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Purification and characterization of urease isolated from the pathogenic fungus *Coccidioides immitis*

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Coccidioides immitis, the causative agent of San Joaquin Valley fever (coccidioidomycosis), produces a urease which has been suggested to contribute to the virulence of this fungal pathogen. Urease catalyzes the hydrolysis of urea and has been proposed to at least partly account for alkalinity of the microenvironment in which *C. immitis* grows due to the release of ammonia and ammonium ions. The *C. immitis* urease was purified to homogeneity (1048-fold) from the mycelial cytosol by chromatographic fractionation. The sequence of 12 *N*-terminal amino-acid residues of the purified, native polypeptide was identical to that predicted by the translated urease gene sequence which has been reported. The isolated enzyme exhibited a specific activity in the presence of urea of 1750 μmol min^{-1} mg^{-1} protein, has a native molecular mass of 450 kDa, revealed a K_m for urea of 4·1 mM, had a pH optimum of 8·0 and is heat stable. Hydroxyurea, acetohydroxamic acid (AHA) and boric acid each inhibited activity of the purified enzyme. Urease activity was enhanced by the presence of 5–10 mM concentrations of Mg^{2+} or Mn^{2+}, but inhibited by Li^{+}, Ni^{2+}, Cu^{2+} or Zn^{2+}. The reversible urease inhibitor, AHA, blocked enzyme activity in the crude mycelial cytosolic fraction when added at a concentration of 10 mM. On the other hand, 10 mM AHA added to 4-day-old mycelial cultures only partially decreased the amount of ammonium detected in the culture medium. It is evident, therefore, that *C. immitis* urease activity does not account for the total amount of ammonia secreted during *in vitro* growth of the pathogen. Other metabolic sources of ammonia, which may also contribute to the virulence of *C. immitis,* are under investigation.

Keywords ammonium production, *Coccidioides immitis*, urease purification

Introduction

Coccidioides immitis is a primary fungal respiratory pathogen of humans, which grows as a saprobe in alkaline desert soil of Southwestern United States, Mexico and parts of Central and South America [1]. When cultured in a glucose–yeast extract broth, the mycelium produced by the saprobic phase releases ammonia and ammonium ions which result in an increase in the pH of the growth medium [2]. The parasitic phase of *C. immitis* can be grown in a defined glucose-salts medium in which ammonium acetate is the sole nitrogen source [3]. The culture medium of the parasitic phase shows a sharp increase in pH and ammonium content during growth over a 7-day period [4]. While several metabolic pathways may be involved in this alkalinization process, urea hydrolysis is known to occur in *C. immitis* and results in ammonia production [5]. Urease (urea amidohydrolase; EC 3·5·1·5) catalyzes the hydrolysis of urea to produce ammonia and carbamate. The latter is further hydrolyzed to yield carbonic acid and ammonia. At physiological pH, the carbonic acid proton dissociates and the ammonia

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molecules become protonated with a resultant increase in pH [6]. Urease activity has been shown to be an important virulence factor in a number of bacterial pathogens of humans, as exemplified by *Helicobacter pylori* [6]. Although urease has also been detected in several genera of medically important fungi (e.g. *Aspergillus*, *Candida*, *Cryptococcus*, *Rhodotorula*, *Trichosporon*), only in the case of *Cryptococcus neoformans* and *C. immitis* has it been suggested that this fungal enzyme plays a role in pathogenicity [4,7,8]. Evidence has been presented that colonization of host tissue by *C. immitis* results in formation of abscesses with a distinctly alkaline microenvironment [4,8]. The latter is proposed to be generated as a result of ammonia secretion by the pathogen, which is at least partly due to intracellular urease activity [4,5]. Here we report the isolation and characterization of the native urease of *C. immitis*.

