Expression dynamics of secreted protease genes in *Trichophyton rubrum* induced by key host’s proteinaceous components

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*Trichophyton rubrum* is the most common agent of dermatophytosis, a disease that affects millions of individuals worldwide. Its molecular pathogenicity mechanisms are still not completely elucidated. It has been widely recognized that proteases secreted by *T. rubrum* are the key virulence factors during host infection. However, our knowledge about the expression of its secreted proteases in host infection is still obscure. This investigation provides the expression patterns and dynamics of secreted protease genes belonging to the subtilisins (SUB) and metalloproteases (MEP) gene families in *T. rubrum*. The data was obtained under simulated host infection conditions through relative quantification of real time PCR. Keratin, collagen, and elastin induced the expression of similar protease genes, and the expression patterns and dynamics of these protease genes in media containing human skin sections were different from those in media containing individual protein substrates. According to the expression dynamics of these protease genes, we conclude that Sub3, Sub4, and Mep4 may be the dominant proteases secreted by *T. rubrum* during host infection, and that these proteases could be good targets for new antifungal chemotherapy and molecular diagnostic markers. This work presents useful molecular details to further our understanding of the pathogenesis of dermatophytosis.

**Keywords** *Trichophyton rubrum*, secreted proteases, subtilisin, metalloprotease, Real time PCR

Introduction

The dermatophytes are a group of closely related fungi that can invade keratinized tissue (skin, hair and nails) causing the infection, dermatophytosis, commonly referred to as ringworm. The global prevalence of dermatomycoses is as high as 20% according to the World Health Organization [1], and approximately 10% of the human population suffers from onychomycosis [2]. Among dermatophytes, *Trichophyton rubrum* is the most frequently isolated agent of dermatophytosis worldwide and is responsible for 90% of chronic dermatophyte infections [3] and approximately 80% of reported cases of onychomycosis [4]. In immunocompromised hosts, *T. rubrum* can achieve deep dermal invasion [5–8]. Although not usually life-threatening, infections caused by *T. rubrum* are often difficult to eliminate completely and compromise patients’ quality of life. Despite the prevalence of this fungal infection, the detailed molecular basis of *T. rubrum* pathogenesis remains unknown.

Historically, the secretion of proteolytic enzymes by dermatophytes has been considered a key factor in the
invasion, utilization, and subsequent dissemination of the fungi through the stratum corneum of the host [9]. Recently, the genes encoding two families of secreted proteolytic enzymes in T. rubrum were sequenced, and characterized as comprising a seven-member subtilisin (SUB) gene family [10] and a five-member metalloprotease (MEP) gene family [11]. Some of these proteases were identified from the culture supernatant of T. rubrum cultured in media containing soy proteins as a sole nitrogen and carbon source, and the activities of several enzymes in natural or recombinant forms were measured. However, the detailed expression patterns and dynamics of these protease genes are still not clear over the course of host infection. During host-pathogen interaction, pathogen gene expression is usually modulated by signals from the host. Understanding pathogen gene expression patterns and dynamics may provide insight into the pathogenetic mechanisms and their importance in the process of host infection.

Real-time PCR is one of the most sensitive and reliably quantitative methods for gene expression analysis, and this method has been broadly applied to microarray verification, pathogen quantification, cancer quantification, transgenic copy number determination, and drug therapy studies [12–15]. In this study, the expression patterns and dynamics of genes encoding two major families of secreted proteases in T. rubrum were measured by real-time PCR under host infection mimicking conditions.

**Material and methods**

**Skin sections**

Normal thigh skin was harvested from surgically excised tissues from women who underwent abdominoplasty. All specimens were prepared by placing pieces of skin, approximately 0.1–0.2 cm², with full epidermal and dermal thickness into small Petri dishes. Skin pieces were immersed in cold sterilized PBS. Whenever possible, skin samples were used within 1 h of removal.

