Isolation of extracellular protease from *Trichophyton mentagrophytes* of Skin isolates

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(Received 28 / 1 / 2008 , Accepted 29 / 5 / 2008)

Abstract:

Dermatophyte fungi of the genera *Trichophyton*, *Epidermophyton*, and *Microsporum* infect human skin, hair, and nails. These types of infections, termed "dermatophytoses," are widespread that affects billions of individuals worldwide. In the present study, we isolated *Trichophyton mentagrophytes* from patients with dermatophytoses. The isolates were grown on Sabouraud agar and were identified on the basis of microscopic examination, for the presence of fungal elements. To promote production of protease, *T. mentagrophytes* isolate cultured in protein liquid medium. Results showed that *T. mentographyte*s have ability to produce extracellular protease which indicated by proteolysis activity 111.9 U/ml against casein when added as substrate to the culture filtrates of *T. mentographyte*s with specific activity 2238U/mg. To optimize protease production by *T. mentographyte*s, the effect of different cultural conditions (production media, incubation period, optimal temperature and pH) on production of extracellular protease were studied. The results showed that *T. mentographyte*s have high ability to produce protease after 9 days of incubation in protein liquid media at 30 °C, and pH 7.0. The extracellular protease synthesized by *T. mentagrophytes* extracted, then partial purified by using Fractionation by Aqueous Two Phase System.

Introduction:

Dermatophytes, consisting of organisms in the *Trichophyton*, *Epidermophyton*, and *Microsporum* genera, these are human and animal pathogenic fungi (1,2,3,4), which are the primary etiologic pathogens of various dermatophytoses, such as tinea capitis, tinea corporis, tinea inguinalis, tinea manus, tinea unguium and tinea pedis (5,6,7). Among approximately 10 human pathogenic species isolated in Europe, *Trichophyton rubrum*, *Trichophyton mentagrophytes* and *Microsprum canis* are most commonly observed, accounting for 71-95% of these species isolated in hospital and private practices (7,8). A characteristic is their ability to grow exclusively in the stratum corneum, nails or hair and to digest components of the cornified cell envelope, and it is evident that secreted proteolytic activity is important for their virulence. All investigated dermatophytes produce proteolytic activity in vitro (9). There are many reports of the isolation and characterization of one or two proteases from an individual species of dermatophyte (10,11,12).

Different studies have shown that these secreted proteinases can not only degrade proteins such as keratin, elastin and collagen to supply nutrients to the fungi, but can also control of host defense mechanisms, and induce delayed-type hypersensitivity (such as with the SUB family and Tri r4), (13,14,15,16). However, although the *T. rubrum* allergen Tri r4 EST shares 98% homology with that in *Trichophyton mentagrophytes*, it is clear that the inflammation mediated by *T. rubrum* is not as severe as that induced by *T. mentagrophytes* . This is probably due, at least in part, to the presence of multiple inflammatory factors in *T. mentagrophytes* (5,16).

The dermaphyte infections are widespread and increasing in prevalence on a global scale. Indeed, in some geographic regions, dermatophyte infection is now considered a major public health concern. Skin and nail infections caused by dermatophyte fungi such as *Trichophyton* have become more common in recent years (17).

For these reasons, we aimed in the present to isolate an extracellular proteinase from a clinical isolate of *T. mentagrophytes*, and we attempt to study the effect of some cultural condition on proteolytic activity of protease.

Materials and Methods

Fungal strain and maintenance of the strain – Clinical isolates of *T. mentagrophytes* were obtained from patients with dermatophytoses attended in Tikrit Hospital. The isolates were grown on Sabouraud agar and were identified on the basis of microscopic examination of the culture (18,19). To obtain spores, the strain were grown on complete solid medium (CSM) at 28°C for 12 days (20).

