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Purification, characterization and analysis of the regulation of three exocellular proteinases from Trichophyton rubrum.

by

Gerard Apodaca

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

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of the

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by
Gérard Apodaca

dedicated to:

my mother and best friend Thérèse Apodaca; my family, Silviano, Christine and Francine; my dearest friends Dan and Geovany

acknowledgments

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ABSTRACT

Purification, characterization and analysis of the regulation of three exocellular proteinases from *Trichophyton rubrum*. - Gérard Apodaca

Trichophyton rubrum is the most common dermatophyte of man and normally colonizes the superficial layers of the epidermis (stratum corneum). Three secreted proteinases with a possible role in the metabolism of host proteins have been purified from conditioned medium (CM) of this fungus. Gel filtration chromatography and chromatofocusing were used to purify two proteinases of M_r 71,000 and 93,000. Both of these enzymes existed as dimers of a smaller subunit, M_r 36,000 and 44,000 respectively, and were serine proteinases with a pH optima of 8.0 and a calcium dependence of 1 mM. They degraded azocoll, elastin, keratin and synthetic peptide substrates with hydrophobic amino acids in their P-1 site, e.g. succinyl-alanyl-alanyl-prolyl-phenylalanyl-p-nitroanilide (pNA). An additional proteinase of M_r 27,000 was purified from *T. rubrum* CM by concanavalin A affinity chromatography followed by anion exchange chromatography. This proteinase had a reduced molecular weight of 44,000, was also a serine proteinase with a pH optimum of 8.0 and degraded azocoll and the tetrapeptide substrates succinyl-alanyl-alanyl-prolyl-phenylalanyl-pNA and t-boc-alanyl-alanyl-leucyl-pNA. The M_r 27,000 proteinase degraded type III collagen, type IV procollagen, laminin and fibronectin, but not types I and V collagen.

The regulation of these enzymes and their role in fungal metabolism was studied at the biochemical level. General proteolytic (azocollytic) activity was repressed when log phase cultures of *T. rubrum* were grown in a minimal medium that contained readily metabolized sources of carbon, nitrogen, sulfur and phosphorous. When either carbon, nitrogen or sulfur was deleted from this minimal medium, azocollytic activity was derepressed. In all cases a high molecular weight activity (> M_r 200,000) was expressed. The M_r 71,000 proteinase was observed in nitrogen depleted cultures, and proteolytic species of M_r 124,000 and 27,000 were secreted in sulfur depleted cultures. The addition of either inorganic ($MgSO_4$, Na_2SO_3, NaS_2O_3) or organic (methionine, cysteine) sulfur to the sulfur depleted medium repressed the expression of azocollytic activity. Glucose repressed azocollytic and elastinolytic activity in log phase cultures of fungus grown in a medium where a single protein served as the sole source of carbon and nitrogen. In contrast, keratinolytic activity was induced when either a protein source was included in the minimal medium, or 0.5% (w/v) glucose was added to a neopeptone or protein medium. Stationary phase cultures of *T. rubrum* secreted all proteolytic activities

constitutively. Unlike log phase cultures, total azocollytic, elastinolytic and keratinolytic activity in the stationary phase cultures fell in the carbon, nitrogen and phosphorous depleted media, but these activities remained high in the minimal and the sulfur depleted media. Stationary phase proteinases were not repressed by the addition of glucose to the fungal medium.

The following model is proposed for the regulation of *T. rubrum* proteolytic activity. In the initial stages of infection, *T. rubrum* grows logarithmically. In this state, proteolytic activity is derepressed whenever carbon, nitrogen or sulfur is lacking in the fungal milieu. The general proteinases produced could act on the non-keratinous proteins in the stratum corneum. There are probably peptidases, as yet unidentified, that would cleave the peptides generated by the initial proteolysis into amino acids. These amino acids would provide the cell with a source of carbon, nitrogen and sulfur. Under these conditions, the expression of general proteinases would be repressed while specific keratinases would be induced in this nutrient rich environment. Disease may occur when the fungus reaches stationary phase, when proteinases are secreted constitutively. These enzymes may directly or indirectly incite a host response, resulting in the inflammatory manifestations of dermatophytosis. Fungal proteinases could also facilitate the disruption of connective tissue barriers in the rare cases of disseminated disease.

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I. INTRODUCTION

1.1 The Dermatophytes and the Diseases They Produce

The dermatophytes, or literally the "skin plants", are a group of fungi that parasitize the keratinized tissues, i.e. skin, hair and nails, of humans and animals. This group is comprised of three genera: *Epidermophyton*, *Microsporum* and *Trichophyton*. The asexual or telomorph stages of these genera have been classified in the following manner (Matsumoto and Ajello, 1987):

Kingdom	Fungi
Phylum	Eumycota
Subphylum	Deuteromycotina
Class	Hyphomycetes
Order	Hyphomycetales
Family	Moniliaceae
Genera	*Epidermophyton, Microsporum, Trichophyton*

These genera are distinguished by their characteristic large, asexual spores or macroconidia. The macroconidia of the genus *Epidermophyton* are club shaped, smooth walled, multiseptate, and borne in clusters. Members of this genus do not produce the small, asexual spores or microconidia characteristic of the other two genera. The macroconidia produced by the species of the genus *Microsporum* are elliptical to fusiform in shape with echinulated, thick cell walls; their microconidia are small and unicellular. The species of the genus *Trichophyton* produce large, pencil shaped macroconidia which are septate and have smooth walls. The microconidia are pyrical or clavate in shape. The species of these genera are differentiated from one another by growth patterns, mycelial and spore morphology, and their requirements for certain nutrients, e.g. thiamine and histidine.

Additionally, these fungi are subdivided into habitat groups depending on the natural reservoir for the species (Table 1). Geophilic species, such as *M. gypseum*, usually live in the soil and rarely cause disease in animals or humans. *M. canis* is a zoophilic organism whose natural reservoirs include dogs and cats. Human infections caused by dermatophytes in this group are usually acute, inflammatory and do not recur. Anthropophilic species, such as *T. rubrum*, are normally isolated from, and parasitize humans. Some anthropophilic species, e.g. *M. audouinii*, appear to be solely transmitted from human to human (Joklik et al., 1984). Infections by anthropophilic species are usually chronic and tend to recur. The zoophilic and anthropophilic dermatophytes are

Anthropophilic	Zoophilic	Geophilic
dermatophytes with a worldwide distribution		
E. floccosum	*M. canis*	*M. cookei*
M. audouinii	*M. equinum*	*M. gypseum*
T. mentagrophytes var. *interdigitale*	*M. gallinae*	*M. fulvum*
T. rubrum	*T. equinum*	*M. nanum*
T. tonsurans	*T. mentagrophytes* var. *mentagrophytes*	
T. violaceum	*T. verrucosum*	
T. shoenleinii		
dermatophytes with a limited geographic distribution		
M. ferrugineum (Africa, Asia, Europe, Far East)	*M. distortum* (Australia, New Zealand)	*M. racemosum* (Europe, N. and S. America)
T. concentricum (C. and S. America, Far East)	*T. mentagrophytes* var. *erinacei* (New Zealand, Europe)	*M. vanbreuseghemii* (Africa, Europe, N. America)
T. gourvilli (Africa)	*T. mentagrophytes* var. *quinckeanum* (England, New Zealand)	
T. megninii (Europe, Asia, Africa)	*T. simii* (India)	
T. soudanense (Africa, Europe, N. America)	*M. persicolor* (Europe)	
T. yaoundei (Africa)		

Table 1. Ecology of the Dermatophytes.

neither isolated from, nor can they live in soil. Although several species of dermatophytes exist, only a few are responsible for disease in humans.

The diseases caused by dermatophytes are referred to as dermatophytoses or ringworm infections. The dermatophytoses are subdivided into various tineas, describing the part of the body infected. For example, tinea pedis is a ringworm infection of the foot. The tineas, and the fungi responsible for these diseases, are described in Table 2. It should be noted that the majority of human disease in the U.S., and other parts of the world, is caused by *T. rubrum* (Rippon, 1982; Georg, 1960). Blank and Mann (1975) found that 79.2% of their cases of dermatophytoses in Philadelphia, PA were caused by *T. rubrum*. In a nationwide survey of infections caused by dermatophytes, 46.8% of the isolates were found to be *T. rubrum* (Sinski and Kelly, 1987). *T. rubrum* usually infects men more often than women and is responsible for most cases of tinea corporis, cruris, pedis, manuum and unguium (Rippon, 1982).

1.2 Proteolysis of Keratins and Associated Proteins

Because *T. rubrum* parasitizes the nutrition-poor cornified tissues of humans , I and others, have proposed that this organism, and dermatophytes in general, requires proteinases to cleave available proteins into metabolically useable nitrogen, carbon and sulfur. Keratins are the major constituent of the stratum corneum, hair and nail. The keratins are highly disulfide bonded, insoluble in aqueous solutions, and highly resistant to proteolytic attack (Noval and Nickerson, 1959; Sun and Green, 1978; Moll et al., 1982; Lynch et al., 1986; Nagle, 1988). The hair is made-up primarily of cortical cells filled with 10 nm keratin filaments and an associated interfilamentous matrix. There are three groups of wool proteins. A small molecular weight group of "high glycine/tyrosine proteins" (M_r 6,000-9,000) which can be further subdivided into proteins that are poor in cystine but rich in phenylalanine, and into a another group with proteins that are poor in phenylalanine but rich in cystine. The low molecular weight proteins along with a group of "high sulfur proteins" (M_r 10,000-25,000) are thought to comprise the interfilamentous matrix. These high sulfur proteins can be subdivided into three major subfamilies of M_r 11,000, 16,000 and 19,000. Finally, there is the group of "low sulfur keratins" that are believed to form the actual 10 nm keratin filament. There are two subfamilies of these low sulfur proteins, based on their relative charge and immunoreactivities: the acidic, type I keratins and the basic, type II keratins. It is probably required that at least one basic and one acidic subunit be expressed for filament assembly to occur (Lynch et al., 1986). Human hair is composed of a triplet of basic, low sulfur keratins (M_r 56,000/59,000/60,000) and an acidic pair of

Clinical Disease	Site of lesions	Dermatophyte involved
Tinea capitis (epidemic)	scalp	*M. audouinii, T. tonsurans, T. violaceum, M. ferrugineum*
Tinea capitis (non epidemic)	scalp	*M. canis, T. verrucosum, M. gypseum*
Tinea favosa	scalp, torso	*T. schoenleinii, T. violaceum*
Tinea barbae	beard	*T. mentagrophytes, T. verrucossum T. rubrum*
Tinea corporis	arms, legs, torso	*T. rubrum, M. canis, T. mentagrophytes*
Tinea cruris	groin	*T. rubrum, T. mentagrophytes E. floccosum*
Tinea pedis and manuum	feet, hands	*T. rubrum, T. mentagrophytes E. floccosum*
Tinea unguium	nails	*T. rubrum, T. mentagrophytes,*
Tinea imbricata	torso	*T. concentricum*

Table 2. Clinical diseases associated with dermatophytic infections.

M_r 44,000/46,000 (Lynch et al., 1986). The high sulfur proteins are found in human hair but the high glycine/tyrosine proteins are not.The major proteins of human nail include those found in human hair and a small but significant (10-20%) amount of keratins usually associated with the "soft" keratins of epidermal tissues (Lynch et al., 1986). The soft keratins include 18-19 different species of M_r 40,000-70,000 and like all keratins these molecules can be subdivided into acidic, type I and basic, type II subfamilies. The keratins of the stratum corneum include major species of M_r 63,000 and 55,000 and several minor species of M_r 40,000-53,000. The keratins account for approximately 95% of the protein contained in this layer of the epidermis and require urea and β-mercaptoethanol to be extracted (Sun and Green, 1978). The other 5% of proteins include a number of non-keratinous proteins of M_r 10,000-68,000 that can be easily extracted with aqueous buffers. The water soluble proteins and nutrients from hair and nails can support the growth of dermatophytes (Raubitschek, 1961). Nevertheless, several investigators, including myself, have looked for proteinases that can degrade keratins as well as the other proteins.

The ability to degrade keratin is restricted to a few insects, bacteria and fungi. The chewing lice of birds (*Mallophaga*), the larvae of some dermestid beetles and the larvae of a few moths, in or related to the family tineidae, are arthropods capable of digesting keratin (Waterhouse, 1957). The digestion of keratin by the demestid and *Tineola* larvae may be related to the reducing conditions present in the midgut of these organisms (Waterhouse, 1957). Once the keratin enters the midgut region its disulfide bonds are broken, and proteinases are then able to act upon the denatured keratin. The proteinases required do not have to be specific to keratin as trypsin will act on the denatured substrate.

Keratin decomposition has also been described in the bacterium *Streptomyces fradiae* (Noval and Nickerson, 1959). When this organism was cultured in a wool containing medium it was able to degrade 90% of the wool substrate in 30 days. Other actinomycetes also had significant activity against wool including *S. griseoluteus*, *Nocardia polychromogenes*, and to a lesser extent *S. griseus* (Noval and Nickerson, 1959). Importantly, the conditioned medium (CM) from *S. fradiae* cultures had little ability to release sulfhydryl containing material from the wool when compared to cells grown directly in contact with the substrate. The authors suggested that there was a reducing mechanism on the surface of the bacterium that was able to disrupt the disulfide bonds present in keratin, leaving a denatured substrate upon which proteinases could act. However, it was later found that a keratinase could be purified from the CM of *S. fradiae* (Nickerson et al., 1963; Nickerson and Durand, 1963). This enzyme digested one third the weight of undenatured wool in twenty four hours. This enzyme did not release sulfhydryl containing

peptides suggesting that it may represent an activity different than that originally described. The crystallized enzyme had a pH optimum of 9.0 and also degraded casein, poly L-lysine and denatured hemoglobin.

Keratinolytic activity has also been described in other bacteria including *Pseudomonas aeruginosa* (Burrell and MacDiarmid, 1984) and various species of cutaneous and oral bacteria including *Staphylococcus epidermidis, S. haemolyticus, Brevibacterium linens, B. epidermidis, Bacteroides gingivalis* and *Treponema denticola* (Mikx and DeJong, 1987). In the case of the normal flora bacteria, keratinolytic activity was assayed using fluorescein isothiocyanate(FITC)-labeled epidermal keratin. The keratin used in these experiments was extracted in a way that would preserve its native structure. There was little degradation of the keratin unless the reaction mixture included the actual bacterial cells and a reducing agent, dithiothreitol (DTT). This data suggests that the keratinolytic activity is in someway associated with the bacterial cell surface, and that either there is a proteinase which requires DTT as an activator, i.e. like thiol proteinases, or that the keratinolytic activity requires a partially denatured substrate. Although these two explanations are not mutually exclusive, it seems the latter is more likely. If the keratin was added directly to the reaction mixture there was a lag of two hours before the release of FITC occurred; however, if the keratin was preincubated with 1 mM DTT before addition to the reaction mix then keratinolysis was linear throughout the incubation. Do these bacteria degrade keratin *in vivo*? That is not clear; however, the bacteria that possess activity against keratin can be found in locations, around sebaceous follicles and periodontal pockets, where low redox potentials are thought to exist (Noble, 1984). Under these reducing conditions, the keratins may be susceptable to proteolysis.

Other members of the normal flora of humans also possess keratinolytic activity. *Candida albicans* can be cultured in a medium with keratin as the sole source of nitrogen (Kapica and Blank, 1957). However, in these experiments the keratin was autoclaved and may have been partially denatured by this process. More recently, Hattori, et al. (1984) reported the existence of a pepstatin A- and chymostatin-sensitive proteinase in the CM of *C. albicans* that could cleave the α-fibrous protein extracted from human stratum corneum. Negi and his coworkers (1984) have also purified a carboxy proteinase with activity against the water insoluble fraction prepared from human stratum corneum. This M_r 42,000 proteinase has little activity against fingernails and showed no digestion of human scalp hair. When *C. albicans* blastoconidia adhere to the stratum corneum they produce cavitations in the keratinocytes (Ray and Payne, 1988). This effect is abrogated by the addition of pepstatin A, arguing that a carboxyproteinase may be responsible for the depressions seen in scanning electron micrographs. Although close contact of the

blastoconidia and keratinocyte are presumably necessary for cavitation to occur, the carboxyproteinase activities are unlike the bacteria of the normal flora that have been described in that proteolysis of keratin does not require the intimate contact of cells with substrate.

1.3 Dermatophytic Fungal Proteinases

Early work with the dermatophytes demonstrated that the following organisms digested ethylene oxide sterilized wool, releasing sulfhydryl containing components into their medium: *M. canis, M. gypseum, T. schoenleinii and T. rubrum* (Weary et al., 1965; Weary and Canby, 1967). Two strains of *T. mentagrophytes* had little keratinolytic activity. When the wool fibers from the *T. rubrum* cultures were examined microscopically it was found that the cuticles of the hair fibers remained intact, while the central, medullary region of the hair shaft was dissolved. The wool fibers from the *T. schoenleinii* cultures were randomly fragmented, with erosion of the fiber shaft. When strains of *T. rubrum* and *T. schoenleinii* were separated from radioactively labeled wool by a permeable membrane, the fungi produced a diffusible activity that could degrade the radioactive substrate (Weary and Canby, 1969). Although these results were suggestive that dermatophytes could indeed degrade keratin, and that this activity was mediated by a diffusible substance, no attempts were made to characterize the keratinolytic activity.

A proteinase with approximate molecular weight of 48,000 was later purified from the CM of *T. mentagrophytes* (Yu et al., 1968; Yu et al.1969a; Yu et al. 1969b). It had a pH optimum of 7.0 and degraded native hair, casein, collagen, elastin, gelatin and a host of other proteins, amides and peptides. The class of proteinase can not be determined from the data presented. The enzymatic activity was somewhat inhibited by ethylenediaminetetraacetic acid (EDTA; 30% less than controls) but no other inhibitors were tested. When hair, treated with the purified keratinase, was examined microscopically it was found that the cortex contained many fissures and the medulla was digested. These results suggested that, in the case of *T. mentagrophytes*, an actual proteolytic enzyme could be purified with keratinolytic activity and that unlike some bacterial keratinolysis there is no need for a direct contact between the cell and its keratin substrate.

Two additional proteinases have been described in *T. mentagrophytes* (Yu et al., 1971). These "cell bound" keratinases were purified from cultures of mycelia that were grown up in a horse hair medium, washed, then allowed to sit for one hour in a pH 7.8 phosphate buffer containing 1 M NaCl. The proteinases were purified from this salt wash. Both of these enzymes degraded guinea pig hair and had estimated molecular weights of 440,000 and 20,300. No attempt was made to determine the class of these proteinases.

Lastly, antisera prepared against these keratinases, and the original M_r 48,000 protein, cross-reacted with one another, but there were no lines of identity in an Ouchterlony assay.

A keratinolytic activity with an acidic pH optimum of 4.5 has recently been partially purified from the CM of keratin grown *T. mentagrophytes* (Tsuboi et al., 1987). The class of this proteinase is not clear as both phenylmethylsulfonylfluoride (PMSF) and EDTA are inhibitory. The activity may be due to both a serine- and metallo-proteinase, or a serine proteinase with a requirement for a divalent cation.

Keratinolytic activity has also been described in *T. gallinae* (Wawrzkiewicz, 1987) and *M. canis* (Takiuchi et al., 1982; Takiuchi et al., 1984). The enzyme from *M. canis* has an estimated molecular weight of M_r 45,000 and is strongly inhibited by 10 mM PMSF. Interestingly, antisera prepared against this proteinase showed that there were immunologically related materials contained in the CMs of *M. gypseum, T. mentagrophytes* and *T. rubrum* (Takiuchi et al., 1983).

Finally, several proteinases have been described and purified from the CM of *T. rubrum*. Meevootisom and Niederpruem (1979) have shown that the CM of keratin grown *T. rubrum* contained proteolytic activity capable of degrading casein, bovine serum albumin, collagen, elastin, guinea pig hair and keratin. However, the incubations were carried out at 45° C; the physiological significance of substrates degraded at this high temperature are questionable, especially for a substrate like collagen with a melting temperature around 37° C. Nonetheless, all of the activities had pH optima of 7.0, with the exception of keratin degradation which showed a pH optimum of 8.5. All of these activities were inhibited by PMSF. A serine proteinase with a molecular weight of M_r 35,000 has been purified from the CM of Sabouraud dextrose broth-grown *T. rubrum* (Sanyal et al., 1985). This enzyme has an alkaline pH optimum and degrades casein, azoalbumin and α-N-benzoyl-L-arginine ethyl ester. This proteinase has no activity against defatted human hair. Lastly, two proteinases with keratinolytic activity have been purified from the CM of *T. rubrum* (Asahi et.al., 1985). These serine proteinases have molecular weights of M_r 44,000 and 36,000 under reducing conditions and their purification, along with that of a previously uncharacterized proteinase, will be described in the following chapter.

It appears that a specialized group of organisms have the ability to catalyze the decomposition of keratin. However, the method of keratinolysis differs from one group of organisms to the next. In the insects, it is thought that the low redox potential of their midgut region promotes the reduction of the disulfide bonds present in the keratins. This denatured substrate can then be acted upon by proteinases. The requirement for a partially denatured keratin substrate may also be true of the normal flora bacteria, as they do not degrade keratin unless a reducing agent is present. The cells must be in close contact to the

substrate for maximal activity to be expressed. The soil bacterium *S. fradiae* may have two systems for the degradation of keratin. One system, like that of the oral and cutaneous bacteria, requires intimate contact of bacteria with substrate; it releases sulfhydryl containing peptides. The other system of proteolysis utilizes an enzyme that does not require the presence of cells, and releases products of hair that do not contain sulfhydryls. The fungi, *C. albicans* and the dermatophytes, secrete keratinases and do not appear to require the apposition of cells and substrates. Because the dermatophytes live in direct contact with their substrate, keratinolysis *in vivo* may involve both the enzymatic degradation of keratin or the denaturation of substrate, either through reduction or mechanically by the growth of hyphae into tissues.

1.4 Possible Role for Proteinases in *T. rubrum* Infections

What is the significance of the proteinases of *T. rubrum* and other dermatophytes? It seems that proteinases are important in two respects; for the organism, the enzymes could free up nutrients from their nutrition-poor environment; in the host, the elaborated proteinases could be important in the pathogenesis of dermatophytosis.

Earlier studies showed that dermatophytes, including *T. rubrum*, could live in a medium where undenatured wool was the only source of carbon and nitrogen (Weary et al., 1965; Weary and Canby, 1967). More recently Meevootisom and Niederpruem (1979) have shown that *T. rubrum* can live in a defined culture medium with no nitrogen or carbon source other than keratin or elastin. With time, proteolytic enzymes were produced, the concentration of substrate decreased and ninhydrin positive material was solubilized. Also, these investigators found that the addition of alternative carbon sources such as glucose of individual amino acids repressed proteinase secretion. Arguing, that as other nutrient sources are made available to the organism, proteinase secretion is not required as long as other nutritive molecules are present. I will present evidence that *T. rubrum*'s proteinases are repressed by carbon, nitrogen and sulfur. When any one of these nutrients are absent from the fungal milieu the fungus responds by secreting proteinases, strongly suggesting that proteinases do play an important role in nutrition gathering by this organism.

The effect of proteinases on the host suggests these enzymes may also be important in the pathogenesis of dermatophyte infections. For example, strains of *T. mentagrophytes* with increased proteolytic activity incite a more acute inflammatory response than those strains that secrete less enzyme (Rippon and Garber, 1969; Minocha et al., 1972). There was a correlation for isolates of *T. rubrum* with high proteolytic activity to have lesions of a shorter duration and to show less cutaneous involvement (Minocha et al., 1972; Rippon and Garber, 1969). Additionally, *T. rubrum* extracts produced erythema when applied to

the stripped skin of human volunteers. When the proteolytic extracts from these organisms were injected intradermally into punch biopsies of human skin or into dogs there was evidence of microscopic spongiosis, intraepidermal splits and in some cases dissolution of the epidermal-dermal junction. These changes probably can not be attributed solely to proteolytic enzymes. However, punch biopsies of skin incubated with proteinases from fungi, as well as elastase and trypsin, showed evidence of epidermal-dermal separation and dissolution of the desmosomes in the spinous layer of the epidermis (Dobson and Bosley, 1963; Einbinder et al. 1966; Hino et al., 1982a; Hino et al., 1982b). While acantholysis, and the inflammation and thinning of the collagen- and elastin-rich regions of the dermis have been noted *in vivo* (Graham et.al., 1972), the epidermal-dermal splits are not seen in biopsy material taken from the sites of actual human infections. The fungal proteinases could be responsible for the acantholysis seen in human infections.

Proteinases could also be responsible for the activation of serum factors such as complement and plasminogen and would account for the neutrophil infiltrates, vasodilation, extravasation of fibrin and general spongiosis seen in dermatophytosis. Recently, investigators have shown that *T. rubrum* is a potent activator of complement, producing C5a and neutrophil aggregation (Swan et al., 1983; Dahl and Carpenter, 1986). Whether proteinases, or some cell wall constituent, are responsible for the activation of complement requires further study. However, Hernandez et al. (1986) have shown that fixed *T. mentagrophytes* mycelia and spores can activate the complement cascade. This might suggest that no elaboration of toxins would be necessary to manifest the cutaneous aspects of dermatophytosis, but this effect is only seen in animals previously infected with the fungus. This indicates that an immune response was mounted against this organism and although the proteins were fixed they may still retain epitopes that are recognized by antibodies made in the immune host which could act as opsonins for the fixation of complement.

Guinea pigs infected with *T. mentagrophytes*, and later immunized with a purified proteinase from the same fungus responded with dermal inflammation, mostly mononuclear, and formation of antibodies capable of inhibiting proteinase activity (Grappel and Blank, 1972). A challenge with the heat inactivated proteinase produced a reaction more intense than that of commercially available trichophytin. The enzyme could be detected in previously infected guinea pig's skin using indirect immunofluorescence (Collins et al., 1973). Anti-keratinase antibodies can retard the growth, decrease the sporulation, and increase the hyphal width of *T. mentagrophytes* (Grappel et al., 1971; Grappel, 1976). Furthermore, migration of peritoneal exudate cells from pigs sensitized to keratinase was inhibited when tested in the capillary tube migration assay (Eleutrio et al.,

1973), as was the migration of peripheral leukocytes (Garcia-de-Lomas et al., 1983). These studies indicate that fungal proteinases are important antigens in dermatophytosis, and may in fact be partially responsible for the acquired resistance to fungal infections described by several authors (Grappel et al., 1974, Jones et al., 1973).

1.5 Invasive Disease

Dermatophyte infections are usually limited to the stratum corneum. This is probably due to a combination of fungal and host factors. Dermatophytes are very sensitive to temperature. Growth of most species and strains of dermatophytes is inhibited at normal body temperature of 37° C (Rippon, 1982). Heating cultures of dermatophytes at 39° C can be lethal when this temperature is maintained for a two week period (Lorincz and Son, 1963). On the host side, serum contains a serum inhibitory factor (SIF) that stays the growth of dermatophytes. This factor has been purified and appears to be transferrin (King et al., 1975). The SIF activity was positively correlated with unbound serum iron binding capacity. The addition of iron to serum neutralized SIF activity, while the addition of purified iron-free transferrin restored this activity. Finally, removal of transferrin from serum by affinity chromatography resulted in a serum that lacked SIF activity. A patient with low levels of SIF showed extensive invasion of his epidermis and dermis by *T. rubrum* (Roth et al., 1959), and when serum is removed from organ cultures of skin, *T. rubrum* is able to invade the cellular areas of the epidermis. Invasive, granulomatous infections by *T. rubrum* and *M. audouinii* have been described in individuals with lymphomas, Cushing's syndrome, abnormalities of carbohydrate metabolism and immune impairment (Rippon, 1982). These facts point out that *T. rubrum*, and other dermatophytes, have the potential to invade and disseminate into human's tissues, even at 37° C, but this potential is not realized in the normal host.

The subject of this dissertation will be the purification, characterization and regulation of three proteolytic activities elaborated by the most common dermatophyte of humans, *T. rubrum*. These proteinases may serve several roles in this host-parasite relationship. In particular, valuable nutrients for the fungus are derived from the proteolysis of susceptible proteins. Although the invasion of fungi into tissues could be purely mechanical, it will be demonstrated that *T. rubrum* possess a battery of proteinases that could facilitate the disruption of connective tissue barriers in cases of disseminated disease.

II. The Purification and Characterization of Three Major Exocellular Proteinases From *T. rubrum* Conditioned Medium.

2.1 Identification of the Major Exocellular Proteinases of *T. rubrum*.

Because *T. rubrum* parasitizes the keratinized tissues of humans it is hypothesized that this organism would secrete proteinases able to cleave available proteins into readily metabolized sources of carbon, nitrogen and sulfur. Some of the goals of this study were to: determine if *T. rubrum* expresses proteolytic activity; determine if these proteinases are capable of degrading proteins contained in the stratum corneum, and other keratinized tissues; determine if proteinases are secreted by this organism in response to nutrient deprivation. Earlier work suggested that *T. rubrum* may secreted proteinases capable of degrading guinea pig hair, keratin, casein, bovine serum albumin, collagen and elastin (Weary and Canby, 1965; Weary and Canby, 1967; Meevootisom and Niederpruem, 1979; Sanyal et al., 1985). In contrast, Rippon and Varadi (1968) were unable to detect clearing of particulate elastin by strains of *T. rubrum* in an agar plate assay. However, they included 2% glucose in their medium, and this concentration of glucose was later shown to be inhibitory to proteinase production (Meevootisom and Niederpruem, 1979).

When a strain of *T. rubrum* (I.F.O. 9185) was cultivated on agar plates containing particulate elastin it was able to clear this substrate (Figure 1). This data indicated that this strain of *T. rubrum* could live on a protein substrate as its sole source of nitrogen and carbon, that it elaborated an elastinolytic activity, and that this activity was a diffusible substance; the fungus grew on the surface of the agar plate, while the elastin settled to the bottom of the plate.

To extend these studies and test the hypothesis that *T. rubrum* secreted proteinases for its nutritional needs, the CM from stationary phase cultures of this organism was assayed for proteinases. It was found that the CM contained enzymes that could degrade azocoll, elastin and keratin. The azocollytic and elastinolytic activities had pH optima of 8.0, while the keratinolytic activity had a pH optimum of 9.0. The azocoll degradation was inhibited 80% by 1 mM PMSF, and 95% by 100 μg of α_1-proteinase inhibitor/ ml. There was no inhibition by 2 mM 1,10 phenanthroline, or 5 mM N-ethylmaleimide. The elastase and keratinase activities were similarly inhibited by serine proteinase inhibitors, and not by metallo- or thiol-proteinase inhibitors. Although elastase activity was strongly inhibited by

Figure 1. Clearing of particulate elastin by *T. rubrum*. Strain I.F.O. 9185 was grown on a medium containing 0.5% (w/v) particulate elastin, 3.4 mM KH_2PO_4 and 5.75 mM K_2HPO_4, 2 mM $MgSO_4$ and 100 µg/l thiamine-HCl as described in Appendix 1. The plate was incubated at 30° C for 2 weeks before being photographed.

PMSF (92%) the α_1-proteinase inhibitor had less of an effect (30%). This was also true of keratinolytic activity. In an *in vitro* model of the extracellular matrix, the R-22 assay, the crude CM was able to degrade 57.7 ± 2.2 % of the total available radioactivity. 24.9 ± 7.1 % of the trypsin-sensitive material, 58.0 ± 3.8 % of the elastase-sensitive material and 88.9 ± 14.4 % of the collagenase-sensitive material were degraded. Control incubations with just buffer released less than 2.3 ± 0.8 % of the total radioactivity present in the matrix.

Were these activities due to one, or several proteolytic enzymes ? To determine how many proteolytic enzymes were present in *T. rubrum* CM, the filtrate of stationary phase cultures of the fungus was analyzed in polyacrylamide gels copolymerized with gelatin. Gelatin is a denatured collagen substrate that is cleaved by many proteinases. Several activities were detected (Figure 2A). Besides several major species of M_r 124,000, 93,000, 71,000, 53,000 and 27,000 there were minor bands of M_r 30,000, 25,000 and 23,000. Additionally, there was a broad band of activity between the M_r 124,000 proteinase and the top of the gel. This might represent several high molecular weight activities or multimers or aggregates of the smaller molecular weight activities. Depending on the age of the cultures the M_r 53,000, 31,000 and 25,000 proteinases were present to a variable degree. Also, the Mr 93,000 proteinase was a major species in original cultures of the fungus, however, with time (3 years) its production has decreased while that of the M_r 124,000 species has increased. When the CM was analyzed on elastin substrate gels, activities of M_r 124,000, 93,000, 71,000, 53,000 and 27,000 were detected (Figure 2B). The high molecular weight clearing was also detected under these conditions. Lysates of stationary phase fungus also contained azocollytic activity, and when analyzed on gelatin containing substrate gels, the lysates were found to contain all of the proteolytic species found in *T. rubrum* CM (data not presented). It is interesting to note that *Neurospora crassa* proteinases are stored in membrane bound vesicles and released when this organism is starved for nutrients (Matile, 1965).

