In vitro, intracellular proteinase and keratinase activity of Pathogenic Dermatophytes and yeasts

*Dr. Adnan H.Aubaid AL-Hamadani

Summary

Intracellular ensymes including proteinase and karateinase produced by 80 isolates belong to 14 species of skin pathogenic fungi (dermatophytes and yeasts) were tested semi_quantiatively by photometric assay. The optimal pH and temperatare ranges of the ensyme activities were determined. The results showed that all of the isolates have the ability to produce the tested enzymes, and the amount ofenzymes wasvaried. The protein content (endocellular protein) of each fungal species was alos determined.

الخلاصة

اختبرت الفعالية الانزيمية داخل خلوية لانزيمي البروتنيز و الكيراتينيز بصورة شبة كمية لـ (80) عزلة تعود لاربعة عشر نوعا من الفطريات الجلدية و الخمائر المرضية باستخدام الطريقة الفوتومترية وحددت ايضا المديات المثلى للدالة الحامضية ودرجة الحرارة للفعلية الانزيمية و اظهرت النتائج ان كل العزلات الفطرية المختبرية لها القابلية على افراز الانزيمين من الاختبار و ان كمية الانزيمين المنتجة كانت متباينة حسب نوع الفطر و حدد ايضا محتوى البروتين داخل خلوى لكل العزلات الفطرية المختبرة .....

Introduction

Dermatophytest and yeast species are a group of ubiquitous pathogenic fungi, which cause a vareity of mycotic disease of thes kin, hair and nails of human and animals' [1]. Recently, more attention has been given to the enzyme production by pathogenic fungi due to their role in human pathogencity [2, 3, 4, 5]. Despite of different techniques have been used for enzymic assay, the use of culture filtrate and cell-free extract, are mostly applied by several workers [6, 7, 8, 9]. However, exoenzyme found to be produced by dermatophytes and yeast were keratinase, protease, and lipase [16, 19]. This study was attempted to determine the activity of intracellular proteinase and keratinase by selected skin pathogens fungi using photometric assay.

Materials and Methods

A.Biolgical views

Nine species of dermatophytes and five species of yeasts were isolated and identified from dermatomycoses patients who attended Dermatology and venereal diseases centre. Teaching of Qadisia Hospital in AL-Qadisia province between October 2001 and September 2002 (Table 1). For each enzymic test , an inoculum of 5mm disc of fungal stock cultures was transferred into pepton broth media [10] and incubated at 29 °C for dermatphytes and 37°C for yeasts .After 14 days of incubation , the contents of the flasks were cooled at 4°C and filtered throughout sintered glass bed filter (pore size 5 Mm) . The mycelia were washed throughly with0.15m NaCl

*Pathology Dept.,College of Medicine,AL-Qadisia University,IRAQ

solution to remove adhering medium. Washed mycelial were taken in a glass mortor containing 5g sea sand along with 1ml Tris-HCL buffer (10MM,pH7.2) and ground manually for 15 min to break the cells.The resulting slurry was diluted with 9ml buffer solution and ground further for 5min the homogenate was centrifuged at 3000g for 10 min. The clear supernatant was removed carefully from the tubes and stored at 4 °C for 10 min before enzyme assay [11]. All the above steps were carried out at 0-4°C, and the reagents used were perchilled to 0°C .Protein concentration (endocellular protein) was detrmined according to the method [12]. Calibration curve was made using bovine serum albumin (Fluka; switrezland). As a standard to determine the protein amount in terms of mg per ml. Different pH (4-10) were tested to determine the optimal enzyme activity by using 0.028 m Tris –HCl buffer for the neutral and basic pH values . The effect of different temperature (15-60) °C on the enzymic activities was also examined under optimal pH condition.

B.Enzymic assays

Proteinase activity against (Sigma; vitamine free from bovine milk) substrate was measured according to method [13]. The reaction mixture contained 0.25 ml substrate (10g/L in 0.05 Mphosphate buffer; pH 7.8); 0.05 ml of 0.3M phosphate buffer, pH7.8; and 0.05 ml of enzyme source (cell-free extract). The reaction was carried out at 40°cfor 30 min, and then stopped by addition of 2.5 ml of 0.34M. Trichloroacetic acid (TCA). The tubes were allowed to stand for 1hr. and the precipitates were removed by centrifugation for 15 min .Abosorbance of the supernatam was measured against a reaction blank at 280 nm. The reaction blank was prepared as above except that TCA was added before the addition of the enzme source . One unit of enzyme activity was defined as the amount of enzyme that could liberate products having an absorbance of 0.1 under these conditions. The specific activity was expressed as the amount of units of activity per mg protein.