Materials and methods

Reagents

Nutrients used in the fungal culture medium were obtained from Difco Laboratories (Detroit, MI, USA). All other chemicals and prepared solutions were purchased from Sigma Chemical Company (St. Louis, MO, USA), unless otherwise specified. The DEAE Sepharose, Phenyl-Sepharose, and Superose 6 HR 10/30 columns were purchased from Pharmacia Biotechnology (Piscataway, NJ, USA), while the Q HyperD 20 column was purchased from Beckman (Fullerton, CA, USA). Both FPLC (Pharmacia) and HPLC systems (BioSystem Model 510; Beckman) were used in this study.

Growth of C. immitis

The saprobic phase of *C. immitis* strain C735 was grown in liquid medium (GYE) which contained 2% glucose (w/v) plus 0·5% yeast extract (w/v) as previously described [9]. Abundant mycelia were produced in Erlenmeyer flasks after incubation in a gyratory shaker (100 rpm, 30 °C) for 4–8 days. The mycelium was separated from the culture medium by filtration through Whatman 1 filter paper (Fisher Scientific Co., Pittsburgh, PA, USA).

Ammonium detection in culture filtrates

The amount of ammonium released into the culture filtrate by *C. immitis* was measured as indophenol produced by a phenol–hypochlorite reaction (Bertholet reaction [10]). The concentration of indophenol was determined colorimetrically by measurement of absorbance at 625 nm. Aliquots of the culture filtrate were assayed and the ammonium content was determined on the basis of a standard curve using NH_4Cl.

Urease assay

One unit of urease activity is defined as the amount of enzyme required to hydrolyze 1 μmol min^{-1} of urea at 37 °C. Urease activity was measured by monitoring the rate of ammonium release from the urea substrate. The concentration of ammonium in the reaction mixture was determined by the Berthelot reaction as described above. The enzyme assay buffer consisted of 50 mM Hepes, 50 mM urea, and 0·5 mM EDTA (pH 7·75). Control assays included detection of ammonium in the (i) reaction mixture at time zero; (ii) boiled enzyme extract, and (iii) reaction mixture in the absence of urea. The purified enzyme was added to reaction mixtures in the presence of various concentrations of substrate to ensure that the urease activity determinations were linear with respect to time.

Purification of urease

All steps of urease isolation and purification were performed at 4 °C, unless otherwise specified. Urease activity in each of the chromatographic fractions described below was measured as the amount of enzyme per mg of protein in the crude cytosolic preparations required to hydrolyze 1 μmol min^{-1} of urea at 37 °C. The urea substrate was added to the cytosolic fractions in the same urease assay buffer as described above. The protein content of the fractions was measured using the Bio-Rad D_c protein assay reagent (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as the standard. Purification of urease from the cytosol of saprobic phase cultures involved the following five steps:

1. Enzyme extraction
The mycelium isolated by culture filtration was washed twice with PE buffer (20 mM K_2HPO_4, 20 mM KH_2PO_4, 1 mM EDTA, pH 8·0), which contained 1 mM phenylmethylsulfonyl fluoride (PMSF), and then frozen and ground in liquid nitrogen using a mortar and pestle. The homogenate was mixed with 20 times its volume of PE buffer to which 1 mM β-mercaptoethanol was added, and then centrifuged (50 000*g*, 30 min). The resultant, clear supernatant is referred to as the crude mycelial cytosol.

2. Primary anion exchange chromatography
The crude mycelial cytosol (500 ml) was passed through a nylon filter (Acrodisc, 0·45-μm pore size; Pall-Gelman, Ann Arbor, MI, USA) to remove particulate material. The filtrate was applied (1 ml min^{-1} flow rate) to a DEAE Fast Flow Sepharose FPLC column (1·5 cm inner

diameter [i.d.]×16 cm) which had been equilibrated with running buffer (RB1; 20 mM K_2HPO_4, 20 mM KH_2PO_4, 1 mM β-mercaptoethanol, pH 8·0). After washing with five column bed volumes of RB1, the urease was eluted from the column with 40 ml of a linear concentration gradient of NaCl (0 to 1·0 M) in RB1. The eluate was collected in 4-ml fractions and those with the highest urease activity were pooled. This pool is referred to as the DEAE-Sepharose fraction.

