Jpn. J. Med. Mycol.
Vol. 48, 19－25, 2007
ISSN 0916－4804

Original Article

Isolation and Characterization of a Novel Acid Proteinase, Tropiase from *Candida tropicalis* IFO 0589

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〔Received: 13, March 2006. Accepted: 21, September 2006〕

Abstract

A novel acid proteinase (Tropiase) was isolated from *Candida tropicalis* IFO 0589 by DE52-cellulose, and DEAE-Cosmogel column chromatographies. The purified tropiase gave a single band on disc polyacrylamide gel electrophoresis, isoelectric focusing and sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. The enzyme preparation had a molecular weight of 23,900, isoelectric point of pH 5.1, optimum pH range of 7 to 9 and possessed 208 amino acid residues. The enzyme hydrolyzed casein, fibrinogen, keratin and collagen. The purified tropiase demonstrated hemorrhagic and capillary permeability-increasing activities. Inhibition of tropiase occurred with leupeptin and N-bromosuccinimide, however, no inhibition was observed with α_2-macroglobulin, soybean trypsin inhibitor, benzamidine-HCl or diisopropyl fluorophosphate.

Key words: *Candida tropicalis*, acid proteinase, purification

Introduction

Candidiasis is mainly induced by *Candida albicans*, *C. glabrata* or *C. tropicalis*. All three are important in immunocompromised host infection. Obata *et al.*[1] reported that the ratio of isolation for yeast, *C. albicans* was 53.8%. The ratios of other yeasts *C. glabrata*, *C. tropicalis* and *C. parapsilosis* were 12.5%, 5.3%, and 3.4%, respectively[1]. Since 1990, infectious diseases related to *C. glabrata*, *C. tropicalis* and *C. parapsilosis* have increased. Hospital mortality rates from candidemia range between 34～57%[2-4], which is considered high. The most predominant causative species of these high rates were found to be *C. albicans* (52%) and *C. tropicalis* (11%) according to Kazama and Furukawa[5]. Fluconazole resistance of *Candida* species has been reported by Rex *et al.*[6]. Vandeputte *et al.*[7] investigated fluconazole and azole resistance of *C. tropicalis* and determined the molecular mechanisms responsible for azole resistance in a clinical isolate of this pathogenic yeast.

There are conflicting reports in these earlier studies on the proteinases from *C. albicans*[8-13] and their intervention in pathogenicity[14-17]. Neely and Holder[18] reported the effect of proteolytic activity on virulence of *C. albicans* in burned mice. A direct correlation exists between the virulence of *C. albicans*, *C. tropicalis*, and *C. parapsilosis* and the secretion of aspartic proteinases[19].

Negi *et al.*[10] purified a proteinase from *C. albicans* possessing casein and hemoglobin hydrolytic activities. Its molecular weight was 42,000 and was inhibited by pepstatin and α_2-macroglobulin. Ray and Payne[20] purified a *Candida* acid proteinase (CAP) from *C. albicans* which possessed casein, hemoglobin and collagen hydrolytic activities. Its molecular weight was

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41,500.

C. tropicalis is detected in candidiasis, although there are few reports concerning the proteinases of *C. tropicalis*[19, 21, 22]. In this paper, we report the isolation of a novel acid proteinase, tropiase from *C. tropicalis* IFO 0589 and the biological properties of the purified enzyme and their relationship with pathogenicity are discussed.

Materials and Methods

Fungal strains and cultivation conditions

C. tropicalis IFO 0589, AHU 4083, *C. albicans* IFO 1060, IFO 1061, *C. krusei* AHU 3993, *C. parapsilosis* AHU 4336, *C. guilliermondii* AHU 4101 and *C. kefyr* AHU 3221 were provided by the stock center (Institute for Fermentation, Osaka; IFO and the Graduate School of Agriculture and the Faculty of Agriculture, Hokkaido University; AHU) of fungal strains. These strains were grown at 37°C in YCB-BSA broth medium (Yeast carbon base (Difco Laboratories, U.S.A.) 11.7 g/*l* and bovine serum albumin (Nacalai Tesque, Inc, Kyoto, Japan) 2 g/*l*).

