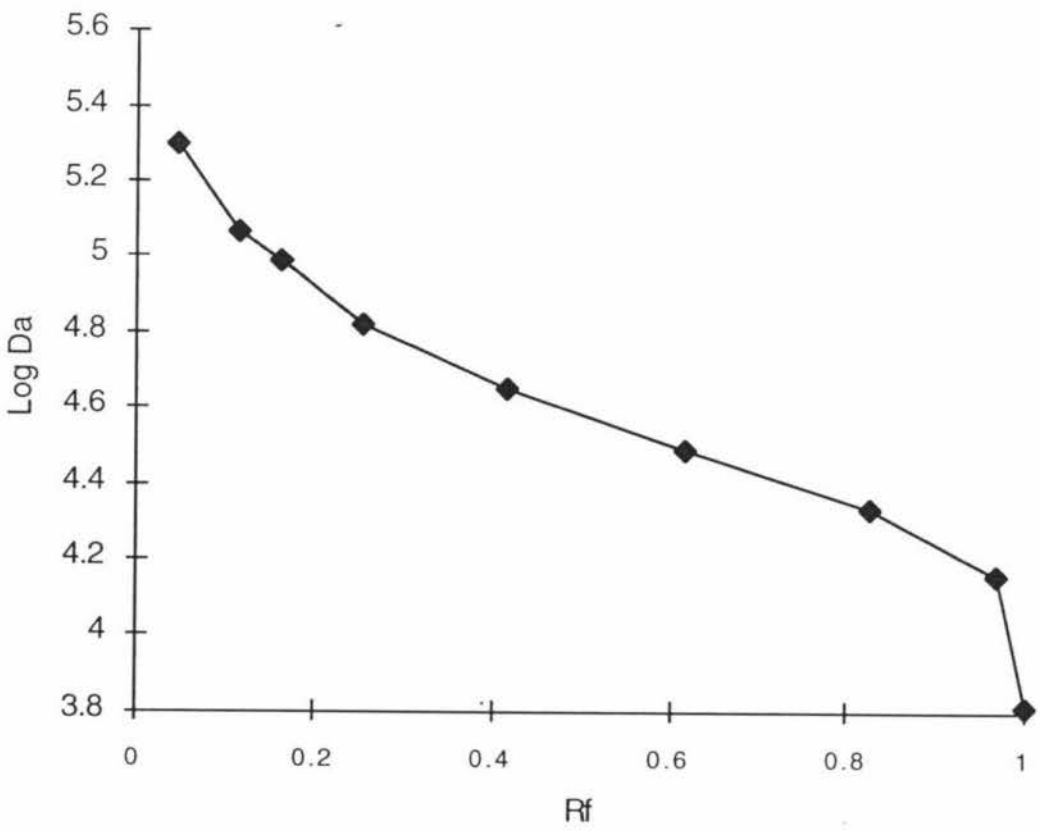
The relative mobilities of known proteins (ie the molecular weight markers) were plotted against log base 10 of molecular weight of the markers & a line of best fit drawn. The Rf's of the proteolytic bands were calculated & read from the same plot.

In early runs, problems with staining of molecular weight markers occurred especially for Ovalbumin, Carbonic anhydrase & Soybean trypsin inhibitor. At first the reason for their absence was unknown , but later it became apparent the problem arose with the incubation of the gels in substrate buffer overnight & resultant loss of markers to the buffer during incubation. This also became a source of error in calculating the molecular weight of the proteinase bands. Separating the markers from the rest of the gel by cutting the gel & staining separately seemed to eliminate this source of error.

Table 2.2: Relative Mobilities of Molecular Weight Markers

Protein	Protein size (Da)	Distance Travelled(cm)	Relative mobility(Rf)
Myosin	200,000	0.061	0.7
ß-galactosidase	116,250	0.175	2.0
Phosphorylase B	97,400	0.210	2.4
Serum albumin	66,200	0.333	3.8
Ovalbumin	45,000	0.517	5.9
Carbonic anhydrase	31,000	0.789	9.0
Trypsin inhibitor	21,500	0.895	10.2
Lysozyme	14,400	0.991	11.3
Aprotinin	6,500	1.0	11.4

Fig 2.1: Standard Curve for Molecular Weight Markers



2.3.3 Characterisation of Gelatin-Degrading Enzymes using Substrate SDS-PAGE with Proteinase Inhibitors.

2.3.3.1 Methods

The Bio-Rad Protean II system allows one or two copolymerised gels to undergo electrophoresis at the same time. This ability was used in an attempt to characterise the proteinase bands separated by electrophoresis, by the addition of specific proteinase inhibitors to samples. One gel acted as a positive control, with samples containing no inhibitor, while the other gel was used as the experimental gel & contained samples with specific inhibitor added.

The same proteinase inhibitors(PMSF, EDTA, p-CMB & PEP) as in section 2.2.3 were used & at the same working concentrations(see Table 2.1).

Filtrates from 14 day shake cultures grown at 25°C were prepared as described in Section 2.1.5,. both SDB & BSW were used to compare proteinase expression in different media.

Electrophoresis was at à constant 40mA to optimise the running of gels. The electrophoretic technique & staining/destaining used were as described in 2.3.2.4.

For the experimental gels containing inhibitor, the incubation buffer also contained inhibitor at the same working concentration as in samples added to gels.

Once stained, the gels were destained until visualisation of the proteolytic bands was reached & then immersed in water for photography. The gel with inhibitor (experimental gel) was compared to the gel without inhibitor (positive control gel) & any differences noted.

CHAPTER 3

RESULTS

3.1 GENERAL ANALYSIS OF PROTEINASES PRODUCED BY *M.CANIS* & *M.COOKEI*

Assays for azocollytic, elastinolytic and keratinolytic activity were performed on culture filtrates prepared as in Section 2.2, following growth of one strain of each species (MB6 & 24H) in the two media Sabouraud Dextrose Broth(SDB) & Basal Salts Wool(BSW) in shake culture at 30°C for two weeks. Each assay was repeated at least twice.

3.1.1 Effect of pH on Proteolytic Activity

3.1.1.1 Azocollytic Activity

The effects of pH on azocollytic activity of both species are shown in Tables 3.1 & 3.2. Azocollytic activity of *M.canis* BSW filtrates was markedly higher over the pH range 5-10 compared to filtrates from SDB. Azocollytic activity of *M.cookei* filtrates was very similar in both SDB & BSW.

The pH optima of azocollytic activity for both species was in the range pH 8-9(Fig 3.1 & 3.2).

Table 3.1:Azocollytic activity(μ/ml hr^{-1}) of *M.canis* culture filtrates(from shake cultures in SDB & BSW) between pH 3-12.

pH	*M.canis*	
	SDB (±SD)	BSW (±SD)
3	0.01±0.01	0.01±.001
4	0.01±0.01	0.01±0.00
5	0.01±0.00	0.16±0.03
6	0.04±0.02	0.27±.004
7	0.06±0.03	0.29±0.04
8	0.07±0.03	0.30±0.04
9	0.03±0.02	0.36±0.05
10	0.02±0.01	0.28±0.04
11	0.01±0.01	0.08±0.02
12	0.01±0.01	0.00±0.00

SD = standard deviation (2 replicates)

Table 3.2:Azocollytic activity(μ/ml hr^{-1})of *M.cookei* culture filtrates(from shake cultures in SDB & BSW) between pH 3-12.

pH	*M.cookei*	
	SDB (±SD)	BSW (±SD)
3	0.00±0.00	0.00±0.00
4	0.00±0.00	0.01±0.01
5	0.05±0.02	0.08±0.03
6	0.16+0.05	0.16±0.03
7	0.18±0.07	0.15±0.06
8	0.20±0.05	0.16±0.03
9	0.19±0.06	0.14±0.05
10	0.19±0.06	0.06±0.02
11	0.03±0.02	0.01±0.01
12	0.00±0.00	0.01±0.01

Fig 3.1:pH optima for azocollytic activity (μ/ml hr^{-1}) of *M.canis* culture filtrates.

The effect the different media had on the expression of azocollytic activity by *M.canis* can clearly be seen. BSW filtrates possessed higher azocollytic activity(0.36μ/ml hr^{-1} at pH 9) than SDB filtrates(0.070μ/ml hr^{-1} at pH 8) .

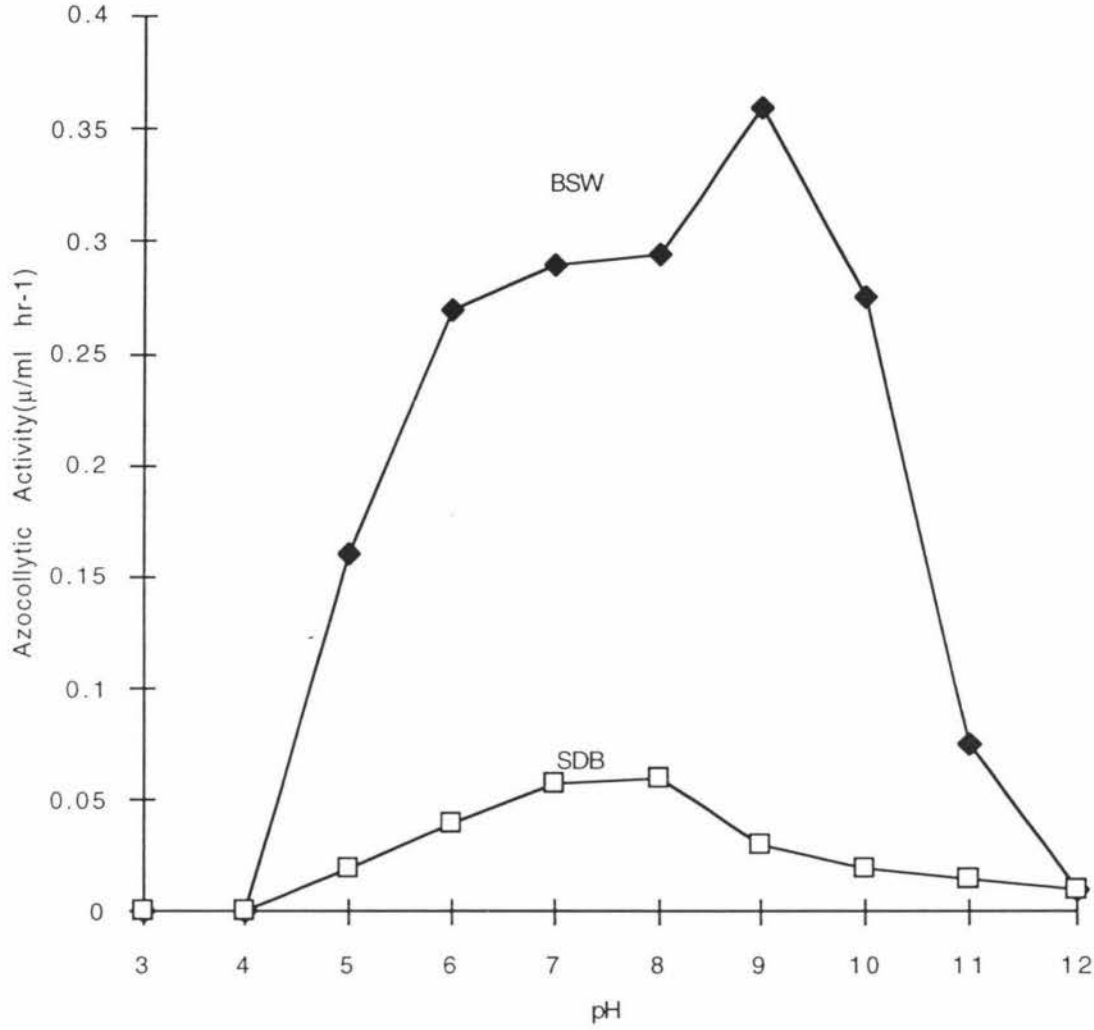
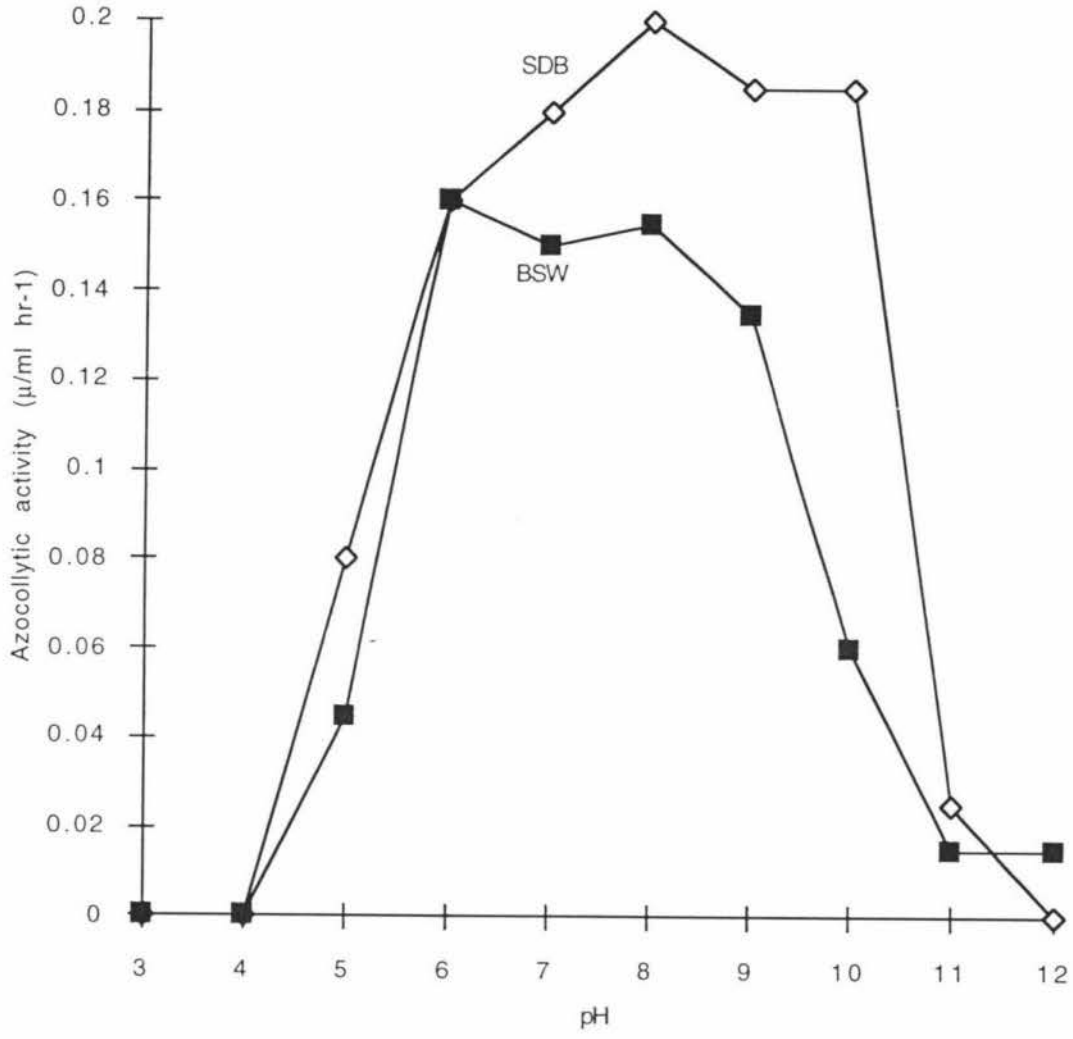

0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Azocollytic Activity(μ/ml hr-1)
BSW
SDB
3
4
5
6
7
8
9
10
11
12
pH

Fig 3.2:pH optima for azocollytic activity(μ/ml hr^{-1}) of *M.cookei* culture filtrates.

The effect the different media had on azocollytic activity was less obvious with *M.cookei*. SDB filtrates produced somewhat higher azocollytic activity (peaking at 0.2 μ/ml hr^{-1} at pH 8) compared to BSW filtrates(0.15-0.16 μ/ml hr^{-1} , pH 6-8).


0.2
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
Azocollytic activity (µ/ml hr-1)
SDB
BSW
3
4
5
6
7
8
9
10
11
12
pH

3.1.1.2 Elastase Activity

The effects of pH on elastase activity of both species are shown in Tables 3.3 & 3.4. Elastase activity of *M.canis* BSW filtrates was markedly higher over the pH range 7-10 compared to SDB filtrates. Elastase activity of *M.cookei* cultured in BSW was higher than when cultured in SDB but the differences in activities of *M.cookei* filtrates was less marked than *M.canis*(Figs 3.3 & 3.4).

Table 3.3:Elastase activity(μ/ml hr^{-1}) of *M.canis* culture filtrates(from shake cultures in SDB & BSW) between pH 3-12.

pH	*M.canis*	
	SDB (±SD)	BSW (±SD)
3	0.04±0.04	0.00±0.00
4	0.04±0.03	0.02±0.01
5	0.08±0.07	0.02±0.01
6	0.03±0.03	0.09±0.05
7	0.06±0.06	0.35±0.15
8	0.08±0.06	0.43±0.13
9	0.04±0.02	1.00±0.40
10	0.05±0.04	0.17±0.05
11	0.03±0.03	0.02±0.01
12	0.03±0.03	0.01±0.01

SD = Standard Deviation(2 replicates)

Table 3.4:Elastase activity(μ/ml hr^{-1}) of *M.cookei* culture filtrates(from shake cultures in SDB & BSW) between pH 3-12.

pH	*M.cookei*	
	SDB (±SD)	BSW (±SD)
3	0.05±0.03	0.01±0.01
4	0.04±0.03	0.00±0.00
5	0.04±0.03	0.02±0.01
6	0.05±0.04	0.02±0.03
7	0.05±0.04	0.24±0.13
8	0.14±0.07	0.15±0.12
9	0.13±0.06	0.23±0.12
10	0.17±0.10	0.02±0.01
11	0.05±0.04	0.02±0.02
12	0.03±0.02	0.00±0.00

Fig 3.3:pH optima for elastase activity(μ/ml hr^{-1}) of *M.canis* culture filtrates.