3. Hydrophobic interaction chromatography

The DEAE-Sepharose fraction (50 ml) was dialyzed overnight against 10 vol of running buffer 2 (RB2; 1·0 M K_2HPO_4, 1·0 M KH_2PO_4, 1 mM β-mercaptoethanol, pH 8·0). The retentate was then filtered through an Acrodisc nylon membrane as above and transferred to a Phenyl-Sepharose FPLC column (1 cm i.d.×10 cm), which had been pre-equilibrated with RB2. After sample application, the column was washed with five column bed volumes of RB2 (flow rate 1 ml min^{-1}). The sample was then eluted with a decreasing concentration gradient of potassium phosphate in RB2 from 1·0 M to 20 mM. Two-milliliter fractions were collected and those with highest urease activity were pooled. The enzyme was concentrated by ultrafiltration using a Centricon YM-10 membrane (Amicon, Beverly, MA, USA), which was permeable to molecules with $M_r < 10\,000$. The concentrated sample is referred to as the Phenyl-Sepharose fraction.

4. Second anion exchange chromatography

The Phenyl-Sepharose fraction was diluted with 3 volumes of running buffer 3 (RB3; 20 mM Tris-HCl plus 1 mM β-mercaptoethanol, pH 8·0). The sample was then filtered through a PVDF membrane (Acrodisc LC [PVDF], 0·2 μm pore size; Pall-Gelman) and applied to a HPLC Q HyperD 20 anion exchange column (4·6 mm i.d.×100 mm) which had been pre-equilibrated with RB3. After sample application, the column was washed with five column bed volumes of RB3 (flow rate 1 ml min^{-1}). The enzyme was eluted from the column with a linear concentration gradient of NaC1 (0–1·0 M) in RB3. Fractions with highest urease activity were pooled and are referred to as the Q HyperD 20 fraction.

5. Size exclusion chromatography

The Q HyperD 20 fraction was concentrated by ultrafiltration (Centricon YM-10), filtered (PVDF membrane), and applied to a Superose 6 HR FPLC column (10 mm i.d.×30 cm) which had been pre-equilibrated with RB3 containing 200 mM NaCl. The flow rate of the column was maintained at 0·2 ml min^{-1}, and 1-ml fractions were collected. The fraction with the highest urease activity was isolated and is referred to here as the Superose 6 HR fraction.

Native molecular mass estimation

The molecular mass of the purified native urease was determined using the Superose 6 HR size exclusion column and the same buffer and flow rate as described above. The molecular mass standards used to calibrate the column included thyroglobulin (669 kDa), apoferritin (443 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), and bovine carbonic anhydrase (31 kDa).

Polyacrylamide gel electrophoresis (PAGE)

Discontinuous gel electrophoresis was conducted with a 12% polyacrylamide resolving gel (w/v) and a 3% polyacrylamide stacking gel (w/v) using the Tris-glycine buffer of Laemmli [11] which contained 0·5% sodium dodecyl sulfate (SDS; w/v). The samples were denatured (100 °C, 5 min) before they were applied to the gels. After electrophoresis, the gels were stained with either Coomassie brilliant blue or silver nitrate [12,13].

N-terminal amino acid sequence analysis

The purified, denatured urease (8·0 μg), which was subjected to SDS–PAGE as described above, was transferred by electroblotting to a Immobilon-P membrane (Millipore Corp., Bedford, MA, USA) and stained with 0·1% Coomassie brilliant blue (w/v) in 50% methanol as previously described [14]. The stained band was excised and the N-terminal amino acid sequence of the isolated polypeptide was determined using an Applied Biosystems pulsed liquid-phase sequenator (ABI Model 477A; Macromolecular Structure Analysis Facility at the University of Kentucky, Lexington, KY, USA).