Protease production media – The cells of *T. mentagrophytes* isolate were grown on Sabouraud broth and protein liquid medium, to promote production of protease. The protein liquid medium prepared by dissolving 0·2 % soy protein in distilled water. No salt was added, and then medium was sterilized by autoclaving at 120 °C for 15 min. A volume of 100 ml liquid medium was inoculated with a plug of freshly growing mycelium in 800 ml tissue-culture flasks. The cultures were incubated for 10 days at 30 °C without shaking (12).

Determination of protease activity - Crude cell-free extracts were prepared as described by Arbesú et al., 1993 (21). The specific activity of protease in crude extracts was measured using 0.02% casein as substrate, and expressed in unit per milligram of protein as described by Jousson et al. (12).

Protein determination – The protein concentrations during extraction and purification stages were estimated according to method used by Lowry et al. (22).

Optimal conditions of protease production - In this work, we report the presence of protease from haploid strain of *T. mentagrophytes*. Activities of protease was measured under several nutritional conditions including, production medium (Sabouraud broth, and Soya protein),

the incubation intervals (3, 5, 7, 9, 11) days, pH of cultural media (5, 5.5, 6, 6.6, 7, 7.5, 8, 8.5, 9), and the incubation temperature (20, 25, 30, 35, 40, 45, 50 °C).

Preparation of PEG solution – Extracts were analyzed by PEG 6000 gel separation, which prepared by adding 8 gm of Dextran T70 gradually to 25 ml heated distilled water (95 °C), then 12.8 gm of PEG 6000 added gradually to obtain homogenous solution, the weight completed to 45 gm by adding distilled water, finally the solution stored at 4 °C (23).

***Partial purification of extracellular protease* (23):**

1.Centrifugation: After propagation of the fungus in production media at 30 °C for 9 days, the mycelium was eparated from the supernatant by centrifugation at 5000g for 15 min.

2. Fractionation by Aqueous Two Phase System: 0.6 gm of PEG 6000 solution and 64 ml of 4 M Nacl were added to 1 ml of the collected supernatant, mixed well then centrifuged at 5000 g for 15 min. The upper phase separated to a test tube and the enzymatic activity was estimated in both phases. The upper phase showed enzymatic activity with no activity found in lower phase, so the enzyme concentration determined in upper phase.

3. Elution of protease: The proteolytic enzyme eluted from upper phase by using different concentrations of ammonium sulfate 14, 20, 30, 40, 50, 60, 70% (w/v) with stirring in magnetic stirrer at 4 °C, and then centrifuged at 5000 g for 15 min, the upper phase separated to a test tube and the enzymatic activity was determined separately in both phases. The phase with proteolysis activity was further concentrated in a dialysis tube against deionized water with continuously stirring at 4 °C, and then the enzyme solution concentrated with sucrose, the enzymatic activity and protein concentration were determined.

Results:

Results in present study showed that *Trichophytone mentographyte*s have ability to produce extracellular protease which indicated by proteolytic activity against casein when added as substrate to the culture filtrates of *T. mentographyte*s. After 9 days of growth, the *T. mentagrophytes* culture supernatants showed proteolytic activity 111.9 U/ml against casein which was 0.050 µg/ml. These results was obtained by monitoring protease production throughout the steps of strain growth.

In this study we investigated the influence of cultural conditions (cultural medium, pH, incubation period, and incubation temperature) on production of proteases by *T. mentographyte*s. The *T. mentographyte*s showed high ability to produce protease 106.4 U/ml in Soya protein media more than Sabouraud media 68.6 U/ml.

Also we studied the effect of different incubation period (3,6,9,12,15 days) on protease production by *T. mentographyte*s, the results showed that high activity of the enzyme obtained when *T. mentographyte*s incubated in Soya protein media for 9 days, reached to 111.9 U/ml, then a progressive decrease in proteolytic activity observed when incubated for 12, and 15 days, as shown in figure (1).

