A New Plate Assay for Detecting Inhibitors of *Candida* Aspartyl Proteinase

VIDULA R. DIKSHIT* and PRAGNA D. DESAI

Department of Microbiology, Bhavan's College,
Munshi Nagar, Andheri (W), Mumbai-400 0053, INDIA

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In the last two decades, there has been a dramatic rise in the number of immunocompromised patients in our population, resulting from infection with HIV or due to aggressive medical practices. Such patients are especially susceptible to infections by opportunistic fungi leading to life threatening mycoses[1]. Scientists worldwide are exploring new fungal targets for safe and effective antifungal therapy. The virulence factors of fungi are now considered as potential therapeutic targets[2]. Many of the opportunistic fungi produce an extracellular aspartyl proteinase which is implicated in their virulence[3,4]. The *Candida* aspartyl proteinase (CAP) facilitates the penetration of *Candida albicans* into keratinized epithelial tissue[5] thus presenting itself as an appealing target. Recently studies were undertaken to assess if pepstatin A, an inhibitor of CAP, could be an effective protective drug[6]. Previous observations showed that pepstatin A inhibited growth of *Candida in vitro*[6] and was found to affect adhesion and invasion of *C. albicans* in human mucosal explants *in vitro*[4]. However, pepstatin A is not used clinically because it is distributed and metabolized in the liver and quickly cleared from the blood after i.v. administration[6]. Therefore it would be useful to search for new water soluble, non-peptide inhibitors of CAP for systemic candidiasis[7]. Novel CAP inhibitors, viz. YF-0200R-A and B and YF-044P-D have been reported from the culture broths of *Streptomyces* sp.[7,8]. CAP is usually assayed spectrophotometrically by using bovine serum albumin or hemoglobin as substrates[9~11]. However, CAP activity in yeast isolates has been monitored using bovine serum albumin (BSA) plates containing nutrient medium by observing a clear zone around the colonies in the absence of any staining procedure[12].

In this paper, we describe a plate assay to observe CAP activity wherein digestion of casein by CAP in casein agar plates, devoid of nutrient medium, formed the basis of the assay. This test required a purified preparation of CAP from *C. albicans*. The enzyme added to agar wells gave a zone of clearing in 18 hours and no zone in presence of pepstatin A. Thus, inhibitors of CAP can be easily detected by this plate assay.

Purification of CAP from *Candida albicans*[9,10]

Microorganism: *C. albicans* was grown overnight in Sabouraud medium, washed twice under sterile conditions with saline to remove traces of medium and cell density was adjusted to O.D. 1.0 at 600 nm (using Spectronic Genesys 5, Milton Roy) for use as inoculum. Medium composed of Yeast Carbon Base (YCB) 1.2% and BSA 0.2% was filter sterilized and dispensed in 250 ml Erlenmeyer flasks containing 50 ml of medium each. 100 μl of the above inoculum was added to each flask and incubated at 37°C using a water bath shaker for 6 days.

Proteinase activity assay: CAP activity in culture supernatants was tested daily. Enzyme activity was determined spectrophotometrically by following the digestion of hemoglobin[11]. The reaction mixture contained 0.4 ml of 0.1 M sodium acetate buffer (pH 3.5), 0.4 ml buffered 1.25% (w/v) hemoglobin and 0.2 ml sample. After incubation at 37°C for 60 minutes, 2.0 ml 5.0% trichloroacetic acid (TCA) was added. The mixture was then centrifuged and filtered through Whatman no. 1.0 paper, after which the A_{280} was measured. CAP was added to the control assay just before the addition of TCA. One unit (U) of enzyme activity was defined as equal to a change in A_{280} of 1.0 ($60\ min^{-1}$) at pH 3.5 and 37°C.

Purification of CAP: The culture broth was centrifuged at 5,000 rpm for 20 minutes, filtered through a 0.45 μm porosity membrane and concentrated by lyophilization. The purification procedures to follow were conducted at 4°C. The lyophilized material was resuspended in 20 mM sodium citrate buffer (NCB) pH 6.3 to a final volume of 25 ml and dialyzed against 20 mM NCB pH 6.3. Dialyzed material was centrifuged at 10,000 rpm for 10 minutes to remove insolubles and applied on a DEAE Sephacel column (1.5× 8 cm) equilibrated with 20 mM NCB pH 6.3. The column was washed with 60 ml 20 mM NCB pH 6.3 and elution was carried out by applying a continuous gradient of 75 ml of 20 mM and 75 ml of 200 mM NCB pH 6.3. Eighty fractions of 3.0 ml each were collected and alternate fractions were estimated for CAP activity by hemoglobin assay[11] and protein content by measuring A_{280}. The active fractions (49~64) were pooled and the protein was concentrated by $NH_4(SO_4)_2$ precipitation (85% saturation). Concentrated protein was resuspended in 0.1 M sodium acetate buffer with 1 M NaCl pH 3.5 and dialyzed against the same buffer.

The dialyzed material was loaded on a pepstatin agarose affinity column (0.7×8 cm) equilibrated with 0.1 M sodium acetate buffer with 1 M NaCl pH 3.5 and the column was washed with the same buffer after loading the sample. Elution was carried out with 0.1 M Tris-HCl with 1 M NaCl, pH 8.6. Two ml fractions were collected in tubes containing 0.5 ml of 0.1 M sodium acetate buffer with 1 M NaCl, pH 3.5 to acidify the samples. Active fractions were pooled and concentrated by aquacide dialysis and dialyzed against 20 mM NCB pH 6.3 containing 50% glycerol at 4°C. Aliquots of the enzyme were stored at −70°C and removed when required for the assay.