Elastase activity was again higher in BSW filtrates (1.0 μ/ml hr^{-1}at pH 9), compared to SDB(0.08 μ/ml hr^{-1} at pH 8) .

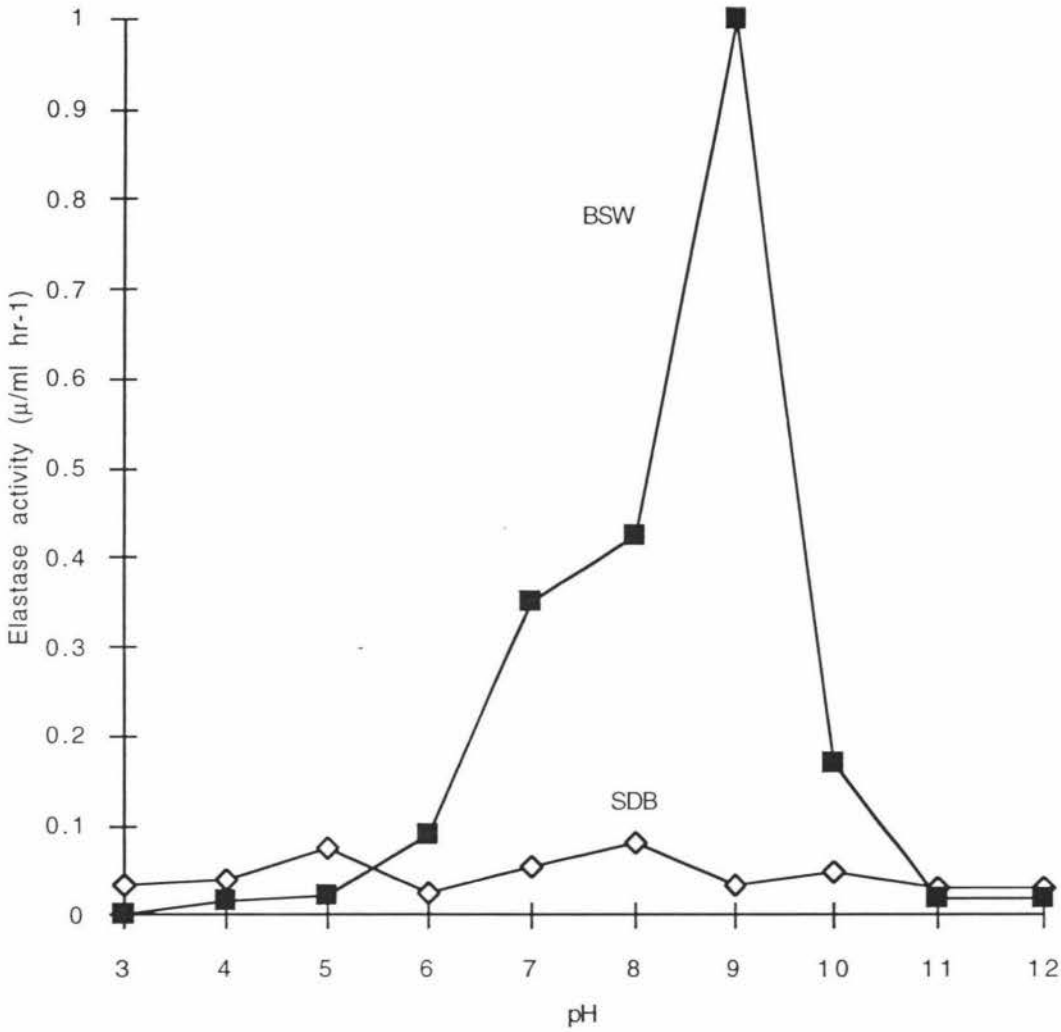
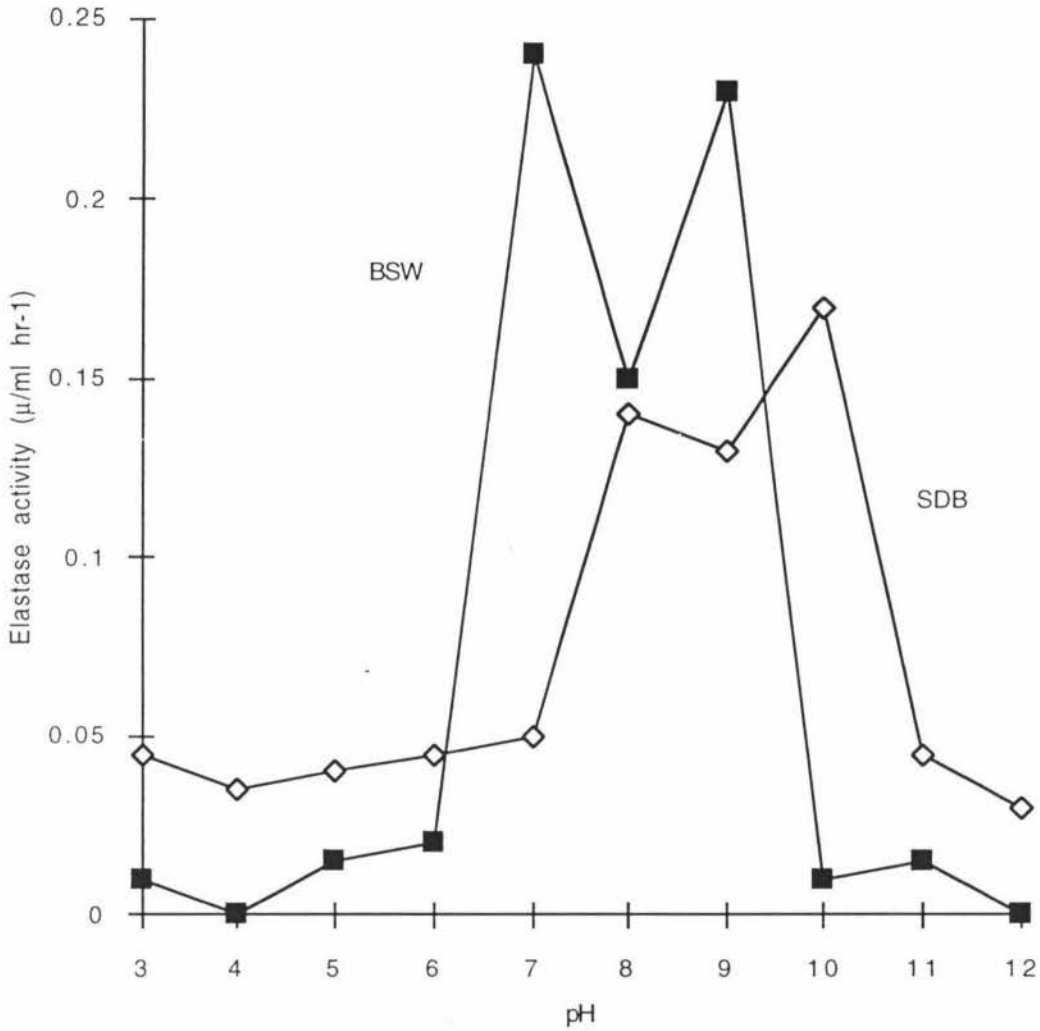

Elastase activity (μ/ml hr-1)
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
BSW
SDB
3
4
5
6
7
8
9
10
11
12
pH

Fig 3.4:pH optima for elastase activity (μ/ml hr^{-1}) of *M.cookei* culture filtrates.

The expression of elastase by *M.cookei* was greatest in BSW filtrates over the pH range 7-9. Peak activity in SDB filtrates appeared at pH 10.


0.25
0.2
0.15
0.1
0.05
0
Elastase activity (µ/ml hr-1)
BSW
SDB
3
4
5
6
7
8
9
10
11
12
pH

3.1.1.3 Keratinolytic Activity

The effect of pH on keratinase activity could only be assesed with filtrates of BSW cultures as when the species were cultured in SDB no recordable keratinase activity was noted. Each assay was repeated four times, as the release of dye from the keratin azure substrate proved to be inconsistent. Table 3.5 & Fig 3.5 present the results.

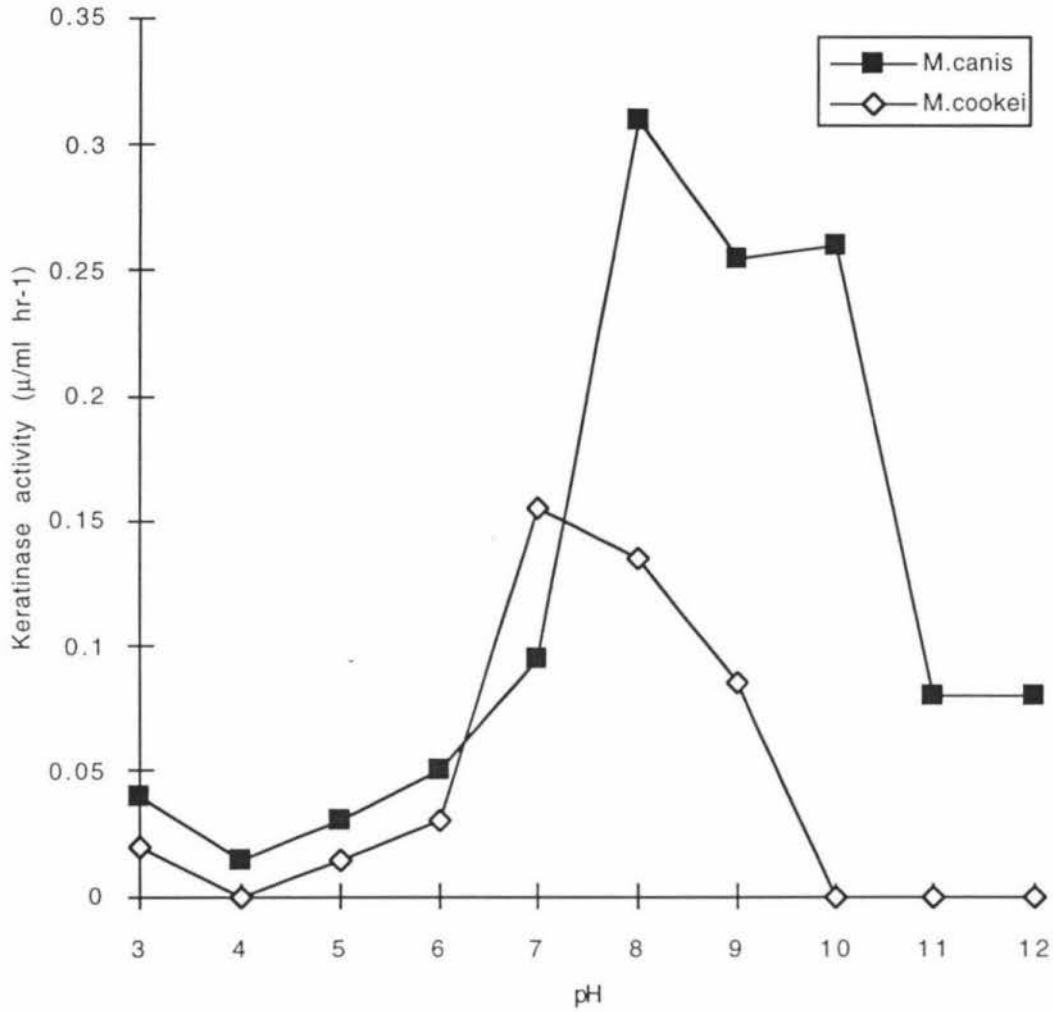
Table 3.5: Keratinase activity(μ/ml hr^{-1}) between pH 3-12 of *M.canis* & *M.cookei* cultures filtrates from BSW .

pH	*M.canis* (±SD)	*M.cookei* (±SD)
3	0.04±0.04	0.02±0.02
4	0.02±0.02	0.00±0.00
5	0.03±0.01	0.02±0.02
6	0.05±0.03	0.03±0.02
7	0.10±0.07	0.16±0.11
8	0.31±0.15	0.14±0.09
9	0.26±0.13	0.09±0.07
10	0.26±0.14	0.00±0.00
11	0.08±0.07	0.00±0.00
12	0.08±0.08	0.00±0.00

SD = Standard Deviation(4 replicates)

Fig 3.5:Keratinase activity(μ/ml hr^{-1}) of *M.canis* & *M.cookei* filtrates culture from shake cultures in BSW.

M.canis expressed the highest keratinase activity(0.31 μ/ml hr^{-1}at pH 8), but peaked over the pH range 8-10. Keratinase activity of *M.cookei* (0.16 μ/ml hr^{-1} pH 7)was about half that of *M.canis*, and peaked over the pH range 7-9.


0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Keratinase activity (µ/ml hr-1)
3
4
5
6
7
8
9
10
11
12
pH
M.canis
M.cookei

3.1.2 Effect of Temperature on Azocollytic Activity

The effect of temperature on azocollytic activity for both species using pH 8 buffer was analysed (Tables 3.6 & 3.7). The assays were incubated for only 6 hours at the appropriate temperature, for fire safety reasons due to water evaporation from the water baths at higher temperatures ie not 24 hours as for normal azocoll assays. Again for *M.canis* BSW filtrates showed markedly higher activity than SDB filtrates & for *M.cookei*, activity in the two filtrates was similar. The azocollytic activity of both species peaked between the temperatures 45°C & 50°C (Fig 3.6).

Table 3.6:Effect of temperature on azocollytic activity(μ/ml hr-1) of *M.canis* filtrates from shake cultures in SDB & BSW.

Temperature	*M.canis*	
	SDB (±SD)	BSW (±SD)
25°C	0.00±0.00	0.05±0.02
30°C	0.00±0.00	0.30±0.11
37°C	0.03±0.01	0.70±0.25
45°C	0.14±0.05	1.10±0.28
50°C	0.00±0.00	0.80±0.21
60°C	0.00±0.00	0.16±.088

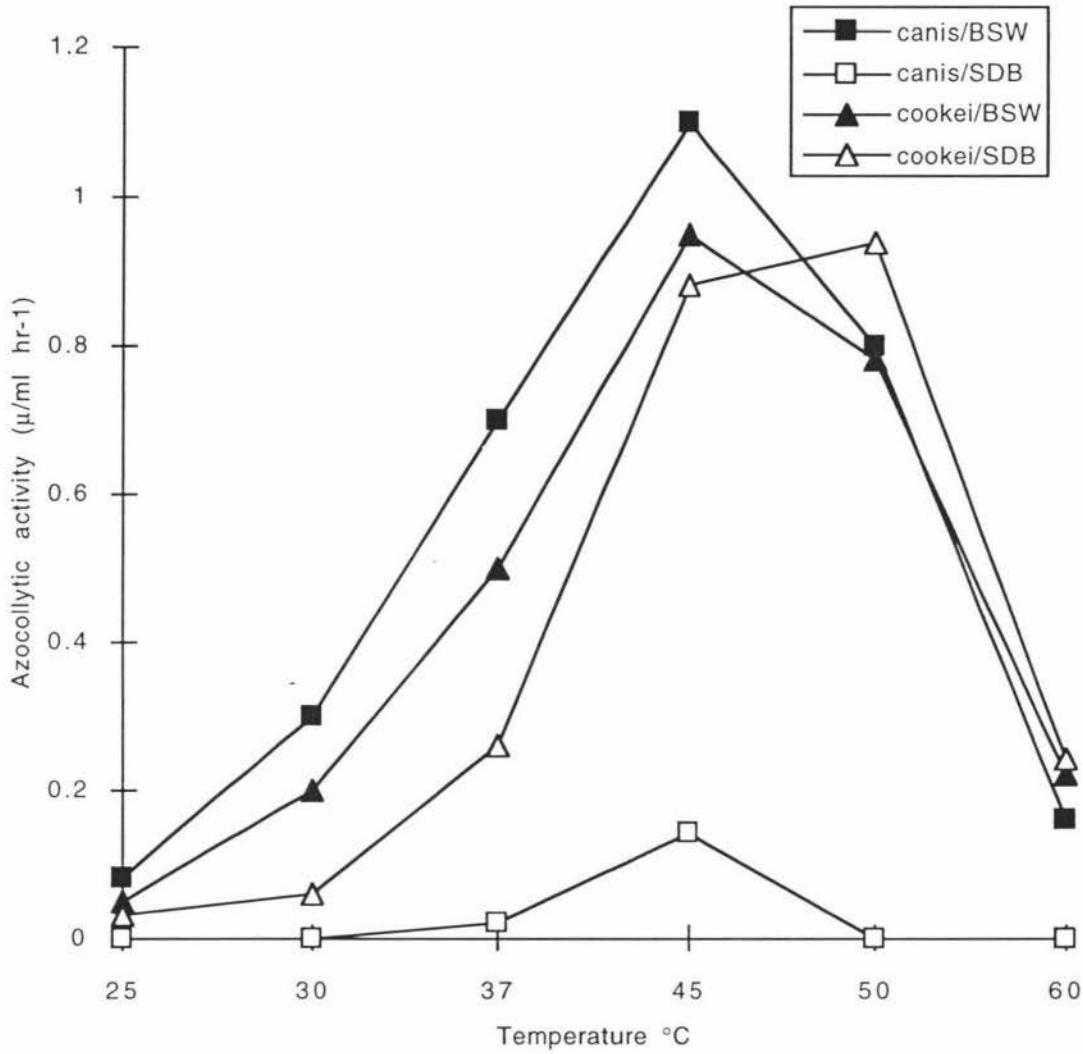
SD = Standard Deviation(2 replicates)

Table 3.7:Effect of temperature on azocollytic activity(μ/ml hr^{-1}) of *M.cookei* filtrates from shake cultures in SDB & BSW.

