Yonsei Medical Journal
Vol. 38, No. 3, pp. 178~186, 1997

Seroreactivities of Proteinases of *Candida albicans*, *C. tropicalis*, and *C. parapsilosis* in Sera from Various *Candida* species-Infected Mice

Kyoung-Ho Lee, Woon-Seob Shin, Hyun-Sook Park, Joo Young Park, and Choon-Myung Koh

From the culture filtrates of C. albicans, C. tropicalis and C. parapsilosis, proteinases were purified using a series of chromatographic steps consisting of DEAE-Sepharose, Sephacryl S-200 and size-exclusion HPLC which removed contaminating mannoproteins and extraneous proteins. Anti-Candida proteinase antibodies in sera from mice infected with various Candida species were detected using ELISA for serodiagnosis of candidiasis. Three proteinases were blotted by homologous and heterologous anti-proteinase antisera on Western blot analysis. All sera from six Candida species-infected mice were reactive with proteinases of C. albicans, C. tropicalis, and C. parapsilosis, although C. glabrata, C. guilliermondii, and C. krusei did not secrete proteinase. The seroreactivities of proteinase with sera from mice infected with homologous C. albicans and C. tropicalis were higher than those with sera from heterologous Candida species-infected mice. These results suggest that three proteinases have at least one common epitope, but its application for diagnosis of candidiasis should be considered with limits of specificity.

Key Words: *Candida* proteinase, seroreactivity, HPLC, common epitope

Several *Candida* species are dimorphic fungi that commensally colonize the cutaneous and mucosal surfaces of humans. In settings in which the epithelial barrier or host immunity, or both, are altered or suppressed, the fungi cause both superficial and deep-seated diseases (Musial *et al.* 1988; Greenfield, 1992). Proposed virulence factors of the pathogenic *Candida* species are the ability to form hyphae (Sobel *et al.* 1984; Shepherd, 1985; Buckley *et al.* 1986; Hubbard *et al.* 1986), to adhere to epithelial cell surfaces (Calderone *et al.* 1985; Lehrer *et al.* 1986; Saxena *et al.* 1989), and to secrete an acid proteinase (Macdonald and Odds, 1983; Kwon-Chung *et al.* 1985; Ross *et al.* 1990; Louie *et al.* 1994). Of those, the proteinase facilitates the organism in invading and colonizing host tissues (Borg and Rüchel, 1988; Ray and Payne, 1988) and in assimilating nitrogen from proteinaceous sources (Negi *et al.* 1984). Among *Candida* species, *C. albicans*, *C. tropicalis* and *C. parapsilosis* are frequently isolated in patients suffering from candidiasis. Proteinases secreted by them have been found and they

Received May 19, 1997
Accepted June 26, 1997
Department of Microbiology and Institute of Basic Medical Science, Yonsei University Wonju College of Medicine, 162 Ilsan-Dong, Wonju, Kangwon-Do, 220-701, Korea
This study was supported by a grant (# HMP-96-M-2-1060) of the '96 Good Health R&D Project, Ministry of Health and Welfare, Republic of Korea.
Address reprint request to Dr. C.M. Koh, Department of Microbiology and Institute of Basic Medical Science, Yonsei University Wonju College of Medicine, 162 Ilsan-Dong, Wonju, Kangwon-Do, 220-701, Korea

produced proteinase to a greater extent than did other *Candida* species (Rüchel *et al.* 1992).

Because of difficulties in distinguishing *Candida* colonization from *Candida* invasion and because of the low rate of positive blood cultures in patients with invasive disease, there has been interest in the serodiagnosis of invasive candidiasis (Matthews, 1996). At present, the detection of anti-*Candida* antibodies, *Candida* antigens and metabolites such as mannose and arabinitol in sera are being studied for the diagnosis of infection due to *Candida* species (Jones, 1990). Of those, the use of proteinase for the immunodiagnosis of candidal infection has been described (Rüchel and Boning, 1983; Rüchel *et al.* 1988). The advantage of a test using a virulence factor as a marker for infection lies in its potential to differentiate between simple colonization and invasive disease. In addition, clinical isolates are more virulent and produce larger amounts of proteinase than commensal isolates (de Bernardis *et al.* 1990; Rüchel *et al.* 1992; Ollert *et al.* 1995). However, antibodies used in these tests are only as specific as the purity of the antigen used to elicit them. Therefore, proteinase should be free of contaminating cell wall protein and extraneous proteins which reduce specificity.

To eliminate the possible complications associated with the presence of contaminating proteins in proteinase preparations, the proteinases of *C. albicans, C. tropicalis* and *C. parapsilosis* were purified from culture filtrate using a chromatographic series consisting of DEAE-Sepharose, Sephacryl S-200 and size-exclusion high performance liquid chromatography (HPLC). With these purified proteinases, we examined antigenic cross-reactivity among the three proteinases using Western blot analysis and also investigated whether *Candida* proteinase could be a diagnostic marker for detecting anti-proteinase antibodies from mice infected with several *Candida* species.

