ELASTASE ACTIVITY OF *COCCIDIOIDES IMMITIS*

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SUMMARY. Twenty-two strains of *Coccidioides immitis* were tested for the ability to hydrolyse elastin. Screening assays with Czapek's or Tryptic Soy Agar supplemented with 0.5% elastin demonstrated that 21 strains (95%) were elastolytic. In broth cultures, elastase activity was induced by incorporation of insoluble elastin into the medium and induction was suppressed by supplementation with yeast extract. *C. immitis* appears to be unique amongst dimorphic fungal pathogens in its digestion of elastin.

INTRODUCTION

The elastolytic activity of fungi has been investigated by Rippon and Varadi (1968) in 70 species and by Hopsu-Havu, Sonck and Tunnela (1972) in 185 isolates. These studies found that many fungi hydrolyse elastin but, with the exception of a single strain of *Coccidioides immitis*, the dimorphic pathogens showed no elastolysis.

Because only a single strain of *C. immitis* was studied, the present investigation was undertaken to determine the frequency of elastolysis by strains of *C. immitis*.

MATERIALS AND METHODS

Fungus strains. The strains of *C. immitis* used were supplied by Dr M. Huppert, Veterans Administration Hospital, San Antonio, TX 78284, USA and maintained on glucose-yeast extract agar (2%:1%:2%).

Screening assays. A survey of elastase activity was undertaken with Czapek's agar containing bovine elastin (Sigma Chemical Co., St Louis, MO, 63178, USA) 0.5%. Tryptic Soy Agar (Difco Labs, Detroit, MI 48216, USA) with elastin 0.5% was also used to determine whether or not organic nutrients affected growth and enzyme production. Bi-petri plates were seeded with the *C. immitis* strains, incubated at 30°C, and examined daily for elastolysis. Digestion was observed as a clearing of particulate elastin in the agar and zones of digestion were measured through the reverse of the plates.

Growth and elastase production. Six strains of *C. immitis* (C-39, C-40, C-46, C-60, C-108 and C-187) were selected to study the relationship between fungal growth and elastase production. Spore and mycelium suspensions in saline were shaken to fragment the mycelium and suspend the arthrospores. Viability was determined by pour plates in glucose-yeast extract agar.

Strains of *C. immitis* were inoculated into 50 ml of Czapek's fluid, Czapek's fluid with bovine elastin 0.5% and Czapek's fluid with elastin and yeast extract 0.1%. Each flask received 2.5×10^4 cfu and 15 flasks were employed for each medium. These were incubated at 35°C on a gyrotory

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(150 rpm) shaker. Three flasks of each medium were harvested at 3-day intervals during a 15-day period. Mycelium and spores were removed by centrifugation (1000 *g* for 15 min) and the supernate sterilised by filtration. The fluid was assayed for elastase activity as detailed below. The culture fluid was stored at -20°C until the total protein could be estimated. The mycelial mats were heated (10 min at 121°C), washed twice with 100-ml volumes of distilled H_2O , and freeze-dried for total carbohydrate estimation.

Elastase assay. This was modified from that of Sachar *et al.* (1955) for mammalian elastase. Duplicate lots of 10 mg of orcein-elastin (Sigma) were placed in acid-cleaned 13×100 mm screw-capped tubes with 1.5 ml of 0.2 M tris-maleate buffer, pH 8.5. A sample (0.5 ml) of the unknown enzyme solution was added, and the tubes were placed on a rotator at 37°C for 2 h. To stop the reaction, 2.0 ml of 0.5 M phosphate buffer, pH 6.0, was added. The tubes were centrifuged at 1000 *g* for 10 min to sediment undigested elastin and the absorbance of the supernate at 590 nm was determined. The units of absorbance were compared with standard curve values obtained by digestion of orcein elastin with porcine elastase (Sigma). Heat treated (10 min at 100°C) control samples were also included in each elastase assay.

Carbohydrate assay. Carbohydrate was taken as a measure of fungal growth and determined by the method of Dubois *et al.* (1956) with dextrose as the standard.

Protein assay. Total protein was determined by the method of Lowry *et al.* (1951). In instances where elastin-digestion products would interfere with the assay, macromolecular protein was precipitated by adding aqueous saturated ammonium sulphate to the culture fluid to achieve a final concentration of 75% saturation. The precipitate was collected by centrifugation (1500 *g* for 10 min), and the supernate discarded. The pellet was dissolved in 1 ml of deionised water, dialysed overnight against deionised water, and assayed for total protein.

RESULTS

Elastase survey

Twenty-one (95%) of the strains tested hydrolysed elastin (table I). In some instances, the zone of elastin digestion was clearly larger than the colony diameter, but more often it was not (fig. 1; table I). Strain C-605 failed to grow on the Czapek's-elastin agar medium (CPK-E). This strain grew on the elastin-supplemented TSA (TSA-E), but failed to digest elastin.