Temperature	*M.cookei*	
	SDB (±SD)	BSW (±SD)
25°C	0.03±0.01	0.05±0.02
30°C	0.06±0.01	0.20±0.06
37°C	0.26±0.02	0.50±0.04
45°C	0.88±0.08	0.95±0.07
50°C	0.94±0.16	0.78±0.08
60°C	0.24±0.08	0.22±0.08

Fig 3.6:Temperature optima for azocollytic activity(μ/ml hr^{-1}) for *M.canis* & *M.cookei* culture filtrates.

The optimal temperature for azocollytic activity for all culture filtrates was 45°C, although *M.cookei* filtrates from SDB extended this peak to 50°C.


canis/BSW
canis/SDB
cookei/BSW
cookei/SDB
Azocollytic activity (µ/ml hr-1)
1.2
1
0.8
0.6
0.4
0.2
0
25
30
37
45
50
60
Temperature °C

3.1.3 Characterisation of Azocollytic & Elastolytic Enzymes

Filtrates from *M.canis* & *M.cookei* cultures in both SDB & BSW had azocollytic & elastase activity. To characterise these enzymes, the proteinase inhibitors listed in Table 2.1(PMSF, EDTA, p-CMB & PEP) were used to determine the types of enzymes involved. One strain from each species was grown in the appropriate medium(SDB or BSW) in shake culture at 30°C for two weeks. Each assay was repeated a minimum of two times.

3.1.3.1 Effects of Proteinase Inhibitors on Azocollytic activity of *M.canis* & *M.cookei.*

The results of these assays are shown in Figs 3.7 & 3.8.

PMSF

The serine proteinase inhibitor PMSF showed significant inhibition of both *M.canis* & *M.cookei* filtrates from SDB cultures(81% & 59% respectively). In BSW filtrates the percentage of inhibition by PMSF was reduced (29% & 24% respectively).

EDTA

The metalloproteinase inhibitor EDTA showed little difference in the inhibition of *M.cookei* azocollytic activity following growth in BSW & SDB (89% & 85% respectively). The effect of EDTA on *M.canis* cultures was greater, in BSW there was 62% inhibition of azocollytic activity, but in SDB 40% inhibition by EDTA.

PEP

The aspartic proteinase inhibitor Pepstatin A, produced a small amount of inhibition against azocollytic activity of *M.canis* & *M.cookei* in SDB cultures(12% & 6% respectively). When *M.canis* & *M.cookei* were grown in BSW no inhibition was noted.

p-CMB

The cysteine proteinase inhibitor produced similar inhibition (≈40%) of azocollytic activity for both species cultured in both substrates.

Fig 3.7: Inhibition of azocollytic activity in *M.canis* & *M.cookei* culture filtrates from SDB.

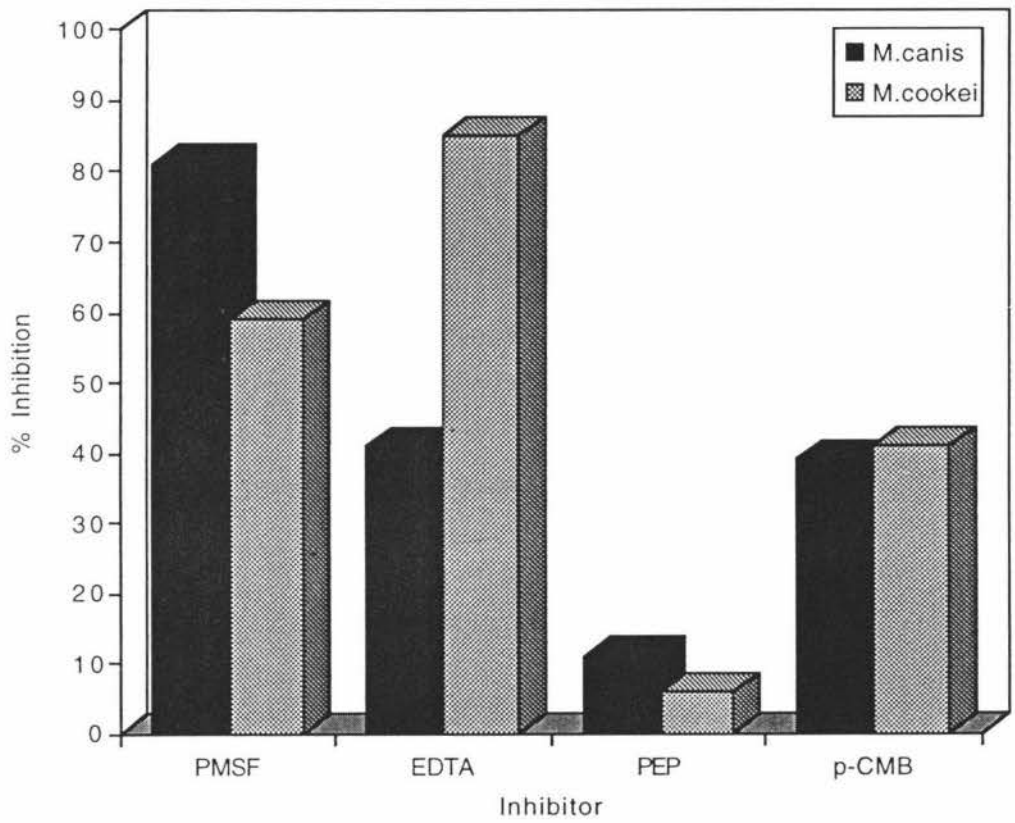
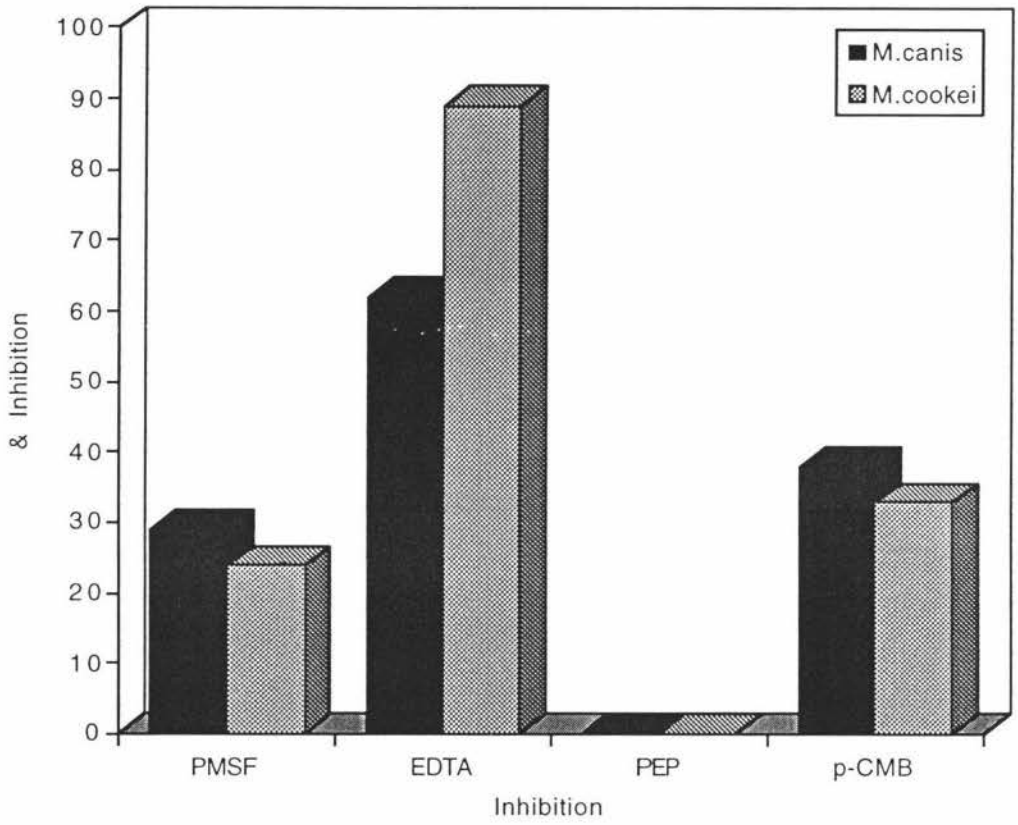


Fig 3.8:Inhibition of azocollytic activity in *M.canis* & *M.cookei* culture filtrates from BSW.



3.1.3.2 Effects of Proteinase Inhibitors on Elastase activity of *M.canis* & *M.cookei*

The results of these are shown in Figs 3.9 & 3.10.

PMSF

PMSF produced high inhibition of elastase activity for both *M.canis* & *M.cookei* filtrates from SDB(100% & 85% respectively). Similarly high inhibition was produced with BSW culture filtrates(76% & 71% respectively).

EDTA

EDTA caused high rates of inhibition for *M.cookei* filtrates from both media(75% for SDB & 84% for BSW) For *M.canis*, EDTA caused 59% inhibition of activity from filtrates of BSW cultures & 46% inhibition of elastase activity from the filtrates of SDB cultures.

PEP

PEP caused inhibition of between 40-70% of elastase activity of the two species in both SDB & BSW filtrates.

p-CMB

p-CMB had little effect on the elastase activity of *M.canis* filtrates from both substrates but greater inhibition with *M.cookei* filtrates(28% BSW & 16% SDB).

Fig 3.9: Inhibition of elastase activity in *M.canis* & *M.cookei* culture filtrates from SDB.

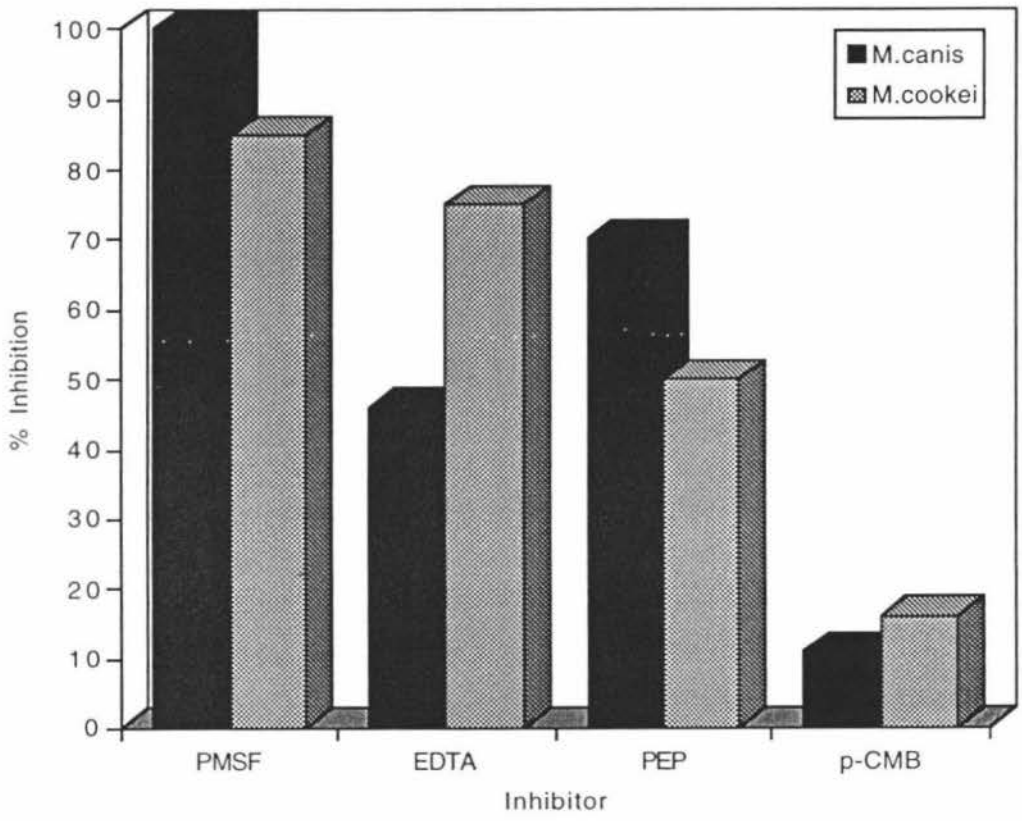
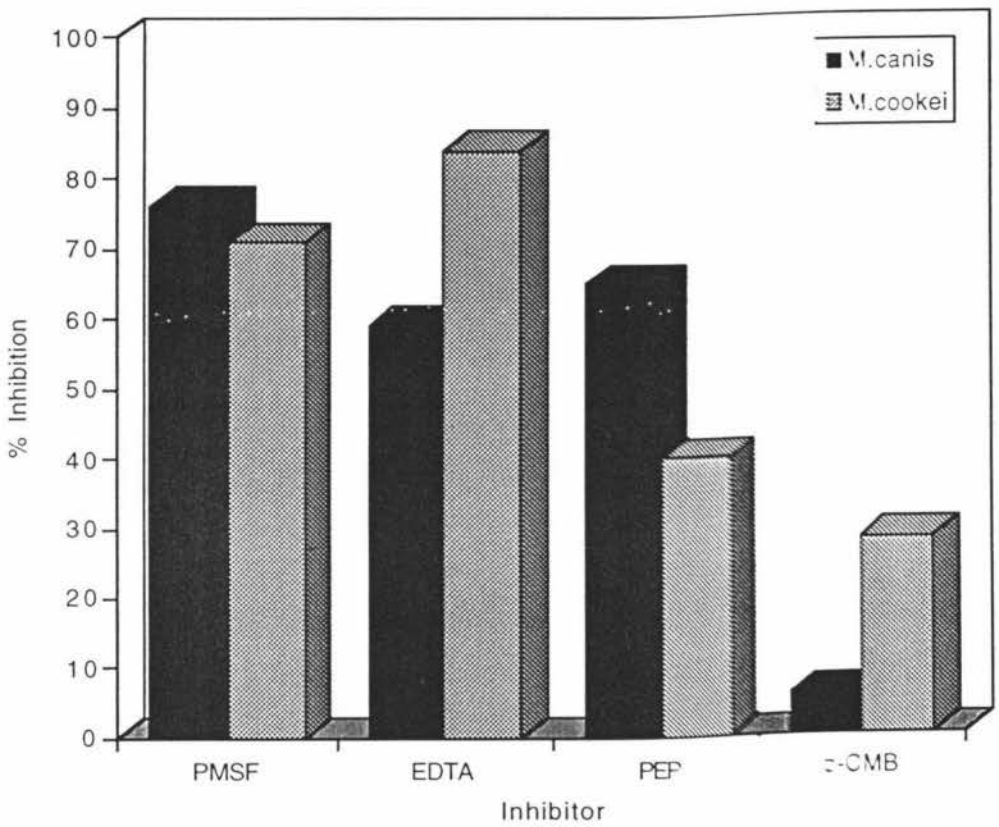


Fig 3.10: Inhibition of elastase activity in *M.canis* & *M.cookei* culture filtrates from BSW.



3.2 ANALYSIS OF PROTEINASES BY SUBSTRATE SDS-PAGE CO-POLYMERISED GELS.

Culture filtrates were prepared as described in Section 2.1. & the proteinases(as gelatinases) present were examined by the Substrate SDS-PAGE technique, which allowed a more detailed investigation of the various components of the proteinase mix. A sample of each filtrate to be tested was added to each well in the gel & each gel was run in duplicate. Since the proteinases were separated under SDS-denaturing conditions, the molecular weight (Mr) of each proteinase could be estimated from their positions relative to standards of known Mr. The molecular weight markers used are listed in Section 2.5.

3.2.1 Effect of Growth Medium on Gelatinase Expression

In this first series of experiments, aimed to provide information on the number of bands & the reproducbility of the technique four strains of each species were grown in both BSW & SDB in shake culture at 25°C for 14 days.

3.2.1.1 *M.canis*

Table 3.9 & Fig 3.11 show the relative banding patterns of gelatinases obtained with filtrates of the four *M.canis* strains. From BSW filtrates, up to six bands ranging from 13,000 Da Mr to 64,500 Da were detected. From SDB filtrates a total of six bands ranging from 16,000 Da to 85,000 Da were obtained.

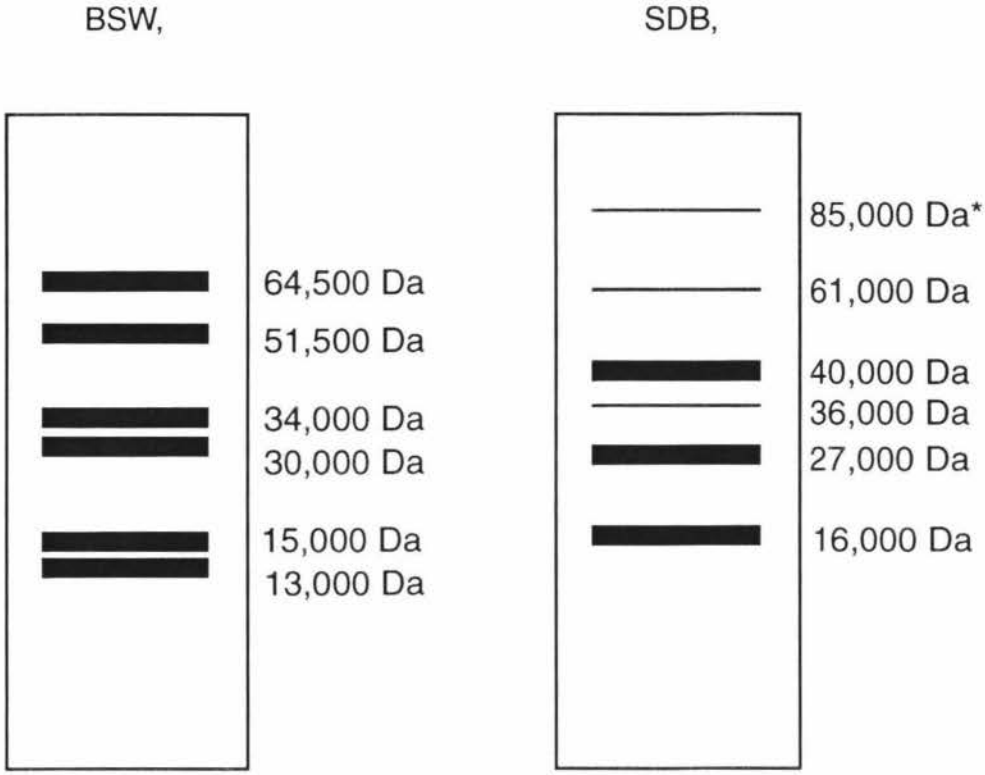
Table 3.8:Gelatinase bands obtained with filtrates of *M.canis* cultured in BSW & in SDB (Mr in Da).