K_m and effects of pH, temperature, metal ions and organic inhibitors on urease activity

The Michaelis–Menten constant (K_m) for the purified urease was calculated on the basis of Michaelis–Menten kinetics using a Lineweaver–Burk plot as previously described [15]. The pH optimum of the purified urease was determined using citrate buffer (pH 3·0–7·0) and Hepes buffer (pH 7·0–10·0), each prepared at a concentration of 50 mM and containing 0·5 mM Na_2EDTA. These assays were performed using 50 ng of purified urease in 1 ml buffer incubated at 37 °C. The effect of different temperatures on enzyme activity was determined as above using the Hepes buffer adjusted to pH 7·75. Different concentrations of metal ions (as

chloride salts), Na_2EDTA, and reported organic inhibitors of urease were used to determine their effects on activity of the purified enzyme. Each metal ion, chelator, or organic inhibitor was mixed with 50 ng of the *C. immitis* urease in 1 ml assay buffer (50 mM Hepes plus 50 mM urea [pH 7·75]), and the rate of ammonium production was determined as described above.

Inhibition of urease activity in vitro

The effects of addition of 10 mM acetohydroxamic acid (AHA) to the medium of 4-day-old cultures of the mycelial phase on growth rate, urease activity and ammonium release were also determined. The concentration of AHA added to mycelial culture medium was chosen on the basis of its inhibitory effect on urease activity in cytosolic fractions isolated from 4-day cultures of the saprobic phase. The mycelial cytosol was obtained by glass bead homogenization of the mycelial mat in enzyme assay buffer (4 °C) followed by centrifugation (40 000 *g*). The assay of urease activity was conducted as described above. The concentration of total protein in each cytosolic fraction was also determined as described above.

Control cultures grown in the absence of AHA were subjected to the same analyses as above. After initial growth of the mycelium in glucose plus yeast extract medium for 4 days, the mycelial mat was harvested, washed twice with growth medium and transferred to freshly prepared medium that contained 10 mM AHA. The cultures were GYE incubated at 31 °C on a gyratory shaker for a subsequent 48-h period. Urease activity in isolated cytosolic fractions and ammonium content of the culture medium were then determined at intervals over the 48-h period of incubation by the same assays as described previously. In addition, cultures (100 ml) of the mycelial phase were collected by filtration on Whatman 1 filter paper at each of the same intervals, washed twice with equal volumes of distilled water, and dried at 105 °C to determine the effect of AHA on growth of the mycelial phase.

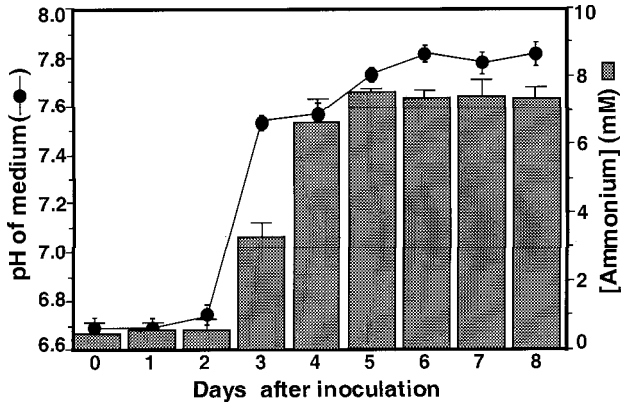


Fig. 1 Changes in pH and $[NH_3/NH_4^+]$ during growth of saprobic phase of *C. immitis*. The concentration of ammonium in the mycelial culture filtrate was measured by the Bertholet reaction. Initial pH and $[NH_3/NH_4^+]$ indicated at day 0 represent values obtained for the culture medium prior to inoculation. Data points represent means±SD of three separate analyses.

Results

Ammonium production in vitro

To confirm that *C. immitis* has the capacity to generate an alkaline culture medium and that it releases ammonia, the culture filtrate of the mycelial phase of the fungus grown in glucose–yeast extract broth was sampled during an incubation period of 8 days. Ammonium production was detected by the Bertholet reaction and an increase of approximately 2 pH units was recorded (Fig. 1). The same growth conditions used in this analysis were employed for purification of urease from the mycelial cytosol described below.

