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MULTIPLE PROTEOLYTIC ENZYME PRODUCTION

IN KERATINOPHILIC FUNGI

(Preliminary Investigations)

A thesis presented in fulfillment of the requirement for
the degree of Master of Science in Microbiology
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ABSTRACT

Superficial fungal infections can be acquired from a number of sources, e.g. animals, humans or from the soil. Many of the fungal species commonly associated with human disease arise from infection by species known commonly as dermatophytes, although infection from other non-dermatophytic keratinophilic fungi is becoming more common.

Other species not commonly regarded as pathogenic have on occasion been found in human infection. Many of these opportunistic species are commonly found in soils.

Isolation procedures employed in these studies were the hairbrush technique for small animals and the keratin-baiting technique for soil with samples being cultured on SDA containing antibiotics. Soil samples yielded 3 keratinophilic genera found in human infection (*Microsporum spp.. Trichophyton spp. Aphanoascus sp.*) while fungi isolated from animals yielded 3 fungal species, *Microsporum canis, Microsporum cookei* and *Scopulariopsis brevicaulis.*

In these studies, various culture parameters e.g. pH, spore numbers and various hydrolysis techniques were examined in order to asses the production of proteolytic enzymes *in vitro.* Also in the course of these studies, the use of lactrimel medium as a suitable recovery agent for strains presenting atypical colony morphology and reduced proteolytic enzyme production was trialed with excellent results.

The gelatin SDS-PAGE technique, mode of culture (shake and stationary) and the effect of substrate were analysed to compare the effects that these have on a range of keratinophilic fungi. Both pathogens and saprophytes were examined in an attempt to detect similarities in enzyme production which could be associated with the ability of various species to invade skin *in vivo.*

A large body of data has been gathered demonstrating that the proteolytic enzymes produced by most keratinophilic fungi encompass a wide range of MW sizes and are not entirely predictable. This strongly suggests that when these fungi come into contact with a particular substrate, the ability of the strain to adapt may depend on the strains ability to produce a proteolytic enzyme capable of breaking down the substrates in the external environment providing nutrients for the growing fungi.

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INTRODUCTION

1.1 Keratinophilic Fungi

Fungi are an integral part of the biomass of any natural environment, including soils, where many species have become specialised in the evolutionary processes to invade, colonise and thereby ensure decomposition of keratinous debris of other organisms (Ajello, 1974). These fungi utilise subsequent protein products as nutrient sources for continued growth and reproduction. They can be termed keratinophilic or keratinolytic, with their lytic abilities attributed in varying degrees to the production of various structures enabling physical invasion of substrates (Davidson & Gregory, 1934, Vanbreuseghem, 1949,1950,1952, English, 1963, Barlow & Chattaway, 1955, etc.) together with the production and secretion of various proteases, lipases and keratinases (Yu *et al.*, 1968, 1969).

The ability to colonise natural keratin is widespread and a considerable number of species within the aquatic fungi (*Chrytridiales, Saprolegniales),* zygomycetes, ascomycetes and deuteromycetes are reported with this attribute. These fungi have been placed into two different groups: 1) dermatophytes and other species able to attack native keratin by penetration (Vanbreuseghem, 1952, English , 1963) as well as enzyme activity (Vanbreuseghem,1953, Mathison, 1964) and 2) saprophytic species which attack the keratin mainly by hyphal penetration to various extents (English, 1963) or simply surface growth, utilising the more easily decomposable compounds of the keratin substrate (English, 1965, Apinis, 1967).

In general, keratinous materials which fall to the ground are successively colonised by differing groups of fungi. The *Chrytidiaceae*, which can be seen microscopically only, may colonise first, followed by non-keratinolytic species, often with representatives from the genera *Fusarium, Penicillium, Mucor & Diheterospora.*

These fungi assimilate the easier to digest substances which are probably present in the intercellular substance of the substrate. A third group is able to break down the more resistant substances found there e.g. *Chaetomium, Gliocladium, Humicola & Scopulariopsis brevicaulis,* while the final group is formed by true keratinolytic species such as *Microsporum, Trichophyton & Chrysosporium spp.* (DeVries, 1962).

Several genera of these fungi include species which are pathogenic to man and other animals. The most important are species of the 'dermatophytes', *Microsporum, Trichophyton & Epidermophyton,* which are able to actively invade the keratinised tissues resulting in 'dermatophytoses', but invasion by other keratinophilic species is not uncommon and such infections are frequently referred to as opportunistic.

1.2 Identification and Classification of Keratinophilic Fungi

Identification of the various species has in the past been based on traditional methods of observation of both gross colony morphology on Sabouraud's Dextrose Agar (SDA) and microscopic examination of spores.

Important colony characteristics for the dermatophytes and other species include rate of growth, topography, texture and reverse and surface pigmentation. As colony appearance and pigmentation depends on the medium used for culture, SDA is used to obtain colonies which may be compared to others reported in the literature (Ajello, 1966).

Also commonly used for dermatophytes is dermatophyte test medium (DTM). This is a selective medium which excludes most bacteria by the incorporation of chloramphenicol, which inhibits the peptidyl transferase activity of the 50S ribosomal subunit in prokaryotes, and also inhibits non-dermatophyte fungi via the use of cycloheximide which blocks the peptidyl transferase activity of the 60S ribosomal subunit in eukaryotes (Stryer, 1995). No sensitivity to this latter has been found among dermatophytes. This medium also contains a yellow - red pH indicator rapidly affected by dermatophytes and related species, allowing early recognition (Rebel &Taplin, 1970).

Other methods employed in identification include the use of selective media based on known physiological characteristics of a particular strain, such as partial or essential nutrient requirements, e.g. *T. equinum* -niacin or *T. meginii* - l-histidine, (Geog *et al.*, 1957) and *T. tonsurans* var. *sulfureum* - thiamine (Padhye *et al.*, 1994).

Microscopic examination of various species shows that most dermatophytes have two types of conidia/aleuriospores: multicelled macrospores (macroaleuriospores) and small unicellular microspores (microaleuriospores).

Macrospores of the genus *Microsporum* tend to have thick, rough walls, while those of *Trichophyton* and *Epidermophyton* are thin, smooth walled, although various strains of *Microsporum spp.* have been known to produce smooth walled macrospores (Rebel & Taplin, 1970).

Microspores are produced by most parasitic dermatophytes on occasion, except for *Epidermophyton floccosum* which is unique in that it does not produce microspores (Rebel & Taplin, 1970).

There is also a range of vegetative structures seen microscopically which can be useful in identifying various strains or species, i.e. chlamydospores, spiral hyphae, antler-shaped hyphae (chandeliers), nodular organs and pycnidia-like structures (Emmons, 1934, Ajello, 1966, Rebel & Taplin, 1970).

Identification criteria of other keratinophilic species are based on gross and microscopic characteristics listed in various publications e.g. for *Chrysosporium & Aphanoascus* -Carmichael (1962), Apinis (1967), Barron (1968), Rippon *et al* (1970) Cano & Guarro (1990), for *Scopulariopsis brevicaulis* - Barron (1968), Gravesen *et al.* (1994), for *Diheterospora* and it's *Verticillium* states - Barron (1968,1985).