BSW	No. of strains showing band	SDB	No. of strains showing band
64,000	4	85,000	3
51,500	4	61,000	1
34,000	4	40,000	4
30,000	4	36,000	2
15,000	4	27,000	1
13,000	4	16,000	1

In BSW culture filtrates two lower molecular weight proteinases (15,000 Da & 13,000 Da) were present whereas in SDB cultures there was only one at 16,000 Da. In repeat runs the 85,000 Da, 61,000 Da & 36,000 Da gelatinase bands detected in SDB filtrates were variable in

their detection & could not be consistently reproduced from culture to culture of the same strain. They are shown as thin lines in Figs 3.11.

Fig 3.11:Relative banding patterns of gelatinases produced by *M.canis* cultured in BSW & SDB



* Thin lines indicate inconsistently demonstrated bands.

Plate 3.1a:Relative Banding Patterns by *M.canis* cultured in BSW
Plate 3.1b:Relative Banding Patterns by *M.canis* cultured in SDB

Plate 3.1a

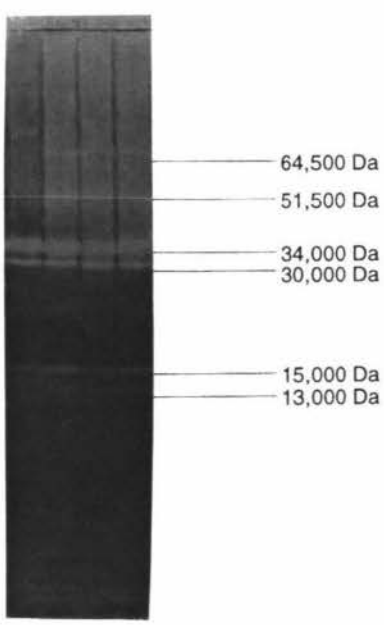
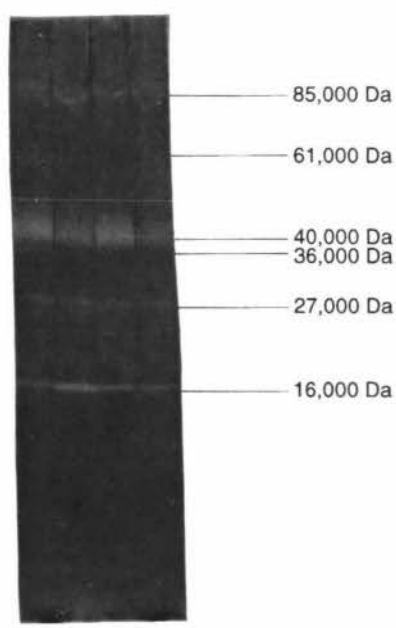


Plate 3.1b



3.2.1.2 *M.cookei*

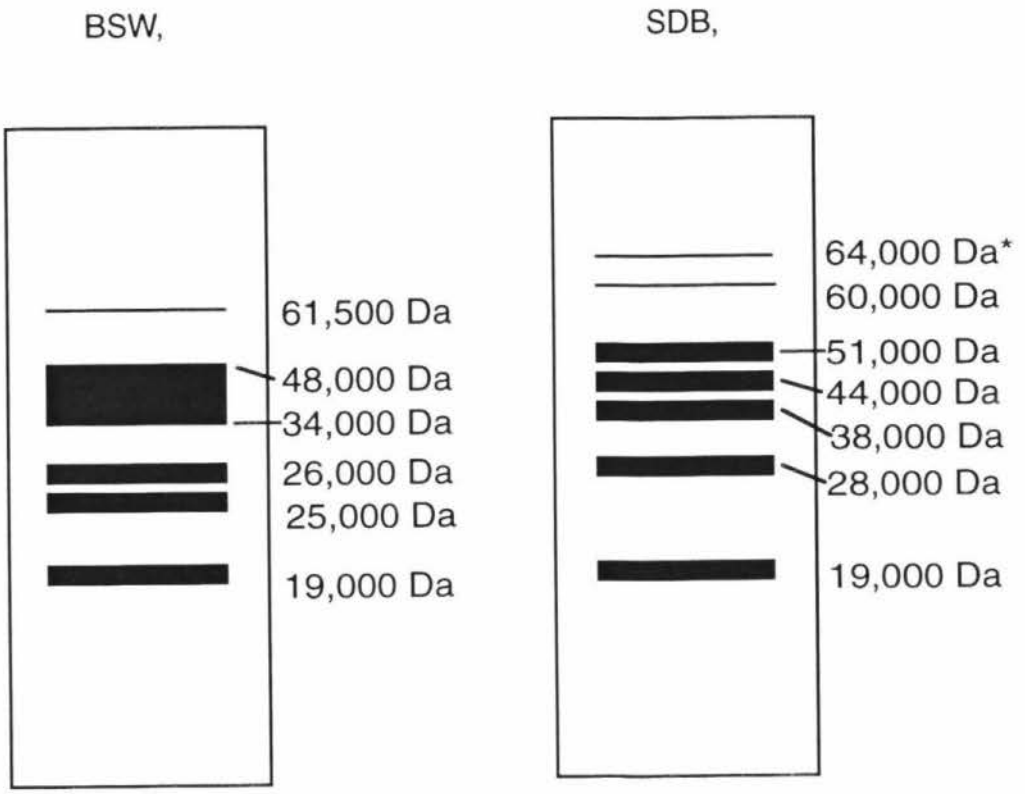
Table 3.10 & Fig 3.12 show the relative banding patterns of gelatinases obtained with filtrates of the four *M.cookei* strains. From BSW filtrates up to five bands ranging in size from 19,000 Da to 61,500 Da were detected & from SDB filtrates up to seven bands were observed, ranging from 19,000 Da to 64,000 Da in size.

Table 3.9:Gelatinase bands obtained with filtrates of *M.cookei* cultured in BSW & SDB(Mr in Da).

BSW	No. of strains showing band	SDB	No. of strains showing band
61,500	4	64,000	1
		60,000	3
34,000-48,000	4	51,000	4
		44,000	4
		38,000	4
26,000	4	28,000	3
25,000	4		
19,000	4	19,000	1

In BSW culture filtrates the 34,000-48,000 Da band was largely smeared & no discrete proteinase bands could be seen within the smear, but at a similar molecular weight up to 3 proteinase bands(51,000, 44,000, 38,000 Da) could be detected in SDB culture filtrates(Fig 3.12). In BSW cultures two bands (25,000 & 26,000 Da) were expressed distinctly but in SDB cultures only one band of a similar size(28,000 Da) was detected. As with *M.canis*, some bands were variable in their appearance from run to run. *M.cookei* grown in SDB expressed two high molecular weight proteinases (60,000 & 64,000 Da) of this type which could not be consistantly demonstrated from culture to culture, as was the 61,500 Da proteinase expressed in BSW.

Fig 3.12:Relative banding patterns of gelatinases produced by *M.cookei* cultured in BSW & SDB.



*Thin lines indicate inconsistently demonstrated bands

Plate 3.2a:Relative Banding Patterns by *M.cookei* cultured in BSW
Plate 3.2b:Relative Banding Patterns by *M.cookei* cultured in SDB

Plate 3.2a

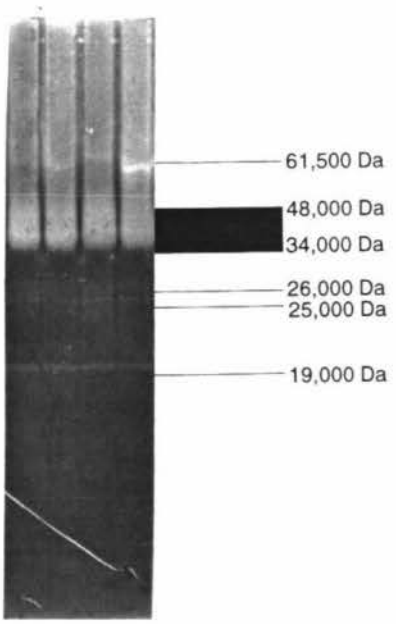
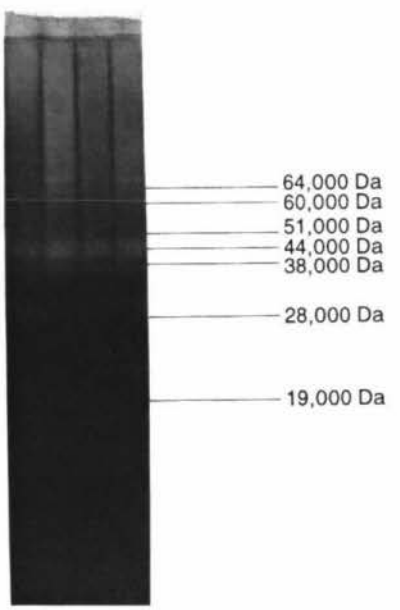


Plate 3.1b



3.2.2 Effect of Time, Culture Method & Temperature on Gelatinase Production.

Figs 3.13 to 3.24 show the banding patterns of gelatinases obtained with filtrates of *M.canis* & *M.cookei* derived from cultures set up (a) at three different temperatures(25°C, 30°C & 33°C), (b) in the shake & stationary modes of culture & (c) in the two media SDB & BSW. Samples were taken weekly for four weeks to determine if time had any effect on the expression of gelatinases. Four different strains from each species were used & the whole experiment was repeated twice. Figures 3.13 to 3.24 reflect the overall appearance of bands which are recorded as -(not detected) or +, with the number before the + referring to the number of strains expressing the band in question.

3.2.2.1 SDB Cultures

Effect of Temperature

The effect of temperature was a reflection of the amount of growth of the fungus. No additional gelatinase bands in either species were observed at higher temperatures. The maximum number of bands detected in *M.canis* filtrates was demonstrated at 25°C & higher temperatures showed a marked reduction in band appearance over the four strains, especially at 33°C, & particularly for the "inconsistent" bands. Good banding was also obtained with *M.cookei* at 25°C but this species did not grow well at 30°C & 33°C & this was reflected in the overall number of bands present on the gels. Of the four *M.cookei* strains one grew particularly poorly at 33°C & no bands were demonstrated with this strain at this temperature. Again, the variable 64,000 Da & 60,000 Da bands were not detected at 33°C & the 19,000 Da was also absent at 33°C. (Figs 3.13 , 3.14 , 3.15 , 3.16 , 3.17 & 3.18).

Effect of Time

The 85,000 Da, 40,000 Da & 36,000 Da gelatinase bands expressed by *M.canis* grown on SDB were the first to be detected after only 7 days of growth & tended to persist for all 28 days (Fig 3.13). The 27,000 Da

& 16,000 Da gelatinase bands from these filtrates began to appear after 14 days & persisted throughout the age of the culture for some strains (Fig 3.13-3.15) but the 61,000 Da band was poorly expressed. *M.cookei* grown on SDB gave a similar picture as *M.canis* on SDB with the 51,000 Da & 44,000 Da gelatinases of *M.cookei* being expressed first (after 7 days at 25°C & 30°C) & persisting throughout the age of the culture. The 28,000 Da & 19,000 Da gelatinase bands first appeared after 14 days of growth & when expressed, persisted throughout the age of the culture (Fig 3.16)

Effect of Shake & Stationary Culture Mode

Differences were noted between shake & stationary cultures of *M.canis* grown in SDB. The 85,000 Da, 27,000 Da & 16,000 Da gelatinase bands appeared within 14 days of growth in shake cultures but in stationary cultures the above gelatinase bands usually did not appear, if at all, until 28 days(Figs 3.13, 3.14 & 3.15).*M.cookei* grown on SDB also demonstrated differences between shake & stationary cultures. The 44,000 Da 28,000 Da & 19,000 Da bands were expressed in shake cultures before, or were more frequently expressed, than in stationary cultures.

3.2.2.2 BSW Cultures

Effect of Temperature

There was strong production of gelatinases in BSW & temperature had a less marked effect. Again no new gelatin degrading bands were demonstrated at any of the three temperatures. The poor growth of *M.cookei* at 33°C was again reflected in the poor detection of bands (Figs 3.22, 3.23 & 3.24). Temperature also had no effect on *M.canis*, with no visible temperature dependent gelatinases being expressed at 25°C, 30°C or 33°C .*M.canis* grew well at 25°C & 30°C but at 33°C growth was relatively slow compared to 25°C but faster than *M.cookei* under the same temperature & growth conditions (Figs 3.19, 3.20 & 3.21).

Effect of Time

M.canis & *M.cookei* grown on BSW cultures expressed all gelatin degrading enzymes from the first week of growth & no new gelatinases were expressed as the cultures grew older & none of the gelatinases expressed at the first week of growth disappeared as the culture aged (Figs 3.19 & 3.24). Even at 33°C, all bands were detected with the majority of strains.

Effect of Shake & Stationary Culture Mode

Differences between shake & stationary cultures when *M.canis* was grown in BSW medium was less apparent than in SDB, although most bands were more strongly expressed in shake culture than in stationary culture.*M.cookei* also gave no clear differences of expression of gelatinase bands between shake & stationary cultures following growth in BSW.

Fig 3.13: Gelatinases expressed by *M.canis* cultured in SDB at 25°C over four weeks in shake & stationary modes of culture.

	W1		W2		W3		W4	
	Sh	St	Sh	St	Sh	St	Sh	St
85,000 Da	3+	-	3+	-	2+	-	2+	3+
61,000 Da	1+	-	1+	1+	1+	-	2+	1+
40,000 Da	4+	3+	4+	4+	4+	4+	4+	4+
36,000 Da	2+	2+	2+	2+	-	2+	-	3+
27,000 Da	-	-	1+	-	2+	-	3+	2+
16,000 Da	-	-	1+	-	1+	-	2+	-
Total no. of bands	4	2	6	3	5	2	5	5

Symbols used in Figs 3.13-3.24

+ = good expression - = no expression, Number before + refers to the number of strains out of 4 expressing that particular band.
Sh = shake mode of culture St = stationary mode of culture
W1 = week 1, W2 = week 2 , W3 = week 3 , W4 = week 4

Fig 3.14:Gelatinases expressed by *M.canis* cultured in SDB at 30°C over four weeks in shake & stationary mode of culture.

	W1		W2		W3		W4	
	Sh	St	Sh	St	Sh	St	Sh	St
85,000 Da	2+	-	2+	-	2+	-	4+	3+
61,000 Da	-	-	-	-	-	-	1+	1+
40,000 Da	4+	3+	4+	4+	4+	4+	4+	4+
36,000 Da	4+	-	1+	-	1+	1+	1+	1+
27,000 Da	-	-	-	-	3+	-	4+	-
16,000 Da	-	-	1+	-	3+	-	4+	-
Total no. of bands	3	1	4	1	5	2	6	5

Fig 3.15:Gelatinases expressed by *M.canis* cultured in SDB at 33°C over four weeks in skake & stationary mode of culture.

	W1		W2		W3		W4	
	Sh	St	Sh	St	Sh	St	Sh	St
85,000 Da	-	-	3+	-	2+	-	2+	-
61,000 Da	-	-	-	-	-	-	-	-
40,000 Da	3+	3+	4+	4+	4+	4+	4+	4+
36,000 Da	-	-	1+	-	1+	-	1+	-
27,000 Da	-	-	1+	-	4+	-	2+	2+
16,000 Da	-	-	2+	1+	4+	2+	4+	1+
Total no. of bands	1	1	5	2	5	2	5	3

Fig 3.16:Gelatinases expressed by *M.cookei* cultured in SDB at 25°C over four weeks in shake & stationary mode of culture.

	W1		W2		W3		W4	
	Sh	St	Sh	St	Sh	St	Sh	St
64,000 Da	-	-	-	-	3+	-	3+	-
60,000 Da	-	-	3+	1+	3+	3+	3+	3+
51,000 Da	4+	2+	4+	3+	4+	4+	3+	4+
44,000 Da	4+	2+	4+	3+	4+	4+	3+	4+
38,000 Da	-	-	4+	3+	4+	4+	2+	4+
28,000 Da	-	-	3+	-	2+	-	2+	3+
19,000 Da	-	-	1+	-	3+	-	3+	1+
Total no. of bands	2	2	6	4	7	4	7	6

Fig 3.17:Gelatinases expressed by *M.cookei* cultured in SDB at 30°C over four weeks in shake & stationary mode of culture.

