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Purification and Characterization of Extracellular Aspartic Proteinase of *Candida albicans*

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An extracellular proteinase of *Candida albicans* was purified by a combination of 0~75% ammonium sulfate precipitation, DEAE Sepharose Fast Flow ion exchange chromatography, and Sephacryl S-200 HR molecular sieve chromatography. Its molecular weight was approximately 41 kDa on SDS-PAGE and isoelectric point was 4.4. The enzyme was inhibited by pepstatin A. Optimum enzyme activity ranged from pH 2.0 to 3.5 with its maximum at pH 2.5 and a temperature of 45°C. The addition of divalent cations, Ca^{2+}, Zn^{2+} and Mg^{2+}, resulted in no significant inhibition of enzymatic activity. However, some inhibitory effects were observed by Fe^{2+}, Ag^{2+} and Cu^{2+}. With BSA as substrate, an apparent K_m was determined to be 7×10^{-7} M and K_i, using pepstatin A as an inhibitor, was 8.05×10^{-8} M. N-terminal amino acid sequence was QAVPVTLXNEQ. Degradation of BSA and fibronectin was shown but not collagen, hemoglobin, immunoglobulin G, or lysozyme. The enzyme preferred peptides with Glu and Leu at the P_1 position, but the enzyme activity was highly reduced when the P_2 position was Phe or Pro. This enzyme showed antigenicity against sera of patients with candidiasis.

Key words: *Candida albicans*, Aspartic proteinase, Purification, Characterization.

Infection with *Candida albicans*, a normal yeast flora of the gastrointestinal tract and mucosa (22, 25), is ever increasing in the immunocompromised host and in patients undergoing invasive surgical procedures (7). *C. albicans* and some other pathogenic *Candida* species secrete an aspartic (acid) proteinase that can degrade a number of host proteins including albumin, collagen, immunoglobulins, keratin, and hemoglobin (8, 19, 27, 28) and is potentially involved in the pathogenicity of the organism. The evidence of this role has been proved by the fact that the deficiency in the aspartic proteinase has been well correlated with a low level of virulence in some strains of *C. albicans* (11, 14, 26). The proteinase appears to facilitate adherence, growth, and epithelium and tissue invasion (9, 32).

In previous studies, extracellular proteinase of *C. albicans* has been partially characterized by the several investigators (8, 16, 19, 24, 27), but a thorough investigation has been hampered by the lack of purified proteinase. In this study, we have purified the extracellular aspartic proteinase from culture filtrate of *C. albicans* and characterized the biochemical and immunological properties.

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Materials and Methods

Strain and culture condition

Twenty five clinical isolates of *C. albicans* were isolated from Korean patients, identified by using API 20 C AUX Kit (BioMerieux Inc., France) and were used in this study. To select a strain producing high level of proteinase, BSA agar medium containing 0.2% BSA, 1.45 g of yeast nitrogen base (YNB; without ammonium sulfate and amino acids, Difco Laboratories, Detroit, Mich.), 20 g of glucose and 20 g of agar per liter was used. The agar solution was autoclaved, and the 0.22 μm filter-sterilized BSA-YNB broth was added to the cooled (45°C) agar solution. The pH of agar medium was adjusted to 4.0. Twenty five strains were streaked on the plates and incubated at 30°C for 3 days. The amount of proteinase produced by each strain was determined by the size of clear zone of each colony. The strain (KIT 1113) which showed the largest clear zone was selected and used for further study. The selected strain was cultured in BSA-YNB broth as described by Homma *et al* (6).

Purification of enzyme

The enzyme activity in the culture filtrate reached its maximum after 2 days growth. At this stage *C. albicans* cells were removed by centrifugation at

10,000 rpm for 10 min. The culture filtrate was passed through 0.22 μm membrane filters (Gelman Sciences, Ann Arbor, MI) to remove any remaining yeast cells. Culture filtrate was precipitated with ammonium sulfate (75%), centrifuged at 10,000 rpm for 10 min and dialyzed against 50 mM sodium phosphate buffer (pH 6.5). The dialysate was applied to a column (1.6 by 13 cm) of DEAE Sepharose Fast Flow equilibrated with 50 mM sodium phosphate buffer (pH 6.5) and eluted with the same buffer at a flow rate of 40 ml/h. Adsorbed proteins were eluted with 0.1, 0.2, 0.3, and 0.5 M NaCl in stepwise gradient. Eluate (2.5 ml/fraction) was collected for measurement of absorbance at 280 nm and enzyme activity. Fractions containing maximal enzyme activity were pooled, dialyzed against distilled water at 4°C and lyophilized. The partially purified enzyme was further purified by using Sephacryl S-200 HR molecular sieve chromatography (16 by 65 cm) equilibrated with 25 mM Tris-HCl buffer (pH 7.0) containing 10 mM NaCl. The protein concentration was measured by the method of Lowry *et al.* (13) using BSA as the standard.