1.2.1 The Teleomorphic States

As the perfect or teleomorphic states of a number of the dermatophytic and other species have been discovered, changes have been made to classifications (Ajello, 1974,1977, Vanbreuseghem, 1977, Takashio, 1979). For example, the perfect states of *Microsporum spp.* have been named as *Nannizia spp.* and the perfect states of *Trichophyton spp.* named as *Arthroderma sp.* (Table 1.1)

The teleomorph genus *Aphanoascus* (anamorph *Chrysosporium spp.*)has suffered taxonomic confusion since its foundation by Zukal (1890). It was extensively reviewed by Apinis (1967), who considered *A. fulvescens* (Cooke) Apinis the type species. The species *A. terreus* was originally identified by Randhawa & Sandhu (1964) who named it *Keratinomyces terreus,* it was later renamed following comparison with a culture from Centraalbureau voor Schimmelcultures, Baarn (Apinis, 1967).

Subsequent mating studies have also revealed that some of the anamorphic states represent a complex of species, e.g. the *M. gypseum-fulvum* complex and the *T. terrestre* complex.

The biological group of dermatophytes and some other fungi e.g. *Ctenomyces serratus*, a species with a strong predilection for feathers, are now classified in the family Arthrodermataceae and it is in this group that keratinophily or a predilection for keratin substrates is fully expressed (Leclerc *et al.*, 1994).

Table 1.1: The major *Trichophyton* & *Microsporum* species with known teleomorphic states.

Teleomorph	Anamorph
Trichophyton spp..	
Arthroderma Berkley, 1860	*Trichophyton* Malmsten, 1845
A. benhamiae Ajello & Cheng 1967b	*T. mentagrophytes var mentagrophytes*
A. insingulare Padhye & Carmicheal, 1972	*T. terrestre*
A. lenticularum Pore, Tsao & Plunkett, 1965	*T. terrestre*
A. quadrifidum Dawson & Gentiles, 1961	*T. terrestre*
A. uncinatum Dawson & Gentiles, 1961	*T. ajelloi*
A. vanbreuseghemii Takashio, 1973	*T. mentagrophytes var. interdigitale*
Microsporum spp.	
Nannizzia Stockdale, 1961	*Microsporum* Gruby, 1843
N. cajetani Ajello, 1961	*M. cookei*
N. grubyia Georg & Ajello Friedman & Brinkman, 1962	*M. vanbreuseghemii*
N.gypsea Stockdale, 1963	*M. gypseum*
N. incurvata Stockdale, 1961	*M. gypseum*
N. obtusa Dawson & Gentiles, 1961	*M. nanum*
N. otae Hasegawa & Usui, 1975	*M. canis*

Modified from Ajello (1968, 1974), Vanbreuseghem *et al.* (1977), Howard (1983) and Simpanya (1994).

1.2.2 Molecular Mycology in Identification Processes

Although fungal taxonomy relies upon traditional morphological examination, several chemotaxonomic methods have been applied to dermatophytes, including fatty acid analysis (Jones & Noble, 1981) and sterol composition (Jones & Mallet, 1983) with the latter method tending to show division of species according to ecological origin. However, clinical mycology laboratories are increasingly utilising molecular based methods for the recognition of pathogenic fungi involved in human disease. Central to this is the design and use of DNA/RNA probes and primers for use with the polymerase chain reaction (PCR).

The basis for investigations of this type is that DNA sequences can be found which are common to all fungi but differ to those found in non-fungal species, in particular humans, eukaryotic parasites and bacteria, thereby addressing the question "Is the infection fungal rather than bacterial?" (Mitchel *et al.*, 1994).

DNA homology studies of dermatophytes tend to support the present classification system (Davidson *et al.*, 1984, Taylor, 1986) although there are still some ambiguous results e.g. unexpected low homologies between anamorphs and teleomorphs. However, using mitochondrial DNA (mtDNA) from the sexual and asexual states, Kawasaki *et al.* (1995, 1996) concluded that there was no distinction between the genera *Arthroderma & Nannizzia*, which supports the work of earlier researchers (Weitzman *et al.*, 1986).

Recently, Cano *et al.* (1996) have felt that they have had some success with using mtDNA Restriction Fragment Length Polymorphism (RFLP) techniques in classifying various strains of *Chrysosporium sp.*, achieved using restriction enzymes with G - C four base recognition. However, they have stated that the phylogenetic value of this technique is limited, due to the numbers of mtDNA able to be studied being few in number.

It is believed that the ribosomal RNA gene (rDNA) region in fungi contains areas with enough sequence conservation for primers and probes at relatively deep taxonomic levels, and other areas with enough variability for some species-specific discrimination. Being a multi copy gene it may be considered a "pre-amplified" target, with further amplification increasing the sensitivity for detecting organisms few in number in a clinical specimen (Mitchel *et al.*, 1994).

Sequence comparisons of various subunits of the rDNA region is enabling researchers to investigate phylogeny of dermatophytes and other fungi, as well as investigate approaches to the detection and identification of pathogenic fungi (Olsen *et al.*, 1986, Leclerc *et al.*, 1994, Harmsen *et al.*, 1995, Haynes *et al.*, 1995).

Although at present there are no well developed probes available for the detection of commonly found dermatophytes, currently available acridinium ester-labelled DNA probes directed against rRNA have been evaluated for other fungi such as *Histoplasma capsulatum* and *Cryptococcus. neoformans*, with specificity and sensitivity for these fungi near 100% (Benek & Rogers, 1996).

1.3 Ecology

It was initially hypothesised in 1893 and 1910 by Sabouraud that dermatophytes live as saprophytes in soil during a part of their life cycle. This hypothesis was taken up by Davidson & Gregory (1932,1934) who assumed that dermatophytes grew on keratinaceous debris in the soil, and the term 'keratinophilic' was later applied to all pathogenic fungal species, not only dermatophytic, which could be isolated on keratin baits (de Vries, 1962).

The use of specialised isolation techniques for the study of keratinophilic fungi was introduced by Karling (1946) for his study of keratinophilic *Chrydiaceae.* He grew these fungi on hairs or skin scales which he had put on the surface of the soil or substrate to be investigated. This technique was used extensively by Vanbreuseghem (1952), and is now commonly known as the "Hair Baiting Technique".

Soil samples being investigated for keratinophilic fungi are usually taken from the surface layer of soils and placed into suitable receptacles. Once back in the laboratory, the soil is placed in a sterile petri dish, moistened with sterile distilled water and "baited" with pieces of sterile keratin such as feathers, wool or hair. The sample is then incubated at 25°C and examined daily for fungal growth (Plate 1.1).

A large number of pathogenic and potentially pathogenic fungi from a range of genera have been isolated from soils in all parts of the world and the presence of an abundant keratinophilic fungal flora in soils has been revealed (Ajello, 1956,1959, Marples, 1965, Chmel *et al.*, 1972, Lee, 1979, Mercatini *et al.*, 1993, Simpanya *et al.*, 1996, Currah *et al.*,1996). Thus the soil is a very important environmental reservoir of pathogenic fungi.

Infections caused by some of these species involves an unlimited host range and demonstrates the wide ranging versatilities of some fungi. But among the dermatophyte group there is a host range in which individual species differ, with the differences in host specificity considered attributable to the molecular differences in keratins of various hosts (Rippon, 1982).

With the probability that some fungal pathogens have evolved from an original natural habitat in the soil and then developed host specificity, dermatophytes were divided by Georg (1959) into three ecological groups:- geophiles, zoophiles and anthropophiles.

Plate 1.1 Soil Plate "Keratin Baited" with Sheep Wool

Fungal growth clearly visible on wool samples

1.3.1 Geophilic Fungi

Keratinophilic fungi in this group occur as saprophytes in soils, particularly those rich in keratinous substrates. Their presence in the soil is determined by a number of factors e.g. pH , temperature and availability of keratin, which influence their ability to grow and survive. A few of these species under suitable conditions have been known to cause dermatophyte infection in animals and humans (Woodgyer, 1994). Although the lesions caused during these infections may be severe, they usually heal spontaneously.