MATERIALS AND METHODS

Organism and proteinase production

Candida albicans ATCC 10231, *C. tropicalis* ATCC 14056, *C. parapsilosis* ATCC 7330, *C. glabrata* ATCC 38326, *C. guilliermondii* ATCC 56822, and *C. krusei* ATCC 2159, were obtained from the National Institute of Health, Seoul, Korea.

These were precultured on YEPD broth composed of 1%(w:v) yeast extract, 2%(w:v) peptone and 2%(w:v) glucose at 37°C for 24 h, transferred into fresh YEPD medium, and incubated at 37°C for 4 h. The culture was centrifuged and cells washed twice with 0.9%(w:v) NaCl. For production of *Candida* proteinase, yeast cells were cultured on bovine serum albumin (BSA) broth composed of yeast carbon base (Difco Laboratories, Detroit, MI, USA), 0.2% (w:v) BSA (Sigma Chemical Co., St Louis, Mo, USA), 0.01%(w:v) yeast extract (Difco), and additional 0.1%(w:v) glucose. Cultures were maintained with slight agitation in a reciprocal shaker at 37°C for up to 72 h. At intervals, *aliquots* were harvested and assayed for growth and proteinase activity.

Proteinase activity assay

Candida proteinase activity was determined spectrophotometrically following the digestion of BSA as substrate as described by Crandall and Edwards (1987). To 100 μl culture supernatant, 300 μl 1% BSA in 50 mM sodium citrate buffer (SCB), pH 3.4, was added, and the mixture was incubated at 37°C for 60 min. The reaction was then stopped by adding 800 μl cold 10% trichloroacetic acid (TCA). Precipitated protein was removed by microcentrifugation at 12,000 rpm for 5 min followed by filtration through a 0.45 μm filter. The amount of proteolysis was determined by measuring the absorbance of the filtrate at 280 nm. Control tubes containing 100 μl culture supernatant were incubated for 60 min at 37°C, followed by the addition of 800 μl TCA and then 300 μl BSA.

Purification of proteinase

When proteinase activity reached a maximum, the culture supernatant was collected by centrifugation at 5,000×g for 10 min, filtered through a 0.45 μm porosity membrane and concentrated by vacuum evaporation. For desalting and further concentration of the filtrate, ultrafiltration (molecular mass cut-off, 10 kDa) was performed by adding distilled water.

A DEAE-Sepharose (Sigma) column (2.5×30 cm) was packed to 90 ml capacity and equilibrated with 20 mM SCB, pH 6.3. Concentrated filtrates were applied to the column and washed with 1,000 ml of column buffer. Elution buffer (200 mM SCB, pH 6.3) was applied and 5 ml fractions were collected. Fractions from the resultant single A280 peak, containing proteinase activity, were pooled and concentrated by vacuum evaporation.

Concentrated, pooled fractions from the DEAE-Sepharose chromatography were applied to a column packed with Sephacryl S-200 (Pharmacia AB Biotechnology, Uppsala, Sweden) gel of 50 ml capacity. The column was equilibrated with 20 mM SCB, pH 6.3, charged with the sample, and eluted with the same buffer. Fractions of 5 ml were collected.

Fractions from a single A280 peak, containing proteinase activity, collected from gel filtration, were applied to a size-exclusion HPLC column, a YMC-Pack Diol-120 column (8.0×500 mm: YMC Co., Kyoto, Japan) that was equilibrated with 20 mM SCB, pH 6.3. HPLC was performed at a flow rate of 1 ml/min. The protein peaks were monitored by A280.

Enzyme-linked immunosorbent assay

An indirect solid phase enzyme-linked immunosorbent assay for the detection of antibodies against *Candida* proteinase was performed as follows: Polystyrene microplates were coated overnight at 4°C with 50 μl of purified proteinase (5 μg/ml in 0.1 M carbonate-bicarbonate buffer, pH 9.8), and blocked by incubation with 1%(w : v) BSA in PBST [50 mM potassium phosphate, pH 7.2, containing 50 mM NaCl and 0.05%(v : v) Tween-20] for 1 h at 37°C. All subsequent steps were performed at room temperature with 50 mM PBS which was employed for dilution and rinsing. Mouse antiserum (50 μl of a 1 in 1,000 dilution) was added and after 2 h at 37°C the wells were rinsed twice with PBS. Rabbit anti-mouse IgG peroxidase conjugate (Sigma; 100 μl of 1 in 2,000 dilution) was added followed by incubation for 1 h at 37°C and rinsing as above. The substrate mixture (100 μl; 50 mM citric acid, 100 mM Na_2HPO_4, 2 mM H_2O_2, 0.4 mg/ml orthophenylenediamine) was added for color reaction. The reaction was stopped after 15 min by addition of 100 μl of 2.5 N sulfuric acid. Absorbance was measured at 490 nm with ELISA reader (Dynatech, Alexandria, VA, USA). Each sample was measured in triplicate and normal mice sera was used as a control.

