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Purification and characterization of a fibrinogenolytic serine proteinase from *Aspergillus fumigatus* culture filtrate

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A fibrinogenolytic proteinase has been isolated from *Aspergillus fumigatus* culture filtrate by ammonium sulfate precipitation followed by successive chromatographies on Sephadex G-75 and immobilized phenylalanine. The purified proteinase exhibited a molecular weight of about 33 kDa. When analysed by SDS-polyacrylamide gels containing co-polymerized fibrinogen, the proteinase appeared as a broad band at the top of the gels, which could correspond to polymerization of the enzyme, as suggested by SDS-PAGE analysis of the unboiled eluate. The isoelectric point was 8.75 and the enzyme was not glycosylated. Proteinase activity was optimum at pH 9 and between 37 and 42°C, although a decrease in activity was observed above 37°C. PMSF and chymostatin markedly inhibited the proteinase activity, and good kinetic constants were obtained for the synthetic substrate, *N*-Suc-Ala-Ala-Pro-Phe-pNA. These results provide direct evidence that this enzyme belongs to the chymotrypsin-like serine proteinase group.

Enzyme purification; Serine proteinase; Fibrinogenolytic; *Aspergillus fumigatus*

1. INTRODUCTION

Aspergillus proteinases are typical enzymes which have been investigated for practical purposes such as medicine additives, fermentation of food products and depilation of skins [1–3]. Moreover, proteinases are now of medical interest because there is presumptive evidence for their role in the pathogenesis of infection. The widespread tissue damage observed in patients with aspergillosis suggests the involvement of extracellular products which could degrade host proteins. The relationship between fungal proteolytic activity and invasive aspergillosis was first considered by Martin and Jönsson [4]. More specific activities, such as elastinolytic and collagenolytic activities have been described in *A. fumigatus* and *A. flavus* [5–9], the main causative agents of aspergillosis in humans [10–12]. In previous studies [13,14], we demonstrated the presence at the surface of conidia, the dissemination form of the fungus, of fibrinogen receptors which could mediate attachment to damaged epithelia via the fibrinogen deposits. In addition, *A. fumigatus* culture filtrate exhibited a fibrinogenolytic activity which might play an important role in the host tissue invasion [15]. Keeping in view the potential role of this exoprotease in the pathogenesis of aspergillosis, the present work was undertaken to purify and characterize this enzyme.

Abbreviations: CBS, Centraalbureau voor Schimmelcultures; MCA, 7-amido-4-methyl-coumarin; PEG, polyethyleneglycol; pNA, para-nitroanilide; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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2. MATERIALS AND METHODS

2.1. *Microorganism and culture conditions*

A. fumigatus CBS 113.26 was used and propagated on Sabouraud agar plates. For enzyme production, the strain was grown in nitrogen-depleted Czapek-Dox liquid medium supplemented with 0.1% pancreatic peptone as sole nitrogen source. Flasks containing 1 litre of medium were inoculated with approximately 10^7 cells per ml and incubated for 72 h at 37°C.

2.2. *Enzyme purification and activity assay*

Step 1. The culture medium was separated from mycelium by filtration. After adding ammonium sulfate to 40% final saturation, the filtrate was stirred for 1 h and then centrifuged (20,000 × *g*, 30 min). The resulting supernatant was then precipitated to 70% ammonium sulfate saturation and centrifuged (20,000 × *g*, 30 min). The fraction that precipitated between 40 and 70% saturation was resuspended in distilled water at 1:10 of the original volume and dialysed against 50 mM Tris-HCl, 100 mM NaCl buffer, pH 7.5 (Tris buffer saline: TBS). After removal of the insoluble material by centrifugation at 45,000 × *g* for 20 min, the supernatant was concentrated with PEG 35,000.

Step 2. The crude enzyme was loaded onto a Sephadex G-75 column (2.5 × 100 cm) equilibrated with TBS. Samples of the effluent, 1 ml, were collected at a flow rate of 6 ml/h. Fractions with high proteinase activity were pooled, concentrated with PEG 35,000 and dialysed against TBS.

Step 3. Finally, the dialysate was subjected to a (*Nα*-CBZ)-D-phenylalanine agarose (Pierce) column (1.14 × 10 cm) equilibrated with TBS. Proteinase was eluted with 0.2 M acetic acid, 0.2 M sodium acetate buffer, pH 5, at a flow rate of 10 ml/h. To avoid enzyme denaturation, 1 ml-collected fractions were supplemented with 200 µl

of 10-fold concentrated 50 mM Tris-HCl buffer, pH 10, to reach neutral pH. Fractions with enzyme activity were pooled, dialysed against 50 mM Tris-HCl, pH 7.5 (Tris buffer), and divided into aliquots which were stored at −20°C.