The more pathogenic strains of geophilic dermatophytes are sometimes found to show characteristic traits of true pathogens e.g. production of arthrospores in invaded skin. The principle virulent geophilic dermatophyte is considered to be *M. gypseum*.

This species has been well documented world-wide as a pathogen of man and animals, with infective propagules originating from saprobic sources transmitted directly or indirectly to the host (Ajello *et al.*, 1966, 1974, De Vroey, 1984,1985, Woodgyer, 1994). More recently, the *M. gypseum* has come to light as a complication in immunocompromised patients such as those with acquired immunodeficiency syndrome, with clinical manifestations being more severe and with a wider variation than those found in non-immunocompromised patients (Porro *et al.*, 1997).

Other species which have been rarely associated with human infection are *M. cookei* (Frey, 1971) and *T. ajelloi* (Presbury & Young, 1978). In addition, infections from non-dermatophytic geophilic fungi or opportunist infections, occurring either singly or as part of a mixed infection, have been recorded with e.g. *S. brevicaulis* and *Chrysosporium sp.*,(Woodgyer, 1994, 1995,1996., Velez *et al.*, 1997) and an infection from an *Aphanoascus sp.* has been recorded (Rippon *et al.*, 1970).

Currently, there are no reported cases of human infection from members of *Diheterospora sp.*

1.3.2 Zoophilic Fungi

Zoophilic fungi are mainly animal pathogens but several are also able to infect humans. The principal dermatophytes in this group are listed in Table 1.2 (Woodgyer, 1994).

Table 1.2 Common zoophilic dermatophyte species in NZ and most common animal host.

Dermatophyte	Cat	Dog	Rodent	Guinea Pigs	Rabbits	Hedgehogs	Cattle	Horses	Pigs
Microsporum canis	F	IF			R			IF*	
M. equinum								R**	
M. nanum									F
Trichophyton equinum (both varieties)		R***					R	F	
T. mentagrophytes var erinacei	R	IF				F			
T.mentagrophytes var mentagrophytes	R	R	F	F	R	R	R	R	
T.verrucosum							F	R	

legend: F = Frequently isolated *** Identified as *M. canis* but possibly *M. equinum***

IF = Infrequently isolated **** Rare cause of equine ringworm in North Island**

R = Rarely isolated ***** Case diagnosed where dog had contact with infected horse**

Humans, particularly children in the age range 5 - 14 yrs, readily acquire infections through direct contact with infected animals or via infected fur and hair shed by domestic pets, particularly kittens and puppies. Infections with one of the above species are highly inflammatory and tend to be self-limiting. Treatment with antifungals in these cases tips the balance in favour of the host and the majority of infections respond favourably to such treatment.

The two most common agents of human infection from the above group of fungi are *M. canis*, which is a frequent cause of tinea capitis in pre-pubertal children accounting for up to 88% of culture diagnosed cases (Woodgyer, 1993) and *T. mentagrophytes*. Although *M. canis* is not found in the soil, *T. mentagrophytes* has occasionally been isolated from soil (Baxter, 1966, Padhye & Carmicheal, 1968) where it can survive for several months.

1.3.3 Anthropophilic Fungi

Anthropophilic species are primarily adapted for parasitism of man, with the 3 main genera involved in human infections being *Epidermophyton, Trichophyton & Microsporum spp.*. These do not form part of the natural fauna of the hair and skin and although humans are the primary host, there have been occasional reports of infections in animals (Kaplan *et al.*, 1957, Georg, 1960). An outstanding feature of these dermatophytes is the large number of species specifically adapted to parasitize humans, larger than the total number of species adapted to parasitize all other mammalia (Tanaka *et al.*, 1992).

These species are commonly associated with community living and transmission is from host to host via direct contact, or by indirect contact via the shedding of infectious propagules into communal areas such as swimming pools, changing rooms, barracks and dormitories. In closed communities, the use of common personal items can lead to the rapid spread of infection throughout that community.

T. rubrum is an extremely common cause of skin infections world wide, affecting all populations and ethnic groups (Rippon, 1985, De Vroey, 1985). It is becoming increasingly more prevalent in urban populations, particularly with populations that commonly wear tight fitting shoes and clothing, which maintain heat and humidity (Philpot, 1977).

T. tonsurans is another species which is emerging as a very common cause of tinea capitis in New Zealand, but due to its highly variable manifestations, infections are frequently misdiagnosed as other dermatologic disorders such as seborrhoea, dandruff and impetigo (Woodgyer, 1993).

Anthropophilic infections tend to produce less inflammatory and more chronic disease states, suggesting that these species are very well adapted to the human host, particularly by comparison to infections caused by most zoophilic or geophilic species (Sohnle, 1989). This aspect can make detection or correct diagnosis difficult and cases of "tinea incognito" are not unknown, with various patients being diagnosed as having bacterial infections. In two such cases the infectious agent was in fact found to be *T. verrucosum* and in others the agent has been found to be *T. tonsurans* (Woodgyer, 1993, 1994).

1.4 Keratin

Keratins are a major constituent of cells and tissues largely unreactive toward the environment and mechanically quite strong (Fraser *et al.*, 1973, Mercer, 1961) but also are substrates which dermatophytes and other keratinophilic fungi are able to utilise as a nutrient source.

They are structural proteins forming cytoplasmic intermediate filaments (IF) in cells such as those found in the epidermis. Keratin is not a single material but a complex of sulphur-containing proteins with a large number of cysteine residues initially existing in the thiol form but, during the keratinization process, these thiol groups are oxidised in pairs leading to the formation of diamino acid cysteine or disulphide bonds. It is thought these linkages are responsible for the insolubility of keratinized tissue and its resistance to enzymatic proteolysis. It is these linkages which are thought to be attacked by secreted proteases of keratinophilic fungi.

Other cytoplasmic intermediate filaments in vertebrate cells are vimentin and vimentin related filaments and neurofilaments. (Alberts *et al.*, 1994). In each type of IF the protein monomers involved are all highly elongated fibrous molecules having an amino-terminal head, a carboxy-terminal tail and a central rod domain. The central rod domain consists of an extended α-helical motif called the heptad repeat. This seven amino acid sequence motif promotes the formation of coiled-coil dimers between two parallel helices.

Although of common embryological origin and sharing a common structure, the keratins cover a very large range of amino acid compositions (Gillespie, 1990), and can be subdivided into two types: acidic (Type I) and neutral/basic(Type II) keratins. Keratin filaments are heteropolymers formed from equal numbers of type I and type II keratin polypeptides (Alberts *et al.*, 1994).

Keratins are commonly divided into three groups on the basis of the structures adopted by their various protein constituents rather than on their physical attributes. These are a) α-keratins which include "Hard" keratins such as hair, nails, claws etc. and the "Soft" epidermal keratins which form the stratum corneum, corns and callouses, b) β-keratins which are not naturally occurring but which are produced by the action of pressure and temperature on native α-keratins and c) "Feather" keratins, which include feathers, scales and also parts of beaks and claws (Parry, 1996).

1.4.1 Hard and Epidermal Keratin

X-ray diffraction patterns of hard and epidermal α-keratin have been interpreted in terms of filamentous assemblies of highly orientated molecules with conformations based on the right-handed α-helix.

