Mycopathologia (2006) 161: 369–375
DOI 10.1007/s11046-006-0019-8

Partial purification and characterization of a 37 kDa extracellular proteinase from *Trichophyton vanbreuseghemii*

Hossein Moallaei[1], Farideh Zaini[1], Gérald Larcher[2], Bertrand Beucher[2] & Jean-Philippe Bouchara[2]

[1]*Department of Medical Mycology and Parasitology, School of Public Health, Tehran University of Medical Sciences, Tehran 14155, Iran;* [2]*Groupe d'Etude des Interactions Hôte-Parasite, UPRES-EA 3142, Laboratoire de Parasitologie-Mycologie, Centre Hospitalier Universitaire, 4, rue Larrey, 49033 Angers, France*

Received 22 February 2006; accepted in revised form 28 February 2006

Abstract

An exocellular proteinase synthesized by the geophilic dermatophyte *Trichophyton vanbreuseghemii* has been purified and characterized. The fungus obtained from soil in Iran was cultivated in modified Czapek–Dox liquid medium containing 0.1% bacteriological peptone and 1% glucose as the nitrogen and carbon sources. Partial purification of the proteinase was accomplished by $(NH_4)_2SO_4$ precipitation, followed by ion exchange chromatography. Analysis of the enzyme by SDS-PAGE revealed a single polypeptide chain with an apparent molecular mass of 37 kDa. Proteinase activity was optimum at pH 8, but remained high in the range of pH 7–11. Moreover, the partially purified enzyme presented a keratinolytic activity as evidenced by the keratin azure test. The inhibition profile and the good activity of the enzyme towards the synthetic substrate *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide suggested that it belonged to the chymotrypsin/subtilisin group of serine proteinases. The keratinolytic properties of *T. vanbreuseghemii* suggest that this fungus may be an alternative for the recycling of industrial keratinic wastes.

Key words: enzyme purification, keratinolytic, serine proteinase, *Trichophyton vanbreuseghemii*

Introduction

Keratin is an insoluble macromolecule requiring the secretion of extracellular enzymes for its degradation. This protein comprises long polypeptide chains, which are resistant to numerous proteases. Adjacent chains are linked by disulphide bonds which are thought to be responsible for its stability and resistance against degradation [1]. However, the food and feed industry generates large quantities of keratinic wastes that constitute a growing problem. At present, their recycling consists in a alkaline hydrolysis at high temperature. Therefore, the development of new methods for the bioconversion of these materials has raised much scientific interest and the processing by keratinolytic microorganisms may be a valuable alternative. The keratinophilic fungi are the major group of organisms capable to use keratin as the sole source of carbon and nitrogen. Secretion of keratinolytic enzymes has been demonstrated in some pathogenic fungi with a high affinity for keratin which can cause infections by invading skin or scalp of mammals [2]. However, such enzymes are not exclusively associated with pathogenic fungi since they have also been found in some geophilic species [3, 4]. Indeed, it has been reported that among ten keratinophilic fungal species, only three were

able to perforate and degrade hair rapidly: *Chrysosporium keratinophilum*, *Microsporum gypseum* and *T. vanbreuseghemii* [4, 5]. Keratinases have been characterized for the first two species [6–8], but nothing is known for the third one, probably in relation with the lack of pathogenicity of this fungus and with its paucity in the environment. The recent isolation by one of us of one strain of *T. vanbreuseghemii* from soil in Iran led us to investigate the proteolytic equipment of this fungus. Here, we report the partial purification and characterization of an extracellular proteinase from *T. vanbreuseghemii* which supports a keratinolytic activity.

Materials and methods

Microorganism and culture conditions

This study was carried out using one isolate of *T. vanbreuseghemii* Ir-84 obtained from soil in Iran. The fungus was propagated on yeast extract/peptone/dextrose (YPD) agar plates at 25 °C, and inoculum was prepared from 7-day-old cultures by flooding with approx. 10 ml of sterile distilled water and scraping off the agar plates.

