Similarity of Acid Proteinases Secreted by *Candida albicans* NIH A-207 and NIH B-792 Strains Cultured in BSA-Supplemented Medium

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Candida albicans NIH A-207 (serotype A) and NIH B-792 (serotype B) strains secreted one acid proteinases (AP) each in a yeast carbon-based medium supplemented with bovine serum albumin (BSA) as the sole nitrogen source. Isolation of AP from the culture filtrates was achieved by dialysis, followed by DEAE-Sepharose and Bio-gel P-100 column chromatographies. It was found that both enzymes from the two strains had very similar properties when examined. The molecular weights and isoelectric points were found to be 43 kDa and pH 4.0, respectively. The amino acid components and first 12 N-terminal amino acid sequences were virtually identical in both enzymes. The optimum pH of the enzymes was 3.5–4.0. The enzymes were heat-labile, and decreases in their activities were found above 37°C. The AP activities were completely inhibited by the addition of pepstatin. No other inhibitor among those tested had any effect. The enzymes degraded all proteins examined, especially host defense factors such as immunoglobulin G and the granulocyte colony stimulating factor. The enzymes also caused similar degrees of enhancement of vascular permeability when they were injected into the dorsal skin of guinea pigs.

Key words *Candida albicans*; serotype A; serotype B; acid proteinase; host defense factor degradation; vascular permeability enhancement

Candidosis is an endogenous infectious disease occurring in a compromised host. Among the various *Candida* species, *C. albicans* is the most medically important. In 1961, Hasenclever and Mitchell^{1,2)} identified two serotypes in *C. albicans* species, A and B, demonstrating that the pathogenicity of both types was identical in mice when the inoculation of the cells was conducted *via* the intravenous route. The acid proteinase (AP) produced by the *C. albicans* species has attracted a great deal of attention as a virulence factor in the pathogenesis of human and animal infections,^{3–10)} though the existence of contradictory findings has been pointed out.^{11,12)} It has been shown that the recovery of the serotype A strains was more pronounced than that of serotype B in mice when both strains were orally inoculated with mixtures of the two strains.¹³⁾ Borg and Rùchel¹⁴⁾ reported that different strains of serotype B, during infection into phagocytes, expressed an AP antigen only on blastoconidia, while filamentous cells of these strains appeared to be nonproteolytic, and that the serotype A strains expressed the antigen on the cells of both types.

During the course of screening the proteinase-producing *Candida* strains, we found that the *C. albicans* NIH B-792 (serotype B) strain produced a large amount of AP in the culture broth compared to the *C. albicans* NIH A-207 (serotype A) strain when cultured in a yeast carbon-based medium supplemented with bovine serum albumin (BSA) as the sole nitrogen source. In this paper, we have compared the properties of the APs produced by the *C. albicans* NIH A-207 and NIH B-792 strains in order to determine the relevance of these enzymes in Candidosis.

MATERIALS AND METHODS

Organisms and Growth Conditions *C. albicans* NIH A-207 (serotype A) and *C. albicans* NIH B-792 (serotype B) strains, abbreviated A-207 and B-792, respectively, were kindly donated by Dr. T. Shinoda, Department of Microbiol-

ogy, Meiji College of Pharmacy, Tokyo, Japan, and were maintained on Sabouraud agar. The organisms were routinely inoculated into 200 ml of 0.5% (w/v) of Sabouraud medium supplemented with yeast extract, and cultured in a rotary shaker (150 rpm) for 48 h at 27°C. After cultivation, the organisms were collected by centrifugation, and washed three times with saline. The washed cells (5×10^8) were then suspended in 1 ml of saline. To obtain the secretory AP, 1 ml of the washed cell suspension was cultured in 200 ml of growth medium containing 0.2% BSA and a 1.17% yeast carbon base in a 500 ml culture flask, and then further cultured for 2–3 d at 27°C on a rotary shaker (150 rpm).

Assay of AP Activity AP activity was determined as described by Kobayashi *et al.*¹⁵⁾ using bovine hemoglobin as the substrate. Briefly, a 0.4 ml portion of enzyme solution was added to 2 ml of substrate solution [6 g of bovine hemoglobin (Wako Pure Chemical Industries, Ltd., Osaka, Japan) in 1 l of 50 mM sodium citrate buffer (pH 3.6)] and the mixture was incubated at 37°C for 60 min. The reaction was stopped by adding 2 ml of 0.44 M trichloroacetic acid. This mixture was then centrifuged and the absorbance of the supernatant was measured at A_{275} nm. One unit of AP activity was defined as that producing an increase in the absorbance value at the A_{275} nm of 0.001/min.