Hard α-keratin IF are embedded in matrix proteins. The matrix consist of three families of proteins: those rich in cysteine residues (the high-sulphur proteins), those extremely rich in cysteine residues (the ultra high-sulphur proteins), and those rich in glycine and tyrosine residues (the high tyrosine proteins). The content and composition of the matrix proteins vary with source, age and nutrition (Parry, 1996, Fraser *et al.*, 1973, Mercer, 1961).

Although the matrix proteins as a group play an important role mechanically, especially in compression, it is the number and disposition of the covalent disuphide bonds formed within and between the matrix proteins and the terminal domains of the IF molecules that dominate the physical attributes of hard α-keratin.

There is no matrix as such in the epidermal keratins, but IF associated proteins (IFAP) such as filaggrin are responsible for aggregating IF into larger assembles that act as the functional unit *in vivo*.

1.4.2 β -Keratin

This particular form of keratin does not occur naturally but can be readily produced when mammalian hard α-keratin is stretched by about 100% in steam over the course of five to six hours. The specimen must then be held in this extended state in the presence of steam for a couple more hours in order to set the structure in the β form, and hence prevent its conversion back to the α-structure. This setting mechanism, in a modified and less severe form, is commonly used in the clothing industry and in hairdressing to maintain shape and form.

β-Keratin provides a neat structural link between the α-keratins and the feather structure. It has also provided insight into the relationship between the chain and molecular structure of epidermal keratin and its filamentous aggregate (IF) (Parry, 1996).

1.4.3 Feather and Reptilian Scale Keratin

The hard keratin in avian epidermal appendages such as feathers, beaks, and claws can largely be accounted for by a single protein species with a molecular weight of about 10.4 Kilodaltons (Kda). While this feature is dissimilar to that seen in the α -keratins, where distinct families of filament-forming and matrix proteins were identified, electron microscopy has shown that feather keratin also consists of filaments embedded in a "matrix", with filaments only about 3.3 nm in diameter. This has been confirmed by X-ray diffraction patterns. Feather keratin protein is unique, however, in that it forms both the filament and the "matrix".

The amino acid sequences of feather and scale keratin display a high degree of homology. But scale keratin molecules have a much higher molecular weight than those of feather keratin (15 Kda vs 10.4 Kda) and a much higher degree of lateral order.

The homology between feather and scale keratin implies that these two proteins have a common ancestor. Since scales occurred well before feathers in an evolutionary time scale, it is likely that feathers evolved from scales as a result of the deletion of the 4 x 13 residue motif found in scales but not in feathers (Parry, 1996).

1.4.4 Physical Properties of Keratins

Each of the keratinous tissues has unique features that enable it to function optimally, for example, the thermal insulation attributes of hair necessitate that it must be flexible and moderately extensible. In addition, water sorption properties are important. In contrast, it is easily seen that the hard α-keratin of rhinoceros horn (which is actually hair) and the epidermal keratin that forms the surface layer of skin must have quite different physical attributes. In contrast yet again, feather keratin must be light and inextensible without being brittle.

The hard α-keratins (but not the soft ones) both have a filament-matrix texture. The 10 nm -diameter intermediate filaments (IF) are of high elastic modulus and provide axial strength, and the matrix of low elastic modulus resists compression. This mixture allows stress in the tissue to be distributed uniformly and, in addition, provides a natural crack-resist mechanism.

The physical properties of keratins do not depend solely on the composite nature of the material. Other aspects that are important include the water content, the lipid content (particularly in epidermal keratins) and the composition and content of the proteins that constitute either the matrix or the proteins associated with the IF (the IFAP).

The highly disulphide-bonded structure of the α-keratins present a firm barrier to the environment, in contrast to the flexible barrier presented by the epidermal keratins. Superficially at least, the IF in the epidermis are similar to those in the hard α-keratins. But the N- and the C- terminal domains of the constituent molecules have very different characteristics, leading to the differing structures with differing physical attributes. The cysteines in hard α-keratins will form disulphide bonds to give rigid and tough structures whereas the glycine and serine residues in epidermal keratin will form many weak van der Waals interactions with other IF molecules and IFAP. The matrix of the hard α-keratins is absent in the epidermal keratins, affecting the properties of the latter and leading to a more flexible ultrastructure.

Orientation of the IF in the aggregates that form are also crucial to the function of a particular cell. For example, in wool and hair, they lie parallel to the axis of the fibre, yet in tactile whiskers a large fraction is tangentially disposed. In human nail the IF lie parallel to the surface but are perpendicular to the direction of growth, whereas in stratum corneum, the bundles of IF, which are roughly parallel to the skin surface, are woven into a fabric-like structure. Therefore orientation can play a leading role in specifying mechanical properties.

The natural resistance of keratin fibres to degradation by chemicals or enzymes is well known and related to their protective function in nature. It is the heterogeneous nature of keratins and the varying compositions of non-keratinous components which governs both the specificity of certain fungi to particular types of keratinous substrates and to the growth-pattern adopted. Furthermore, the structure of the keratin can be markedly altered by pre-treatment such as autoclaving e.g. hair and wool are richer in the "tougher" keratins than nail, hooves, horns and the stratum corneum of the skin.

All these factors make the choice of substrate to be used in studies of the legitimate breakdown of keratin difficult.

1.5 Colonisation of Keratins by Fungi

One of the distinctive properties of keratinophilic fungi is the ability to colonise and to breakdown keratin and there have been many investigations concerning the mechanisms involved. The colonisation process itself is a dynamic one with substrate, environment and fungal factors involved. This holds true where invasion involves a dermatophyte or a non-dermatophyte opportunist propagule. (Raubitschek, 1961, Kunert, 1972, Minocha *et al.*, 1972, Ruffin *et al.*, 1976, Meevootisom *et al.*, 1979).

During the initial phase of colonisation a propagule must make contact with a surface and adherence must occur in order for germination, colonisation and invasion to proceed. *In vitro* studies by Zurita *et al.*,(1987) showed that adherence of both saprophytic macro and microspores and parasitic arthrospores to human keratinocytes occurs in a time dependant fashion, with maximum adherence of both microspores and arthrospores occurring within 3 - 4 hours.

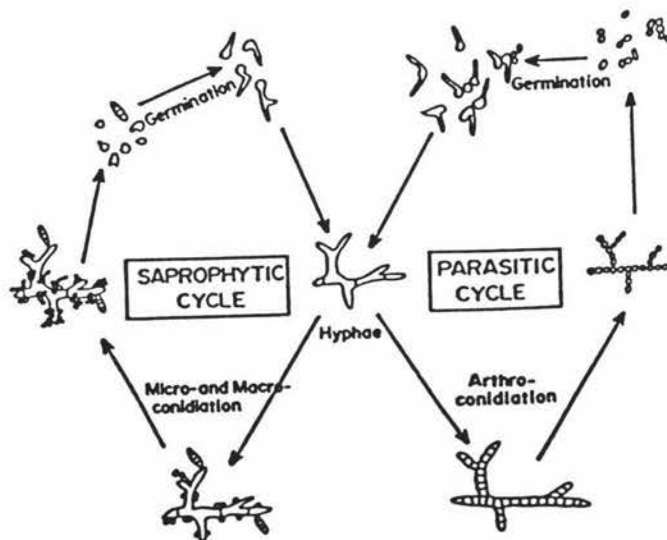
The adherence mechanism is not well understood due to a lack of satisfactory models with which to study early colonisation events (Bhattacharya *et al.*, 1998). However, it is known that adherence is enhanced by factors such as hydration, with water considered a suitable vehicle for the transfer of a propagule from host to host.