2.2 Purification of the M_r 93,000 and 71,000 Proteinases

The purification of the M_r 93,000 and 71,000 proteinases was attempted first because of their preponderance in the CM of early cultures of *T. rubrum*. The CM of stationary phase cultures of fungus was concentrated by $(NH_4)_2SO_4$ precipitation and then chromatographed on a Sephadex G-100 column (Figure 3). A broad peak (fractions 90-130) of azocoll and elastin degrading activity was separated from the majority of A_{280} absorbing material by this first purification step. A small peak of elastase activity (fractions 70-80) was resolved from the major peak of activity. This minor peak contained The M_r

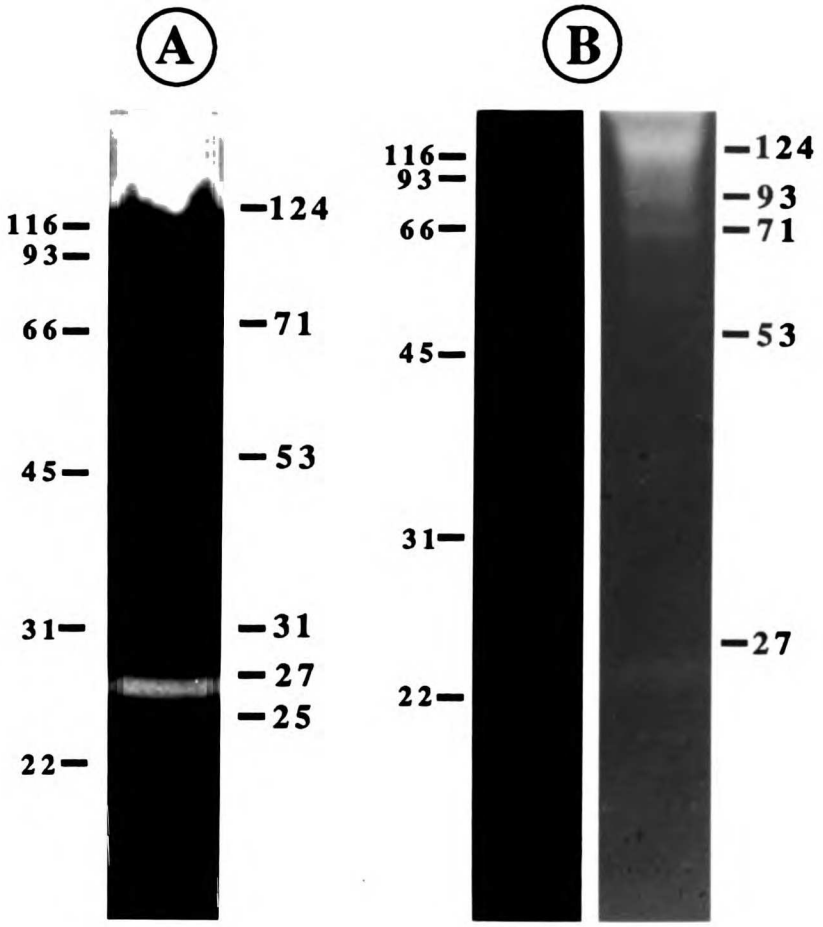

Figure 2. Detection of proteinase activity in the CM of *T. rubrum* by SDS-substrate gel analysis. **(A)** An aliquot (10 µl) of CM from stationary phase cultures of fungus grown in Sabouraud dextrose broth was mixed with an equal volume of 2 X nonreducing sample buffer and proteolytic activity resolved in a 10% polyacrylamide gel copolymerized with 0.12% gelatin as described in Appendix 1. Following electrophoresis, the gel was soaked in 2.5% (v/v) Triton X-100 for 30 min, then incubated in 100 mM Tris-HCl, pH 8.0, 1 mM $CaCl_2$ for 4 h at 37° C. The gel was then stained with Coomassie R-250, then destained. Zones of clearing represent proteolytic activity. **(B)** Elastinolytic enzymes present in the Crude CM were detected on 12% polyacrylamide gels copolymerized with 0.12% α-elastin and treated as described above. Relative molecular mass (x 10^{-3}) of the proteinases is indicated at the right. Molecular mass markers (x 10^{-3}) are indicated at the left.

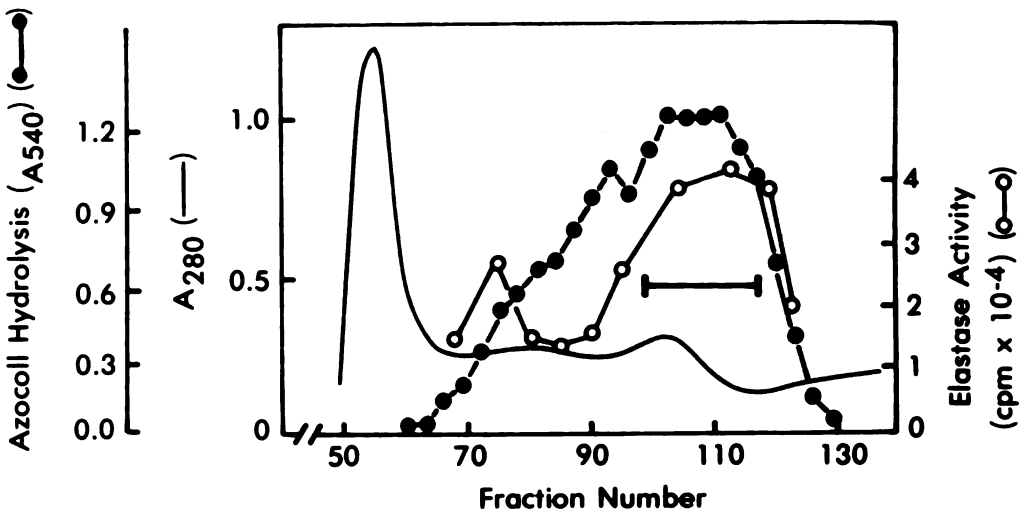

Figure 3. Sephadex G-100 chromatography of crude *T. rubrum* CM. Lyophilized samples of CM (50-70 mg) were dissolved in 20 mM Tris-HCl, pH 8.0, 150 mM NaCl and applied to a Sephadex G-100 column (1.9 cm x 100 cm). Samples were eluted at a flow rate of 15 ml/min and 2 ml fractions were collected. Aliquots of the fractions were assayed for azocollytic (●) and elastinolytic (o) activity as described in Appendix 1. The solid line represents absorption at 280 nm and the bar indicates fractions pooled for chromatofocusing.

27,000 activity, and the major peak contained the higher activities and the M_r 27,000 proteolytic species. To further purify the proteinases contained in the major peak, these fractions were concentrated and resolved by chromatofocusing on a Mono P column. A pH 8.3 to pH 5.0 gradient was chosen. Three peaks of proteolytic activity were resolved (Figure 4). The first peak of activity eluted at the void and thus had a pI greater than 8.0. The second peak eluted at an approximate pI of 7.8 and contained the M_r 93,000 proteinase (Figure 5). The third peak of activity contained the M_r 71,000 proteinase.When the fractions containing the M_r 93,000 proteinase were reduced, resolved by electrophoresis and silver stained, a single band of M_r 44,000 was visualized (Figure 6). The Mr 71,000 proteinase was also purified to homogeneity by this purification step and had a reduced molecular weight of M_r 36,000 (Figures 5 and 6). When these purified proteinases were analyzed by electrophoresis under non-reducing conditions they migrated with M_r values that were approximately doubled. It appears that these proteinases exist as dimers in their "native" states. Activity also eluted from the column when it was washed with 2 M NaCl. This peak of proteolytic activity was later shown to contain the M_r 27,000 proteinase, indicating that this activity has a pI less than 5.0.

Table 3 summarizes the purification of the M_r 93,000 and 71,000 proteinases. The M_r 93,000 activity was purified 42 fold with a 20.4 % recovery while the M_r 71,000 proteinase was purified 2.5 fold. These enzymes degraded azocoll, elastin and keratin (Table 4). The M_r 71,000 species had a lower specific activity than that of the M_r 93,000 activity for all of the substrates tested. The pH optimum of the two enzymes was broad, peaking at a pH of 8.0 (Figure 7). Both enzymes were inhibited by the serine proteinase inhibitors α_1-proteinase inhibitor, PMSF and Trasylol, while 1,10 phenanthroline and N-ethylmaleimide were not inhibitory (Table 5). There was inhibition of the M_r 93,000 proteinase by EDTA, markedly less so for the M_r 71,000 proteinase. This EDTA inhibition could be related to the Ca^{2+} dependance of both these activities (1 mM). Lastly, three chloromethylketone inhibitors were tested. ala-ala-pro-ala-CH_2Cl is an excellent inhibitor of leukocyte and pancreatic elastase (Powers and Tuhy, 1973), and had limited activity against the M_r 93,000 proteinase, and some activity against the M_r 71,000 proteinase. The ala-ala-pro-leu-CH_2Cl and phe-gly-ala-leu-CH_2Cl are excellent inhibitors of subtilisin (Powers et al., 1977). phe-gly-ala-leu-CH_2Cl was the most effective inhibitor for both proteinases, but the absolute inhibition was greater for the M_r 93,000 proteinase than for the M_r 71,000 activity.

Based on the chloromethylketone inhibition, several synthetic peptide substrates with hydrophobic amino acids in the P-1 site were tested (Table 6). Peptide substrates with

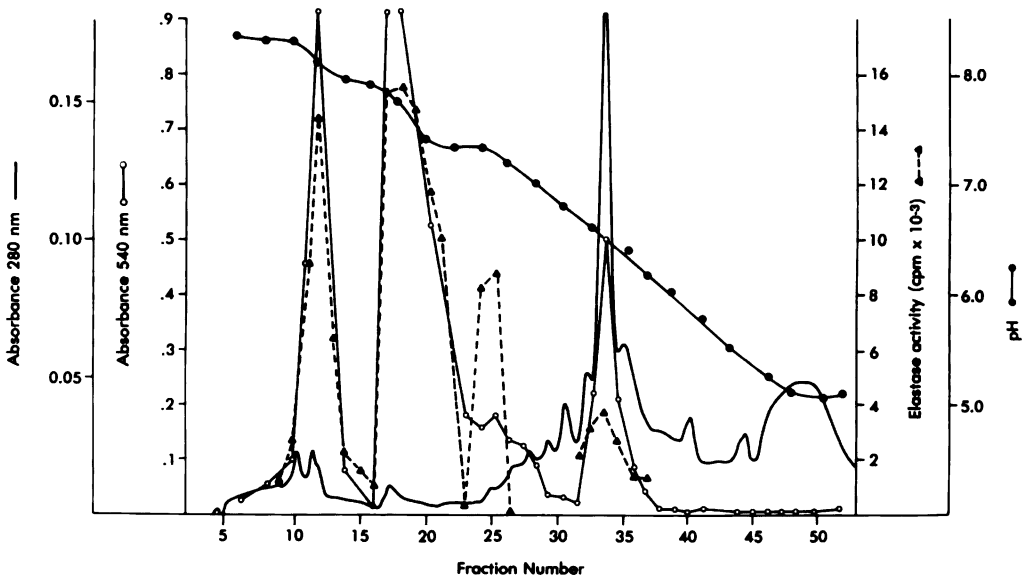

Figure 4. Chromatofocusing of pooled fractions from Sephadex G-100 chromatography. The peak fractions of proteolytic activity from the Sephadex G-100 column were pooled and dialyzed against 20 mM Tris-acetate, pH 8.3. The proteolytic activity was further resolved on a Mono P column equilibrated with the same buffer. The pH gradient was generated with Polybuffer, pH 5.0. Samples were eluted at a flow rate of 0.5 ml/min and 1 ml fractions were collected. An aliquot was assayed for azocoll degradation (o), elastin degradation (▲) and pH (●). The solid line represents absorption at 280 nm.

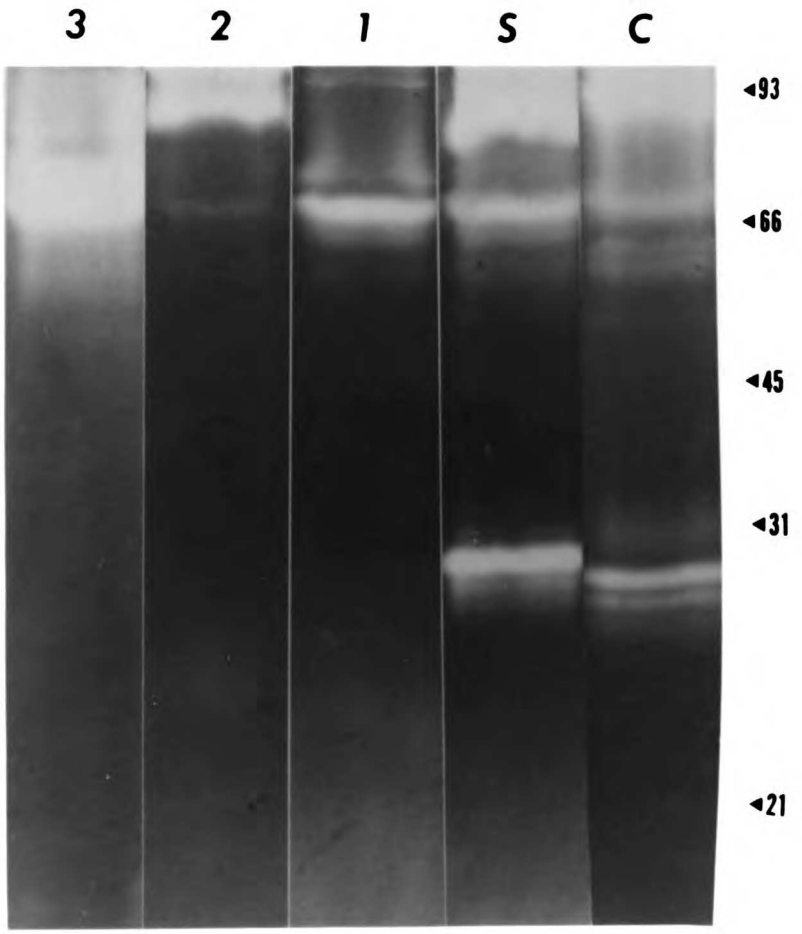

Figure 5. Gelatin substrate gel of fractions from the purification of the M_r 93,000 and 71,000 proteinases. Approximately 5 μg of protein was loaded onto each lane of the gel. Lane C, crude CM; lane S, pool of active fractions from Sephadex G-100 chromatography; lanes 1, 2 and 3 are peaks 1, 2 and 3 respectively from chromatofocusing. Molecular mass markers (x 10^{-3}) are indicated at the right.

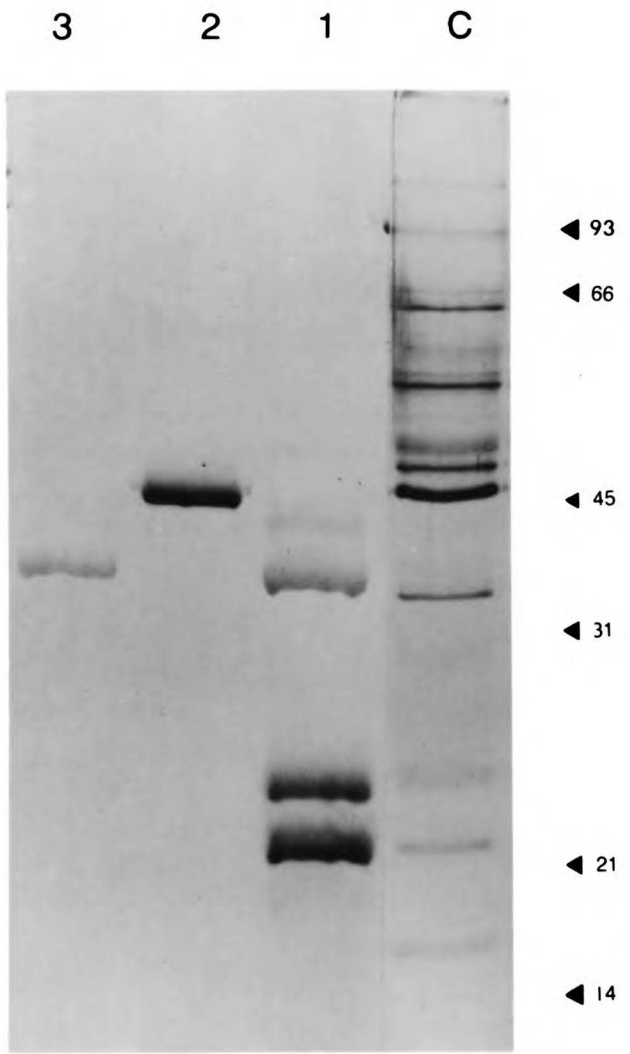

Figure 6. SDS-polyacrylamide gel electrophoresis of fractions from the purification of the M_r 93,000 and 71,000 proteinases. Fractions from the purification steps were reduced and resolved on a 10% polyacrylamide gel. The gel was stained with silver. Lane C, crude CM; lanes 1, 2 and 3 are peaks 1, 2 and 3 respectively from the chromatofocusing column. Molecular mass markers (x 10^{-3}) are indicated at the right of the figure.

purification step	total protein (mg)	specific activity (units/mg)	total activity (units)	purification factor	yield (%)
culture filtrate [70% $(NH_4)_2SO_4$ fraction]	66.0	302.0	19932.0	1.0	100.0
gel filtration (Sephadex G-100)	12.0	972.0	11664.0	3.2	58.5
chromatofocusing	0.3*	12820.0*	4075.0*	42.0*	20.4*
(Mono P)	1.0†	735.0†	735.0†	2.5†	3.7†

*M_r 93,000 proteinase †M_r 71,000 proteinase

Table 3. Summary of purification of the M_r 93,000 and 71,000 proteinases from the CM of stationary phase *T. rubrum* cultures. For experimental details see Appendix 1. One unit of enzyme is defined as that amount of enzyme needed to give a change of A_{540} of 0.1/h in the azocoll degradation assay.

substrate	M_r 93,000 proteinase (units/mg)	M_r 71,000 proteinase (units/mg)
azocoll	128,000.0	735.0
elastin	59,000.0	19,000.0
keratin	200.0	32.0

Table 4. Specific activities of the M_r 93,000 and 71,000 proteinases. One unit of azocoll degrading activity is that amount of enzyme needed to give a change in A_{540} of 0.1/h. One unit of elastin degrading activity will solubilize 1 μg of [^{3}H]elastin/h, and one unit of keratinase will degrade 1 μg of [^{14}C]keratin/h.

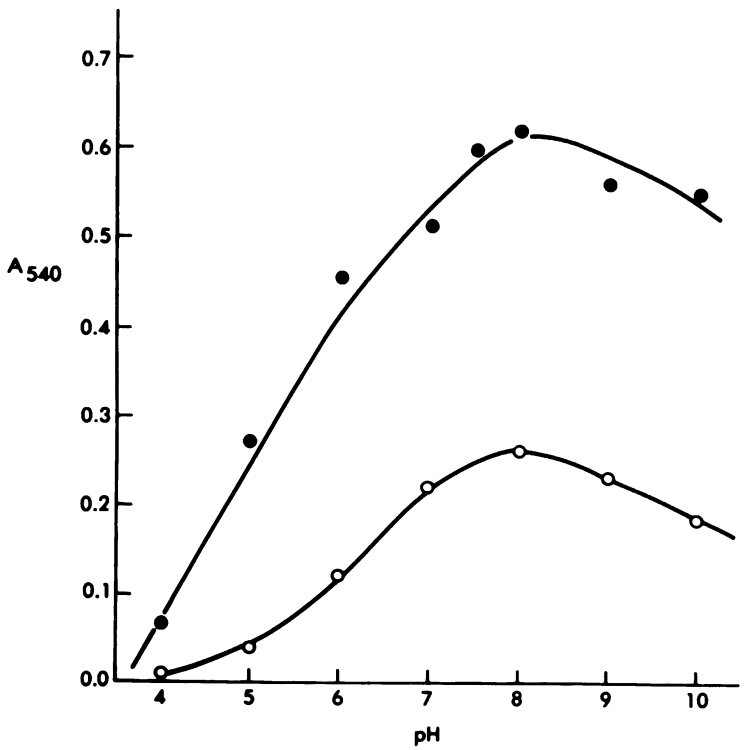

Figure 7. pH optimum of the M_r 93,000 and 71,000 proteinases. The buffer in the pH range of 4-6 was 100 mM sodium acetate-HCl, 1 mM $CaCl_2$ and that in the pH range of 7-10 was 100 mM Tris-HCl, 1 mM $CaCl_2$. Azocoll was used as the substrate for the M_r 93,000 (●) and 71,000 (o) proteinases.

inhibitor	final concentration	% inhibition	
		M_r 93,000 activity	M_r 71,000 activity
α_1-proteinase inhibitor	75 μg/ml	99.4	99.4
PMSF	1 mM	96.9	99.9
Trasylol (aprotinin)	150 μg/ml	96.1	96.5
EDTA	10 mM	66.1	15.3
N-ethylmaleimide	5 mM	4.1	14.7
1,10 phenanthroline	1mM	2.6	24.0
phe-gly-ala-leu-CH_2Cl	0.05 μM	92.7	42.6
	0.10 μM	95.8	53.8
ala-ala-pro-leu-CH_2Cl	0.10 μM	56.8	39.3
	0.20 μM	71.4	61.7
ala-ala-pro-ala-CH_2Cl	1.0 μM	27.1	44.3
	2.0 μM	45.3	53.2

Table 5. Inhibition profile of the M_r 93,000 and 71,000 proteolytic species. For these studies the inhibitors were preincubated with enzyme for 20 min at ambient temperature before azocoll substrate was added to the reaction mixture as described in Appendix 1. Data is reported as percentage inhibition of activity as compared to a positive, enzyme control. For inhibitors requiring ethanol or dimethylsulfoxide in stock solutions, an equivalent amount of solvent plus enzyme was run as a control.

proteolytic species	substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)
M_r 93,000	Suc-ala-ala-pro-phe-pNA	0.35	9.46	27028
	Suc-ala-ala-pro-met-pNA	0.73	11.21	15354
	Suc-ala-ala-pro-nle-pNA	0.52	4.67	8974
	Suc-ala-ala-pro-ile-pNA	no activity*		
	Suc-ala-ala-pro-val-pNA	no activity*		
M_r 71,000	Suc-ala-ala-pro-phe-pNA	0.71	2.72	3837
	Suc-ala-ala-pro-met-pNA	0.24	0.38	1580
	Suc-ala-ala-pro-nle-pNA	0.41	0.56	1360
	Suc-ala-ala-pro-ile-pNA	no activity*		
	Suc-ala-ala-pro-val-pNA	no activity*		
M_r 93,000	MeOSuc-ala-ala-pro-val-SBzl	0.56	3.04	5410
M_r 71,000	MeOSuc-ala-ala-pro-val-SBzl	0.80	1.12	1408
M_r 93,000	MeOSuc-ala-phe-AMC	0.40	0.16	400
	Suc-ala-ala-pro-phe-AMC	1.13	0.31	274
	Suc-phe-gly-ala-leu-AMC	0.33	0.03	86

* < 0.002 A/10 min at 1 mM substrate

Table 6. Kinetic studies of the hydrolysis of tetrapeptide substrates by the M_r 93,000 and 71,000 proteolytic species. Assay conditions are described in Appendix 1.

phenylalanine or methionine at the P-1 site gave the highest k_{cat}/K_m for the para-nitroanilide (pNA), 7-amino-4-methylcoumarin (AMC) and S-benzyl (SBzl) substrates. β-branching of the P-1 side chain decreased activity against the peptide-pNA substrates. However, activity with valine at the P-1 site could be measured with use of the more reactive SBzl substrates. As a general trend, the k_{cat}/K_m for the M_r 93,000 proteinase was always greater than that of the M_r 71,000 enzyme. In addition, three AMC substrates were tried with the M_r 93,000 proteinase. They were significantly less reactive than the pNA and SBzl substrates.

2.3 Purification of the M_r 27,000 Proteinase

The major gelatinolytic activity in *T. rubrum* CM is the M_r 27,000 proteinase (Figure 2). Fungal CM was concentrated by $(NH_4)_2SO_4$ precipitation and loaded onto a concanavalin A column (Figure 8). The flow-through of this column contained the majority of A_{280} absorbing material. When these fractions were analyzed on gelatin substrate gels it was found that they contained the M_r 124,000, 71,000 and 27,000 activities (Figure 9). The M_r 53,000 activity was not found in any of these fractions; however, this activity was detected when the the concanavalin A column was eluted with α-methyl mannoside (Figure 9), as were the M_r 124,000, 71,000 and 27,000 activities.

To further resolve the activities contained in the flow-through of the concanavalin A column, these fractions (typically numbers 1-10) were concentrated and then loaded on a Polyanion SI anion-exchange column equilibrated with 50 mM Bis-Tris, pH 5.8 (Figure 10). The column was developed with a linear gradient of salt from 0 to 500 mM NaCl. Three peaks of azocollytic activity were resolved. The flow-through contained the high molecular weight activities including the M_r 124,000 proteinase. The second peak contained the M_r 71,000 proteinase and in the third peak the M_r 27,000 activity was detected (Figure 9). The salt wash contained the M_r 27,000 activity and two faster migrating species of M_r 25,000 and 23,000. It is not clear if these lower molecular weight activities represent different proteinases or proteolytic fragments of the M_r 27,000 activity. If the material that sticks to the concanavalin A column was chromatographed on the Polyanion SI column a similar elution profile was observed. With one exception, there was an additional peak of activity around fraction 24 that contained the M_r 53,000 proteinase. However, this activity was always contaminated with the M_r 27,000 species. The M_r 53,000 activity is inhibited by PMSF on gelatin substrate gels and is able to cleave the substrate Z-ala-ala-phe-amino-fluorocoumarin impregnated in cellulose acetate membranes (data not shown).

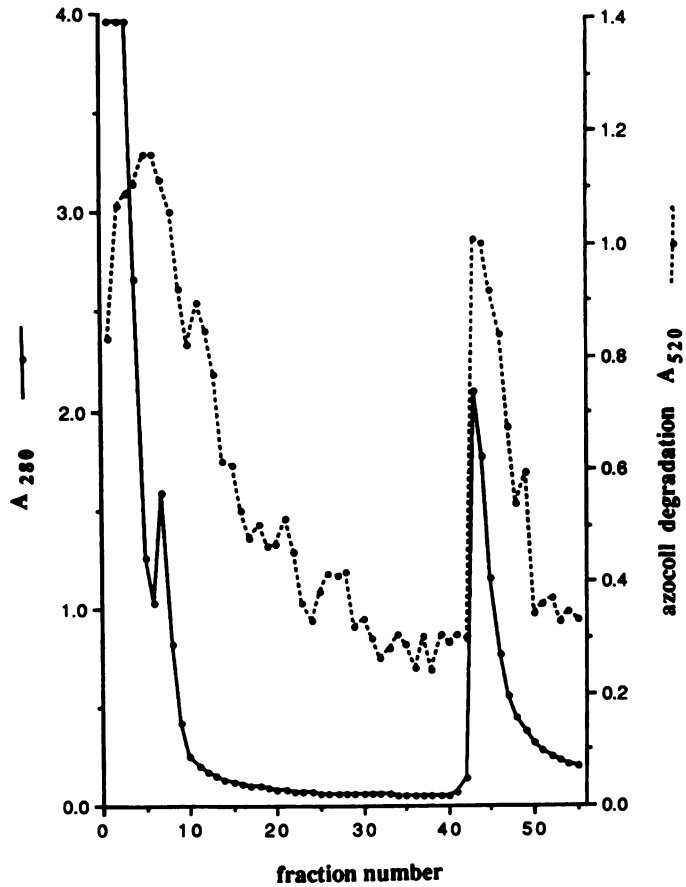

Figure 8. Concanavalin A affinity-chromatography of *T. rubrum* CM. Fungal CM (20-50 mg) was dissolved in 100 mM Tris-HCl, pH 7.4, 500 mM NaCl, 0.02% NaN_3 and applied to a 11 cc disposable column packed with 8 ml of concanavalin A sepharose 4B equilibrated with the same buffer. The proteins were allowed to interact with the column for 30 min, then unbound proteins were eluted with the loading buffer. After fraction 40 (100 ml), the bound proteins were eluted with 100 mM Tris-HCl, pH 7.4, 500 mM NaCl, 200 mM α-methyl mannoside, 0.02% NaN_3 and an aliquot assayed for absorption at 280 nm and for degradation of azocoll. Samples were eluted at a flow-rate of 45 ml/h and 2.5 ml fractions were collected.

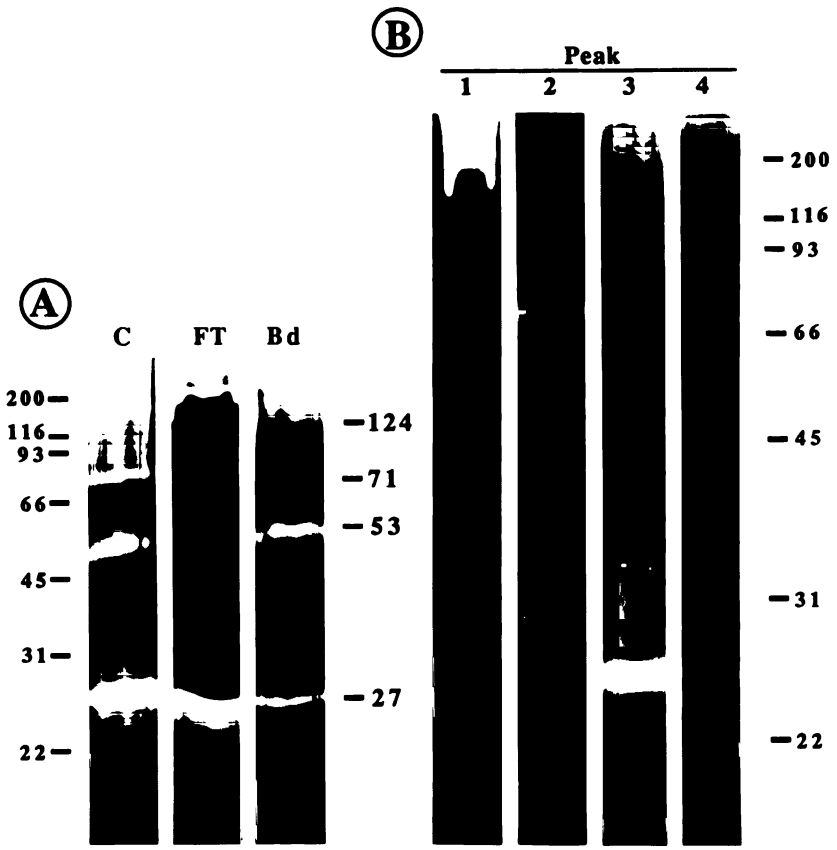

Figure 9. Gelatin substrate gel of fractions from the purification of the M_r 27,000 proteinase. **(A)** The crude CM (C), and samples from the flow-through (FT) and bound (Bd) fractions of a concanavalin A column were analyzed on gelatin containing substrate gels. Molecular mass markers (x 10^{-3}) are indicated at the left and relative molecular mass (x 10^{-3}) of the major proteolytic species is indicated at the right. **(B)** The lanes marked 1, 2, 3 and 4 are aliquots of peaks I, II, III and IV respectively of a Polyanion SI column analyzed on gelatin substrate gels. Molecular mass markers (x 10^{-3}) are indicated at the right.

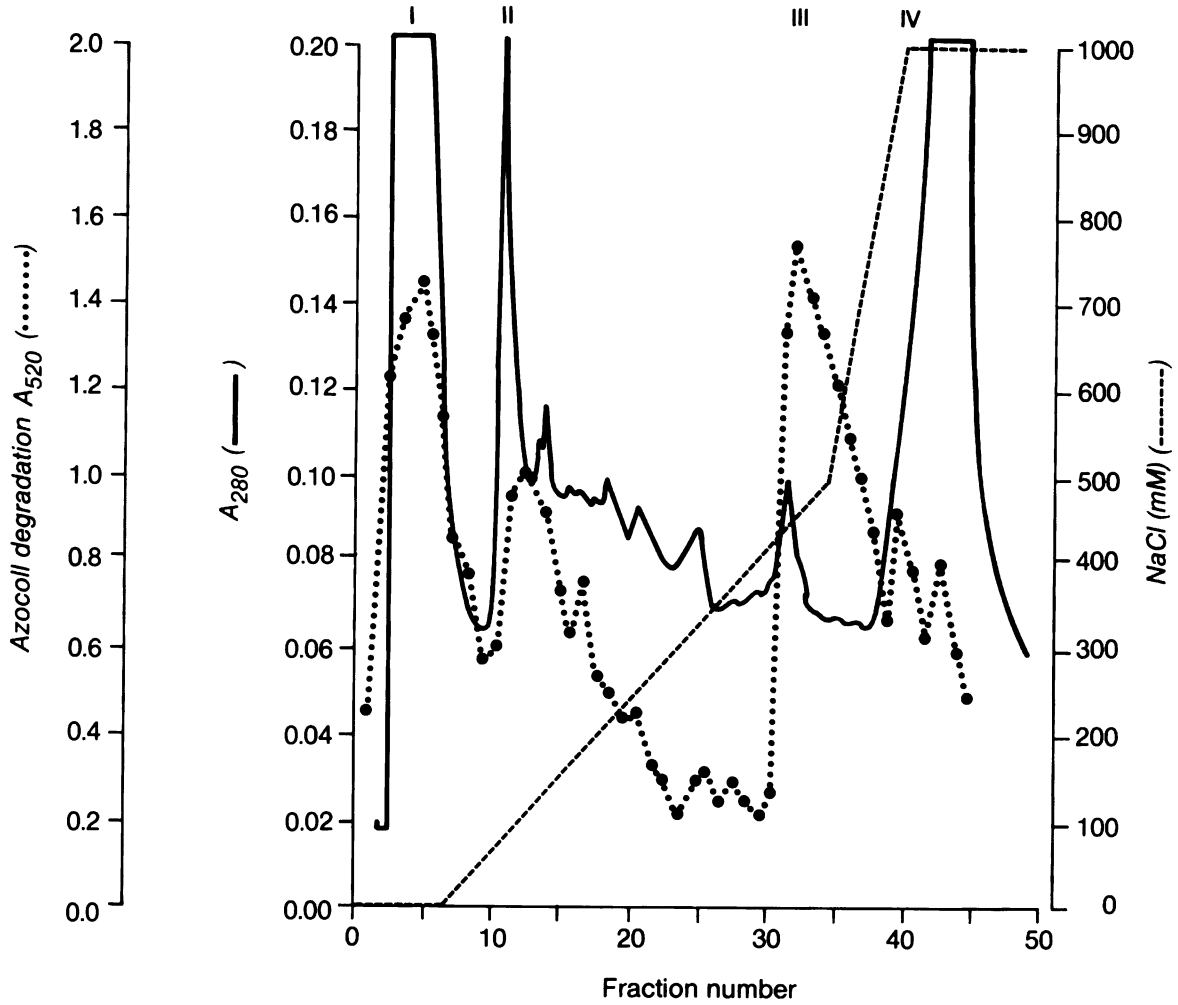

Figure 10. Anion-exchange chromatography of pooled fractions from the flow-through of a concanavalin A column. Active fractions from the flow-through of a concanavalin A column (typically fractions 1-10) were pooled, concentrated and dialyzed against water. The concentrated proteins (in 0.5 ml) were mixed with an equal volume of 50 mM Bis-Tris-HCl, pH 5.8 and applied to a Polyanion SI column, that had previously been equilibrated with the same buffer. After 6 ml of the start buffer had flowed through the column, proteins that interacted with the column were eluted off the matrix by a 30 ml linear gradient of salt (---) from 0-500 mM NaCl, in the same buffer. Samples were eluted at a flow rate of 0.5 ml/min and 1 ml fractions were collected. Aliquots from each fraction were assayed for azocollytic activity (●). The solid line represents absorption at 280 nm.