Enzyme purification

After separation of the mycelial mat from the culture filtrate, the mycelium was frozen, homogenized and the cytosolic fraction was isolated and assayed for urease activity. The results of the enzyme purification steps are summarized in Table 1. The isolation strategy recovered 10·5% of the original enzyme activity and achieved a

Table 1 Purification of urease from the mycelial cytosol of *C. immitis*

Fraction	Specific activity (μmol urea min^{-1} mg^{-1})*	Purification (fold)	Total activity (μmol urea min^{-1})	Total protein (mg)	Yield (%)
Crude mycelial cytosol	1·67	1	3340	2000	100
DEAE-Sepharose	12·23	7·32	2813	230	84·2
Phenyl-Sepharose	172	103	2150	12·5	62·8
Q HyperD 20	1339	802	1607	1·2	48·1
Superose 6 HR	1750	1048	350	0·2	10·5

*One unit of urease activity is defined as the amount of enzyme (mg) required to hydrolyze 1 μmol urea min^{-1} at 37 °C.

final specific activity in the presence of urea of 1750 μmol min^{-1} mg^{-1} protein. After application of the crude mycelial cytosol to the DEAE fast-flow Sepharose column, an estimated 99% of the active urease was retained on the column after washing with RB1. The bound fraction was eluted with a linear salt gradient and urease was released as a single peak at 1 M NaCl (Fig. 2a). Almost all the active urease present in the DEAE-Sepharose fraction was subsequently bound to the RB2-equilibrated Phenyl-Sepharose column, but was then released when the concentration of K_2HPO_4/KH_2PO_4 in the eluate was reduced to approximately 20 mM (Fig. 2b). The active urease fraction isolated after Phenyl-Sepharose chromatographic separation was then applied to the Q HyperD 20 HPLC column. The latter was washed with a linear salt gradient and the active urease was eluted as a single peak at 500 mM NaC1 (Fig. 2c). The final step in purification of the fungal urease was conducted by gel filtration chromatography using a Superose 6 FPLC column. The isolated fraction obtained from the Q HyperD 20 column was further fractionated to yield a single, symmetrical absorbance peak composed of urease-active fractions (Fig. 3a). Analysis of each of these fractions by SDS–PAGE revealed a single protein band by silver stain in fraction 15 (M_r=101 000; Fig. 3b). The protein appeared to be purified to homogeneity. On the basis of the elution profile of the Superose 6 HR chromatographic separation, plus the results obtained by SDS–PAGE, we suggest that the 101-kDa fraction represents the purified monomer of the *C. immitis* urease.