Work done by Tsuboi *et al.*,(1994) showed that with *T. mentagrophytes*, adherence appeared mediated by germling outer cell fibrils while further work by Ogawa *et al.*, (1998) demonstrated very close contact between dermatophyte arthroconidia and corneocytes in ultrastructural studies. Where contact is made, a fibrillar-floccular material, the nature and origin of which is yet to be determined, is seen in the space between the arthroconidial outer wall and the corneocyte membrane.

The above findings suggest that germination itself may be a virulence factor involved in disease initiation, particularly as a spore which fails to germinate will be removed by the normal and continuous desquamation of the epithelium. (Tsuboi *et al.*, 1994, Ogawa *et al.*, 1998).

In vivo dermatophyte infection usually follows indirect or direct contact with infected scales or hairs containing arthrospores and the chances of successful transmission is considerably improved by the prolonged viability of these arthrospores in exfoliated skin (Dvorak *et al.*, 1968, Sohnle, 1989). Successful invasion is followed by proliferation of the organisms with the extension of the infection into the surrounding area and at times from one structure to another e.g. skin to hair or nail.

Depending on the environment in which the fungus grows, one of two types of asexual propagule can be produced, the saprophytic and parasitic spores/conidia. Under non-parasitic conditions often two distinct spores are seen, saprophytic macrospores and microspores, both formed in the holothallic mode by conversion of a terminal or intercalary segment of a fertile hypha into a propagule surrounded by a multilayered wall. The parasitic arthrospores however, are formed in the thallic-arthric mode by consecutive segmentation and fragmentation of an existing hypha (Cole & Samson, 1979).



The asexual life cycle of the dermatophyte *T. mentagrophytes*. When fertile hyphae enter the saprophytic cycle, they proliferate by apical elongation and lateral branching forming macro- or microconidiogenous hyphae. Swelling of the apical tips of the conidiogenous hyphae is the initial event that leads to macro- or microconidium formation. Under parasitic conditions, fertile hyphae are repeatedly segmented and eventually fragmented to form arthroconidia.

Fig 1.1 Asexual cycle of *T. mentagrophytes.*

Fig by Emaynitoff and Hashimoto (Cole & Hoch, 1991)

The regulatory elements of development of one state in favour of another are largely unknown. However, it is recognised that the mechanism controlling saprophytic sporogenesis is suppressed during states of active invasion or parasitization of a host, with the production of parasitic arthrospores being the only asexual propagules formed in these circumstances (Cole & Hoch, 1991). It is notable, that while in theory infection can arise from the initial germination of either a saprophytic or parasitic propagule on a suitable host, it is commonly held that the arthrospore form is the major mediator responsible for disease transmission by anthropophilic dermatophytes.

However, infections caused by geophilic dermatophytes or by opportunistic non-dermatophytic keratinophilic fungi involves the uptake of a "saprophytic" propagule, e.g. infections caused by *M. gypseum*, a geophile, would almost certainly arise from the continued growth of a segment of vegetative hypha or the germination of a conidium on a suitable host. Although growth in the case of dermatophytes will be in the arthrospore form, in the case of opportunistic infections e.g. by *S. brevicaulis*, microscopic examination of samples directly from the lesion may reveal conidia, indicating that an "arthrospore" (or hyphal-yeast morphology such as that found in *Malasezzia furfur* and *Candida spp.*.) is not always required for fungal invasion.

Exposure to and uptake of an infectious fungal propagule by human or animal skin *in vivo* does not always result in infection and should infection occur, it may heal spontaneously or persist as a chronic infection. The type and severity of any given infection will depend upon the relationship occurring between the invading fungal species and host. The length and degree of infection is dependent upon the characteristics of the invading organism, the site of infection and both the innate and aquired components of the host immunological system (Sohnle, 1989, Ogawa *et al.*, 1998).

The stratum corneum itself *in vivo* represents an effective barrier to invasion by a fungus, with epidermal cell proliferation and keratinization processes presenting an important initial defence mechanism against infection. Furthermore, keratinocytes in the periphery of annular dermatophytosis lesions have been found to have an increased rate of cell division (Sohnle, 1989, Ogawa *et al.*, 1998). On the other hand, if infection has occurred the stratum corneum, due to its physical structure, may protect the invading organism from direct contact with effector cells of the immune system.

The major infiltrates of the cell-mediated immune response are generally confined to the dermis but some neutrophils and lymphocytes may enter the epidermis and are able to directly attack and kill pathogens by a variety of mechanisms e.g. the production of the antimicrobial protein, calprotectin which can inhibit fungal growth by competing with the microbes for zinc (Ogawa *et al.*, 1998).

Also found superficially are substances such as lipids containing saturated fatty acids that are active against organisms causing tinea capitis and others. More recently, the sphingosines have been characterized as an antimicrobial barrier in the skin (Ogawa *et al.*, 1998).

The invading organism must be able to obtain nutrients required for growth and the variety of proteinases, including keratinases, produced by dermatophytes, for example, facilitate penetration of the cornified tissues. In response to this, host proteins such as transferrin and complement contribute to resistance by restricting or inhibiting the growth of the organisms.

Cell-mediated responses can be an important line of defence with a number of cell types with immunological potential in the skin e.g. epidermal Langerhan's cells, dermal dendritic cells and epidermal T-lymphocytes, with these cells thought to interact with each other by means of surface markers, adhesion molecules, cytokines and eicosanoids to generate an inflammatory response. All of these aid in the development of resistance to infection.

However, defects in T-cell mediated immunity or in phagocytosis have been found to be important with fungal infection of all types, including dermatophytoses. Disease is more frequent and severe in patients with immunological defects (Ogawa *et al.*, 1998). Any number of systemic factors have been associated with decreased effectiveness of certain immunological functions, many of which are inheritable (Sohnle, 1989). Important also is the increasing incidence within the worldwide human population of AIDs. This condition involves the compromising of the immune system leaving victims vulnerable to secondary infection, a large number of which are caused by fungal agents, including superficial fungi (Porro *et al.*, 1997).

Overall, while deep infections with dermatophytes and opportunistic fungi do occurr, such infections are rare. Skin *in vivo* is a very effective barrier to invasion by these organisms with this barrier function consisting of a variety of components which provides an overall defence system, with the dual role of confining invading fungi to superficial epidermal sites and then eliminating them from the skin. Thus the body surface offers a variety of restraints on fungal growth *in vivo* which would not be operating *in vitro.*

The specialised category of anthropophilic dermatophytes, however, are particularly well adapted to the locations they parasitize as well as utilising keratins and other structural proteins as nutrient sources. They fulfill the requirements of a successful pathogen i.e. they have the ability to adhere to the stratum corneum or other surfaces, can penetrate those surfaces and can multiply *in vivo.* In addition to their thermotolerance and the ability to adapt to the physiochemical conditions of the host, these pathogenic fungi can also avoid the host defence mechanisms and so damage the host.

1.5.1 Invasion Processes

All dermatophyte species have the capacity to invade the stratum corneum of the epidermis and the follicular ostium of hairs while most other keratinophilic fungi have only limited ability to do so.

But in all, following the uptake, adherence and subsequent germination of an infectious propagule to a host substrate, colonisation of the affected substrate proceeds via both mechanical and enzymatic means. Several species exhibit differences in growth patterns thought to be related to differing nutritional factors and colonisation *in vivo* and *in vitro* can involve contrasing mechanical and enzymatic processes. The net result, especially in hair, can be very different.