When aliquots of the fractions containing the M_r 27,000 proteinase were electrophoresed on a nonreducing sodium dodecyl sulfate (SDS)-polyacrylamide gel, a single band at approximately M_r 27,000 was visualized (Figure 11). Interestingly, when reduced the M_r 27,000 proteinase ran at a molecular weight of M_r 44,000. To confirm this result, the M_r 27,000 proteinase was labeled with [^{3}H]diisopropyl fluorophosphate (DFP) and electrophoresed under reducing and nonreducing conditions. Again, under nonreducing conditions the proteinase ran as a M_r 27,000 protein and under reducing conditions the protein ran at M_r 44,000 (Figure 12A). No labeling was detected in control reactions that contained buffer and no enzyme. The purification of this proteinase is summarized in Table 7. A purification of approximately 32-fold with a yield of 9.4% was achieved. The yield and purification are artificially low as there are many activities in *T. rubrum* CM that degrade azocoll and some of the proteinases have a higher specific activity for azocoll than the M_r 27,000 proteinase (e.g. the M_r 93,000 enzyme). At times two additional bands of protein at M_r 32,000 and 15,000 were detected in the purified fractions. These bands could represent contaminants, or the unfolded products of an endoproteolytic cut of the M_r 44,000 proteinase. The sum of 32,000 and 15,000 is 47,000, which is reasonably close to the 44,000 Dalton molecular weight of this proteinase. Alternatively, these two additional proteins may represent different conformations of the M_r 27,000 proteinase. To determine which of these models was correct, the [^{3}H]DFP labeled proteinase was electrophoresed under nonreducing conditions, localized by Coomassie blue staining, then cut out of the gel. After electroelution from the gel slice, the eluted protein was again subjected to electrophoresis and then analyzed by autoradiography. Several radioactive bands of M_r 35,000, 32,000 and 15,000 were detected in the nonreduced lanes (Figure 12B). When an aliquot of the same sample was reduced, and ran next to the nonreduced lanes, a single band of M_r 44,000 was detected.

The purified proteinase degraded azocoll (2520 U/mg), elastin (30.2 U/mg) and keratin azure (77 U/mg). Additionally, this enzyme degraded several components of the extracellular matrix (ECM) including laminin, fibronectin, type III collagen and type IV procollagen (Figure 13). This degradation was prevented by the addition of 1.0 μM phe-gly-ala-leu-CH_2CL to the reaction mix, except in the case of the degradation of fibronectin. There was no degradation of Types I and V collagen. When an *in vitro* model of the ECM, the R-22 matrix, was degraded with this enzyme, 20.8 ± 2.1 % of the available counts were released. A significant amount of the trypsin sensitive proteins (43.9 ± 2.8%), a small amount of the elastase sensitive material (14.0 ± 5.0%) and a variable amount of the collagenase sensitive molecules (6.2 ± 6.3%) were degraded.

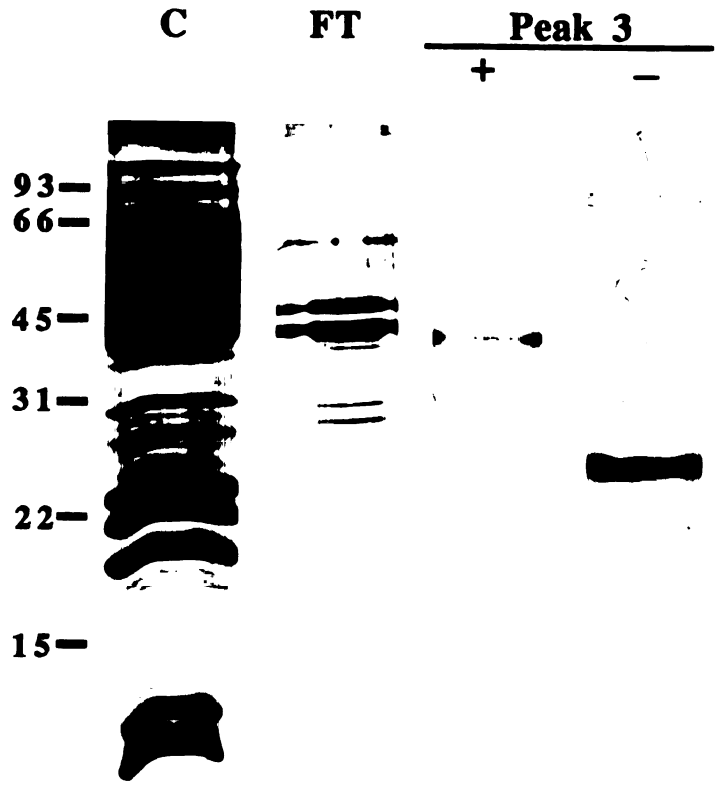

Figure 11. SDS-polyacrylamide gel electrophoresis of fractions from the purification of the M_r 27,000 proteinase. Fractions from the purification steps were resolved on a 14% polyacrylamide gel. The gel was stained with silver. Lane C, crude CM; lane FT, flow-through from the concanavalin A column; Peak 3 fractions from the Polyanion SI column were either reduced (+) or left unreduced (-) prior to being resolved by electrophoresis. Molecular mass markers (x 10^{-3}) are indicated at the left of the figure.

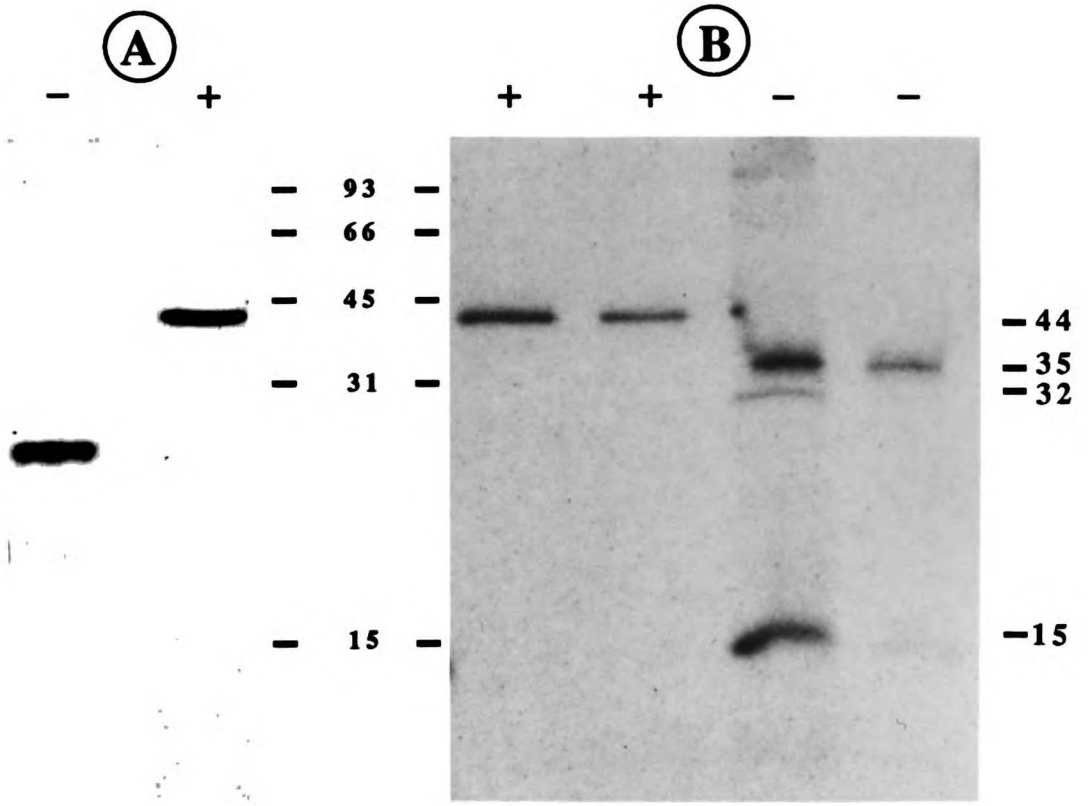

Figure 12. [^{3}H]DFP labeling of the M_r 27,000 proteinase and electroelution. **(A)** The purified M_r 27,000 proteinase was labeled with [^{3}H]DFP as described in Appendix 1. The reduced (+) or unreduced (-) proteinase was analyzed on a 4% stacking/14% resolving gel. The gel was fluorographed and labeled proteinase was detected by autoradiography. Molecular mass markers (x 10^{-3}) are indicated at the right. **(B)** The labeled proteinase (nonreduced) was electrophoresed, the gel stained with Coomassie R-250, destained and the single protein band (at M_r 27,000) was sliced out of the gel. The protein was electroeluted out of the gel slice, reduced (+) or left unreduced (-), and again analyzed by autoradiography of fluorographed 14% SDS-polyacrylamide gels. Equal amounts of the electroeluted, labeled proteinase were loaded into each lane. Molecular mass markers (x 10^{-3}) are indicated at the left and relative molecular mass (x 10^{-3}) of the major radiolabeled protein species is indicated at the right.

purification step	total protein (mg)	specific activity (units/mg)	total activity (units)	purification factor	yield (%)
culture filtrate [70% $(NH_4)_2SO_4$ fraction]	45.90	79.7	3660.1	1.0	100.0
affinity chromatography (con A column)	8.32	120.7	1004.5	1.5	27.4
anion exchange (Polyanion SI)	0.14	2520.1	352.8	31.6	9.6

Table 7. Summary of purification of the M_r 27,000 proteinase from the CM of stationary phase *T. rubrum* cultures. For experimental details see Appendix 1. One unit of enzyme is defined as that amount of enzyme needed to give a change of A_{540} of 0.1/h in the azocoll degradation assay.

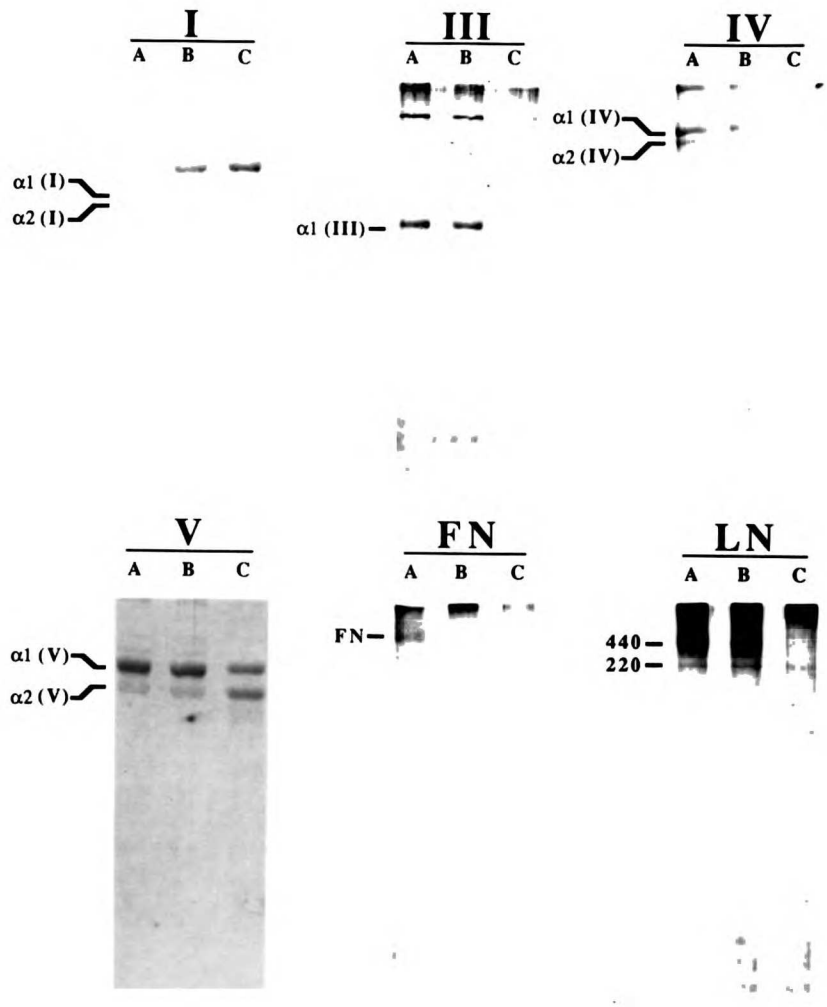

Figure 13. Degradation of macromolecular substrates by the purified M_r 27,000 proteinase. Five μg of the following substrates were incubated with 1.1 μg of the M_r 27,000 proteinase in 100 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM $CaCl_2$ at 34° C for 18 h: type I collagen (I), type III collagen (III), type IV procollagen (IV), type V collagen (V), fibronectin (FN), or laminin (LN) . An aliquot from the reactions were reduced and proteins resolved by SDS-polyacrylamide gel electrophoresis on 4% stacking/ 7% resolving gels and stained with silver. Lane A, substrate alone; lane B, substrate plus enzyme that had been preincubated with 1.0 μM phe-gly-ala-leu-CH_2Cl; lane C, substrate plus enzyme. The locations of the collagen chains and the subunits of laminin are indicated at the left of each substrate.

The M_r 27,000 proteinase had a pH optimum which peaked at pH 8.0 (Figure 14), and a calcium dependance of 2 mM. This activity was inhibited strongly by inhibitors of serine proteinases, including 1 mM PMSF (94%), 0.2 μM phe-gly-ala-leu-CH_2Cl (92%) and of course this proteinase also labeled with [^{3}H]DFP (Table 8). There was inhibition by the thiol proteinase inhibitor NEM (53%) but little by the metalloproteinase inhibitor 1,10 phenanthroline (7%). There was significant inhibition by chloromethylketone inhibitors that contained a large, hydrophobic amino acid next to the chloromethyl group, while ala-ala-pro-ala-CH_2Cl had little inhibitory activity.

The chloromethylketone inhibition data indicated that the M_r 27,000 activity was a serine proteinase that preferred a leucine or a phenylalanine in the P-1 site of peptide substrates. Like the M_r 93,000 and 71,000 proteinases the M_r 27,000 activity cleaved peptide substrates with hydrophobic amino acids in the P-1 site, i.e. leucine and phenylalanine (Table 9). The highest k_{cat}/K_m was for the Z-ala-ala-leu-pNA substrate (1614 $M^{-1}s^{-1}$). There was neither activity against a pancreatic elastase substrate, Suc-ala-ala-ala-pNA, nor against a trypsin substrate, Z-lys-glu-arg-pNA.

2.4 Discussion

Three of the major azocollytic proteinases from the CM of stationary phase cultures of *T. rubrum* have been purified to homogeneity. In addition to azocoll the M_r 93,000 and 71,000 proteinases degrade elastin and keratin. These enzymes exist as dimers. In the presence of reducing agents these proteinases have molecular weights of M_r 44,000 and 36,000, respectively. Because of their existence as dimers and similar substrate specificities, pH optima, calcium dependencies, inhibition profiles and kinetic constants, it is hypothesized that these are different forms of the same enzyme. The size differences could be attributed to differential splicing of the proteinase message or to some post-translational modification, such as glycosylation or proteolytic trimming of the larger precursor. If glycosylation accounts for the size difference, then the polysaccharide chains attached to the M_r 93,000 proteinase must be free of terminal mannose residues because they have poor affinity for the concanavalin A column. The proteolytic cleavage of a M_r 8,000 polypeptide from the M_r 44,000 proteinase could result in a conformational change that would account for the similar, but lower specific activities the M_r 71,000 proteinase has for all of the substrates tested. Loss of the hydrophobic binding site at P-4 would explain the differences exhibited by the proteins when inhibited by phe-gly-ala-leu-CH_2Cl. Of course an alternative hypothesis would be that these activities represent the products of different genes.

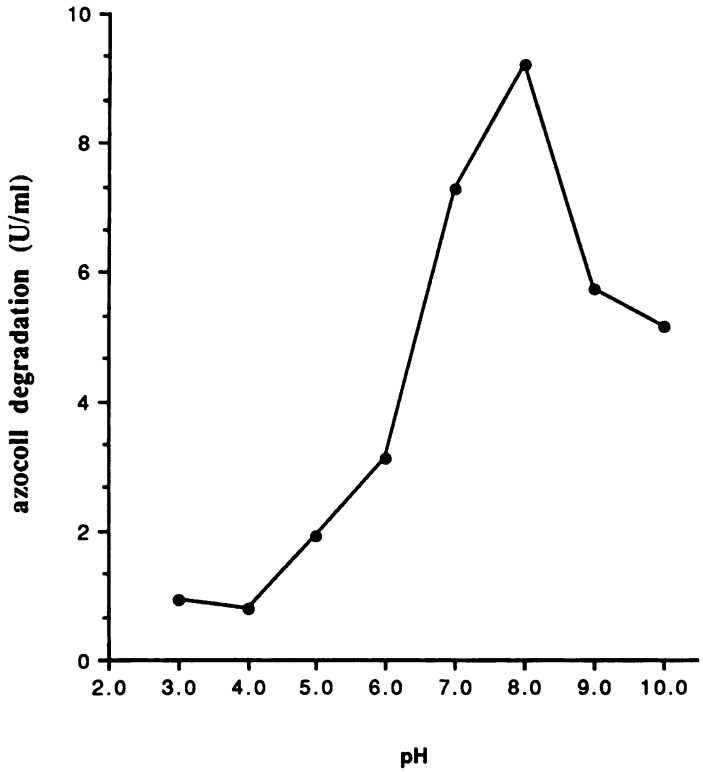

Figure 14. pH optimum of the M_r 27,000 proteinase. The buffer in the pH range of 3-6 was 100 mM sodium acetate-HCl, 1 mM $CaCl_2$, that in the pH range of 7-8 was 100 mM Tris-HCl, 1 mM $CaCl_2$, and that in the pH range of 9-10 was 100 mM glycine-NaOH, 1 mM $CaCl_2$. Azocoll was used as the substrate.

inhibitor	final concentration	% inhibition
α_1-proteinase inhibitor	75 μg/ml	69
PMSF	1 mM	94
N-ethylmaleimide	5 mM	53
1,10 phenanthroline	1mM	7
phe-gly-ala-leu-CH_2Cl	0.2 μM	92
ala-ala-pro-leu-CH_2Cl	0.2 μM	77
ala-ala-pro-phe-CH_2Cl	0.2 μM	59
ala-ala-pro-ala-CH_2Cl	0.2 μM	6

Table 8. Inhibition profile of the M_r 27,000 proteinase. For these studies the inhibitors were preincubated with enzyme for 20 min at ambient temperature before azocoll substrate was added to the reaction mixture as described in Appendix 1. Data is reported as percentage inhibition of activity as compared to a positive, enzyme control. For inhibitors requiring ethanol or dimethylsulfoxide in stock solutions, an equivalent amount of solvent plus enzyme was run as a control.

substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)
Suc-ala-ala-pro-phe-pNA	4.68	7.36	1573
Suc-ala-ala-pro-ile-pNA	no activity*		
Suc-ala-ala-pro-val-pNA	no activity*		
Z-ala-ala-leu-pNA	1.01	1.63	1614
Suc-ala-ala-ala-pNA	no activity*		
Z-gly-gly-gly-pNA	no activity*		
Z-lys-glu-arg-pNA	no activity*		

* < 0.01 A/10 min at 1 mM substrate

Table 9. Kinetic studies of the hydrolysis of tetrapeptide substrates by the M_r 27,000 proteinase. Assay conditions are described in Appendix 1.

The M_r 27,000 proteinase shares a number of characteristics with the M_r 93,000 and 71,000 species. It is a serine proteinase with a pH optimum of 8.0, that degrades azocoll and it prefers hydrophobic amino acids in the P-1 site of peptide substrates. Although the M_r 93,000 and M_r 27,000 proteinases share a similar molecular weight under reducing conditions, they do not appear to be the same enzyme. A summary of the characteristics of the three enzymes that have been purified can be found in Table 10. The M_r 27,000 proteinase is a poor elastase and keratinase, has a pI less than 5.0 (compared to the M_r 93,000's pI of 7.8), cleaves the peptide substrate ala-ala-pro-phe-pNA with a specific activity 18 fold less than the M_r 93,000 species, and is not formed by the dimerization of two smaller subunits. Finally, as will be discussed, the M_r 27,000 proteinase is under a different set of regulatory signals than those for the other proteinases.

The M_r 27,000 proteinase appears to result from the tight folding of a M_r 44,000 protein, since the nonreduced molecular weight is less than the reduced molecular weight. This folding could be mediated by intrachain disulfide bonds. This is one mechanism thought to account for the faster migration of nonreduced proteins, as compared to their reduced counterparts, and has been described for several proteins including pig skeletal muscle actin, kappa light chains and trypsin (Allore and Barber, 1984; personal communication, Dr. C. Craik). Tight folding of the M_r 44,000 protein may also explain the M_r 15,000, 32,000 and 35,000 species seen following electroelution of the M_r 27,000 proteinase. All of these proteins resolve to a single M_r 44,000 species following reduction.

The other M_r 23,000, 25,000, 53,000, 124,000 and the high molecular weight activities that have been identified have unknown substrate specificities and relationships with the other proteinases that have been purified. The smaller species could represent proteolytic cleavages of the M_r 27,000 proteinases. The high molecular weight smear could represent multimers of lower molecular weight proteinases or actual large proteinase species. It appears that the M_r 53,000 proteinase is a glycoprotein because it interacts strongly with the concanavalin A column. It is not found in the flow-through of the column, but is eluted with α-methyl mannoside. An alternative explanation would be that it interacts weakly with an inhibitor that is a glycoprotein. However, this would have to be a very specific inhibitor of the M_r 53,000 proteinase because this M_r 53,000 species is a serine proteinase that cleaves substrates with a hydrophobic amino acid in the P-1 site, and is thus very similar to the other gelatinolytic enzymes I have characterized. The purification of this enzyme would allow for the characterization of not only its interaction with lectin columns, but its general substrate specificities and regulation as well.
It is not clear if the other proteinases are glycoproteins. Their elution with the concanavalin A-binding material may represent a nonspecific interaction with the large number of

	Proteolytic enzyme		
	M_r 93,000	M_r 71,000	M_r 27,000
Reduced M_r	44,000	36,000	44,000
pI	7.8	6.5	<5.0
pH optimum	8.0	8.0	8.0
Ca^{2+} dependence (mM)	1.0	1.0	2.0
class	serine	serine	serine
A-A-P-F-pNA (M^{-1} s^{-1})	27,028	3,837	1,573
substrate (U/mg)			
azocoll	128,000	735	2,520
elastin	59,000	19,000	30
keratin*	200	32	77

Table 10. Summary of the characteristics of three proteinases purified from the CM of *T. rubrum*. * All assays are comparable except in the case of the keratin degradation assays, in which a different substrate was used for the M_r 27,000 proteinase (see Appendix 1).

proteins that are contained in these fractions. It could also represent a nonspecific interaction with the column. The M_r 27,000 proteinase, and a high molecular weight activity (around M_r 200,000), were detected in all of the fractions that came after the void. Lastly, the other proteinases could contain a few, or sterically hindered, mannose residues that would prevent them from interacting as strongly with the column as those of the M_r 53,000 proteinase, or the interaction could be due to the weak binding of the concanavalin A with other carbohydrate moieties.

The primary function of the proteinases I have purified is probably to degrade the keratins, and other proteins associated with the stratum corneum and nails, into nitrogen, carbon and sulfur sources easily assimilated by the fungus. Although the M_r 27,000 activity is a poor keratinase, it still could act on the 5% of proteins contained in the stratum corneum that are easily extracted by aqueous buffers (Sun and Green, 1978). In this sense, the M_r 27,000 proteinase can be thought of as a general proteinase akin to trypsin or chymotrypsin. This proteinase could also catalyze the hydrolysis of keratins that had previously been attacked and denatured by actual keratinases, including the M_r 93,000 and 71,000 activities. Finally, these proteinases have the ability to degrade components of the ECM, and could mediate the tissue invasion and destruction seen when *T. rubrum* infects an immunocompromised host (Rippon, 1982). For example, the M_r 71,000 and 93,000 proteinases are excellent elastases and could degrade the elastin present in the dermal layers of the skin. The M_r 27,000 proteinase can degrade fibronectin, laminin, type III collagen and type IV collagen. Although this proteinase does not degrade type I collagen, there is probably an activity secreted by *T. rubrum* that can. Crude CM from this fungus was able to degrade the majority of collagen contained in an *in vitro* model of the dermis, the R-22 matrix. The identification of the actual collagenase(s) will require further experimentation.

III. The Regulation of *T. rubrum* Proteolytic Activity

3.1 Clues From Other Fungi

In this chapter data will be presented that shows *T. rubrum* produces exocellular proteinases to utilize host proteins as a nutrient source. Data will also be presented that helps to define how extracellular proteinase expression is regulated. Previous work suggested that the level of fungal proteolytic enzymes are reduced when the organisms are grown in a rich medium, or one that contains easily metabolized substrates such as glucose, amino acids, nitrate or ammonium (Matile, 1965; Cohen, 1972; Drucker, 1972; Cohen, 1973a; Meevootisom and Niederpruem, 1979; this work). In contrast, nutrient depletion, or growth as stationary phase cultures increases production of proteinases (Matile, 1965; this work). Early studies with *Aspergillus niger* showed that the depletion of sulfur from its medium resulted in the secretion of an acid proteinase (Tomonaga et al., 1964). The control of extracellular proteinases has since been described in a number of fungi including *A. nidulans, N. crassa* and *T. rubrum.*

A. nidulans responds to ammonium deprivation by derepressing the synthesis of several enzymes related to nitrogen metabolism including nitrate reductase, xanthine dehydrogenase, uricase, acetimidase, glutamate and urea permeases and extracellular proteinases (Cohen, 1972; Marzluf, 1977). A mutant exists, *xpr*D1, that leads to the loss of ammonium repression for all of these enzymes (Cohen, 1972). This mutant was later reclassified as a mutant of the *are*A^{d} (derepressed allele of ammonium repression) locus (Marzluf, 1977). In addition, a pleiotropic proteinaseless mutant (*xpr*C1) was isolated that has been instrumental in defining the significance of extracellular proteinases (Cohen, 1973b). This mutant helped show that expression of proteinases is required for growth of *A. nidulans* in a medium that contains protein as a sole source of nitrogen and carbon; however, proteinases are not required when the fungus is cultured on medium containing easily metabolized sources of nitrogen and carbon.

A number of extracellular proteolytic enzymes, labeled α, γ, ε, are produced by *A. nidulans* (Cohen, 1973a). There is a precursor of γ, referred to as δ, which is present in both extracts and CM from this fungus. Additionally, there is a β proteinase and its two precursors, β^1 and β^2, that is found only in extracts of mycelium. When *A. nidulans* cultures are starved for carbon, nitrogen or sulfur the extracellular proteinases α, δ–γ and ε are found in the CM of the fungus. Repression of the conversion of β^1 and β^2 to the intracellular β enzyme is also mediated by low molecular weight carbon, nitrogen and sulfur

sources. In addition, this intracellular proteinase is also repressed when phosphorus is present in the fungal milieu. The regulation of the extracellular and intracellular proteinases is independent although they can respond to similar growth conditions (Cohen, 1973a). For example, if the *xpr*D1 mutants were grown in a glucose-ammonium medium the extracellular proteinases were secreted, while the intracellular proteinase β occurred in its repressed, precursor form. If the mutant *xpr*D1 cells were shifted to growth in a nitrogen and carbon free medium, then activation of the β^1 and β^2 precursors to the β proteinase occurred. Also, the response of the intracellular enzyme to phosphorus starvation remains derepressible in the *xpr*D1 mutant. The mutation in the *xpr*D1 locus appears to affect the repression and secretion of extracellular proteinases and not the derepression of the conversion of β^1 and β^2 to β. Finally, the effect of *xpr*D1 is pleiotropic. The release of extracellular proteinases if derepressed whether carbon, nitrogen or sulfur are present in the fungal medium. This indicates that the carbon, nitrogen and sulfur repression of proteinases is interrelated.

N. crassa's proteinases are regulated in a similar manner, with one difference. Under conditions of carbon, nitrogen and sulfur limitation extracellular proteinases from this organism are expressed, but only in the presence of a high molecular weight protein in its medium (Matile, 1965; Drucker, 1972; Hanson and Marzluf, 1973; Drucker, 1973; Drucker, 1975). The induction is not seen when peptides or amino acids are added to the medium. The major extracellular, alkaline proteinase (Mr 31,000) of *N. crassa* has been purified and is expressed whenever carbon, nitrogen or sulfur are limited (Hanson and Marzluf, 1973; Hanson and Marzluf, 1975; Lindberg et al., 1981).

The structural gene of this proteinase is thought to be regulated by signals arising from one of three circuits that control carbon, nitrogen and sulfur assimilation. The carbon circuit effect is hypothetical, as no one has isolated *Neurospora* equivalents of the *A. nidulans cre*A^d mutant (derepressed allele of the carbon repression A locus) which is defective in carbon catabolite repression (Marzluf, 1977). However, the control of this proteinase by the regulatory gene *nit*-2 has been described (Hanson and Marzluf, 1975). Whenever glutamine or ammonia are not available to the fungus, the nitrogen metabolism locus, *nit*-2, acts as a positive regulator of several genes involved in the utilization of secondary nitrogen sources including nitrate and nitrite reductase, L-amino acid oxidase, phenylalanine ammonia lyase, the purine catabolic enzymes and of course extracellular proteinase (Hanson and Marzluf, 1975; Fu and Marzluf, 1987). Typically, these enzymes require an inducer in addition to the product of the *nit*-2 locus. For example, nitrate and nitrite reductase requires the induction by nitrate, mediated by the minor control gene *nit*-4, and the lifting of nitrogen metabolite repression by *nit*-2. The *nit*-2 gene has been cloned

and is regulated and expression of its mRNA is increased three fold under conditions of nitrogen starvation (Fu and Marzluf, 1987).

The sulfur assimilating enzymes of *N. crassa* are also under the control of a master regulatory circuit. The expression of sulfate permeases, aryl sulfatase, choline sulfatase, choline sulfate permease, a methionine specific permease and an extracellular proteinase is repressed in the presence of methionine or inorganic sulfate, and derepressed whenever sulfur is limited (Hanson and Marzluf, 1973; Marzluf, 1977). The *cys*-3 (cysteine) locus encodes a positive regulatory protein that is required for the expression of all of these activities (Marzluf and Metzenberg, 1968; Hanson and Marzluf, 1975; Marzluf, 1977; Ketter and Marzluf, 1988). The gene encoding *cys*-3 has been cloned and like the *nit*-2 gene it is regulated. Expression of its mRNA is increased when wild type cells are cultured in a sulfur depleted medium (Paietta et al., 1987). A second unlinked regulatory gene, *scon*, has been described in the sulfur regulatory circuit. Its effect is opposite that of *cys-3*; it represses the expression of these genes (Burton and Metzenberg, 1972). The mutant allele, *scon*^{c}, results in a phenotype where the various enzymes are constitutively expressed. The two loci, *scon* and *cys*-3, may act in concert. The product of the *scon* gene could respond to the amount of sulfur present in the cell. If the levels of sulfur were low the product of the *scon* locus would act to derepress or induce the *cys*-3 gene, resulting in the derepression of the sulfur metabolizing enzymes.