**Strain and culture conditions**

The T. rubrum clinical isolate BMU 01672 used in the present experiments was obtained from Professor Ruoyu Li (Research Center for Medical Mycology, Peking University). This isolate was confirmed as T. rubrum by morphologic identification as well as by PCR amplification and sequencing of the 18S ribosomal DNA and internal transcribed spacer (ITS) regions.

BMU 01672 was cultivated for 10 days at 28°C on Sabouraud dextrose agar (Difco Laboratories, Detroit, MI) slants. A 5-litre conical flask containing 2 litres of yeast extract peptone dextrose (YPD) liquid medium (Difco laboratories) was inoculated with 20 plugs (7 mm in diameter) of freshly growing mycelium, and incubated at 28°C and 250 rpm on a rotary shaker for 3 days. Following this culture period, the resulting T. rubrum mycelia were washed three times with PBS, transferred to 2 l of fresh glucose medium, and incubated at 28°C and 250 rpm for another 24 h. The purpose of this additional incubation in glucose medium was to eliminate potential interferences from YPD medium which contains protein components and may induce expression of the protease genes measured in this study. T. rubrum were harvested and washed three times with PBS. The washed T. rubrum mycelia were filtered with a sterile stainless steel cell dissociation sieve. The remaining liquid was sucked as much as possible with sterile tissue paper. Then 20 fractions of mycelia were weighed from the above treated mycelia, each fraction weighed one gram. The remaining mycelia were stored in liquid nitrogen. The weighed mycelia were transferred to 20 250 ml flasks containing 100 ml of one of the following five media: collagen salt medium, elastin salt medium, keratin salt medium, human skin sections medium, and glucose medium (four flasks per media type). Of the five media, glucose medium served as ‘control’ medium, while the other served as ‘test’ media, also referred to as inducing media. All 20 flasks were incubated at 28°C and 250 rpm for 24 h, and a sample from each of the five media was harvested and stored in liquid nitrogen at 1, 3, 12, and 24 h incubation time points.

Glucose medium contained the following components (per litre): 2.5 g dextrose, 0.5 g MgSO₄·7H₂O, 0.46 g KH₂PO₄, 1 g K₂HPO₄, and 100 μg thiamine hydrochloride (Sigma-Aldrich, St. Louis, MO). The four other media contained the same components as the glucose medium, but dextrose was replaced by 0.5% (w/v) keratin powder, 0.5% (w/v) collagen powder, 0.5% (w/v) elastin powder (all obtained from Sigma-Aldrich), and 0.5% (w/v) human skin sections.

**RNA isolation and cDNA synthesis**

Total RNA of mycelia from all samples were extracted with the NucleoSpin® RNA Plant kit (MACHEREY-NAGEL GmbH & Co. KG, Düren Germany) following the manufacturer’s instructions. The purity and integrity of the RNA samples was confirmed by agarose gel electrophoresis, and sample concentration and purity were determined spectrophotometrically by measuring absorbance at 230, 260, 280, and 320 nm. Total RNA (5 μg) from each sample was reverse transcribed into cDNA using Superscript™II RT (Life Technologies/Invitrogen, Carlsbad, CA).
Quantitative real-time PCR

All quantitative real-time PCR experiments were performed on ABI PRISM® 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). Each gene was measured in triplicate in a single experiment. Specific primers (see Table 1) were designed for the genes of interest and for the gene encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which served as an endogenous control, by the Primer Premier 5.0 software (PREMIER Biosoft International, Palo Alto, CA). The reaction mixtures contained 1 μl of cDNA, 25 μl of Power SYBR® Green Universal Master Mix (Applied Biosystems), and 400 nM of each primer, and the volume was brought to 50 μl with nuclease-free water. The PCR program was 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and the annealing/extension at 60°C for 1 min. Following PCR, dissociation curve analysis was performed to verify that a single product was amplified. Independent PCRs were performed using the same cDNA for both the genes of interest and for GAPDH.

