Chapter - 4

Keratin Digestion Profile
4. KERATIN DIGESTION PROFILE

4.1. INTRODUCTION

Keratinophilic fungi in nature are microorganisms, limited to the decomposition of keratinaceous substrates, though in culture they can utilize other scleroproteins (e.g. collagen and elastin) and many soluble proteins. Since the production of extracellular proteases (Cole 1996) and the presence of Lowry-positive peptides (Kunert 1989) are not enough to show that keratin has been degraded. It is, in fact, uncertain whether peptides are actually released from keratin or other keratin-associated proteins (Kunert 1989; Odds 1991). Experiments with purified dermatophytic proteases on native keratin have shown the breaking of disulphide bonds of the keratin. Earlier works have suggested (Majchrowicz and Dominik 1969; Dominik et al. 1973) that the term keratinolytic be reserved for colonizers that are really capable of attacking and demolishing keratin, whereas those that accompany them and only use the more readily degradable substances should be called keratinophilic. The literal meaning of Keratinophilic is derived from Greek and simply means “endowed with an affinity for keratinic substrates”. Keratinophilic fungi, therefore, can be said to include all natural colonizers of these substrates, whereas keratinolytic fungi are only those that have been shown to attack keratin itself in some way.

However the most active keratinolytic fungi are dermatophytes and their correlates, especially *Microsporum*, *Trichophyton*, *Aphanoascus*, *Chrysosporium*, *Geomycetes*, *Gymnoascus*, *Malbranchea* and *Myceliophthora* species, though forms of attack have
equally been reported for some species of Alternaria, Beauveria, Cladosporium, Mucor, Paecilomyces, Penicillium and Scopulariopsis (Marchisio et al. 1986 & 1991 & 1994a & 1994b). It should also be borne in mind that keratinolysis, like many other fungal biochemical activities, does not seem either a constant or a species-specific character (Marchisio et al. 1986, 1991, 1994b). Both active and nonactive isolates actually occur within a given species in the same environmental conditions. Variations may also be observed in the manner and intensity in which each isolate attacks the substrate and differentiates specialised structures for this purpose. For the reasons mentioned above, the simple growth of a fungus on keratin residues, often visible with the naked eye, is not a sufficient demonstration of keratinolytic activity. Thus it is important to study the nature of degradation by analyzing the products and its co-relation between species of different ecological groups of dermatophytes - anthropophilic, geophilic and zoophilic. The digestion profile of keratin by the keratinase enzyme isolated from different species of dermatophytes belonging to different ecological groups was studied.

4.2. METHODOLOGY

4.2.1. DIGESTION PROFILE OF KERATIN BY KERATINASE

(Yoo et al. 2004; Jousson 2004b)

Five ml of the crude enzyme extract of each isolates of each strain were pooled individually and incubated with 5 ml of the 1% pure keratin powder (Hi-media, India) in separate tubes for 5 hours at 26°C. After incubation the reaction mixture was centrifuged at 5000x g for 10 min. The supernatant was taken in a separate 25 ml falcon
tube and the peptides and proteins present in the solution was precipitated by adding 2.5 ml of TCA (1 volume of TCA stock to 4 volumes of protein sample) and incubated for 10 min at 4°C. After incubation the tube was spinned at 14000 rpm for 5 min. The supernatant was removed. The whitish fluffy pellet was then dispersed in 1 ml of cold acetone taken in a 2 ml eppendorf tube and the tube was spin at 14000 rpm for 5 min. The supernatant was removed. Once again the pellet was washed with 1 ml of acetone. The final pellet was dried by placing the tube in a 95°C heat block for 5-10 min to remove the acetone fully. This dried pellet was diluted in 2x sample buffer (0.25 M Tris hydrochloride [pH 6.8], 20% [v/v] glycerol, 0.2% [w/v] sodium dodecyl sulfate, and 0.005% [w/v] Bromophenol Blue) and left on the 95°C heat block for 10 min before loading sample onto polyacrylamide gel. 4% Stacking and 8% Resolving SDS-PAGE gel of 8 x 7 cm x 0.75 mm thickness was prepared on a slab-gel apparatus (Helini-mini slab gel apparatus & Mini power pack). Tris/ Glycine buffer (pH 8.3) was used as running buffer. Electrophoresis was run at 20 mA/ gel. After electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250 in water-methanol-acetic acid (5:5:1, v/v) for 30 min and then destained in 45% (v/v) methanol/ 3% (v/v) acetic acid until the bands are clearly visible for photography. For calculation of MW molecular weight standards viz. 116.00 kDa, 66.00 kDa, 45.00 kDa, 35.00 kDa, 25.00 kDa, 18.40 kDa, 14.40 kDa were run simultaneously in a separate lane of the gel. Using Genetools software (PerkinElmer, USA) the molecular weight of the digested peptide fragments and band matching analysis were determined.
4.3. RESULTS