Purification was monitored for protein by measuring the absorbance at 280 nm. The enzyme activity was determined by measuring the rate of hydrolysis of a synthetic peptide, *N*-Suc-Ala-Ala-Pro-Phe-pNA (Sigma), which was 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 Tris buffer with 20 μl of substrate. After 20 min of incubation at 37°C, the reaction was stopped by the addition of 100 μl of 2% v/v acetic acid and the amount of paranitroaniline released was measured at 405 nm using a Titertek Multiskan spectrophotometer (Flow Laboratories). One unit of activity (U) was defined as the amount of enzyme that liberated 1 nmol of paranitroaniline per min under the assay conditions. Protein concentrations were determined by the method of Bradford [16] using bovine serum albumin as a standard.

2.3. *Electrophoretic procedures*

SDS-PAGE was carried out according to Laemmli [17] using 10% polyacrylamide gels which were stained with Coomassie brilliant blue R-250, destained in 10% v/v acetic acid and re-stained with silver reagent [18]. The electrophoretic migration of the purified proteinase was compared to molecular weight markers from Pharmacia. Purified proteinase was analysed by substrate-gel electrophoresis adapted from the procedure of Heussen and Dowdle [19]. The enzyme extract was run on a 10% polyacrylamide gel co-polymerized with 0.02% human fibrinogen. The gel was rinsed, 3 × 15 min, with distilled water and then incubated in TBS for 16 h at room temperature. Isoelectric focusing using LKB-IEF gels (3.5–9.5) was done with an LKB Multiphor apparatus according to the company's recommendations. The appropriate LKB calibration kit was used and silver staining was performed. Proteinase activity was detected by incubation of the gel in Tris buffer containing 0.5 mM of the fluorogenic substrate, *N*-Suc-Ala-Ala-Pro-Phe-MCA (Sigma), for 5 min at room temperature. Dig Protein and Glycan Detection Kits from Boehringer were used after electrotransfer of the enzyme onto Immobilon membranes (Millipore).

2.4. *Characterization of the enzyme*

All assays were run in triplicate. The proteinase activity was measured over a range of pH values from 3.5 to 12, using (i) 0.2 M sodium acetate, pH 3.5–5, (ii) 0.2 M Tris-maleate–NaOH, pH 6–8, and (iii) 0.1 M glycine–NaOH, pH 9–12, buffers. The optimum temperature for enzyme activity and heat stability of the purified proteinase were determined at temperatures ranging from 37 to 100°C. The effect of various reagents on enzyme activity was studied. These included metal chelating agents (EDTA, EGTA), reducing or alkylating reagents such as dithiothreitol (DTT), 2-mercaptoethanol (2-ME), *N*-ethylmaleimide (NEM) and iodoacetamide, detergents (SDS, Triton X-100, Nonidet P-40), organic solvents (methanol, ethanol, DMSO) and commercially available enzyme inhibitors (phenylmethylsulfonyl fluoride (PMSF), tosyl-L-phenylalanine chloromethylketone (TPCK), *N*-tosyl-L-lysine chloromethylketone hydrochloride (TLCK), soybean trypsin inhibitor (SBTI), pepstatin, leupeptin, chymostatin, bestatin, elastatinal). Aliquots of the purified proteinase solution (160 μl at 2 μg/ml in Tris buffer) were pre-incubated for 10 min at 37°C with 20 μl of 10-fold concentrated stock solutions of each reagent. Then, 20 μl of chromogenic substrate (5 mM) were added and proteinase activity was assayed as described above. PMSF was prepared as a stock solution in methanol, TLCK and TPCK in ethanol, and chymostatin in DMSO. Residual activity was determined as a percentage of the activity in control samples without reagent. For inhibitors requiring methanol, ethanol or DMSO in stock solutions, an equivalent amount of solvent was run as a control. Kinetic constants were determined using various synthetic chromogenic substrates purchased from Sigma. Substrates were prepared in DMSO to yield concentrations in the reaction mixture of 0.125–4 mM. Proteinase activity was assayed as described above at different times of incubation (5, 10, 15 and 20 min). Kinetic constants for each substrate were calculated from Lineweaver–Burk plots [20].