SDS-PAGE and immunoblotting

SDS-PAGE was performed by the method of Laemmli (1970) using 10%(w : v) polyacrylamide gels. After electrophoresis, a piece of wet nitrocellulose membrane was laid on top of the gel and the layers were assembled into a sandwich between six sheets of transfer-buffer soaked filter paper. The SDS-PAGE resolved bands were electrotransferred onto the nitrocellulose membrane at 100 V for 1.5 h. Following this, the nitrocellulose membrane was blocked by incubation with 2%(w:v) BSA in PBST. The membrane was probed for 2 h with an 1 in 500 dilution of mouse anti-proteinase antiserum raised against purified proteinase from *C. albicans, C. tropicalis* and *C. parapsilosis.* Following rinsing with PBST, the blot was incubated with a 1 in 2,000 dilution of secondary horse-radish peroxidase conjugated rabbit anti-mouse IgG. After the membrane was rinsed as above, the blot was developed with 4-chloro-1-naphthol (Bio-Rad Laboratories, Richmond, CA, USA) and H_2O_2.

Experimental infection

Specific pathogen-free female ICR mice, 10 to 12

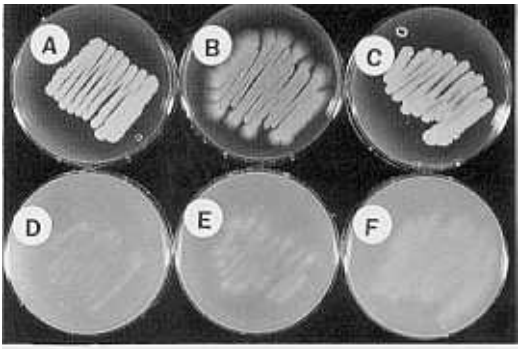

Fig. 1. Photograph of proteolysis on BSA agar plate by Candida species. C. albicans (A), C. tropicalis (B), C. parapsilosis (C), C. glabrata (D), C. krusei (E) and C. guilliermondii. Each Candida species was incubated at 37°C for 15 days.

weeks of age, were infected by subcutaneous injection with 1×10^5 colony-forming units (CFU) of various *Candida* species (*C. albicans, C. glabrata, C. guilliermondii, C. krusei C. tropicalis,* and *C. parapsilosis;* n=2) in 0.2 ml of PBS. Sera were collected by tail-bleeding at 3, 4, and 5 weeks after infection and seroreactivity was tested by ELISA.

General signs of infection, e.g. decrease in activity, loss of hair, were shown in the infected mice. After termination of blood sampling, establishment of *Candida* infection was verified by isolation of microorganisms in culture and touch smearing from the infected site.

Immunization of mice with proteinases

Purified proteinases, in complete adjuvant, were used to immunize specific pathogen-free, female BALB/c mice at an age of 10 to 12 weeks. Mice were immunized by intraperitoneal injection (0.5 ml) and subcutaneous injection (0.2 ml) of the antigen-complete Freund's adjuvant immersion containing 50 μg purified proteinase and boosted twice by intraperitoneal injection of 20 μg purified proteinase in incomplete Freund's adjuvant at an interval of 2 weeks. After 2 weeks, immunoblotting was done for specific anti-*Candida* proteinases.