Isolation and Purification of AP The culture broths (A-207: culture medium 5 l, 27°C, 72 h; B-792: culture medium 4 l, 27°C, 48 h) of each *C. albicans* strain were separately centrifuged for 10 min at 8880×g, and 1 M citrate buffer (pH 6.7) was added to the supernatants to obtain a final concentration of 1 mM. The solutions were then dialyzed against 20 mM citrate buffer (pH 6.7). After concentration by an evaporator at 30°C, the AP activity in the concentrate was eluted from the DEAE-Sepharose column (1.8×36 cm) using sodium citrate buffer (pH 6.7) with a linear increase in the ionic strength ranging from 20 to 800 mM after washing with the 20 mM buffer. The fractions exhibiting AP activity were pooled and concentrated *in vacuo*. The fractions were applied

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to a Biogel P-100 column (1.8×65 cm) using 50 mM citrate buffer (pH 6.7). The fractions exhibiting AP activity were used in this experiment and some were stored in the presence of 5% (w/v) glycerol at -80°C . The molecular weight determinations of the AP specimens were performed by SDS-PAGE as described by Laemmli and Favre.¹⁶⁾ Before application of the gels, samples were boiled for 2 min in 70 mM Tris-HCl buffer (pH 6.8) containing 2% SDS, 5% 2-mercaptoethanol, and 10% glycerol. Electrophoresis was performed in Tris-glycine buffer (pH 8.8) using a discontinuous gel consisting of a 5% stacking gel and the 15% separating gel. The molecular weight standards (BIO-RAD) used were phosphorylase B (97400), bovine serum albumin (66200), ovalbumin (45000), carbonic anhydrase (31000), soybean trypsin inhibitor (21500), and lysozyme (14400). The gels were stained with Coomassie brilliant blue R-250. Isoelectric focusing was done using the techniques of Vesterberg and Svensson.¹⁷⁾ The protein concentration was determined with the bicinchoninic acid protein assay reagent (BCA, PIERCE) of Redinbaugh and Turley¹⁸⁾ with bovine serum albumin as the standard.

Amino Acid Analysis Samples containing 0.3–0.5 mg of purified AP were hydrolyzed in 1 ml of 6 N HCl *in vacuo* for 24 h at 110°C . Amino acid analysis was performed using an amino acid analyzer (Hitachi Model 835).

N-Terminal Amino Acid Sequencing The purified AP was first separated on SDS-PAGE and then transferred onto PVDF-Immobilon membranes (Millipore). After staining with Coomassie brilliant blue, the protein band was cut out and analyzed using a protein sequencing system (Model HP G1005A, Hewlett-Packard, Palo Alto, CA, U.S.A.).

Optimum pH and pH Denaturation The optimum pH for AP activity was determined at pHs 2.3 to 7.0 with 50 mM citrate buffer, at pHs 5.0 to 7.0 with 50 mM phosphate buffer, and at pHs 7.0 to 9.0 with 50 mM Tris-HCl buffer. The effect of pH on the denaturation of the AP was determined by the same method as the optimum pH determination at pH 3.6 after incubation of the AP for 15 h at 37°C in the same buffers (pHs 3.0 to 9.0).

Optimum Temperature and Heat Stability The optimum temperature for the AP activity was determined by measuring the activity at temperatures ranging from 4 to 100°C in 50 mM citrate buffer (pH 3.6). The heat stability was determined by measuring the activity in a 50 mM citrate buffer (pH 3.6) after storing the AP solution at temperatures of 4 to 100°C for 30 min in a 50 mM citrate buffer (pH 6.7).