Purification of proteinase

C. tropicalis IFO 0589 strain was grown for 7 days at 37°C in YCB-BSA broth medium. Purification of tropiase was achieved by ion-exchange chromatography at 4°C. An entire culture was chromatographed on a DE52-cellulose (Whatman Biosystems, Ltd, England) column (Fig. 1A). The column was eluted with 0.01 M Tris-HCl buffer (pH 7.2) and 0.01 M Tris-HCl buffer (pH 7.2) containing 0.5 M NaCl. Proteinase activity was found in fractions 245-276. These fractions were dialyzed against 0.01 M Tris-HCl buffer (pH 7.2). The dialysate was subjected to column chromatography on DE52-cellulose. The column was eluted with a linear gradient from 0 to 0.5 M NaCl in 0.01 M Tris-HCl buffer (pH 7.2), (Fig. 1B). Proteinase activity was found in fractions 239-262. These fractions were dialyzed against 0.01 M Tris-HCl buffer (pH 7.2), and then concentrated by lyophilization. The lyophilized sample was applied onto DEAE-Cosmogel (Pharmacia, Uppsala, Sweden) (Fig. 1C). The column was eluted with a linear gradient from 0 to 0.5 M NaCl in 0.01 M Tris-HCl buffer (pH 7.2). Proteinase activity was found in fraction 1 and shown to be electrophoretically homogeneous by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis[23].

Fibrinogenase, keratinase and collagenase activities

Fibrinogenase, keratinase and collagenase activities were measured according to the method of Ouyang and Teng[24]. The purified enzyme in 10 mM Tris-HCl buffer (pH 7.2)

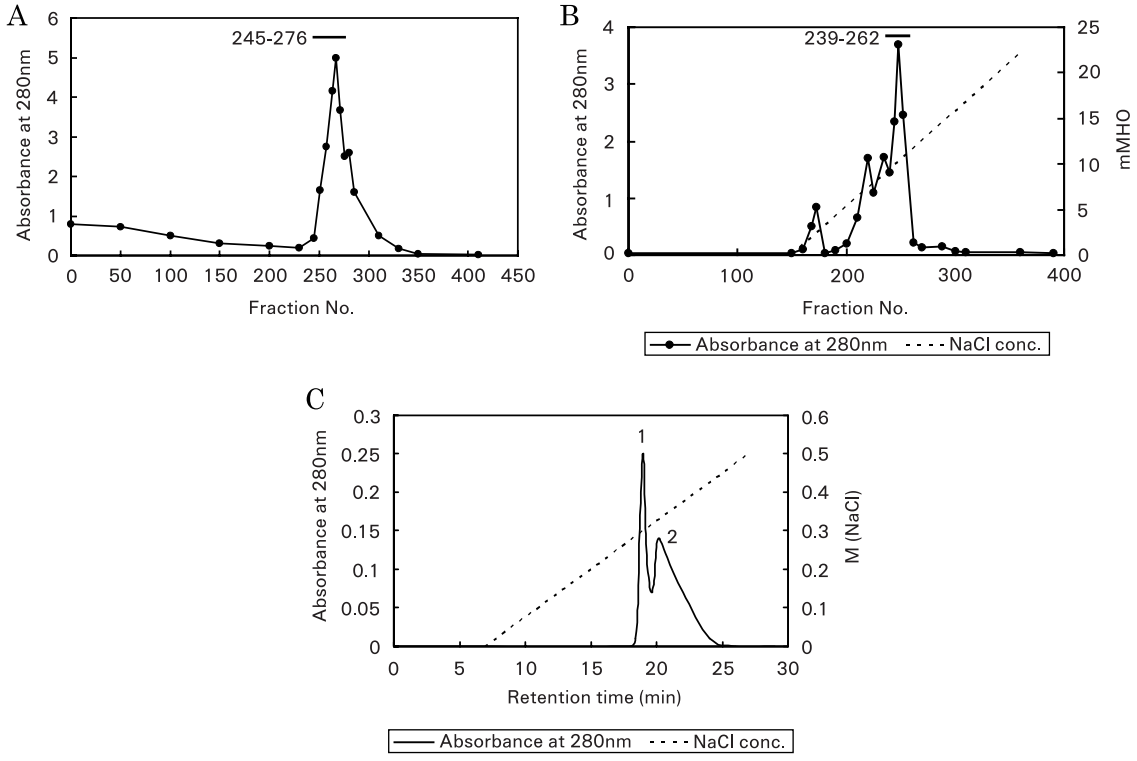

Fig. 1. Column chromatography of cultured medium from *C. tropicalis* IFO 0589. (A) First step: DE52-cellulose column chromatography (B) Second step: DE52-cellulose column chromatography (C) Third step: DEAE-Cosmogel column chromatography. Proteinase activity was found in peak 1.

containing 20 mM NaCl was incubated with fibrinogen (Daiichi Pure Chemicals Co., Tokyo, Japan) (1 mg), keratin (Sigma Chemical Co., St Louis, MO, U.S.A.) (1 mg) or collagen (Wako Pure Chemical Industries, Ltd., Osaka, Japan) (1 mg) at 37°C for various time intervals. Meanwhile, 0.1 m*l* of the incubation mixture was added to 0.1 m*l* of a 10 mM phosphate buffer (pH 7.2) solution containing 10 M urea, 4% sodium dodecyl sulfate and 4% β-mercaptoethanol. This solution was incubated at 37°C overnight followed by electrophoresis on a 12% polyacrylamide slab gel. Electrophoresis was carried out for 4 hrs, starting with a constant current of 25 mA/slab gel, until the tracking dye reached the separating gel, then the current was increased to 30 mA for the remainder of the run.