Proteinase activity assay

Enzyme activity was determined spectrophotometrically following the digestion of BSA as substrate as described by Crandall and Edwards (2). To 30 μl enzyme solution, 270 μl 1% (w/v) BSA in 50 mM KCl-HCl buffer (pH 2.5) was added, and the mixture incubated at 37°C for 2 h. The reaction was then stopped by adding 700 μl ice-cold 10% (w/v) trichloroacetic acid. Precipitated protein was removed by centrifugation at 10,000 rpm for 5 min. The amount of proteolysis was determined by measuring the A_{280} of the supernatant. One unit of enzyme activity was defined as the amount of enzyme needed to increase 0.1 O.D. unit in A_{280}.

SDS-PAGE

SDS-PAGE was performed by the method of Laemmli (12) using 10% (w/v) polyacrylamide gels. Proteins in the gel were stained with 0.1% Coomassie Brilliant Blue R-250 (CBB-R250) in methanol/acetic acid/water (1:2:1, by vol.). To evaluate the reactivity of purified enzyme to concanavalin A (Con A), electrotransfer blot of enzyme was reacted with peroxidase-conjugated Con A (Sigma Chemicals Co., St. Louis, Mo.) diluted 1:200 in PBST for 4 h. The reaction was visualized by incubating the blot with substrate solution of 0.05% 3,3'-diaminobenzidine (DAB) and 0.01% H_2O_2.

Determination of molecular weight

The molecular weight of the purified enzyme was determined by SDS-PAGE. The reference proteins were phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20, 100) and α-lactalbumin (14,400) (Sigma). The native molecular weight of the purified enzyme was determined by molecular sieve chromatography. The purified enzyme was applied to a column (1.6 by 50 cm) of Sephacryl S-200 HR precalibrated with calibration standards. Standard proteins were aldolase (158,000), bovine serum albumin (67,000), and chymotrypsinogen A (25,000) (Pharmacia, Uppsala, Sweden).

Determination of isoelectric point

Isoelectric focusing was carried out on a Pharmacia Phast gel (Pharmacia) containing ampholine of the pH range of 3-10 and calibration kit proteins (Pharmacia) were used. After electrofocusing, the gel was stained and destained.

Determination of optimal pH

The pH optimum of purified enzyme was determined in various buffers (pH range 1.0~7.0). Thirty microliters of enzyme solution was added to 270 μl of 50 mM KCl-HCl buffers (pH 1.0~2.5), 50 mM sodium citrate buffers (pH 3.0~3.5), 50 mM sodium acetate buffers (pH 4.0~5.5), and 50 mM sodium phosphate buffers (pH 6.0~7.0), which then were incubated for 2 h at 37°C with BSA as the substrate. For each pH step, blanks were measured separately.

Determination of optimal temperature and heat stability

To determine the optimal temperature of enzyme activity, thirty microliters of enzyme solution was added to 270 μl of 50 mM KCl-HCl buffer (pH 2.5) and incubated for 2 h at various temperatures from 10°C to 70°C with BSA as the substrate. Heat stability of purified enzyme was assessed by the following method. The purified enzyme in 50 mM KCl-HCl buffer (pH 2.5) was preincubated in a sealed tube for various time intervals at 45°C and 60°C prior to the assay. And then residual enzymatic activity was measured.

Effect of proteinase inhibitors and divalent cations on enzyme activity

The effect of proteinase inhibitors on enzyme activity was examined. The purified enzyme was preincubated at 37°C for 30 min in 50 mM KCl-HCl buffer (pH 2.5) containing inhibitors. Substrate was then added. The reaction mixtures were incubated at 37°C for 2 h and enzyme activity was