4.3.1. DIGESTION PROFILE OF KERATIN BY KERATINASE

The keratin digestion profile by the keratinase enzyme isolated from the different species of dermatophytes taken for the present study viz. *T. rubrum*, *E. floccosum*, *M. canis* and *M. gypseum* showed some unique and related banding patterns. *T. rubrum* showed a total of 8 protein bands ranging from 57.30 kDa to 15.75 kDa. *E. floccosum* showed total of 7 protein bands ranging from 51.84 kDa to 16.15 kDa. *M. gypseum* showed total of 11 protein bands ranging from 121.28 kDa to 15.12 kDa. *M. canis* showed total of 11 protein bands ranging from 50.63 kDa to 14.17 kDa. Band matching analysis showed that the banding pattern of *T. rubrum* and *E. floccosum* were similar with co-efficient values of 0.400. On the other hand banding pattern of *M. gypseum* and *M. canis* were similar with co-efficient values of 0.636 (Fig. 19; Graph 21, 22, 23, 24 & 25; Table 9 & 10).
**Fig 19: Digestion profile of keratin by keratinase secreted by different dermatophyte species**

**Track-1:** Digestion by keratinase from *T. rubrum*

**Track-2:** Digestion by keratinase from *E. floccosum*

**Track-3:** Marker lane

**Track-4:** Digestion by keratinase from *M. canis*

**Track-5:** Digestion by keratinase from *M. gypseum*

---

**Table 9: Molecular Weight (in kDa) and raw intensity (pixel intensity) of the peaks identified in the digestion profile of keratin by keratinase secreted by different dermatophyte species**

<table>
<thead>
<tr>
<th>Peak No.</th>
<th><strong>T. rubrum</strong> (Track 1)</th>
<th><strong>E. floccosum</strong> (Track 2)</th>
<th><strong>M. canis</strong> (Track 4)</th>
<th><strong>M. gypseum</strong> (Track 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MW (kDa)</td>
<td>Pixel Intensity</td>
<td>MW (kDa)</td>
<td>Pixel Intensity</td>
</tr>
<tr>
<td>1</td>
<td>57.30</td>
<td>123654.02</td>
<td>51.84</td>
<td>113333.02</td>
</tr>
<tr>
<td>2</td>
<td>40.59</td>
<td>4510.45</td>
<td>37.09</td>
<td>6625.41</td>
</tr>
<tr>
<td>3</td>
<td>34.12</td>
<td>850.12</td>
<td>32.89</td>
<td>2766.76</td>
</tr>
<tr>
<td>4</td>
<td>30.24</td>
<td>19683.38</td>
<td>29.58</td>
<td>35820.07</td>
</tr>
<tr>
<td>5</td>
<td>25.93</td>
<td>1874.46</td>
<td>25.84</td>
<td>1924.17</td>
</tr>
<tr>
<td>6</td>
<td>22.35</td>
<td>4373.43</td>
<td>23.47</td>
<td>584.55</td>
</tr>
<tr>
<td>7</td>
<td>19.79</td>
<td>3740.59</td>
<td>16.15</td>
<td>60634.67</td>
</tr>
<tr>
<td>8</td>
<td>15.75</td>
<td>27372.21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td></td>
<td></td>
<td>19.99</td>
<td>3793.34</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td></td>
<td>17.66</td>
<td>2510.00</td>
</tr>
<tr>
<td>11</td>
<td></td>
<td></td>
<td>14.17</td>
<td>23318.09</td>
</tr>
</tbody>
</table>