Assay for proteolytic activity

Casein hydrolytic activity was assayed by the method of Murata *et al.*[25]. Casein (Sigma Chemical Co.) (1.0 g in 50 m*l* of 0.4 M Tris-HCl buffer, pH 8.5) was dissolved by heating for 15 min in a boiling water bath. For determination of casein digestion, 0.5 m*l* of the enzyme solution and the 2% casein solution were incubated for 15 min at 37°C. The reaction was stopped by adding 1 m*l* of 0.44 M trichloroacetic acid, and after standing for 30 min, the mixture was filtered through filter paper. Sodium carbonate (2.5 m*l* of 0.4 M) and Folin reagent (0.5 m*l* diluted to one-third of the original strength) were added to 0.5 m*l* of the filtrate and the developed color was read at 660 nm. One unit is expressed as units/mg protein.

Determination of amino acid composition

The amino acid composition of the carboxyamidemethylated tropiase was determined with an amino acid analyzer JEOL, JLC-300. Samples were hydrolyzed with constant boiling HCl at 110°C for 24, 48 and 72 hrs. Tryptophan content was determined by the method of Edelhoch[26].

N-terminal sequence analysis

N-terminal analysis of the purified enzyme was performed with an Applied Biosystems 491 protein sequencer. The phenylthiohydantoin (PTH) derivatives of amino acids were identified with an Applied Biosystems Model 120A PTH analyzer.

Determination of isoelectric point

The isoelectric point of tropiase was estimated by isoelectric focusing/polyacrylamide gel electrophoresis. The ampholyte concentration was 4% (w/v) with a pH range of 3.5-10.5. Protein standards used were acetylated cytochrome c, pI 10.6, 9.7, 8.3, 6.4, 4.9 and 4.1. Isoelectric focusing was carried out using a constant potential of 200V at 4°C for 4 hrs.

Assay for hemorrhagic activity

A guinea pig was injected subcutaneously in the back with one dose in 0.1 m*l* of 0.9% saline and sacrificed after 1 hr.

Assay for hemolytic activity

Indirect hemolytic activity was assayed by the method of Hendon and Fraenkle-Conrat[27].

Other methods

Hydrolase activities on azocasein (Sigma Chemical Co.) and azoalbumin (Sigma Chemical Co.) were measured by the method of Charney and Tomarelli[28]. Hide powder azure (Sigma Chemical Co.) hydrolytic activity was determined

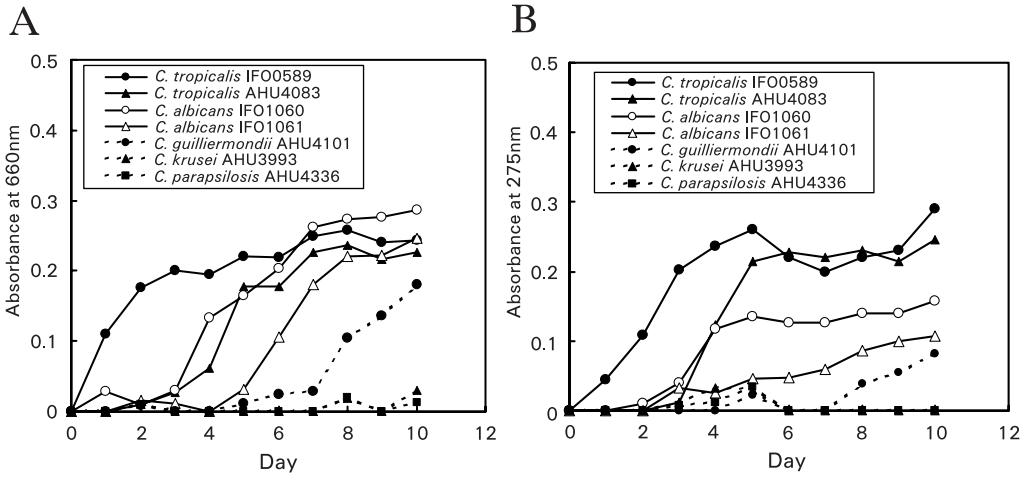

Fig. 2 Proteinase activity of culture filtrate from *Candida* spp. (A) Casein hydrolytic activity. (B) Hemoglobin hydrolytic activity.

according to Rinderknecht *et al.*[29]. Arginine ester hydrolase activity was assayed using tosyl-L-arginine methyl ester as the substrate by the method of Roberts[30].