Inhibition Studies of AP Activity This was measured in the presence of the following inhibitors and concentrations: 100 $\mu\text{g/ml}$ Pepstatin A, 100 $\mu\text{g/ml}$ chymostatin, 100 $\mu\text{g/ml}$ α 2-macroglobulin, 100 $\mu\text{g/ml}$ leupeptin, 10 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM ethylenediamine *N,N,N',N'*-tetraacetic acid (EDTA), 10 mM ethyleneglycol-bis-(β -aminoethylether)*N,N,N',N'*-tetraacetic acid (EGTA), 100 $\mu\text{g/ml}$ 1,2-epoxy-3-*p*-nitro-phenoxypropane (EPNP), 100 $\mu\text{g/ml}$ diazoacetylornithine methyl ester (DAN), 50 mM cysteine, 100 $\mu\text{g/ml}$ *N*-ethylmaleimide (NEM), 100 $\mu\text{g/ml}$ soybean trypsin inhibitor (SBTI), 10 mM *p*-chloromercuribenzoate (PCMB), and 50 mM 2-mercaptoethanol (2-ME). Pepstatin A and PMSF were solubilized in ethanol and methanol, respectively. The reaction mixture, containing 50 μl each of the enzyme solution and inhibitor solution, was

stored at room temperature for 30 min. The remaining activity was then measured and compared with that (100%) of the control without inhibitors.

Substrate Specificity The AP activity was measured using the respective substrates instead of the bovine hemoglobin in the ordinary assay method. The substrates used were bovine hemoglobin (HG-2500), insoluble collagen type 1, albumin bovine (A-7030), albumin bovine (A-4378), albumin bovine fraction V (A-8022), and fibrinogen fraction 1 from human plasma, which were purchased from Sigma Co., Ltd. Keratin powder was purchased from Nacalai Tesque (Tokyo, Japan). Casein (purified, Difco) and casein (Hammerstein, MERCK) were also used in this experiment.

Denaturation of Bioactive Proteins A polyclonal rabbit anti-Candida factor ("Candida Check"; Iatron, Tokyo, Japan) corresponding to antigen 1, the definition of which was given by Fukazawa *et al.*,¹⁹⁾ was used as an example of immunoglobulin G. The factor serum 1 (2 μl) was treated with purified AP in 500 μl of 50 mM citrate buffer (pH 3.6) for 15 h at 37°C . The remaining antibody activity in the treated-serum dilution was checked by an ELISA system using *C. albicans* serotype A mannan as the antigen. Human granulocyte colony stimulating factor (G-CSF) was used as a cytokine example. G-CSF activity was measured using an ELISA kit (Amersham) after treatment of G-CSF (6.25 pg) with purified AP in 400 μl of 50 mM citrate buffer (pH 3.6) for 15 h at 37°C . Both activities were expressed by comparing the control without AP treatment.

Vascular Permeability Enhancement Reaction in Guinea Pig Skin The permeability assay was performed with guinea pig skin as described by Kaminishi *et al.*⁹⁾ Briefly, the purified AP was diluted to the desired concentration with 20 mM acetate buffer (pH 4.0). A 100 μl of sample was intradermally injected into the back of a guinea pig immediately after the intravenous injection of 20 mg of Evans blue (5% solution in saline) per kg. The reaction intensity was expressed by taking photographs 20 min after the injection.

RESULTS

The AP activity in the culture supernatants was 895 U/ml for A-207 and 2710 U/ml for B-792. The APs were purified from the supernatants as follows. By gradient elutions during DEAE-Sepharose column chromatography of the dialyzed culture supernatants of the A-207 and B-792 strains, the AP activities were eluted from the columns by citrate buffer between 0.4 and 0.6 M. The active fractions were applied to a Biogel P-100 column. The patterns of the activity and protein concentration showed a single symmetrical peak. The fractions containing the activity were found to contain a homogeneous protein by SDS-PAGE when stained with Coomassie brilliant blue R-250 (Fig. 1). The molecular weights of both enzymes were identical, *i.e.*, 43 kDa. The isoelectric point of the enzymes, as determined by isoelectric focusing, was at pH 4.0. The amino acid composition of the enzymes showed the presence of a large amount of Asp and/or Asn (Table 1). The sequence of the first 12 N-terminal amino acids of both enzymes was determined to be Gln-Ala-Val-Pro-Val-Thr-Leu-His-Asn-Glu-Gln-Val-.

The optimum pH for both enzyme activities was 3.5–4.0

Fig. 1. SDS-PAGE of Purified AP

1, molecular weight standards; 2, purified AP of A-207; 3, purified AP of B-792.

Table 1. Amino Acid Composition of Purified AP

Amino acid	Molar ratio ^{a)}	
	A-207-AP	B-792-AP
Asx	18.7	19.1
Glx	8.8	8.7
Ser	10.4	10.8
Gly	9.9	10.1
His	0.4	0.3
Arg	1.0	1.0
Thr	7.2	7.0
Ala	7.8	7.9
Pro	1.5	1.4
Tyr	2.7	2.8
Val	8.5	8.7
Met	0	0
Cysteine	0.4	0.4
Ile	5.1	5.3
Leu	9.2	9.5
Phe	2.8	2.6
Lys	2.5	2.4

a) Purified APs prepared from A-207 and B-792 were hydrolyzed and analyzed as described in Materials and Methods. Data are expressed as the molar ratio against arginine (1.0).