measured. The inhibitors used in this study were diisopropyl fluorophosphate (DFP, 100 M), phenylmethyl sulfonylfluoride (PMSF, 1 mM), trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64, 10 M), N-α-p-tosyl-L-lysine-chloromethyl ketone (TLCK, 100 μM), N-tosyl-L-phenylalanine-chloromethyl ketone (TPCK, 100 μM), iodoacetic acid (100 μM), pepstatin A (1 μM), and ethylenediaminetetraacetic acid (EDTA, 5 mM). All the inhibitors were purchased from Sigma. The effect of metal ions on enzyme activity was examined by the following method. The purified enzyme was incubated in 50 mM KCl-HCl buffer (pH 2.5) containing 2, 5 and 10 mM $MgCl_2$, $CaCl_2$, $ZnCl_2$, $FeCl_4$, $CuSO_4$, and $AgNO_3$ at 37°C for 2 h, respectively. Appropriate blanks were treated identically without adding divalent cations.

Kinetic studies

Kinetics were measured at pH 2.5 with BSA as the substrate at concentration between 10^{-3} and 10^{-8} M. The enzyme concentration was constant at 10^{-9} M. K_m and V_{max} were calculated from the Lineweaver-Burk plot of the results. Inhibition kinetics were measured at constant enzyme concentration of 10^{-9} M, and by varying substrate and pepstatin A concentration, ranging between 10^{-3} and 10^{-8} M and 10^{-5} and 10^{-9} M, respectively. K_i was calculated from the Lineweaver-Burk plot of the result.

N-terminal amino acid sequencing

The purified enzyme was subjected to SDS-PAGE. Electrotransfer of proteins to polyvinylidene difluoride (PVDF) membrane was carried out according to the method described previously (15). Analysis of the N-terminal sequence was carried out by the automated Edman degradation with a MilliGen/Biosearch 6600 Prosequence system (Millipore, Bedford, MA).

Substrate specificity

Purified collagen, type I (from bovine achilles tendon), fibronectin (from human plasma), hemoglobin (from human blood), bovine serum albumin and lysozyme (from human) were purchased from Sigma. Immunoglobulin G was purchased from Organon Teknika N.V. Cappel Products. These proteins were dissolved in 50 mM KCl-HCl buffer (pH 2.5) at a concentration of 1 mg/ml. These substrates were incubated with purified enzyme at an enzyme : substrate ratio (1:150) for various time intervals (0 to 12 h) at 45°C. The reactions were stopped by adding an equal volume of denaturing sample buffer (0.125 M Tris-HCl (pH 6.8), 2% SDS, 2% sucrose, 0.1% β-mercaptoethanol) followed by boiling the sample for 2 min. SDS-PAGE was performed by the method described previously. Substrate specificity was further determined by using various chromogenic peptide substrates. The peptide substrates used here were N-Acetyl-Ala-Ala-Pro-Ala β-Naphthylamide, Nα-Benzoyl-Arg-Gly-Phe-Phe-Leu β-Naphthylamide, N-CBZ-Leu-Leu-Glu β-Naphthylamide, N-Succinyl-Gly-Gly-Phe p-Nitroanilide, N-CBZ-Gly-Gly-Leu p-Nitroanilide, N-Acethyl-Ile-Glu-Ala-Arg p-Nitroanilide, Gly-Pro-Leu β-Naphthylamide, N-Succinyl-Ala-Ala-Pro-Phe p-Nitroanilide, N-Succinyl-Ala-Ala-Val p-Nitroanilide, N-Methoxysuccinyl-Ala-Ala-Pro-Met p-Nitroanilide and Nα-Benzoyl-$_{DL}$-Arg β-Naphthylamide. Purified enzyme (7 μg) and peptide substrate (final concentration at 100 μM) were incubated in 50 mM KCl-HCl buffer (pH 2.5) at 37°C for 1 h. After then, changes in absorbance at 400 nm, for p-Nitroanilide, and at 340 nm, for β-Naphthylamide, were determined with spectrophotometer (Beckerman, DU-600).

Immunoblotting

Electrophoretic transfer of proteinase from polyacrylamide gels to nitrocellulose membrane was performed in Tris-Glycine buffer (pH 8.3), as described previously (31). Non-specific sites were blocked with 3% skim milk in PBS-0.5% Tween 20 (PBST, pH 7.2). The blocked sheets were immersed in antiserum diluted 1:1,000 in 3% skim milk in PBST for 2 h with gentle shaking and washed with PBST three times (10 min each wash). The sheets were incubated with peroxidase-conjugated anti-human goat IgG (Sigma) diluted 1:1,000 in PBST for 2 h. The reaction was visualized by incubating the sheets with substrate solution of 0.05% 3,3'-diaminobenzidine (DAB) and 0.01% H_2O_2.