Enzyme production

Trichophyton vanbreuseghemii Ir-84 was grown in modified Czapek–Dox liquid medium (MCLM) containing glucose, 10 g; K_2HPO_4, 1 g; $MgSO_4$, 0.5 g; KCl, 0.5 g; $FeSO_4{\cdot}7H_2O$, 0.01 g; chloramphenicol, 10 g; and bacteriological peptone 0.1% (w/v) as the nitrogen source. For kinetic study of enzyme production, triplicate cultures of *T. vanbreuseghemii* Ir-84 were carried out in 100-ml sterile flasks containing each 50 ml of MCLM which were incubated at 25 °C for up to 3 weeks. After incubation, they were filtered through 0.45 μm-pore-size pre-tared membranes (Millipore) which were then lyophilized for determination of the mycelial dry weight, and proteolytic activity was assayed in the culture filtrates using the chromogenic substrate *N*-Suc-Ala-Ala-Pro-Phe-*p*NA (Sigma) [9]. Specific activities which correspond to the enzyme activity in nkat per mg of mycelial dry weight, were determined. One katal is being defined as the amount of enzyme which releases one mole of *p*NA in one second.

Enzyme purification

Flasks (2 l) containing 1 l of MCLM were inoculated with the fungal suspension, and incubated for 15 days at 25 °C. Cultures were then filtered successively through filter paper no. 3 (Whatman) and 0.2 μm-pore-size filters (Millipore). To limit enzyme autolysis, all procedures were carried out at 4 °C. Enzyme purification was realized using a two-step procedure consisting in ammonium sulphate precipitation followed by ion-exchange chromatography. To do this, solid $(NH_4)_2SO_4$ was added to the culture supernatant to 80% saturation. After 1 h of stirring at 4 °C, the suspension was centrifuged at 4 °C for 30 min at $20{,}000 \times g$. The pellet was then resuspended in minimum volume of distilled water and dialyzed against three changes of 20 mM Tris–HCl buffer, pH 8 (buffer A). Insoluble material was removed by centrifugation at $10{,}000 \times g$ for 10 min, then the dialysate was loaded onto a column (2×20 cm) containing 50 ml of DEAE–Trisacryl M gel (Biosepra) equilibrated with buffer A. After washing the column with five volumes of buffer A, 100 ml of 0.1 M NaCl in buffer A was applied to the column at a flow rate of 60 ml/h, followed by a linear gradient of NaCl (0.1–0.3 M NaCl, 300 ml) in the same buffer. Eluate was collected by 3-ml fractions. Fractions with enzyme activity were pooled, dialyzed against 20 mM Tris–HCl buffer, pH 8 and stored as aliquots at −20 °C.

The last step of purification was monitored for protein by measuring the absorbance at 280 nm. The proteolytic activity of the different chromatographic fractions was assayed using *N*-Suc-Ala-Ala-Pro-Phe-*p*NA, and their protein concentration was determined as described by Bradford [10], using BSA as standard.

Enzyme activity assay

Substrates were dissolved in dimethyl sulfoxide (DMSO) at a stock concentration of 5 mM. Unless otherwise stated, the assay was performed on polystyrene microtiter plates and the reaction mixture contained, per well, 180 μl of a suitably diluted proteinase solution in buffer A and 20 μl of chromogenic substrate (0.5 mM final concentration). After 30 min of incubation at 37 °C, the amount of *p*-nitroaniline (*p*NA) released was

measured at 405 nm using a Titertek Multiscan spectrophotometer (Labsystem). Three different chromogenic substrates were used: *N*-Suc-Ala-Ala-Pro-Phe-*p*NA, *N*-Suc-Ala-Ala-Pro-Leu-*p*NA and *N*-Bz-Phe-Val-Arg-*p*NA (Sigma) (where Suc is succinyl and Bz, benzoyl) titrating the chymotrypsin/subtilisin, elastase and trypsin activities respectively. Enzyme activity was expressed in nkat/ml. Keratinolytic activity was measured using keratin azure (Sigma). Samples (1 ml) were incubated with keratin azure (5 mg) at 37 °C for 8 h in buffer A. Keratinolytic activity was determined by measuring the absorbance at 595 nm, the enzyme unit being defined as the amount of enzyme producing an increase in A_{595} of 0.001 unit per hour.

Electrophoretic analysis

Protein purity and the molecular mass of the enzyme were evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) using the discontinuous buffer system of Laemmli [11]. Samples (20 μg of protein per lane) were analyzed on 1.5-mm-thick slab gels [12% (w/v) polyacrylamide resolving gel; 3% (w/v) polyacrylamide stacking gel]. Gels were stained with Coomassie brilliant blue R-250 and the electrophoretic migration of the proteinase was compared with that of low-molecular-mass protein markers (Pharmacia).