(data not shown). AP activity began to decline above pH 4.5 and was not observed at pH 6.0 or higher, although the most stable pH for the enzymes was 6.0. Approximately 80% of the AP activity was lost either at pH 2.3 or at pH 8.0 (data not shown). The optimum temperature was 45 °C. Only 10% of the AP activity remained at 65 °C (data not shown). When the enzymes were heated for 30 min at 37, 45, 55, 70, and 100 °C, decreases in activity due to protein denaturation were observed at temperatures above 37 °C (data not shown).

The inhibitory effects of various substances on the AP activities are shown in Table 2. Of the inhibitors used, only pepstatin A completely inhibited (100%) the AP activities. From these inhibitory profiles, both enzymes were identified to be a carboxyl proteinase similar to cathepsin D. The enzymes had a broad substrate specificity (Table 3). Hemoglobin was the most susceptible substrate to these enzymes. Fibrinogen and BSA were substrates with a relatively high susceptibility to these enzymes. All other proteins used in this experiment were also slowly hydrolyzed by the enzymes.

Both enzymes clearly decreased the antibody activity of immunoglobulin G in the factor serum 1 of "Candida Check" (Fig. 2-1). The disappearance of the activity of human G-CSF was also observed by treatment of these enzymes (Fig.

Table 2. Effects of Various Inhibitors on AP Activity

Inhibitor	Conc.	Relative activity (%)	
		A-207-AP	B-792-AP
None		100	100
Pepstatin A	100 µg/ml	0	0
Chymostatin	100 µg/ml	100	100
α2-Macroglobulin	100 µg/ml	100	100
Leupeptin	100 µg/ml	100	100
PMSF	10 mM	100	100
EDTA	10 mM	100	100
EPNP	100 µg/ml	100	98
DAN	100 µg/ml	100	96
Cysteine	50 mM	100	100
NEM	50 mM	100	100
SBTI	100 mM	100	100
PCMB	100 mM	100	98
2-ME	50 mM	100	99

AP activity was determined in the presence or absence of the inhibitors as described in Materials and Methods. Relative activity (%) is expressed by comparing with those (100%) in a matched control lacking inhibitor.

Fig. 2. Degradation of Polyclonal Factor Serum 1 (1) and Human G-CSF (2) Activities by Purified AP from A-207 (A) and B-792 (B)

The factor serum 1 and human G-CSF were treated with purified AP, and the remaining activities were measured as described in Materials and Methods. The control shows activity without enzyme treatment.

Table 3. Substrate Specificity of Purified AP

Substrate	Relative activity (%)	
	A-207-AP	B-792-AP
Bovine hemoglobin (H-2500)	100.0	100.0
Collagen Type 1 insoluble	2.4	1.2
Albumin bovine (A-7033)	19.2	19.9
Albumin bovine (A-4378)	20.4	24.7
Albumin bovine (A-8022)	21.5	29.8
Keratin powder	1.8	1.2
Casein (purified)	5.6	4.6
Casein (Hammersten)	5.9	5.1
Fibrinogen	49.8	71.1

AP activity was measured using the respective substrates as described in Materials and Methods. Relative activity (%) is expressed by comparing with those (100%) measured using hemoglobin.

2-2). Intradermal injection of both enzymes (3, 10, 30 µg) significantly enhanced the permeability in the guinea pig (Fig. 3). The response varied according to the amount of enzyme used. Because the heat-treated enzyme (30 µg, 100 °C,

Fig. 3. Permeability-Enhancing Reaction of Purified AP from A-207 (A) and B-792 (B)

AP at doses 30 (1), 10 (2), and 3 (3) μ g was injected into the dorsal skin of a guinea pig. The results with 30 μ g of heated (100 $^{\circ}$ C, 5 min) AP (4) and 100 μ l of 20 mM acetate buffer (pH 4.0) (5) are also shown. Details of this experiment are described in Materials and Methods.

5 min) did not cause the permeability enhancement, the increased vein permeability was attributable to the enzymic action of both proteinases.