Results

Purification of extracellular proteinase

Purification of an extracellular proteinase from culture filtrate of *C. albicans* was performed with ammonium sulfate precipitation and a series of chromatographic steps. DEAE Sepharose Fast Flow ion exchange chromatography of the ammonium sulfate precipitates yielded one peak of proteolytic activity (Fig. 1). The fractions which showed proteolytic activity were pooled, concentrated and applied to Sephacryl S-200 HR molecular sieve chromatography. The active fractions were pooled, concentrated, and used for further studies. A typical purification step is summarized in Table 1. Fig. 2 shows purified enzyme

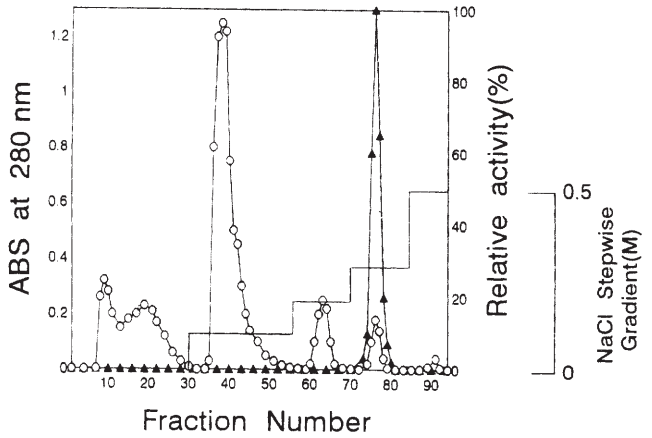

Fig. 1. Elution profile of proteinase on the DEAE Sepharose Fast Flow ion exchange chromatography. The concentrated crude extract obtained from culture filtrate of *C. albicans* was applied to DEAE column (1.6 by 15 cm) preequilibrated with 50 mM sodium phosphate buffer (pH 6.5). The column was eluted with 0.1, 0.2, 0.3 and 0.5 M NaCl in stepwise gradient (–). Fraction volume was 2.5 ml and proteolytic activity assayed (▲) and protein concentration (○) was monitored at 280 nm.

Table 1. Purification scheme of aspartic proteinase of *C. albicans*

	Total volume (ml)	Total protein (mg)	Total activity (Unit)	Specific activity (Unit/mg)	Purification fold	Yield (%)
C.F.[1]	2,000	297.5	17,800	18.3	1	100
A.S.[2]	100	684.4	16,357	23.9	1.3	91.9
DEAE[3]	13	4.7	8,155	1,735.1	94.8	45.8
Gel[4]	3	2.4	6,621	2,758.8	150.8	37.2

[1] Culture filtrate
[2] Ammonium sulfate precipitation
[3] DEAE Sepharose Fast Flow ion exchange chromatography
[4] Sephacryl S-200 HR molecular sieve chromatography

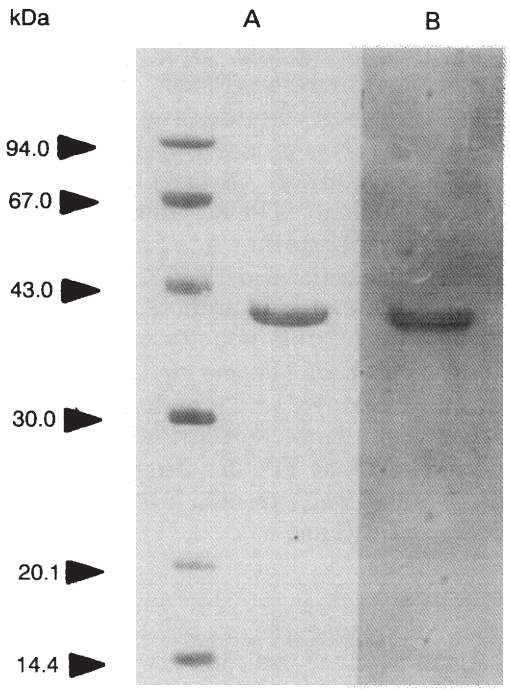

Fig. 2. SDS-PAGE analysis of purified proteinase of *C. albicans*. Lane A, Coomassie blue stain; Lane B, Reaction with peroxidase-conjugated Con A.

on SDS-PAGE. The enzyme had reactivity with Con A; therefore, this enzyme was thought to be a glycoprotein. The molecular weight of the enzyme is approximately 41 kDa on SDS-PAGE and the native molecular weight was about 43 kDa when determined by Sephacryl S-200 HR molecular sieve chromatography (Fig. 3). The isoelectric point was determined to be 4.4 (data not shown).