An average digestion zone diameter of 2.7 cm was obtained on the CPK-E medium while identical strains grown on the enriched agar medium (TSA-E) had a significantly ($p < 0.05$) smaller average diameter (2.26 cm).

The colonies of *C. immitis* had a better growth rate on TSA-E (table I); however, elastase activity, as measured by zones of digestion, was decreased. Furthermore, strains (C-62, C-80, C-238, C-606, and C-607) that were positive on the minimal medium (CPK-E) gave either questionable or negative results (table I) on the enriched medium (TSA-E).

When the CPK-E and TSA-E bi-petri plates were held for an additional 2 weeks at 4°C , an apparent increase in elastase activity was noted (fig. 2; table II). The average digestion zone on both media was significantly larger than the zone observed after the initial incubation. Moreover, the doubtful strains (C-62, C-80, and C-607) were easily discerned as positive which suggested that supplemental incubation at 4°C was necessary for verifying the elastase activity of questionable or presumably negative strains.

Elastase production in liquid culture

Cultivation on CPK-E and TSA-E suggested that the presence of extra nutrients

FIG. 1.—Elastase activity of six strains (C39, C40, C42, C46, C48, C49) of *C. immitis* cultured on Czapek's elastin agar (left half of plate) and Tryptic Soy elastin agar (right half of plate) after incubation at 30°C for 1 week. Each culture medium was supplemented with elastin 0.5% which is particulate and insoluble. Elastase activity is indicated by clearing of insoluble elastin in the agar under each fungal colony.

TABLE I

Elastase activity of C. immitis after incubation for 1 week at 30°C on Czapek's and Tryptic Soy Agar media supplemented with elastin 0.5%.

Strain no.	Growth and activity of strain on			
	Czapek's Agar		Tryptic Soy Agar	
	Colony diameter (cm)	Elastin hydrolysis diameter (cm)	Colony diameter (cm)	Elastin hydrolysis diameter (cm)
C-39	3.7	3.7	3.8	2.5
C-40	3.2	3.2	3.7	1.5
C-42	2.5	2.0	2.8	1.7
C-46	2.6	2.4	3.5	3.0
C-48	2.5	2.3	3.4	2.8
C-49	2.5	2.5	3.5	2.2
C-54	3.0	3.0	4.0	1.8
C-56	2.4	2.4	3.2	2.1
C-60	2.9	2.9	3.6	2.3
C-62	2.3	1.2	3.8	±*
C-80	2.7	2.1	3.3	0
C-108	2.6	2.9	3.2	3.2
C-155	2.6	3.5	3.8	1.8
C-180	3.2	3.2	4.4	1.9
C-187	3.9	3.3	4.0	2.4
C-238	2.4	1.4	3.3	0
C-239	2.6	3.1	3.0	3.1
C-249	2.2	1.9	3.0	2.0
C-396	2.8	2.5	3.7	1.9
C-605	0	0	2.5	0
C-606	3.8	3.3	3.8	±*
C-607	4.3	3.3	3.6	±*
Mean†	2.89	2.67	3.48	2.26
(95% C.I.)	(2.64-3.14)	(2.29-3.05)	(3.23-3.73)	(1.89-2.63)

* Elastin digestion zone < 1.0 cm.

† The mean value is the average value of the measurable zones only.

FIG. 2.—Elastase activity of six strains (C39, C40, C42, C46, C48, C49) of *C. immitis* cultured on Czapek's elastin agar (left half of plate) and Tryptic Soy elastin agar (right half of plate) after incubation at 30°C for 1 week and 4°C for 2 weeks. Each culture medium was supplemented with bovine elastin 0.5%. Overall increase in elastin hydrolysis at 4°C is noticeable when compared to results shown in fig. 1.

TABLE II

Elastase activity of C. immitis after incubation for 1 week at 30°C and 2 weeks at 4°C on Czapek's and Tryptic Soy Agar media supplemented with elastin 0.5%