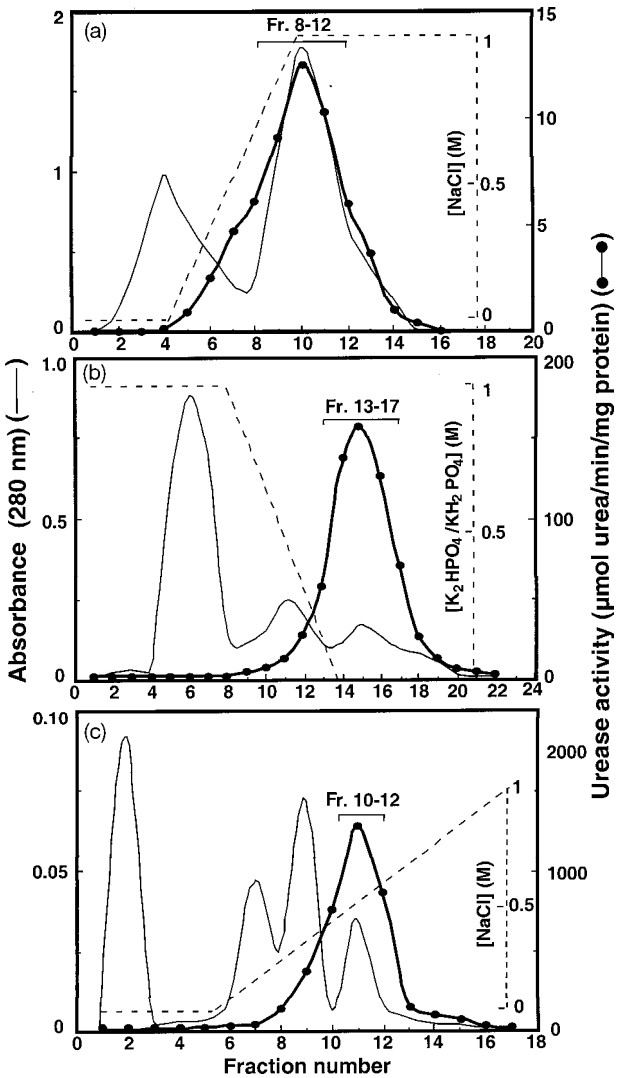


Fig. 2 Chromatographic fractionation of *C. immitis* mycelial cytosol. The mycelial cytosol preparation was chromatographically separated (a) on a DEAE-Sepharose (FPLC) column, followed by separation on (b) Phenyl-Sepharose (FPLC), and (c) Q HyperD 20 (HPLC) columns. Eluted fractions were assayed for urease activity as described in the Methods section. Urease activity (●—●), NaCl concentration (- - -), and absorbance at 280 nm (—) are indicated. Fractions that were collected and pooled after each step are indicated by brackets.

Estimated molecular mass of the native urease

The native molecular mass of the *C. immitis* urease was estimated by gel filtration using protein standards with molecular sizes in the range 29–669 kDa. The approximate molecular size of the native urease is 450 kDa. Since only a single protein band was detected in the Superose 6 chromatographic fraction 15 by SDS–PAGE under reducing conditions, we suggest that the native enzyme is a tetramer.

Amino acid sequence analysis

The N-terminal amino acid sequence of the purified 101-kDa polypeptide isolated from the SDS–PAGE gel is Met–Gln–Leu–Val–Pro–Arg–Glu–Ile–Asp–Lys–Leu–Thr. This sequence is identical to the previously reported N-terminal sequence encoded by the *URE* gene (GenBank accession U81509 [5]). Comparison of this sequence to that of reported fungal and nonfungal ureases was performed using the CLUSTAL W alignment program [16]. The results revealed that the *C. immitis* enzyme is 58% identical to the *Cryptococcus neoformans* urease (GenBank accession AF006062) [7], 61% identical to the *Schizosaccharomyces pombe* urease (GenBank accession AB002590) [17], and 55% identical to the Jack bean urease (GenBank accession PO7374) [18].

K_m *estimation*

The rate of urea hydrolysis was monitored as a function of urea concentration (0–100 mM) (Fig. 4). The purified

