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Is the Difference in Keratinase Activity of Dermatophytes to Different Keratinaceous Substrates an Attribute of Adaptation to Parasitism?

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Abstract

Dermatophytes can digest keratin and other proteinaceous substrates present in skin and its appendages such as nail, hair, and feather and use it as its sole source of carbon and nitrogen. Proteolytic and keratinolytic activities of dermatophytes have been a subject of interest for several years to understand the pathogenicity of infection. In this study we intend to elucidate the keratinase activity profile among the three ecological groups viz. geophilic, zoophilic and anthrophophilic of dermatophytes. Six isolates of each species of T. rubrum and E. floccosum, M. gypseum and M. canis were grown on mineral medium consist of human hair, human nail and chicken feather individually. The keratinolytic activity was measured spectrophotometrically at 660 nm following Folin-Ciocalteu method. The data collected was analyzed using ANOVA followed Duncan Multiple Range test. A statistically significant difference (P= 0.000**) was noted in the enzyme activity of different ecological groups of dermatophytes. T. rubrum and E. floccosum (anthrophophilic deramtophytes) recorded the lowest activity for all the substrates when compared to the geophilic (M. gypesum) and zoophilic (M. canis) counterparts. We presume that the enzyme moderation could be an attribute for obligate Anthropization in certain dermatophytes.

Introduction

Keratins are the most abundant proteins in epithelial cells of vertebrates and represent the major constituent of skin and its appendages such as nail, hair, feather and wool. Keratin is a stable insoluble protein with rigid structure due to the presence of several cross-linking disulfide bonds. Dermatophytes can digest keratin and other proteinaceous substrates present in the skin, nail, hair etc. and use it as its sole source of carbon and nitrogen [1].

Proteolytic and keratinolytic activities of dermatophytes have been a subject of interest for several years to understand the pathogenicity of infection [2]. Dermatophytes of different ecological groups have evolved owing to their differences in host specificity. However a detailed comparative study of the selective preference of the different native keratin substrates among the ecological groups of dermatophytes and its possible co-relation to pathogenicity is lacking. Therefore in the current study we intended to elucidate the difference in the keratinase activity of Trichophyton rubrum & Epidermophyton floccosum (Anthrophophilic) Microsporum gypseum (Geophilic) and Microsporum canis (Zoophilic) on keratin substrates such as human hair, human nail and chicken feather.

Materials And Methods

Six isolates each of T. rubrum and E. floccosum (clinical origin), M. gypseum

(2 clinical, 4 soil origin) and M. canis (from pet dogs) grown on Sabouraud Dextrose broth were used in the current study. Twenty micro liters of the fungal spore suspension of the dermatophytes viz. T. rubrum, E. floccosum, M. of $MgSO_4.7H_2O$, 0.1g of $Cacl_2.2H_2O$, pH 7.5 gypseum, and M. canis prepared in distilled water and adjusted to an absorbance of 0.6 at 450 nm were used as inoculums for the present study. Nine sets of 10 ml mineral medium (g/l of 0.5g KCl, 1.0g K_2HPO_4, 0.5g $MgSO_4$, 2.0g $NaNO_3$, 0.01g $FeSO_4.7H_2O$, 30g Dextrose) were prepared in 25 ml conical flasks containing 1% of different native keratin substrates viz. human hair, human nail and chicken feather individually for each isolate of each species. The above substrates viz. human hair, human nail and chicken feather were washed with ethanol, dried, and hammer milled individually prior to addition to the medium. The inoculated conical flasks were incubated on a rotary shaker (130rpm) at room temperature. From 4th day of incubation one flask (1/9) per strain per substrate was taken for keratinase enzyme assay. Keratinase assay was done on every alternate day from 4th day of incubation till 20^{th} day [3].

One and half ml of culture was transferred to Eppendorf tube and centrifuged at 5000 rpm for 10 minutes. The culture supernatant (crude enzyme extract) was used for enzyme assay. A known volume (500ul) of the supernatant was incubated with 1% (w/v) keratin powder (Hi-media, India) in 20 mM Tris-HCl buffer (pH 8.0) at 30°C for 5h. The reaction was terminated with 6% (w/v) Tri Chloro Acetic acid (TCA) and then allowed to stand for 30min followed by centrifugation at 15,000xg for 10min. The resultant supernatant was mixed with 1:1 dilution of Folin-Ciocalteu reagent and 0.5 M NaOH and then incubated at room temperature for 1hour. The keratinolytic activity was measured spectrophotometrically (PerkinElmer) at 660 nm. The keratinolytic activity was expressed in keratin units (KU), and 1KU is defined as an increase of 0.01 OD at 660 nm in 1h [3,4]. The data collected was analyzed using ANOVA followed Duncan Multiple Range test.