---

85
Graph 21: Keratin digestion profile by keratinase of *T. rubrum*

X axis - Rf Distance

Y axis - Profile height

Graph 22: Keratin digestion profile by keratinase of *E. floccosum*

X axis - Rf Distance

Y axis - Profile height

Graph 23: Keratin digestion profile by keratinase of *M. canis*

X axis - Rf Distance

Y axis - Profile height
Table 10: Matching coefficient values of the keratin digestion profile of different dermatophytes

<table>
<thead>
<tr>
<th>Track</th>
<th>Track 1</th>
<th>Track 2</th>
<th>Track 3</th>
<th>Track 4</th>
<th>Track 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Track 1</td>
<td>1.000</td>
<td>0.400</td>
<td>0.133</td>
<td>0.421</td>
<td>0.421</td>
</tr>
<tr>
<td>Track 2</td>
<td>0.400</td>
<td>1.000</td>
<td>0.143</td>
<td>0.444</td>
<td>0.444</td>
</tr>
<tr>
<td>Track 3</td>
<td>0.133</td>
<td>0.143</td>
<td>1.000</td>
<td>0.222</td>
<td>0.333</td>
</tr>
<tr>
<td>Track 4</td>
<td>0.421</td>
<td>0.444</td>
<td>0.222</td>
<td>1.000</td>
<td>0.636</td>
</tr>
<tr>
<td>Track 5</td>
<td>0.421</td>
<td>0.444</td>
<td>0.333</td>
<td>0.636</td>
<td>1.000</td>
</tr>
</tbody>
</table>

Graph 25: Dendrogram based on Molecular Weight matching of keratin digestion profile of different dermatophytes

Track-1: Digestion by keratinase from *T. rubrum*
Track-2: Digestion by keratinase from *E. floccosum*
Track-3: Marker lane
Track-4: Digestion by keratinase from *M. canis*
Track-5: Digestion by keratinase from *M. gypseum*
4.4. DISCUSSION

4.4.1. DIGESTION PROFILE OF KERATIN BY KERATINASE

The dermatophytes are adapted to the use keratin as the source of carbon. Keratin were utilized preferentially from the medium even in the presence of glucose (Ziegler et al. 1962 & 1964 & 1969). Many researchers worked on the in-vitro keratinolytic activity of dermatophytes on keratinaceous substrates.

In dermatophytes, keratinolytic abilities were compared in species differing in the degree of adaptation to parasitism (Kunert 1989). Keratin degradation in a liquid medium is accompanied by the release of cleavage products in the culture. Several earlier workers have studied the degradation products of different protein substrates with respect to individual species (Kamalam and Thambiah 1981; Safranek and Goos 1982; Shrivastava et al. 1996; Yoo 2004). Monod et al. (2005) reported the separation of 8 different molecular weight proteins from the culture supernatant of T. rubrum grown on liquid Keratin medium. Kunert (1976) reported the isolation of many fragments with higher as well as lower molecular weight proteins from the keratin digestion supernatant of M. gypseum. But, a comparative study on the keratin digestion profile by the keratinase enzyme secreted by the dermatophytes belonging to different ecological groups was not studied earlier. Hence, in the present study the electrophoresis study of digestion profile of keratin by the keratinase enzyme isolated from different species belonging to different ecological groups such as geophilic, zoophilic and anthropophilic was studied individually.
to find out the co-relation between the keratinase activity among the ecological groups and actual degradation of the keratin.

Present electrophoresis analysis showed a number of bands at different molecular weights across the strains. Though the banding patterns of the different test species were almost similar, they differed in the intensities (quantity). The profile shows different fragments ranging from MW (Molecular Weight) 121.28 to 14.40 kDa. The MW (in kDa) of some of these protein bands irrespective of the species (22.35, 25.65, 25.84, 32.89, 33.99, 33.85, 45.00, 51.23, 51.84, 64.84, 121.28) closely resembles various proteases and its dimers isolated from different dermatophytes earlier (Dei Cas and Vernes 1986; Simpanya and Baxter 1995). Production of multiple proteases had also been reported in other dermatophytes, such as *T. rubrum* and *T. mentagrophytes* (Asahi *et al.* 1985; Sanyal *et al.* 1985; Apodaca *et al.* 1989, & 1989, & 1990).