It is hypothesized (Figure 15) that the *N. crassa* alkaline proteinase gene contains upstream regions that could be recognized by the products of the major regulatory genes nit-2, cys-3 and an unidentified carbon-catabolite repression locus (Hanson and Marzluf, 1975; Marzluf, 1977). The proteins encoded by these master regulatory genes would not only have an affinity for these upstream sequences but also for the specific repressor metabolite or repressor molecule such as *scon* that would reflect the carbon (glucose or cAMP), nitrogen (ammonia) or sulfur (cysteine) content in or around the cell. Other *N. crassa* proteinases are expressed in response to nutrient starvation including two chelator sensitive proteinases, and an acid proteinase (Lindberg et al., 1982).

Little is known how the proteinases of *T. rubrum*, or the other dermatophytes, are regulated. Meevootisom and Niederpruem (1979) have reported that glucose or amino acids can repress the expression of extracellular proteinase activity. Clearing of particulate elastin in an agar-plate assay occurred for all dermatophytes tested as long as glucose was left out of the medium. The dermatophytes they studied included *M. gypseum, M. cookei, M. canis, T. mentagrophytes, T. simii, T. terrestre and T. rubrum.* The addition of 2% glucose to their agar medium prevented the clearing of the elastin for all strains of fungus tested

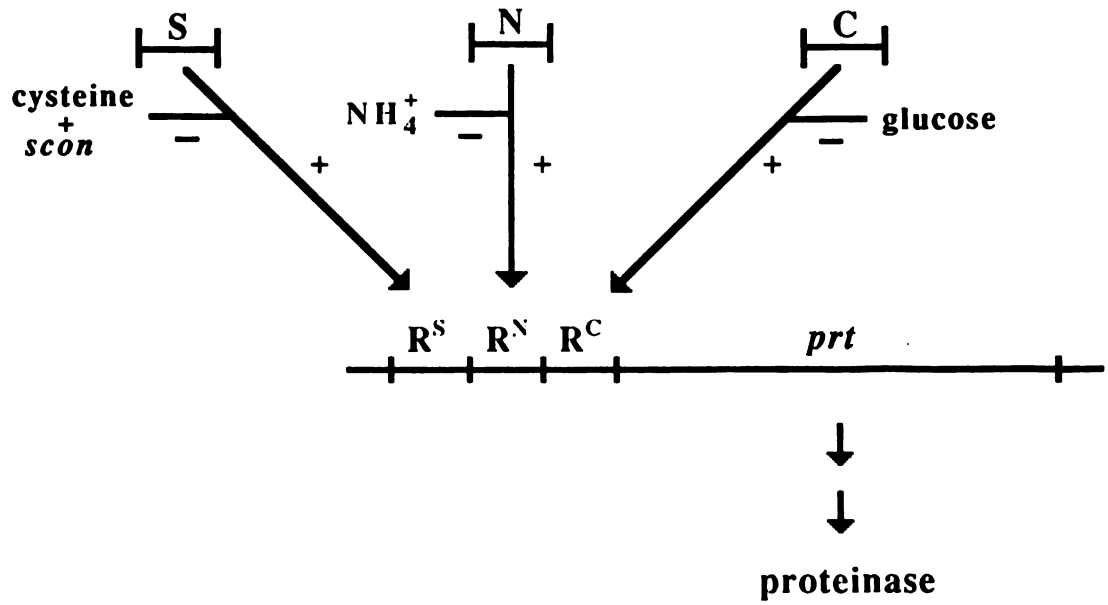

Figure 15. Proposed model for regulation of extracellular proteinase in *Neurospora* (Hanson and Marzluf, 1975). S represents the *cys*-3 regulatory gene; N represents the *nit*-2 locus. The other genetic locus (C) is unidentified. R^S, R^N and R^C are the receptor sites postulated to exist in a promoter region adjacent to *prt*, the structural gene for the enzyme. Negative effectors are cysteine (also *scon*), NH_4^+ and either glucose or cAMP for repression of sulfur, nitrogen and carbon regulated enzymes, respectively.

with the exception of *T. mentagrophytes* and *M. gypseum*. In these fungi there was a 50% reduction of elastin clearing.

Glucose also repressed the production of guinea pig hair hydrolyzing enzymes for cultures of liquid grown fungi. After two weeks of growth in a keratin salts medium that contained 0.5% (w/v) glucose, strains of *M. gypseum, T. mentagrophytes*, and *T. rubrum* produced 80% less proteinase. After four weeks, proteinase activity in cultures of *T. mentagrophytes* and *M. gypseum* did increase, but this was not the case for *T. rubrum*. Addition of 1-2% glucose to the dermatophyte medium obliterated proteinase production by these fungi when the CM was assayed after two or four weeks.The effect of sugars and amino acids on proteinase production by *T. rubrum* was also studied. When erythrose, sorbitol, galactose, glucose, fructose or mannitol where added to the fungal medium there was a complete inhibition of guinea pig hair hydrolysis when measured at two weeks. After four weeks the arabinose, galactose and lactose cultures produced proteolytic enzymes. Amino acids were also inhibitory, suppressing proteinase expression except in the case of isoleucine, serine and a complex of casamino acids.

The results of this study indicate that as easily metabolized substrates are made available to *T. rubrum*, proteinase secretion is not required. To confirm and extend these studies I have looked at the effects of glucose and neopeptone on proteinase production in cultures of *T. rubrum* grown on elastin agar, and in liquid cultures of log phase and stationary phase fungus. I have done additional work that shows that some of the proteinases produced by log phase *T. rubrum* cells are under carbon, nitrogen and sulfur repression.

3.2 Effects of Glucose and Neopeptone on Clearing of Elastin by *T. rubrum*

Several researchers have looked at the clearing of particulate elastin by strains of dermatophytes grown on agar containing this substrate (Rippon and Varadi, 1968; Meevootisom and Niederpruem, 1979). I have tested 12 clinical isolates of *T. rubrum* in this assay and found that only one strain, TR-13, was incapable of clearing particulate elastin (Table 11). On the whole, the addition of glucose repressed proteolytic activity, in agreement with previous reports. Three strains of *T. rubrum*, I.F.O. 9185, TR-3 and TR-5 were capable of clearing elastin when their medium was supplemented with 0.5% glucose. Strain TR-3 was unaffected by glucose concentrations as high as 2.0%. Clearing of elastin was usually seen within the first two weeks of growth if no glucose was added to the

strain	elastin salts (ES)	ES+0.5% glucose	ES+2.0% glucose
I.F.O. 9185	++++	+++	-
TR-3	++++	++++	++++
TR-4	++	-	-
TR-5	+++	+	-
TR-6	+	-	-
TR-7	+	-	-
TR-8	++++	-	-
TR-9	++++	-	-
TR-10	++++	-	-
TR-11	+++	-	-
TR-12	++++	-	-
TR-13	-	-	-

Table 11. Clearing of elastin salts agar, with and without the addition of 0.5% or 2.0% glucose, by various strains of *T. rubrum*. The plates were incubated for 4 weeks at 30° C. Clearing of the elastin was scored as a positive (+) result. *key*: (+), slight clearing of elastin under a few of the colonies; (++), clearing of the elastin under less than 1/2 of the colonies; (+++), clearing of less than 50% of the elastin in the plate; (++++), clearing of more than 50% of the elastin in the plate.

medium. The clearing of elastin in the 0.5% glucose cultures required a four week period before clearing was detected. The one exception was TR-3 which degraded the elastin usually within the first week.

Interestingly, potent proteolytic activity, including elastinolytic activity, has been described in strains of *T. rubrum* that were cultured in Sabouraud dextrose broth, which is composed of 1% neopeptone and 2% glucose (Sanyal et al., 1985; Asahi et al., 1985; this work). This is a glucose concentration that is inhibitory to clearing of elastin in the agar-plate assay. To test if strains of *T. rubrum* could clear elastin in this medium, the fungi were replated onto Sabouraud dextrose agar plates containing elastin (Table 12). Other combinations of glucose and neopeptone were also tested. Several strains of *T. rubrum* were able to clear elastin in the Sabouraud dextrose medium. This effect did take three to four weeks to be realized. After four weeks there was also clearing of elastin in the 1% neopeptone, 0.5% glucose medium by strains I.F.O. 9185, TR-5 and to a lesser extant TR-4. One strain, TR-3, cleared the elastin under all conditions and usually within one week.

In agreement with Meevootisom and Niederpruem (1979), in the presence of a readily accessible nitrogen and carbon source (neopeptone) or carbon source (2% glucose), there was no clearing of elastin (except in the case of TR-3). However, the combination of these two repressive substances appeared to stimulate the clearing of elastin. To confirm this result, 1% neopeptone was added to the original elastin salts (± glucose) medium and the fungi were replated under these conditions (Table 13). Whereas glucose inhibited clearing in the original elastin salts medium (Table 11), the addition of neopeptone reversed this effect in several of the strains. *T. rubrum* strain I.F.O. 9185, TR-5, TR-10, TR-13 and of course TR-3 were all capable of clearing elastin in salts plus 1% neopeptone, 2% glucose medium. Strains I.F.O. 9185, TR-3, TR-5 and TR-13 also cleared elastin in salts plus 1% neopeptone, 0.5% glucose or salts plus 1% neopeptone (with the exception of TR-13). These latter reactions required three to four weeks to be seen.

3.3 Log Phase Cultures Grown Under Conditions of Glucose Repression

The studies of *T. rubrum*'s proteinase expression were extended to include an analysis of proteinases contained in the CM of *T. rubrum* grown in liquid cultures. Log phase cultures of strain I.F.O. 9185 were grown in neopeptone, elastin salts or keratin salts media and the effect of glucose on three proteolytic activities was assessed (Table 14). Unless otherwise stated Strain I.F.O. 9185 was used for the rest of the studies described in this manuscript. It appears that when this fungus was grown as log phase cultures, easily metabolized substances such as glucose and neopeptone inhibit proteinase production. The

strain	1% neopeptone (neo)	neo+0.5% glucose	neo+2.0% glucose
I.F.O. 9185	-	++++	++++
TR-3	++++	++++	++++
TR-4	-	+	++++
TR-5	-	++++	++++
TR-6	-	-	+++
TR-7	-	-	-
TR-8	-	-	+
TR-9	-	-	+
TR-10	-	-	++++
TR-11	-	-	+++
TR-12	-	-	-
TR-13	-	-	-

Table 12. Clearing of 1% neopeptone, 0.5% (w/v) elastin agar, with and without the addition of 0.5% or 2.0% glucose, by various strains of *T. rubrum*. The plates were incubated for 4 weeks at 30° C. Clearing of the elastin was scored as a positive (+) result. *key*: (+), slight clearing of elastin under a few of the colonies; (++), clearing of the elastin under less than 1/2 of the colonies; (+++), clearing of less than 50% of the elastin in the plate; (++++), clearing of more than 50% of the elastin in the plate.

strain	elastin salts + 1% neopeptone (ESN)	ESN+0.5% glucose	ESN+2.0% glucose
I.F.O. 9185	++	++++	++++
TR-3	++++	++++	++++
TR-4	-	-	-
TR-5	++++	++++	++
TR-6	-	-	-
TR-7	-	-	-
TR-8	-	-	-
TR-9	-	-	-
TR-10	-	-	++++
TR-11	-	-	-
TR-12	-	-	-
TR-13	-	++	++

Table 13. Clearing of 1% neopeptone, elastin salts (ESN) agar, with and without the addition of 0.5% or 2.0% glucose, by various strains of *T. rubrum*. The plates were incubated for 4 weeks at 30° C. Clearing of the elastin was scored as a positive (+) result. *key*: (+), slight clearing of elastin under a few of the colonies; (++), clearing of the elastin under less than 1/2 of the colonies; (+++), clearing of less than 50% of the elastin in the plate; (++++), clearing of more than 50% of the elastin in the plate.

growth medium	substrate degraded (units/ml)		
	azocoll	elastin	keratin
1% neopeptone (neo)	0.26 ± 0.05	0.73 ± 0.14	0.41 ± 0.37
neo + 0.5% glucose	0.26 ± 0.13	0.50 ± 0.05	1.55 ± 0.49
neo + 2.0% glucose	0.43 ± 0.19	0.35 ± 0.21	0.28 ± 0.12
elastin salts (ES)	5.27 ± 1.12	9.18 ± 4.71	0.64 ± 0.17
ES + 0.5% glucose	0.36 ± 0.13	0.36 ± 0.13	1.18 ± 0.79
ES + 2.0% glucose	0.37 ± 0.09	0.41 ± 0.22	1.35 ± 0.71
keratin salts (KS)	4.09 ± 0.36	6.73 ± 1.28	0.19 ± 0.26
KS + 0.5% glucose	0.30 ± 0.08	0.66 ± 0.40	2.11 ± 0.56
KS + 2.0% glucose	1.10 ± 0.25	0.39 ± 0.03	0.88 ± 0.44

Table 14. Effect of glucose and neopeptone on the expression of azocoll, elastin and keratin degrading activities in log phase cultures of *T. rubrum*. Log phase hyphae of *T. rubrum* were inoculated into 10 ml of the above media as described in Appendix 1. After a 1 week incubation at 30° C an aliquot of the CM was analyzed for proteolytic activity (units/ml ± s.d.; n=3).

addition of either 0.5% or 2.0% glucose significantly decreased the amounts of azocollytic and elastinolytic activities present in *T. rubrum* CM. In contrast, the degradation of keratin azure seemed to be stimulated by the addition of 0.5% glucose to the neopeptone, elastin salts or keratin salts media. This effect was also seen when 2% glucose was added to elastin salts, and to a lesser extent when 2% glucose was added to keratin salts.

The proteinases present in the fungal CMs were analyzed in gelatin substrate gels (Figure 16). Under all conditions a high molecular weight activity (>200,000) was detected. The M_r 53,000 proteinase was detected in the neopeptone grown cultures, whether glucose was added or not. The elastin salts cultures produced an additional band around M_r 200,000 and a smear that could include the M_r 93,000 proteinase. The keratin salts cultures secreted the M_r 200,000 species and the M_r 27,000 proteinase. The keratin salts, 2.0% glucose cultures also secreted the M_r 200,000 species and the M_r 27,000 proteinase.

3.4 Stationary Phase Cultures Grown Under Glucose Repressive Conditions

Because three proteinases have now been purified from stationary phase cultures of *T. rubrum*, the effect of glucose on these stationary phase cultures was assessed. The fungus was grown up to stationary phase in Sabouraud dextrose broth, the mycelia was washed and the original broth was replaced with neopeptone, elastin salts or keratin salts media (± glucose). Each week, an aliquot from each culture was taken and assayed for azocollytic, elastinolytic and keratinolytic activity. The CMs were also analyzed by electrophoresis on gelatin or elastin substrate gels. There was no clear cut effect of glucose on proteinase production by stationary phase cultures. In the neopeptone medium, glucose repressed azocollytic activity but only in the first week (Figure 17A). During the second week, azocollytic activity fell in the neopeptone and neopeptone, 0.5% glucose cultures but again peaked in the third week. Proteinases in Sabouraud dextrose cultures peaked in the third week. Activity in all of the media fell in the fourth week.

Cultures of fungus grown in an elastin or keratin salts medium responded differently to glucose (Figure 17B and C). In the case of the elastin salts cultures, azocollytic activity was highest in the first week for medium that contained 0.5% glucose. The activity from elastin salts cultures peaked in the third week. Azocollytic activity rose approximately two-fold from the first to the fourth weeks in the elastin salts, 2% glucose cultures. Fungus

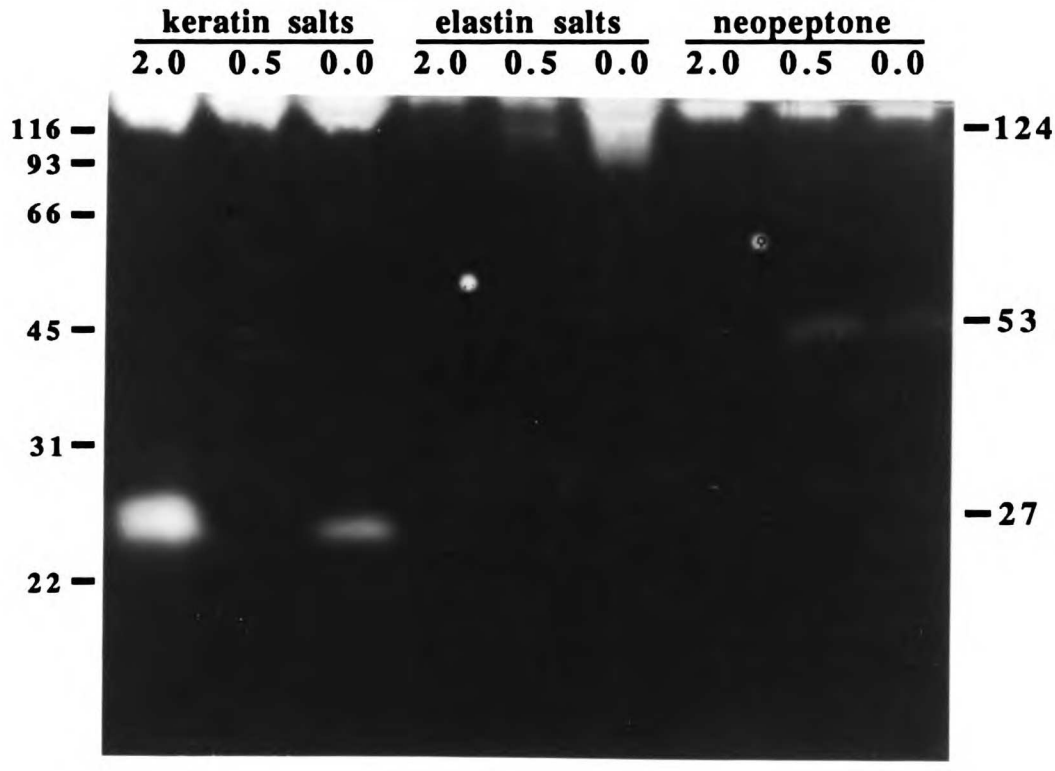

Figure 16. Effect of glucose on the expression of log phase gelatin-degrading enzymes. CM from log phase cultures of *T. rubrum* grown on keratin salts, elastin salts and neopeptone media appended with no glucose (0.0), 0.5% (w/v) glucose (0.5) or 2.0% (w/v) glucose (2.0), was analyzed on 12% gelatin containing substrate gels. Molecular mass markers (x 10^{-3}) are indicated at the left and relative molecular mass (x 10^{-3}) of the major proteolytic species is indicated at the right.

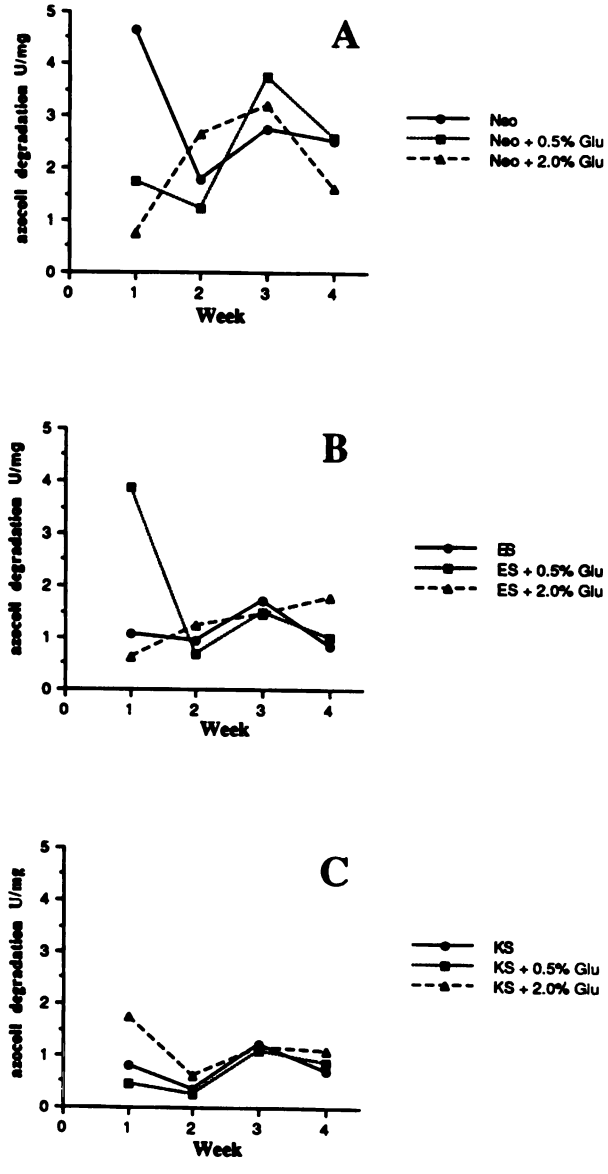

Figure 17. Effect of glucose on the expression of azocollytic activity in stationary phase cultures of *T. rubrum*. **(A)** Stationary phase cultures of *T. rubrum* were cultivated in a neopeptone medium (neo; ± glucose) and aliquots of the CM were assayed for azocollytic activity and protein content. **(B)** Fungus was cultivated in elastin salts medium (ES; ± glucose) and aliquots of the CM were assayed for azocollytic activity and protein content. **(C)** Fungus was cultivated in keratin salts medium (KS; ± glucose) and aliquots of the CM were assayed for azocollytic activity and protein content. The experiments were performed in triplicate and the average of the three values is reported.

grown in keratin salts produced proteinases that had higher specific activity in the first week (2% glucose cultures were the highest), which dropped in the second week, peaked in the case of keratin salts and keratin salts, 0.5% glucose in the third week and decreased somewhat in the fourth week. On the whole, *T. rubrum* grown in the elastin salts or keratin salts (± glucose) media had less azocollytic activity than fungus grown in the neopeptone (± glucose) media.

When CM from *T. rubrum* cultured in elastin salts, keratin salts or neopeptone media (± glucose) was analyzed on gelatin substrate gels several major activities could be detected at M_r 27,000, 53,000, 71,000 and a broad smear of activity from approximately M_r 93,000 to the top of the gel (Figure 18A, B and C). Although this smear was more visible on 10% gels no individual species could be distinguished (see figure 18B). The neopeptone ± glucose cultures expressed all of the major activities. There was a trend for the azocoll degrading activity to be mimicked by increased gelatinase activity when the same CMs were analyzed on gelatin substrate gels (Figure 18A). For example, if one looks at the neopeptone, 2% glucose culture, there was relatively low azocoll specific activity and low amounts of gelatin degrading proteinases during the first week. In the second week the M_r 27,000 proteinase activity was up as was the specific activity of azocoll degradation. The peak of specific activity occurred in the third week, and there also appeared to be increased gelatin clearing on substrate gels during this week. During the fourth week the specific activity decreased, while the intensity of substrate gel clearing remained almost constant. For each medium, the amount of M_r 27,000 proteinases appeared to increase with time. There was also the production of several low molecular weight activities of M_r 25,000 and 23,000 as the incubations were continued.

The elastin salts ± glucose cultures expressed the M_r 27,000, 53,000 proteinases and the broad smear of high molecular weight activity that sometimes included the M_r 71,000 activity (Figure 18B). Like the neopeptone cultures there was a tendency for the clearing of gelatin on substrate gels to grossly mimic the temporal pattern of azocoll degrading activity. For example, if one looks at the elastin salts, 2% glucose culture, there was an increase of azocoll degrading specific activity throughout the four week period, accompanied by an increased clearing of gelatin on substrate gels during the same four week period of time. The keratin salts ± glucose cultures, like the neopeptone cultures secreted the M_r 27,000, 53,000, 71,000 species and the high molecular weight smear of activity (Figure 18C). CM from fungus grown on keratin also had azocoll degrading specific activities that were mimicked by clearing of gelatin in substrate gels. This is pointed out in the keratin salts,

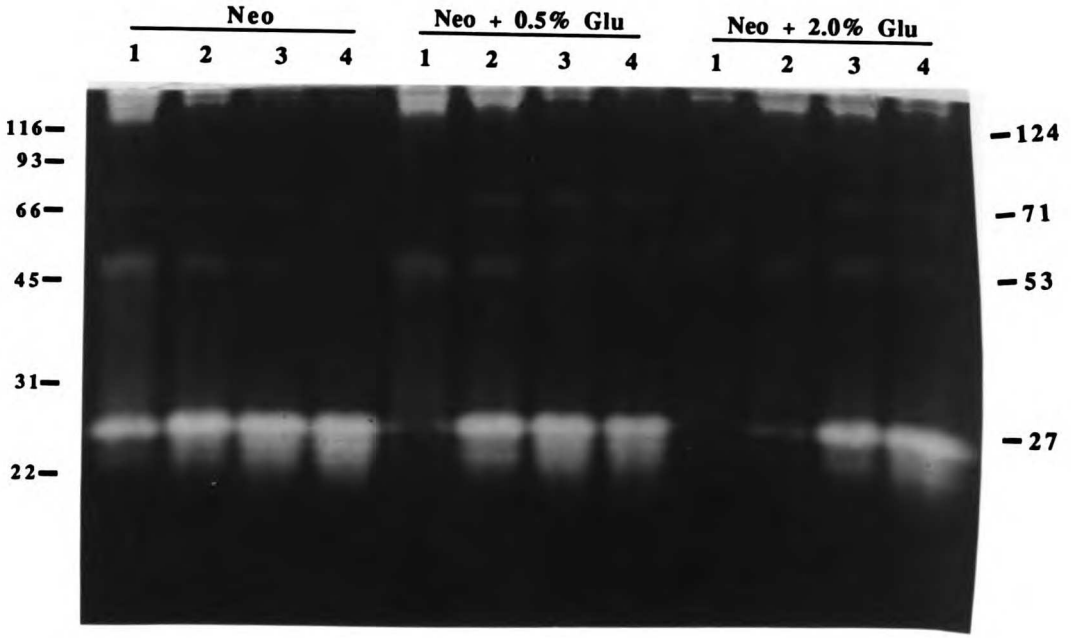

Figure 18. Effect of glucose on the expression of stationary phase gelatin-degrading enzymes. CM from stationary phase cultures of *T. rubrum* grown on **(A)** neopeptone media (neo; ± glucose), **(B)** elastin salts media (ES ± glucose), **(C)** or keratin salts medium (KS; ± glucose), was analyzed on 12% gelatin containing substrate gels (10% gel in the case of Figure 18B). Molecular mass markers (x 10^{-3}) are indicated at the left and relative molecular mass (x 10^{-3}) of the major proteolytic species is indicated at the right. The numbers underneath the media represent the week from which the CM was sampled.

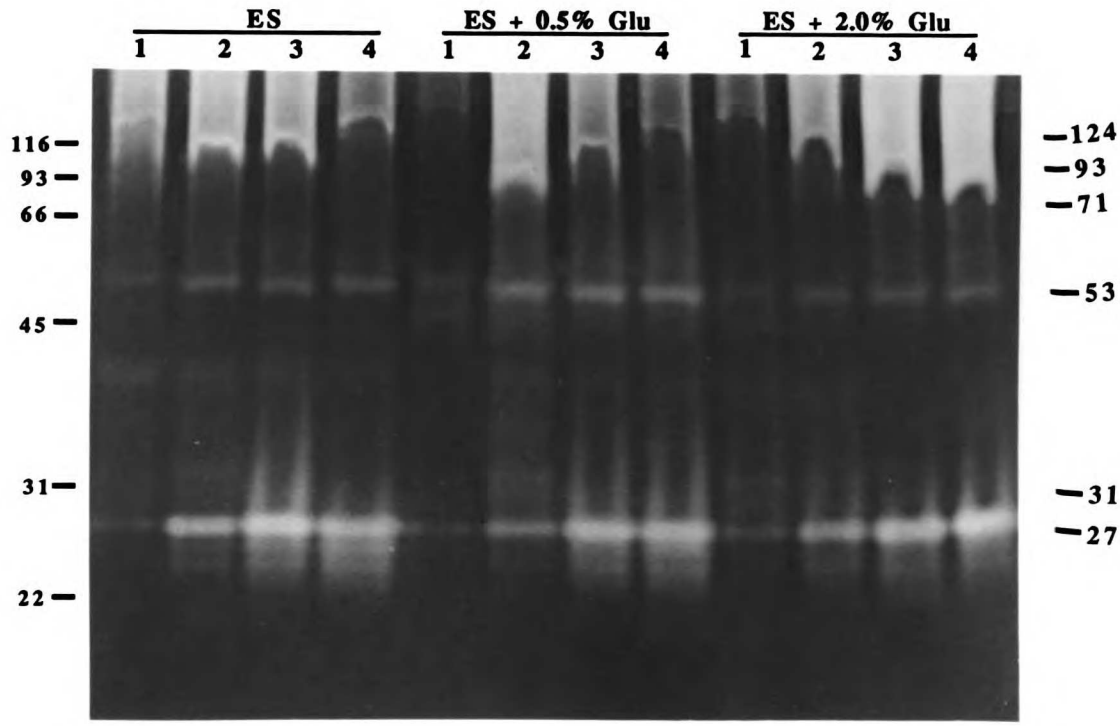
ES
ES + 0.5% Glu
ES + 2.0% Glu
1 2 3 4
1 2 3 4
1 2 3 4
116
93
66
45
31
22
124
93
71
53
31
27

Figure 18B

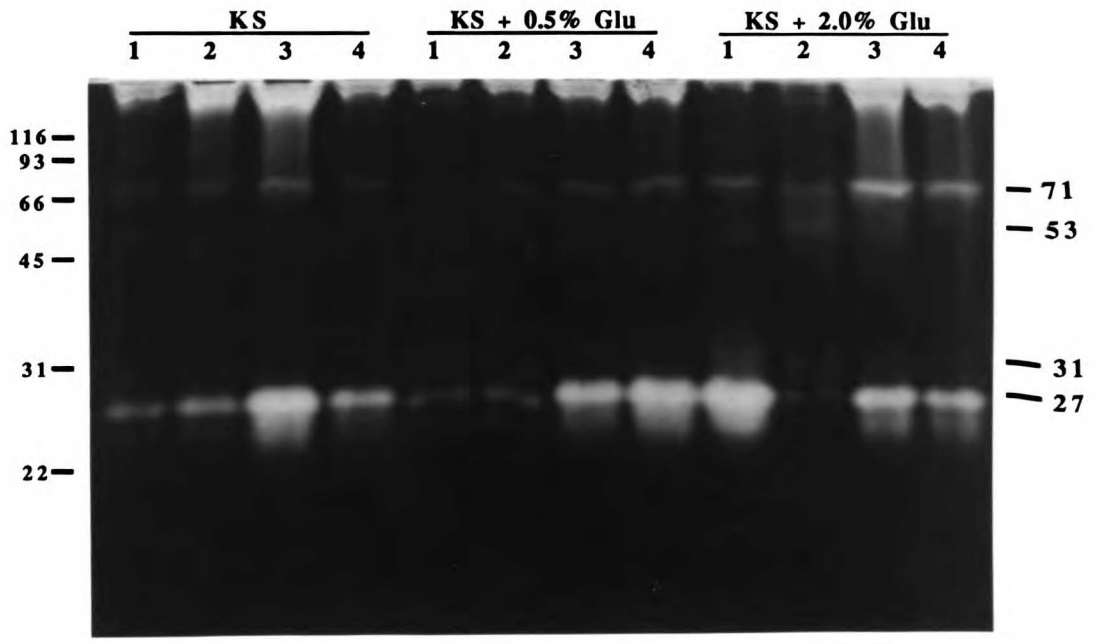

Figure 18C

2% glucose cultures. There was high specific activity and clearing in the first week, both of which fell during the second week. Both specific activity and gelatin clearing remained approximately constant during the third and fourth weeks. For both the elastin and keratin salts ± glucose cultures there appeared to be increased amounts of the M_r 27,000 activity during peaks of specific activity. Several lower molecular weight species that migrated faster than the M_r 27,000 proteinase were also detected.

The secretion of elastases appeared to be affected differently by the addition of glucose when compared to the expression of azocoll degrading proteinases. Whereas azocollytic activity was typically high in the first and third weeks, elastase activity peaked in different weeks depending on the composition of the medium (Figure 19A, B, C). For example, neopeptone and neopeptone, 0.5% glucose cultures had high elastase activity in the first week, while Sabouraud dextrose broth grown fungus produced very high elastase activity in the second week. Once elastase activity peaked in the cultures, it decreased to minimal levels by the fourth week. Elastase activity peaked in the second week for elastin salts, and elastin salts, 0.5% glucose grown fungus, eventually dropping in the third and fourth weeks. The elastin salts, 2% glucose cultures produced the most elastinolytic activity in the second and third weeks. Elastase activity was low in keratin salts ± glucose grown *T. rubrum* cultures. Elastase activity was highest in the second week for keratin salts and keratin salts, 0.5% glucose grown cultures. The keratin salts, 2% glucose cultures had very low levels of elastase activity in the first and second weeks, and relatively higher activity in the third and fourth weeks. Like the azocollytic activity , the peaks of elastase activity were highest in the neopeptone ± glucose cultures.