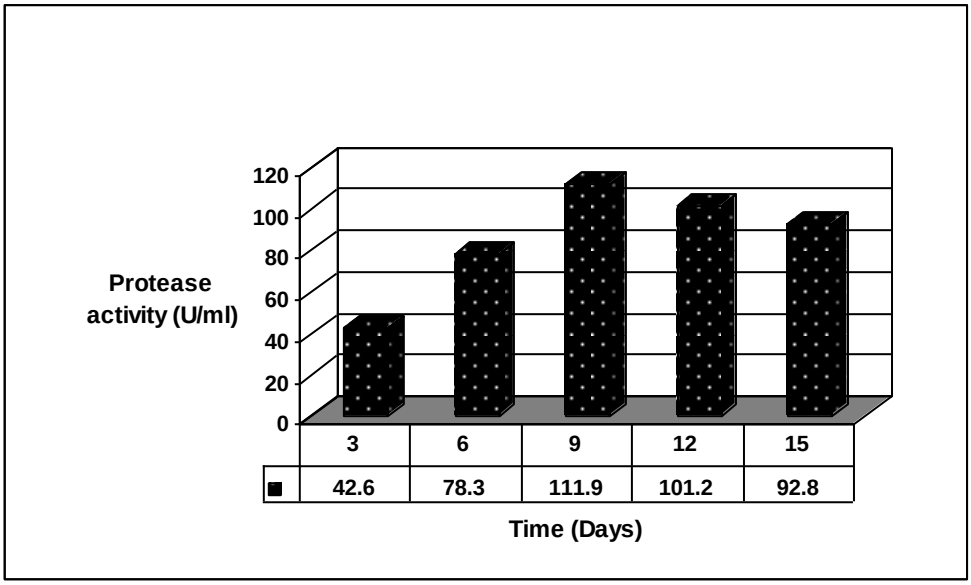

Figure (1): Effect of incubation intervals on protease production by *T. mentographyte*s.

The media pH changes influence production of proteases, results in this study showed that the proteolytic activities of strain *T. mentographyte*s were rapidly increased, reaching a maximum 115.3 U/ml at pH 7, then a progressive decrease in protease production occurred with increasing pH values which was 30 U/ml at pH 9, also protease production reduced at low pH values, which reached to 26 U/ml at pH 5, as shown in figure (2).

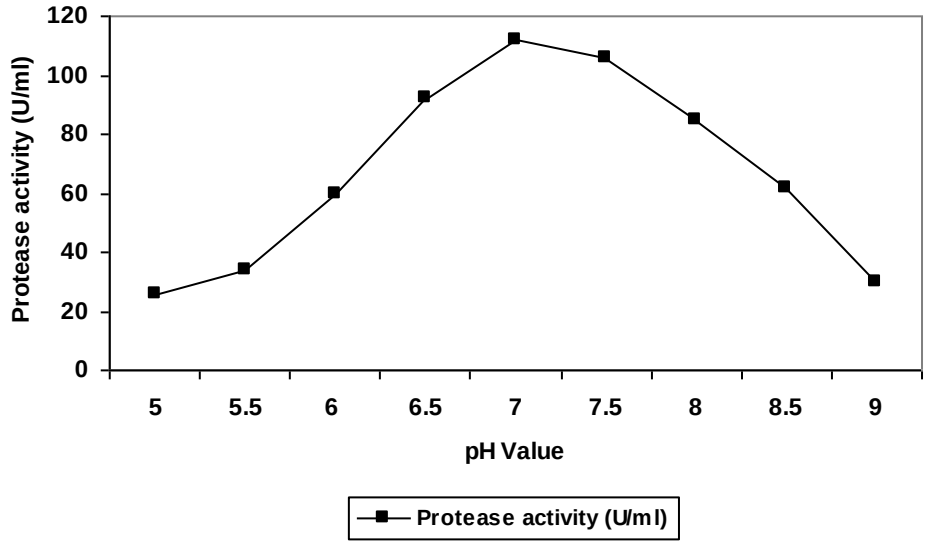

Figure (2): Effect of pH on protease production by *T. mentographytes*.