RESULTS

Screening of proteinase-secreting *Candida* species

C. albicans, C. tropicalis, C. parapsilosis, C. gla-

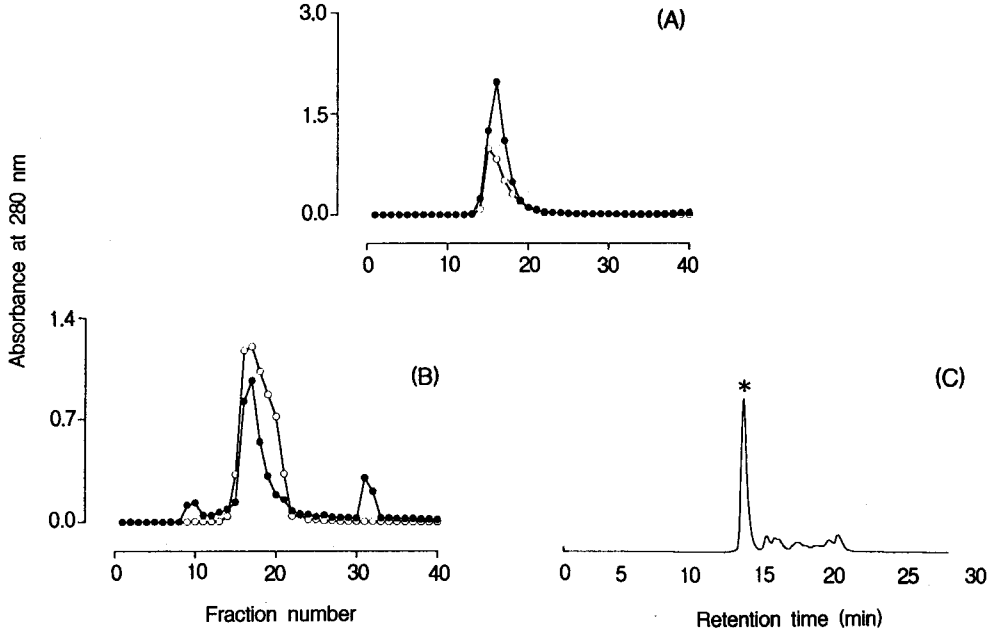

Fig. 2. *Chromatographic profiles of the proteinase from culture filtrate of C. albicans. (A) DEAE-Sepharose column chromatography. The column (2.5×30 cm; 90 ml of packed resin) was equilibrated with 20 mM SCB, pH 6.3 and eluted with 200 mM SCB, pH 6.3. Fractions of 5 ml were collected. Protein concentration (●) was measured at 280 nm. Proteolytic activity (○) was measured by the amount of albumin digested. (B) Sephacryl S-200 column chromatography of pooled proteinase fractions from DEAE-Sepharose column chromatography. The column (1.0×75 cm; 50 ml of packed gel) was equilibrated with 20 mM SCB, pH 6.3 and eluted with the same buffer. Fractions of 5 ml were collected. (C) Size-exclusion HPLC profile of pooled proteinase fractions from Sephacryl S-200 column chromatography. The YMC-Pack Diol-120 column was equilibrated with 20 mM SCB, pH 6.3. HPLC was performed with the same buffer at a flow rate of 1.0 ml per min. The peak fractions monitored by absorbance at 280 nm were collected and measured for proteolytic activity. Star symbol (*) represents the proteinase peak which had a retention time of 13.99 min and molecular mass of 43 kDa.*

brata, C. guilliermondii, and *C. krusei* were cultured on BSA agar plate at 37°C for 15 days. Proteinases secreted by *C. albicans, C. tropicalis and C. parapsilosis* allowed a clear zone to form around colonies on the BSA agar plate. In *C. glabrata, C. guilliermondii,* and *C. krusei,* the clear zone was not demonstrated (Fig. 1).

Purification of *Candida* proteinases

The proteinases of three *Candida* species were purified from culture filtrate using a chromatographic series consisting of DEAE-Sepharose, Sephacryl S-200 and size-exclusion HPLC (Fig. 2~4).

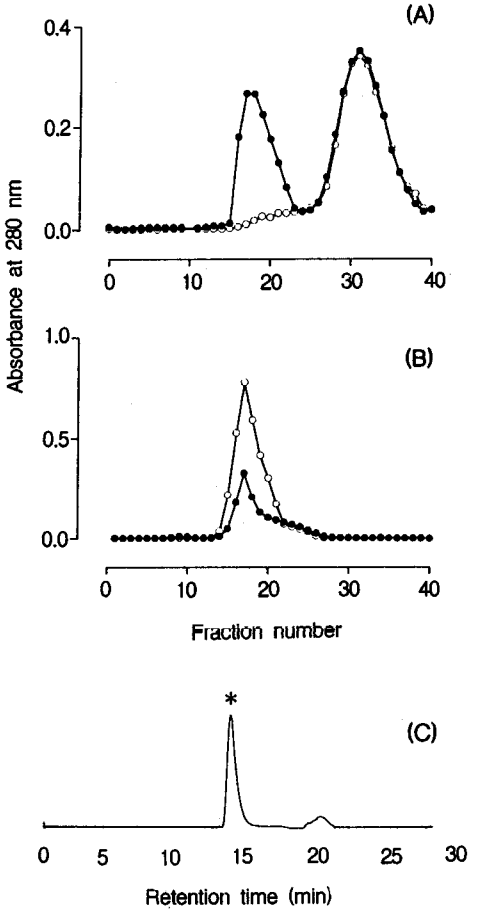

Fig. 3. Chromatographic profiles of the proteinase from culture filtrate of C. tropicalis. (A) DEAE-Sepharose column chromatography. (B) Sephacryl S-200 column chromatography of pooled proteinase fractions from DEAE-Sepharose column chromatography. (C) Size-exclusion HPLC profile of pooled proteinase fractions from Sephacryl S-200 column chromatography. Star symbol (*) represents the proteinase peak which had a retention time of 14.62 min and molecular mass of 36 kDa. Methods are described in Fig. 2. ●, Protein concentration; ○, Proteolytic activity.