	W1		W2		W3		W4	
	Sh	St	Sh	St	Sh	St	Sh	St
64,000 Da	-	-	-	-	-	-	-	-
60,000 Da	-	-	3+	3+	-	-	-	-
51,000 Da	3+	-	3+	3+	4+	4+	4+	4+
44,000 Da	3+	-	3+	-	4+	4+	4+	4+
38,000 Da	-	-	-	-	4+	4+	4+	4+
28,000 Da	1+	-	-	-	3+	-	3+	2+
19,000 Da	-	-	1+	-	2+	-	4+	-
Total no. of bands	3	0	4	2	5	3	5	4

Fig 3.18:Gelatinases expressed by *M.cookei* cultured in SDB at 33°C over four weeks in shake & stationary mode of culture.

	W1		W2		W3		W4	
	Sh	St	Sh	St	Sh	St	Sh	St
64,000 Da	-	-	-	-	-	-	-	-
60,000 Da	-	-	-	-	-	-	-	-
51,000 Da	-	-	3+	1+	3+	3+	3+	3+
44,000 Da	-	-	3+	-	3+	-	3+	3+
38,000 Da	-	-	3+	-	3+	-	3+	-
28,000 Da	-	-	3+	-	3+	-	3+	-
19,000 Da	-	-	-	-	-	-	-	-
Total no. of bands	0	0	4	1	4	1	4	2

Fig 3.19:Gelatinases expressed by *M.canis* cultured in BSW at 25°C over four weeks in shake & stationary mode of culture.

	W1		W2		W3		W4	
	Sh	St	Sh	St	Sh	St	Sh	St
64,500 Da	4+	4+	4+	4+	2+	2+	2+	2+
51,500 Da	4+	4+	4+	4+	2+	3+	3+	4+
34,000 Da	4+	4+	4+	4+	4+	4+	4+	4+
30,000 Da	4+	4+	4+	4+	4+	4+	4+	4+
15,000 Da	4+	4+	4+	4+	4+	4+	4+	4+
13,000 Da	4+	2+	4+	2+	4+	3+	4+	4+
Total no. of bands	6	6	6	6	6	6	6	6

Fig 3.20:Gelatinases expressed by *M.canis* cultured in BSW at 30°C over four weeks in shake & stationary mode of culture.

	W1		W2		W3		W4	
	Sh	St	Sh	St	Sh	St	Sh	St
64,500 Da	4+	4+	3+	4+	-	4+	4+	2+
51,500 Da	4+	4+	3+	4+	2+	4+	3+	4+
34,000 Da	4+	4+	4+	4+	4+	4+	4+	4+
30,000 Da	4+	4+	4+	4+	4+	4+	3+	4+
15,000 Da	4+	4+	4+	4+	4+	4+	4+	4+
13,000 Da	3+	2+	3+	4+	3+	2+	4+	4+
Total no. of bands	6	6	6	6	6	6	6	6

Fig 3.21:Gelatinases expressed by *M.canis* cultured in BSW at 33°C over four weeks in shake & stationary mode of culture.

	W1		W2		W3		W4	
	Sh	St	Sh	St	Sh	St	Sh	St
64,500 Da	4+	4+	4+	2+	2+	2+	3+	2+
51,500Da	4+	4+	4+	1+	-	2+	3+	2+
34,000 Da	4+	2+	4+	2+	3+	3+	4+	3+
30,000 Da	4+	2+	4+	2+	3+	2+	4+	2+
15,000 Da	4+	4+	4+	4+	4+	4+	4+	4+
13,000 Da	-	-	-	-	2+	2+	3+	2+
Total no. of bands	5	5	5	5	5	6	6	6

Fig 3.22:Gelatinases expressed by *M.cookei* cultured in BSW at 25°C over four weeks in shake & stationary mode of culture.

	W1		W2		W3		W4	
	Sh	St	Sh	St	Sh	St	Sh	St
61,500 Da	2+	3+	4+	4+	2+	3+	2+	3+
34,000 Da	3+	3+	4+	4+	4+	4+	4+	4+
26,000 Da	3+	3+	4+	4+	4+	4+	4+	4+
25,000 Da	3+	3+	4+	4+	4+	4+	4+	4+
19,000 Da	3+	3+	4+	4+	4+	4+	4+	4+
Total no. of bands	5	5	5	5	5	5	5	5

Fig 3.23:Gelatinases expressed by *M.cookei* cultured in BSW at 30°C over four weeks in shake & stationary mode of culture.

	W1		W2		W3		W4	
	Sh	St	Sh	St	Sh	St	Sh	St
61,500 Da	3+	3+	3+	3+	3+	2+	-	3+
34,000 Da	3+	3+	3+	3+	3+	3+	3+	3+
26,000 Da	3+	3+	3+	3+	3+	3+	3+	3+
25,000 Da	3+	3+	3+	3+	3+	3+	3+	3+
19,000 Da	3+	2+	3+	3+	3+	3+	3+	3+
Total no. of bands	5	5	5	5	5	5	4	5

Fig 3.24:Gelatinases expressed by *M.cookei* cultured in BSW at 33°C over four weeks in shake & stationary mode of culture.

	W1		W2		W3		W4	
	Sh	St	Sh	St	Sh	St	Sh	St
61,500 Da	2+	-	2+	2+	2+	2+	2+	3+
34,000KDa	2+	-	2+	-	2+	2+	3+	3+
26,000KDa	-	-	-	-	2+	2+	3+	3+
25,000 Da	-	-	-	-	2+	2+	3+	3+
19,000 Da	-	-	-	-	2+	-	2+	3+
Total no. of bands	2	0	2	1	5	3	5	5

3.2.3 Comparison of *M.canis* & *M.cookei*

For the two species the detected proteinase bands for comparative purposes could be arbitrarily separated into a number of molecular weight "groups"numbered 1-6 (see in Figs 3.11 & 3.12), to allow for possible experimential variation in the running of the gels & the calculation of Mrs. Within each group, proteinases from the two species had somewhat similar molecular weights, suggesting the possibility of genus specific proteinases having been expressed. The validity of this grouping is investigated in the next section.

Table 3.10:Comparison of gelatinase groups produced by *M.canis* & *M.cookei* (Mr in Da).

	M.canis		*M.cookei*	
	SDB	BSW	SDB	BSW
Group 1	85,000*	-	-	-
Group 2	61,000*	64,500	64,000* 60,000*	61,500*
Group 3	-	51,000	51,000	-
Group 4	40,000 36,000*	34,000 30,000	44,000 38,000	34,000-48,000†
Group 5	27,000	-	28,000	26,000 25,000
Group 6	16,000	15,000 13,000	19,000	19,000

* inconsistently detected

†smear

In BSW the large smear produced by *M.cookei* (34,000-48,000 Da), made it difficult to estimate if one or more proteinases were present. *M.cookei* expressed proteinases of molecular weight 26,000 Da & 25,000 Da but *M.canis* produced no equivalent bands in this range in this medium.

3.2.4 Characterisation of *M.canis* & *M.cookei* Gelatinases using Substrate SDS-PAGE with Proteinase Inhibitors.

Samples of culture filtrate were prepared from four strains of *M.canis* & four strains of *M.cookei* grown in shake culture in both media, following incubation at 25°C for 14 days. A number of proteinase inibitors were added to the samples in an attempt to characterise the gelatinase bands expressed by both species(see Section 2.2).

3.2.4.1 Effect of inhibitors on *M.canis* gelatinases from SDB & BSW cultures.

PMSF

The serine proteinase inhibitor PMSF added to both sample & incubation buffers at 2mM concentration caused complete inhibition of the 40,000 Da group 3 & 16,000 Da group 5 gelatinases from SDB culture filtrates & also the 15,000 Da & 13,000 Da group 4 bands from BSW culture filtrates (Plates 3.3a, 3.3b , 3.4a & 3.4b).

EDTA

The metalloproteinase inhibitor EDTA was added to both sample & incubation buffers at 5mM concentration & caused partial inhibition of the 40,000 Da group 2 gelatinase band from SDB culture filtrate(plates 3.5a & 3.5b). From BSW cultures EDTA caused partial inhibition of the 64,500 Da group 1 & 34,000 Da plus 30,000 Da group 2 gelatinase bands (Plates 3.6a & 3.6b).

PEP

The aspartic proteinase inhibitor pepstatin A , added to both sample & incubation buffers caused no visible inhibition of gelatinases expressed by *M.canis* cultured on SDB or BSW

p-CMB

The cysteine proteinase inhibitor p-CMB caused partial inhibition of the 40,000 Da group 3 gelatinase expressed by *M.canis* cultured in SDB (Plates 3.7a & 3.7b). From BSW cultures p-CMB caused partial inhibition of the 64,500 Da group 1 gelatinase (Plates 3.8a & 3.8b).

Plate 3.3a:Effect of PMSF on gelatinases of *M.canis* cultured in SDB.

Plate 3.3b:Control with no PMSF added

Plate 3.4a:Effect of PMSF on gelatinases of *M.canis* cultured in BSW.

Plate 3.4b:Control with no PMSF added.

Plate 3.3a

Plate 3.3b

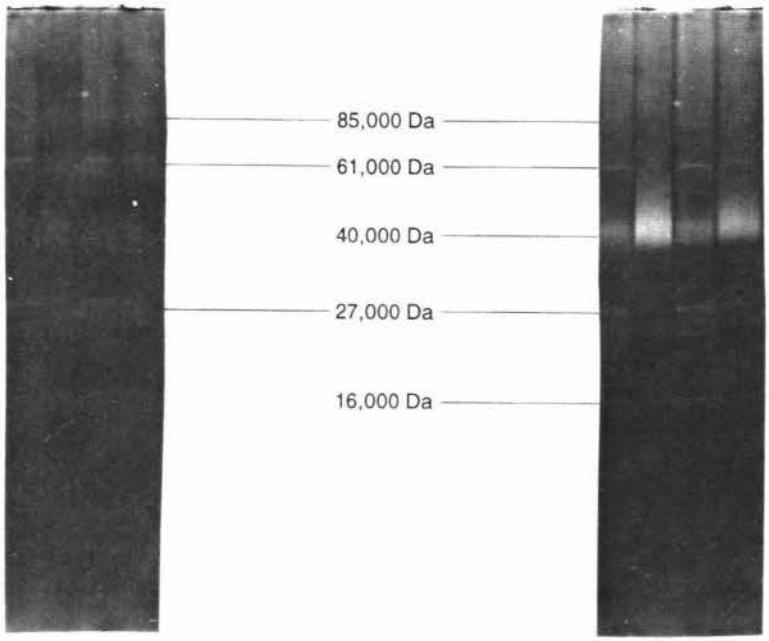


Plate 3.4a

Plate 3.4b

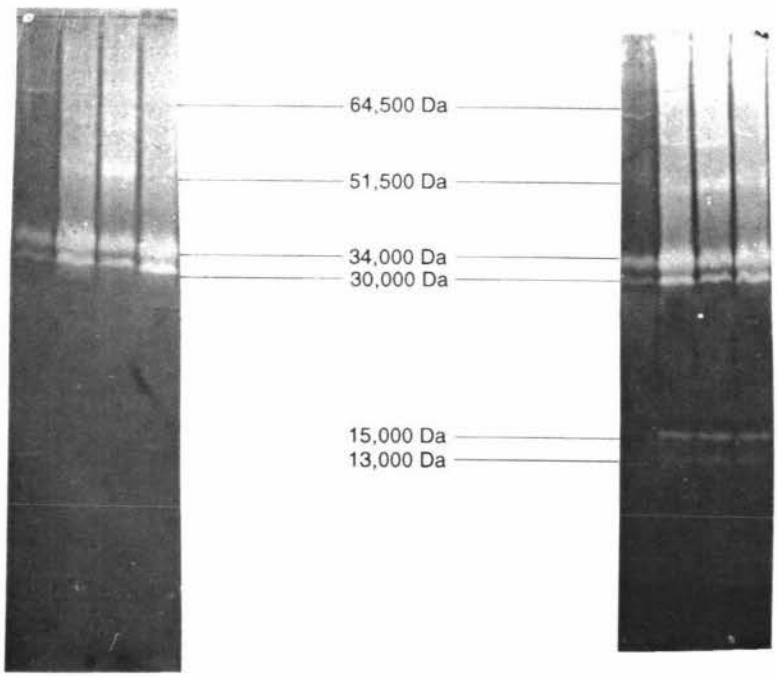


Plate 3.5a:Effect of EDTA on gelatinases of *M.canis* cultured in SDB.

Plate 3.5b:Control with no EDTA added.

Plate 3.6a:Effect of EDTA on gelatinases of *M.canis* cultured in BSW

Plate 3.6b:Control with no EDTA added.

Plate 3.5a

Plate 3.5b

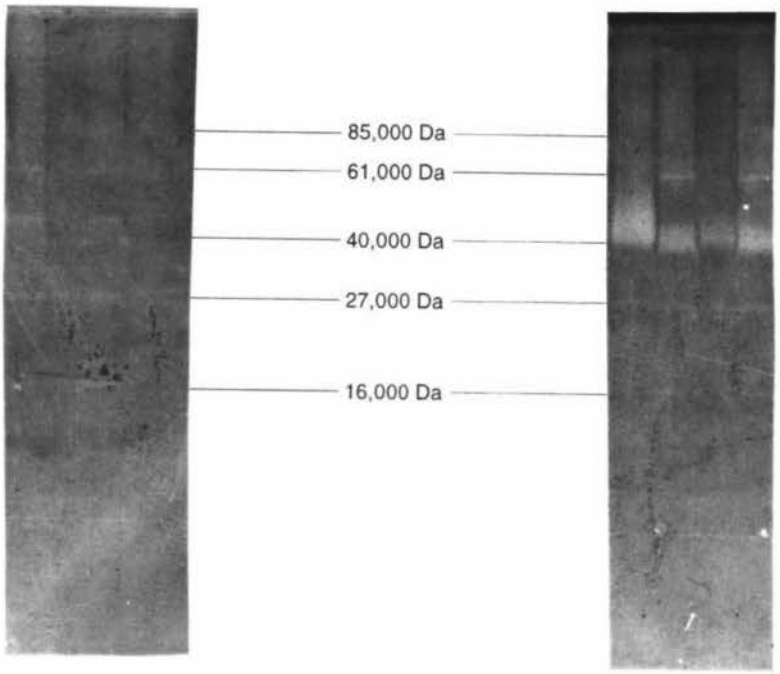


Plate 3.6a

Plate 3.6b

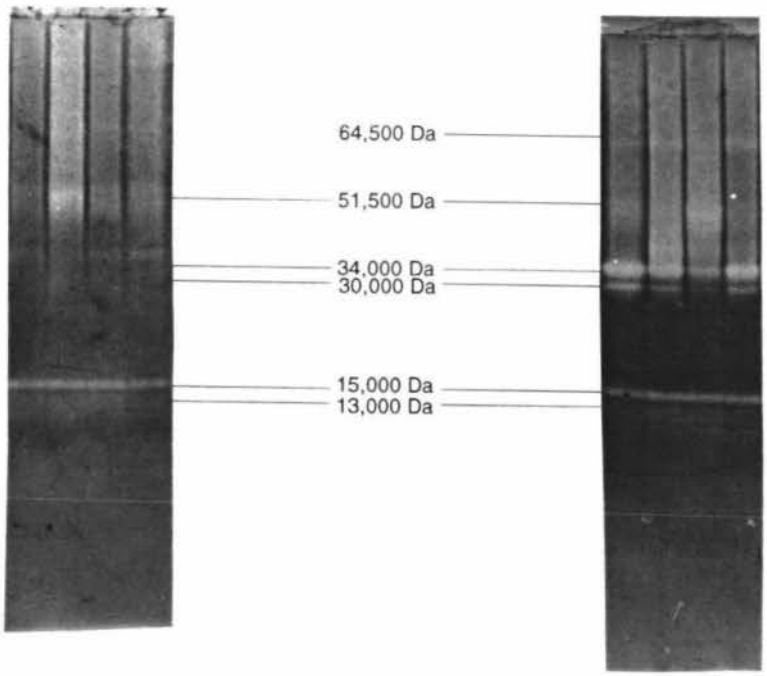


Plate 3.7a:Effect of p-CMB on gelatinases of *M.canis* cultured in SDB

Plate 3.7b:Control with no p-CMB added

Plate 3.8a:Effect of p-CMB on gelatinases of *M.canis* cultured in BSW

Plate 3.8b:Control with no p-CMB added

Plate 3.7a

Plate 3.7b

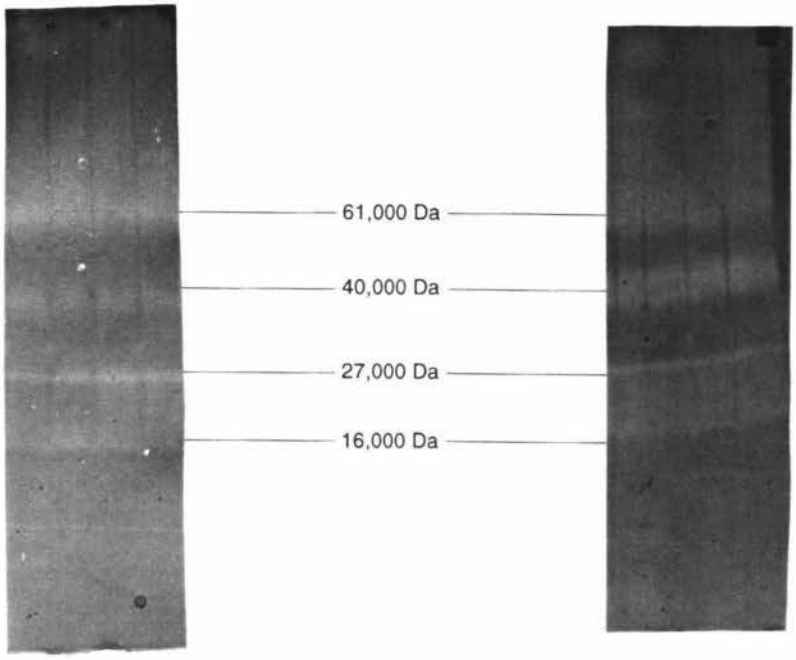
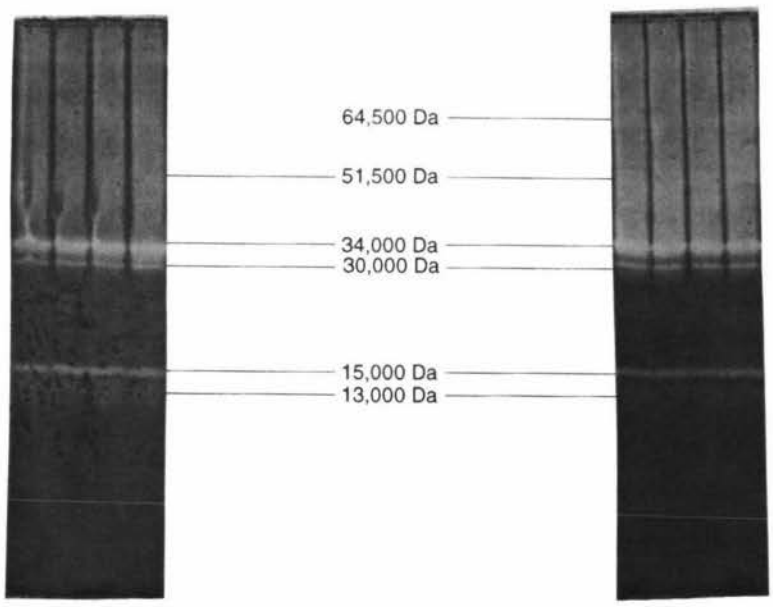


