Detection of the basement membrane-degrading proteolytic activity of *Paracoccidioides brasiliensis* after SDS-PAGE using agarose overlays containing Abz-MKALTLQ-EDDnp

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Abstract

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We have characterized, in the *Paracoccidioides brasiliensis* yeast phase, an exocellular SH-dependent serine proteinase activity against Abz-MKRLTL-EDDnp and analogous fluorescent-quenched peptides, and showed that it is also active against constituents of the basement membrane *in vitro*. In the present study, we separated the components of *P. brasiliensis* culture filtrates by electrophoresis and demonstrated that the serine-thiol exocellular proteinase has a diffuse and heterogeneous migration by SDS-PAGE, localizing in a region between 69 and 43 kDa. The hydrolytic activity was demonstrable after SDS-PAGE using buffered agarose overlays of Abz-MKALTLQ-EDDnp, following incubation at 37ºC, and detection of fluorescent bands with a UV transilluminator. Hydrolysis was more intense when incubation was carried out at basic pH, and was completely inhibited with 2.5 mM PMSF and partially with sodium 7-hydroxymercuribenzoate (2.5 mM *p*-HMB), suggesting its serine-thiol nature. A proteolytic band with similar characteristics was observed in conventional gelatin zymograms, but could not be correlated with a silver-stained component. Detection of the serine-thiol proteinase in substrate gels after SDS-PAGE provides a useful way of monitoring purification of the basement membrane degrading enzyme.

Paracoccidioides brasiliensis is a dimorphic fungus that causes human paracoccidioidomycosis (PCM), a systemic mycosis endemic in Latin America, which can be fatal in immunocompromised individuals if not successfully treated. Patients with PCM can have multiple clinical forms which are dependent on the relationship between the host's resistance mechanisms and fungal pathogenicity (1). The yeast cell wall α-glucan (2) and the gp43 antigen (3,4), which is protective in experimental murine PCM (5), have been implicated in fungal virulence. Other virulence factors in *P. brasiliensis* probably exist.

We have characterized a subtilisin-like,

SH-dependent serine proteinase activity in *P. brasiliensis* grown in the yeast phase (6). Internally quenched fluorescent peptides derived from MKRLTL and flanked by Abz (*ortho*-aminobenzoyl) and EDDnp (ethylenediaminedinitrophenyl) were cleaved at the L/T bond when incubated with a *P. brasiliensis* culture filtrate. The exocellular proteinase activity had an optimum pH of >9.0 and was irreversibly inhibited by PMSF, mercuric acetate and sodium 7-hydroxymercuribenzoate (*p*-HMB). Moreover, the enzyme was able to selectively cleave, *in vitro*, components of the basal membrane or associated with it, including laminin, fibronectin, type IV collagen and proteoglycans. Such activity could be of biological significance in tissue invasion by the fungus (7).

The serine-thiol proteolytic activity involves an enzyme of very high specific activity, which is rather unstable and is lost after several chromatographic steps. So far, a single silver-stainable band in SDS-PAGE has not been observed in fractionated preparations, which has hampered the purification and characterization of the enzyme. The objective of the present study was to detect the exocellular serine-thiol proteinase activity in SDS-PAGE gels using overlays of Abz-MKALTLQ-EDDnp. This is a substrate analogous to Abz-MKRLTL-EDDnp but with a better V_{max}/K_m value (6), and was synthesized as described previously (8,9). A conventional gelatin zymogram was used in parallel.

P. brasiliensis strain 339 (originally provided by Dr. Angela Restrepo-Moreno, Medellin, Colombia) was cultivated at 35°C, with shaking, in modified YPD (0.5% Bacto-yeast extract, 0.5% casein peptone and 1.5% glucose), and the culture supernatant was collected by paper filtration at the peak of proteinase activity against Abz-MKALTLQ-EDDnp (7). Ammonium sulfate was added to the culture filtrate at 40% saturation and the precipitate separated by centrifugation (16300 *g*, 30 min). The supernatant (50 ml)

was concentrated by FPLC in a Phenyl Superose HR 5/5 (Pharmacia/LKB, Uppsala, Sweden) column, according to standard procedures. The proteolytic activity against Abz-MKALTLQ-EDDnp was concentrated in two fractions, denoted F3 and F4, which were analyzed on substrate gels. This concentration procedure using Phenyl Superose, although more laborious, resulted in a better yield of the active enzyme than precipitation of culture filtrates with 50% ammonium sulfate (6), since very little of the hydrolytic activity is precipitated with salt.

For SDS-PAGE analysis, F3 and F4 were electrophoresed (100 V, at 4°C) in conventional linear 10% gels (10), using sample buffer containing SDS at 0.5% final concentration without boiling. Pre-stained low molecular weight protein markers (Gibco BRL, Gaithersburg, MD, USA) were applied to the gels, and their migration position was marked with a needle after running as a reference for localization of the proteolytic bands. The gels were then washed for one hour in 0.05 M Tris-HCl, pH 8.5, 10% MeOH (11), with three changes, and finally in buffer without MeOH. Abz-MKALTLQ-EDDnp (100 µg/ml) overlays were prepared in 1% buffered agarose (0.75 mm thick), placed on the washed SDS-PAGE gels, and incubated at 37°C in a moist chamber. Fluorescent green bands were visualized with a UV transilluminator (302 nm) and the results were recorded on Polaroid films using a yellow filter.