Results

Human Hair: M. gypseum recorded maximum keratinase activity (80.75KU) which was closely followed by M. canis (78.72KU) on the 20th day. E. floccosum recorded relatively lower activity of 56.90KU, while T. rubrum recorded the least activity of 45.43KU on the 20th day. (P value = 0.01**) **(Table 1, 2 & fig 1, 4)**

Human Nail: T. rubrum recorded the least keratinase activity of 38.07KU on the 20th day, while M. gypseum recorded the maximum activity of 70.68KU. M. canis and E. floccosum recorded moderate activity of 64.55 and 66.28 KU respectively for the human nail on the 20th day (P value = 0.01**). **(Table 1, 2 & fig. 2, 4)**

Chicken Feather: M. gypseum (77.65KU) and M. canis (76.20KU) recorded higher keratinase activity, while that of E. floccosum is moderate (48.37KU) on the 20th day. T. rubrum recorded the lowest (25.75KU) keratinase activity among the tested organisms on the 20th Day (P value = 0.01**). **(Table 1, 2 & fig. 3, 4)**

Days	Human Hair				Human Nail				Chicken Feather			
	Fungal Strains				Fungal Strains				Fungal Strains			
	T. rubrum	*M. gypseum*	*M. Canis*	*E. floccosum*	*T. rubrum*	*M. gypseum*	*M. Canis*	*E. floccosum*	*T. rubrum*	*M. gypseum*	*M. Canis*	*E. floccosum*
4	0.18	10.25	1.15	0	0.13	8.62	0	0.47	0	10.9	0.52	0
6	6.47	23.55	4.4	2.3	6.62	14.93	2.32	4.28	4.37	24.97	4.12	2.17
8	13.53	42.37	9.38	10.33	10.1	30.45	10.25	14.52	9.12	42.37	13.92	9.37
10	23.52	60.45	20.53	18.57	16.23	50.05	18.4	30.15	16.05	60.4	29.97	16.3
12	36.88	71.12	38.75	30.48	29.03	62.57	32.08	51	19.9	68.87	47.77	21.28
14	42.1	76.33	58.23	40.83	31.65	66.63	42.17	60.4	21.53	73.68	58.1	30.95
16	42.8	79.67	76.97	52.35	34.55	67.98	58.58	64.92	23	75.28	65.42	43.37
18	44.6	80.22	78.52	55.92	37.68	70.48	62.58	65.82	25.03	76.95	74.7	48.2
20	45.43	80.75	78.72	56.9	38.07	70.68	64.55	66.28	25.75	77.65	76.2	48.37

Table 1: Keratinase Activity (KU) by dermatophytes at different time interval in response to keratin substrates

	T. rubrum		*M. gypseum*		*M. canis*		*E. floccosum*		
Substrate	Mean (KU)	SD	Mean (KU)	SD	Mean (KU)	SD	Mean (KU)	SD	p-value
Human Hair	45.43[a]	1.89	80.75[d]	0.95	78.72[c]	0.73	56.90[b]	1.09	0.01**
Human Nail	38.07[a]	2.55	70.68[c]	1.79	64.55[b]	2.73	66.28[b]	1.28	0.01**
Chicken Feather	25.75[a]	2.11	77.65[d]	1.22	76.20[c]	1.44	48.37[b]	0.44	0.01**

Table 2: Peak Keratinase Activity of dermatophytes on different substrates
** Denotes Significant at 1% level.
Different Alphabets (a/b/c/d) between the organisms denotes significant above 5% level.
The Order of alphabet denotes lower to higher enzyme activity between organisms

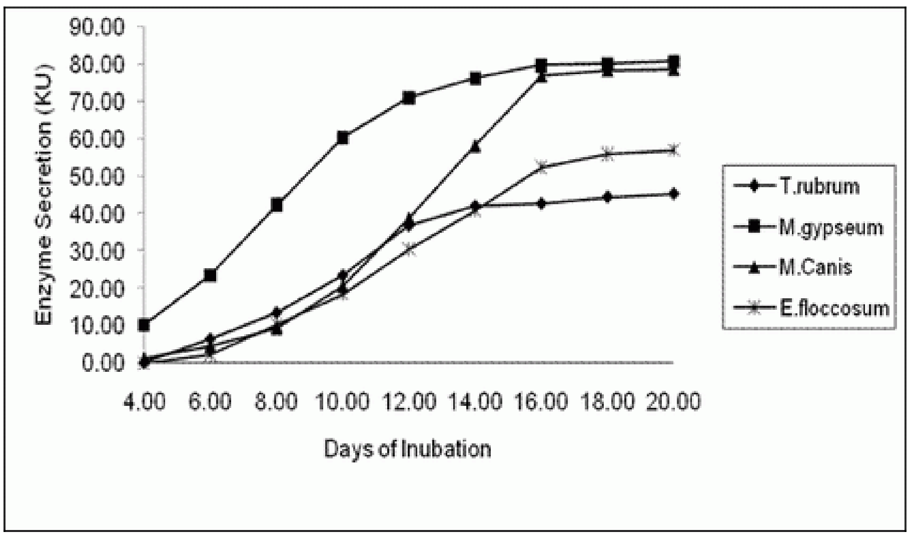

Fig 1: Keratinase Activity by dermatophytes at different time interval in response to Human Hair