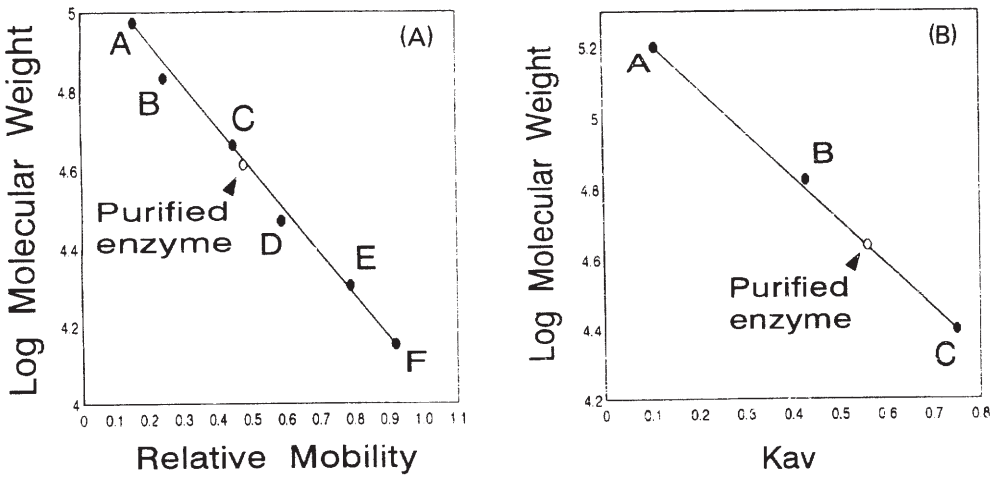

Fig. 3. Molecular weight determination of purified proteinase. (A) Molecular weight determination by SDS-PAGE. A-F are standard proteins, phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100) and α-lactalbumin (14,400), respectively. (B) Molecular weight determination by Sephacryl S-200 HR molecular sieve chromatography. A-C are standard proteins, aldolase (158,000), bovine serum albumin (67,000) and chymotrypsinogen A (25,000), respectively.

Optimal pH

The purified enzyme was most active at pH 2.5 in various buffers (pH 1.0~7.0). The enzyme was active over a narrow pH range of 2.0 to 3.5 and showed no activity below pH 2.0 and above pH 4.0 (Fig. 4A). The enzyme was denaturated and inactivated at alkaline conditions (data not shown). Alkaline denaturation was observed definitely above pH 8.0 with some denaturation at neutral pH. The enzyme activity was not recoverable after alkaline denaturation.