1.5.1.1 Hair

The physical stages by which hairs are attacked *in vitro* by keratinophilic fungi are well documented (Barlow & Chattaway, 1955, Vanbreuseghem, 1950, English, 1963) with the principal mechanical mechanisms summarised as 1) cuticle lifting, 2) cortical erosion, 3) penetrating organs and colonisation of the medulla.

Following spore germination on or near a hair, germ tubes, following the edges of scales of the cuticle, encircle the hair, developing into a wide main hypha oriented longitudally along the hair. From this, lateral branches are sent out encircling the hair and arising from these lateral branches come flattened fronds of 'eroding mycelium' which penetrate between the cuticular cells and the outer layers of the cortex.

Immediately following lifting of the cuticle, cortical ersion occurs, carried out by the fronds of eroding mycelia with new fronds formed beneath the level of the original ones. Perforating organs, where formed, are basically a modified eroding mycelium consisting essentially of a column of short, wide cells, the lowest one cone-shaped and penetrating tangentially or radially into the hair, often projecting backwards from it.

In contrast, *in vivo* colonisation of the hair may occur in one of a number of invasion patterns. These patterns are commonly known as "Endothrix", where invasion by hyphae occurs within the hair shaft itself or "Ectothrix", where invasion also occurs within the hair follicle.

There are no eroding hyphae or perforating organs formed *in vivo* but the pattern of hair invasion along with the size of arthrospores formed by disarticulation of hyphae can often suggest the identity of the infecting agent.

1.5.1.2 Skin

The arthroconidia of dermatophytes appear able to adhere to all body surfaces including the face, back of hand, palm, leg and sole. Infection is established following contact, adherence and germination of a propagule. The germination of a suitable propagule may be seen by the formation of germ tubes and penetration longitudinally between layers and transversely between the thickness of the stratum corneum followed by the formation of hyphae.

In vivo the microscopic morphology of the invader is commonly of long, septate and branched hyphae which eventually disarticulate into arthrospores, completing the cycle. It is the horizontal extension which results in the clinically observable signs of peripheral expansion of the lesion. In contrast, *in vitro* colonisation leads to the development of the typical saprophytic morphology of the fungus (Baxter, pers. comm).

It should be recognised that the skin consists of two distinct parts, the epidermis or outer layer, which is the area to which invading dermatophyte species are generally physically confined and the dermis or inner layer. These are joined at the dermal-epidermal junction, via which *in vivo* the cells of the immune system must cross if they are to be effective. It has been observed that following the initial invasion of the skin, the fungus remains confined to the stratum corneum, while pathological changes occur in deeper layers of the epidermis and the dermis (Pilsbury *et a.,l* 1956), suggesting that pathological reactions are mediated by diffusible product(s) of the fungus. Cruickshank and Trotter, (1956) found that culture filtrates from *T. mentagrophytes* and *T. rubrum* affected bonds in the epidermal-dermal junction resulting in separation of the epidermis from the dermis. Thus the net result *in vivo* is the development of scales and vesicles.

1.5.1.3 Nails

The nails of humans are made up of a strong plate of hard keratin that grows out from a curved transverse groove. The floor of this groove is the germinal region of the nail plate and is therefore known as the nail matrix.

As the progeny of proliferating cells approach the dorsal surface of the nail, they are displaced distally and gradually transform into hard keratin, causing the nail plate to lengthen and strengthen (Cormack, 1993).

The horny layer of the distal nail plate and hyponychial groove may assist trapping of dermatophyte infective particles (Tsuboi *et al.*, 1994). The most common type of fungal nail infection is distal and lateral subungual tinea unguium, usually originating from fungal infection of the plantar and palmar surfaces of the feet and/or hands. Invasion of the hyponychial epidermis and subsequent invasion of the nail bed epidermis occurs. This in turn is followed by invasion of the most distal and lateral aspects of the nail plate (Ogawa *et al.*, 1998).

Infection of the nail bed epidermis results in thickening of the stratum corneum beneath the nail plate which causes a slight elevation of the nail plate from the nail bed. It is this change which clinically gives the involved nail an altered cream colour rather than the normal transparent appearance. Initally the dense keratin of the nail plate is not involved but becomes infected later (Ogawa *et al.*, 1998).

Dermatophytes tend to be orientated at random in hyponychial keratin, but lie parallel to the surface in the nail plate. Long septate and branched hyphae, with arthrospores, are the main microscopic features found in dermatophytic nail infections *in vivo*. Over time, dermatophytes create tunnels containing air within the nail plate and where the network of lacunae is sufficiently dense , the nail is opaque (Simpanya, 1994).

The dermatophytes commonly associated with tinea unguium are *T. rubrum* and *T. mentagrophytes*. The latter is normally seen only in toenail infections (Ogawa *et al.*, 1998). The main non-dermatophyte moulds involved in onychomycosis appear to be *S. brevicaulis & Scytalidium*, (Ellis, 1996) . However, there is considerable controversy on the significance of these moulds when identified in the presence of a dermatophyte (Ellis, 1996).

In vitro, nails are colonised by the 'saprophytic' phase of the fungus, although under certain cultural conditions, arthrospores can be induced (Baxter, pers. comm.).

1.5.2 Enymatic Invasion

1.5.2.1 Proteolytic Enzyme Production

The evidence for proteolytic enzyme involvement in the processes involved in colonisation of keratin has been established for some time (Davidson *et al.*, 1934). Observations of empty areas around perforating organs e.g. the mouth of a pit that is 2 or 3 times as wide as the actual organ within it, or a trough extending beyond the eroding mycelium it contains (English, 1963) are other examples.

Baxter & Mann (1961) examined the pattern of invasion of cut human hair *in vitro* by three dermatophytes (*T. mentagrophytes*, *T. rubrum and T. ajelloi*) and found variations in the keratinolytic ability of these species. *T. mentagrophytes* was the most keratinolytic and was seen to breakdown the hair keratin as evidenced by "gaps" around fungal structures. It has also been shown that the fungus was capable of causing extensive breakdown of keratin in the saprophytic stages and large numbers of granules and mitochondria were observed in the hyphae which were not observed in the other species. The authors suggested that this could be due to the higher enzymatic activity of this species. Mercer & Verma, (1963) looked at the invasion of sterile cut human hair *in vitro* by *T. mentagrophytes*. They also found that the process of hair invasion involved an enzymatic breakdown of keratin bundles with complete loss of the keratin.

Studies by Rashid *et al.*, (1996) confirmed the work of earlier researchers. *T. mentagrophytes* was seen to digest the endocuticle, causing detachment of cells from the underlying cortex.

Kunert and Krajci, (1981) studied the process of hair invasion by *M. gypseum in vitro* and found that the process of keratin degradation also had features of enzymatic breakdown, with some evidence of a mechanical effect of the hyphae on the cuticular cells.

The pattern of invasion of human hair by non-dermatophytic fungi shows many similar features. *Chrysosporium tropicum* is a soil-inhabiting fungus which when inoculated onto autoclaved hair formed perforating hyphae and showed all the characteristics suggestive of enzyme digestion (Yu *et al.*, 1972, A. Rashid *et al.*, 1996).