Several elastin degrading proteins were detected on elastin substrate gels (Figure 20). Activities detected included bands at M_r 27,000, 53,000, 71,000, 93,000, 124,000 and a high molecular weight smear to the top of the gel. Like the gelatin gels, there was a similarity in the pattern of elastase specific activity, and in the gross clearing of elastin in substrate gels. For example, in the neopeptone cultures the specific activity and clearing of elastin were highest in the first weeks. Both of these activities fell off during the following three weeks. The amount of clearing due to the M_r 27,000 and 53,000 proteinases was minimal, most of the elastase activity seemed to be due to the higher molecular weight activities. Keratinolytic activity was similar to azocollytic activity, peaking typically in the first and third weeks (Figure 21). However, in the neopeptone, 2% glucose cultures keratinase activity peaked in the second week. The 1% neopeptone cultures had high keratinolytic activity in the first week, lower activity in the second, and high activity in the

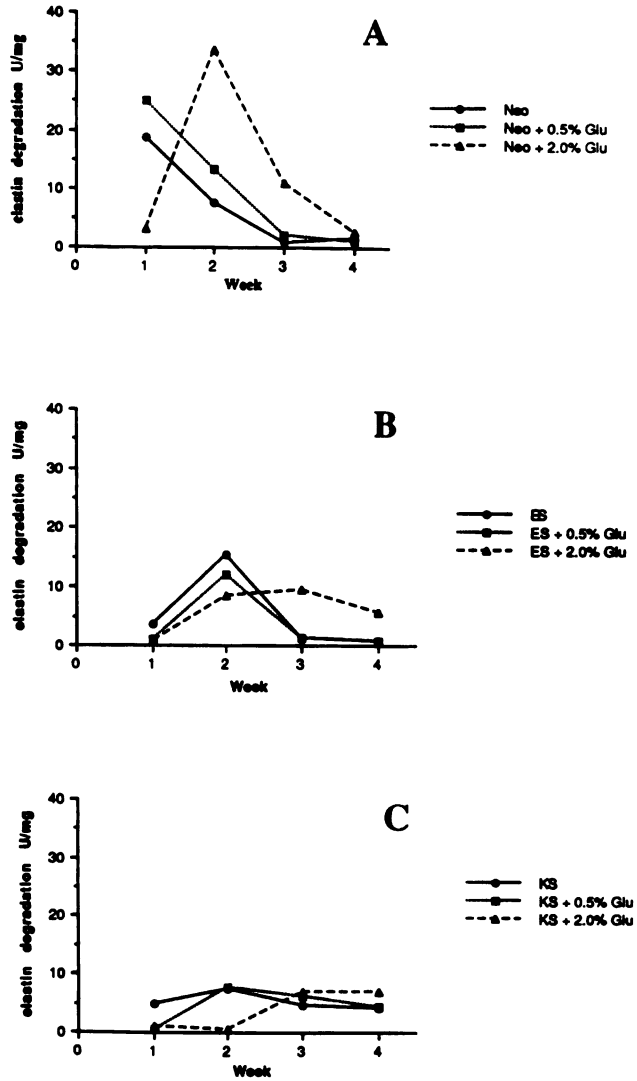

Figure 19. Effect of glucose on the expression of elastinolytic activity in stationary phase cultures of *T. rubrum*. (A) Stationary phase cultures of *T. rubrum* were cultivated in a neopeptone medium (neo; ± glucose) and aliquots of the CM were assayed for elastinolytic activity and protein content. (B) Fungus was cultivated in elastin salts medium (ES; ± glucose) and aliquots of the CM were assayed for elastinolytic activity and protein content. (C) Fungus was cultivated in keratin salts medium (KS; ± glucose) and aliquots of the CM were assayed for elastinolytic activity and protein content. The experiments were performed in triplicate and the average of the three values is reported.

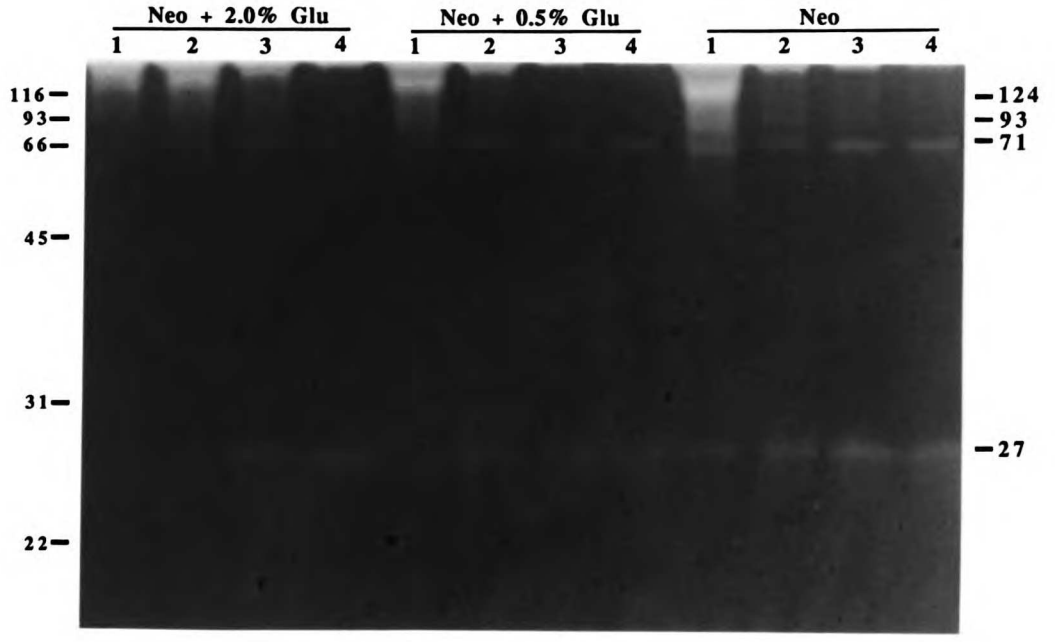

Figure 20. Effect of glucose on the expression of stationary phase elastin-degrading enzymes. CM from stationary phase cultures of *T. rubrum* grown on **(A)** neopeptone media (neo; ± glucose), **(B)** elastin salts media (ES ± glucose), **(C)** or keratin salts medium (KS; ± glucose), was analyzed on 12% elastin containing substrate gels. Molecular mass markers (x 10^{-3}) are indicated at the left and relative molecular mass (x 10^{-3}) of the major proteolytic species is indicated at the right. The numbers underneath the media represent the week from which the CM was sampled.

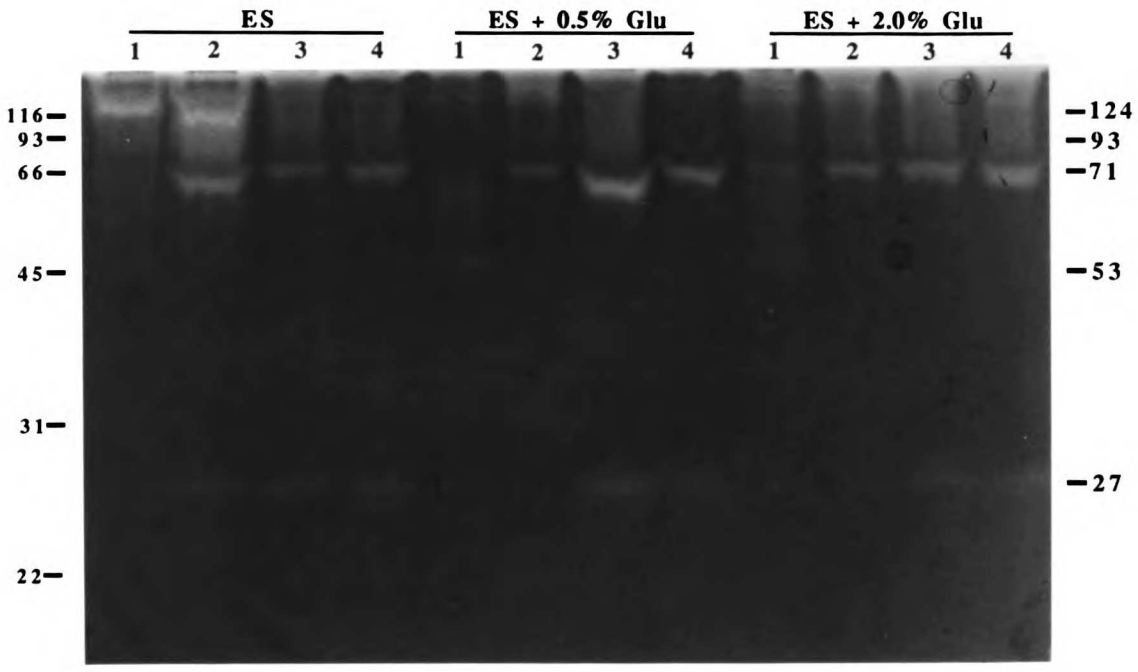
ES
ES + 0.5% Glu
ES + 2.0% Glu
1 2 3 4 1 2 3 4 1 2 3 4
116
93
66
45
31
22
124
93
71
53
27

Figure 20B

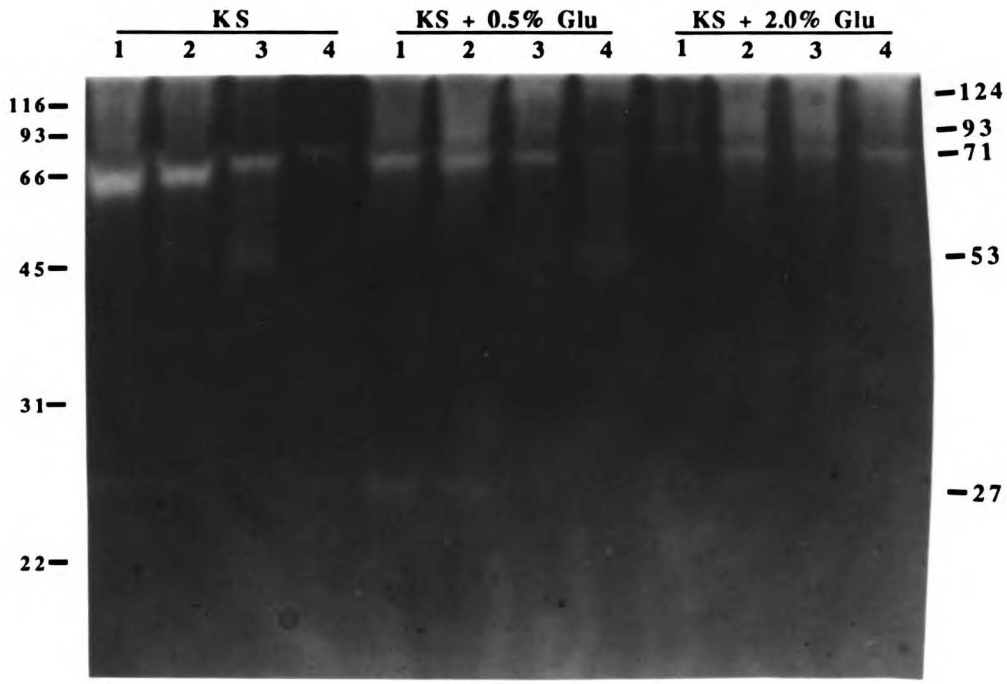
KS
KS + 0.5% Glu
KS + 2.0% Glu
1 2 3 4 1 2 3 4 1 2 3 4
116
93
66
45
31
22
124
93
71
53
27

Figure 20C

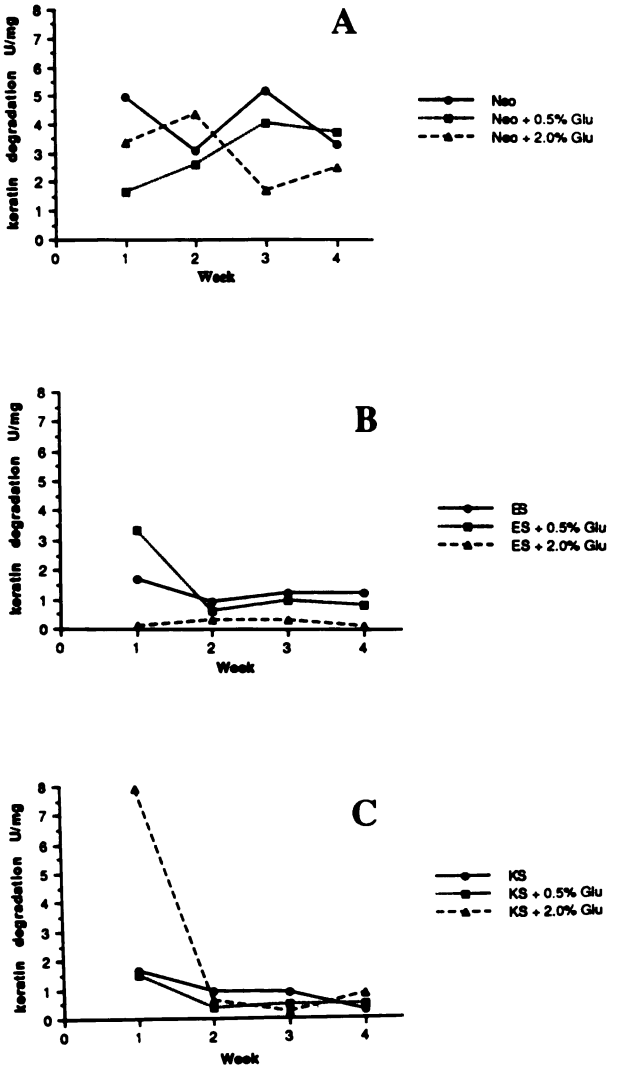

Figure 21. Effect of glucose on the expression of keratinolytic activity in stationary phase cultures of *T. rubrum*. (A) Stationary phase cultures of *T. rubrum* were cultivated in a neopeptone medium (neo; ± glucose) and aliquots of the CM were assayed for keratinolytic activity and protein content. (B) Fungus was cultivated in elastin salts medium (ES; ± glucose) and aliquots of the CM were assayed for keratinolytic activity and protein content. (C) Fungus was cultivated in keratin salts medium (KS; ± glucose) and aliquots of the CM were assayed for keratinolytic activity and protein content. The experiments were performed in duplicate and the average of the two values is reported.

third that decreased in the fourth. In the neopeptone, 0.5% glucose cultures keratinolytic activity peaked in the third week. The elastin salts and elastin salts, 0.5% cultures had the highest keratinolytic activity in the first week. The elastin salts, 2% glucose cultures had low activity throughout the four week period. In the keratin salts ± glucose cultures keratinolytic activity was highest in the first week (especially in the keratin salts, 2% glucose cultures) and remained at a constant, low level for the rest of the four week period.

3.5 Repression of Log Phase Proteinase Expression

As described in the introduction to this chapter, fungi are able to sense a lack of carbon, nitrogen, sulfur and phosphorus in their environment. They respond by producing a number of activities responsible for the assimilation of important nutrients, including proteinases. Log phase fungi were washed, and then cultured in minimal medium (0.25% glucose as a carbon source, 50 mM $(NH_4)_2PO_4$ as a nitrogen source, 3.4 mM KH_2PO_4 and 5.75 mM K_2HPO_4 as phosphorus sources and 2 mM $MgSO_4$ as a sulfur source) or a medium lacking a carbon, nitrogen, sulfur or phosphorus source. The results of nutrient depletion on proteinase production are shown in Table 15.

Very little azocollytic or elastinolytic activity was produced in the minimal medium, although there was some keratinase activity produced. Clearly, *T. rubrum* responds to nutrient deprivation by elaborating proteinases. When either nitrogen, carbon or sulfur is deleted from the minimal medium this fungus responds by secreting proteinases capable of degrading azocoll. Little elastase or keratinase was produced under these conditions, except for slightly higher amounts of elastase produced in the nitrogen and carbon depleted cultures, when compared to the minimal medium control. However, because of the large variations, this data was probably not significant. The addition of keratin or elastin to the minimal medium did not induce azocollytic or elastinolytic activities, however, it did induce keratinolytic activity. It should be noted that in early experiments the nitrogen and carbon depleted cultures died out before the CMs could be assayed. These experiments were repeated using five times the inoculum of fungus,which yielded positive results. The concentration of glucose in the minimal medium did not appear to matter as similar results were obtained if the glucose concentration was increased to either 1% or 2%. Growth of *T. rubrum* was greatest in the minimal medium, the other conditions were inhibitory to mycelial production. Although the sulfur depleted cultures of fungus did appear to still be growing as their mass increased from 1.5 to 4 mg in the one week incubation period. Finally the the same derepression of proteolytic activity was detected if the fungus was incubated for only 3 days before the CMs were assayed.

growth medium	substrate degraded (units/ml)			weight (mg)
	azocoll	elastin	keratin	
minimal	0.03 ± 0.01	0.14 ± 0.12	0.57 ± 0.31	12
minimal + elastin	0.11 ± 0.02	0.25 ± 0.19	1.20 ± 1.02	n.d.
minimal + keratin	0.04 ± 0.05	0.60 ± 0.22	2.15 ± 1.45	n.d.
(-) carbon	1.00 ± 0.06	0.10 ± 0.14	0.08 ± 0.14	5
(-) nitrogen	1.35 ± 0.14	0.57 ± 0.58	0.30 ± 0.30	5
(-) sulfur	1.62 ± 0.16	0.32 ± 0.10	0.08 ± 0.14	4
(-) phosphorus	0.16 ± 0.07	0.19 ± 0.09	0.20 ± 0.20	n.d.

n.d. = not determined

Table 15. Effect of carbon, nitrogen, sulfur and phosphorus deprivation and proteins on the expression of azocoll, elastin and keratin degrading activities in log phase cultures of *T. rubrum*. Log phase hyphae of *T. rubrum* (approximately 1.5 mg of fungus, except in the case of the carbon or nitrogen depleted media in which case 2.9 mg of fungus were used) were inoculated into 10 ml of the above media as described in Appendix 1. After a 1 week incubation at 30° C an aliquot of the CM was analyzed for proteolytic activity (units/ml ± s.d.; n=3), and the mycelial mat was dried and weighed (mean; n=3).

When the CMs of the nutrient starved cultures were analyzed in gelatin substrate gels the following activities were detected (Figure 22). In the minimal medium a high molecular weight activity (M_r > 200,000) and the M_r 53,000 activity were detected. The carbon depleted cultures produced the high molecular weight activity, as did the nitrogen depleted cultures. The nitrogen depleted cultures also secreted the M_r 71,000 proteinase. The high molecular weight activity, the M_r 124,000 species and the M_r 27,000 proteinase were all secreted when the medium contained no sulfur. Finally, the phosphorus depleted cultures secreted the high molecular weight activity and the M_r 124,000 activity.

3.6 Effects of Organic and Inorganic Sulfur Sources on Proteinase Production by Sulfur Depleted Cultures

The repression of *T. rubrum*'s proteolytic activities by sulfur was particularly interesting because one of the activities that has been purified, the M_r 27,000 proteinase, was derepressed when sulfur was depleted from the fungus's medium. It would be interesting to determine if both organic and inorganic sources of sulfur could repress this proteolytic activity, and what proteinases would be expressed if the fungus was grown in a medium were a single protein served as the sole source of carbon, nitrogen and sulfur. Cultures of log phase *T. rubrum* were washed, then cultured in the minimal medium without $MgSO_4$. Various amino acids and inorganic sources of sulfur were added to these cultures.

The addition of methionine or cysteine to the sulfur depleted medium repressed proteinase production by fungus grown under these conditions (Table 16). Concentrations of sulfur containing amino acids as low as 0.5 mM were effective. Although all concentrations of methionine and 0.5 mM cysteine had no visible effect on fungal growth, the 5 mM and 50 mM cysteine cultures were growth inhibited. Addition of any of the other amino acids tested actually appeared to stimulate the production of proteolytic activity. Grossly, these cultures did not grow better than the minimal or methionine appended cultures. When the fungus was cultured in sulfur depleted medium that contained casamino acids, a blend of amino acids that contains methionine and cysteine, expression of proteolytic activity was repressed. Inorganic sources of sulfur, e.g. $MgSO_4$, NaS_2O_3 and Na_2SO_3 were equally as effective as organic sources of sulfur in repressing proteinase activity. On gelatin substrate gels, the M_r 27,000 proteinase and a zone of hydrolysis from M_r 124,000 to the top of the gel, was detected under conditions of derepression, but not under conditions of sulfur repression (Figure 23).

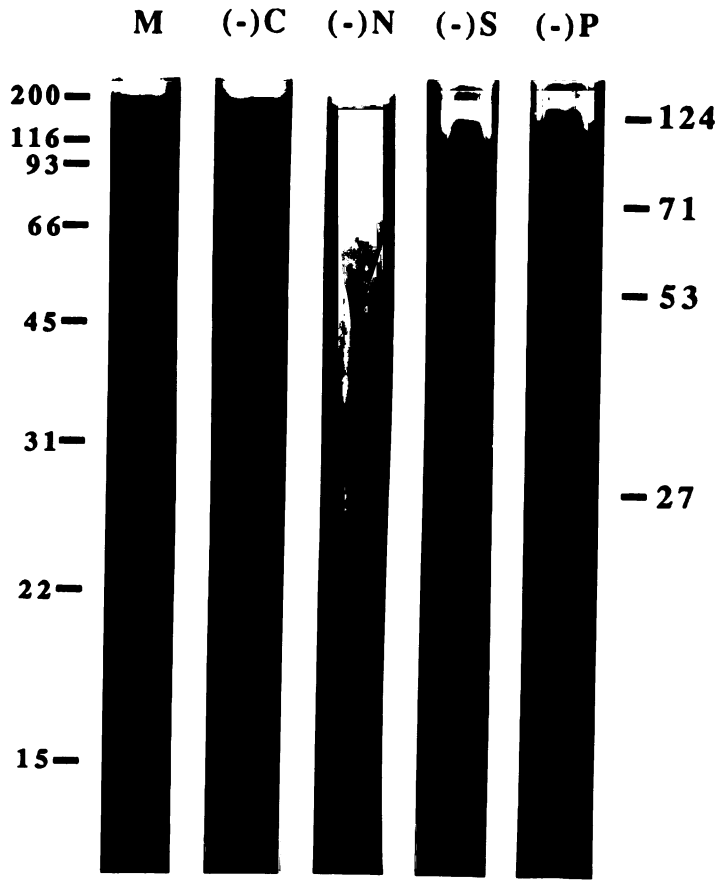

Figure 22. Expression of gelatin-degrading enzymes in log phase cultures of *T. rubrum* depleted of carbon, nitrogen, sulfur or phosphorus. Log phase *T. rubrum* was cultivated in a minimal medium (M) that contained 0.25% (w/v) glucose as a carbon source, 50 mM $(NH_4)_2PO_4$ as a nitrogen source, 3.4 mM KH_2PO_4 and 5.75 mM K_2HPO_4 as phosphorus sources and 2 mM $MgSO_4$ -7 H_2O as a source of sulfur. The carbon, nitrogen, sulfur or phosphorus depleted media (- C, N, S or P) were made by deleting the appropriate compound from the minimal medium, with the following changes: In the phosphorus depleted medium the potassium phosphate salts were left out of the medium and 50 mM NH_4HCO_3 was substituted for the $(NH_4)_2PO_4$. The 2 mM $MgSO_4$ was replaced by 2 mM $MgCl_2$ in the sulfur depleted medium. After 1 week incubation at 30° C an aliquot of CM from each of the cultures was analyzed by electrophoresis on gelatin containing substrate gels. Molecular mass markers (x 10^{-3}) are indicated at the left and relative molecular mass (x 10^{-3}) of the proteolytic species is indicated at the right.

growth medium	substrate degraded (units/ml)		
	azocoll	elastin	keratin
minimal	0.19 ± 0.03	*	*
(-) sulfur (S)	2.41 ± 0.18	*	*
(-) S + 2 mM NaS_2O_3	0.21 ± 0.15	*	*
(-) S + 2 mM Na_2SO_3	0.16 ± 0.03	*	*
(-) S + 50 mM met	0.62 ± 0.06	*	*
(-) S + 5 mM met	0.34 ± 0.05	*	*
(-) S + 0.5 mM met	0.27 ± 0.18	*	*
(-) S + 50 mM cys	0.31 ± 0.05	*	*
(-) S + 5 mM cys	0.40 ± 0.02	*	*
(-) S + 0.5 mM cys	0.50 ± 0.10	*	*
(-) S + 50 mM gly	3.04 ± 0.60	*	*
(-) S + 50 mM ile	4.13 ± 0.43	*	*
(-) S + 50 mM pro	2.99 ± 0.13	*	*
(-) S + 50 mM ala	3.29 ± 0.36	*	*
(-) S + 50 mM phe	3.04 ± 0.14	*	*
(-) S + 50 mM ser	2.54 ± 0.06	*	*
(-) S + casamino acids	0.77 ± 0.24	*	*
elastin (-) C,N,S	6.44 ± 0.76	30.50 ± 5.25	1.11 ± 0.59
keratin (-) C,N,S	1.03 ± 0.40	0.96 ± 0.12	0.95 ± 0.30
gelatin (-) C,N,S	0.79 ± 0.00	0.68 ± 0.09	0.00 ± 0.00
albumin (-) C,N,S	7.34 ± 0.49	31.99 ± 2.52	3.29 ± 0.93

* not determined

Table 16. Effect of inorganic and organic sulfur sources on the expression of proteolytic activity in log phase cultures of *T. rubrum*. Log phase hyphae of *T. rubrum* were inoculated into 10 ml of the sulfur depleted medium [(-)S] appended with inorganic sources of sulfur or amino acids as described in Appendix 1. The log phase hyphae were also cultured in a medium were a protein served as the sole source of carbon (C), nitrogen (N) and sulfur. After a 1 week incubation at 30° C an aliquot of the CM was analyzed for proteolytic activity (units/ml ± s.d.; n=3).

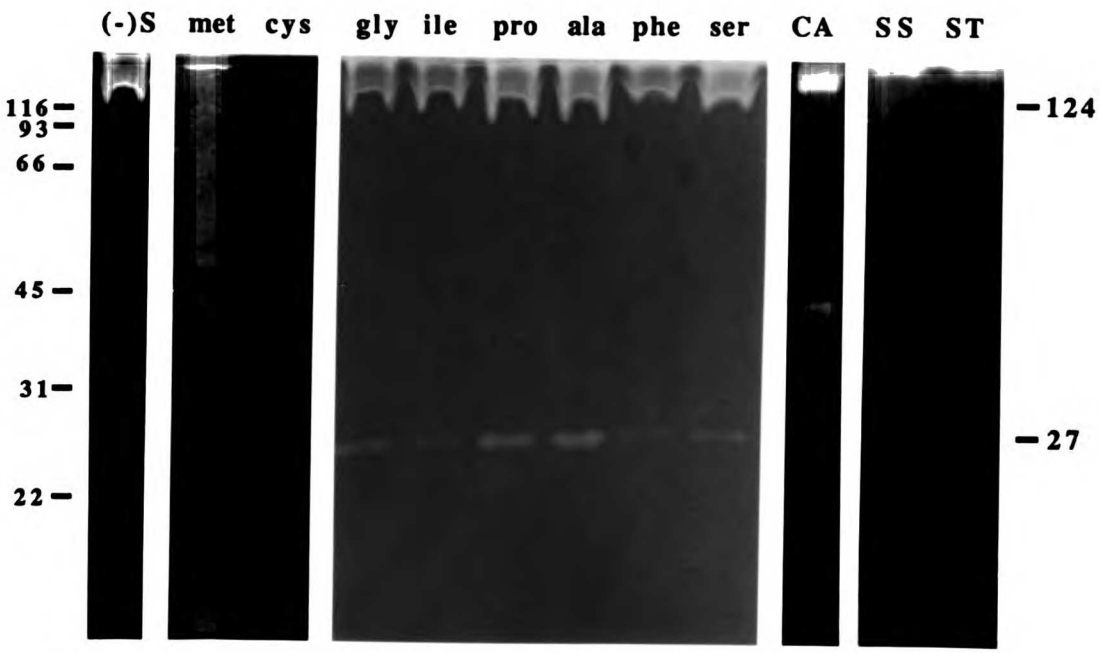

Figure 23. Expression of gelatin-degrading enzymes in sulfur depleted medium supplemented with amino acids and inorganic sources of sulfur. Log phase *T. rubrum* was cultured in sulfur depleted medium (-S) or sulfur depleted medium supplemented with 2.5% (w/v) casamino acids (CA) or 2 mM Na_2SO_3 (SS) or NaS_2O_3 (ST) or any of the following amino acids (50 mM): L-methionine, L-cysteine, L-glycine, L-isoleucine, L-proline, L-alanine, L-phenylalanine, L-serine. After 1 weeks incubation at 30° C an aliquot of CM from each of the cultures was analyzed by electrophoresis on gelatin containing substrate gels. Molecular mass markers (x 10^{-3}) are indicated at the left and relative molecular mass (x 10^{-3}) of the proteolytic species is indicated at the right.

Both the purified M_r 27,000 proteinase and the low molecular weight activity (also M_r 27,000) present in the sulfur depleted medium migrated in a similar fashion when analyzed on gelatin substrate gels. Both the purified M_r 27,000 enzyme and the sulfur depleted medium degraded azocoll and had low activity against elastin. The log phase sulfur depleted CM, like its M_r 27,000 proteinase counterpart, was inhibited by phe-gly-ala-leu-CH_2Cl (77% inhibition) and not by 1,10 phenanthroline or NEM. Finally, both the purified enzyme and the sulfur depleted CM degraded ala-ala-pro-phe-pNA (5.7 μM/min-ml of sulfur depleted CM) and ala-ala-leu-pNA (4.5 μM/min-ml of sulfur depleted CM) substrates.

If *T. rubrum* was grown under conditions where a single protein served as the sole source of carbon, nitrogen and sulfur, proteinases were derepressed in the case of elastin, which contains no methionines or cysteines. Proteinases were also derepressed when BSA was the sole source of carbon, nitrogen and sulfur. On gelatin substrate gels the M_r 27,000 activity was expressed as was the high molecular weight activities (data not shown). Although keratin, carbon, nitrogen, sulfur depleted cultures had repressed azocollytic and elastinolytic activity they still had significant keratinolytic activity.

3.7 Effects of Nutrient Depletion on Stationary Phase Cultures

Stationary phase cultures of *T. rubrum* were cultured in minimal medium or a medium lacking a source of carbon, nitrogen, sulfur or phosphorus, and proteolytic activity was assayed (Table 17). Unlike the log phase cultures, addition of elastin or keratin to the minimal medium of stationary phase cultures did not induce any activity above the controls. These stationary phase cultures responded poorly to depletion of phosphorus, carbon and especially nitrogen from their medium. Proteolytic activity was decreased under these conditions. The only exception was when sulfur was left out of the minimal medium. In this situation large amounts of azocoll, elastin and keratin degrading activity was detected.

The CM from these various culture conditions was analyzed on gelatin substrate gels (Figure 24). The minimal, minimal plus elastin, minimal plus keratin and phosphorus depleted cultures all had the same proteinase pattern. There was a broad area of activity from M_r 71,000 to the top of the gel, a doublet of activity around M_r 53,000 and additional activities at M_r 31,000, 27,000 and 25,000. The carbon and nitrogen depleted cultures of fungus secreted a series of high molecular weight activities (M_r 93,000 to the top of the gel), and two activities of M_r 53,000 and 45,000. A broad smear of activity from M_r 71,000 to the top of the gel, and the M_r 27,000 proteinase were detected in the (-) sulfur cultures.

growth medium	substrate degraded (units/ml)		
	azocoll	elastin	keratin
minimal	7.65 ± 0.22	57.54 ± 12.37	1.59 ± 0.67
minimal + elastin	6.84 ± 0.82	33.10 ± 19.89	0.77 ± 0.57
minimal + keratin	6.20 ± 1.19	7.50 ± 3.69	1.07 ± 1.10
(-) carbon	2.40 ± 0.66	2.99 ± 0.61	1.44 ± 1.36
(-) nitrogen	0.58 ± 0.29	0.81 ± 0.42	0.28 ± 0.36
(-) sulfur	7.94 ± 0.20	77.49 ± 21.08	1.82 ± 1.39
(-) phosphorus	4.16 ± 0.99	12.89± 6.09	0.41 ± 0.33

Table 17. Effect of carbon, nitrogen, sulfur and phosphorus deprivation or proteins on the expression of azocoll, elastin and keratin degrading activities in stationary phase cultures of *T. rubrum*. Stationary phase mats of *T. rubrum* were inoculated into 1.0 ml of the above media in a 24 well plate as described in Appendix 1. After a 1 week incubation at 30° C an aliquot of the CM was analyzed for proteolytic activity (units/ml ± s.d.; n=4).

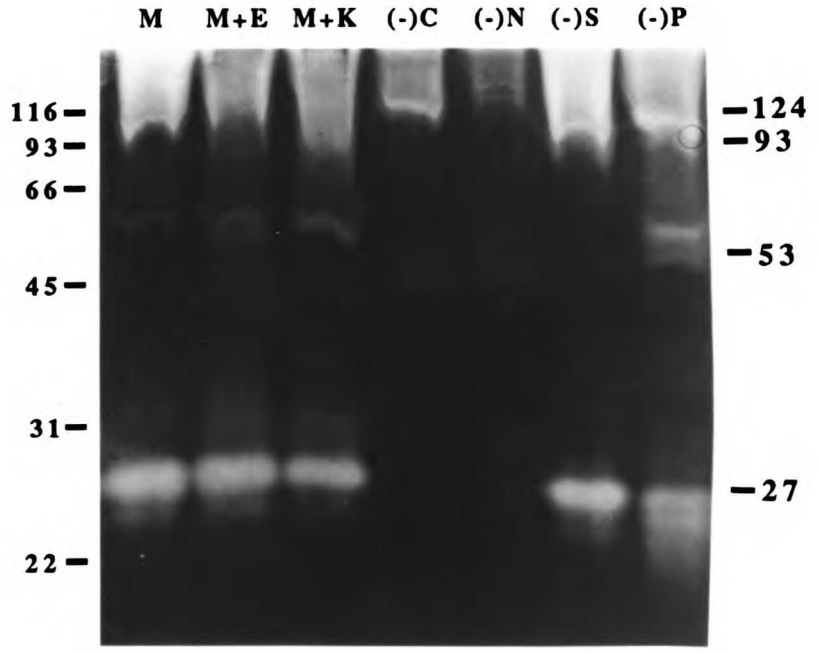

Figure 24. Expression of gelatin-degrading enzymes in stationary phase cultures of *T. rubrum* depleted of carbon, nitrogen, sulfur or phosphorus. Fungus, in stationary phase, was cultivated in a minimal medium (M) that contained 0.25% (w/v) glucose as a carbon source, 50 mM $(NH_4)_2PO_4$ as a nitrogen source, 3.4 mM KH_2PO_4 and 5.75 mM K_2HPO_4 as phosphorus sources and 2 mM $MgSO_4$ -7 H_2O as a source of sulfur. The carbon, nitrogen, sulfur or phosphorus depleted media (- C, N, S or P) were made by deleting the appropriate compound from the minimal medium, with the following changes: In the phosphorus depleted medium the potassium phosphate salts were left out of the medium and 50 mM NH_4HCO_3 was substituted for the $(NH_4)_2PO_4$. The 2 mM $MgSO_4$ was replaced by 2 mM $MgCl_2$ in the sulfur depleted medium. After 1 weeks incubation at 30° C an aliquot of CM from each of the cultures was analyzed by electrophoresis on gelatin containing substrate gels. Molecular mass markers (x 10^{-3}) are indicated at the left and relative molecular mass (x 10^{-3}) of the proteolytic species is indicated at the right.