The highest proteolytic activity 123.5 Unit/ml achieved by strain *T. mentographytes* when incubated in Soya protein media at 30 °C, and then decreased to 31 Unit/ml in 45 °C, while the strain lost its ability to produce proteolytic enzyme when incubated at 50 °C (figure.3).

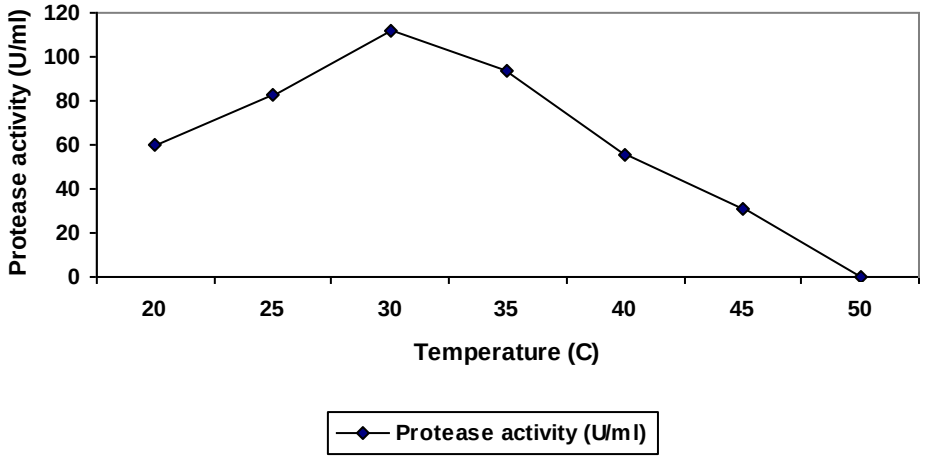

Figure (3): Effect of temperature on protease production by *T. mentographytes*.

The extracellular protease synthesized by *T. mentagrophytes* extracted, and then partial purified using Fractionation by Aqueous Two Phase System.

Table (1) determines the proteolytic activity of the enzyme during purification stages, the crud extract showed proteolytic activity 111.9 U/ml with specific activity 2238 U/mg. In the next stage of purification which we used Aqueous Tow phase System, the PEG phase showed proteolytic activity 66.2 U/ml with specific activity of enzyme 2648 U/mg, at this stage the fold of purification was 1.18 with elution 94.6% of the enzyme. The highest specific activity of the proteolytic enzyme 3600 U/mg observed when we used ammonium sulfide 50% to elute the enzyme from PEG phase, also the volume of enzyme solution reduced at this stage. After dialysis of the upper phase of ammonium sulfide, the enzyme solution concentrated to 25 ml and determined the specific activity of the enzyme which was 3600 U/mg and the purification fold was 1.35 with elution 33.9%.

Table (1): The protease activity and protein concentration during Purification Steps.

Purification Steps	Volume (ml)	Protease activity (U/ml)	Total Protein (mg/ml)	Specific Activity (U/mg)	Total Activity (U)	Purification Fold	Recovery (%)
Crude filtrate	**50**	**111.9**	**0.050**	**2238**	**5595**	**1**	**100**
Two phase System	**80**	**66.2**	**0.025**	**2648**	**5296**	**1.18**	**94.6**
Ammonium Sulfate	**25**	**72**	**0.02**	**3600**	**1800**	**1.35**	**33.9**

Discussion:

Dermatophyte infections are one of the earliest known fungal infections of mankind and are very common throughout the world. *Trichophyton* species play a major role in dermatophyte, is now considered a major public health problem (8,24,25). In the present study we attempt to isolate extracellular protease produced by clinical isolate of *T. mentagrophytes*, which indicated by proteolytic activity against casein. The caseinolytic activity of the *T. mentagrophytes* culture filtrates in present study is mainly due to the presence of protease that is capable of hydrolyzing casein. Several researchers found that the proteolytic activity in skin and nail infections caused by dermatophyte fungi such as *Trichophyton*, is due to the action of protease, in addition to having caseinolytic being indicated as one of the factors responsible for the virulence of the fungus, also they found that production of elastase has been associated with inflammatory dermatophytosis (1,24,26,27,28). The secreted proteinases of dermatophytes play an important role in the process of infection, and represent the pathogenic feature that differentiates dermatophytes from other fungi (29).