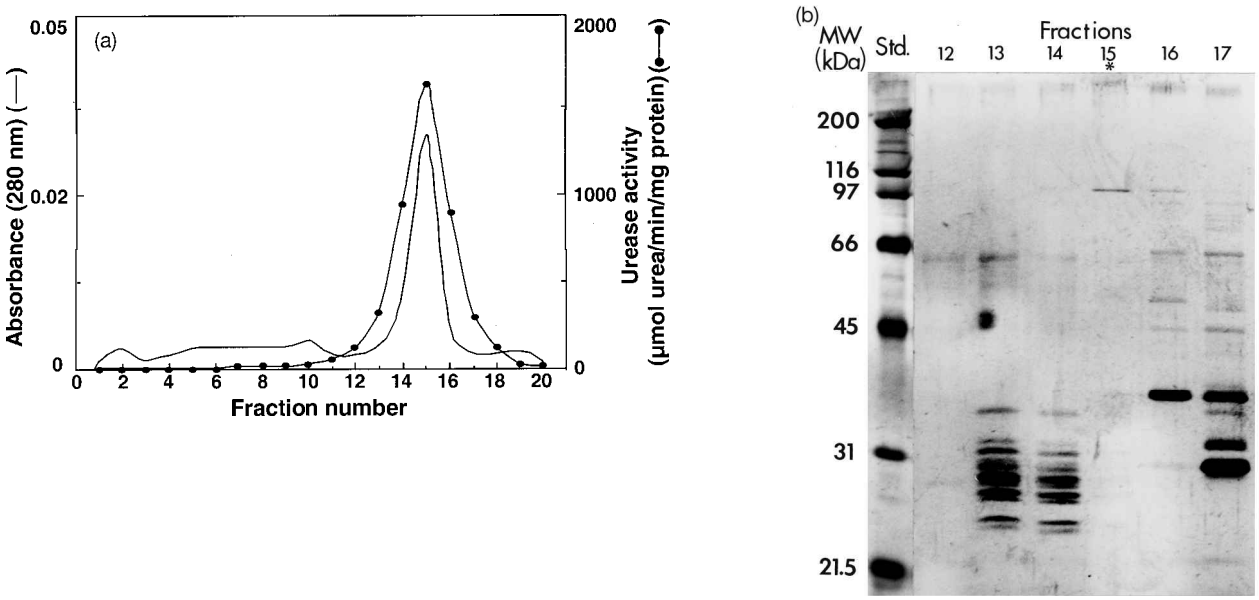


Fig. 3 Purification of *C. immitis* urease. The active fraction from the Q HyperD (HPLC) column was applied to (a) a Superose 6 HR (FPLC) column as described in the Methods section. Column effluent (1-ml fractions) was monitored by absorbance at 280 nm (—) and results of urease activity assays were recorded (●—●). b, Chromatographic fractions were further separated by SDS–PAGE and components were revealed by silver stain. Numbers above lanes represent individual, concentrated chromatographic fractions. Asterisk indicates lane containing purified 101-kDa protein. Molecular mass standards used include myosin (200 kDa), β-galactosidase (116·2 kDa), phosphorylase B (97·4 kDa), bovine serum albumin (66·2 kDa), ovalbumin (45 kDa), bovine carbonic anhydrase (31 kDa), and soybean trypsin inhibitor (21·5 kDa).

enzyme showed high specificity for urea, with an estimated K_m of 4·1 mM (Fig. 4, inset).

pH optimum

The purified enzyme demonstrated activity over a broad pH range (5·0–8·0), with optimum activity at pH 8·0 (Fig. 5a). The pH range and optimum for activity of the *C. immitis* urease is similar to that of other reported ureases [6,19,20].

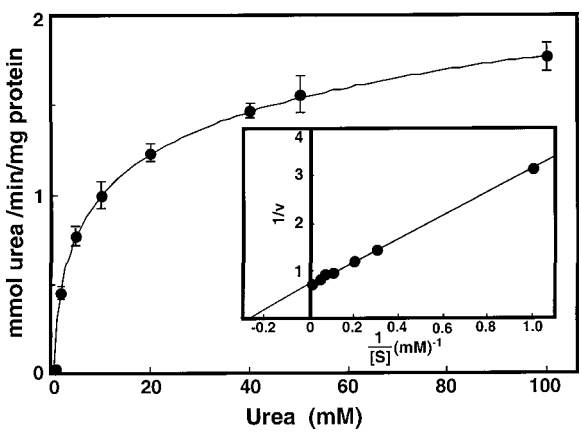


Fig. 4 Effect of substrate concentration on urease activity. The purified enzyme (50 ng protein, fraction 15 from Fig. 3a) was incubated with 0–100 mM urea in assay buffer as described in the Methods section. Inset shows a Lineweaver–Burk plot of urease activity. Data points represent means±SD values for three separate determinations.

Thermostability

After incubation of the purified urease at 37, 65, 80 or 100 °C, the sample was rapidly cooled to 4 °C and then assayed for enzyme activity at 37 °C. The urease appeared to be heat resistant. Its activity decreased by only 35% after 30 min at 65 °C compared to the control at 37 °C (Fig. 5b). However, the enzyme rapidly lost activity at higher temperatures (78 and 100% loss of activity after 2·5 min at 80 and 100 °C, respectively).