Keratinase activity was meathod against guinea pig hair as a substrate according to the method (14)as following ; 0.6 ml enzyme source (cell-free extract);50 mg guinea pig hair (Washed with distilled water, ethanol and byaceton);5.4 ml. 0.028 M phosphate (pH7.2)were mixed and incubated at 37°C with gentle shaking for 2hr . The control samples were made in the same way except that enzyme source had been kept at 100°C for 10 min. The incubated solutions were hammersed in an ice water bath for 10 min to stop the reaction. After that, the hair was removed by filtration with filtration with filter paper (man what No.1), and the absorbance of filtrates were measured spectrophotometrically at 280 nm.The keratinase activity was expressed as one kerationase unit corresponded to an increase in the absorbance value by 0.1. The specific activity was expressed as anumber of units per mg protein.

Results

All the tested isolates of dermatophytes and yeasts showed proteolytic activity against casein (Table2).Highest proteinase activity was rendered by *Trichphyton mentagrophytes* var *.mentagrophytes* (6.70units/ml) followed by

T.verrucosum (6.11units/ml).A minimum production (1.25units/ml) of this enzyme was by yeast Malassezna fur .Among dermatophytes species,*T. mentagrophytes* var .mentagrophytes showed the highest specific activity of proteinase (57.77 units/mg) protein while *Geotrichum candidum* revealedthe highest enzyme specific activity (25.17units/mg)protein among the yeasts(Table2). The higest activity of keratinase was produced by the fungus *Microsprum canis* followedby the *Trichophyton mentagrophytes* var *interdigetile* and the lowest amount produced by the fungus *Malassezia furfur*.Also *T.metagrophytes* gave high specific activity (48.15units/mg protain) followed by Microsprum canis (36.10units/mg protin)while the lowest specific activity was produced by Malassezia furfur (7.55units/mg protein).while *G. candidum* showed lowest specific activity of keratinase(Table2). The results indicated that the protien contents (mg/ml) were varied among the dermatophytes and yeast isolates (Table2). The optimal enzymic activity of the examined fungal species was differed under different pH and temperature (Table3).Keratinase was highly produced at PH7and 34 °C , while the proteinase was highly produces in pH7.8 and 40°C.

Discussion

The application of photometric assay to detect enzyme production clearly demonstrated that pathogenic fungi having the ability to produce the intracellular.enzymes. The photome trichniqe was more precise and give a relaible semiquantitatively values of detected enzymes between different fungal isolates as previously stated [15]. However, the amount of enzymic production by fungi is dependent upon the source of isolates and the virulence factor [16]. It appeared that the proteinase and keratinase activities of different pathogenic fungi were varied. This is mainly due to the type of substrate incorported to the media. Among the examined enzymes, proteinase was frequently produced by different dermatophytes and yeast agents. This may reflect the necessity of pathogenic fungifor this enzyme for penetration the skin or utilize protein for their nutrition[17] the high activity of keratinase of *M.canis*, *T. mentagrophytes* var. *mentagrophytes* and *T . verrucosum* in comparison with the related species can be attributed to their ability to invade keratinized hair and skin. This is in concomitant with some investigations[18,19]. On contrary , this enzyme showed low activity among the yeast isolates. The optimal pH and temperature values were differed according to the enzymic reaction [20,11]. Thus , any changes in these factors would affeected the enzyme stabillity and limit or inhibit its production.

Table(1) List of dermatophytes and yeasts isolated and identified from patients Of dermatomyses.

Fingal isolates	source	Code No.	No. of isolates
Epidermophyton floccosum (Harz) Langeron&Miochev	Skin	EF22	6
Microsporum canis (Bodin) Bodin	Hair	MC1111	8
M. gypseum (Bodin) Guiort & Grig	Hair	MG792	8
Trichophyton mentagrophytes var. erinacei (English & Stock) Smith& Marples	Skin	TME220	6
T. mentagrophytes var . interdigiale Mochizuk	Skin	TMI128	5
T. mentagrophytes var mentagrophytes (Robin) Blanch	Skin	TMM186	10
T.rubrum (Castell) Sab .	Skin	TR345	8
T.verrucosum Bodin	Skin	TVE577	5
T.violeceum Bodin	Hair	TV1961	5
Candida albicans (Robin) Berk.	Nail	CA1066	8
C. parapilosis (Ashford) Lengeron&	Nail	CB156	5
C.tropicalis (Castell)Berk.	Nail	CT2	5
Geotrichum candidum Robin	Skin	GC628	3
Malassezia fur fur(Robin) Baillon	Skin	MV15	8

Table(2) Enzymic and specific activity of intracellular proteinase and keratinase and intrcellular protein contents produced by selected dermatophytes and yeasts.