Strain no.	Growth and activity of strain on			
	Czapek's Agar		Tryptic Soy Agar	
	Colony diameter	Elastin hydrolysis diameter	Colony diameter	Elastin hydrolysis diameter
C-39	3.7 ^a	5.3	3.9	5.2
C-40	3.4	4.5	3.7	3.9
C-42	2.5	3.3	2.8	3.5
C-46	2.7	3.2	3.9	5.4
C-48	2.5	3.5	3.5	4.7
C-49	2.6	3.8	3.4	3.8
C-54	...	...	...	...
C-56	2.5	3.4	3.2	3.6
C-60	3.3	4.7	3.7	5.2
C-62	2.3	1.8	3.8	1.7
C-80	2.8	2.9	3.3	2.8
C-108	2.6	4.2	3.3	5.2
C-155	2.8	3.4	3.8	3.5
C-180	3.5	4.2	4.3	3.7
C-187	3.9	4.6	4.0	4.0
C-238	2.3	2.0	3.5	0
C-239	2.7	4.4	3.1	5.7
C-249	2.2	2.8	3.0	3.9
C-396	3.0	3.4	3.8	3.0
C-605	0	0	2.5	0
C-606	3.8	4.3	3.7	0
C-607	4.4	4.5	3.6	2.1
Mean	2.98	3.71	3.51	3.94
(95% C.I.)	(2.81-3.15)	(3.29-4.13)	(3.16-3.86)	(3.37-4.51)

See footnote to table I.

FIG. 3.—Growth of *C. immitis* strain C-60 in Czapek's fluid (A), Czapek's fluid plus elastin 0.5% (B), and Czapek's fluid plus elastin 0.5% and yeast extract 0.1% (C). Each point represents the average of three culture flasks. —Δ— = Total carbohydrate, —O— = elastase activity and —□— = total macromolecular protein in supernate of culture broth.

suppressed elastase production. To test this, three sets of fluid cultures containing either CPK only, CPK plus elastin 0.5% (CPK-E), and CPK plus elastin 0.5% and yeast extract 0.1% (CPK-E-YE) were seeded with six different strains. The results of the growth of *C. immitis* strain C-60 are shown in fig. 3.

All strains gave visible growth after approximately 3 days; however, the growth in CPK fluid was measured only with difficulty and elastase activity was at baseline levels. In CPK-E fluid (fig. 3, B), strain C-60 started with an early burst of growth that declined by day 6 but returned to maximal levels by day 12. The later peak of growth was accompanied by an increase in elastase activity and in exocellular protein. The initial peak was probably not a result of elastin digestion because elastase activity was only minimal during this period. A similar pattern occurred with the five remaining strains (data not shown).

The greatest amount of growth occurred in the CPK-E-YE fluid (fig. 3C). The initial peak at 6 days coincided with a decrease in extracellular protein. The extracellular protein consumed was presumably that provided by the yeast extract because elastase activity was not apparent until day 6. The depression in growth (day 9) and subsequent return to maximal levels (day 12) was concomitantly associated with an increase in elastase activity and extracellular protein. It appeared that strain C-60 utilised the readily available nutrients from the yeast extract before altering its metabolism to hydrolyse the particulate elastin. These results suggest that coccidioidal elastase is inducible and that *C. immitis* would likely utilise other substrates before digesting elastin.

DISCUSSION

We have confirmed the findings of Rippon and Varadi (1968) who examined the activity of a single strain of *C. immitis*. The results of surveys for fungal elastase (Rippon and Varadi, 1968; Hopsu-Havu *et al.*, 1972) show that *C. immitis* is unique among the dimorphic pathogenic fungi in its ability to digest elastin.

The production of coccidioidal elastase is inducible. When *C. immitis* was cultured in Czapek's fluid without elastin, very little elastase could be detected by spectrophoto-

metric assay. However, when elastin was added to the medium, all six strains tested produced elastase. This in-vitro induction does not appear to occur until other nutrients become limiting. This interpretation follows from growth culture analysis in which incorporation of yeast extract into the Czapek's fluid resulted in a delay in elastase production. Similarly, cultivation of *C. immitis* on an enriched semi-solid medium suppressed elastase production and a number of *C. immitis* strains would have been considered negative had they not been also cultured on a minimal (Czapek's) medium.

The delayed elastase activity seen in the enriched (Tryptic Soy) screening agar and in the yeast extract supplemented Czapek's fluid medium suggested that elastase was suppressed by exogenous nutrients.

We have referred to the enzyme digesting elastin as coccidioidal elastase. At present, we do not have sufficient data about the specificity of the elastase of *C. immitis* to determine whether the enzyme is a specific elastase or a general protease which digests elastin.

Elastin is the major structural protein of the lung and comprises a significant portion of other tissues (Frisell, 1982). Elastases have been shown in several animal models of infection to be critical components of bacterial virulence (Holder and Haidaris, 1979; Pavlovskis and Wretling, 1979; Stewart, 1979; Woods *et al.*, 1982). A similar correlation has been reported for the elastase of the fungus *Aspergillus fumigatus* by Kothary, Chase, and Macmillan (1984). Such an association has not been made for coccidioidal elastase, nor has it been determined whether coccidioidal elastase is produced by the parasitic form of the fungus. The significance and specificity of coccidioidal elastase will be the subject of future investigations.

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