Preparation of Casein Plates

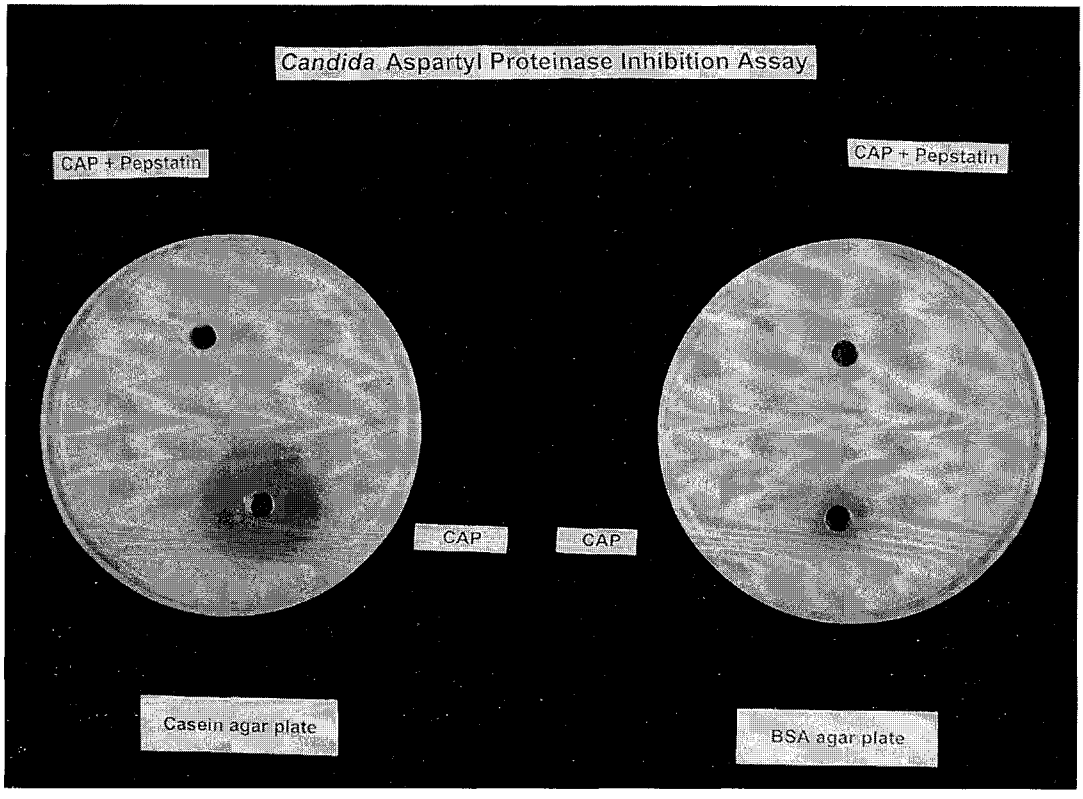
400 mg of casein (Sigma Laboratories) were dissolved in 40 ml of distilled water. 50~100 μl of 1 N NaOH were added to solublize the casein. 3.0 g of agar were added to 160 ml of 0.2 M Na-acetate buffer pH 3.8 and digested in boiling water. The casein solution was added to the molten agar and plates were poured (40 ml medium/15 cm diameter plate). Twenty-five wells of 6.0 mm diameter were punched in each plate. Similarly, BSA agar plates were also prepared to test for CAP activity.

Test for Detecting Inhibitors of CAP

To each of the wells of 6 mm diameter in the casein agar plate, 50 μl of the various test samples obtained from microbial sources or chemical synthesis were added. The plates were left open in the laminar flow hood to allow evaporation of methanol or any other solvent from the test sample. Then to each well, 50 μl of the purified CAP with activity of 1 U were added and the plates were incubated at 37°C for 18 hours. Two controls were included in each plate. Addition of the CAP preparation alone as a negative control gave a 15 mm zone of clearance in casein agar plates which appear opaque as casein is insoluble. Pepstatin A (10 μM) along with CAP was used as a positive control which inhibited the development of the zone of clearance. With most antimicrobial agents, a 50% loss of activity results in a 2 or 3 mm decrease in zone size[13]. Hence, test samples giving a reduced zone (minimum of 2 to 3 mm reduction) or no zone of clearance can be considered to be inhibitors of CAP.

Thus, the casein agar plate assay described here can be used for screening of antifungal agents targeted at CAP. Use of BSA in place of casein was found to give smaller zones of clearance and hence not recommended (Fig. 1).

Fig. 1. Plate assay showing zone of clearance in casein agar plate due to CAP after 18 hours and its inhibition in presence of pepstatin. Similar results in BSA agar plate but giving smaller zone with CAP.



Microbial growth would be unlikely in these plates due to low pH and absence of any nutrients except casein. Also, samples containing solvents can be assayed. Using higher concentrations of CAP can reduce the incubation period of this assay. As compared to the spectrophotometric assay of CAP, this is an inexpensive, rapid and easy to handle method. Also, the results can be photographed for keeping records.

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