Inhibition studies

Proteinase inhibitors were tested for activity against proteinase with the optimized protocol (i.e. pH 8.0). Aliquots of the proteinase solutions (160 μl at 3.3 μg/ml) in buffer A) were preincubated for 10 min at 37 °C with 20 μl of 10-fold concentrated stock solutions of each reagent. Then, 20 μl samples of chromogenic substrate, *N*-Suc-Ala-Ala-Pro-Phe-*p*NA (5 mM) were added, and proteinase activity was assayed as described above. Phenyl methyl sulfonyl fluoride (PMSF), 7-amino-1-chloro-3-L-tosylamidoheptan-2-one (Tos-Lys-CH_2Cl, TLCK) and 1-chloro-4-phenyl-3-L-tosylamidobutan-2-one (Tos-Phe-CH_2Cl, TPCK) were prepared as stock solutions in methanol, and chymostatin was prepared in DMSO. Stock solutions of the other potential protease inhibitors were prepared in distilled water. The effects of several ions at 10 mM final concentrations on proteinase activity were investigated as described above. Residual activities were calculated from triplicate determinations as percentages of the activities in control samples without reagent. Appropriate solvent controls were run in parallel when required.

Optimum pH

The influence of pH on proteinase activity was determined by using the standard proteinase assay with *N*-Suc-Ala-Ala-Pro-Phe-*p*NA chromogenic substrate. Determination of the optimum pH was performed at 37 °C with the following buffer systems: 0.2 M Tris/HCl buffer (pH 7–9) and 0.2 M carbonate buffer (pH 10–11). Activities were estimated as percentages of the maximum.

Results

Study of the kinetics of enzyme synthesis demonstrated that production of the enzyme reached a maximum at day 15, and then remained stable until day 21 (Figure 1). Thus, the fungus was cultivated in MCLM for two weeks for all subsequent experiments. Purification of the protease was then undertaken and Figure 2 shows the elution profile obtained after DEAE chromatography. Proteins bound to the column were eluted using a step of 0.1 M NaCl which provided two major protein

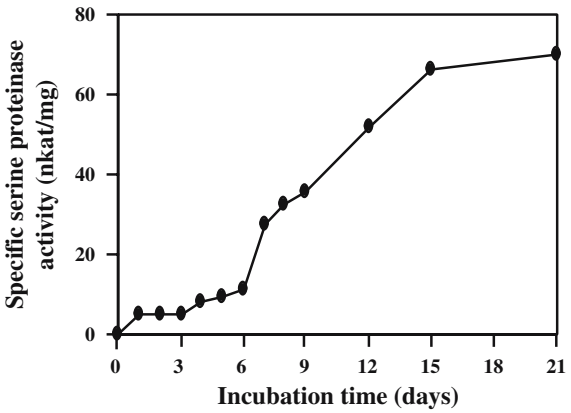

Figure 1. Time course of serine proteinase production of *T. vanbreuseghemii*. Proteolytic activity was determined by measuring the rate of hydrolysis of the chromogenic substrate *N*-Suc-Ala-Ala-Pro-Phe-*p*NA. Specific activity corresponds to activity in nkat per mg of mycelial dry weight.