3.8 Discussion

Clearing of elastin by strains of *T. rubrum* was repressed when 0.5% - 2% glucose was added to elastin salts agar plates containing this particulate substrate. With time (3-4 weeks) some of the strains were able to clear elastin even when 0.5% glucose was added to the elastin salts agar. This may represent the amount of time necessary to utilize the available glucose, or the time required for the production of an inducer of proteolytic enzymes. If this is true, it may be possible to detect clearing of elastin by most strains of *T. rubrum* if the plates are allowed to incubate longer than 4 weeks. Thick plates were poured, but not thick enough to prevent the dehydration of the agar that occurs over these long periods of time. There are strain differences, as only a few strains were able to clear elastin in the presence of 0.5% glucose. One strain, TR-3, seemed unaffected by concentrations of glucose as high as 2.0%. In fact, this strain secreted elastases under all conditions tested and could represent a mutant with constitutive expression of proteinases like the *xpr*D1 mutant of *A. nidulans*. Alternatively, the amount of proteinase produced could be related to the fast growth of this strain of *T. rubrum*.

The addition of a readily accessible nitrogen and carbon source (neopeptone) to agar plates containing elastin also inhibited elastase activity. But as low concentrations of glucose (0.5%) were added to the neopeptone cultures, some clearing of elastin was detected. Interestingly, the addition of 2.0% glucose stimulated the clearing of elastin. The positive effect of neopeptone and 2.0% glucose was also seen if these two substances were added to the original elastin salts plates, clearing of elastin was detected after 3-4 weeks.

Why the combination of two mediators of repression, neopeptone and glucose, act positively when in concert is not understood. It does explain why under certain conditions proteinases can be detected in the CM of *T. rubrum* cultures grown in Sabouraud dextrose broth (Sanyal et al., 1985; Asahi et al., 1985; this work). It should be noted that the derepression of proteolytic activity was not immediate and required 3-4 weeks for the clearing to occur. It could be that clearing of elastin is not detected until the glucose and neopeptone are utilized by the fungus or until the fungus reaches a stationary phase of growth when proteinase expression appears to be constitutive. The combination of 2% glucose and 1% neopeptone could facilitate their relatively rapid uptake or metabolism, not usually seen when only neopeptone or glucose is present in the fungal milieu. Clearing might be seen under all conditions if the incubations are extended. As already mentioned this is hard to do as the agar is prone to dehydration during these long periods of incubation. An alternative hypothesis would be that there is a positive regulator of

elastinolytic enzymes that is only expressed when both the levels of carbon and nitrogen are low.

Like the agar plate assay, glucose and neopeptone were repressive to azocollytic and elastinolytic activity in log phase, liquid cultures. (A summary of this data can be found in Table 18). The major difference being that Sabouraud dextrose broth was not stimulatory to proteinase production in log phase cultures. This difference may be related to the conditions of growth, i.e. on top of an agar surface as opposed to liquid culture. Additionally, the differences may be related to the different states of development of the fungus. The agar plate cultures sporulate, while liquid cultures do not sporulate as well. In contrast to the azocoll and elastin degradation, the proteolysis of keratin appeared to be stimulated by the addition of 0.5% glucose to the media. In gelatin substrate gels, no additional bands of activity were detected in the 0.5% cultures when these CMs are compared with those of the fungus grown in the other media. This would indicate that the proteinase responsible for keratinase activity does not degrade gelatin, or has very little activity against this substrate.

The presence of keratinolytic activity in 0.5% glucose cultures is significantly different from data presented by Meevootisom and Niederpruem (1979). These investigators reported that glucose concentrations as low as 0.5% were inhibitory to the hydrolysis of guinea pig hair by *T. rubrum*. The differences between our two reports may reflect the different substrates used in these studies and the pH at which activity was assayed, assuming that the hydrolysis of guinea pig hair or keratin azure (a dyed wool product) both represent keratinolytic activity. Meevootisom and Niederpruem reported degradation of guinea pig hair at pH 7.0, which incidentally is the same pH which their caseinolytic, elastinolytic, collagenolytic, etc. activities were assayed and detected. The ninhydrin-positive material released from the hair could represent the action of the neutral pH caseinolytic and elastinolytic enzymes on the non-keratinous components of hair. This argument is further strengthened by Meevootisom and Niederpruem's report that the actual degradation of keratins had a pH optimum of 8.5, close to the optimum I found for keratin azure degradation.

There were no clear cut effects of glucose or neopeptone on stationary phase cultures. Proteolytic activity was detected under all conditions. There was a trend for *T. rubrum* grown in neopeptone media ± glucose to have higher levels of azocollytic, elastinolytic and keratinolytic activity, when compared to fungus grown in the other media. The gelatin substrate gel patterns were similar for each culture medium and time point, suggesting that variations in the levels of total proteinase expression, or variations in the amount of actual

	substrate			proteinase M_r (x 10^{-3})					
	azocoll	elastin	keratin	>200	124	93	71	53	27
log phase cultures									
1% neo	-	-	-	+	-	-	-	+	-
1% neo, 0.5% glu	-	-	+	+	-	-	-	+	-
1% neo. 2.0% glu	-	-	-	+	-	-	-	+	-
ES	+	+	-	+	+	+	-	-	-
ES, 0.5% glu	-	-	+	+	-	-	-	-	-
ES, 2.0% glu	-	-	+	+	-	-	-	-	-
KS	+	+	-	+	-	-	-	-	+
KS, 0.5% glu	-	-	+	+	-	-	-	-	-
KS, 2.0% glu	+	-	+	+	-	-	-	-	+
(-) carbon	+	-	-	+	-	-	-	-	-
(-) nitrogen	+	-	-	+	-	-	+	-	-
(-) sulfur	+	-	-	+	+	-	-	-	+
(-) phosphorus	-	-	-	+	+	-	-	-	-
minimal	-	-	-	+	-	-	-	+	-
minimal + elastin	-	-	+	+	-	-	-	+	-
minimal + kerain	-	-	+	+	-	-	-	+	-
stationary phase cultures									
1% neo	+	+	+	+	+	(+)	+	[+]	+
1% neo, 0.5% glu	+	+	+	+	(+)	(+)	+	[+]	+
1% neo. 2.0% glu	+	+	+	+	(+)	-	+	[+]	+
ES	+	+	+	+	+	+	+	[+]	+
ES, 0.5% glu	+	+	+	+	+	+	+	+	+
ES, 2.0% glu	+	+	+	+	+	+	+	+	+
KS	+	+	+	+	(+)	+	+	+	+
KS, 0.5% glu	+	+	+	+	(+)	(+)	+	(+)	+
KS, 2.0% glu	+	+	+	+	(+)	(+)	+	+	+
(-) carbon	+	+	±	+	+	-	-	-	-
(-) nitrogen	-	±	-	+	+	-	-	-	-
(-) sulfur	+	+	+	+	+	+	-	-	+
(-) phosphorus	+	+	-	+	+	+	-	+	+
minimal	+	+	+	+	+	+	-	+	+
minimal + elastin	+	+	±	+	+	+	-	+	+
minimal + kerain	+	+	±	+	+	+	-	+	+

Table 18. Summary of the effects of glucose and nutrient deprivation on the expression of proteolytic activity in log phase and stationary phase *T. rubrum* cultures. *key*: neo, neopeptone; glu, glucose; ES, elastin salts; KS, keratin salts; +, ability to degrade a substrate, or presence of a particular proteolytic species; -, no degradation of the substrate or no detection of a particular proteolytic species; ±, variable degradation of a substrate; (+), activity observed only on elastin substrate gels; [+], activity observed only on gelatin substrate gels.

enzyme present in the total secreted protein, or presence of an inhibitor of proteinases accounted for the changes in the specific activity of azocoll degradation from week to week. In fact for many of the media the amount of clearing on the gelatin substrate gels was mimicked by the changes in azocoll degrading specific activities, i.e. there was increased clearing of gelatin on substrate gels during peaks of specific activity and decreased clearing during lulls in the azocoll degrading specific activity. The patterns of azocollytic and keratinolytic specific activities were similar for each individual medium tested. For example, if one looks at the elastin salts, 0.5% glucose cultures, both azocollytic and keratinolytic specific activities were highest in the first week, they dropped in the second, rose slightly in the third, and dropped slightly in the fourth.

The similarities in the peaks of azocoll and keratin degradation could suggest that the same or similar proteinases were responsible for both activities. Some of the proteinases that I have purified, the M_r 93,000 and 71,000 species, do have both keratinolytic and azocollytic activity and may be partially responsible for the keratin degradation I have observed. However, as noted for the log phase cultures there was a keratinase activity that was not detected by gelatin substrate gels and that was regulated differently than the azocoll and elastin degrading enzymes. The expression of this activity was stimulated by the addition of 0.5% glucose, while azocollytic and elastinolytic activities were repressed at this concentration of glucose. This uncharacterized keratinase activity could be the same activity seen in stationary phase cultures and deserves further study since it may represent the major keratinolytic activity of *T. rubrum*.

The pattern of elastase activity was different from the patterns of keratinase and azocollase activity. It typically peaked in the first or second weeks. Although, the M_r 93,000, 71,000 and 27,000 proteinases have elastinolytic activity, they probably only account for a portion of the total elastin degrading activity. This was demonstrated on elastin substrate gels, where higher molecular weight species with elastin degrading activity can be detected. Importantly, there seemed to be a more intense clearing of elastin in these gels during peaks of elastase specific activity. Like the keratinolytic activity there appears to be other elastin degrading proteinases that deserve further study.

Like *A. nidulans*, *A. niger* and *N. crassa*, log phase cultures of *T. rubrum* respond to a lack of carbon, nitrogen and sulfur in their environment by secreting proteinases (see Table 18). Under carbon, nitrogen and sulfur deprivation a high molecular weight activity (M_r > 200,000) was secreted. An additional M_r 71,000 proteinase was also produced by the nitrogen depleted cultures. The M_r 27,000 proteinase was secreted when this fungus was deprived of sulfur, as was a M_r 124,000 species. The secretion of different proteolytic

activities in response to nitrogen, carbon and sulfur starvation is different than what is seen in other fungi. *A. nidulans* and *N. crassa* secrete the same activities when proteinases are derepressed by lack of sulfur, carbon and nitrogen in the fungal environment. In the case of *A. nidulans*, three proteinases, α, δ–γ and ε, are secreted (Cohen, 1973b). *N. crassa* secreted an M_r 31,000 proteinase under carbon, nitrogen and sulfur depleted conditions (Hanson and Marzluf, 1975). This organism also expresses several acid proteinases under the same conditions (Lindberg et al., 1982).

Although the CM from fungus grown in the minimal medium had little proteolytic activity, the M_r 53,000 proteinase and a high molecular weight activity were detected by substrate gel analysis. There also was a small amount of detectable keratinolytic activity. By definition these proteinases are constitutively expressed, albeit at low levels. In addition, keratinolytic activity was induced when either elastin or keratin was included in the minimal medium. In contrast, azocollytic and elastinolytic activities were not induced by the addition of these proteins to the minimal medium. If this protein induced keratinase is the same as the activity seen in the log phase neopeptone, 0.5% glucose cultures it would suggest that protein induction of keratinase is only one way that *T. rubrum* regulates this activity, and unlike *N. crassa,* proteins are not necessary for proteinase induction.

Because one of the proteinases that has been purified, the M_r 27,000 species, was repressed by sulfur these results were pursued further. Is this the same activity that was purified from stationary phase cultures? There are several pieces of data that suggest this is true. Beside both activities migrating in a similar fashion when analyzed on gelatin substrate gels, both the purified enzyme and the CM from the sulfur depleted cultures degraded azocoll and had low activity against elastin. The log phase sulfur depleted CM, like its M_r 27,000 proteinase counterpart, was inhibited by phe-gly-ala-leu-CH_2Cl and not by 1,10 phenanthroline or NEM. Finally, both the purified enzyme and the sulfur depleted CM degraded ala-ala-pro-phe-pNA and ala-ala-leu-pNA substrates. Production of antibodies to the purified M_r 27,000 proteinase will allow me to determine if this activity is actually present in the sulfur depleted medium.

The following model for control of proteolytic activity in log phase cultures of *T. rubrum* is proposed. Under conditions of carbon, nitrogen or sulfur deprivation the fungus derepresses general proteinase (azocollytic) activity. For example, when *T. rubrum* was grown in a medium where a single protein served as the sole source of carbon or nitrogen, the fungus responded by secreting general proteinases capable of cleaving proteins into peptides. There are probably peptidases secreted by *T. rubrum* that would cleave the peptides into amino acids. The amino acids would then be recognized either directly, or metabolized into intermediates that could act as mediators of repression of not only

proteolytic activity, but other enzymes responsible for the assimilation of carbon and nitrogen.

If this model is correct, the addition of individual amino acids to the carbon or nitrogen depleted media should result in the repression of proteolytic activity. In addition, if the proposed model is correct it should be possible to detect exopeptidase activity in *T. rubrum* CM. I have shown that either a carbon source (glucose) or a carbon and nitrogen source (neopeptone) were inhibitory to azocoll and elastin degrading activities. Additionally, Meevootisom and Niederpruem (1979) have shown that the addition of individual amino acids repressed degradation of guinea pig hair. This proteolysis is probably not mediated by keratinases but rather by the other general proteolytic activities they described. It should be noted that the amount of proteolytic activity derepressed by carbon or nitrogen deprivation was much less than that seen when the fungus was cultured in a medium such as elastin salts which contains elastin as a sole source of carbon and nitrogen. In this medium greater amounts of azocoll and elastin degrading proteinases were expressed. It appears that the derepression of proteolytic activity is greater if the fungus is deprived of both carbon and nitrogen.

T. rubrum also expresses azocollytic activity when its medium is depleted of sulfur (Figure 25). Under this condition the fungus responds by turning on the expression of the M_r 27,000 and 124,000 proteinases. Again, these proteinases would cleave extracellular proteins, presumably the nonkeratinous proteins found in the stratum corneum and nails, into peptides. The proposed peptidases would cleave the peptides into individual amino acids. When the fungus takes up sulfur containing amino acids, i.e. methionine and cysteine, they would either be utilized directly, e.g. as amino acids for protein synthesis, or metabolized into other sulfur containing intermediates. It is hypothesized that like *N. crassa* and *A. nidulans* there are mediators of catabolite repression that would sense the increased concentration of sulfur in the cell, down regulating several enzymes responsible for sulfur assimilation, including proteinases.

In confirmation of this model of sulfur regulation, the addition of methionine or cysteine in concentrations as low as 0.5 mM to the sulfur depleted medium repressed proteinase production by fungus grown under these conditions. Addition of several other amino acids, including glycine, isoleucine, proline, alanine, phenylalanine and serine actually stimulated the production of proteolytic activity. When the fungus was cultured in sulfur depleted medium that contained casamino acids, a blend of amino acids that contains methionines and cysteines, expression of proteinases was also repressed. It appeared that the effect of sulfur containing amino acids, even in a blend of amino acids, are still repressive to proteinase expression. If the fungus was grown under conditions where a

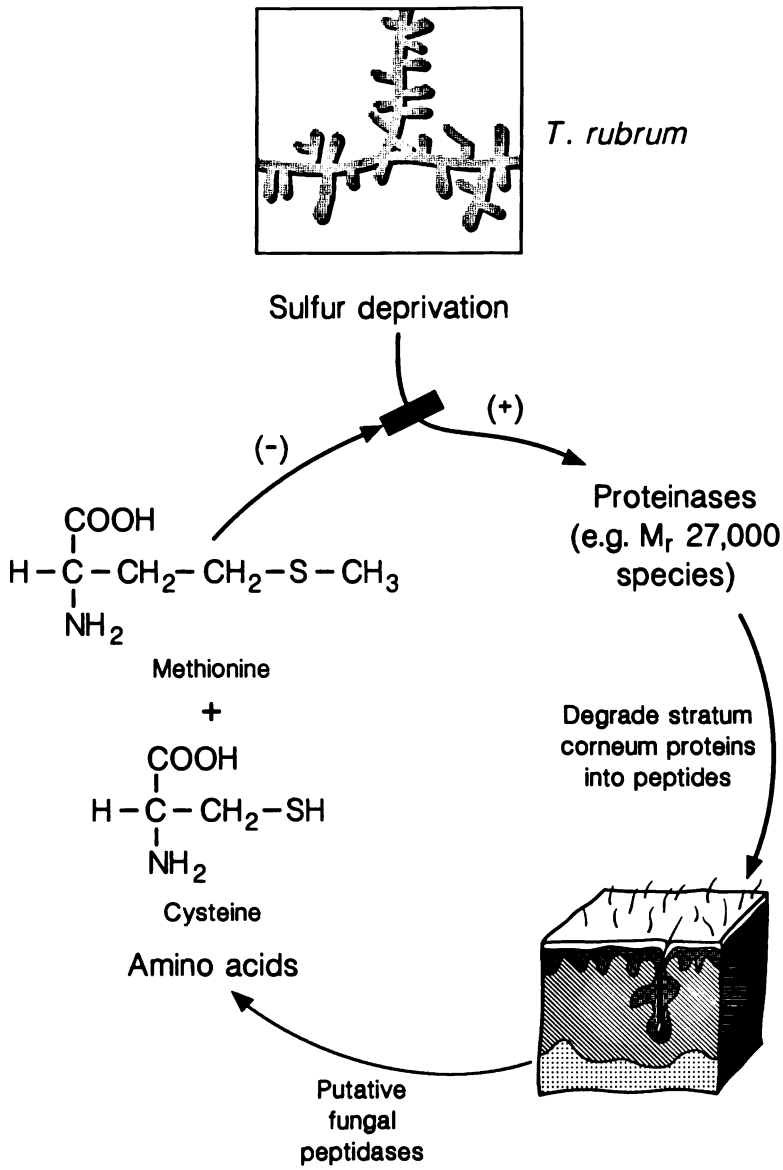

Figure 25. Proposed model for the regulation of the sulfur repressed proteinases of *T. rubrum*. Under conditions of sulfur deprivation, *T. rubrum* responds by secreting azocoll degrading proteinases, including the M_r 27,000 species. These proteinases would act on proteins contained in the stratum corneum of the skin releasing peptides into the fungal environment. There are putative peptidases that would cleave the peptides into amino acids including methionine and cysteine. These sulfur containing amino acids would then act as direct or indirect mediators of proteinase repression.

single protein, such as gelatin, elastin or keratin, was the sole source of carbon, nitrogen and sulfur, proteinase were derepressed only in the case of elastin which contains no methionines or cysteines. To confirm that only non sulfur containing proteinases would allow for the expression of the proteinase, BSA was also tried under the same conditions. Surprisingly, large amounts of azocoll, elastin and keratin degrading activities were expressed under these conditions. It may be that the proteolysis of BSA results in specific peptides that would stimulate the expression of proteinases. Another possibility is that BSA is not a good substrate for the fungal proteinases, such that no amino acids, sulfur containing or not, would be released, and proteinase activity would remain derepressed. Clearly, other proteins need to be studied. On substrate gels, the M_r 27,000 proteinase and a high molecular weight activity were detected when a protein serves as the sole source of carbon, nitrogen and sulfur. Inorganic sources of sulfur were equally effective at repressing proteinase expression. For example 2 mM $MgSO_4$, NaS_2O_3 or Na_2SO_3 quelled azocollytic activities.

While azocollytic and elastinolytic activities were repressed by carbon, nitrogen and sulfur, keratinolytic activity was not, indicating that it is regulated by a different mechanism. This activity was induced when insoluble proteins, such as elastin and keratin, were added to the minimal medium. The soluble protein, gelatin, had no effect in this regard (unpublished data). Although relatively little keratinase activity was expressed in the neopeptone, elastin salts or keratin salts medium, the addition of 0.5% or 2.0% glucose to these media stimulated expression of keratinolytic activity. The data suggests that keratinase is induced whenever there is a source of available carbon, nitrogen and sulfur. This induction can occur in the presence or absence of protein. How then is the keratinase activity induced if it requires carbon, nitrogen and sulfur to be present in the fungal medium? It is proposed that at the onset of a *T. rubrum* infection the carbon, nitrogen and sulfur repressed activities would become derepressed and act on the proteins other than keratins present in the stratum corneum. Once cleaved, peptides derived from these proteins would be further degraded by peptidases into individual amino acids. These would provide the cell with a source of carbon, nitrogen and sulfur. Under these conditions, the azocollytic and elastinolytic activities would be repressed while keratinases could be secreted.

Proteinase activity in stationary phase cultures appeared to be regulated differently than proteolytic activity in log phase cultures (see Table 18). This has already been pointed out in the discussion of the proteinase expression by *T. rubrum* cultures grown in glucose appended neopeptone, elastin salts and keratin salts media. Stationary phase cultures also respond differently to nutrient starvation. Unlike the log phase cultures, the stationary

phase fungus produced azocollytic, elastinolytic and keratinolytic activity in the minimal medium. The addition of protein to this medium did not induce any proteolytic activity. Proteinases were not derepressed when sources of carbon, nitrogen or phosphorus were deleted from the minimal medium. In fact, all proteolytic activities decreased under these conditions. The exception was in the sulfur depleted cultures. In this case, there was an increase in all activities. In stationary phase cultures all activities were derepressed and secreted constitutively.

IV. CONCLUSIONS AND FUTURE STUDIES

4.1 The Major Proteinases of *T. rubrum*

Because *T. rubrum* parasitizes the cornified tissues of man, several authors, have proposed that this organism requires proteinases to cleave available proteins into metabolically useable nitrogen, carbon and sulfur. In fact, *T. rubrum* CM has proteinases that not only degrade azocoll and keratin but also elastin and other proteins present in a model of the ECM, the R-22 matrix. On gelatin and elastin substrate gels several activities were detected, suggesting that there might be several proteinases secreted by this organism. Three of the major gelatinolytic enzymes present in *T. rubrum* CM have been purified. Gel filtration of the CM, followed by chromatofocusing was used to purify a M_r 93,000 proteinase 42-fold from crude fungal culture filtrate. This enzyme has a pI of 7.8 and a subunit of M_r 44,000, but forms a dimer of approximately M_r 93,000 in the absence of reducing agents. Using the same purification scheme a second enzyme of M_r 71,000 but with a lower pI of 6.5 was also purified. This enzyme has a reduced molecular weight of M_r 36,000 and exists as a dimer of M_r 71,000. It has a similar substrate specificity to that of the M_r 93,000 activity but has a lower specific activity for all of the substrates tested. Both species has pH optima of 8.0, calcium dependencies of 1mM, and were inhibited by serine proteinase inhibitors, α_1-proteinase inhibitor and PMSF. Kinetic studies showed that tetrapeptides containing aromatic or hydrophobic residues in the P-1 site were the best substrates. A k_{cat}/K_m of 27,000 $M^{-1}s^{-1}$ was calculated for the substrate succinyl-ala-ala-pro-phe-pNA when assayed with the Mr 93,000 proteinase. Both enzymes had significant activity against keratin, elastin and denatured type I collagen (azocoll).

The relationship of these two enzymes is not known, but because of their similar characteristics one could propose that the M_r 71,000 proteinase could be created by a posttranscriptional event such as mRNA splicing or a post translational event such as proteolysis of a larger precursor. Alternatively, the proteinases could be encoded by two separate genes. At present, the genes for these proteinases have not been identified so assessing the mRNA splicing hypothesis is not possible. To test the post-translational processing hypothesis would involve making antibodies to the enzymes. Immunoprecipitations of pulse-chase experiments may allow one to determine if there is a precursoral relationship between the two enzymes. The M_r 71,000 proteinase could represent a truncated version of the M_r 93,000 species, as such, its relative amount would increase with time, while that of the M_r 93,000 proteinase would decrease with time in a pulse-chase experiment. The M_r 93,000 enzyme could be the glycosylated or acylated version of the M_r 71,000 activity. If this were true, one would expect that in a pulse-chase

experiment the M_r 71,000 enzyme would be converted to the higher molecular weight M_r 93,000 species. Another experiment would be to cleave the M_r 93,000 proteinase with a glycosidase or a lipase, this could result in a molecule with a smaller molecular weight.

The major gelatinolytic enzyme secreted by *T. rubrum*, the M_r 27,000 proteinase, has been purified 32-fold from crude CM. The proteolytic activity in the flow-through of a concanavalin A column was fractionated on an anion exchange column (Polyanion SI). The M_r 27,000 proteinase was resolved from the higher molecular weight activities. When aliquots of the M_r 27,000 proteinase fractions were run on a SDS-polyacrylamide gel, without reduction, and silver stained, a single band at M_r 27,000 was visualized. Surprisingly, when reduced the M_r 27,000 band ran at a molecular weight of M_r 44,000. To confirm this result, the M_r 27,000 proteinase was labeled with [^{3}H]DFP and again analyzed by electrophoresis under reducing and nonreducing conditions. Again, under nonreducing conditions the proteinase migrated as a M_r 27,000 protein and under reducing conditions the protein migrated at M_r 44,000. At times two additional bands of protein at M_r 32,000 and 15,000 were detected in purified fractions. These bands could have represented contaminants, the unfolded products of an endoproteolytic cut, or different conformations of the M_r 27,000 proteinase. [^{3}H]DFP-labeled proteinase was electrophoresed on a preparative gel, electroeluted, resolved by electrophoresis and radioactive proteins were detected by autoradiography. Several radioactive proteins of M_r 35,000, 32,000 and 15,000 were detected, all of which resolved to a M_r 44,000 species following reduction. It appears that this M_r 44,000 proteinase is usually folded into a compact molecule with a preferred M_r of 27,000. This folding may be partially mediated by intrachain disulfide bonds.

The M_r 27,000 proteinase had a pH optimum of 8.0 and a calcium dependence of 2 mM. It was strongly inhibited by inhibitors of serine proteinases including 1 mM PMSF (94% inhibition), 0.2 μM phe-gly-ala-leu-CH_2Cl (92% inhibition) and was labeled with [^{3}H]DFP. This enzyme also cleaved peptide substrates with hydrophobic amino acids in the P-1 site, e.g. succiny-ala-ala-pro-phe-pNA (k_{cat}/K_m of 1573 M-1s-1) or Z-ala-ala-leu-pNA (k_{cat}/K_m of 1614 M-1s-1). It also degraded azocoll, laminin, fibronectin, type III collagen and type IV collagen. It had little activity against elastin and keratin and no activity against types I and V collagen.

Although the M_r 93,000 and 27,000 proteinases share a similar reduced molecular weight, they do not appear to be the same enzyme. The M_r 27,000 proteinase is a poor elastase and keratinase, has a pI less than 5.0, cleaves the substrate ala-ala-pro-phe-pNA with a specific activity 18-fold less than the M_r 93,000 species, and is not formed by the

dimerization of two smaller subunits. Also, the M_r 27,000 proteinase is repressed by sulfur, unlike the other proteinases.

The other M_r 23,000, 25,000, 53,000, 124,000 and the high molecular weight activities that have been identified have unknown substrate specificities and relations with the other proteinases that have been purified. The smaller species could represent proteolytic cleavages of the M_r 27,000 proteinases. The high molecular weight smear could represent multimers of lower molecular weight proteinases or unique, large proteinase species. It appears that the M_r 53,000 proteinase is a glycoprotein since it interacts strongly with the concanavalin A column. It is not found in the flow-through of the column, but is eluted with α-methyl mannoside.

4.2 Proteinases and Keratinolysis

The primary function of the proteinases that have been purified is to degrade the keratins and other proteins associated with the stratum corneum and nails, into nitrogen, carbon and sulfur sources easily assimilated by the fungus. The M_r 27,000 proteinase was a poor keratinase when assayed with keratin azure, a dyed wool substrate. In contrast, the M_r 93,000 and 71,000 proteinases degraded keratins extracted from the stratum corneum. Even if the M_r 27,000 proteinase is a poor keratinase it could still act on the 5% of proteins contained in the stratum corneum that are easily extracted by aqueous buffers (Sun and Green, 1978). In this sense, the M_r 27,000 proteinase can be thought of as a general proteinase akin to trypsin or chymotrypsin. This proteinase could also catalyze the hydrolysis of keratins that had previously been attacked and denatured by keratinases, such as the M_r 93,000 and 71,000 proteinases.

An alternative strategy of keratinolysis would be for proteinases like the M_r 27,000 species to act on the more readily degraded reduced keratin substrates. In the insects, e.g. the chewing lice of birds, the demestid beetles and some moths, it is thought that the low redox potential of their midgut region promotes the reduction of the disulfide bonds present in the keratins (Waterhouse, 1957). This denatured substrate can then be easily degraded by proteinases. The requirement for a partially denatured keratin substrate may also be true of some of the cutaneous and oral bacteria that colonize humans, as they do not degrade keratin unless a reducing agent is present (Mikx and DeJong, 1987). The cells must be in close contact to the substrate for maximal activity to be expressed. The soil bacterium *S. fradiae* may have two systems for the degradation of keratin (Noval and Nickerson, 1959). One system, like that of the oral and cutaneous bacteria, requires intimate contact of bacteria with substrate; it releases sulfhydryl containing peptides. The other system of proteolysis utilizes an enzyme that does not require the presence of cells, and releases products of hair

that do not contain sulfhydryls. Because the dermatophytes live in direct contact with their substrate, keratinolysis *in vivo* may involve both the enzymatic degradation of intact keratin and the denaturation of this substrate through reduction. It would be interesting to know if there is a reducing environment created, either by the host and/or fungus, around penetrating fungal hyphae.

Another possibility is that the keratins present in the deeper layers of the epidermis, still accessible to the fungus, are not as highly disulfide bonded as those of the stratum corneum. Whereas the majority (approximately 95%) of stratum corneum keratins require urea and β-mercaptoethanol to be extracted, the majority of live keratinocyte keratin can be extracted with urea alone (Sun and Green, 1979). These urea extracted keratins from keratinocytes can form 80 nm tonofilaments when the urea is dialyzed out of the extraction buffer, even in the absence of disulfide bond formation or the presence of DTT. Furthermore, these tonofilaments became crosslinked if the filaments are exposed to oxygen. Sun and Green (1979) have hypothesized that in living keratinocytes there is a reducing environment that prevents disulfide bonding of the keratins present in the cells. It would be an interesting experiment to determine if these reduced keratins are more susceptible to keratinolysis. If this were so, more proteins would be accessible to *T. rubrum*'s "general" proteinases than just a few easily extracted proteins in the stratum corneum.

How many keratinases are expressed by *T. rubrum*? At this time it is not possible to answer this question. It is clear that there are more keratinolytic activities present in *T. rubrum* CM than just the M_r 93,000 and 71,000 proteinases. For example, in log phase cultures of *T. rubrum* azocoll and elastin degrading activities were inhibited by glucose while the production of keratinolytic proteinases was stimulated by the addition of 0.5% glucose to protein or neopeptone media. On gelatin substrate gels, no additional bands of activity were detected in the 0.5% glucose cultures when these CMs were compared to those of the fungus grown in other media. Also keratinase activity was induced by the addition of keratin or elastin to a minimal medium; however, azocollytic and elastinolytic activity was not induced. This would indicate that there is an undetected keratinase(s) that does not degrade gelatin. Because the M_r 93,000 and 71,000 proteinases degrade both of these latter substrates it is unlikely they are responsible for the keratinase activity detected. It would be important to purify this activity to define its relationship to the other proteinases, and to better understand the expression of keratinolytic enzymes by this organism. Finally, it is also important to determine what domains of the keratin molecules are susceptible to *T. rubrum* keratinases. This organism's proteinases and the state the keratin molecule must be in to be degraded (e.g. reduced or nonreduced) may help us to

better understand both the substrate specificity of the fungal proteinases and how keratins are turned over *in vivo*.

4.3 Fungal Elastases

The production of proteolytic enzymes by *T. rubrum* is not limited to proteinases with a specificity for keratins. For example, the M_r 71,000 and 93,000 proteinases are excellent elastases and could degrade the elastin present in the dermal layers of the skin. These are probably not the only elastases, as several higher molecular weight species with elastinolytic activity can be detected on elastin substrate gels.