Plate 3.8a

Plate 3.8b



3.2.3.2 Effect of Inhibitors on *M.cookei* gelatinases from SDB & BSW cultures.

PMSF

The serine proteinase inhibitor PMSF was added to both sample & incubation buffers to give a final working concentration of 2mM.The 61,500 Da from BSW group 1 gelatinases were inhibited by PMSF & also the 51,000 Da group 2 band from SDB. PMSF also caused inhibition of group 4 gelatinases from both SDB & BSW culture filtrates. as well as the group 3 gelatinases from BSW culture filtrates(see plates 3.9a, 3.9b, 3.10a & 3.10b)

EDTA

The metalloproteinase inhibitor EDTA was added to both sample & incubation buffers to give a final working concentration of 5mM. The 64,000 Da group 1 gelatinase from SDB culture filtrate was not present on the gel incubated with EDTA(see Plates 3.11a & 3.11b).From BSW culture filtrates the 34,000-48,000 Da group 2 gelatinase smear was partially inhibited by the EDTA(see Plates 3.12a & 3.12b).

PEP

The aspartic proteinase inhibitor Pepstatin A was added to both sample & incubation buffers to give a final working concentration of 50µg/ml. No observable differences were seen between the two gels from both SDB & BSW cultrate filtrates.

p-CMB

The cysteine proteinase inhibitor p-CMB was added to both sample & incubation buffers to give a final working concentration of 2mM. No observable differences were seen between the two gels for both SDB & BSW culture filtrates.

Plate 3.9a:Effect of PMSF on gelatinases of *M.cookei* cultured in SDB

Plate 3.9b:Control with no PMSF added

Plate 3.10a:Effect of PMSF on gelatinases of *M.cookei* cultured in BSW

Plate 3.10b:Control with no PMSF added

Plate 3.9a

Plate 3.9b

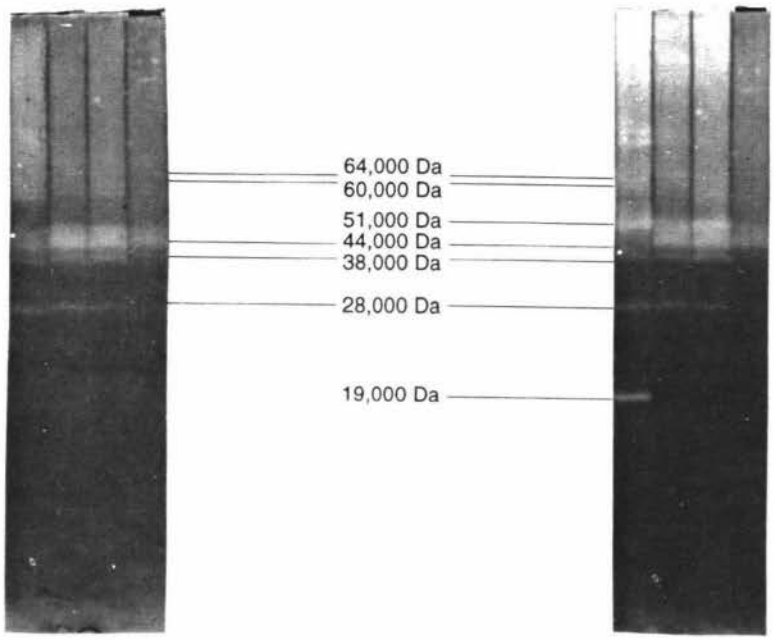


Plate 3.10a

Plate 3.10b

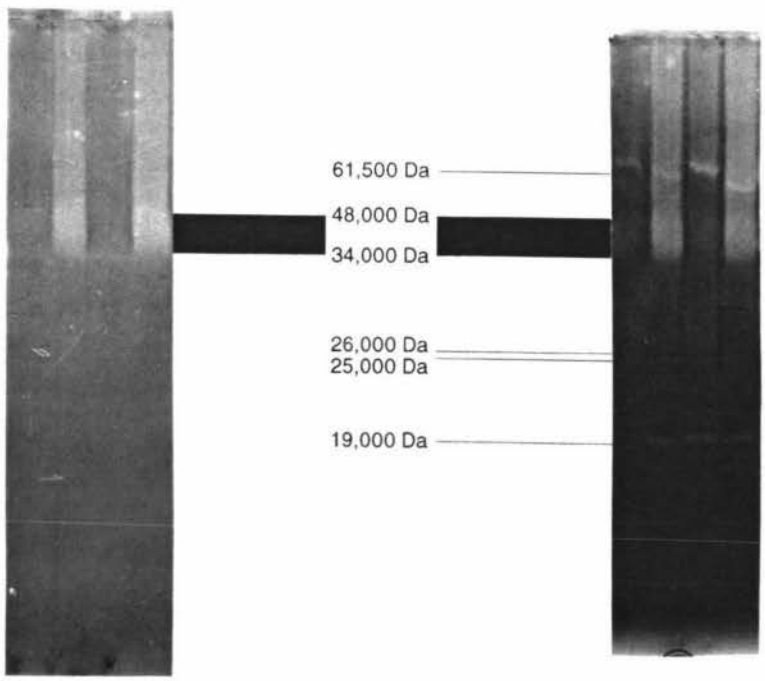


Plate 3.11a:Effect of EDTA on gelatinases of *M.cookei* cultured in SDB

Plate 3.11b:Control with no EDTA added

Plate 3.12a:Effect of EDTA on gelatinases of *M.cookei* cultured in BSW

Plate 3.12b:Control with no EDTA added

Plate 3.11a

Plate 3.11b

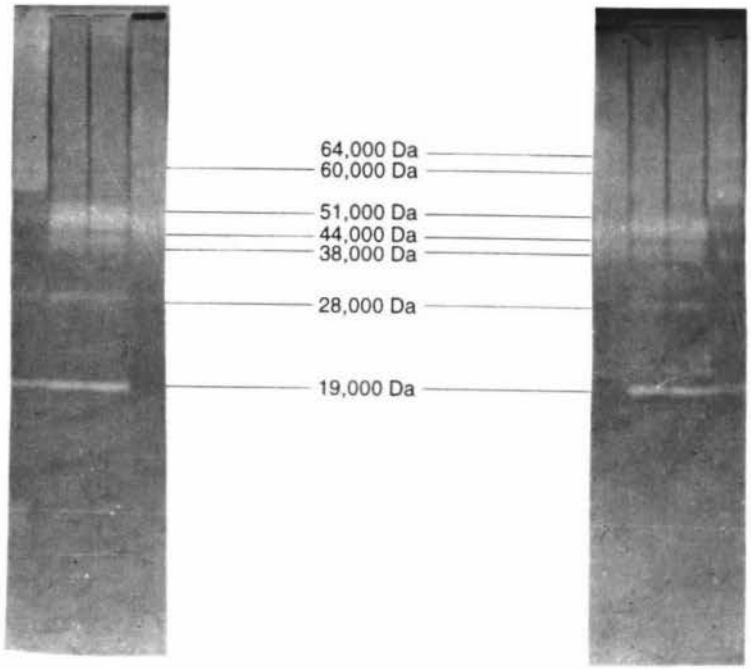
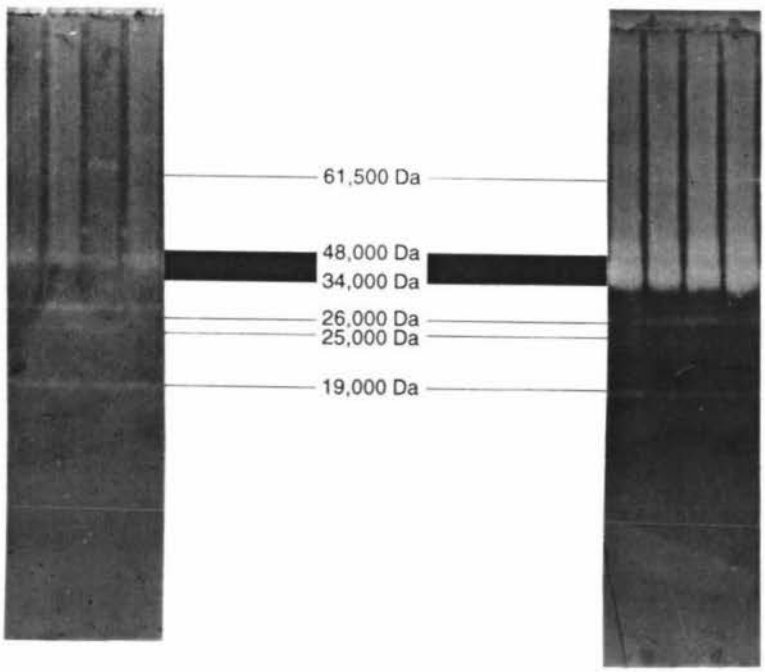


Plate 3.12a

Plate 3.12b



CHAPTER 4

DISCUSSION

Factors Affecting Proteinase Expression

The studies of the effects of cultural conditions on the expression of proteolytic enzymes by the two species examined were aimed at determining if any had a particular effect on enzymes which might be associated with the growth of the fungus on keratin. As *M.canis* is a common zoophilic dermatophyte causing infections of a wide range of host species & *M.cookei* a non-pathogenic geophilic dermatophyte, but still possessing considerable keratinolytic powers, it was considered possible that differences in proteolytic enzyme production between the two species may also reflect possible virulence determinants of importance for *M.canis*.

Thoughout these studies one factor which had a notable effect on the expression of proteolytic enzymes was substrate. BSW medium can be considered to simulate the nutritional conditions that the fungus would encounter on the skin *in vivo* or on keratinous substrates *in vitro*. On the other hand, SDB medium contains peptone & glucose which are rich sources of peptides & sugars for the fungus to grow on. *M.canis* showed higher azocollytic & elastase activity in BSW than in SDB, unlike *M.cookei*, which showed relatively similar azocollytic & elastase activity in both media(Fig 3.1, 3.2, 3.3 & 3.4). Thus *M.canis* demonstrated a greater ability to respond to keratin substrates than *M.cookei,* at least in shake culture. Both species also showed some keratinase activity in BSW shake culture(Fig 3.5) but in SDB no keratinase activity was detected.

BSW was also found to be the better medium to detect gelatinases & allowed consistent demonstration of the component bands(section 3.2.1). Furthermore on the basis of Mr, apparently different proteinases were present in BSW & SDB, although up to six or seven were found in both. Recently Tsuboi (1994) using a liquid medium supplemented with human hair showed *M.canis* expressed three keratinases of molecular

weights 48,000, 34,000 & 32,500 Da but did not comment on the characterisation of the enzymes.

When comparing the proteinases expressed by the same species but in different media, differences were noted, for example *M.canis* grown in BSW expressed 13,000 Da & 15,000 Da gelatinase bands but when grown in SDB only one band of a similar size, 16,000 Da, was expressed. Also in SDB, *M.canis* expressed a 27,000 Da gelatinase but in BSW no such band was detected. Similarly *M.cookei* grown in BSW expressed two group 5 bands at 25,000 Da & 26,000 Da but when grown in SDB only one band of a similar size was found. In BSW cultures of *M.cookei* the 34,000-48,000 Da band was largely smeared & no discrete gelatinase bands could be seen within the smear, but at a similar molecular weight for gelatinases expressed on SDB cultures, up to three bands could be detected. The 34,000 Da smear from BSW cultures could be a combination of the three gelatinase bands expressed in SDB cultures, which at the higher concentration found in BSW cultures have combined to form a single smear as seen, for example, in Plate 3.2a. Only careful separation of the gelatinases & their characterisation would allow an answer to this question, but the use of inhibitors(to be discussed later) sheds some light on the specificity of the bands. In an attempt to compare gelatinase bands produced by both species the similarly sized gelatinase bands were grouped together into six proteinase "groups"(see Table 3.11). From this arrangement it is interesting to note between the two species what appear to be genus specific proteinases produced by both, for example in SDB medium the groups 2, 4, 5 & 6 proteinases & in SDB medium the groups 2, 4 & 6. The inhibition studies aided in the assessment of the validity of the group concept.

However, substrate-SDS-PAGE gels cannot be relied upon to produce accurate size estimations as demonstrated by Apodaca & McKerrow (1989a) when they purified an unreduced 27,000 Da proteinase from *T.rubrum* but under reducing conditions using standard SDS-PAGE calculated its size to be 44,000 Da. Apodaca & McKerrow considered this size difference to be mediated by intrachain disulphide bonds. The above problem of size estimation could hold true for the bands

expressed by the *Microsporum* species and it is difficult to judge which bands are expressed in both media.

Other researchers(Takiuchi 1982, O'Sullivan & Mathison 1971) have also reported that dermatophytes cultured on a protein source such as hair or wool produce much higher general proteinase activity than when cultured on a nutrient rich medium such as SDB. Either the dermatophyte increases proteinase expression of existing proteinases or as shown here, new & distinct proteinases are induced by the presence of the protein source. Apodaca & McKerrow(1989a) suggested dermatophytes express certain proteinases in response to different levels of sulphur, phosphorus, carbon & nitrogen. In this study, *M.canis* cultured in BSW produced much higher levels of azocollytic & elastase activity than when cultured on SDB, in addition to the expression of gelatinases as detected by SDS-PAGE(Plate 3.1). *M.cookei* on the other hand, produced higher elastase activity when cultured in BSW but less azocollytic activity. This difference in azocollytic activity between the two species may be an important clue to the role of proteinases in virulence.

When the effect of time was examined it was noted for both species cultured in BSW medium that all gelatinases expressed after the first seven days of growth were expressed throughout the age of the culture(ie up to 28 days). On the other hand, when cultured in SDB certain gelatinase bands appeared first. For *M.canis,* the 85,000 Da(group 1), 40,000 Da & 36,000 Da(group 4) gelatinase bands were expressed after seven days of growth & persisted for the whole age of the culture. The 61,000 Da(group 2), 27,000 Da(group 5) & 16,000 Da(group 6) gelatinases, in contrast, tended to appear only after fourteen days, but then also persisted throughout the rest of the incubation period. *M.cookei* cultured in SDB produced a similar result, with the 51,000, 44,000 & 19,000 Da gelatinases being expressed after seven days of growth & persisting throughout the age of the culture & the rest of the gelatinase bands expressed after fourteen days of growth. This pattern of enzyme expression could be due to the concentration of gelatinases in the culture medium, the sensitivity of the SDS-PAGE technique, or could be the expression of specific proteolytic enzymes by the fungus in response to availability of amino acids &

peptides in the medium. Interestingly, Tanaka et al (1992) reported that in a keratin-supplemented medium *T.rubrum* changed relative activities of acidic & neutral proteinases during the age of the culture, with acidic proteinases being highest during the first two weeks of the culture and neutral proteinases increasing after the third week as the culture pH increased from 6 to 7. Hence the proteinases expressed by both *Microsporum* species after seven days growth may be the more acidic proteinases.

The effect of incubation temperature on gelatinase expression by the two species was limiting, in the sense that at higher temperatures such as 33°C, *M.canis* & *M.cookei* produced less growth so the concentration of gelatinases in the culture medium was less, resulting in sensitivity problems when analysed by substrate SDS-PAGE. However, it is of interest to note that *M.canis* cultured in either SDB or BSW produced much more growth than *M.cookei* when incubated at these higher temperatures, which simulate skin temperature. So the ability to grow at higher temperatures must be an important pathogenic determinant for *M.canis*.

In this study both species expressed enzymes with optimal temperatures of 45°C-50°C, Fig 3.6) but both species did not grow well at higher temperatures such as 33°C. Lorincz & Sun (1963) reported exposure to 43°C for 72 hr of all dermatophyte strains tested proved lethal, so the question remains why does the dermatophyte produce enzymes with temperature optima above its survival threshold. Sei (1978) purified two keratinases from *M.canis* which also had optima of 47°C & a pH optimum of 7.8, which mirrors results presented in this study for keratinase activity(Fig 3.5).