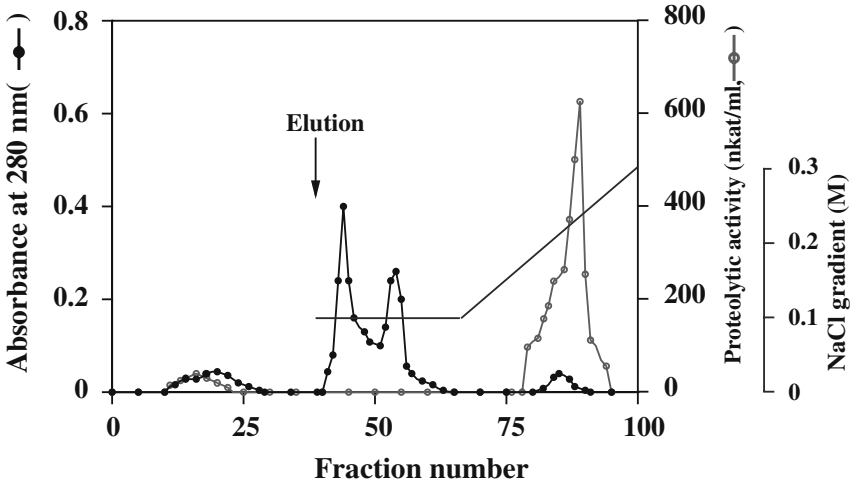

Figure 2. Purification by DEAE chromatography of *T. vanbreuseghemii* serine proteinase. Purification was monitored for protein by measuring the absorbance at 280 nm, and for enzyme activity by measuring the rate of hydrolysis of the chromogenic substrate *N*-Suc-Ala-Ala-Pro-Phe- *p*NA.

peaks, followed by a linear gradient of NaCl (0.1–0.3 M) leading to a very weak protein peak (Figure 2). A weak proteinase activity was detected in the protein peak corresponding to the exclusion step. Enzyme detection in the eluate revealed a second peak with high proteinase activity corresponding to 0.24 M NaCl which superimposed with the last protein peak. The purification steps are summarized in Table 1 with an overall recovery of 25.8% and 3.6-fold purification. Analysis by SDS-PAGE of the enzymatic peak disclosed a major protein band with an apparent molecular mass of 37 kDa and several minor bands (Figure 3, lane 3). Only weak activities were detected on *N*-Bz-Phe-Val-Arg-*p*NA (119 nkat/ml) and *N*-Suc-Ala-Ala-Pro-Leu-*p*NA (92 nkat/ml) which are specific substrates for trypsin and elastase-like serine proteinases, respectively. In contrast, the partially purified enzyme proved to be efficient towards the synthetic substrate *N*-Suc-Ala-Ala-Pro-Phe-*p*NA (533 nkat/ml), suggesting that it belongs to the chymotrypsin/subtilisin group of serine proteases. In addition, an activity of 126 U/ml was observed using the keratin azure test, thus confirming the keratinolytic activity of the enzyme.

The effects of different reagents were tested on the activity of *T. vanbreuseghemii* proteinase (Table 2). The enzyme was inactivated by PMSF, TPCK and chymostatin. This last compound which acts specifically on chymotrypsin/subtilisin-like serine proteinases caused a drastic decrease in enzyme activity with 8% residual activity. TLCK and SBTI, which are trypsin inhibitors, had a significant effect on proteinase activity. The proteinase was also inhibited by the reducing agent 2-ME. A slight effect was observed for SDS whereas methanol and ethanol partially affected the proteinase activity. In contrast, proteinase was not inhibited by elastatinal, benzamidine, bestatin, leupeptin, E-64, iodoacetamide, NEM, pepstatin A, EDTA and DMSO. Finally, the proteinase activity was partially inhibited by the presence of

Table 1. Purification of the serine-protease from *T. vanbreuseghemii*

Purification steps	Volume (ml)	Total protein (mg)	Total activity (nkat)	Specific activity (nkat/mg)	Yield (%)	Purification (fold)
Culture filtrate	4870	53.5	1,165,878	21,792	100	1
$(NH_4)_2SO_4$ precipitation	71	21.9	598,032	27,307	51.3	1.25
DEAE (Trisacryl M	42	3.8	300,873	79,177	25.8	3.63

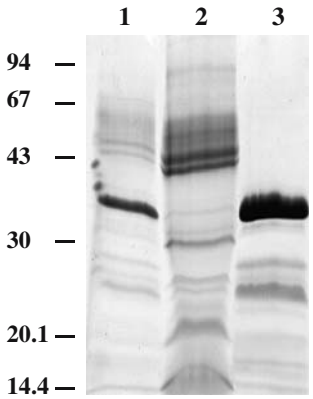

Figure. 3. SDS-PAGE pattern of *T. vanbreuseghemii* serine proteinase. Lane 1: culture filtrate; lane 2: first protein peak eluted from DEAE–Trisacryl column with 0.1 M NaCl; and lane 3: partially purified enzyme eluted from the column with 0.24 M NaCl. Molecular mass (MM) of standard proteins (94, 67, 43, 30, 20.1 and 14.4 kDa) are indicated in the left.