The function of the fungal elastases is not known. One characteristic of elastases is their broad substrate specificity (Banda et al., 1987). They have the ability to degrade several molecules including fibronectin, laminin, gamma globulins, α_1-proteinase inhibitor, keratin and type IV collagen (Banda et al., 1987; McKerrow et al., 1985a). In this sense, elastin is only one substrate an elastase can degrade. For example, the M_r 93,000 and 71,000 proteinases can degrade both keratin and elastin and may also degrade other molecules associated with the ECM. This latter possibility requires additional experimentation. If it is true that proteinases are required for the parasitism of what are basically proteinaceous tissues, one might imagine that there would be a evolutionary selection for organisms that produced enzymes with broad substrate specificities. In the case of *T. rubrum*, several proteinases are expressed, at least three of which have a relatively broad substrate specificity.

Fungal elastases could also have evolved to serve a function in the saprophytic existence of dermatophytes. Since elastin does not accumulate in the environment, one might suggest that there exists an ecological niche for organisms that can degrade elastin. As an embellishment of the model proposed for the evolution of the dermatophytes as parasites of keratinized tissues (Rippon, 1982) one might imagine that the ancient geophilic species of dermatophytes would be responsible for the degradation of proteins like keratins and elastin from dead animal tissue. Because of their ability to degrade keratins these geophilic species would have been admirably suited to later live on the hair and skin of living, soil inhabiting rodents. Although in this setting elastase production could be thought of as a vestigial process, it should be remembered that elastases appear to have broad substrate specificities and could be useful to the fungus when substrates other than keratin were encountered. The leap from rodent to humans would not be that great, and with time the host (humans) and the dermatophyte (*T. rubrum*) would reach a state of equilibrium when superficial infection and not invasive disease is observed. Ultimately, some of these dermatophytes become solely anthropophilic.

Besides elastin, *T. rubrum* also secretes enzymes that can degrade other components of the ECM. For example, the crude CM of this fungus can degrade significant amounts of both the trypsin- and elastase-sensitive components of the R-22 matrix, an *in vitro* model of the skin. In addition, the crude CM degraded almost all of the collagen present in this matrix. Some of the enzymes I have purified are able to degrade individual components of the ECM. As already mentioned, the M_r 93,000 and 71,000 proteinases can degrade elastin. The M_r 27,000 proteinase can degrade laminin, fibronectin, type IV procollagen (all substrates found in the basement membranes of cells) type III collagen and denatured type I collagen (azocoll). Although the M_r 27,000 proteinase does not degrade type I collagen, there is an activity in *T. rubrum* CM that can. Crude CM from this fungus was able to degrade the majority of collagen contained in the R-22 matrix. The identification of the actual collagenase(s) will require further experimentation. This fungus therefore possesses a battery of proteinases that could facilitate the disruption of connective tissue barriers in cases of disseminated disease. Such cases are rare (Rippon, 1982) reflecting the normal inhibition of *T. rubrum* growth at temperatures above that of the stratum corneum. However, Rippon and Scherr (1959) have shown that *T. rubrum* can be adapted to grow at 37° C.

4.4 Regulation of *T. rubrum* Proteinase Expression

I have suggested that proteinases are important to *T. rubrum* as a means of releasing carbon, nitrogen and sulfur sources from the keratinized tissues of humans. Two lines of evidence suggest that this may be true. First, easily metabolized substrates such as glucose and amino acids can repress proteinase secretion, arguing that as other nutrient sources are made available to this organism, proteinase secretion is not required as long as other nutritive molecules are present in its environment. Secondly, when either carbon, nitrogen or sulfur is absent from the fungal milieu the fungus responds by expressing proteinases, strongly suggesting that proteinases do play an important role in nutrition gathering by this organism.

The repressive effect of glucose or neopeptone on proteinase expression was seen under several conditions. For example, 0.5-2.0% glucose was repressive to the elastases produced by several strains of *T. rubrum* when added to elastin agar. With time (3-4 weeks), some of the strains were able to clear elastin even when 0.5% glucose was added to the elastin salts agar. An agar medium composed of neopeptone and particulate elastin also inhibited elastase production. Elastase activity could be detected on Sabouraud dextrose elastin agar plates, however, this clearing required several weeks to be visualized. One strain of fungus, TR-3, secreted elastases under all conditions tested and could

represent a mutant with constitutive expression of proteinases somewhat akin to the *xpr*D1 mutant of *A. nidulans*. Alternatively, the amount of proteinase produced could be related to the fast and luxuriant growth of this strain of *T. rubrum*. It would be interesting to define why proteinase production by this strain of fungus is so insensitive to repression by glucose and neopeptone, and if keratinase activity is also expressed in the absence of induction. Because of its quick growth and increased proteinase production, this strain may also be useful for the purification of several uncharacterized activities in *T. rubrum* CM. The azocollytic and elastinolytic activities of log phase cultures were also repressed by glucose and neopeptone when a single protein served as the sole source of carbon and nitrogen. Meevootisom and Niederpruem (1979) have reported similar results and have shown that a wide spectrum of sugars and single amino acids are repressive to proteinase production by this organism.

Like a number of fungi including *A. nidulans*, *A. niger* and *N. crassa*, log phase cultures of *T. rubrum* respond to a lack of carbon, nitrogen and sulfur in its environment by secreting proteinases. In this case azocollytic enzymes are expressed. A high molecular weight activity (M_r > 200,000) was secreted under all conditions of starvation. An additional M_r 71,000 proteinase was produced by nitrogen depleted cultures and the M_r 27,000 proteinase was secreted when this fungus was deprived of sulfur, as was a M_r 124,000 proteinase. The secretion of different proteolytic activities in response to carbon, nitrogen and sulfur starvation is different than what is seen in other fungi. *A. nidulans* and *N. crassa* secrete the same activities when proteinases are derepressed by lack of carbon, nitrogen and sulfur. It would be interesting to test various combinations of nutrient starvation on *T. rubrum*, e.g. carbon and nitrogen depletion or carbon and sulfur depletion, etc., to see what effect this would have on proteinase expression. One might expect to see something like what is observed when log phase cultures were cultivated on a protein such as keratin or elastin as the sole source of carbon and nitrogen. Instead of just the derepression of azocollytic activity, elastase activity could also be derepressed.

Why only azocollytic activity is derepressed in the face of nutrient deprivation is not known, but it does suggest that under these conditions the proteinases that are expressed are sufficient to cleave available proteins into carbon, nitrogen and sulfur sources. Importantly, the effect carbon and nitrogen depletion required a good deal of fungus to be detectable, five times more than required to see an equivalent response with the sulfur depleted cultures. The carbon and nitrogen depleted cultures responded poorly to nutrient starvation. The sulfur depleted cultures survived, albeit they assumed a bizarre morphology. The cells became atypically vacuolated and large. Although they did not grow rapidly their mass did increase slightly with time. The proteolytic activity expressed by the

sulfur depleted cultures, including the M_r 27,000 proteinase, was repressed by inorganic ($MgSO_4$, Na_2SO_3, NaS_2O_3) and organic (methionine and cysteine) sources of sulfur, but not by individual amino acids that do not contain sulfur.

This is not the complete picture. The M_r 53,000 proteinase was secreted constitutively in a minimal medium composed of readily metabolized sources of carbon, nitrogen, sulfur and phosphorus, albeit at low levels. No proteolytic activity could be detected in this minimal medium. However, keratinase activity was induced when either keratin or elastin was added to the minimal medium. This reiterates the fact that there are keratinases other the M_r 93,000 and 71,000 proteinases expressed by *T. rubrum*, and that the keratinases are under a different set of regulatory signals than those for the azocollytic and elastinolytic activities. Although azocollytic and elastinolytic enzymes from log phase cultures of *T. rubrum* were repressed by the addition of glucose to a protein media, keratinase activity was induced when 0.5% glucose was added to these protein media or a neopeptone medium.

The following model for control of proteolytic activity in log phase cultures of *T. rubrum* is proposed. Under conditions of carbon, nitrogen or sulfur deprivation the fungus derepresses proteinase activity. For example, when *T. rubrum* was grown in a medium where a single protein served as the sole source of carbon or nitrogen, the fungus responds by secreting proteinases capable of cleaving the proteins into peptides. There are peptidases secreted by *T. rubrum* that would cleave the peptides into amino acids. The amino acids would then be recognized either directly, or metabolized into intermediates that could act as mediators of repression of not only proteolytic activity, but other enzymes responsible for the assimilation of carbon and nitrogen.

If this model is correct, the addition of individual amino acids to the carbon or nitrogen depleted media should result in the repression of proteolytic activity. In addition, if this model is true it should be possible to detect exopeptidase activity in *T. rubrum* CM. It has been shown that either a carbon source (glucose) or a carbon and nitrogen source (neopeptone) were inhibitory to azocoll and elastin degrading activities. Additionally, Meevootisom and Niederpruem (1979) have shown that the addition of individual amino acids repressed degradation of guinea pig hair. One could argue that this proteolysis is mediated not by keratinases but rather by the other general proteolytic activities they described. It should be noted that the amount of proteolytic activity derepressed by carbon or nitrogen deprivation was much less than that seen when the fungus was cultured in a medium such as elastin salts which contains elastin as a sole source of carbon and nitrogen. In this medium greater amounts of azocoll and elastin degrading proteinases were

expressed. It appears that the derepression of proteolytic activity is greater if the fungus is deprived of both carbon and nitrogen.

T. rubrum also expresses azocollytic activity when sulfur is depleted from its medium. Under this condition the fungus responds by turning on the expression of the M_r 27,000 and 124,000 proteinases. Again, these proteinases would cleave extracellular proteins, presumably the non keratinous proteins found in the stratum corneum and nails, into peptides. The proposed peptidases would cleave the peptides into individual amino acids. When the fungus takes up sulfur containing amino acids, i.e. methionine and cysteine, they would either be utilized directly, e.g. as amino acids for protein synthesis, or metabolized into other sulfur containing intermediates. It is hypothesized that as in *N. crassa* and *A. nidulans* there are mediators of catabolite repression that would sense the increased concentration of sulfur in the cell, down regulating several enzymes responsible for sulfur assimilation, including proteinases.

In confirmation of this model of sulfur regulation, the addition of methionine or cysteine in concentrations as low as 0.5 mM to the sulfur depleted medium repressed proteinase production by fungus grown under these conditions. Addition of several other amino acids, including glycine, isoleucine, proline, alanine, phenylalanine and serine actually stimulated the production of proteolytic activity. When the fungus was cultured in a sulfur depleted medium that contained casamino acids, a blend of amino acids that contains methionines and cysteines, expression of proteinases was also repressed. It appeared that the effect of sulfur containing amino acids, even in a blend of amino acids, are still repressive to proteinase expression. If the fungus was grown under conditions where a single protein, such as gelatin, elastin or keratin, was the sole source of carbon, nitrogen and sulfur, proteinase were derepressed only in the case of elastin which contains no methionines or cysteines. To confirm that only nonsulfur containing proteins would allow for the expression of the proteinase BSA (a protein that contains sulfur containing amino acids) was also tried under the same conditions. Surprisingly, large amounts of azocoll, elastin and keratin degrading activities were expressed under these conditions. It may be that the proteolysis of BSA results in specific peptides that would stimulate the expression of proteinases. Another possibility is that BSA is not a good substrate for the fungus's proteinases, such that no amino acids, sulfur containing or not, would be released, and proteinase activity would remain derepressed. Clearly, other proteins need to be studied. On substrate gels, the M_r 27,000 proteinase and a high molecular weight activity were detected when a protein serves as the sole source of carbon, nitrogen and sulfur. Inorganic sources of sulfur were equally effective at repressing proteinase expression. For example 2 mM $MgSO_4$, NaS_2O_3 or Na_2SO_3 quelled azocollytic activities.

While azocollytic and elastinolytic activities were repressed by carbon, nitrogen and sulfur, keratinolytic activity was not, indicating that it is regulated by a different mechanism. This activity was induced when insoluble proteins, such as elastin and keratin, were added to the minimal medium. The soluble protein, gelatin, had no effect in this regard. Although relatively little keratinase activity was expressed in the neopeptone, elastin salts or keratin salts medium, the addition of 0.5% or 2.0% glucose to these media stimulated expression of keratinolytic activity. The data suggests that keratinase is induced whenever there is a source of available carbon, nitrogen and sulfur. This induction can occur in the presence or absence of protein. How then is the keratinase activity induced if it requires carbon, nitrogen and sulfur to be present in the fungal medium? At the onset of a *T. rubrum* infection the carbon, nitrogen and sulfur repressed activities would become derepressed and act on the proteins other than keratins present in the stratum corneum. Once cleaved, peptides derived from these proteins would be further degraded by peptidases into individual amino acids. These would provide the cell with a source of carbon, nitrogen and sulfur. Under these conditions, the azocollytic and elastinolytic activities would be repressed while keratinases could be secreted.

Proteinase activity in stationary phase cultures appeared to be regulated differently than proteolytic activity in log phase cultures. Azocollytic, elastinolytic and keratinolytic enzymes were not repressed when glucose was appended neopeptone, elastin salts and keratin salts media. Stationary phase cultures also respond differently to nutrient starvation. Unlike the log phase cultures, the stationary phase fungus produced lots of azocollytic, elastinolytic and keratinolytic activity in the minimal medium. The addition of protein to this medium did not induce any proteolytic activity. Proteinases were not derepressed when sources of carbon, nitrogen or phosphorus were deleted from the minimal medium. In fact, all proteolytic activities decreased under these conditions. The exception was in the sulfur depleted cultures. In this case, there was an increase in all activities. It seemed as though in stationary phase cultures all activities were derepressed and secreted constitutively.

4.5 Future Molecular Analysis of Proteinase Regulation

Further testing of my model for the regulation of *T. rubrum* proteinases would benefit from the use of cloned proteinase genes as a probes to further dissect the regulation of these proteinases. Is the regulation of proteinase expression at the transcriptional or translational level, or in a step following these events? The proteinase genes could contain upstream sequences such as promoters or operators that recognize master regulatory molecules equivalent to *N. crassa*'s *nit*-2 and *cys*-3 gene products. Analysis of *T. rubrum* proteinase genes may allow for the detection of regulatory sequences, and these cloned genes would

also be useful as probes, e.g. in northern blots to detect regulation of mRNA content in the cell.

The cloning *T. rubrum*'s proteinase genes would be useful for several other reasons as well. One of my goals was to determine the relationship of the purified proteinases with one another and with those of other *T. rubrum* proteinases yet to be purified. It would be interesting to know if these proteins are the products of a few or several genes. Some of the heterogeneity could be due to post transcriptional processing such as alternative splicing or due to a post translational event such as glycosylation, acylation or proteolysis of a larger precursor. For example, if some of the species were the result of alternative splicing then it might be possible to detect more than one message encoding a cloned gene if a northern blot were probed with a cloned piece of DNA encoding the proteinase of interest. It would be interesting to determine if there is any similarity in the purified proteinases and other serine proteinases. These enzymes could have conserved active site regions more closely related to the mammalian trypsin-like serine proteinases or alternatively they could be more similar to the procaryotic subtilisin family of serine proteinases.

Finally, the most important analysis would be to determine what is the role of proteinases in *T. rubrum* metabolism and infection. One could hypothesize that proteinases have a central role in the metabolism and invasion of host tissues by this organism. With the proteinase genes identified and sequenced it may be possible to alter the wild type genes, creating proteinase-deficient mutants. It is predicted that *T. rubrum* would no longer be able to grow on a medium unless it contained easily metabolized sources of carbon, nitrogen or sulfur, i.e. the fungus would not grow on a protein medium. Also, this organism would not be able to parasitize human keratinized tissues and would be less likely to cause invasive disease in the immunocompromised host.

One approach to cloning these proteinase genes would be to have probes, such as an oligonucleotide complimentary to a region of the protein (e.g. the N-terminus) or antibodies to the proteinases, that could be used to screen cDNA expression libraries (Young and Davis, 1983; Huynh et al., 1985).The N-terminal sequences of the purified proteinases have yet to be determined. These could be used to define synthetic oligonucleotides that could be used as probes to screen both genomic and cDNA libraries that have been constructed for this organism (see Appendix 2). One caveat, the cDNA libraries were created before the studies of proteinase regulation were begun. Because the organism was grown up as log phase cultures in Sabouraud dextrose broth, it is highly probable that very little proteinase other than the M_r 53,000 and keratinases were being expressed. The isolation of RNA from a medium such as BSA depleted of carbon, nitrogen

and sulfur or the sulfur depleted medium would therefore be advisable for future cloning attempts.

Another possible approach would be to create a library of *T. rubrum* DNA cloned into a *Neurospora* or *Aspergillus* vector that would allow for the complementation of *Neurospora* and *Aspergillus* proteinaseless mutants (Weiss et al., 1985).

Heterologous probes may also be useful in cloning proteinase genes. Several serine proteinase cDNAs, such as rat anionic trypsin, rat elastase or the *Schistosoma mansoni* cercarial elastase have been used to probe Southern blots of *T. rubrum* DNA. A few small molecular weight fragments (around 0.6 Kb) were detected on blots of EcoRI cut fungal DNA (Figure 26). There was also binding of the probes to higher molecular weight DNA fragments. The trypsin probe also hybridized when the stringency of hybridization was raised.

Unfortunately, no bands of hybridization were detected on northern blots probed with the same cDNAs and conditions used for the Southern blots (data not shown). This could be due to the relative lack of proteolytic activity expressed by the fungus grown in the Sabouraud dextrose medium. If RNA, isolated from fungus grown in a media lacking an easily metabolized substrate, were used it might be possible to detect hybridization of the heterologous probes to a northern blot. Alternatively, the Southern blot hybridization could represent the nonspecific interaction of probe and *T. rubrum* DNA or hybridization of nonspecific domains to similar sequences present in the *T. rubrum* genome. If the interaction is true then the trypsin probe could be used to screen the fungal genomic library I have constructed.

Lastly, Dr. Judy Sakanari has recently used degenerate oligonucleotide probes, coding for sequences conserved around the active site serine and histidine of the trypsin family of serine proteinases, to prime polymerase chain reactions (manuscript in preparation). Using this technique, it has been possible to amplify gene fragments of several serine proteinases from such diverse sources as protozoa and rat DNA. These probes could be used to screen libraries for the cDNAs or genes coding for these proteinases. Clearly N-terminal sequences, or an internal sequence of the enzymes, would be one way to unambiguously assign the cloned genes to a particular activity. Unfortunately, no amplified fragments have been detected in *T. rubrum* DNA using these eucaryotic serine proteinase consensus sequence probes. This could be due to the degeneracy of the probes used, or the conditions of hybridization and denaturation employed in these reactions. Another possibility is that the activities present in *T. rubrum*

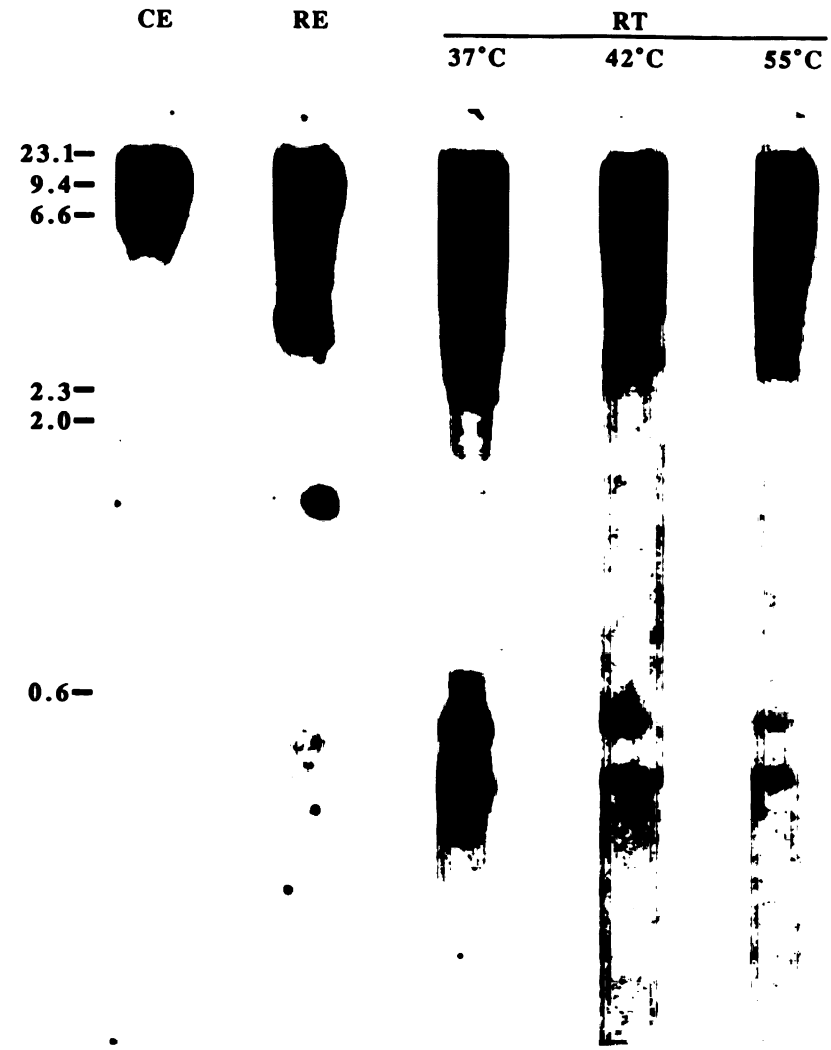

Figure 26. Southern blot of *T. rubrum* DNA probed with serine proteinase probes. *T. rubrum* DNA was purified, cut with EcoRI and blotted onto nitrocellulose as described in Appendix 2. The 900 bp cDNA fragment encoding the majority of the *Schistosoma mansoni* cercarial elastase (CE), the rat elastase cDNA (RE) or the Pst I cut rat anionic trypsin cDNA (RT) were hybridized to *T. rubrum* DNA. The Southern blots were prehybridized in 5 X SSC, 5 X Denhardt's, 100 mg/ml denatured calf thymus DNA, 30% deionized formamide, 50 mM sodium phosphate buffer, pH 6.8 and 0.1% (w/v) SDS for 1-2 h at 37° C. The filters were then hybridized in the same hybridization mix used for prehybridization but supplemented with 10^6 cpm [^{32}P]labeled probe per ml. Probes (200 ng) were random primed and labeled with [^{32}P]dCTP. Filters were washed three times for 30 min at 37° C in 4 X SSC, 0.1% (w/v) SDS. In the case of the hybridization with rat trypsin, following the 37° C wash the filters were washed for an additional 15 min at 42° C or 55° C in 4 X SSC, 0.1% (w/v) SDS.

CM are more closely related to the subtilisin family of serine proteinases. It may be possible to use probes encoding the consensus sites of subtilisin-like enzymes to prime the polymerase chain reactions. If *T. rubrum* proteinases are more like subtilisin than trypsin it would suggest that the hybridization of the trypsin probe to the fungal Southern is non-specific or that this organism has some serine proteinases that are like trypsin and some proteinases related to subtilisin.

The determination of which proteinases are actually being expressed *in vivo*, during a typical *T. rubrum* infection, will have to wait for production of probes, such as cloned proteinase genes or antibodies to purified *T. rubrum* proteinases. Also, at this time there is no way to tell if during infection the fungus is in a log phase or stationary phase growth cycle. Presumably, in the initial stages of infection the fungus is growing logarithmically. This log phase growth may actually continue for long periods of time, as the fungus can be continually shed as old keratinocytes are replaced by new cornified tissues. There would be neither a lack of substrate upon which proteinases could act, nor a build up of toxic substances under these conditions. In this log phase state of growth, general proteolytic (azocollytic) activity would be derepressed whenever carbon, nitrogen or sulfur is lacking in the fungal environment (Figure 27). These proteinases would act on the nonkeratinous proteins in the stratum corneum. There are probably peptidases, as yet unidentified, that would cleave the peptides generated by the initial proteolysis into amino acids. These amino acids would provide the cell with a source of carbon, nitrogen and sulfur. Under these conditions, the expression of general proteinases would be repressed while keratinases would be induced in this nutrient rich envionment. Disease may occur when the fungus reaches a stationary phase growth condition. In this state proteolytic activity appears to be constitutively expressed and could act to incite an inflammatory response. There is a correlation of high proteolytic activity and the more acute and inflammatory infections of *T. mentagrophytes* (Rippon and Garber, 1969; Minocha et al., 1972). Based on this model it should be possible to prevent the colonization of the stratum corneum and the ensuing disease by inhibiting the proteinases produced by *T. rubrum* with specific inhibitors. Regardless of the stage of growth that *T. rubrum* is in during human infections, this organism clearly possesses the proteolytic armamentarium necessary to parasitize the cornified tissues of the body.

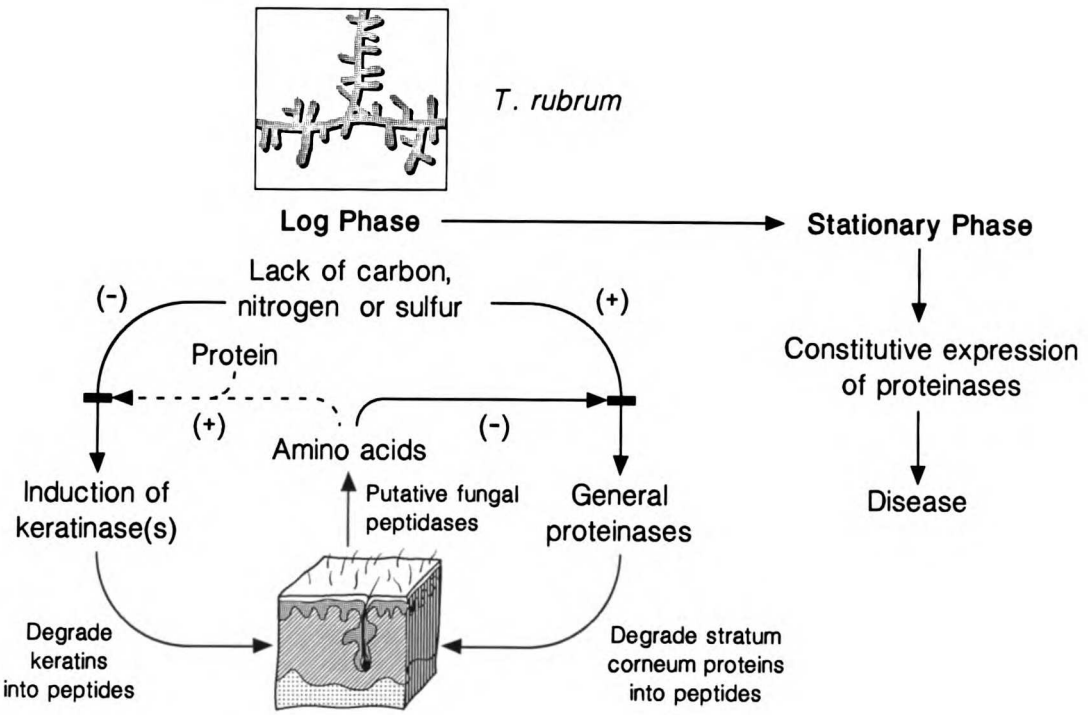

Figure 27. Proposed model for the regulation of *T. rubrum* proteolytic activity. In the initial stages of infection, *T. rubrum* grows logarithmically. In this state, general proteolytic activity is derepressed whenever carbon, nitrogen or sulfur is lacking in the fungal milieu. These proteinases would act on the non-keratinous proteins contained in the stratum corneum of the skin, releasing peptides into the fungal environment. There are putative peptidases that would cleave the peptides into amino acids. These amino acids would provide the cell with a source of carbon, nitrogen and sulfur. Under these conditions, the general proteolytic activity would be repressed while keratinases would be induced in the presence of protein. Disease may occur when the fungus reaches stationary phase, when proteinases are secreted constitutively. These enzymes may directly or indirectly incite a host response, resulting in the inflammatory manifestations of dermatophtosis

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APPENDIX 1

Culture of *T. rubrum* - *T. rubrum* strain I.F.O. 9185 is a stock culture from Kyushu University, Fukuoka, Japan and was obtained from Dr. Asahi, Kyushu University. Strains TR-3-12 were obtained from clinical material at UCSF and identified as strains of *T. rubrum* by Dr. Carlyn Halde, UCSF. All strains were routinely maintained on Sabouraud dextrose agar (Difco, Detroit, MI) slants stored at 4° C . The fungus was subcultured by streaking the fungus onto the same medium every two months. *T. rubrum* spore suspensions were also maintained as stock cultures. To obtain the spore suspensions the fungus was grown on Sabouraud dextrose agar slants at 30° C and when the fungus completely covered the surface of the slant the tube was flooded with approximately 5 ml of sterile distilled water. The water was pipetted rapidly up and down to dislodge the spores, but careful attention was paid to not disrupt the integrity of the actual agar surface. If necessary, this spore suspension was filtered through sterile gauze to remove bits of agar and mycelium. These spore suspensions were stored at ambient temperature in tightly stoppered, sterile containers.

These spore suspensions were used to start stationary and log phase cultures of *T. rubrum*. To obtain stationary phase cultures of this fungus an aliquot of the spore suspension, typically 500 µl, was added to 100 ml of Sabouraud dextrose broth in 75 cm^2 plastic tissue culture flasks (Costar, Cambridge, MA) and incubated at 30° C for approximately 3-4 weeks. After this amount of time, a thick white mat of fungus had formed on the surface of the medium. The CM from these cultures was retained for purification of proteinases. In some experiments, the fungal mat was washed with sterile water and the CM was replaced with 50 ml of Sabouraud dextrose broth or 50 ml of several other media including 1% (w/v) neopeptone (Difco)± glucose (Sigma, St. Louis, MO), elastin salts ± glucose or keratin salts ± glucose. The elastin salts medium (Rippon and Varadi, 1968) contained per liter of distilled water: elastin powder (Sigma), 5g; $MgSO_4$-7 H_2O (Sigma), 0.5 g; KH_2PO_4 (Sigma), 0.46 g; K_2HPO_4 (Sigma), 1g; thiamine-HCl (Sigma), 100 µg. Keratin salts contained the same salts and thiamine as the elastin salts broth except 2.5 g of keratin powder (I.C.N., Cleveland, OH) was substituted for the elastin powder (Meevootisom and Niederpruem, 1979). The cultures were incubated at 30° C for periods up to one 1 month. The CM from these cultures served as a source of proteinases. An alternative method used circles (diameter of approximately 1 cm) of stationary phase fungus that had been cut out of stationary phase mats (grown in Sabouraud dextrose broth in 75 cm^2 tissue culture flasks as described above) with the open

end of a sterile 120 X 17 mm conical centrifuge tube (Sarstedt, Hayward, CA). These 1 cm diameter circles of fungus were placed in 24 well tissue culture plates (Corning, Corning, NY) that contained 1ml of the following media: minimal, or a medium depleted of either sulfur, carbon, nitrogen or phosphorus . "Minimal medium" contained: 0.25% (w/v) glucose as a carbon source, 50 mM $(NH_4)_2PO_4$ (Sigma) as a nitrogen source, 3.4 mM KH_2PO_4 and 5.75 mM K_2HPO_4 as phosphorus sources and 2 mM $MgSO_4$ -7 H_2O as a source of sulfur. The carbon, nitrogen, sulfur or phosphorus depleted media were made by deleting the appropriate compound from the minimal medium, with the following changes: in the phosphorus depleted medium the potassium phosphate salts were left out of the medium and 50 mM NH_4HCO_3 was substituted for the $(NH_4)_2PO_4$. The 2 mM $MgSO_4$ was replaced by 2 mM $MgCl_2$ in the sulfur depleted medium. At times, proteins such as 0.5% (w/v) elastin powder, 0.25% (w/v) keratin powder were added to the minimal medium. These cultures were incubated at 30° C for periods up to 1 week and aliquots of CM were assayed for proteolytic activity.

Lysates of stationary phase fungi were also produced. *T. rubrum* was cultivated in Sabouraud dextrose broth in 75 cm^2 tissue culture flasks as described above. The fungal medium was changed once, and after an additional 2 week incubation period, the mat of fungus was removed from the CM and washed five times with 200 ml of 10 mM tris(hydroxymethyl)aminomethane (tris; BioRad, Richmond, CA)-HCl, 150 mM NaCl (Sigma). Approximately 1 g, wet weight, of fungus was quick-frozen with liquid nitrogen and ground in a mortar to a fine powder. The ground mycelium was resuspended in 4 ml of the above buffer and after a 10 min incubation period the particulate material was removed by centrifugation and the supernatant stored at -20° C. This supernatant, the CM and the washes were all assayed for proteolytic activity in the azocoll degradation assay and by gelatin substrate gel electrophoresis.

To culture log phase fungus, a 2 l Erlenmeyer flask, containing a spin bar, was filled with 1 l of sterile keratin salts or minimal medium and inoculated with 5 ml of a spore suspension. The fungus was incubated at ambient temperature with constant stirring on a magnetic stir plate (Corning) for 2-3 weeks. An aliquot, typically 30 ml of this culture, was placed in a 50 ml sterile plastic centrifuge tube (Corning) and spun at a setting of 7 in a International Machine Corp. table-top centrifuge (Needham Heights, MA), for 10 min at ambient temperature. The supernatant was discarded and the pellet resuspended in 45 ml of sterile distilled water, and recentrifuged for an additional 10 min at the same setting. After two additional washes the pellet was finally resuspended in 5 ml of sterile distilled H_2O, which gave an A_{600} of approximately 2.6/ml. An aliquot of the sterile washed hyphae, typically 300 µl (equivalent to 1.5 mg, dry weight of fungus; or 1.5 ml in the case of the

carbon or nitrogen depleted cultures), was added to 10 ml or the above mentioned media in 60 X 15 mm sterile plastic petri dishes (Fisher, Pittsburgh, PA), and incubated at 30° C for periods up to a week. In some experiments the sulfur depleted medium was supplemented with 0.5-50 mM L-methionine or L-cysteine, or 50 mM L-glycine, L-isoleucine, L-proline, L-alanine, L-phenylalanine, L-serine or 2.5% (w/v) casamino acids (all obtained from Sigma). At times, proteins such as 0.5% (w/v) elastin powder, 0.25% (w/v) keratin powder, 0.5% (w/v) type I swine skin gelatin (Sigma), or 0.5% (w/v) pentax fraction V bovine serum albumin (Sigma) were added to the minimal medium or a medium that lacked a source of carbon, nitrogen and sulfur , i.e. the protein plus the potassium phosphate buffer plus 2 mM $MgCl_2$. These cultures were incubated at 30° C for periods up to 1 week and aliquots of CM were assayed for proteolytic activity. All culture media were sterilized by autoclaving, and when appended with glucose, proteins or amino acids all ingredients were autoclaved at the same time.