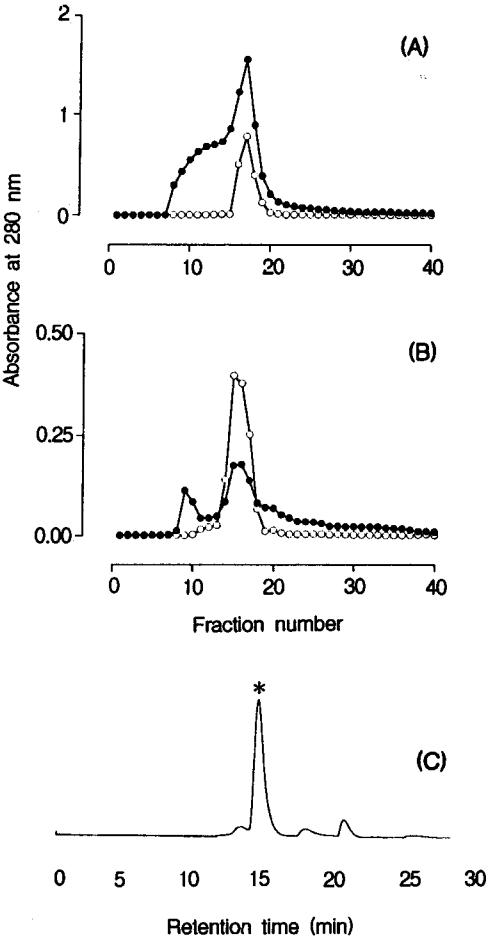

Fig. 4. Chromatographic profiles of the proteinase from culture filtrate of C. parapsilosis. (A) DEAE-Sepharose column chromatography. (B) Sephacryl S-200 column chromatography of pooled proteinase fractions from DEAE-Sepharose column chromatography. (C) Size-exclusion HPLC profile of pooled proteinase fractions from Sephacryl S-200 column chromatography. Star symbol (*) represents the proteinase peak which had a retention time of 14.97 min and molecular mass of 33 kDa. Methods are described in Fig. 2. ●, Protein concentration; ○, Proteolytic activity.

DEAE-Sepharose chromatography gave a single peak of proteinase activity eluting at 75~95 ml in *C. albicans*, 140~180 ml in *C. tropicalis* and 80~100 ml in *C. parapsilosis*. The fractions containing proteolytic activity were pooled and applied to a Sephacryl S-200 column chromatography and a single peak of proteinase activity was eluted at 75~105 ml in *C. albicans*, 80~110 ml in *C. tropicalis* and 85~120 ml in *C. parapsilosis*. Finally, purified proteinases were obtained through size-exclusion HPLC of the highest proteinase fractions on DEAE-Sepharose chromatography. The retention time of proteinase was 13.99 min in *C. albicans*, 14.62 min in *C. tropicalis* and 14.97 min in *C. parapsilosis*.

Difference in molecular mass between HPLC and SDS-PAGE

The molecular mass of proteinase was calculated by retention time on size-exclusion HPLC and was 43 kDa in *C. albicans*, 36 kDa in *C. tropicalis* and 33 kDa in *C. parapsilosis* (Fig. 2~4). However, on SDS-PAGE, the molecular mass was 45 kDa in *C. albicans*, 48 kDa in *C. tropicalis* and 38 kDa in *C. parapsilosis* (Fig. 5). The molecular mass on SDS-PAGE was larger than that on HPLC and the proteinase sizes of *C. albicans* and *C. tropicalis* were reversed.

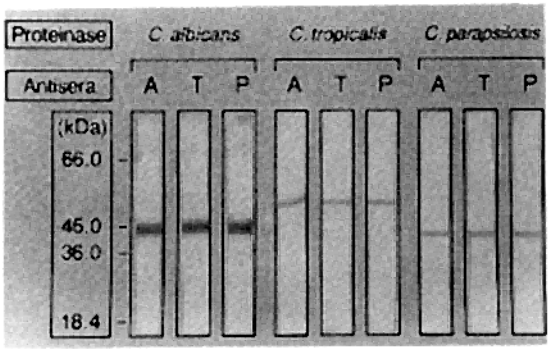

Fig. 5. Immunoblot analysis of the purified proteinases as antigen tested with homologous and heterologous anti-proteinase antibodies. Lane: A, anti-C. albicans proteinase antibody; T, anti-C. tropicalis proteinase antibody; P, anti-C. parapsilosis proteinase antibody.

Immunoblot analysis of *Candida* proteinases.