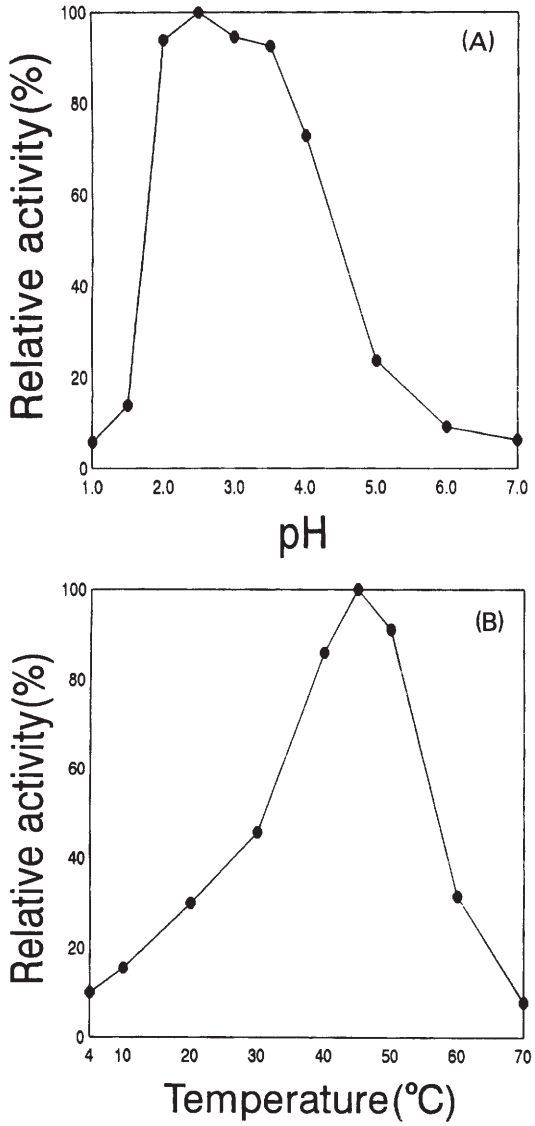

Fig. 4. Optimal pH and optimal temperature of purified enzyme. (A) Optimal pH of purified enzyme. The activity was assayed in 50 mM KCl-HCl buffers (pH 1.0~2.5), 50 mM sodium citrate buffers (pH 3.0~3.5), 50 mM sodium acetate buffers (pH 4.0~5.5) and 50 mM sodium phosphate buffers (pH 6.0~7.0). Maximal activity was shown as 100%. (B) Optimal temperature of purified enzyme. The purified enzyme was incubated at various temperatures for 2 h and then the proteinase activity assayed. Maximal activity was shown as 100%.

Optimal temperature and heat stability

The enzyme exhibited a narrow temperature optimum with a maximum activity at 45°C with inactivation rapidly occurring below 40°C and above 50°C; yet it was not completely inactivated (Fig. 4B). The enzyme became unstable at 45°C and residual activity after 12 h incubation was 22%. At 60°C, the enzyme was completely inactivated after 8 h (data not shown).

Effect of proteinase inhibitors and divalent cations

The effect of a wide spectrum of inhibitors on the activity of enzyme was determined by measuring residual activity following preincubation of the enzyme with inhibitors in 50 mM KCl-HCl buffer (pH 2.5) at 37°C for 30 min (Table 2). The enzyme was inhibited by pepstatin A. However, no significant inhibitions were observed with DFP, PMSF, TPCK and TLCK (inhibitors of serine proteinases), E-64 and iodoacetic acid (inhibitors of cysteine proteinases) and EDTA (inhibitor of metalloproteinases). While the diavalent cations, Ca^{2+}, Zn^{2+}, and Mg^{2+} showed no significant inhibition, the Cu^{2+}, Ag^{2+}, and Fe^{2+} exhibited some inhibitory effect, especially the Fe^{2+} cation. At a concentration of 10 mM of Fe^{2+}, enzyme activity was completely inhibited (Table 3).

Substrate specificity

The purified enzyme degraded BSA and fibronectin in a time dependent manner (Fig. 5). BSA was rapidly degraded; however, the degra-

Table 2. Effect of inhibitors on the activity of proteinase of *C. albicans*

Inhibitor	Concentration (mM)	Relative activity (%)[2]
Control[1]		100.0
PMSF	1	104.7
DFP	0.1	105.8
TPCK	0.1	95.0
TLCK	0.1	91.8
E-64	0.01	102.7
Iodoacetic acid	0.1	104.3
Pepstatin A	0.001	9.9
EDTA	5	87.6

[1] Control represents the activity tested without any inhibitors.
[2] % of control
PMSF, phenylmethyl sulfonylfluoride; DFP, diisopropyl fluorophosphate; TPCK, N-tosyl-L-phenylalanine-chloromethyl ketone; TLCK, N-α-p-tosyl-L-lysine-chloromethyl ketone; E-64, trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane; EDTA, ethylenediaminetetraacetic acid

Table 3. Effect of divalent cations on the activity of proteinase purified from culture filtrate of *C. albicans*

Metal ions	Concentration (mM)	Relative activity (%)[2]
Control[1]		100
$CuSO_4$	2.5	89.8
	5.0	75.6
	10.0	59.5
$AgNO_3$	2.5	112.8
	5.0	94.9
	10.0	75.4
$CaCl_2$	2.5	104.2
	5.0	108.2
	10.0	108.3
$ZnCl_2$	2.5	102.9
	5.0	103.5
	10.0	98.6
$MgCl_2$	2.5	105.2
	5.0	108.1
	10.0	103.1
$FeCl_4$	2.5	73.9
	5.0	50.2
	10.0	0

[1] Control represents the activity tested without any metal ions.
[2] % of control.

dation of fibrinonectin was slow in which the first definite degradation products appeared after 12 h. Immunoglobulin G, collagen type I, lysozyme and hemoglobin were not degraded significantly. The enzyme preferred peptides with Glu and Leu at the P_1 position; however, the enzyme activity was highly reduced when the P_2 position was Phe or Pro (Table 4).