Species	proteinase		keratinase		Intracellular Protein
	*enzyme activity	**specific activity	enzyme activity	specific activity	(ug/ml)
E.floccosum	3.17	19.24	3.09	22.61	0.206
M.canis	3.99	23.46	5.62	36.10	0.204
M.gypsum	4.15	29.77	2.33	23.77	0.168
T.mentagrophytes var. Erinacei	4.17	27.40	4.12	31.62	0.182
T.mentagrophytes var. interdigitale	3.72	22.13	4.88	31.72	0.204
T.mentagrophytes var. mentagrophytes	6.70	57.77	4.57	48.15	0.131
T.rubrum	4.93	27.23	1.25	13.47	0.210
T.verrucosum	6.11	26.13	3.98	20.82	0.262
T.violaceum	3.19	19.53	4.61	30.40	0.204
C.albicans	4.47	22.27	3.30	19.82	0.245
C.parapsilosis	2.55	21.18	2.35	25.18	0.160
C.tropicalis	2.45	18.49	2.14	21.38	0.177
G.candidum	2.22	25.17	0.16	8.43	0.233
M.fur fur	1.25	19.08	0.07	7.55	0.220

(*)Units/ml; (**)Units/mg protein.

Table (3) Optimal pH and temperature values of the enzymic activities of intracellular proteinase and keratinase of dermatephytes and yeasts.

Enzymes	PH	Temperature °C	*Enzyme activity(units/ml) x±SD
Protinase	7.8	40	3.98±1.2
Keratinase	7.0	34	3.12±_1.0

*x±SD=represent the means and standard deuiation of activity of 80 isolates of dermatophytes and yeasts.

References

1- Ajello.L.(1968).Ataxonomic review of dermatophytes and yeast species . Saboraudia 6,147-159.

2- Apodoca, G.and Mckerrow.J.H.F (1990).Expression of proteolytic activity by cultres of Trichophyton rubrum . J.Med. Vet . Mycol.28,159-71.

3- Brasch, J; Martins ,B.S. and christophers, E.(1991). Enzyme release by Trichophyton rubrum depends on nutritional conditions. Mycoses 34,356-8.
4- Brasch,J.and Zaldua,M.(1994).Enzyme patterns of dermatophytes.Mycoses37.11-6.
5- Ghannoum,M.andAbu-Elteen,K.(1986).Correlative relationship between proteinase production . adheronce and pathogenic of various strains of candida albicans. J.Med.Vet. Mycol.24.407-413.
6- Chattaway,F.;Elliis,D.A.and Barlow,A.J.E(1963).peptidases of dermatophtyes.J.Invest.Dermatol. 41,31-37.
7- Danew,P.;Friedrch,E.and Mannsfeld,T,T.H.J(1971).Studies on the peptidase activity of fungi pathogenic to skin. Dermatologisches Monatsschrift. 157,232-238.
8- Davies .R.R.and Zaiani. F.(1984).Enzymic activities of Trichophyton rubrum and the chemotaxis of polymorphonuclear leucocytes. Saboraudia 22,235-241.
9- Yu,R.J.;Harmon,S.R;Wachter ,P.E.and Blank ,F.(1969).Hair digestion by a keratinase of Trichophyton mentagrophytes .J.I nvest.Dermatol. 53,166-171.
10- Mushin. I.M.; Aubaid. A.HandAL-Dobon. A.H. (1998).Extracellularenzyme of dermatophytes and yeast isolates in solid media. Mycoses 45, 1-5.
11- Sanal. A.K.; DAs, S.K.and Banerjee. A.B. (1985).Purification and partial characterization of an exocellular proteinase from Trichophyton rubrum.Sabouraudia 23,165-178.
12- Lowry. O.H.; Rasebrough. N.J; Farr. A.L.and Randall. R.J. (1951).protein measure-ment with Folin phenol reagent. Journal of biological chemistry 193,265-267.
13- Hanlon,G.Wand Hogeds. N.A.(1981). Reqirement for glucose during production of extracellular serine protease by cultures of Bacillus Licheniformis. FEMS Microbiology leters 11,51-54.
14- Takiuchi. I.and Higuchi. D.(1977) . Isolation , purification and biochemical properties of keratinaseelaborated from Microsprum gypseum the Japanese journal of Dermatology 87,305-309.
15- Hankin. L.and AnagnostKIS. s.l (1975). The use of sold media for detection of enzyme production by fungi . mycologia67,597-607.
16- Kunert,J(1972).The digestion of human hair by the dermatophyte microsporum gypseum in a subemerged culture. Mykosen 15,59-71.
17- North ,M.(1982).Comparative biochemistry of the proteinase ofeuocaryotic microoganisms , microbiol .Rev. 46,308-340.
18- Yu,R.J.;Harmon,S.R.;Grapel,S.F.and Blank,F.(1971).Two cell-bound kerati-nase of trichophyton mentagrophytes.J.Invest.Dermatol.56,27-32.
19- Qin.L.M.;. Dekio. S.and Jidio ,J.(1992).Some biochemical characteristics of a partially purified extracellular keratinase from trichophyton schoenlenii. Zbl.Bakt.277,236-244.
20- Tsubboi,R.I.;Ko,J.;Matsoda,K.and Ogawa ,H .(1978)Anew keratinolytic proteinase from clinical isolates of trichophyton mentagrophytes.J.Dermatol .Rev.(Tokyo)14,506-503.