3. RESULTS

3.1. *Purification of the fungal proteinase and its molecular properties*

The enzyme was purified from 3-day-old culture filtrate of *A. fumigatus* by sequential chromatographies on Sephadex G-75 and immobilized phenylalanine. In the appropriate fractions from each chromatography, a single peak with proteinase activity was observed (Fig. 1). The purification steps are summarized in Table I with an overall recovery of 22% and 39-fold purification. The fact that only a 39-fold purification was sufficient to achieve homogeneity confirms that the proteinase is one of the major secreted proteins in *A. fumigatus* culture medium. SDS-PAGE analysis of the purified enzyme disclosed, after silver staining, a major protein band with an estimated molecular weight of 33 kDa and minor bands of 21 and 26 kDa (Fig. 2, lane 2). Similar results were obtained under reducing conditions suggesting the absence of disulfide bonds (data not shown). To check the fibrinogenolytic activity, the purified proteinase was analysed in SDS-polyacrylamide gels con-

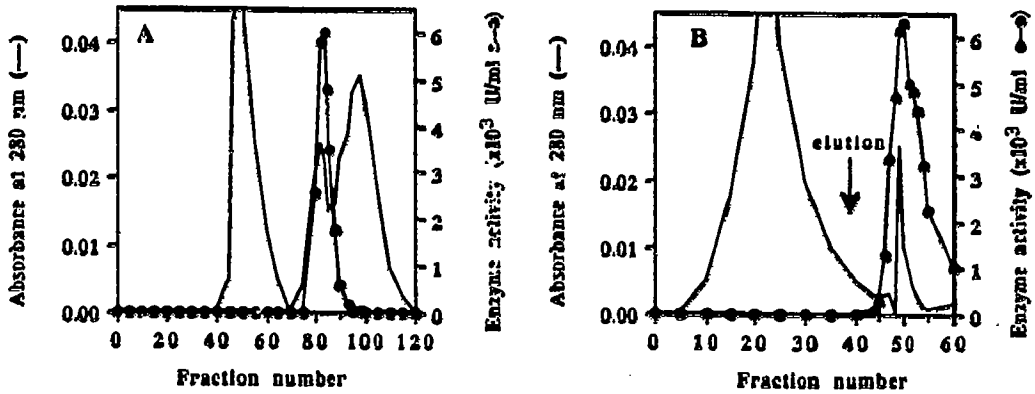

Fig. 1. Elution profiles of protein (—) and proteinase activity (●—●) from Sephadex G-75 column chromatography (A) and (*N*α-CBZ)-D-phenylalanine agarose column (B). The conditions were as described in section 2.

Table I

Summary of purification of the proteinase from *A. fumigatus*

Purification	Total volume (ml)	Total protein (mg)	Total activity ($\times 10^4$ U)	Recovery (%)	Specific activity ($\times 10^4$ U/mg)	Purification (fold)
Culture filtrate	4,900	24.50	224	100	9.14	1.0
Fractionated ammonium sulfate precipitation						
0–40%	200	1.70	4.54	2	2.67	0.3
40–70%	200	7.24	104	46	14.4	1.6
Sephadex G-75	12	2.50	50.2	22	20	2.2
Affinity chromatography on immobilized phenylalanine	17	0.14	50	22	357	39

taining co-polymerized human fibrinogen. Curiously a clear zone appeared on the top of the gel (Fig. 2, lane 3) contrasting with the 33-kDa molecular weight estimated previously. However, analysis of the unboiled enzyme by SDS-PAGE gave a single protein band with similar relative mobility (Fig. 2, lane 5). On isoelectric focusing, the enzyme migrated as a single sharp band with a pI of 8.75 (Fig. 2D), indicating the homogeneity of the preparation. An investigation of the carbohydrate residues on the molecule failed, showing that the proteinase was not glycosylated (data not shown).

3.2. *Enzymatic properties*

The proteinase exhibited maximal activity at pH 9 and was inactive below pH 3.5 (Fig. 3). Proteinase activity decreased drastically above pH 11. However, inactivation by acid or alkaline pH was reversible. Titrating back to neutral pH after pre-incubation of the enzyme for 20 min in 0.2 M sodium acetate, pH 3.5, or 0.1 M glycine–NaOH, pH 12, buffers restored the enzyme activity. The optimum temperature, as determined by the synthetic substrate assay, lay between 37 and 42°C. Only 38% of the enzyme activity remained at 56°C and no activity was detected at 75°C (Fig. 4). To determine its heat stability, the proteinase was subjected for 20 min at temperatures ranging from 37 to 100°C, and a decrease in activity due to protein denaturation was observed above 37°C (Fig. 4).