DISCUSSION

C. albicans secretes an AP when grown with a protein, especially BSA, as the sole nitrogen source.^{4,20–23} The AP is believed to be an important virulence factor.^{3–10} The serotype A strains were shown to display stronger virulence activity compared to the B strains in mice as described by Auger *et al.*¹³ In this paper, we have found that the B-792 (serotype B) strain had much higher AP production than A-207 (serotype A) strain. Thus, we did not find a good correlation for the relationship between pathogenicity and AP producibility in the A-207 and B-792 strains (in preparation). With respect to the cause of these contradictory findings, we speculate that the APs produced by the A-207 and B-792 strains have different properties. This concept can be substantiated by the finding of Rchel *et al.*²¹ that each single strain-specific proteinase, which is capable of conveying long-range effects in the host, is exhibited in the respective *C. albicans* strains.

A *C. albicans* proteinase produced by the induction of BSA has been characterized,^{9,11,12,20–26} but a comparison of the APs from the serotype A and B strains has not been reported. In this experiment, we found that in media containing BSA as the sole nitrogen source, secretory APs from both the serotype A (A-207) and B (B-792) strains of *C. albicans* had very similar properties when examined, though the AP producibility of the B-792 strain is greater than that of the A-207 strain. The same results were also obtained when cultured in media containing collagen as the sole nitrogen

source (unpublished data). Most of the properties of the AP were very similar or identical with those of the proteinases already reported.^{22,26–29} However, the molecular weight (43 kDa) and isoelectric point (pH 4.0) were different from those of the CAP,^{23,26,27} KPase,²⁸ and collagenolytic enzyme.²⁹ The stability (100%) to chymostatin and α 2-macroglobulin, and to cysteine of the AP was different from those of the KPase²⁸ and the collagenolytic enzyme.²⁹

Four kinds of N-terminal amino acid sequences in *C. albicans* enzymes have been separately reported.^{30–33} Our result (Gln–Ala–Val–Pro–Val–Thr–Leu–His–Asn–Glu–Gln–Val–) was the same as that of the description by Ganesan *et al.*³¹ It has been reported recently that *C. albicans* secretes nine aspartic proteinases (Saps).³⁴ From the N-terminal amino acid sequences, the APs from the serotype A (A-207) and B (B-792) strains seem to be the Sap 2.

Though *C. albicans* proteinase can attack several kinds of biologically important proteins such as immunoglobulins, α 2-macroglobulin, and zymogens of the regulatory serine proteinases, and complement C3,^{35,36} it has not been reported for the cytokines. In this report, the AP clearly decreased the activities of immunoglobulin G and G-CSF, which is used as an example of a cytokine. Both enzymes also caused a permeability enhancement with the same intensity when injected into guinea pig skin.⁹ Overall, these results show that the serotype A and B strains of *C. albicans* used in this experiment produced an AP with the same physicochemical and biological properties in the culture medium supplemented with BSA as the sole nitrogen source. Therefore, it is presumed that pathogenicity determinants which confer virulence on *C. albicans* include factors related to the species and strains, adherence, dimorphism, toxin and enzyme productions, and cell surface composition.³⁷ These descriptions imply that the virulence of *C. albicans* is a function of multiple factors working jointly to overcome the host defenses. We suggest that the *Candida* extracellular proteinases are one of the important virulence factors, as already pointed out by several authors,^{3–10,14,15,21,22,28,34–37} though the relative importance differs with the strains because of the producibility *in vivo*.¹⁴ Whether this similarity of AP for these serotypes (A and B) can be expanded to all other strains³⁸ remains to be investigated.