Figure 1A shows the fluorescence obtained after increasing incubation times with Abz-MKALTLQ-EDDnp in agarose overlays. The proteolytic activity appeared as a diffuse zone in the gel between bovine serum albumin (BSA) and egg ovalbumin (OVA), or between 69 and 43 kDa apparent molecular masses. The fluorescent band at 43 kDa (gp43) seen in F4 was not due to proteolysis, but to natural fluorescence emitted by the molecule, and could still be seen without the peptide overlay, or when the

sample was previously boiled in SDS for protease inactivation (data not shown). The protein profiles of F3 and F4 (Figure 2A) indicated that F4 was rich in the gp43 component, which could barely be seen in F3 by silver staining. This fractionation after Phenyl Superose chromatography has been advantageous in that only F3 fractions are now being processed for proteinase purification. This procedure avoids the gp43 depletion step by affinity chromatography which was used before (6). On the other hand, F3 is usually twice more active than F4 against Abz-MKALTLQ-EDDnp in solution.

In the experiment shown in Figure 1A, maximum fluorescence arising from the hydrolyzed substrate was achieved after 1 h and 45 min of incubation at 37ºC. After 3 h, it diffused and was not as intense. The fluorescence intensity and the incubation time necessary to reach a maximum of fluorescence varied with the activity of the sample in a dose-dependent fashion (data not shown). The result shown in Figure 1A was similar when the samples were incubated with reduced or nonreduced SDS-PAGE sample buffer.

The specificity of the activity against Abz-MKALTLQ-EDDnp in agarose overlays was assessed by pre-incubation (30 min, room temperature) of F3 with known protease inhibitors before addition of the sample buffer and electrophoresis. The result is illustrated in Figure 1B, where proteolysis was completely inactivated by 2.5 mM PMSF (lane 4), and by boiling in SDS buffer (lane 2); *p*-HMB (0.15 and 2.5 mM) was partially inhibitory, especially at higher concentrations (lane 7*). Since this is not an active site inhibitor, it could have been partly dissociated during electrophoresis. In addition, the activity was more intense upon incubation at pH 8.5 than at pH 6.5 (data not shown). These results strongly suggest that the exocellular serine-thiol activity previously characterized in *P. brasiliensis* (6) is due to a proteinase which migrates diffusely between 69 and 43 kDa in
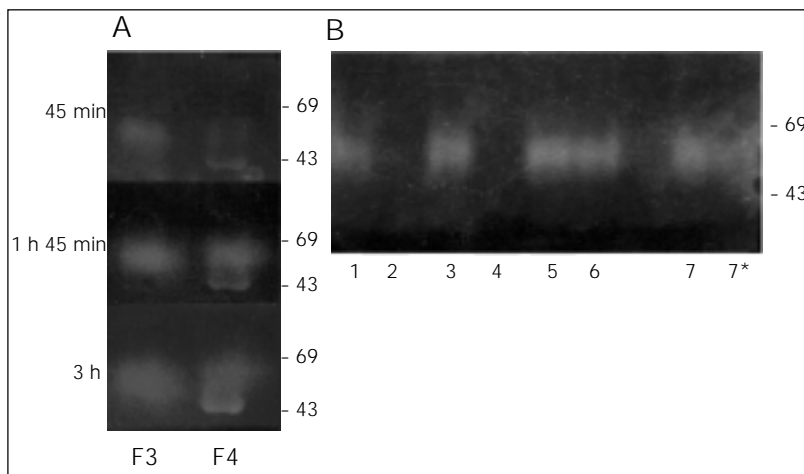