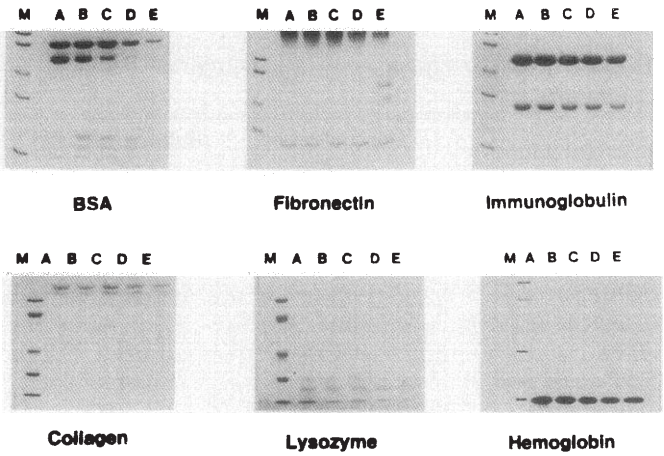

Fig. 5. Substrate specificity of purified proteinase. Each reaction mixture containing proteinase was run under the condition described in materials and methods. Lane A-E, each substrate was incubated with proteinase for 0, 2, 4, 6 and 12 h at 37°C, respectively. Lane M, standard marker proteins.

Table 4. Substrate specifiifty of purified proteinase from *C. albicans* toward various chromogenic peptides

Peptide substrate	Relative activity(%)
N-Acetyl-Ala-Ala-Pro-Ala β-NA	6.4
Nα-Benzoyl-Arg-Gly-Phe-Phe-Leu β-Na	46.2
N-Succinyl-Gly-Gly-Phe p-NA	13.3
N-CBZ-Leu-Leu-Glu β-NA	100.0
N-Acethyl-Ile-Glu-Ala-Arg p-NA	0
N-Succinyl-Ala-Ala-Pro-Phe p-NA	7.0
N-Methoxysuccinyl-Ala-Ala-Pro-Met p-NA	15.3
N-Succinyl-Ala-Ala-Val p-NA	0
N-CBZ-Gly-Gly-Leu p-NA	76.1
Gly-Pro-Leu β-NA	8.3
Nα-Benzoyl-$_{DL}$-Arg β-NA	6.2

Dehydrated chromogenic β-Naphthylamide(β-NA) subsrates and p-Nitroanilide(p-NA) substrates(100 μM) were incubated with purified enzyme(7 μg in 50 mM KCl-HCl, pH 2.5) from *C. albicans* at 37°C for 1 h.

Kinetic studies

Kinetic studies were done with the purified enzyme. V_{max} *and* K_m for BSA were determined by carring out enzyme assays with various concentration of BSA at a constant concentration of enzyme. V_{max} was 1.5×10^{-6} M and K_m was 7.5×10^{-7} M. Inhibitory effect of pepstatin A was also investigated. Pepstatin A exhibited competitive inhibitory effect and *Ki* was 8.05×10^{-8} M (data not shown).

N-terminal amino acid sequence

The result of N-terminal amino acid sequencing of the enzyme is shown in Table 5. N-terminal amino acid sequence was determined as QAVPVTLXNEQ. This showed high homology with the N-terminal amino acid sequence of aspartic proteinases of *C. albicans* ATCC 10231 and *C. albicans* ATCC 10261, but, not with those of *C. albicans* ATCC 2730, *C. tropicalis*, and *C. parapsilosis*.

Immunoblot analysis

Table 5. Comparison of the N-terminal amino acid sequences of extracellular aspartic proteinases from clinically isolated *C. albicans* and other strains

Strain	N-terminal sequences	Reference
C. albicans		
KIT 1113	QAVPVTLXNEQ	In this study
ATCC 10231	QALPVTLNNEHVSYA	Hube *et al.*(5)
ATCC 10261	QAVPVTLHNEQVTYA	Wright *et al.*(34)
CBS 2730	GTVQTSLINE	Morrison *et al.*(17)
C. tropicalis	SDVPTTLINEGP	Togni *et al.*(30)
C. parapsilosis	SSPSSPLYFNGP	De Viragh *et al*(4)

An X in the sequence represents a residue that could not be determined.