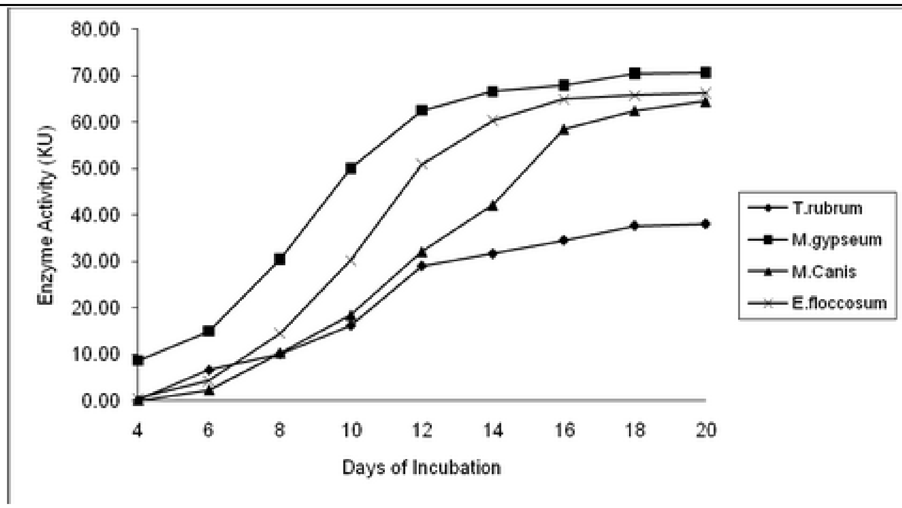

Fig 2: Keratinase Activity by dermatophytes at different time interval in response to Human Nail

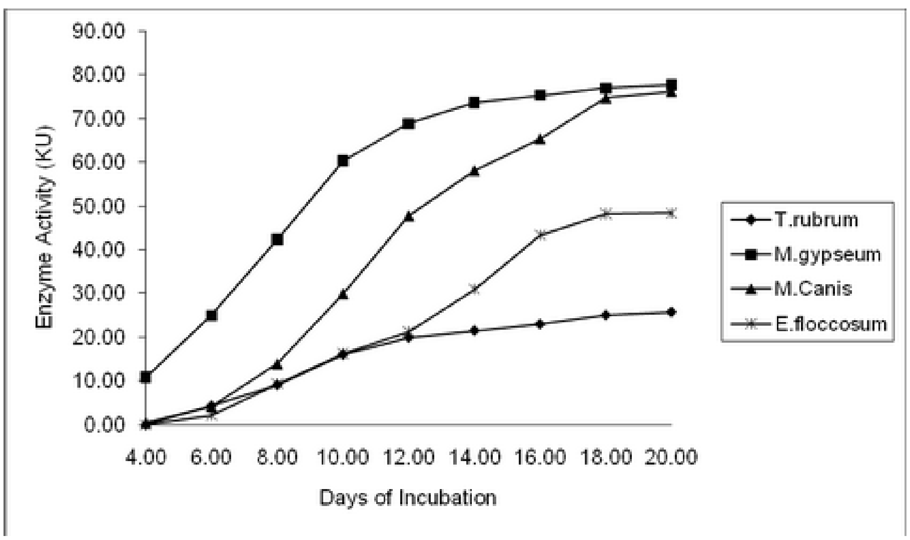

Fig 3: Keratinase Activity by dermatophytes at different time interval in response to Chicken Feather