The influence of a series of compounds used for enzymological characterization is summarized in Table II. Organic solvents such as methanol, ethanol and DMSO, or non-ionic detergents (Triton X-100, Nonidet P-40) had no significant effect on proteinase activity. In contrast, partial inactivation was observed at 1% SDS and increased at higher concentrations (5% and 10%). Among the protease inhibitors, PMSF and chymostatin strongly inhibited enzyme activity. This inhibition profile allows us to classify the enzyme to the category of serine proteinases. Moreover, synthetic substrates for chymotrypsin proved to be excellent substrates for the fungal enzyme. The K_m of the proteinase was estimated to be 0.62 mM against *N*-Suc-Ala-Ala-Pro-Phe-pNA and 1.2 mM against *N*-Suc-Ala-Ala-Pro-Leu-pNA. High k_{cat}/K_m values were obtained for both synthetic substrates (Table III).

4. DISCUSSION

Optimum pH, inhibition profile and kinetic constants for various synthetic substrates revealed the enzyme to be a chymotrypsin-like serine proteinase. The enzyme presented an inhibition profile and a substrate specificity similar to those obtained for bovine pancreatic chymotrypsin (data not shown). As with chymotrypsin, the fungal proteinase was sensitive to PMSF and chymostatin. Both enzymes presented a high affinity for *N*-Suc-Ala-Ala-Pro-Phe-pNA, a lower affinity for *N*-Suc-Ala-Ala-Pro-Leu-pNA, which is more specific for elastase-like serine proteinases, and did not hydrolyse substrates for subtilisins, such as *N*-Cbz-Gly-Gly-Leu-pNA and *N*-Cbz-Ala-Ala-Leu-pNA [21]. Other experi-

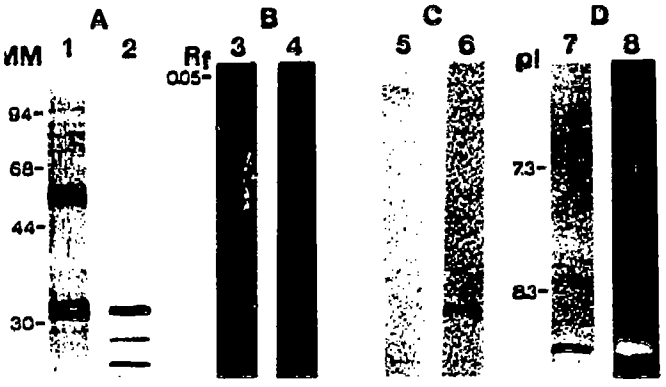

Fig. 2. Electrophoretic analyses of the proteinase. Panel A: SDS-PAGE pattern of culture filtrate (1) and purified proteinase (2) after silver staining. Panel B: fibrinogen substrate-gel electrophoresis of the unboiled (3) and boiled (4) purified proteinase. Panel C: SDS-PAGE analysis of the unboiled (5) and boiled (6) purified proteinase after Coomassie blue staining. Panel D: isoelectric focusing of the enzyme after silver staining (7) and its detection with a fluorogenic substrate (8). The apparent molecular mass (MM) was estimated using the data shown in panel A: phosphorylase *b* (94 kDa), bovine serum albumin (68 kDa), ovalbumin (44 kDa), carbonic anhydrase (30 kDa) and soybean trypsin inhibitor (20 kDa). The isoelectric point was determined by co-migration with isoelectric focusing standards (pH 3.5–9.5) indicated in panel D.

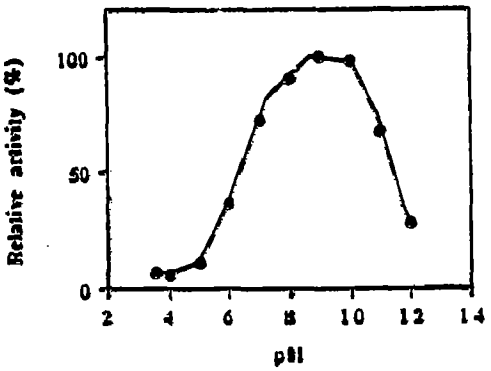

Fig. 3. Effect of pH on the proteinase activity. The maximal activity observed was set as 100% relative enzyme activity. Buffers were 0.2 M sodium acetate, pH 3.5–5, 0.2 M Tris-maleate–NaOH pH 6–8, 0.1 M glycine–NaOH, pH 9–12. Purified proteinase was diluted in the appropriate buffer and the assay was carried out under standard conditions.

ments demonstrated that this monomeric enzyme was not glycosylated. This absence of glycosylation seems to be the case for other fungal proteinases. For example, the elastinolytic proteinases of *A. flavus* and *A. fumigatus* do not include carbohydrate residues according to recently published reports [9,22].