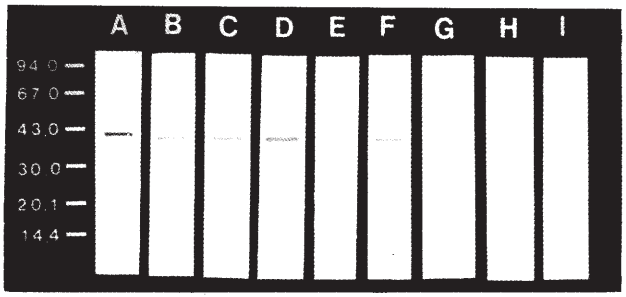

Fig. 6. Immunoblot analysis of purified proteinase of *C. albicans* by antibodies in different serum samples. Lane A-F, serum samples from patients with candidiasis; Lane G-I, normal human sera.

The immunoblot result is shown in Fig. 6. The purified enzyme showed strong antigenicity against sera of candidiasis patients. However, normal sera had no reactivity with the enzyme.

Discussion

Among the member of the genus *Candida, Candida albicans* is of the most important opportunistic pathogen in human. *Candida* invade oral or vaginal mucosa causing oral thrush or vaginal candidiasis, which is commonly encountered as fungal infection (20). A few virulence factors of *Candida* have been described in connection with the invasive candidiasis. The extracellular proteinase is one of the virulence factors responsible for proteolytic invasion by these yeasts (3, 18, 21, 33). Several purification schemes and characterizations by a number of investigators have been described in that the extracellular proteolytic activity of *C. albicans* is largely attributed to a aspartic proteinase, possessing pepsin-like and cathepsin D-like properties (16, 19, 27, 28).

In this study, we have purified aspartic proteinase of *C. albicans* with high purity by using a simplified purification scheme. It was shown to be a glycoprotein with a molecular weight of 41 kDa on SDS-PAGE and isoelectric point of 4.4. The enzyme was inhibited by pepstatin A, an inhibitor of pepsin-like proteinase. The addition of divalent cations, Ca^{2+}, Zn^{2+} and Mg^{2+} showed no significant inhibition at low concentrations. However, Cu^{2+} and Ag^{2+} had some inhibitory effects on enzyme activity at high concentrations. And Fe^{2+} showed a strong inhibitory effect in which at the concentration of 10 mM, a ccomplete inhibition resulted. Neutral pH showed inactivity and alkaline conditions denatured it irreversibly. These properties coincide with the previous studies (16, 19, 23, 27, 29). However, its biochemical properties are highly different from previous reports in two aspects.

First, optimum activity was shown at pH 2.0 to 3. 5 with a maximum at pH 2.5 and ware inactivated rapidly below pH 2.0 and above pH 4.0, while the previous reports revealed optimum pH of 4.0 to 4.5. Second, the enzyme showed narrow substrate specificity. *Candida* aspartic proteinase is a general proteinase with broad substrate specificity; substrates include albumin, hemoglobin, transferrin, casein, immunoglobulins and collagen (24). However, the enzyme purified in this study was able to degrade BSA and fibronectin, but not collagen, hemoglobin, immunoglobulins or lysozyme.

Porcine pepsin, one of the most well known aspartic proteinases, has a strong primary preference for aromatic and hydrophobic amino acids at the P_1 site, especially Phe and Leu. However, this enzyme showed low preference for Phe at the P_1 the site. This enzyme showed high preference for Glu and Leu at the P_1 site and the enzyme activity was highly reduced when the P_2 site was Phe or Pro. When the P_1 site was Arg or Val, no enzymatic activity was detected. Ala and Met showed low preference for enzyme activity. These suggest that this enzyme has some different substrate specificity with pepsin.

Diagnosis of candidiasis, especially systemic candidiasis, is not easy using the serological method due to high cross reactivity with other yeasts and fungi. The fact that the aspartic proteinase of *C. albicans* is an important virulence factor and normally produced by most of pathogenic strains of *C. albicans* suggested that the proteinase of *C. albicans* can be used to serodiagnosis of candidiasis. We investigated the antigenicity of purified enzyme using sera of twenty candidiasis patients by immunoblot. The purified enzyme reacted with all patients' sera but not the sera of normal human. Although, there was a weak reactivity with sera of aspergillosis, another medically important fungal infection (our unpublished data). This may be due to the cross reactivity with aspartic proteinase produced by *Aspergillus* spp. (1, 10). Therefore, more sensitive and specific diagnosis of candidiasis is necessary and may be possible, if highly specific monoclonal antibodies reacting with only aspartic proteinase of *C. albicans* are produced.

Acknowledgment

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