Another factor which was found to obviously influence expression of proteolytic enzymes was the method of culture(ie shake or stationary). In the study of gelatinases, all bands expressed by *M.canis* when cultured in SDB were consistently more strongly expressed in shake cultures than in stationary cultures. This was particularly noticeable for the 85,000 Da, 27,000 Da & 16,000 Da bands & leads one to think these gelatinases may be induced by the so called parasitic-like morphology assumed in shake culture Simpanya (1994) also noted

strains of *M.canis* cultured in SDB expressed a significantly greater number of bands in shake culture than in stationary culture. For *M.cookei* in SDB the effect was less marked. However, when the fungi were cultured in BSW medium, a medium which presumably more closely resembles the *in vivo* situation because of its keratin component, there was little difference between shake & stationary cultures. Noteably with *M.canis,*. the 27,000 Da band did not appear in BSW but a second low molecular weight gelatinase (13,000 Da) was expressed, more strongly in shake culture. This may be an important pathogenic factor for the fungus.

Raubitschek (1955), Evon-Maoz & Raubitschek(1960) & later Roberts et al (1984), noted that during the parasitic phase *in vivo*, dermatophytes only produce mycelium & arthrospores but when cultured on agar media or in stationary broth culture they produce mycelium, macro- & microconidia. When dermatophytes are grown in liquid shake culture, on the other hand, they more closely resemble the parasitic morphology in that they produce only mycelium, chlamydospores & arthrospores. Figs 4.1, 4.2, 4.3, 4.4 & 4.5 illustrate the morphological differences between growth in shake & stationary liquid cultures. Such a change in morphology was associated with a stronger expression of proteolytic enzymes & it could be postulated that the stronger expression in shake culture, particularly in keratin-containing media, reflects the situation *in vivo* for *M.canis. M.cookei*, on the other hand, although undergoing similar morphology changes in shake culture, did not show this effect of stronger enzyme expression to any notable degree & this could be a reflection of its saprophytic, geophilic growth. It is considered that it would be appropriate for the finer details of the mycelial morphology in relation enzyme production & pathogenesis to be investigated in more detail in the future.

Plate 4.1:Sample of *M.canis* growth in shake mode of culture

Plate 4.2:Sample of *M.canis* growth in stationary mode of culture

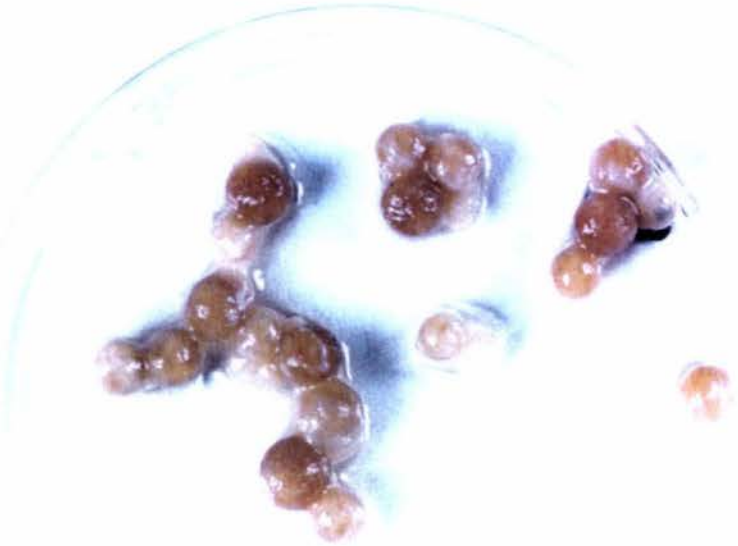


Plate 4.3:Mycelial morphology of *M.canis* in shake culture in SDB(magnification X400). NB:Note chlamydospores

Plate 4.4:Morphology of the surface mycelium of *M.canis* in stationary culture in SDB(magnification X400).
NB:Note macroconidia

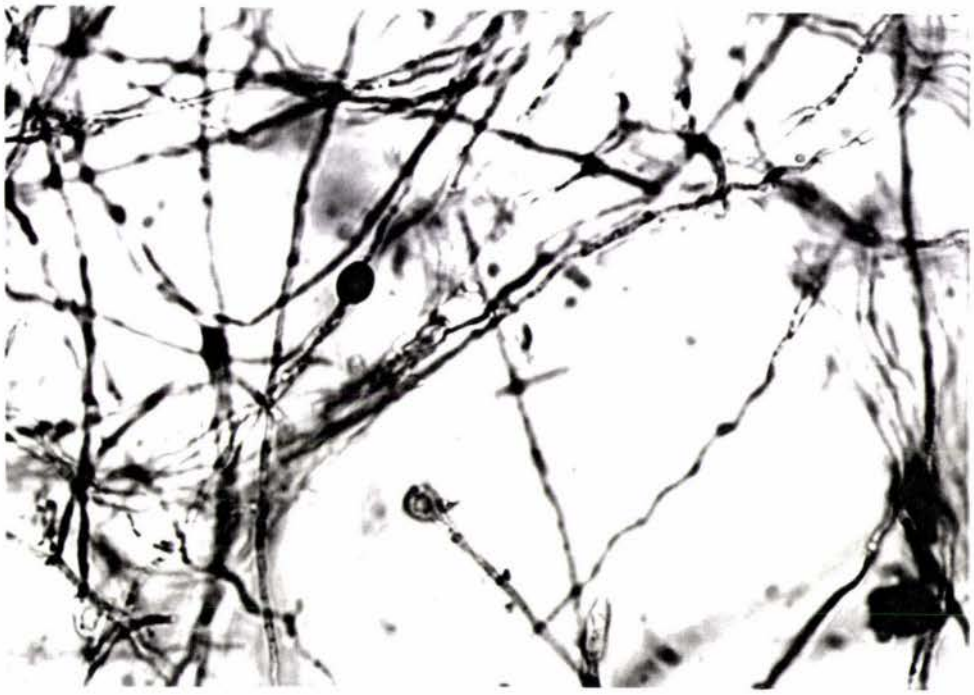
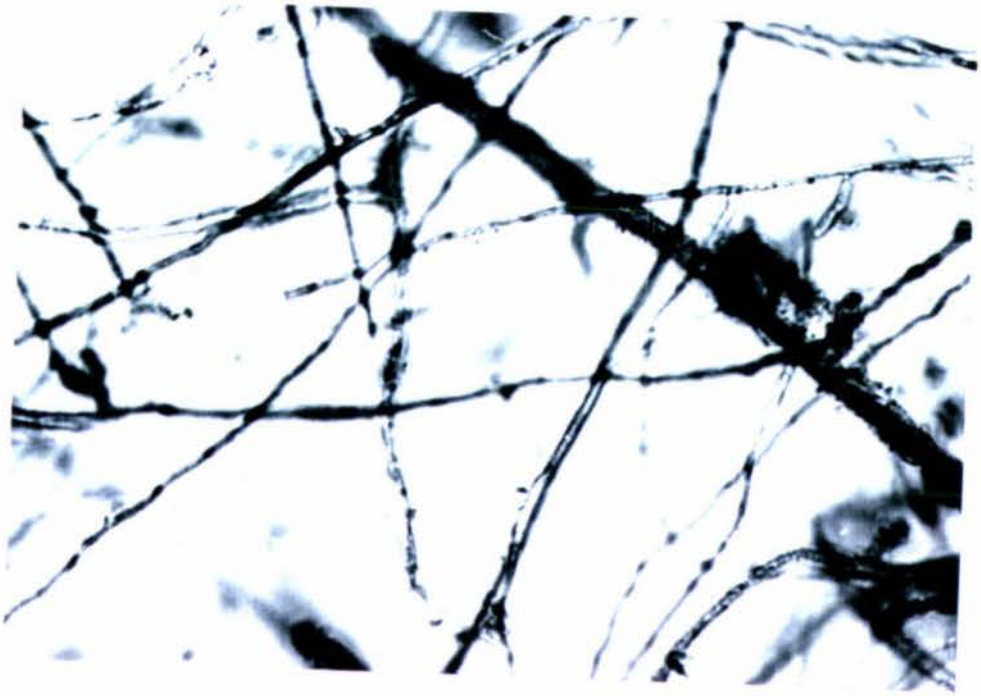


Plate 4.5:Morphology of the submerged mycelium of *M.canis* in stationary culture in SDB(magnification X400).



Characterisation of Proteinases Using Specific Inhibitors

The use of azocollytic & elastase assays & of specific enzyme inhibitors gave some idea of the relative importance of the four different types of proteinases in the degradation of azocoll & elastin, & thus their possible role in dermatophyte growth. Also, in an attempt to partially characterise the gelatinase bands detected by substrate-SDS-PAGE, specific proteinase inhibitors were employed & added to the samples & gels to analyse which bands showed inhibition. Similar investigations have recently been reported by Rodier et al(1994), where gelatinases expressed by *Candida albicans* were characterised by the addition of inhibitors to the substrate gels. Rodier et al reported two cytoplasmic proteinases(50,000 Da & 60,000 Da), common to all five isolates of *C. albicans* examined which were sensitive to the serine proteinase inhibitor PMSF. Interestingly, an attempt to detect extracellular gelatinase activity in supernatants of *C. albicans* was also made by Rodier, but no activity was detected even when incubated in different buffers from pH 2 to 9. Robertson et al (1990) had also investigated the proteinases expressed by *Trypanosoma brucei* & were able to characterise several cysteine proteinases by their sensitivity to inhibitors using the substrate SDS-PAGE technique.

Azocollytic activity of *M.canis* cultured in SDB had high sensitivity to PMSF & moderate sensitivity to EDTA & p-CMB but low sensitivity to PEP (Fig 3.7). Azocollytic activity of *M.canis* cultured in BSW, on the other hand, had high sensitivity to EDTA & moderate sensitivity to PMSF & p-CMB but no sensitivity to PEP(Fig 3.8). Thus relative expresson of the various enzyme mixes must be different in the two media ie be a reflection of substrate availability. Azocollytic activity of *M.cookei* cultured in SDB had high sensitivity to EDTA, PMSF & p-CMB, & no sensitivity to PEP. Inhibition of azocollytic activity of *M.cookei* cultured in BSW showed similar results as when cultured in SDB except lower inhibition by PMSF. From this data & the SDS-PAGE analysis with the same inhibitors, it is suggested that when *M.canis* is cultured in SDB, the two PMSF sensitive(serine) gelatinases(40,000 Da & 16,000 Da) are responsible for most of the azocollytic activity(81%), but when cultured in BSW, serine proteinases are responsible for only

29% of azocollytic activity. The EDTA sensitive(metalloproteinases) 34,000 Da & 30,000 Da gelatinases similarly are responsible for 62% of azocollytic activity on BSW, but are not expressed by *M.canis* in SDB, being replaced by the 40,000 Da enzyme. Thus it is possible that the 34,000 Da & 30,000 Da metalloproteinases could play an important role in the ability of *M.canis* to grow on keratin, & hence also in the pathogenicity of *M.canis*.

The inhibitors PMSF & EDTA produced high inhibition of elastase activity of both *M.canis* & *M.cookei* in both media(Figs 3.9 & 3.10). The aspartic proteinase inhibitor PEP also caused high inhibition unlike its action on azocollytic activity, which suggests *M.canis* produces one or more aspartic proteinase(s) in the mix of proteinases expressed, with high elastase specificity but which did not appear on the substrate SDS-PAGE gels. This may be due to irreversible inactiviation by SDS, inability to separate the bands possibly due to similar sized gelatinases masking their presence, low activity or its high substrate specificity, ie lack of ability to degrade the gelatin present in the substrate gels. Rippon (1968) isolated an extracellular proteinase from *T.schoenleinii*, with high activity against collagen but low activity towards azocoll, which suggests dermatophytes do produce proteinases with high substrate specificity. The inhibitor p-CMB caused low inhibition of elastase activity, indicating cysteine proteinases produced by dermatophytes have low activity towards elastin.

In the substrate SDS-PAGE experiments the serine proteinase inhibitor PMSF caused inhibition of the 40,000 Da(group 4) & 16,000 Da(group 6) gelatinases of *M.canis* cultured in SDB. When *M.canis* was cultured in BSW, PMSF caused inhibition of the 15,000 Da & 13,000 Da(group 6) bands, but did not cause inhibition of the 30,000 Da or 34,000 Da(group 4) bands. This result indicates that the 30,000 Da & 34,000 Da bands expressed by *M.canis* cultured in BSW are not the same as those group 4 bands expressed when cultured in SDB & are different from the 40,000 Da band. Takiuchi et al (1982, 1984) reported the expression of a serine proteinase by *M.canis* & calculated its reduced size to be 45,000 Da. They cultured the fungus in a medium containing glucose & human hair, but it is difficult to relate their findings to the present study due to the effect of size calculations of bands & of

reduced & non-reduced enzymes & the effect different substrates have on proteinase expression.

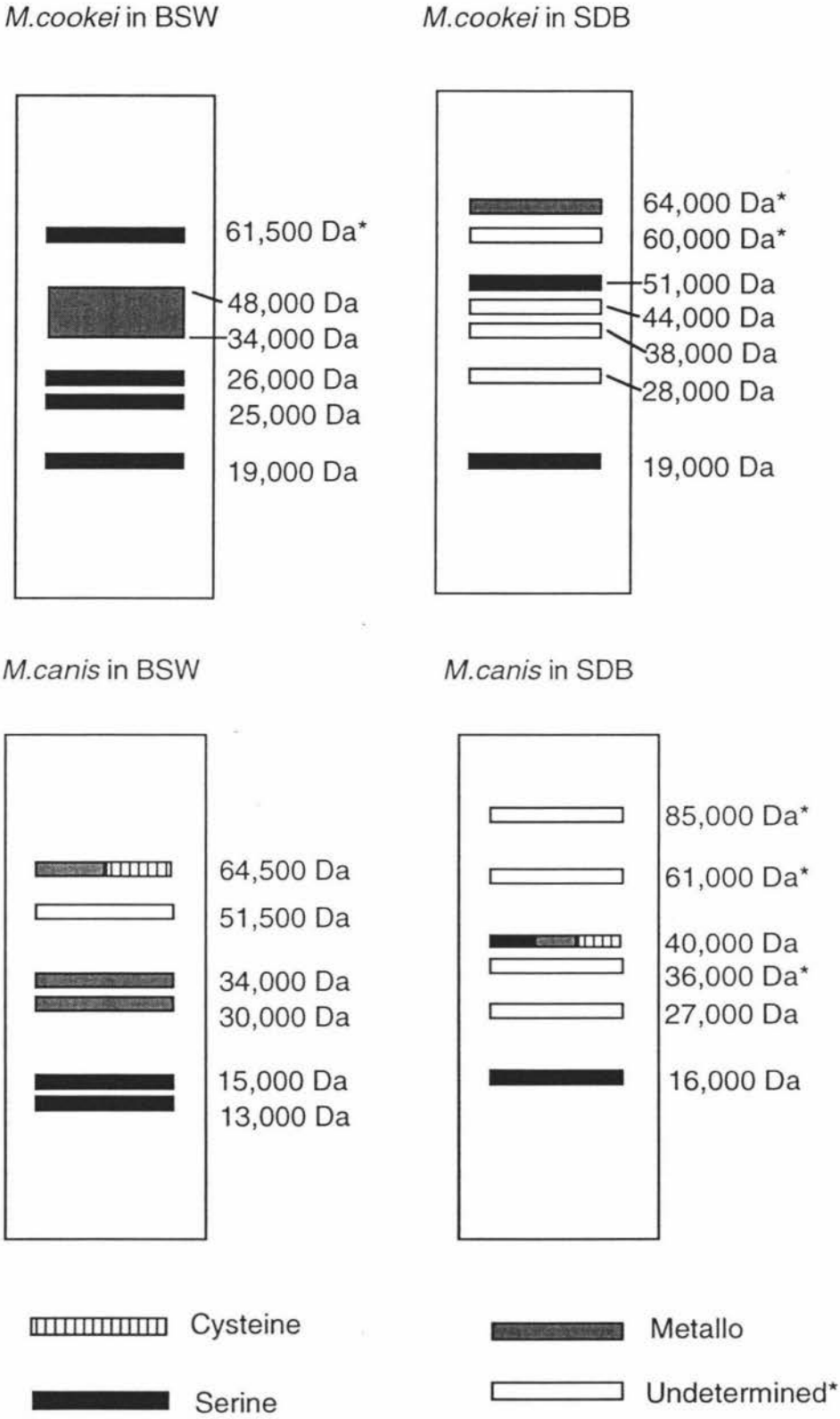
Studies with PMSF on *M.cookei* gelatinases showed four PMSF sensitive bands (61,500 Da, 26,000 Da, 25,000 Da & 19,000 Da) when *M.cookei* was cultured in BSW(Plates 3.17 & 3.18). When cultured in SDB, two PMSF sensitive bands (51,000 Da & 19,000 Da) were expressed(Plates 3.15 & 3.16). From these results it appears the two 19,000 Da group 6 bands expressed in both media are possibly the same serine gelatinases

The metalloproteinase inhibitor, EDTA, caused partial inhibition of the 40,000 Da(group 4) gelatinase band from *M.canis* cultured in SDB & inhibited the 64,500 Da(partial inhibition)(group 2), 34,000 Da & 30,000 Da(group 4) in BSW culture filtrates. EDTA also caused the inhibition of the 64,000 Da(group 2) gelatinase band expressed by *M.cookei* cultured in SDB & caused the inhibition of the 34,000-48,000 Da(group 4) smear expressed by *M.cookei* cultured in BSW medium. Dunn (1989) commented that the diagnosis of metallo-proteinases is best determined on their insensitivity to the other three classes of inhibitors plus the observation of inhibition by EDTA. From the above results the group 4 gelatinases expressed by both species cultured in BSW are possibly genus specific gelatinases expressed in the presence of keratin substrates.