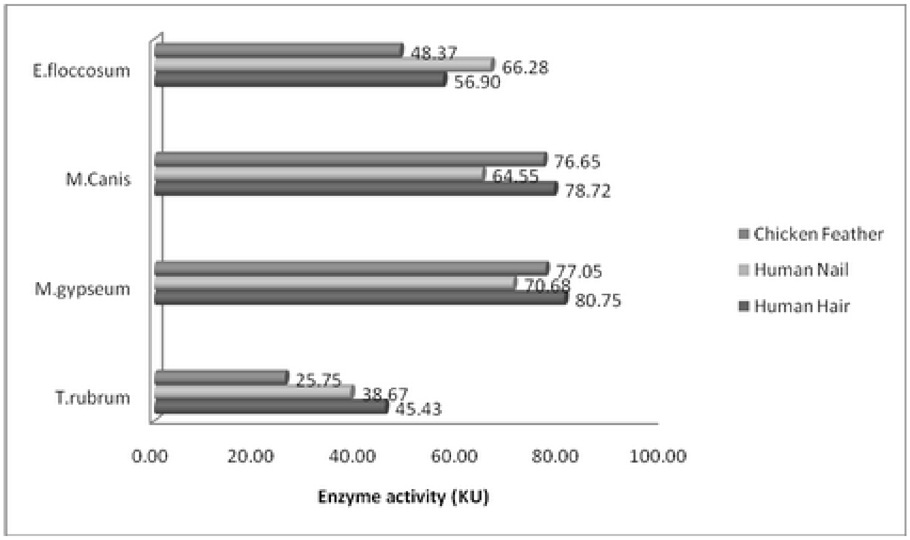

Fig 4: Peak Keratinase Activity (KU) of dermatophytes on different substrates

Discussion

Among the three keratinaceous sources viz. human hair, human nail and chicken feather, human hair was the most preferred keratin source for isolates of T. rubrum, M. gypseum and M. canis as all these organisms showed maximum keratinase activity for human hair. Among these three organisms it was also observed that M. gypseum started the keratinase secretion earlier (4^{th} day of incubation) than others and it showed higher keratinase secretion of 80.75KU. A similar finding was recorded by Page and Stock [5]. Their experiments also showed that keratinase secretion started early in M. gypseum on the 3rd day of incubation itself. This might be because of the presence of perforating organs in M. gypseum which 'facilitates' the mechanical disruption of keratin and allows the growth of mycelium faster [6].

Though T. rubrum showed its preference for human hair among the different substrates, the keratinase activity was however much lower than other organisms. The keratinase activity of T. rubrum showed a steady increase up to 14 days of incubation. Between 14^{th} and 20^{th} day, there was a very little increase in enzyme secretion and a pattern of near to stationary phase was observed. Earlier work has recorded a similar finding [7]. Current study also showed that the keratinase secretion by T. rubrum was the least (25.75KU) in chicken feather and moderate (38.07KU) in human nail when compared to human hair which recorded a peak activity of 45.43KU.

It was also observed that M. gypseum achieved keratinase secretion of 79.67KU on the 16^{th} day of incubation in human hair and reaches its maximum activity of 80.75KU on 20^{th} day of incubation. The results also showed that the secretion pattern of M. canis was similar to M. gypseum. i.e. M. canis also preferred human hair than other keratinaceous substrates with peak enzyme activity of 78.72KU. But the amount of enzyme secretion was relatively lower in M. canis (a zoophilic dermatophyte) than

M. gypseum (a geophilic dermatophyte). The preference for human hair keratin by M. canis and M. gypseum could be the reason for severity of infection caused by these organisms on encountering the human host.

E. floccosum was the only organism in the present study which showed preference towards human nail with maximum keratinase activity of 66.28KU which was followed by human hair (56.90KU). This could be supported by our earlier study [8] and others [9] with the isolation of E. floccosum from human nail infections. The utilization of human hair keratin by E. floccosum was recorded in the present study. Though, clinical infection of human hair (Tinea captis) by E. floccosum is not a common phenomenon, the in vitro utilization of human hair keratin when used as a sole keratin source cannot be ruled out. This current finding was supported by Cabanes et al. [10] and Macedo et al. [11].

It was also noted that the severity of infection and keratinase secretion could be going hand in hand together. The current study revealed that keratinase secretion of T. rubrum was slow and low compared to other strains. This in turn denotes that T. rubrum infections would be passive, chronic and not severe, whereas human infection by M. gypseum or M. canis would be of severe in nature. Current study supports the above mentioned fact, because the keratinase enzyme secretion by M. gypseum and M. canis was very high and very fast compared to T. rubrum. Viani et al. [12,13] reported that the keratinase secretion was statistically higher in animal isolates of M.canis isolated from symptomatic dogs and cats. This is strikingly evident because the isolates of M. canis used in the present study were of animal origin (dogs). When the keratinase secretion is high the manifestation of the infection would also be high, in turn the human immune response to M. gypseum or M. canis infection would also be relatively higher compared to passive T. rubrum infections. Thus T. rubrum may not get eliminated from human skin faster, as chronic mild lesions would be mostly ignored/ untreated by the host. This is the classical feature which facilitates obligate parasitism and complete anthropization of T. rubrum. We have reported in our earlier study [14] that protease moderation in dermatophytes as an attribute to anthropization and the current study is a resurgence of our hypothesis. Further work is being carried out to understand the co-relation between the keratinase digestion products of different substrates by different strains to find out its link to the molecular structure of the different keratin substrates.

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