REFERENCES

- 1) Hasenclever H. F., Mitchell W. O., *J. Bacteriol.*, **82**, 570–573 (1961).
- 2) Hasenclever H. F., Mitchell W. O., *J. Bacteriol.*, **82**, 578–581 (1961).
- 3) Staib F., *Mycopathol. Mycol. Appl.*, **37**, 345–348 (1969).
- 4) Macdonald F., Odds F. C., *J. Med. Microbiol.*, **13**, 423–435 (1980).
- 5) Rchel R., *Zbl. Bakt. Hyg., I. Abt. Orig. A*, **255**, 524–536 (1983).
- 6) Kwon-Chung K. J., Lehman D., Good C., Magee P. T., *Infect. Immun.*, **49**, 571–575 (1985).
- 7) Ghannoum M., Elteen K. A., *J. Med. Vet. Mycol.*, **24**, 407–413 (1986).
- 8) Cassone A., Bernardis F. D., Mondello F., Ceddia T., Agatensi L., *J. Infect. Dis.*, **156**, 777–783 (1987).
- 9) Kaminishi H., Tanaka M., Cho T., Maeda H., Hagihara Y., *Infect. Immun.*, **58**, 2139–2143 (1990).
- 10) Kretschmar M., Hube B., Bertsch T., Sangland D., Merker R., Schrder M., Hof H., Nichterlein T., *Infect. Immun.*, **67**, 6637–6642 (1999).
- 11) Schreiber B., Lyman C. A., Gurevich J., Needham C. A., *Diagn. Microbiol. Infect. Dis.*, **3**, 1–5 (1985).
- 12) Edison A. M., Manning-Zweirink M., *Infect. Immun.*, **56**, 1388–1390

- (1988).
- 13) Auger P., Dumas C., Joly J., *Sabouraudia*, **21**, 173—178 (1983).
- 14) Borg M., Rüchel R., *J. Med. Vet. Mycol.*, **28**, 3—14 (1990).
- 15) Kobayashi I., Kondoh Y., Shimizu K., Tanaka K., *Microbiol. Immunol.*, **33**, 709—719 (1989).
- 16) Laemmli U. K., Favre M., *J. Mol. Biol.*, **80**, 575—599 (1973).
- 17) Vesterberg O., Svensson H., *Acta Chem. Scand.*, **20**, 820—834 (1966).
- 18) Redinbaugh M. G., Turley R. B., *Anal. Biochem.*, **153**, 267—271 (1986).
- 19) Fukazawa Y., Shinoda T., Tsuchiya T., *J. Bacteriol.*, **95**, 754—763 (1968).
- 20) Staib F., *Sabouraudia*, **4**, 187—193 (1965).
- 21) Rüchel R., Tegeler R., Trost M., *Sabouraudia*, **20**, 233—244 (1982).
- 22) Shimizu K., Kondoh Y., Tanaka K., *Microbiol. Immunol.*, **31**, 1045—1060 (1987).
- 23) Ray T., Payne C. D., *Infect. Immun.*, **58**, 508—514 (1990).
- 24) Ross I. K., Bernardis F. D., Emerson G. W., Cassone A., Sullivan P. A., *J. Gen. Microbiol.*, **136**, 687—694 (1990).
- 25) Banerjee A., Ganesan K., Datta A., *J. Gen. Microbiol.*, **137**, 2455—2461 (1991).
- 26) Remold H., Fasold H., Staib F., *Biochim. Biophys. Acta*, **167**, 399—406 (1968).
- 27) Rüchel R., *Biochim. Biophys. Acta*, **659**, 99—113 (1981).
- 28) Negi M., Tsuboi R., Matsui T., Ogawa H., *J. Invest. Dermatol.*, **83**, 32—36 (1984).
- 29) Kaminishi H., Hagihara Y., Hayashi S., Cho T., *Infect. Immun.*, **53**, 312—316 (1986).
- 30) Lott T. J., Page L. S., Boiron P., Benson J., Reiss E., *Nucleic Acids Res.*, **17**, 1779 (1989).
- 31) Ganesan K., Banerjee A., Datta A., *Infect. Immun.*, **59**, 2972—2977 (1991).
- 32) Hube B., Turver C. J., Odds F. C., Eifert H., Boulnois G. J., Köchel H., Rüchel R., *J. Med. Vet. Mycol.*, **29**, 129—132 (1991).
- 33) Togni G., Sanglard D., Falchetto R., Monod M., *FEBS Lett.*, **286**, 181—185 (1991).
- 34) Monod M., Hube B., Hess D., Sanglard D., *Microbiology*, **144**, 2731—2737 (1998).
- 35) Rüchel R., *Zbl. Bakt. Hyg. A*, **257**, 266—274 (1984).
- 36) Kaminishi H., Miyaguchi H., Tamaki T., Suenaga N., Hisamatsu M., Mihashi I., Matsumoto H., Maeda H., Hagihara Y., *Infect. Immun.*, **63**, 984—988 (1995).
- 37) Ghannoum M. A., Abu-Elteen K. H., *Mycoses*, **33**, 265—282 (1990).
- 38) Fusek M., Smith E. A., Monod M., Dunn B. M., Foundling S. I., *Biochemistry*, **33**, 9791—9799 (1994).