Elastin-Agar Plate Assay - Strains of *T. rubrum* were plated onto elastin salts or 1% (w/v) neopeptone, that had been modified by the addition of 2% (w/v) agar (Difco) and 0.5% (w/v) elastin powder or 1% neopeptone where necessary. The 10 cm diameter sterile plastic petri dishes (Fisher) were filled with the agar media to a height of 1 cm to allow for the extended incubations of 4 weeks at 30° C . Clearing of the particulate elastin was considered a positive demonstration of elastase activity.

Azocoll Degradation - Conditioned medium or column fractions, typically 50-100 µl, was incubated with 3-5 mg of azocoll (Sigma; Calbiochem, La Jolla, CA)in a 100 mM tris-HCl, pH 8.0, 1 mM $CaCl_2$ buffer in a 1.6 ml Eppendorf tube for 4-24 hours at 37° C . The final reaction volume was 1 ml. Following incubation, the samples were centrifuged for 5 min in a Beckman (Palo Alto, CA) microfuge. Degradation of azocoll was measured by determining the A_{520} of the supernatant in a Gilford (Oberlin, OH) spectrophotometer. A unit of azocoll degrading activity was defined as a change of 0.1 A_{520} units per hour.

Elastin Degradation- Purified ox ligamentum nuchae elastin (60 mesh; Elastin Products Corp,. Pacific, MO) was labeled with $NaB[^3H]_4$ and assayed as described by McKerrow et al. (1985b). The radiolabeled elastin was resuspended to a final concentration of 2 mg/ml in a 300 mM Tris-HCl, pH 8.0, 1 mM $CaCl_2$ buffer. A 100 µl aliquot was mixed with 50-100 µl of CM or column fractions and sufficient H_2O to bring the reaction volume up to 300 µl, and incubated at 37° C for 18-24 h. After this incubation period the undigested elastin was spun down for 5 min in a microfuge and 100 µl of the supernatant was mixed

with 7 ml of Opti-Fluor (Packard Instruments Co., Downers Grove, IL.) scintillation fluid and the radioactivity counted in a liquid scintillation spectrophotometer (Beckman model LS 100C). Control incubations with water were included with each assay. The total amount of radioactive elastin available for degradation was determined by adding 10 µl (160 µg) of 2X crystallized porcine pancreatic elastase (Sigma) to a control reaction. One unit of elastase activity will degrade 1 µg of elastin per hour as compared to the positive control.

Keratin Degradation - Epidermal keratin was extracted from neonatal rats (Morozuka et al., 1979) and labeled with [^{14}C]acetic anhydride by the method of Gisslow and McBride (1975). [^{14}C]keratin degradation was assayed by the method of Tzeng et al. (1983). One unit of keratinase activity will degrade 1 µg of keratin/h. Keratin degradation was also assayed using keratin azure (Sigma), a dyed wool product. CM, 50-100 µl, was incubated with 3-5 mg of keratin azure in a 100 mM glycine (BioRad) - NaOH, pH 9.0, 1 mM $CaCl_2$ buffer for 48-72 h at 37° C . The final reaction volume was 1 ml. Following incubation, the reaction was spun in a microfuge for 5 min. The degradation of keratin azure was measured by determining the A_{595} of the supernatant in a spectrophotometer. A change of 0.01 A_{595} units/h equalled one unit of keratinase activity.

Inhibition Assays - For these studies inhibitors were preincubated with enzyme for 20 min at ambient temperature before the azocoll, elastin or keratin substrates were added to the reaction mixture. PMSF, 1,10 phenanthroline and NEM (all from Sigma) were made up as 150 mM stocks in ETOH, while the chloromethylketone inhibitors (Enzyme System Products, Livermore, CA) were made up as 1 mg/ml stocks in dimethylsulfoxide (Sigma). Protein inhibitors such as Trasylol (Sigma) and α_1-proteinase inhibitor (Sigma) were made up as 2 mg/ml stocks in H_2O. For inhibitors requiring ethanol or dimethylsulfoxide in stock solutions, an equivalent amount of solvent was run as a control.

R-22 Assay - [^{3}H]proline labeled R-22 cell ECM was prepared and analyzed for degradation of its glycoprotein, elastin and collagen components as previously described (Jones and DeClerck, 1980; McKerrow et al., 1985a). Concentrated *T. rubrum* CM from stationary phase cultures (25 µl; 250 µg of protein) or purified M_r 27,000 proteinase (1 µg of protein in 25 µl of buffer) were added to wells with 475 µl of 100 mM Tris-HCl, pH 8.0, 1 mM $CaCl_2$ and incubated for 18 h at 37° C . Fifty µl aliquots of these samples were mixed with 7 ml of scintillation fluid and counted by liquid scintillation spectroscopy to determine the amount of matrix degraded. After the last time point was taken the enzyme solution was aspirated from the plate, and 500 µl of 100 µg/ml L-1-tosylamide-2-phenyl-

alaninechloromethylketone-treated trypsin (Worthington, Freehold, NJ) in the same buffer was added to the plate. After 4-6 h at 37° C a 50 µl aliquot was counted and this enzyme solution was replaced with 500 µl of a 16 µg/ml pancreatic elastase solution in the above buffer. Incubation was continued overnight at 37° C , a 50 µl sample was counted and then this elastase solution was exchanged with 500 µl of a 100 µg/ml clostridial collagenase (Sigma), dissolved in the above buffer. After 12-18 h incubation at 37° C , a 50 µl aliquot was again counted. The degradation of the various constituents of the matrix were compared to control wells that contained only buffer in the original proteinase digestion. Eight wells of each sample were digested, and the data was analyzed by a computer program, R-22 degradation, designed by William Keene, UC, Berkeley.

Substrate Gel Analysis - General proteinase activity was also assayed in SDS-polyacrylamide gels copolymerized with substrate (Asahi et al., 1985), in which proteinases can be detected and molecular weights determined simultaneously. The resolving gel, 10 or 12%, was polymerized in the presence of 0.12% pig skin, type I gelatin or α-elastin (Elastin Products Corp.). The samples were not boiled or reduced, and the stacking gel (4%) did not contain substrate. The gels (0.75 mm thick) were prepared in a Hoeffer (San Francisco, CA) small-slab-gel apparatus. CM was diluted with an equal volume of sample buffer (0.25 M Tris-HCl, pH 6.8, 20% [v/v] glycerol, 0.2%[w/v] SDS, and 0.005% [w/v] bromophenol blue) and 15-25 µl were loaded on the gel. After electrophoresis, the gels were washed in 100 ml of 2.5% (v/v) Triton X-100 (Sigma) for 30 min to remove the SDS. The gels were then incubated for 12-24 h at 37° C in the same buffered solution used in the azocoll assay. It was later found that this incubation period could be cut down to a 2-4 h period with no significant loss of visualization of proteinase activity. The gels were stained with 0.1% Coomassie brilliant blue R-250 (BioRad) in water:methanol:acetic acid (5:5:1 v/v) for 30 min and then destained in 45% (v/v) methanol, 3% (v) acetic acid until the proteolytic bands could be visualized. Molecular weight standards (BioRad) were myosin (200,000), β-galactosidase (116,300), phosphorylase b (92,500), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500) and lysozyme (14,500).

Purification of the M_r 93,000 and 71,000 Proteinases - These proteinases were purified according to methods developed by my collaborators and myself (Asahi et. al ., 1985). Proteins in the stationary phase CM (400 ml) of *T. rubrum* strain I.F.O. 9185 grown in Sabouraud dextrose broth were precipitated with 70%-saturated $(NH_4)_2SO_4$ (Sigma), collected by centrifugation at 20,000 x *g* for 20 min, resuspended in 15 ml of

water, dialyzed against 4 l of water overnight, and then lyopholized. The freeze-dried samples (50-70 mg of protein) were dissolved in 3 ml of 150 mM NaCl, 20 mM Tris-HCl, pH 8.0 and applied to a 1.9 cm x 110 cm Sephadex G-100 column (Pharmacia, Piscataway, NJ). Samples were eluted at a flow rate of 15 ml/h, and 2 ml fractions were collected. Fractions (typically 90-130) containing the main peak of proteolytic activity from Sephadex chromatography were pooled and dialyzed against 20 mM Tris-acetate, pH 8.3 buffer. The proteolytic activity was further resolved on a Mono P Chromatofocusing column (Pharmacia) using a fast-protein-liquid-chromatography gradient programmer (Pharmacia). A gradient of pH 8-5 was generated with the 20 mM Tris-acetate, pH 8.3 buffer and Polybuffer, pH 5.0 (Pharmacia). Samples were eluted at a flow rate of 0.5 ml/min, and 1 ml fractions were collected. All procedures were carried out at 4° C .

Purification was monitored by SDS-polyacrylamide gel electrophoresis (Laemmli and Favre, 1973). A 4% stacking gel and 10-14% resolving gel were used. Sample buffer was the same as that used in the substrate gel analysis except it contained 5% (v/v) β-mercaptoethanol (Sigma) or 100 mM DTT (Sigma), and samples were boiled for 5 min before electrophoresis. Protein bands were identified by staining with silver (Wray et al., 1981). Protein standards were the same as those used for substrate gel analysis.

Protein was determined by the method of Bradford (1976), with chymotrypsin as a standard.

Hydrolysis of Peptide Substrates - The rates of hydrolysis of the peptide-pNA substrates (suc-A-A-P-F-pNA was obtained from Vega, Tucson, AZ; other pNA substrates were kindly provided by Dr. C. Largeman, V. A. Hospital, Martinez, CA)were measured at 410 nm on a Gilford spectrophotometer, fitted with a model 6050 chart recorder. The reactions for the M_r 93,000 and 71,000 proteinases were initiated by adding 10 µl of the enzyme solution (4-29 nmol) to 0.5 ml of a solution containing substrate in 100 mM Tris-HCl, pH 7.8, 1mM $CaCl_2$. The reactions for the M_r 27,000 proteinase were initiated by adding 30 µl (25 pmol) of the enzyme to 0.5 ml of a solution containing substrate in 100 mM Tris-HCl, pH 8.0, 1 mM $CaCl_2$. Substrate concentrations were varied over a 10-fold range of 0.1-1.0 mM. Because of solubility problems associated with Z-ala-ala-leu-pNA (Peninsula Laboratories, Belmont, CA), the conditions were different for this substrate. For these reactions, 200 µl of substrate in dimethylformamide (Sigma) was added slowly to 925 µl of 100 mM Tris-HCl, pH 8.0, 1 mM $CaCl_2$, and then 75 µl of the M_r 27,000 proteinase (75 pmol) was added to this buffered solution of substrate. The substrate, made up as a 0.5 mg/ml stock in dimethylformamide, was varied over a 10-fold range of 0.02 to 0.2 mM. An ε value of 8800 M^{-1} cm^{-1} at 410 nm was used for the liberated 4-nitroaniline

(Erlanger et al., 1961), and the kinetic constants were determined from the initial rates of 4-nitroaniline appearance. The data were plotted, with least-squares analysis, by the method of Lineweaver and Burk (1934). Five to seven points were measured for each plot and correlation coefficients were calculated. All but three correlation coefficients were greater than 0.99, and no value was lower than 0.97.

The rates of hydrolysis of several peptide-AMC substrates (obtained from Enzyme System Products, Livermore, CA) were measured with an Aminco SPF-500 spectrofluorimeter. The cleavage of substrates was measured as the increase in fluorescence of liberated aminomethylcoumarin (Castillo et al., 1979), by using a 380 nm excitation wavelength and monitoring the emission at 460 nm. The fluorescence scale was calibrated with a standard AMC solution (1.3 μM), and the rate of substrate hydrolysis was determined from the continuously monitored slope of the AMC increase in the presence of 20 μl of the enzyme sample. A non-enzymic control was obtained from the substrate reaction before addition of the enzyme. The AMC substrate solution (20-40 mM in dimethyl sulfoxide) was added to a solution of 100 mM Tris-HCl, pH 7.8, 1 mM $CaCl_2$, containing dimethyl sulfoxide to give a volume of 2.0 ml, including 100 μl of total dimethyl sulfoxide. Substrate concentrations were in the range 0.06-1.2 mM. The data were plotted as in the nitroaniline assays.

The rates of the hydrolysis of the thioester substrates (obtained from Enzyme System Products) were measured by adding substrate in dimethyl sulfoxide and 4,4'-dithiopyridine in dimethyl sulfoxide to 1.8 ml of 100 mM Tris-HCl, pH 7.8, 1 mM $CaCl_2$, to give a total volume of 2.0 ml (10% dimethyl sulfoxide). A 20 μl enzyme sample was added to start the reaction. The increase in absorbance at 324 nm was monitored with a Gilford 260 spectrophotometer. An ε value of 19,800 M^{-1} cm^{-1} was used for the thiopyridone product (Grassetti and Murray, 1967). The substrate concentration range was 0.1-1.0 mM. The data were plotted as in the nitroaniline assays.

Purification of the M_r 27,000 Proteinase - To purify the M_r 27,000 activity, proteins in the stationary phase CM (1100 ml) of *T. rubrum* strain I.F.O. 9185 grown in Sabouraud dextrose broth were precipitated with 70%-saturated $(NH_4)_2SO_4$, collected by centrifugation at 20,000 x *g* for 20 min, resuspended in 15 ml of water, dialyzed against 4 l of water overnight, and then lyopholized. The freeze-dried samples (20-50 mg of protein) were dissolved in 4 ml of loading buffer (20 mM Tris-HCl, pH 7.4, 500 mM NaCl, 0.02% NaN_3), centrifuged in a microfuge to remove particulate matter, and the supernatant applied to a 11 ml disposable Econo column (BioRad) packed with 8 ml of Con A sepharose 4B (Sigma) that had been washed with 10 column volumes of the above buffer.

The mannose containing molecules were allowed to interact with the column for 25-30 minutes before the column was washed with 100 ml (2.5 ml/fraction) of the same buffer at a flow rate of 45 ml/min. After the fourtieth fraction, the loading buffer was changed to the elution buffer (20 mM Tris-HCl, pH 7.4, 500 mM NaCl, 200 mM α-methyl mannoside [Sigma], 0.02% NaN_3), and an additional 15 fractions were collected. Active fractions from the flow-through of the Con A column (typically fractions 1-10) were pooled then concentrated and dialyzed against H_2O in a Pro-Di-Con apparatus (Pierce, Rockford, IL). The concentrated proteins (in 0.5 ml) were mixed with an equal volume of 50 mM Bis-Tris-HCl, pH 5.8, and applied to a Polyanion SI column (Pharmacia), that had previously been equilibrated with the same buffer. Samples were eluted at a flow rate of 0.5 ml/min and 1 ml fractions were collected. After 6 ml of the 50 mM Bis-Tris-HCl, pH 5.8 buffer had flowed through the column, proteins that interacted with the column were eluted off the matrix by a 30 ml linear gradient of salt from 0 to 500 mM NaCl, in the same buffer. From fractions 36 to 40 the concentration of salt was linearaly increased to 1 M, and maintained at this same concentration for an additional 10 fractions. All procedures were carried out at 4° C.

[^{3}H]DFP Labeling of the M_r 27,000 Proteinase and Electroelution - A 150 µl sample of the purified M_r 27,000 proteinase (about 5.2 µg of protein), was mixed with 10X concentrated buffer (1 M Tris-HCl, pH 8.0, 10 mM $CaCl_2$) and water to give a final reaction volume containing 1X buffer in 300 µl. A reaction with no enzyme was run as a control. To each of the reaction mixtures 25 µCi of [^{3}H]DFP (Amersham, Arlington Heights, IL; 5 mCi/ml, 3.5 Curies/mmol) was added, and the reaction was allowed to proceed for 2 h at 37° C. A 12 µl aliquot of each sample was mixed with 2X sample buffer (± DTT and boiling) and electrophoresed in a 4% stacking/14% resolving SDS-polyacrylamide gel. Labeled proteinase was visualized by autoradiography of the EN^{3}HANCE (NEN Research Products, Boston, MA) fluorographed gels. Some of the remaining labeled proteinase (200 µl) was mixed with an equal volume of nonreducing sample buffer and electrophoresed in a 14% resolving gel. Molecular weight markers were run in an outside lane. The gel was stained with Coomassie brilliant blue R-250 and destained as described for the substrate gels, and the single protein band (at M_r 27,000) was sliced out of the gel. The protein was electroeluted out of the gel slice into an EluTrap device (Schleicher and Schuell, Keene, NH) using a 50 mM NH_4HCO_3, 0.1% (w/v) SDS buffer. A 15 µl aliquot of the 200 µl of electroeluted protein was mixed with 2X sample buffer (± DTT and boiling), and analyzed by autoradiography of fluorographed 14% SDS-polyacrylamide electrophoretic gels. [^{14}C]Standards (Amersham) were run in adjacent

lanes to the samples. These standards have the same molecular weights as those used in substrate gel analysis.

Degradation of Macromolecular Substrates - Five micrograms of type I collagen (from human fetal membrane; Cal Biochem, San Diego, CA), type III collagen (from human fetal membrane; Cal Biochem), type IV collagen (from EHS tumor; Collaborative Research, Lexington, MA), type V collagen (from human fetal membrane; Cal Biochem), laminin (murine; Collaborative Research) or fibronectin (human; Collaborative Research) were incubated with 25 µl of the purified M_r 27,000 proteinase (1.1 µg of protein). In some experiments the enzyme was preincubated for 20 min with 1.0 µM phe-gly-ala-leu-CH_2Cl before it was added to the reaction mixture. The reaction volume (50 µl) was brought up to a final concentration of 100 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1mM $CaCl_2$, and incubated at 34° C for 18 h. An aliquot of the reactions, 10 µl, was mixed with an equal volume of 2X reducing sample buffer, boiled, and the proteins were resolved by SDS-polyacrylamide gel electrophoresis on a 4% stacking/7% resolving gels and stained with silver.

Detection of Proteolytic Activity Using Substrate Impregnated Cellulose Acetate Membranes - Samples of concentrated crude *T. rubrum* CM (10 µl) were mixed with 2X non-reducing sample buffer and electrophoresed on 12% SDS-polyacrylamide gels. Colored molecular weight markers were run in adjacent lanes (Rainbow Markers, Amersham). The gel was soaked in 2.5% (v/v) Triton X-100 for 30 min and then overlayed with a cellulose acetate membrane, impregnated with ala-ala-phe-AFC (EOM membrane, Enzyme System Products), that had been prewetted with 100 mM Tris-HCl, pH 8.0, 1 mM $CaCl_2$ buffer. Following a 10-20 min incubation at 37° C the position of the molecular weight markers was marked on the membrane, and proteolysis of the substrate was detected by shining a hand-held long-wave ultraviolet lamp (Fisher) over the surface of, or underneath, the membrane. A light blue fluorescence was detected wherever the substrate had been cleaved, releasing ultraviolet absorbing, free AFC.

APPENDIX 2

Isolation of *T. rubrum* DNA - An autoclaved 2 l Erlenmeyer flask, containing a spin bar, was filled with 1 l of sterile Sabouraud dextrose broth and inoculated with 5 ml of a spore suspension. The fungus was incubated at ambient temperature with constant stirring on a magnetic stir plate. After 4-5 days, the culture (now filled with fungal balls) was passed through either a fine meshed screen or several layers of sterile cheese cloth. The remaining liquid was squeezed out of the mycelium and discarded. The fungal mat was unfolded, quick frozen by pouring liquid nitrogen on it, and lyopholized for 3-4 h. The freeze-dried fungus was placed in a pre-cooled mortar, refrozen with liquid nitrogen and ground vigorously to a fine powder. The addition of liquid nitrogen followed by grinding was repeated at least ten times. The following procedure was adapted from a protocol for DNA isolation from *Cochliobolus heterostrophus* (O. C. Yoder, Cornell University, Ithaca, NY, personal communication). The ground fungus was suspended in 24 ml of isolation buffer which contains: 150 mM EDTA (Sigma), 50 mM Tris-HCl, pH 8.0, 1% (w/v) sodium sarkosyl (Sigma) and 300 µg proteinase K/ ml (Sigma). This suspension was divided into two autoclaved 50 ml centrifuge tubes (Sarstedt, Hayward, CA) and centrifuged at 2,000 x ***g*** in a Sorvall RC-5 centrifuge for 5 min at 20° C. The supernatant was transferred to several autoclaved 10 ml glass centrifuge tubes (Sarstedt) and 8 µl of heat-treated RNAase A (1 mg/ml; Sigma) was added per ml of extraction buffer. The DNA containing solution was extracted 3 times with an equal volume of phenol:chloroform:isoamyl alcohol, (25:24:1; all from Fisher), that had been saturated with 10 mM Tris-HCl, pH 7.4. After each extraction, the aqueous phase was separated from the organic layer by centrifugation (5 min at 5,000 x ***g***) and transferred to a fresh tube. The DNA was precipitated by adding 0.1 volume of 3 M sodium acetate, pH 5.4 (Sigma) and 2 volumes of absolute ethanol (Fisher), to the aqueous phase and incubating the mixture on ice for 10 min. The DNA was pelleted by centrifugation at 8,000 x ***g*** for 20 min at 4° C, and the pellets dissolved in a total volume of 1.6 ml of H_2O. To this aqueous solution of DNA was added 1.1 ml of 20% polyethylene glycol 8000 (Sigma) in 2.5 M NaCl (Sigma). The solution was mixed, and incubated in ice water for 1 h. The DNA was again pelleted by centrifugation , (20 min, 4° C, 8,000 x ***g***), the supernatant discarded, and the tube dried with the end of a cotton swab. The pellet was washed with cold 70% ethanol and the DNA dissolved in TE (10 mM Tris-HCl, pH 7.4, 1 mM EDTA) and 10 µg/ml heat-treated RNAase A.

Southern Blots - Because of the high concentration of small molecular weight nucleic

acids present in the DNA sample, a 50 μg sample of DNA per lane, in 1 X EcoRI reaction buffer (Boeringer Manheim, Indianapolis, IN), was digested with 1 μl (90 units) of high concentration EcoRI (Boeringer Manheim) and incubated at 37° C for 2 h. The reaction was terminated by adding EDTA to a final concentration of 25 mM. Sample buffer (6X = 0.25% [w/v] bromophenol blue, 30% [w/v] glycerol in H_2O) was added to the reaction mix and the fragments were resolved in a 15 X 15 cm 1.0% (w/v) agarose (BioRad) gel, made up in TBE (0.089 M Tris, 0.089 M borate, 0.002 M EDTA), that had been poured in a horizontal gel apparatus (BioRad). The running buffer was TBE and electrophoresis was performed at 85 V, constant voltage, for 4-5 h at 4° C. HindIII cut λ markers (BioRad) were run in an outside lane. The DNA was stained with ethidium bromide (Sigma), photographed, then washed, denatured, neutralized and blotted onto nitrocellulose (Schleicher and Schuell) by capillary action as described by Maniatis et al. (1982). Following blotting, the nitrocellulose was washed with 6 X SSC (1 X SSC = 150 mM NaCl, 150 mM trisodium citrate, pH 7.0), dried and baked at 80° C for 2 h in a vacuum oven.

Hybridization with Serine Proteinase Probes - The probes used in these studies included the 900 bp cDNA fragment encoding the majority of the *Shistosoma mansoni* cercarial elastase (Newport et al., 1988), the Pst I cut rat anionic trypsin cDNA (kindly provided by Dr. C. Craik, UCSF) and the rat elastase cDNA (Swift et al., 1984). The Southern blots were prehybridized in 5 X SSC, 5 X Denhardt's (50 X = 1% [w/v] ficoll, 1% [w/v] bovine serum albumin, 1% [w/v] polyvinyl pyrrolidone; all from Sigma), 100 μg/ml denatured calf thymus DNA (Sigma), 30% deionized formamide (Sigma), 50 mM sodium phosphate buffer, pH 6.8, 0.1% (w/v) SDS, for 1-2 h at 37° C. The filters were then hybridized at 37° C for 18 h in the same hybridization mix used for prehybridization but supplemented with 10^6 cpm [^{32}P]labeled probe per ml. Probes (200 ng) were random primed and labeled with [^{32}P]dCTP (Amersham; 6000 Ci/mmol, 10 mCi/ml) using the method of Fineberg and Vogelstein (1983). Filters were washed three times for 30 min at 37° C in 4 X SSC, 0.1% (w/v) SDS. In some cases, following the 37° C wash, the filters were washed for an additional 15 min at 42° C or 55° C in 4 X SSC, 0.1% (w/v) SDS.

Isolation of total RNA and mRNA - *T. rubrum* mycelia were grown, harvested and ground to a fine powder with liquid nitrogen as described in the DNA extraction procedure. Total RNA was extracted from approximately 2 g of the ground fungus using the low-temperature guanidinium thiocyanate extraction procedure as described by Han et al. (1987). Approximately 2-3 mg of total RNA was obtained using this procedure. Poly A

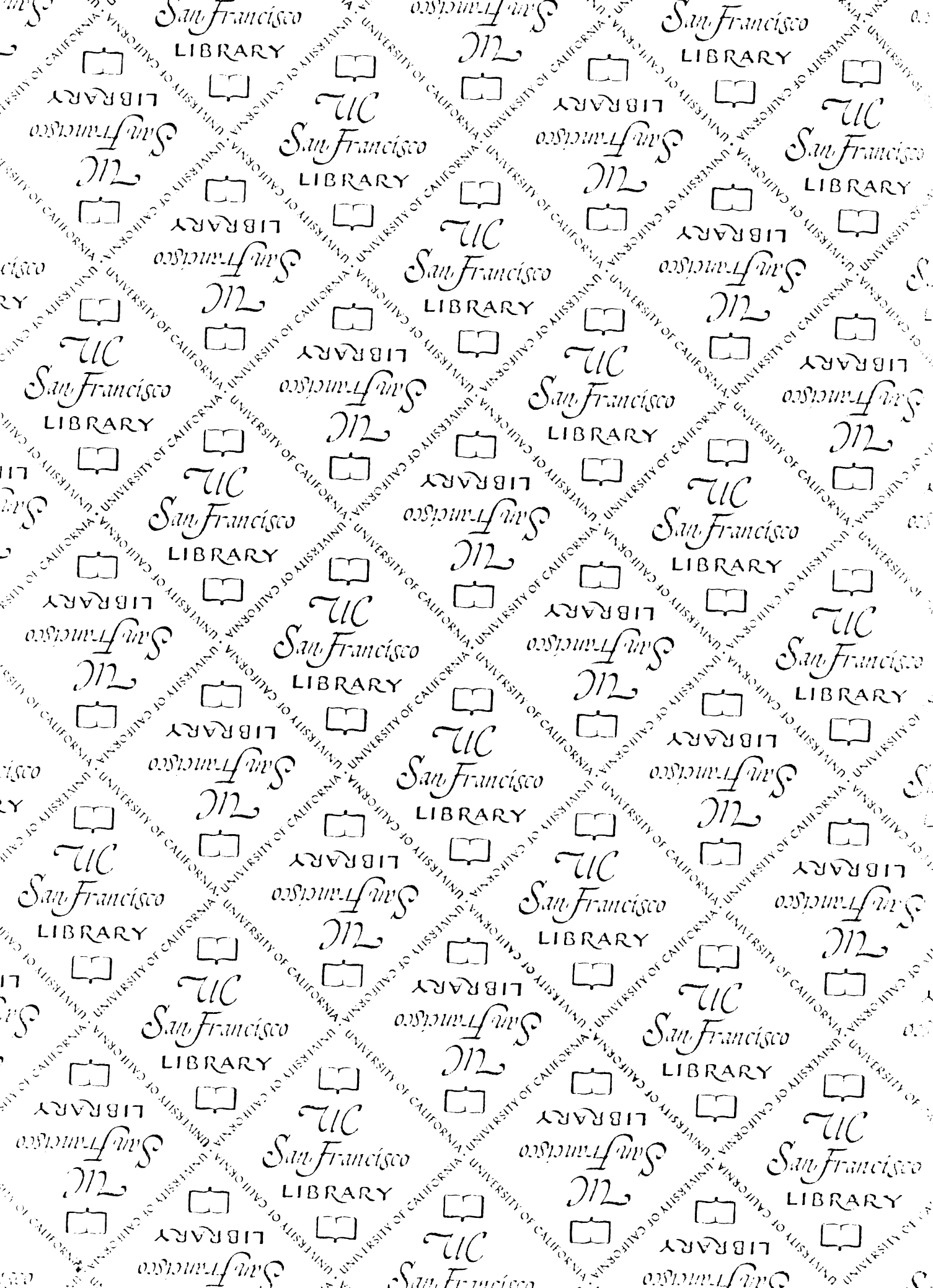
RNA was purified from 1 mg of total RNA according to standard protocols (Aviv and Leder, 1972 as described by Maniatis et al., 1982). An oligo dT Avidchrom cartridge (BioProbe Intl., Tustin, CA) was used and the solutions were injected into the column using a 5 cc syringe. Total RNA was loaded onto a column equilibrated with 20 mM Tris-HCl, pH 7.6, 0.5 M NaCl, 1 mM EDTA, 0.1% SDS. Non poly-A RNA was further eluted from the column by reducing the salt concentration in the above buffer to 0.1 M NaCl. The poly-A RNA was eluted with 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.05% SDS.

Hybridization of Northern Blots with Proteinase Probes - Total RNA (25 μg/lane) or mRNA (1 μg/lane) was fractionated on a 15 X 15 cm 1.25 % (w/v) agarose gel containing formaldehyde as described by Maniatis et. al . (1982). The running buffer was MOPS (40 mM morpholinopropanesulfonic acid, pH 7.0, 10 mM sodium acetate, 1 mM EDTA; all from Sigma)and electrophoresis was carried out at 85 V, constant voltage, in a 4° C cold room. The gel was washed for 1 h in two changes of 20 X SSC and RNA transferred to nitrocellulose by capillary action using 20 X SSC. Following transfer the nitrocellulose was rinsed in 6 X SSC, dried, then baked in a vacuum oven for 2 h at 80° C. RNA standards (BioRad) were run in an adjacent lane, and following transfer, were detected by staining the nitrocellulose with methylene blue (Maniatis et. al ., 1982). The dried filters were prehybridized and hybridized using the same probes and protocols as described for the Southern blots.

Construction of a Genomic Library - DNA (75 μg) was sonicated briefly to generate random fragments that were resolved on a 1% (w/v) agarose TAE (40 mM Tris-acetate, 1 mM EDTA) gel. Hind III cut λ markers were run as standards. The gel was stained with ethidium bromide and fragments of 6.0 - 1.5 kb were transferred onto DEAE paper (Schleicher and Schuell) by electrophoresis, then eluted in 2.5 M NaCl containing 50 mM arginine (Sigma). The DNA was blunt ended, methylated with EcoRI methylase, ligated to excess EcoRI linkers, digested with EcoRI, and ligated to phosphatase-treated, EcoRI-digested bacteriophage lambda gt11 DNA (Promega Biotech, Madison, WI; Stephens et al., 1985). After *in vitro* packaging of the DNA fragments, the recombinant phage were plated on *Escherichia coli* strain Y1088 (Young and Davis, 1983) and maintained as liquid stocks (Maniatis et al., 1982). Approximately 2.8 - 5.7 x 10^8 recombinant phage per ml were obtained from the original packaging reactions.

Construction of cDNA Libraries - Total RNA (50 μg) or mRNA (5 μg) was made into cDNA using a cDNA synthesis kit (Amersham). The Amersham protocol was

followed using the amount of reagents necessary for a "5 μg reaction". Fifty μCi of ^{32}[P]dCTP was included in the first strand reaction so that the products could be followed in subsequent reactions. First strand synthesis was primed with either oligo dT or random hexanucleotide primers. Following second strand synthesis the reaction was terminated, and brought to 0.5 ml with H_2O and then extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1). The organic layer was back extracted with TE until the majority of radioactive material was found in the aqueous layer. The labeled cDNA was ethanol precipitated and resuspended in 200 μl of TE, 150 mM NaCl. The unincorporated nucleotides were further separated from cDNA by chromatography over a 5 ml column (sterile, disposable, serological pipette; 5 ml in 1/10) of G-150 (Pharmacia) that had been equilibrated with TE, 150 mM NaCl. The labeled cDNAs, which eluted with the void of the column, were ethanol precipitated and resuspended in 100 μl of H_2O. This cDNA was blunt ended, methylated, ligated to EcoRI linkers, cut with EcoRI, ligated into λ gt 11, packaged and amplified as described for the construction of the genomic library. Approximately 0.5-7.0 x 10^6 recombinant phage/ml were obtained from the original packaging reactions.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