There are many reports of the isolation and characterization of one or two proteases from an individual species of dermatophyte (4,30). In *T. rubrum*, some keratinases have been isolated and a subtilisin gene family was identified at the genetic level (31). Jousson et al., isolated a five-member secreted metalloproteases family from *T. rubrum*, *T. mentagrophytes* and *M. canis* (12). The varieties of *T. mentagrophytes* produce small amounts of protease activity in skin from hosts to which they are well adapted but produce large amounts on material from an unfamiliar potential host species (18).

In this study, we also studied the influences of the cultural conditions on the production of protease from *T. mentagrophytes*. According to the effect of cultural medium on protease production, the *T. mentagrophytes* showed high ability to produce protease in Soya protein media more than Sabouraud media. Among several tested protein sources, soy proteins were found to be the best for growing and promoting proteolytic activity of dermatophyte species in liquid medium. Where no to poor growth was obtained in liquid medium containing 0·2 % keratin as the sole nitrogen source (12, 16). In culture medium containing soy proteins as a sole nitrogen source, *T. rubrum*, *T. mentagrophytes* and *M. canis* secreted two major metalloproteases accounted for 19–36 % of total secreted protein extracts (12). Day et al., (32) and Yu et al., (33) purified two extracellular and two cell-bound keratinases from the same isolate of *T. mentagrophytes*. They used a growth medium that contained defatted hair as a nitrogen source.

pH changes influence production of proteases. (31). The results in this study showed that the optimum pH to produce protease by *T. mentagrophytes* was 7. These results agreed with other studies, which indicate that the extracellular proteinases of *T. mentagrophytes and T. rubrum* also have optimal proteolytic activity at neutral pH (10,34). Where in other studies with *T. rubrum*, proteinase with a pH optimum of 4.5 was detected (18,35). *T. rubrum* has recently been extensively studied by Apodaca et al., (36), and shown to produce two strongly keratinolytic proteinases, as well as a poorly keratinolytic, trypsin- or chymotrypsin-like general proteinase. These proteinases all have a pH optimum of approximately 8. In a study of protease production during autolysis in different species of filamentous fungi, observed that autolysis occurred at pH values between 6.5 and 8 (37).

Human skin has a weakly acidic pH, and it is noteworthy that proteinases with an optimal activity under acidic conditions are reported to be important virulence factors in *T. mentagrophytes* (38,39). Dermatophyte proteolysis results in the liberation of excess ammonium ion, raising the pH of the growth medium (31,40). This reaction, an attribute relatively uncommon in fungi isolated clinically, has been used as the basis of screening media such as Dermatophyte Test Medium (41).

The optimum temperature for protease production by *T. mentagrophytes* in present study was at 30 °C which reached to 123.5 U/ml then reduced with temperature increasing. These results agreed with other studies which deals that most enzyme denaturated and lost its activity when temperature exceeds 35 °C (42), also high temperatures have effect on fungus growth and protease production, the optimum temperature for proteases production by *Aspergillus* spp. varies between 28-30 °C, also these studies indicated the fungus ability to protease production decreases when incubation temperature was

less or high than 30 °C (43,44). Recent investigations have shown that proteases secreted by dermatophytes are similar to those of other fungi such as *Aspergillus* spp. (4,11,16).