Capillary permeability-increasing activity was conducted using white rabbits weighing 2-2.5 kg by the method described by Miles and Wilhelm[31]. The assay for kinin-releasing activity was by the method of Trautschold[32] who used the rat uterus.

Results

Casein and hemoglobin hydrolytic activities of *Candida* spp.

C. tropicalis IFO 0589, AHU 4083, *C. albicans* IFO 1060, IFO 1061, *C. krusei* AHU 3993, *C. parapsilosis* AHU 4336, *C. guilliermondii* AHU 4101 and *C. kefyr* AHU 3221 were grown for 10 days at 37°C in YCB-BSA broth medium (YCB 11.7 g/*l* and BSA 2 g/*l*). Each day, 1 m*l* of the culture broth was filtered aseptically through a 0.22 μm pore size membrane filter. The cell-free culture supernatant was used as the source of crude enzyme from which the casein and hemoglobin hydrolytic activities of the culture supernatant were measured (Fig. 2A, B). *C. tropicalis* IFO 0589, AHU 4083 and *C. albicans* IFO 1060, IFO 1061 demonstrated stronger activity for both assays than the other species.

Properties

Properties of tropiase are shown in Table 1. The molecular weight of tropiase was determined to be 23,900 by SDS-polyacrylamide gel electrophoresis with β-mercaptoethanol. The isoelectric point was determined to be 5.1 and the final preparation was called tropiase. The enzyme was incubated at 4°C for 18 hrs at various pHs (pH 3 and 4: 0.1 M citrate buffer, pH 5 and 6: 0.1 M acetate buffer, pH 7, 8, 9 and 10: 0.1 M Tris-HCl buffer, pH 11 and 12: 0.1 M sodium hydrogen carbonate buffer), and the enzymatic activity was determined. The enzyme was found to be stable in the pH range 3-12. The enzyme was incubated at 37, 50, 60, and 100°C for 10 min. Proteinase activity of tropiase was determined to be stable after heating at 100°C for 10 min (pH 7.2). For the determination of carbohydrate content, the anthron reaction of Morris[33] was used. The results were positive, indicating that tropiase is a glycoprotein.

The amino acid composition of reduced and carboxymethylated tropiase and N-terminal amino acid sequence are shown in Table 2a and 2b. Tropiase is composed of 208 amino acid residues based on a molecular weight of 23,900. The N-terminal amino acid of tropiase is leucine.

Tropiase possesses casein (1.7 units/mg), hemoglobin (0.7 units/mg), azocasein (0.2 units/mg), azocoll (0.4 units/mg), fibrinogen, keratin and collagen hydrolytic activities. However, none of the azoalbumin hydrolytic, hide powder azure hydrolytic, arginine methyl ester hydrolase, hemolytic, or kinin-releasing activities were observed. When fibrinogen was incubated with purified tropiase (200 μg), the Aα band disappeared first (at 30 min), followed at a significant time interval by the Bβ band (at 120 min). The γ band was not digested. This

Table 1. Biological propertes of tropiase

Biological properties	Tropiase
Molecular weight	23,900
pI	5.1
Heat stability	Stable (100°C, 10min)
pH stability	Stable (pH 3～12)
Optimum pH	pH 7～9
Sugar	+

Table 2. Amino acid composition and N-terminal amino acid sequence of tropiase

a) Amino acid composition of tropiase

Amino acid	Residue
Cm[1]-Cys	20
Asp	36
Thr[2]	9
Ser[2]	7
Glu	31
Gly	9
Ala	24
Val	8
Met	1
Ile	3
Leu	8
Tyr	7
Phe	8
Lys	14
His	8
Arg	2
Pro	12
Trp[3]	1
Total	208

1) Carboxymethyl-
2) The hydrolysis values for threonine and serine were extrapolated to time zero.
3) Tryptophan content was determined by the method of Edelhoch.