Figure 1 - Demonstration of P. brasiliensis exocellular proteolytic activity in SDS-PAGE gels using Abz-MKALTLQ-EDDnp in agarose overlays, and enzyme characterization with protease inhibitors. A, The hydrolytic activity in F3 (1.25 µg) and F4 (5.0 µg) was recorded after 45 min, 1 h and 45 min, and 3 h of incubation at 37ºC. Fluorescence of the 43-kDa component was intrinsic, and was observed without the peptide overlay. B, F3 (1.25 µg) alone (lane 1), or inactivated by boiling (lane 2), pre-incubated with 2.5 mM ortho-phenanthroline and 5 mM EDTA (lane 3), 2.5 mM PMSF (lane 4), 0.13 mM trans-epoxy-succinyl-L-leucylamido-(4-guanido)-butene, E-64 (lane 5), 2.5% DMSO (lane 6), 0.15 mM (lane 7) and 2.5 mM (lane 7*) p-HMB. Molecular mass markers (kDa) are indicated on the right side.
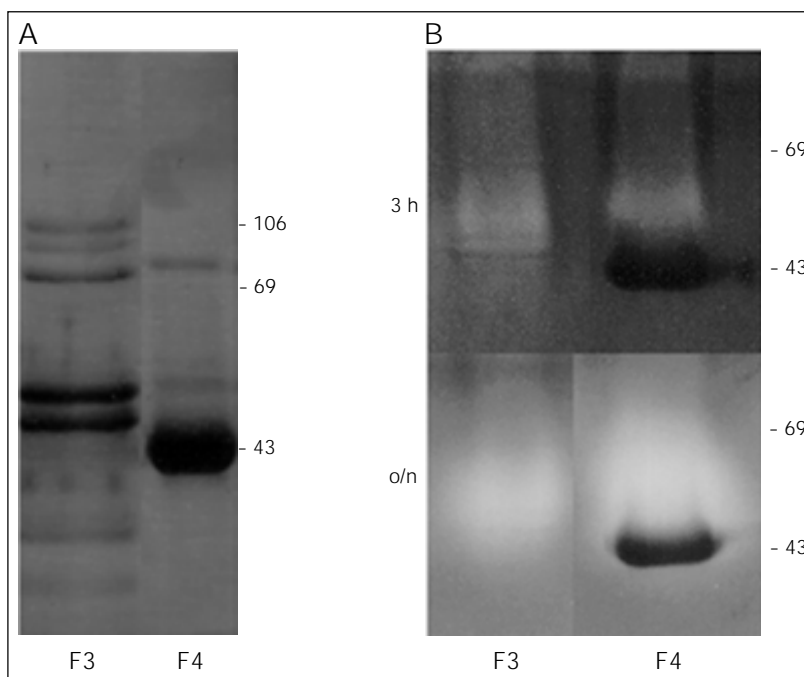

Figure 2 - Silver-stained SDS-PAGE (A) and zymogram (B) profiles of F3 and F4 fractions of P. brasiliensis exocellular components. For the zymograms, the polyacrylamide gels were impregnated with 0.1% gelatin. After the electrophoretic run, the gels were washed with buffered MeOH, as indicated in the text, and incubated at 37ºC in 50 mM Tris-HCl, pH 8.5, for 3 h or overnight (o/n), as indicated. Proteolytic bands were visualized by staining with Coomassie brilliant blue. The amounts loaded onto the gels were: 0.5 µg of F3 and 1 µg of F4 for silver staining and 5 µg of F4, 1.25 µg (3 h) or 0.25 µg (o/n) of F3 for zymograms. Molecular weight markers (kDa) are indicated on the right side.

SDS-PAGE gels. This is also in accordance with our previous data (6) showing that the exocellular serine-thiol activity against Abz-MKRLTL-EDDnp was eluted in a single peak near ovalbumin. Furthermore, the diffuse SDS-PAGE migration of the proteinase is probably due to heterogeneous and/or abundant glycosylation, as inferred from binding of the enzymatic activity to concanavalin A (Puccia R and Carmona AK, unpublished results).

The same results were obtained when F3 and F4 were analyzed for gelatinase activity in a zymogram (Figure 2B), where a fuzzy proteolytic band between 69 and 43 kDa could be seen even after 3 h of incubation at 37°C. After overnight incubation, the proteolytic zone was very clear and broad. The proteolysis inhibition pattern and the pH dependence in zymograms were similar to those with Abz-MKALTLQ-EDDnp in agarose overlays. Additional results obtained with gelatin zymograms showed that the serine-thiol proteinase activity was not altered by heating at 45°C or by lyophilization, and could not be detected after incubation at room temperature (data not shown). Thermostability was checked in protein-rich preparations due to the unstable nature of the proteinase in more purified fractions.

Gelatinase activity in culture filtrates of *P. brasiliensis* concentrated by heat evapo-ration at 45°C and lyophilization has been reported (12). For strain 339, the authors showed what seems to be two overlapping broad bands between 43 and 78 kDa. This gelatinase activity may have been due to the serine-thiol proteinase, on the basis of similarity in molecular weight and heat-stability. However, this is probably not the case, since that activity was very strong upon incubation at room temperature. However, it is unlikely that the gelatinase activity mentioned above (12) was due to gp43, as attributed by the authors without any evidence, since there were a number of components migrating in the same broad region of the proteolytic zone. Moreover, protease motifs have not been reported in the gp43 gene sequence (13) and the proteolytic properties associated with gp43 in the past (14) were later shown to be due to aggregation (6).

In the present work, we show for the first time the usefulness of internally quenched fluorescent peptides flanked by Abz (*ortho*-aminobenzoyl) and EDDnp (ethylenediaminedinitrophenyl) in agarose overlays for the detection of proteolytic activities after SDS-PAGE. For *P. brasiliensis* serine-thiol proteinase, visualization of the activity zone in substrate gels is essential for further characterization and purification of the basement membrane degrading enzyme.

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