The breakdown of various compounds in keratin known to be recalcitrant to chemical proteolysis have been observed frequently and have confirmed that the dermatophytes posses the ability to degrade wool or hair and release sulphydryl compounds into the medium. When *T. rubrum & T. schoenleinii,* in separate experiments, were separated in the medium from ^{35}S radio-labelled wool by a permiable membrane Weary & Canby, (1969) showed that diffusable products produced by the fungi degraded the radio labelled wool

Further to this, other researchers were able to demonstrate keratinolytic abilities in a number of fungi e.g. Deshmukh *et al.,* (1982) were able to demonstrate via specrtrophotomeric methods, keratinolytic abilities in *Auxarthron conjugatum, Chrysosporium indicum, C. pannicola, T. ajelloi and M. gypseum in vitro* using human hair as the sole carbon and nitrogen source and later Apodaca *et al.,* (1989,1990) demonstrated, also via spectrophotomeric methods and SDS-PAGE, the keratinolytic abilities of *T. rubrum* in a keratin salts medium.

Evidence that a true secretion is involved has been established via measurements of alkaline phosphatase in culture filtrates (O'Sullivan & Matheson, 1971) as this enzyme can be used as an indicator of cell lysis. Studies have shown the appearance of the enzyme lags some 30 hours behind the appearance of keratinolytic proteases. When it appeared the levels were much less than those measured in cultures which had been allowed to proceed to the autolytic phase (O'Sullivan, 1970).

Following the confirmation of proteolytic enzyme production by dermatophytes, many researchers have reported purifying enzymes from a range of dermatophytes with many of the enzymes isolated found able to hydrolyse various proteins such as casein, elastin, collagen and keratin e.g Yu *et al.,* (1969) using spectrophotometric methods were able to measure the keratinolytic activity of proeteinases of *T. mentagrophytes* which was found able to hydrolyse guinea pig hair.

Further to this, other researchers have begun characterisation of enzymes produced in investigations of these proteinases as virulence factors during the pathogenesis of infection. (Simpanya, 1994, Palmer, 1995).

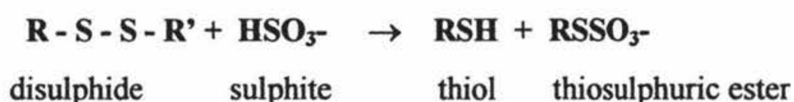
A number of these may be serine proteinses, which act by forming covalent complexes between the enzyme and substrate with the hydroxyl (OH) group from the serine amino acid making a nucleophilic attack on an acyl of the substrate leading to the formation of an ester bond between enzyme and substrate. Via hydrolysis this ester bond is broken along with the peptide bond present in the protein. In one recent example Mignon *et al.*, (1998) isolated via affinity chromatography and SDS-PAGE techniques a 31.5 kda protease from a clinical isolate *M. canis.* This protease was found to belong to the subtilisin-like serine protease family.

Some are cysteine proteinases which also form covalent bonds between enzyme and substrate but the attacking nucleophile is a sulphur atom from the cysteine side chain, with the reactions occurring being the same as those occurring for serine proteases.

Aspartic proteinases and metalloproteinases do not form covalent intermediates, with the catalytic apparatus of the aspartic proteinases thought to be two aspartic acid side chain residues with the carboxyl groups thought to cause cleavage of the peptide bond at low pH e.g.3-5. Metalloproteases however, contain a metal ion such as zinc which provides a strong electrophilic pull which participates in the attraction of a water molecule causing cleavage of the peptide bond (Stryer, 1995).

Evidence suggesting that the primary attack of dermatophytes on keratin rich material is not on keratin itself but on more readily degradable substances has been reported (Raubitschek, 1961, Ruffin *et al.*, 1976). Stahl and co-workers (1950) showed that the first wool proteins degraded by *M.gypseum* are the non-sulphur containing molecules. This was later confirmed by Hose (1976) who showed that release of ^{35}S from guinea pig hair previously radio-labeled *in vivo* occurred after the maximum release of peptide material assayed by chemical methods (Odds, 1991).

Substrate reduction as a key reaction in keratinolysis with thiosulphuric ester synthesis, has been deduced from histochemical tests made in hair perforations (Kunert, 1972) and has been shown to be a key reaction in the degradation of keratin by dermatophyte species. Synthesis of thiosulphuric esters from the disulphide bonds of the keratin can be explained by sulphitolysis reactions (Swan, 1957).



$$\mathbf{R\text{-}S\text{-}S\text{-}R' + HSO_3^- \rightarrow RSH + RSSO_3^-}$$

disulphide sulphite thiol thiosulphuric ester

According to the above equation, cystine (Cy - S - S - Cy) would be converted to S - sulphocysteine (Ruffin *et al.*, 1976, Kunert, 1972).

As long as sulphite is produced from protein-incorporated cystine, the disulphide bonds of keratin can be split according to the above equation and keratin denatured in this way.

However, regardless of the mechanism used, it is clear that the lytic capability of dermatophytes to produce proteinases for keratin substrates goes beyond that of other fungi. Chesters and Mathison (1963) showed that *T. ajelloi* could release amino acids from animal wool whereas trypsin had almost no activity in that assay. Other studies have provided strong indications of a molecular basis for host specifities and affinities between dermatophyte species and keratinaceous substrates (Odds, 1991).

Also under investigation is the number of proteases produced by a strain or strains of a species at any point in time, with some schools of thought indicating that a set number of enzymes are produced for a given strain (Simpanya, 1994, Palmer, 1995) while other researchers have found that the number of enzymes produced per strain will vary depending upon the culture conditions (Tucker *et al.*, 1991, Sparkes *et al.*, 1994, Papini *et al.*, 1996)

Some of the above findings strongly suggest that fungi are able to quickly adapt to given environmental conditions although little is known about the regulation of protease expression. Clues to regulation have been sought from the more extensive examination of proteinase regulation in other filamentous fungi and this led to the proposal of a model for the regulation of *T. rubrum* proteolytic activity by Apodaca and McKerrow (1989). This model suggests that whenever the fungus lacks carbon, nitrogen or sulphur, a group of general proteinases is expressed which initially act upon the non-keratinous proteins within the substrate, providing the above elements, which in turn has a feed back effect repressing general proteinases with keratinases induced by the presence of insoluble proteins such as keratin.