Table 1 Specific primers used for genes encoding secreted subtilisins, metalloproteases, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

<table>
<thead>
<tr>
<th>Target</th>
<th>Primersa</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>F 5′-ACGGCTTCCGTCGATATTGG R 5′-AGTATTCGCGCATTTTGCT</td>
<td>112</td>
</tr>
<tr>
<td>SUB1</td>
<td>F 5′-AGGTGGTGTTCTCCACTGCC</td>
<td>77</td>
</tr>
<tr>
<td>SUB2</td>
<td>F 5′-CCTTGTGGCAGCTGCTATGA R 5′-AGTCCGTGACCTTGCTGATG</td>
<td>84</td>
</tr>
<tr>
<td>SUB3</td>
<td>F 5′-AGGGGAGGGGTGTTGTTGTTGTTGTC</td>
<td>121</td>
</tr>
<tr>
<td>SUB4</td>
<td>F 5′-ACCCCTCTATCCCTTATCCTTCCC R 5′-CATTTCGGCTGTCTTGTGGC</td>
<td>140</td>
</tr>
<tr>
<td>SUB5</td>
<td>F 5′-CTGACGCTCCAGGATGTTCAAGG R 5′-CGGGACGATCAAGGTCACCG</td>
<td>183</td>
</tr>
<tr>
<td>SUB6</td>
<td>F 5′-TGCCTCTTGAGCCATCC</td>
<td>60</td>
</tr>
<tr>
<td>SUB7</td>
<td>F 5′-TGGTGGTCCGTCTTGTTGA</td>
<td>151</td>
</tr>
<tr>
<td>MEP1</td>
<td>F 5′-GCCCATGCGGTGTCGCTTAA R 5′-CCTGGGGGTGTCGCTGTTTCC</td>
<td>83</td>
</tr>
<tr>
<td>MEP2</td>
<td>F 5′-ACCTCACCACATCACTCTTCTTCAT</td>
<td>80</td>
</tr>
<tr>
<td>MEP3</td>
<td>F 5′-AGCCGAGCGAGCGAGAGAGAA</td>
<td>60</td>
</tr>
<tr>
<td>MEP4</td>
<td>F 5′-AGTCGTCGACTGACTGCTTAGC CAG</td>
<td>105</td>
</tr>
<tr>
<td>MEP5</td>
<td>F 5′-GGCAGAGGTCGATTTAGGCTAGGCTA</td>
<td>88</td>
</tr>
</tbody>
</table>

aF and R represent forward and reverse primers, respectively.

Statistical analysis of data

Data obtained from real time PCR experiments were analyzed by the 2^-ΔΔCT method [16] and student’s t-test through SPSS version 13 software package [17].

Result and discussion

Selection of inducing media and time points for measuring protease genes expression

We report the expression patterns and dynamics of genes encoding two major families of secreted proteases, the subtilisins and metalloproteases, in T. rubrum under conditions that mimic host infection. To simulate host infection, we cultured T. rubrum in four different media containing keratin, elastin, collagen, or human skin sections, individually. Keratin is the major protein component of the stratum corneum, the superficial layer of the skin, where dermatophyte infections typically occur. Elastin and collagen are the major extracellular matrix proteins in the dermis. In immunocompromised hosts, deeper penetration by dermatophytes was observed. This suggests that dermatophytes can encounter elastin and collagen during deep dermal invasions and that such exposure may affect the gene expression of dermatophytes, especially expression of secreted protease genes. Moreover, culturing T. rubrum and T. mentagrophytes in media containing keratin or human skin sections is an approach that has been used to mimic host infection conditions. Such conditions are essential for identifying which expressed genes are involved in virulence and adaptation to the host, and for investigating the interaction between dermatophytes and skin cells in dermatophytosis [18–22]. After inoculation, we selected four time points (1, 3, 12, and 24 h) for determining the immediate and early changes in the expression of two families of secreted protease genes in T. rubrum treated with each tested substrate.