Immunoblot analysis was used to investigate antigenic differences among *Candida* proteinases. Proteinases secreted by the three *Candida* species

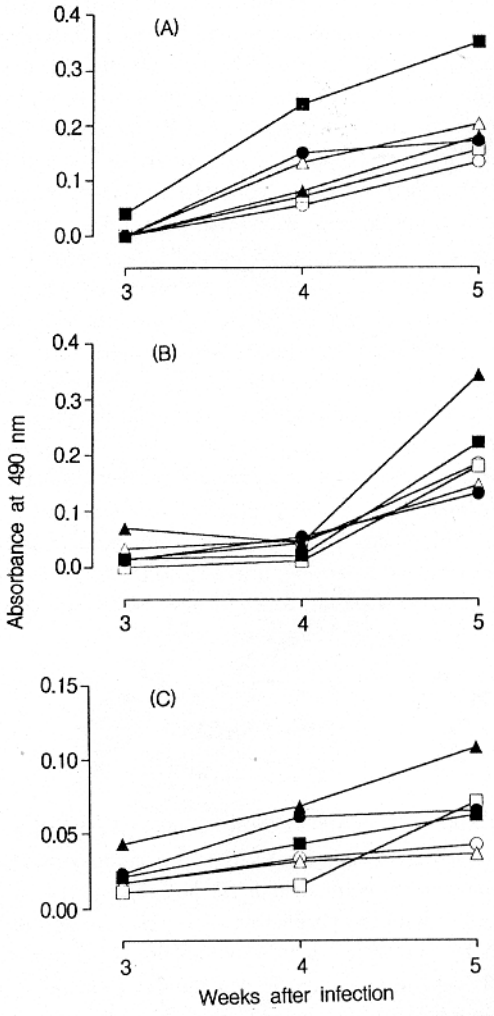

Fig. 6. Seroreactivity of Candida proteinase with sera from mice infected with various Candida species in ELISA. (A) C. albicans proteinase as antigen; (B) C. tropicalis proteinase as antigen; (C) C. parapsilosis proteinase as antigen. Sera obtained from ICR mice (n = 2) infected with C. albicans (■), C. tropicalis (▲), C. parapsilosis (●), C. glabrata (□), C. guilliermondii (△) and C. krusei (○) by subcutaneous injection (1x105 CFU / 0.2 ml). Absorbance was measured at 490 nm. Each sample was measured in triplicate and was corrected for background with normal mice sera.

were related to each other antigenically since they were blotted by homologous and heterologous anti-proteinase antisera (Fig. 5).

Detection of antibody reacting *candida* proteinases in various *candida* species infected mice sera

In the mouse model, anti-*Candida* proteinase antibodies were detected in sera using an ELISA (Fig. 6). Sera from six *Candida* species-infected mice were reactive with proteinases of *C. albicans, C. tropicalis* and *C. parapsilosis* although only three *Candida* species (*C. albicans, C. tropicalis* and *C. parapsilosis*) secreted proteinases. The seroreactivities of proteinase with sera from mice infected with homologous *C. albicans* and *C. tropicalis* were higher than those with sera from mice infected with heterologous *Candida* species. With sera from *C. parapsilosis*-infected mice, however, the seroreactivities of homologous proteinase were similar to those with sera from mice infected with the different *Candida* species.

The seroreactivities increased in the course of infection after inoculation with *Candida* species. The differences in seroreactivity between homologous sera and heterologous sera also increased as the infection processed. The greatest difference was shown at the fifth week of infection.

DISCUSSION

Unpurified culture filtrate contained significant amounts of contaminating cell wall mannoprotein and extraneous proteins which, if not removed, could interfere with the production of *Candida* proteinase-specific antibodies. It is important to use a purified proteinase in studying the detection of proteinase or anti-proteinase in infected tissues, body fluids including sera, or on the surface of the *Candida* cell because the results of the study may be misunderstood.

The purification method of *Candida* proteinase used a combination of molecular sieve chromatography and ion-exchange chromatography, or either of these individually. But there were contaminating proteins in the resultant through these chromatographic series from *C. albicans* (Rüchel *et al.* 1982; Negi *et al.* 1984; Ray and Payne, 1990). After the above purification scheme, rechromatography using anion-exchange column (DEAE-Sephadex A25) resulted in the removal of detectable mannoproteins and extraneous proteins by dot blot and Western blot analysis (Morrison *et al.* 1993).

We adopted HPLC instead of the anion exchange rechromatography for purifying *Candida* proteinases. HPLC produces separation with considerably higher resolutions than conventional column matrices and is used for difficult separations requiring high resolution for success, such as purification of proteins or polypeptides to homogeneity. And the time required for purifying is shorter per cycle on HPLC. Size-exclusion HPLC was performed followed by the commonly used chromatographic scheme to eliminate possible complications associated with the presence of other contaminating proteins in proteinase preparations, and we finally purified three proteinases from culture filtrates of *C. albicans, C. tropicalis* and *C. parapsilosis* by removing the mannoprotein mentioned previously. When anion-exchange HPLC using a DEAE-5PW column was substituted for size-exclusion HPLC, contaminating matter was also successfully removed. However, it was difficult to remove autolytic products of proteinases using a DEAE-5PW column if autolysis proceeded in the course of the purification because of the similarity of ionic strength between proteinase and its fragments (data not shown). As a result, our purification scheme, including the chromatographic series composed of gel filtration and anion-exchange chromatography following size-exclusion HPLC, is recommended to purify *Candida* proteinases.