The cysteine proteinase inhibitor, p-CMB, caused the inhibition of the 64,000 Da group 2 gelatinase expressed in BSW & partial inhibition of the 40,000 Da group 4 band expressed by *M.canis* grown in SDB. p-CMB caused no inhibition of gelatinase bands expressed by *M.cookei* in BSW or SDB, indicating no cysteine proteinases were present. Thus again the cysteine gelatinase may be of significance in the ability of *M.canis* to grow *in vivo*.

Fig 4.1 summaries the presence of the various proteinases detected

Fig 4.1:Summary of the characterisation of gelatinase bands expressed by both species cultured in BSW & SDB



* inconsistent expression or not affected by any inhibitors.

Conclusion

In the past, various groups have reported on the isolation of proteinases from dermatophytes (Roberts 1967, Takiuchi 1982, 1984, Sanyal 1985, Asahi 1985) or of a keratinase(Weary 1965, Yu 1968 & 1969, Tsuboi 1989, Quin 1992), but no one other than Apodaca & McKerrow (1989a, 1989b, 1990) have attempted to study all proteinases produced by dermatophytes by using substrate SDS-PAGE. By comparing the proteinases of the geophilic species *M.cookei* & the zoophilic species *M.canis* it was hoped that differences in the expression of proteinases between the two species would be apparent which might reflect potential virulence factors that *M.canis* possesses to enable it to cause disease. O'Sullivan 1971 & Takiuchi 1982 reported higher proteinase activity of dermatophytes when cultured in protein sources such as hair or feathers compared to when cultured in a rich medium such as SDB. In this study it was also noted cultures grown in BSW, especially those of *M.canis,* produced higher azocollytic, elastase, keratinase & gelatinase activites than when grown in SDB. Other possible environmental factors that could affect gelatinase expression, such as temperature, time & form of culture(ie shake or stationary) caused some variation in the expression of some gelatinase bands, but none of the three factors caused induction of any new bands. However, *M.canis* demonstrated greater ability to grow & produce enzymes at 33°C suggesting the ability to grow at higher temperatures such as that encountered on skin surfaces could be an important virulence factor for *M.canis.*

The enzyme assays conducted with proteinase inhibitors revealed that elastase activity of both species was inhibited by PEP, suggesting aspartic elastase enzymes had been expressed. The minimal inhibitory effects of PEP on azocollytic activity indicates the aspartic elastase enzymes have a narrow substrate specificity, which could also be the reason for their absence on substrate SDS-PAGE gels.

Enzyme inhibitors studies revealed that *M.canis* & *M.cookei* expressed different types of proteinases in different media. The most significant & possibly, in terms of virulence, the most important, was that *M.canis* cultured in SDB produced 40,000 Da & 16,000 Da serine gelatinases

but when cultured in BSW, 34,000 Da & 30,000 Da metalloproteinases & a further small Mr serine gelatinase enzyme were expressed. Perhaps these proteinases expressed by *M.canis* on keratin substrates act as virulence factors in the hydrolysis of skin proteins.

REFERENCES

Ajello, L., 1974. Natural history of the dermatophytes & related fungi. Mycopathol.Mycol.Appl. 53:93-110.

Apodaca, G. and McKerrow, J.H., 1989a. Purification and characterisation of a 27,000-Mr extracellular proteinase from *Trichophyton rubrum* . Infect.Immun., 57(10):3072-3080.

Apodaca, G. and McKerrow, J.H., 1989b. Regulation of *Trichophyton rubrum* proteolytic activity. Infect.Immun., 57(10):3081-3090.

Apodaca, G. and McKerrow, J.H., 1990. Expression of proteolytic activity by cultures of *Trichophyton rubrum*. J.Med.Vet.Mycol., 28: 159-171.

Asahi, M., Lindquist, R., Fukuyama, K.,Espstein, W.L, & McKerrow., 1985. Purification & characterisation of major extracellular proteinases from *Trichophyton rubrum*. J.Biochem., 232:139-144.

Blank, H., Taplin, D, & Zaies, N., 1969. Cutaneous *Trichophyton mentagrophytes* infections in Vietnam. Arch.Dermatol., 99:135-140

Borg, M. and Rüchel, R., 1988. Expression of extracellular acid proteinase by *Candida* spp, during experimental infection of oral mucosa. Infect.Immun., 56(3):626-631.

Brasch, J., Martins, B. and Christophers, E., 1991. Enzyme release by *Trichophyton rubrum* depends upon nutritional conditions. Mycoses., 34:465-368.

Chattaway, F.W., Ellis, D.A. and Barlow, A.J.E., 1963. Peptidases of dermatophytes. J.Invest.Dermatol., 41:31-37.

Day,W.C., Toncic,P., Stratman,S.L., Leeman,U, & Harmon S.R., 1968. Isolation & properties of an extracellular proteinase of *Trichophyton granulosum*. Biochim.Biophys.Acta., 167:597-606.

De Vroey, C., 1985. Epidemiology of ringworm(dermatophytosis) Seminars in dermatology., 4(3):185-200.

Dunn, B.M., 1989. Determination of protease mechanism. In: Beynon, R.J. and Bond, J.S.(eds.), Proteolytic Enzymes: A Practical Approach. pp.57-81.

Emmons, C.W., 1934. Dermatophytes: Natural grouping based on the form of spores & accessory organs. Arch.Dermatol.Syphil., 30(3):337-362.

Frey, D., 1971. Isolation of *M.cookei* from a human case. Sabouraudia., 9:146-148.

Georg, L.K., 1960. Epidemiology of dermatophytes sources of infection, modes of transmission & epidemicity. Ann.N.Y.Acad.Sci., 89:69-77.

Grappel, S.F. and Blank, F.,1972. Role of keratinases in dermatophytosis , 1: Immune response of guinea pigs infected with *Trichophyton mentagrophytes* & guinea pigs immunized with keratinases. Dermatologica., 145:245-255.

Grappel, S.F., Bishop, C.T. and Blank,F., 1974. Immunology of dermatophytes and dermatophytosis. Bacteriological Reviews., 38(2):222-250.

Hames, D.B. and Rickwood, D., 1991. Gel electrophoresis of proteins: A Practical Approach(2nd edition).

Hellgren, L. and Vincent, J., 1980. Lipolytic activity of some dermatophytes. J.Med.Micro., 13:155-157.

Huessen, C. and Dowdle, E.B., 1980. Electrophoretic analysis of plasminogen activators in polyacrylamide gels containing sodium dodecyl sulphate and co-polymerised substrates. Anal.Biochem., 102:196-202.

James, G.T., 1978. Inactivation of the proteinase inhibitor phenylmethylsulfonyl fluoride in buffers. Anal.Biochem., 86:574-579.

Kolattukudy, P.E., Lee, J.D., Rodgers, L.M., Zimmerman, P., Ceselski, S., Fox, B., Stein, B. and Copelan, E.A., 1993. Evidence for possible involvement of a elastolytic serine proteinase in Aspergillosis. Infect.Imm., 61(6):2357-2368.

Kunert, J., 1972. Thiosulphate esters in keratin attacked by dermatophytes *in vitro*. Sabouraudia., 10:6-13.

Kunert, J., 1992. Effect of reducing agents on proteolytic & keratinolytic activity of enzymes of *Microsporium gypseum*. Mycoses., 35:343-348.

Kwon-Chung, K.J., Lehman, D., Good, C. and Magee, P.T., 1985. Genetic evidence for the role of extracellular proteinase in virulence of *Candida albicans*.Infect.Imm., 49(3):571-575.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227:680-685.

Lepper, A.W.D. and Anger, H.S., 1976. Experimental bovine *Trichophyton verrucosum* infection. Comparison of the rate of epidermal cell proliferation and keratinisation in non-infected and reinoculated cattle. Res.Vet.Sc., 20:117-121.

Lorincz, A.L. & Sun, H.S., 1963. Dermatophyte viability at modestly raised temperatures. Arch.Dermatol.,88:393-402.

MacDonald, F. and Odds, F.C., 1983. Virulence for mice of a proteinase secreting strain of *Candida albicans* and a proteinase deficient mutant. J.Gen.Microbiol., 129:431-438.

Marples, M.J., 1965. The distribution of keratinophilic fungi in soils from New Zealand and from two Polynesian islands. Mycopathol.Mycol.Appl., 25:361-372.

Meevootisom, V. and Niederpruem, D.J., 1979. Control of exocellular proteinases in dermatophytes especially *Trichophyton rubrum*. Sabouraudia., 17:91-106.

Minocha, Y., Pasricha, L.S., Mohapatra, L.N. and Kandhan, K.C., 1971. Proteolytic activity of dermatophytes and its role in the pathogensis of skin lesions. Sabouraudia., 10:79-85.

Monod, M., Paris,S., Sarfati, J., Jaton-Ogay, K., Ave, P. and Latgé, J.P., 1993. Virulence of alkaline proteinase deficient mutants of *Aspergillus fumigatus*. FEMS.Microbiol.letters., 106:39-46.

Moser, M., Menz,G., Blaser, K. and Crameri, R., 1994. Recombinant expression and antigenic properties of a 32-kilodalton extracellular proteinase, Representing a possible virulence factor from *Aspergillus fumigatus*. Infect.Imm.62(3):936-942.

North , M.J., Scott, K.T. and Lockwood, B.C., 1988. Multiple cysteine proteinase forms during the life cycle of *Dictyostelium discoideum* revealed by electroporetic analysis. Biochem.J., 254:261-268.

O'Sullivan, J. and Mathison, G.E., 1971. The localization and secretion of a proteolytic enzyme complex by the dermatophyte fungus *Microsporum canis*. J.Gen.Microbiol., 68:319-326.

Philpot, C.M., 1977. Some aspects of the epidemiology of tinea. Mycopathologia., 62:3-13.

Pillsbury, D.M., Shelley, W.B. and Kligman, A.M., 1956. Text book of dermatology. W.B.Saunders Co., Philadelphia pp.105-106.

Quin, L.M., Dekio, S. and Jidoi, J., 1992. Some biochemical characteristics of a partially purified extracellular keratinase from *Trichophyton schoenleinii.* Zbl.Bakt., 277:236-244.

Raubitschek, F., 1955. Nutritional requirements of dermatophytes in continous shake culture. J.Invest.Dermatol., 25(2):83-87.

Raubitschek, F., 1961. Mechanical verus chemical keratinolysis by dermatophytes. Sabouraudia.,1(2):87-90.

Riddel, R., 1958. The role of fungi as human pathogens. In: Proceeding of the Royal Society of Medicine., 51:491-493.

Rippon, J.W., 1968. Extracellular collagenase from *Trichphyton schoenleinii.* J.Bacteriol., 95(1):43-46.

Rippon, J.W., 1982a. Medical Mycology: The Pathogenic Fungi and the Pathogenic Actinomycetes. W.B.Sanders, London. pp.154-248.

Rippon, J.W., 1982b. Host specificity in dermatophytoses. In: Baxter, M. (ed.), Proceedings of the Eighth Congress of the International Society for Human and Animal Mycology, Massey University, Palmerston North, pp.28-33.

Rippon, J.W.,1985. The changing epidemiology & emerging patterns of dermatophyte species. In: McGinnis,M.R.(ed) Current Topics in Medical Mycology, pp.208-234.

Rippon, J,W, and Varadi, D.P., 1968. The elastases of pathogenic fungi and Actinomycetes. J.Invest.Dermatol., 50:54-58.

Roberts, F.F. and Doetsch, R.N., 1967. Purification of a highly active proteinase from a *Microsporum* species. Antonie van Leeuwenhoek., 33:145-152.

Roberts, S.O.B., Hay, R.J. and MacKenzie, D.W.R., 1984. A clinicians guide to fungal disease. In: Haroid, C.H., Whelton, A., Diestag, J.L. (eds.), Infectious Diseases and Antimicrobial agents, pp.55-91.

Roberston, C.D., North, M.J., Lockwood, B.C. and Coombs, G.H., 1990. Analysis of the proteinases of *Trypanosoma brucei.* J.Gen.Microbial., 136:921-925.

Rodier, M., Moudui, B., Gharali, M., Lacroix, C. and Jacquemin, J., 1994. Electrophoretic detection of cytoplasmic serine proteinases(gelatinases) in *Candida albicans.* Expt.Mycology., 18:267-270.

Ross, I.K., DeBernardis, F., Emerson, G.W., Cassone, A. and Sullivan, P.A., 1990. The secreted aspartic proteinase of *Candida albicans*: Physiology of secretion and virulence of a proteinase deficient mutant. J.Gen.Microbial., 136:687-694.

Ruffin, P., Andrieu, S., Biserte,G. and Biguet.J., 1976. Sulphitolysis in keratinolysis. biochemical proof. Sabouraudia., 14:181-184.

Rüchel, R., DeBernardis, F., Ray, T.L., Sullivan, P.A. and Cole, G.T., 1992. *Candida* acid proteinases. J.Med.Vet.Mycol., 30(Suppl.1):123-132.

Simpanya, M.F., 1994. Compartive enzyme studies of *Microsporum canis* & *Microsporum cookei* in relation to their Pathogenicity & Diversity. PhD Thesis. Massey University, New Zealand.

Sanyal, A.K., Das, S.K. and Banerjee, A.B., 1985. Purification and partial characterisation of an exocellular proteinase from *Trichophyton rubrum*. J.Med.Vet. Mycol., 23:165-178.

Sei, Y., Higuchi, D. and Takiuchi, I., 1978. Biochemical qualities of Keratinase of *Microsporum canis*(In Japanese). Magazine for Showa Medical Society., 39(1):49-54.

Sohne, P.G., 1989. Dermatophytosis. In: Cox, R.A.(ed.), Immunology of fungal diseases. pp1-27.

Sohnle, P.G., 1993. Dermatophytosis. In: Murphy, J.W., Freidum, H. and Bendwell, M. (eds.), Fungal Infections and Immune Response, pp27-47.

Stahl, W.H., McQue, B., Mandels, G.R. and Sue R.G.H., 1950. Studies on the microbiological degradation of wool. Tex.Res.J., pp.570-579.

Tanaka, S., Summerbell, R.C., Tsuboi, R., Kaaman,T., Schnle,P.G., Matsumoto, T. and Ray, T.C., 1992. Advances in dermatophytes and dermatophytosis. J.Med.Vet.Mycol., 30(suppl.1):29-39.

Takiuchi, I., Higuchi, D., Sei, Y. and Koga, M., 1982. Isolation of an extracellular proteinase(keratinase) from *Microsporum canis*. Sabouraudia., 20:281-288.

Takiuchi, I., Sei, Y., Takagi, H. and Negi, M., 1984. Partial characterisation of the extracellular keratinase from *Microsporium canis*. Saubouraudia., 22:219-224.

Tang, C.M., Cohen, J. and Holden, D.W., 1993. An *Aspergillus fumigatus* alkaline proteinase mutant constructed by gene disruption is deficient in extracellular elastase activity. Mol.Microbiol., 6(12):1663-1671.

Tang, C.M., Cohen, J., Krausz, T., Van Noorden, S. and Holden, D.W., 1993. The alkaline proteinase of *Aspergillus fumigatus* is not a virulence determinant in two mouse models of invasive pulmonary Aspergillosis. Infect.Immun., 61(5):1650-1656.

Tsuboi, R., Kurita, Y., Negi, M. and Ogawa, H., 1985. A specific inhibitor of keratinolytic proteinase from *Candida albicans* could inhibit cell growth of *Candida albicans*. J.Invest.Dermatol., 85:438-440.

Tsuboi, R., Ko, I., Takamori, K., Ogawa, H., 1989. Isolation of a keratinolytic proteinase from *Trichophyton mentagrophytes* with enzymatic activity at acidic pH. Infect.Immun., 57(11):3479-3483.

Tsuboi, R., Ogawa, H., Bramono, K., Richardson, M.D., Shankland, G.S., Crozier, W.J., Sei, Y., Ninomiya, J., Nakabayashi, A., Takaiuchi, I., Payne, C.D.& Ray ,T.L., 1994. Pathogenesis of superficial mycoses. J.Med.Vet.Mycol., 32(Suppl.1):91-104.

Walter Hans-Emar, 1984. Method with haemoglobin, casein & azocoll as substrate. In: Bergmeyer H.U. (editor in chief), Bergmeyer Jürgen & Grabi Marianne (eds.), Methods of Enzymatic Analysis: Enzymes 3: Peptidases, Proteinases & their Inhibitors. Verlag Chemie, Florida. pp 270-277.

Weary, P.E., Canby, C.M. and Cawley, E.P., 1965. Keratinolytic activity of *Microsporum canis* and *Microsporum gypseum*. J.Invest.Dermatol., 44:300-310.

Weary, P.E. and Canby, C.M., 1969. Further observations on keratinolytic activity of *Trichophyton schoenleinii* and *Trichophyton rubrum*. J.Invest.Dermatol., 53(1):58-63.

Yu, R.J., Harmon, S.R. and Blank, F., 1968. Isolation and purification of extracellular keratinase of *Trichophyton mentagrophytes*. J.Bacteriol., 96(4):1435-1436.

Yu, R.J., Harmon, S.R., Wachter, P.E. and Blank, F., 1969. Amino acid composition and specificity of a keratinase of *Trichophyton mentagrophytes*. Arch.Biochem.Biophys., 135:363-370.

Yu, R.J., Harmon, S.R., Grappel, S.F. and Blank, F., 1971. Two cell bound keratinases of *Trichophyton mentagropytes*. J.Invest.Dermatol., 56(1):27-32.