Expression patterns and dynamics of secreted protease genes in control and four inducing media

All data obtained through real time PCR experiments are presented in Supplementary Fig. 1 (online version only). Statistical analysis results are available in Supplementary Figs. 2 and 3 (online version only). Representative dissociation curves for all measured genes are provided in Supplementary Fig. 4 (online version only). The expression patterns and dynamics of genes in the two families in control medium and each of the inducing media are shown in Figs. 1 and 2. The three figures were plotted according to relative quantification data in the Supplementary Fig. 1. Because T. rubrum cultured in both control and
As shown in Figs. 2 and 3, the expression patterns and dynamics of genes in the two secreted protease families differ among the four inducing media. At 1 h of incubation, expression of most of protease genes from the two families, including SUB1-SUB4 and SUB7 from the SUB family and MEP1-MEP4 from the MEP family, were induced more than two-fold in keratin- and collagen-containing media. Thereafter, expression levels of some protease genes further increased; the others gradually decreased. While expression of these protease genes was also induced in elastin-containing medium at 1 h of incubation, their expression levels were lower than those induced by keratin- or collagen-containing media and further increased at 3 h of incubation. After 3 h, expression of these protease genes in elastin medium displayed different dynamic trends like in keratin- and collagen-containing media. These results indicate that keratin, collagen, and elastin, although components of separate skin regions, can induce the expression of a similar suite of protease genes, and that the inducing effects occur immediately upon *T. rubrum* exposure to these proteins. The time-dependent gene expression dynamics of the induced proteases may reflect their distinct roles in the digestion of the various substrate proteins.

Because keratin, elastin, and collagen are the major extracellular matrix proteins in skin, human skin sections might be expected to have inducing effects similar to those of the individual components on the measured protease genes. However, the expression patterns and dynamics of these protease genes in media containing human skin sections differed from those observed in media containing keratin, collagen, or elastin, individually. When *T. rubrum* were exposed to human skin sections, all of the protease genes induced by the three skin proteins except SUB4 were down-regulated at 1 h. At the following two time points, only expression of SUB4 increased more than two-fold relative to control levels, and expression of both MEP3 and MEP4 was lower than control levels. The expression of MEP1 and MEP3 significantly increased at the last time point, and at the same time point, the expression of SUB3, SUB4, and MEP4 dramatically decreased. Distinct expression dynamics for these protease genes in media containing individual skin proteins versus medium containing human skin sections indicate that regulatory signals from skin proteins and human skin sections differ. Signals from individual skin proteins first induced the expression of protease genes, and then selectivity that resulted in further increase in expression of SUB3, SUB4, MEP3, and MEP4 and gradual decrease in expression of SUB2, SUB7, MEP1, and MEP2 was observed. Regulatory signals from human skin sections initially suppressed
Expression dynamics of secreted protease genes in Trichophyton rubrum

The expression dynamics of secreted protease genes in Trichophyton rubrum were investigated. Previous studies showed that Sub3, Sub4, Mep3, and Mep4 were dominant proteases secreted by T. rubrum grown in soy protein containing media. Proteolytic assays in these studies revealed that Sub3 and Sub4 act on a wide range of substrates, including three skin extracellular matrix proteins, and that secreted metalloproteases were responsible for dominant proteolytic activity in the culture supernatant [10, 11]. In another study on host-pathogen interactions, SUB3, MEP3, and MEP4 were identified as remarkably up-regulated genes by subtractive suppression hybridization after incubation of T. rubrum with keratin for 72 h [21]. These results indicate that Sub3, Sub4, Mep3, and Mep4 may be the dominant proteases secreted by T. rubrum for the digestion of external proteins in both infection host and other natural environments and that they may be key virulence factors related to the processes of invasion and nutrient acquisition during host infection.

In media containing individual skin proteins, expression of SUB2, SUB7, and MEP2 initially increased, but their expression levels except SUB7 and MEP2 in elastin-containing medium at 3 h did not significantly further expression of the protease genes, with the exception of SUB4. Then, selective induction for expression of SUB3, SUB4, MEP1, MEP3, and MEP4 was observed. Human skin is a complex structure, containing a lot of substances other than keratin, elastin, and collagen. Among these substances, some are thought to be active against dermatophytes, for example, 2-macroglobulin keratinase inhibitor, unsaturated transferrin etc [24]. Therefore, it is reasonable to infer that some substances in human skin could suppress expression of the secreted protease genes when T. rubrum were exposed to human skin sections. With the extension of incubation, T. rubrum may produce something to neutralize or decompose the suppressing substances in human skin sections. Then inducing effects of keratin, elastin, and collagen in skin on expression of the protease genes begin to emerge.