With three purified proteinases, we examined antigenic relationships among them using Western blot analysis. Western blot analysis showed that all three proteinases were blotted by homologous and heterologous mice antisera. It indicates that three *Candida* proteinases are antigenically related to each other and have at least one common epitope. In the mouse model, we detected anti-proteinase antibodies from mice infected with several *Candida* species. Sera from various *Candida* species infected mice were reactive with proteinases of *C. albicans, C. tropicalis* and *C. parapsilosis.* The seroreactivities of proteinase with sera from mice infected with homologous *C. albicans* and *C. tropicalis* were higher

than those with sera from mice infected with heterologous *Candida* species. This indicates that the detection of anti-*Candida* proteinase antibody in sera could be useful in the diagnosis of candidal infection in establishing an appropriate cut-off value for distinguishing between *Candida* species.

In the present study, however, sera from mice infected with *Candida* species which did not secrete a proteinase were also reactive with proteinases. And *C. parapsilosis* proteinase was reactive with sera from the different *Candida* species-infected mice as well as sera from mice infected with the same organism. This arose from one, or from a combination, of the following reasons why sera from proteinase-nonsecreting organism infections were reactive with *Candida* proteinases. First, in another study with a sensitive, fluorescent assay rather than BSA digestion assay for proteinase activity which we adopted, the level of proteinase was found to be in the following order *C. albicans/stellatoidae* > *C. tropicalis* > *C. kefyr* > *C. lusitaniae* > *C.* krusei (Capobianco *et al.* 1992). And *C. glabrata* produces only small amounts of extracellular proteinase (Rüchel *et al.* 1992). But in our result, using BSA digestion assay, a relatively insensitive method, proteinases secreted by *C. glabrata* and *C. krusei* were not demonstrated. Besides *C. albicans, C. tropicalis* and *C. parapsilosis,* the other *Candida* species may produce small amounts of proteinase undetected by the BSA digestion method. Second, if there was an insufficient amount of proteinase to be detected using an extremely sensitive assay, it could elicit its antibody in vivo. Third, if glycosylated, this cross-reaction between *Candida* proteinases could occur. The purified proteinase demonstrated carbohydrate positivity either through anthrone reactivity (Macdonald and Odds, 1980) or periodic acid-Schiff staining (Negi *et al.* 1984; Ray and Payne, 1990). The molecular mass on SDS-PAGE was larger than that on HPLC and the proteinase sizes of *C. albicans* and *C. tropicalis* were reversed. This difference in estimated molecular mass could be derived not only from an array of amino acid sequences, structural changes - native or denature form- but also from the degree of glycosylation. Thus, further study on glycosylation of the proteinases is required.

The usefulness of proteinase to diagnose candidiasis should be considered as an alternative. Any anti-*Candida* proteinase antibody can be detected with a kind of proteinase so that the possibility of detection in proteinase-secreting *Candida* infection could be increased. On the other hand, cross reaction between *Candida* proteinase and antiserum in other *Candida* species infection reduces specificity. But it is noticeable that seroreactivities of homologous proteinases were higher than those of heterologous proteinases, and the seroreactivities increased in the course of infection after inoculation with *Candida* species.

We hope the low specificity can be overcome with distinct monoclonal antibodies to species- specific proteinase epitopes and can be producing monoclonal antibodies for each proteinase. However, the application of *Candida* proteinases for diagnosis of candidiasis should be considered with limits of specificity.

REFERENCES

Borg M, Rüchel R: Expression of extracellular acid proteinase by proteolytic *Candida* spp. during experimental infection of oral mucosa. *Infect Immun 56: 626-631, 1988*

Buckley HR, Daneo-Moore L, Ahrens JC, Sobel JD: Isolation of a germ-tube-forming revertant from *Candida albicans* B311V6. *Infect Immun 53: 13-15, 1986*

Calderone RA, Cihlar RL, Lee DD, Hoberg K, Scheld WM: Yeast adhesion in the pathogenesis of endocarditis due to *Candida albicans:* studies with adherence-negative mutants. *J Infect Dis 152: 710-715, 1985*

Capobianco JO, Lerner CG, Goldman RC: Application of a fluorogenic substrate in the assay of proteolytic activity and in the discovery of a potent inhibitor of *Candida albicans* aspartic proteinase. *Anal Biochem 204: 96-102, 1992*

Crandall M, Edwards JE Jr: Segregation of proteinase-negative mutants from heterozygous *Candida albicans. J Gen Microbiol 133: 2817-2824, 1987*

de Bernardis F, Agatensi L, Ross IK, Emerson GW, Lorenzini R, Sullivan PA, Cassone A: Evidence for a role for secreted aspartate proteinase of *Candida albicans* in vulvovaginal candidiasis. *J Infect Dis 161: 1276-1283, 1990*