This serine proteinase supports the fibrinogenolytic activity previously found in *A. fumigatus* culture filtrate [15]. Analysis of the enzyme by substrate-gel electrophoresis gave a clear zone at the top of the gel. Likewise, a single protein band with similar relative mobility was observed by SDS-PAGE analysis of the unboiled enzyme. These results suggested for the proteinase a high hydrophobic character and its polymerization, as recently reported by Frosco et al. [9]. Heating the sample would disintegrate the polymer and release the 33-kDa molecule.

Our results are consistent with the findings of other

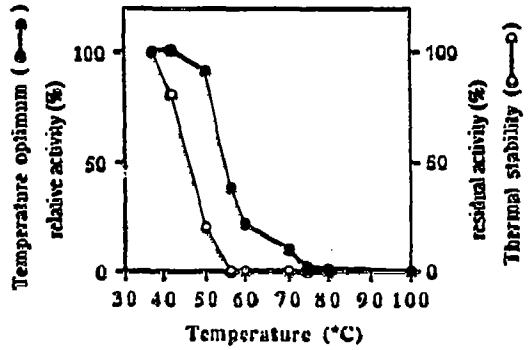

Fig. 4. Effect of temperature on the proteinase activity. Maximal activities observed were set as 100% relative enzyme activity. The optimum temperature was determined by assaying enzyme activity in Tris buffer in a water bath at temperatures ranging from 37 to 100°C. Heat stability of the purified proteinase was determined by incubating the enzyme diluted in Tris buffer at 37, 42, 50, 56, 60, 70, 75, 80 and 100°C for 20 min prior to assaying the activity under standard conditions.

Table II

Effect of various substances on the activity of the *A. fumigatus* proteinase

Reagent	Final concentration	% residual activity
PMSF	1 mM	0
Chymostatin	100 μM	3
Elastatinal	10 μM	78
Iodoacetamide	10 mM	76
SDS	1%	62
	5%	29
	10%	25
Nonidet P-40	1%	76
Ethanol	10%	79

Note: the following substances did not inhibit the enzyme activity significantly (residual activity above 80%) at the concentrations tested and indicated in parentheses: EDTA, EGTA (10 mM); *N*-ethylmaleimide (2 mM); TPCK, TLCK, pepstatin, DTT (1 mM); leupeptin (100 μM); SBTI (50 μM); bestatin (10 μM); DMSO, methanol (10%); 2-mercaptoethanol, Triton X-100 (1%).

laboratories who used different strains of *A. fumigatus* and other purification procedures. In spite of the different approaches, identical results have been obtained regarding a serine proteinase with similar molecular weight between 20 and 35 kDa from culture filtrate [7–9] or mycelial extracts [23] of *A. fumigatus*. In addition, several minor bands of molecular weight less than 32–33 kDa were detected by SDS-PAGE, attesting to the high autolytic potential of this proteinase [7–9]. Monod et al. [8] demonstrated a decrease in this autolytic activity by pre-incubation of this enzyme with PMSF. Authors diverge on the classification of this serine proteinase, which could belong to the chymotrypsin [7,23] or subtilisin [8] group. However, amino acid sequence deduced from the gene encoding for this proteinase, which has recently been cloned by Jaton-Ogay et al. [24] and its comparison with those of other fungal chymotrypsins or subtilisins suggest that it belongs to the subtilisin

Table III

Kinetic constants for the hydrolysis of chromogenic substrates by *A. fumigatus* proteinase

Substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1} \cdot M^{-1}$)
Suc-Ala-Ala-Pro-Phe-pNA	0.62	11.02×10^3	1.78×10^7
Suc-Ala-Ala-Pro-Leu-pNA	1.2	4.41×10^3	0.37×10^7
Suc-Tyr-Leu-Val-pNA		ND*	
Suc-L-Phe-pNA		ND	
Ac-Ile-Glu-Ala-Arg-pNA		ND	
Ac-DL-Phe-pNA		ND	
Bz-L-Tyr-pNA		ND	
Cbz-Gly-Gly-Leu-pNA		ND	
Cbz-Ala-Ala-Leu-pNA		ND	

* ND, hydrolysis not detected. Suc, Succinyl; Ac, Acetyl; Bz, Benzoyl; Cbz, Carbobenzoxy; pNA, Paranitroanilide.

group. Furthermore, the ability of this enzyme to degrade elastin [7–9] or collagen [8] and to detach Vero cells in culture from plastic substrate [7] was reported. Likewise, Robinson et al. [23] have demonstrated that a somatic extract from *A. fumigatus* induced the detachment of human epithelial cells from basement membrane.