Table 2. Effect of various compounds on the activity of *Trichophyton vanbreuseghemii* proteinase

Reagent	Final concentration	Residual activity (%)
PMSF	1 mM	31
Chymostatin	100 μM	8
TPCK	1 mM	33
TLCK	1 mM	41
SBTI	50 μM	53
2-ME	1%	22
SDS	1%	71
Methanol	10%	47
Ethanol	10%	39

The following compounds had no effect on the enzyme activity at the concentrations tested and indicated in parentheses: EDTA, iodoacetamide (10 mM); *N*-ethylmaleimide (2 mM); pepstatin A, benzamidine (1 mM); leupeptin (100 μM); E-64, bestatin, elastatinal (10 μM) and DMSO (10%).

NH_4Cl and $NaNO_2$, but not by $NaNO_3$ at a final concentration of 10 mM (Table 3). Moreover, the enzyme was completely inhibited by some cations such as Ag^+, Al^{3+}, Cu^{2+} and Hg^{2+}, whereas Co^{2+}, Li^+, Mg^{2+}, Mn^{2+} caused only partial inhibitions (residual activity comprised between 40 and 67%). No effect was observed for Ca^{2+}, Fe^{2+} and Zn^{2+}. For optimum pH, enzyme assays were performed from pH 7 to 11. The proteinase was found to have an alkaline optimum pH of 8, but it remained active until pH 11 (data not shown).

Table 3. Effect of ions at 10 mM final concentration on the activity of *Trichophyton vanbreuseghemii* proteinase

Reagent	Residual activity (%)
Ag^+	0
Al^{3+}	0
Co^{2+}	67
Cu^{2+}	2
Hg^{2+}	0
Li^+	46
Mg^{2+}	46
Mn^{2+}	40
NH_4^+	33
NO_2^-	50

The following ions had no effect on the enzyme activity: Ca^{2+}, Fe^{2+}, Zn^{2+} and NO_3^-.

Discussion

Trichophyton vanbreuseghemii was isolated from soil in Tunisia by Rioux, Jarry and Juminer at 1964 for first time. Since this date, this fungus has been found in different parts of the world like Canada, Europe and North Africa, and it is now considered to be world-wide distributed. Although *T. vanbreuseghemii* shows great capabilities to perforate hair *in vitro*, probably due to proteolytic enzymes [4], it is considered as a non-pathogenic fungus, only one case of human infection having been reported as yet [12]. Therefore, this fungus may be interesting for recycling the keratin wastes from food and feed industry. However, to our knowledge the proteolytic equipment of *T. vanbreuseghemii* has never been studied.

Previous studies performed on other filamentous fungi using various protein-free culture media allowed us to define the best culture conditions for protease synthesis [13, 14]. These culture conditions were applied to a *T. vanbreuseghemii* isolate from soil in Iran, and cultivation of the fungus in modified Czapek–Dox liquid medium containing 0.1% bacteriological peptone and 1% glucose gave a high enzyme level after incubation for two weeks. The proteinase was then partially purified from the culture filtrate by a simple two-step method involving ammonium sulphate precipitation and anion-exchange chromatography. Inhibition profile of the enzyme, as well as the determination of its activity towards various synthetic substrates and of its optimum pH, demonstrated that it belonged to the chymotrypsin/subtilisin family of serine

proteinases. As all chymotrypsins/subtilisins, the fungal proteinase was highly sensitive to PMSF and chymostatin. In addition, hydrolysis rate of different synthetic substrates of serine-proteinases was the highest with *N*-Suc-Ala-Ala-Pro-Phe-*p*NA. The enzyme activity was lower for *N*-Bz-Phe-Val-Arg-*p*NA which is more specific for trypsin-like serine proteinase, and very weak for *N*-Suc-Ala-Ala-Pro-Leu-*p*NA, a substrate of elastase-like serine proteinase. The fact that the proteinase was not totally inefficient on these two last substrates seems to be a common feature of fungal subtilisins, since it has also been reported for similar chymotrypsin/subtilisin-like serine proteinases purified from *Aspergillus fumigatus* [13] or *Scedosporium apiospermum* culture filtrate [14]. Likewise, the enzyme was totally inhibited by heavy-metal cations such as Ag^{+}, Al^{3+}, Cu^{2+} and Hg^{2+}, which is also in agreement with similar findings on serine proteinases from *A. fumigatus* and *S. apiospermum* [13, 14]. Moreover, as other subtilisins, the serine proteinase of *T. vanbreuseghemii* which supports a keratinolytic activity as demonstrated by the keratin azure test, presented an alkaline optimum pH.