In the present investigation, the degradation of keratin using keratinase enzyme isolated from *M. gypseum* showed 11 protein bands with MW (in kDa) of 121.28, 64.84, 51.23, 41.92, 33.99, 30.46, 27.59, 26.51, 25.00, 20.78, 15.12 respectively. Several workers had demonstrated the *in-vitro* degradation of keratinaceous substrates by *M. gypseum* and confirmed the presence of various degraded products and enzymes in the culture supernatant (Kamalam and Thambiah 1981; Safranek and Goos 1982; Shrivastava *et al.* 1996). Kunert (1976) reported the isolation of two major fractions from Keratin digestion supernatant by *M. gypseum*, one fraction with many fragment in higher molecular weight
and another fraction with fragments in lower molecular weight around 13 kDa. This corresponds to the findings in our present study.

In the present study *M. canis* showed 11 protein bands with MW (in kDa) of 50.63, 40.59, 36.85, 33.74, 29.91, 26.9, 25.65, 23.35, 19.99, 17.66, and 14.17 respectively. This banding pattern resembles the earlier findings with saprophytic and Pseudo-parasitic strains of *M. canis*, which showed six different MW (in kDa) proteases, namely, 122 kDa, 64 kDa, 62 kDa, 45 kDa, 31 kDa, and 25 kDa (Simpanya and Baxter 1995).

In the present study *T. rubrum* showed 8 protein bands with MW (in kDa) of 57.30, 40.59, 34.12, 30.24, 25.93, 22.35, 19.79 and 15.75 respectively. Monad *et al.* (2005) also showed the separation of 8 different MW protein bands from the *T. rubrum* culture supernatant grown on liquid keratin medium, which includes proteins with MW of 31, 33, 45, 66 and 97 kDa. In addition other workers had also showed the presence of different proteins in *T. rubrum* culture supernatant with MW (in kDa) of 25, 31, 45, 53, 71, 93 and 124 (Asahi *et al.* 1985; Apodaca and McKerrow 1989a & 1989b). Rad *et al.* (2001) isolated twenty-two protein bands from *T. rubrum* culture with molecular weights in the range of 23.2 to 131.8 kDa. Some of the proteins reported in the present study (34.12, 30.24, 25.93 and 22.35 kDa) also showed the same molecular weight and that supports the earlier reports.

In the present study *E. floccosum* showed 7 protein bands with MW (in kDa) of 51.84, 37.09, 32.89, 29.58, 25.84, 23.47 and 16.15 respectively. Cabanes *et al.* (1987) reported
the keratinolytic activity of *E. floccosum* in-vitro with the production of different protein fragments.

All these proteases reported in dermatophyte species have even similarities with other pathogenic organisms including *Trypanosoma, Aspergillus* and *Serratia* (North 1982). The presence of more than one type of protease had also been reported in a number of other species. Multiple forms (isoforms) of a protease with same activity but of different MW had also been reported in the same organism (North 1982).

In the present study, presence of more low MW fragments <35 kDa were observed. Kunert (1976) also reported the presence of low MW peptides and proteins along with free amino acids and high molecular weight proteins. In addition we have also noted that the intensity (quantity of protein) of the fragment varies species to species. This is because of the fact that the rate and intensity of keratinase activity varies among different ecological groups and correlated with the activity profile of the protease and keratinolytic enzymes.

Protein band matching study based on the MW of the protein bands showed that the digestion profile of *M. gypseum* (Geophile) and *M. canis* (Zoophile) were similar and closely related with a matching coefficient value of 0.636. On the other hand the digestion and banding pattern of *T. rubrum* and *E. floccosum* (Anthropophiles) were similar with a matching coefficient value of 0.400. This fact confirms the reported findings on the similarities between the enzyme activities of *M. gypseum* with *M. canis* and the
similarities between the enzyme activities of *T. rubrum* with *E. floccosum* (mentioned in Chapter-2).

The present report showed that the protease and keratinase activity profile of *M. gypseum* (geophilic) and *M. canis* (zoophilic) were higher than that of *T. rubrum* and *E. floccosum* (anthropophilic). In addition the results also showed that the intensity of bands of the digestion profile of *M. gypseum* and *M. canis* were higher with 11 protein bands while that of *T. rubrum* and *E. floccosum* with 7 protein bands. This was related to the higher keratinase activity in *M. gypseum* and *M. canis*. 