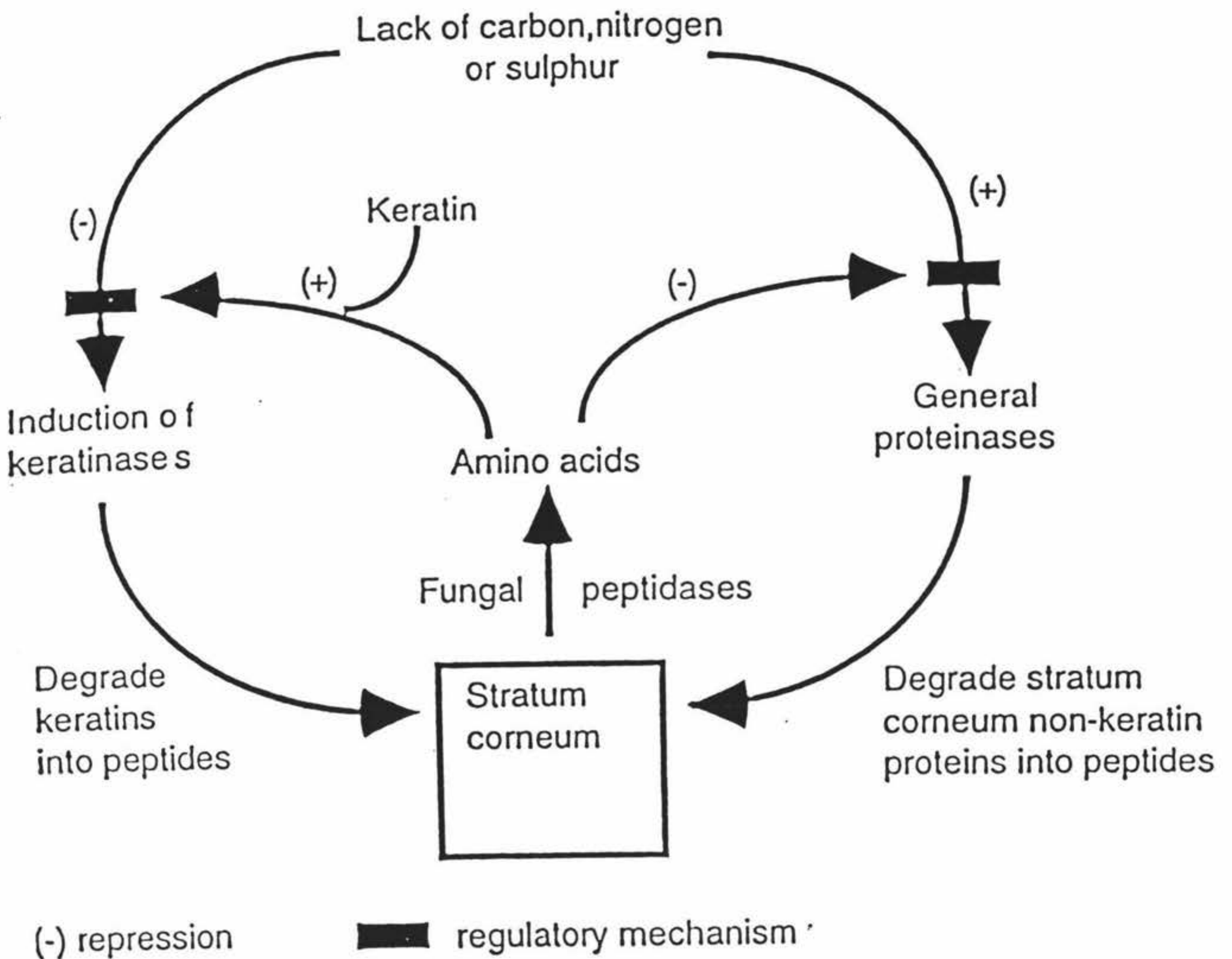


Fig 1.2 Proposed model for regulation of proteolytic activity by *T. rubrum*.

Apodaca & McKerrow (1989) also proposed that there were differences between the proteolytic enzyme expression between log and stationary phase cultures with those initially produced acting upon the non-keratinous proteins in the substrate. Once the stationary phase of growth occurs, proteinases are expressed constitutively, coinciding with disease which may directly or indirectly incite a host response such as inflammation & dermatophytosis.

While substrate specificity and regulation of proteolytic enzyme production is not well understood, the model proposed by Apodaca & McKerrow allows that the fungus is able to respond to its immediate environment, altering the initial production and secretion of proteases such that the immediate nutritional needs of the fungus are met from the substrates most readily available, as opposed to the production of one rigidly defined group of proteases.

1.5.2.2 SDS-PAGE Technique for Enzyme Analysis

Of the various techniques which can be used in enzyme analysis work, polyacrylamide gel electrophoresis (PAGE) has been used by a number of investigators (Takiuchi *et al.*, 1983,1984, Sanyal *et al.*, 1985, Apodaca *et al.*, 1989a, 1989b, 1990, Brahim-Granet *et al.*, 1996.). This technique gives good resolution of separate proteins obtained from samples of only 5 - 25 μg. Electrophoretic methods are able to simultaneously exploit differences in the molecular size of the protein involved and, if appropriate to the work being done, the charge on the enzyme/s of interest. Therefore PAGE separates out proteins on the basis of either size, charge or both.

In the past, high resolution has been achieved via disc electrophoresis utilising a synthetic gel medium of cross-linked polyacrylamide and systems of discontinuous buffers (Ornstein *et al.*, 1964, Arcus, 1970). Nowadays, the electrophoretic method most commonly used is thin-slab gel electrophoresis, on which several samples can be run at the same time allowing for a direct comparison of mobility (Scopes, 1987, Osterman, 1984). PAGE is advantageous as it can be carried out at a wide range of temperatures, and environmental conditions can be specified such that minimal damage occurs to the enzyme/s being examined.

The studies which have been reported utilise denaturing gels, the enzyme extract being treated with sodium dodecyl sulphate (SDS) prior to electrophoresis, so conferring an overall negative charge on the enzyme/s, with subsequent separations using this method thus carried out on the basis of molecular weight. This high-resolution method has two major advantages compared to native electrophoresis. One is that aggregates and insoluble particles which often cause bad results with native gels are solubilised by the SDS, the other is that mobility is related to polypeptide size, so an immediate indication of the molecular weight for each component is provided (Scopes, 1996).

Of particular value is the ability to utilise substrate co-polymerised polyacrylamide gels. These are a modification of Laemmli's (1970) procedure whereby suitable substrate is co-polymerised with the gel in order to assist visualising of enzyme activity. Again enzyme activity is localised by molecular weight.

These gels differ from the standard SDS-Laemmli gels in two ways (1) as mentioned earlier, the gels are made incorporating a suitable substrate of interest e.g. gelatin, within the polyacrylamide matrix and (2) the sample is mixed with a higher concentration of SDS to which is added sucrose and bromophenol blue as a tracking dye.

Despite the fact that the potential of separation of proteins of identical size is not possible with this system, it does give the sharpest overall resolution and cleanest zones of any method and by making a comparison with a mixture of standard polypeptides of known molecular weight, the whole gel can be calibrated in terms of mobility against size (Scopes ,1996).

There are no reducing agents such as β-mercaptoethanol or dithiothreitol added to the samples being analysed, nor are samples boiled, leading to irreversible denaturation of the enzymes of interest. However, standard polypeptides of known molecular weight are prepared using β-mercaptoethanol and boiled for approximately five minutes, then run on the same gel as the protein sample. This allows for the simultaneous detection of the enzymes of interest, along with determination of their molecular weight.

Following electrophoresis, the SDS is removed and the gel washed in the non-ionic detergent triton X-100 for 2 hours to allow for renaturation of the enzymes. (Heussen *et al.*, 1980, Asahi *et al.*, 1985) The gels are then incubated in a suitable buffer allowing for the breakdown of the gelatin by enzyme activity for 12 to 24 hrs., after which the protein band must be visualised.

Visualisation is commonly done by staining with dye of either the protein/s of interest (positive staining) or staining of the gel background with no staining of the protein/s of interest (negative staining) e.g. coommassie brilliant blue R-250 can be used, utilising a negative staining technique in which the main body of the gel retains the stain while areas where proteolytic activity occur in the gel matrix do not, leaving cleared areas visible as bands on the gel. Following the construction of a standard curve using the known standards, the molecular weight of each band can be calculated.

AIMS

1. To compare mycelial morphology of keratinophilic fungi in relation to spore production in stationary and shake culture and assess the possible establishment of a "pseudo-parasitic" condition.

2. To extend earlier studies of Simpanya (1994) and Palmer (1995) concerning proteolytic enzyme production by *Microsporum spp.*. to a range of keratinophilic fungi, dermatophytic and non-dermatophytic, to investigate and possible similarities in relation to keratin breakdown.

3. To assess the reproducibility of the substrate co-polymerised SDS-PAGE technique in the investigation of proteinases; to refine the technique as necessary and enquire into the consistency of results.

4. To investigate the influence of medium and culture conditions on proteinase production, particularly using wool containing media.