Results in present study showed that *T. mentographyte*s have high ability to protease when incubated in protein liquid media for 9 days, then a progressive decrease in protease production observed when incubated for 12, and 15 days. Protease production by strain 22 of *M. anisopliae* increases reaching a peak at 9 days, already during the autolysis phase, and then decreasing slowly, where the proteolytic activities of strain CLII of *M. anisopliae* were rapidly increased, reaching a maximum at about 5 days. A progressive decrease in proteolytic activity occurred thereafter (31). Studies on other species of fungi have shown that in most cases proteolytic activity increases with the beginning of autolysis (41,45,46,47). Apodaca and McKerrow (13,36), and Paveia, have recently shown that during log-phase growth most of the proteolytic enzymes of *T. rubrum* are repressible in vitro by small molecules and are likely repressed during early growth in vivo. The entire complement of proteinases in *T. rubrum* tends to be produced constitutively during the stationary phase of growth in vitro.

The Two Phase System used in this study to remove proteins, cell sediments, and interference substances from *T. mentagrophytes* culture filtrate (27). Many studies used ammonium sulfate for purification of protease, Ingham et al. (24), and Schleif (48), used ammonium sulfate (38-52%) as first step of protease purification. 1 M ammonium sulfate used to purification of keratinase produced by D. microsporus and *P. marquandii* were cultivated in a submerged fermentation (49). The crude enzyme was first saturated up to 30% with solid (NH4)2SO4 and then centrifuged at 5,000 × g for 15 min. The supernatant obtained was further saturated up to 70% with solid (NH4)2SO4 and again centrifuged. The pellets obtained were dissolved in minimum volume of 0.1 M phosphate buffer, pH 6.0 (buffer A), and dialyzed against the same buffer extensively and then concentrated through Amicon Diaflo Ultra-filtration cell using YM-10 membrane (50).

Recommendation: We recommend further studies on purification of protease produced by *T. mentagrophytes*, and characterization of the enzyme.

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عزل انزيم البروتيز من الشعراويات الجوزية من عزلات الجلد

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(تاريخ الاستلام: ٢٨ / ١ /٢٠٠٨ ، تاريخ القبول: ٢٩ / ٥ / ٢٠٠٨)

الملخص:

يعد جنس الشعراويات من الفطريات الجلدية الذي يصيب الملايين من البشر في العالم. ويضم جنس الشعراويات الذي يصيب الإنسان ثلاثة أنواع من الفطريات التي تصيب الجلد، الشعر ،ألاظافر، وهي *Trichophyton* و *Epidermophyton* و *Microsporum* .في هذه الدراسة تم عزل الشعراويات الجوزية ***Trichophyton mentagrophytes*** من المرضى المصابين بالفطريات الجلدية ونميت العزلة على وسط الزرعي سابروبد وفحصت بالمجهر لغرض التأكد من الصفات المظهرية للفطر.

تم زرع العزلة ***Trichophyton mentagrophytes*** في وسط الزرعي سابروبد وفي وسط الزرعي السائل للكشف عن قابلية العزلة في إنتاج الأنزيم بروتيز، وأظهرت نتائج الدراسة أن للعزلة القدرة على إنتاج الأنزيم وذالك بواسطة حل بروتين الكازائين في الوسط الزرعي السائل، إذ بلغت فعالية الأنزيم في الراشح المزرعي للعزلة ***T. mentagrophytes*** ضد المادة الأساسية الكازائين ١١١،٩ وحدة /مل أما الفعالية النوعية للأنزيم فقد بلغت ٢٢٣٨ وحدة/ملغم.

أيضا تمت متابعة إنتاج الأنزيم بروتيز من العزلة ***T. mentagrophytes*** تحت تأثير بعض الظروف المزرعية (نوع الوسط الزرعي، فترة الحضن، درجة الحرارة، والرقم الهيدروجيني) ومدى تأثير التغيرات بهذه العوامل على أنتاج الأنزيم. فقد أظهرت النتائج أن العزلة لها القدرة على إنتاج الأنزيم بعد مرور ٩ أيام من الحضن في الوسط الزرعي السائل وعلى درجة حرارة الحضن ٣٠ مْ وبرقم الهيدروجيني ٧،٥. ومن ثم تم استخلاص الأنزيم بروتيز من الفطر وتنقيته جزئيا باستخدام نظام التجزئة المائي ثنائي الطور.