Greenfield RA: Host defence system in interactions with *Candida. J Med Vet Mycol 30: 89-104, 1992*

Hubbard MJ, Markie D, Poulter RT: Isolation and morphological characterization of a mycelial mutant

of *Candida albicans. J Bacteriol 165: 61-65, 1986*

Jones JM: Laboratory diagnosis of invasive candidiasis. *Clin Microbiol Rev 3: 32-45, 1990*

Kwon-Chung KJ, Lehman D, Good C, Magee PT: Genetic evidence for role of extracellular proteinase in virulence of *Candida albicans. Infect Immun 49: 571-575, 1985*

Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature 227: 680-685, 1970*

Lehrer N, Segal E, Cihlar RL, Calderone RA: Pathogenesis of vaginal candidiasis: studies with a mutant which has reduced ability to adhere *in vitro. J Med Vet Mycol 24: 127-131, 1986*

Louie A, Dixon DM, el-Maghrabi EA, Burnett JW, Baltch AL, Smith RP: Relationship between *Candida albicans* epidermolytic proteinase activity and virulence in mice. *J Med Vet Mycol 32: 59-64, 1994*

MacDonald F, Odds FC: Inducible proteinase of *Candida albicans* in diagnostic serology and in the pathogenesis of systemic candidosis. *J Med Microbiol 13: 423-435, 1980*

MacDonald F, Odds FC: Virulence for mice of a proteinase-secreting strain of *Candida albicans* and a proteinase-deficient mutant. *J Gen Microbiol 129: 431-438, 1983*

Matthews RC: Comparative assessment of the detection of candidal antigens as a diagnostic tool. *J Med Vet Mycol 34:1-10, 1996*

Morrison CJ, Hurst SF, Bragg SL, Kuykendall RJ, Diaz H, McLaughlin DW, Reiss E: Purification and characterization of the extracellular aspartyl proteinase of *Candida albicans:* removal of extraneous proteins and cell wall mannoprotein and evidence for lack of glycosylation. *J Gen Microbiol 139: 1177-1186, 1993*

Musial CE, Cockerill FR, Roberts GD: Fungal infections of the immunocompromised host: clinical and laboratory aspects. *Clin Microbiol Rev 1: 349-364, 1988*

Negi M, Tsuboi R, Matsui T, Ogawa H: Isolation and characterization of proteinase from *Candida albicans:* substrate specificity. *J Invest Dermatol 83: 32-36, 1984*

Ollert MW, Wende C, Grlich M, McMullan-Vogel CG, Borg-von Zepelin M, Vogel CW, Korting HC: Increased expression of *Candida albicans* secretory proteinase, a putative virulence factor, in isolates from human immunodeficiency virus-positive patients. *J Clin Microbiol 33: 2543-2549, 1995*

Ray TL, Payne CD: Scanning electron microscopy of epidermal adherence and cavitation in murine candidiasis: a role for *Candida* acid proteinase. *Infect Immun 56: 1942-1949, 1988*

Ray TL, Payne CD: Comparative production and rapid purification of *Candida* acid proteinase from protein-supplemented cultures. *Infect Immun 58: 508-514, 1990*

Rüchel R, Boning B: Detection of *Candida* proteinase by enzyme immunoassay and interaction of the enzyme with alpha-2-macroglobulin. *J Immunol Methods 61: 107-116, 1983*

Rüchel R, Boning-Stutzer B, Mari A: A synoptical approach to the diagnosis of candidosis, relying on serological antigen and antibody tests, on culture, and on evaluation of clinical data. *Mycoses 31: 87-106, 1988*

Rüchel R, de Bernardis F, Ray TL, Sullivan PA, Cole GT: *Candida* acid proteinases. *J Med Vet Mycol 30(suppl 1): 123-132, 1992*

Rüchel R, Tegeler R, Trost M: A comparison of secretory proteinases from different strains of *Candida albicans. Sabouraudia 20: 233-244, 1982*

Ross IK, de Bernardis F, Emerson GW, Cassone A, Sullivan PA: The secreted aspartate proteinase of *Candida albicans:* physiology of secretion and virulence of a proteinase-deficient mutant. *J Gen Microbiol 136: 687-694, 1990*

Saxena A, Hammer CF, Cihlar RL: Analysis of mannans of two relatively avirulent mutant strains of *Candida albicans. Infect Immun 57: 413-419, 1989*

Shepherd MG: Pathogenicity of morphological and auxotrophic mutants of *Candida albicans* in experimental infections. *Infect Immun 50: 541-544, 1985*

Sobel JD, Muller G, Buckley HR: Critical role of germ tube formation in the pathogenesis of candidal vaginitis. *Infect Immun 44: 576-580, 1984*