All these results are therefore consistent with previous findings from other groups working on keratinolytic fungi. Usually, fungal keratinases belong to the class of serine proteinases. For instance, it is well established that serine proteinases produced by *Trichophyton rubrum* [15, 16], *Trichophyton schoenleinii* [17], *Trichophyton mentagrophytes* [18], *Doratomyces microsporus* [19], *Microsporum canis* [20], *Scopulariopsis brevicaulis* [21] and *Hendersonula toruloidea* [22] support a keratinolytic activity. However, a few keratinolytic enzymes belong to the class of metalloproteases such as some keratinases from *M. canis* [23] and *C. keratinophilum* [6] or the fungalysins that have been isolated in *T. rubrum*, *T. mentagrophytes* and *M. canis* [24]. Optimum pH of the different keratinases that have been characterized so far, may vary from acidic pH, 4.5 for *T. mentagrophytes* [18], 5.5 for *T. mentagrophytes* var. *erinacei* [25] and *T. schoenleinii* [17], to alkaline pH, 8 for *M. canis* metalloprotease [23] and *T. rubrum* [16], 8–9 for *D. microsporus* [19] and 9 for *M. canis* subtilisin serine protease [20], *C. keratinophilum* [6] and *H. toruloidea* [22]. In addition, most of these enzymes have similar molecular mass, about 30 kDa [16–22]. The serine proteinase from *T. vanbreuseghemii* presented a molecular mass of 37 kDa, but several minor bands of lower molecular mass were also detected by SDS-PAGE, probably in relation with the high autolytic potential already reported for such enzymes [26] since no proteinase inhibitors could be used during the purification procedure.

Keratinolytic enzymes may be of great interest particularly for industries of leather, food and poultry that produce large quantities of keratinic wastes [27–29]. Here, a serine proteinase from *T. vanbreuseghemii* has been partially purified and its keratinolytic activity has been established. The potential use of this geophilic fungus or of this extracellular proteinase in the bioconversion of industrial keratinic wastes is worthy of the attention of environmentalists.

Acknowledgement

This research has been supported by School of Public Health, Tehran University of Medical Sciences grant 240.2502.

References

1. Safranek WW, Goos RD. Degradation of wool by saprophytic fungi. Can. J. Microbiol. 1982; 28: 137–140.
2. Matsumoto T. Fungal diseases in dermatology. In: Kibbler CC, Mackenzie DW, Odds FCJ, eds. Principle and Practice of Clinical Mycology, Wiley and Sons Ltd New York, 1996 : 103–129.
3. Kushwaha RKS, Nigam N. Keratinase production by some geophilic fungi. Proc Indian Natl Sci Acad B Biol Sci 1996; 19: 107–109.
4. Bahuguna S, Kushwaha RK. Hair perforation by keratinophilic fungi. Mycoses 1989; 32: 340–343.
5. Scott JA, Untereiner WA. Determination of keratin degradation by fungi using keratin azure. Med. Mycol. 2004; 42: 239–246.
6. Dozie INS, Okeke CN, Unaeze NC. A thermostable, alkaline-active, keratinolytic proteinase from *Chrysosporium keratinophilum*. World J Microbiol Biotechnol 1994; 10: 563–567.
7. Muhsin TM, Aubaid AH, al-Duboon AH. Extracellular enzyme activities of dermatophytes and yeast isolates on solid media. Mycoses 1997; 40: 465–469.
8. Muhsin TM, Salih TH. Exocellular enzyme activity of dermatophytes and other fungi isolated from ruminants in Southern Iraq. Mycopathologia 2001; 150: 49–52.
9. DelMar EG, Largman C, Brodrick JW, Geokas MC. A sensitive new substrate for chymotrypsin. Anal. Biochem. 1979; 99: 316–320.
10. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing

the principle of protein–dye binding. Anal Biochem 1976; 72: 248–254.
11. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970; 227: 680–685.
12. Lopes JO, Salebian A, Alves SH, Benevenga JP. First case of human infection by *Trichophyton vanbreuseghemii* in Brazil. Rev Inst Med Trop Sao Paulo 1994; 36: 379–380.
13. Larcher G, Bouchara JP, Annaix V, Symoens F, Chabasse D, Tronchin G. Purification and characterization of a fibrinogenolytic serine proteinase from *Aspergillus fumigatus* culture filtrate. FEBS Lett. 1992; 308: 65–69.
14. Larcher G, Cimon B, Symoens F, Tronchin G, Chabasse D, Bouchara JP. A 33 kDa serine proteinase from *Scedosporium apiospermum*. Biochem. J. 1996; 315: 119–126.
15. Jousson O, Léchenne B, Bontems O, Mignon B, Reichard U, Barblan J, Quadroni M, Monod M. Secreted subtilisin gene family in *Trichophyton rubrum*. Gene 2004; 339: 79–88.
16. Asahi M, Lindquist R, Fukuyama K, Apodaca G, Epstein WL, McKerrow JH. Purification and characterization of major extracellular proteinases from *Trichophyton rubrum*. Biochem J 1985; 232: 139–144.
17. Qin LM, Dekio S, Jidoi J. Some biochemical characteristics of a partially purified extracellular keratinase from *Trichophyton schoenleinii*. Zentralbl Bakteriol 1992; 277: 236–244.
18. Tsuboi R, Ko IJ, Takamori K, Ogawa H. Isolation of a keratinolytic proteinase from *Trichophyton mentagrophytes* with enzymatic activity at acidic pH. Infect Immun 1989; 57: 3479–3483.
19. Gradisar H, Kern S, Friedrich J. Keratinase of *Doratomyces microsporus*. Appl. Microbiol. Biotechnol. 2000; 53: 196–200.
20. Mignon B, Swinnen M, Bouchara JP, Hofinger M, Nikkels A, Pierard G, Gerday C, Losson B. Purification and characterization of 31.5 kDa keratinolytic subtilisin-like serine protease from *Microsporum canis* and evidence of its secretion in naturally infected cats. Med. Mycol 1998; 36: 395–404.
21. Malviya HK, Rajak RC, Hasija SK. Purification and partial characterization of two extracellular keratinases of *Scopulariopsis brevicaulis*. Mycopathologia 1992; 119: 161–165.
22. Rojanavanich V, Yoshiike T, Tsuboi R, Takamori K, Ogawa H. Purification and characterization of an extracellular proteinase from *Hendersonula toruloidea*. Infect Immun 1990; 58: 2856–2861.
23. Brouta F, Descamps F, Fett T, Losson B, Mignon B. Purification and characterization of a 43.5 kDa keratinolytic metalloprotease from *Microsporum canis*. Med. Mycol 2001; 39: 269–275.
24. Jousson O, Léchenne B, Bontems O, Capoccia S, Mignon B, Barblan J, Quadroni M, Monod M. Multiplication of an ancestral gene encoding secreted fungalysin preceded species differentiation in the dermatophytes *Trichophyton* and *Microsporum*. Microbiology 2004; 150: 301–310.
25. Muhsin TM, Aubaid AH. Partial purification and some biochemical characteristics of exocellular keratinase from *T. mentagrophytes* var. *erinacei*. Mycopathologia 2001; 150: 121–125.
26. Monod M, Togni G, Rahalison L, Frenk E. Isolation and characterisation of an extracellular alkaline protease of *Aspergillus fumigatus*. J. Med. Microbiol. 1991; 35: 23–28.
27. Gupta R, Beg QK, Lorenz P. Bacterial alkaline proteases: molecular approaches and industrial applications. Appl. Microbiol. Biotechnol. 2002; 59: 15–32.
28. EI-Naghy MA, El-Ktatny MS, Fadl-Allah EM, Nazeer WW. Degradation of chicken feathers by *Chrysosporium georgiae*. Mycopathologia 1998; 143: 77–84.
29. Singh CJ. Optimization of an extracellular protease of *Chrysosporium keratinophilum* and its potential in bioremediation of keratinic wastes. Mycopathologia 2002; 156: 151–156.

Address for correspondence: Dr. Farideh Zaini, Department of Medical Mycology and Parasitology, School of Public Health, Tehran University of Medical Sciences, Tehran, 14155, Iran
Phone: + 98-21-8951583; Fax: +98-21-6462267
E-mail: za14_28@yahoo.com