Of two families of secreted protease genes, SUB3, SUB4, and MEP4 were the most significantly induced in simulated infection conditions used in the present study. Although expression level of MEP3 was lower than that of SUB3, SUB4, and MEP4, it was another noticeable secreted protease gene due to its continuous increasing expression dynamics in media containing each skin proteins and the significant increase in its expression level at the last experimental time point in media containing human skin sections. Previous studies showed that Sub3, Sub4, Mep3, and Mep4 were dominant proteases secreted by T. rubrum grown in soy proteins containing media. Proteolytic assays in these studies revealed that Sub3 and Sub4 act on a wide range of substrates, including three skin extracellular matrix proteins, and that secreted metalloproteases were responsible for dominant proteolytic activity in the culture supernatant [10,11]. In another study on host-pathogen interactions, SUB3, MEP3, and MEP4 were identified as remarkably up-regulated genes by subtractive suppression hybridization after incubation of T. rubrum with keratin for 72 h [21]. These results indicate that Sub3, Sub4, Mep3, and Mep4 may be the dominant proteases secreted by T. rubrum for the digestion of external proteins in both infection host and other natural environments and that they may be key virulence factors related to the processes of invasion and nutrient acquisition during host infection.

In media containing individual skin proteins, expression of SUB2, SUB7, and MEP2 initially increased, but their expression levels except SUB7 and MEP2 in elastin-containing medium at 3 h did not significantly further
increased with extension of incubation time. Especially, at the last two experimental time points, expression levels of the three protease genes were far lower than those observed for SUB3, SUB4, and MEP4. In medium with human skin sections, expression levels of SUB2, SUB7, and MEP2 never increased more than two-fold of the control levels. The expression dynamics of SUB2, SUB7, and MEP2 observed in the four inducing media suggest that they are unlikely the primary secreted proteases that degrade skin proteins during infection. Maybe they play other functions than protein degradation.

The expression dynamics of SUB1 in media containing individual skin proteins imply that Sub1 may prefer keratin and elastin over collagen. Expression of SUB1 was not significantly induced in media containing human skin sections, indicating that this protease may not be a key virulence factor in the early stage of host infection and, instead, may be involved in other stages of infection. The expression dynamics of MEP1 in media with individual skin proteins was similar to that observed for MEP2. MEP1 was significantly induced only at the last experimental time point in medium containing human skin sections.

Also, Mep1 was identified in the culture supernatant of *T. rubrum* cultured in media containing soy proteins [11]. These combined results suggest that, while Mep1 may digest host proteins, it is unlikely a dominant protease in *T. rubrum* infection. The observed expression dynamics of SUBS, SUB6, and MEP5 indicate that they may be not involved in host protein digestion but may have other functions during infection. Indeed, recombinant Sub5 from *P. pastoris* was not active on either casein or keratin [10]. Sub6, previously described as *Tri r 2* allergen, induced both immediate and delayed type hypersensitivity skin reactions [25].

**Conclusion**

To our knowledge, this study provides the first comprehensive analysis of the expression patterns and dynamics of two major families of secreted protease genes in *T. rubrum* under simulated host infection conditions. Our results suggest that Sub3, Sub4, and Mep4 may be the dominant secreted proteases responsible for invasion and the use of host proteins as nutrients during infection by *T. rubrum*. The extremely high expression levels of the
three protease genes observed when *T. rubrum* was incubated with human skin sections imply that such proteases could be good targets for new antifungal chemotherapy and molecular diagnostic markers. Our results present useful molecular details for further understanding the pathogenesis of dermatophytes and also provide a valuable framework for studying the expression dynamics of secreted protease genes in other dermatophytes.

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**References**


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