b) N-terminal amino acid sequence of tropiase

NH_2-Leu-Val-Asp-Met-Ala-Lys-X-Asp-Pro-X-Ala-X-X-Tyr-

Table 3. Effect of some inhibitors on tropiase

Inhibitor	Final concentration	Residual activity (%)
α_2-macroglobulin	0.2 mg/ml	97.2
SBTI	0.2 mg/ml	98.6
Leupeptin	0.5 mM	10.5
NBS	1.0 mM	51.8
Benzamidine-HCl	5.0 mM	100.0
DFP	1.0 mM	99.3

SBTI: soybean trypsin inhibitor
NBS: N-bromosuccinimide
DFP: Diisopropyl fluorophosphate

indicates that the purified tropiase degrades the Aα chain first, followed by the Bβ chain, and that both are degraded without the formation of fibrin. Purified tropiase (200 μg in 0.1 m*l* of saline) was injected s.c. into a guinea pig resulting in hemorrhage. When the purified enzyme (200 μg) was injected intradermally, an increase in capillary permeability was observed.

The effects of various reagents (incubated for 10 min, 37°C) on the casein hydrolytic activity of tropiase were investigated (Table 3). Inhibition was observed by leupeptin (inhibition of 89.5%) and NBS (48.2%), but were unaffected by α_2-macroglobulin, soybean trypsin inhibitor, benzamidine-HCl or diisopropyl fluorophosphate.

Discussion

The final preparation had the same molecular weight by SDS-polyacrylamide gel electrophoresis with and without β-mercaptoethanol. This result indicates that tropiase consists of one polypeptide chain. Tropiase is composed of 208 amino acid residues based on a molecular weight of 23,900. The inhibition test indicated that serine (by leupeptin) and tryptophan (by NBS) play an important role in the activity of this proteinase. Tropiase degrades the Aα chain of fibrinogen first, followed by the Bβ chain, and then both are degraded without the formation of fibrin. Fibrinogenase from *C. albicans* also degraded the Aα chain and Bβ chain[34]. This indicates that the cleavage sites of Aα and Bβ chains are different from thrombin. Fibrinogenase from *C. albicans*[34] and tropiase from *C. tropicalis* exhibit fibrinogen hydrolytic activity, however, neither demonstrate clotting activity. From these data it is apparent that when the proteases enter the bloodstream considerable amounts of fibrinogen are degraded. This would result in a tendency for excessive bleeding. Tropiase is stable in heat (100°C, 10 min) and pH (pH 3～12), and hydrolyzed collagen type I, II, III and keratin. The number of carboxymethyl cysteines was 20 and appeared to possess a stable structure for heat or pH. The enzyme might intervene in the pathogenic process of candidiasis caused by the strain IFO0589.

Zaugg *et al.*[22] isolated four secreted aspartic proteinases from *C. tropicalis* (Sapt1p, Sapt2p, Sapt3p, Sapt4p), which had optimum pHs of 3.5 (Sapt1p) and 5.0 (Sapt2p, Sapt3p). These enzymes were inhibited by pepstatin A. Their molecular weights were 44,000 (Sapt1p), 48,000 (Sapt2p) and 49,000 (Sapt3p), respectively. The N-Terminal amino acid of Sapt1p, Sapt2p, Sapt3p and Sapt4p is methionine. Pichova *et al.*[35] purified an aspartic proteinase from *C. tropicalis* and its N-terminal amino acid was determined to be serine. However, the N-terminal amino acid of tropiase was leucine which differs from the proteinases that have been investigated. The primary structure of the aspartic proteinase, SAPT, isolated from *C. tropicalis* was determined by Symersky *et al.*[36]. The N-terminal amino acid sequence tropiase has no homology in the amino acid sequences of Sapt1p, Sapt2p, Sapt3p, Sapt4p and SAPT. From the results shown by amino acid sequence and heat stability, tropiase should not be an autolysate of SAPT. Additionally, the biochemical properties, the N-terminal amino acid sequence, the biological activities between tropiase and the enzymes isolated from *C. albicans* or *C. tropicalis* differ dramatically leading to the conclusion that tropiase is a novel proteinase.

At this time it is unclear whether tropiase is specific to IFO 0589. In order to resolve this question, proteinases from multiple strains would need to be investigated to determine the presence of tropiase. More extensive research is required to investigate the relationship between pathogenicity and the biological properties of tropiase in order to fully understand the physiological functioning of this enzyme in *C. tropicalis*.

Acknowledgements

This research was financially supported by a Grant-in-Aid for a High-Tech Research Center Project from the Ministry of Education, Culture, Sports, Science and Technology of Japan, which is gratefully acknowledged. We are also grateful to Dr. Kazuhito Kamiya (Aichi Prefectural College of Nursing & Health) for support.

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