The primary function of such an extracellular proteinase is to digest proteins as a nutrient source to allow the fungus to grow. However, degradation of proteins, like fibrinogen, elastin [7–9], collagen [8] and laminin (unpublished data), would also facilitate this organism to invade the host tissues. Indeed, some authors have closely related the ability of *A. fumigatus* to cause cerebral [25] or pulmonary [26] mycoses with the proteinase production by the fungus. Studies are under way to determine if this extracellular proteinase plays an important role in parasitism.

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REFERENCES

[1] Botton, B., Breton, A., Fevre, M., Guy, Ph., Larpent, J.P. and Veau, P. (1990) Moisissures utiles et nuisibles. Importance industrielle, Masson, Paris.
[2] Prescott, S.C. and Dunn, C.G. (1949) Industrial Microbiology, McGraw-Hill, New York.
[3] Malathi, S. and Chakrabory, R. (1991) Appl. Env. Microbiol. 42, 712–716.
[4] Martin, S.M. and Jönsson, A.G. (1965) Can. J. Biochem. 43, 1745–1753.
[5] Rhodes, J.C., Bode, R.B. and McCuan-Kirsch, C.M. (1988) Diagn. Microbiol. Infect. Dis. 10, 165–170.
[6] Rhodes, J.C., Amlung, T.W. and Miller, M.S. (1990) Infect. Immun. 58, 2529–2534.
[7] Reichard, U., Büttner, S., Eiffert, H., Staib, H. and Rüchel, R. (1990) J. Med. Microbiol. 33, 243–251.
[8] Monod, M., Togni, G., Rahalison, L. and Frenk, E. (1991) J. Med. Microbiol. 35, 23–28.
[9] Froco, M., Chase Jr., T. and McMillan, J.D. (1992) Infect. Immun. 60, 728–734.
[10] Bodey, G.P. (1988) J. Hosp. Infect. 11, Suppl. A, 411–426.
[11] Reingold, A.L., Lu, X.D., Plikaytis, B.D. and Ajello, L. (1986) J. Med. Vet. Mycol. 24, 433–436.
[12] Yu, V.L., Muder, R.R. and Poorsattar, A. (1986) Am. J. Med. 81, 249–254.
[13] Bouchara, J.P., Bouali, A., Tronchin, G., Robert, R., Chabasse, D. and Senet, J.M. (1988) J. Med. Vet. Mycol. 26, 327–334.
[14] Annaix, V., Bouchara, J.P., Larcher, G., Chabasse, D. and Tronchin, G. (1992) Infect. Immun. 60, 1747–1755.
[15] Bouchara, J.P., Larcher, G., Annaix, V., Bucher, P., Tronchin, G. and Chabasse, D. (1991) J. Mycol. Med. 1, 105–111.
[16] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[17] Laemmli, U.K. (1970) Nature 227, 680–685.
[18] Merril, C.R., Goldman, D., Sedman, S.A. and Ebert, M.H. (1981) Science 211, 1437–1438.
[19] Heussen, C. and Dowdle, E.B. (1980) Anal. Biochem. 102, 196–202.
[20] Lineweaver, M. and Burk, D. (1934) J. Am. Chem. Soc. 56, 658–666.
[21] Dunn, B.M. (1989) in: Proteolytic Enzymes: A Practical Approach (Beynon, R.J. and Bond, J.S. eds.) pp. 57–81, IRL Press, Oxford.
[22] Rhodes, J.C. and Amlung, T.W. (1991) J. Med. Vet. Mycol. 29, 407–411.
[23] Robinson, B.W.S., Venaille, T.J., Mendis, A.H.W. and McAleer, R. (1990) J. Allergy Clin. Immunol. 86, 726–731.
[24] Jaton-Ogay, K., Suter, M., Crameri, R., Falchetto, R., Fatih, A. and Monod, M. (1992) FEMS Microbiol. Lett. 92, 163–168.
[25] Miyaji, M. and Nishimura, K. (1977) Mycopathologia 62, 161–166.
[26] Kothary, M.H., Chase Jr., T. and McMillan, J.D. (1984) Infect. Immun. 43, 320–325.