Influence of metal ions and Na_2EDTA on urease activity

The effects of selected metal ions and EDTA on *C. immitis* urease activity are summarized in Table 2. The enzyme buffer mixture was supplemented with either selected cations or Na_2EDTA at 5 or 10 mM. Ca^{2+}, K^+, Na^+ and Na_2EDTA had essentially no effect on urease activity. On the other hand, the activity of the purified enzyme was partially inhibited by Li^+, and strongly inhibited by Ni^{2+}, Cu^{2+}, Zn^{2+}. Stimulation of urease activity was observed when either Mn^{2+} or Mg^{2+} was added to the assay mixture.

Table 2 Effect of metal ions and Na_2EDTA on activity of the purified *C. immitis* urease

Supplement	Urease activity (%)*	
	5 mM	10 mM
Mn^{2+}	119±3	115±4
Mg^{2+}	106±2	110±4
Ca^{2+}	102±3	96±7
K^{+}	98±2	100±3
Na^{+}	90±3	95±3
Li^{+}	87±5	88±2
Ni^{2+}	38±4	31±2
Cu^{2+}	32±3	27±4
Zn^{2+}	33±6	31±3
Na_2EDTA	94±4	91±6

*Percentage activity of urease incubated in the standard assay mixture (assay buffer, pH 7·75, 37 °C) was considered to be 100%. The data typically showed less than 10% variation of the mean of six assays using two separately purified enzyme preparations.

Table 3 Effect of selected organic inhibitors on activity of the purified *C. immitis* urease

Supplement to assay mixture	Urease activity (%)*				
	1 mM	2 mM	3 mM	5 mM	10 mM
Hydroxyurea	68±3	49±4	34±2	ND	ND
Acetohydroxamic acid	53±4	48±3	17±5	ND	ND
Boric acid	76±6	60±2	55±4	ND	ND
Thiourea	ND	ND	ND	76±6	61±2

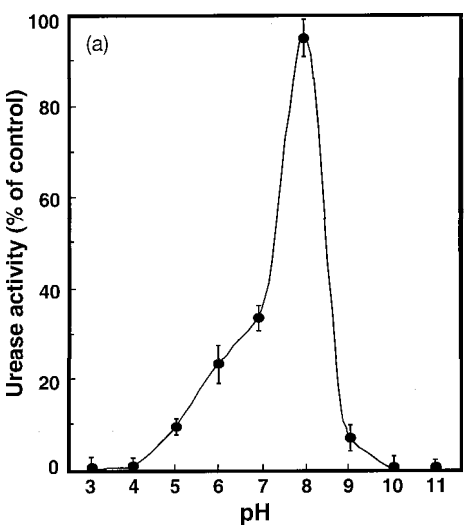
*Percentage activity of the enzyme in the standard assay mixture was considered to be 100%, and variation of the mean values for test samples was less than 10% as described in Table 2.
ND, not determined.

Action of urease inhibitors

C. immitis urease activity was also influenced by organic compounds previously reported to be urease inhibitors (Table 3). AHA, hydroxyurea, and boric acid inhibited activity of the fungal urease, with hydroxyurea being the most effective at the lowest concentration (1 mM). Thiourea showed only partial inhibition of enzyme activity at concentrations of 5 and 10 mM.

Effect of inhibition of urease activity on ammonia production in vitro

The data presented in Table 4 show that addition of 10 mM AHA to cytosolic fractions of *C. immitis* isolated from 4-day-old mycelial cultures resulted in total inhibition of urease activity. On this basis, 10 mM AHA was added to fresh media inoculated with the washed mycelial mat obtained from 4-day GYE cultures to determine the effect of inhibition of urease activity on secretion of ammonia *in vitro*. Control cultures were inoculated in the same manner without addition of AHA. The freshly inoculated cultures were then incubated for 48 h at 31 °C (Fig. 6a, b). The urease activity of the control culture remained constant, and AHA had no apparent effect on mycelial growth rate



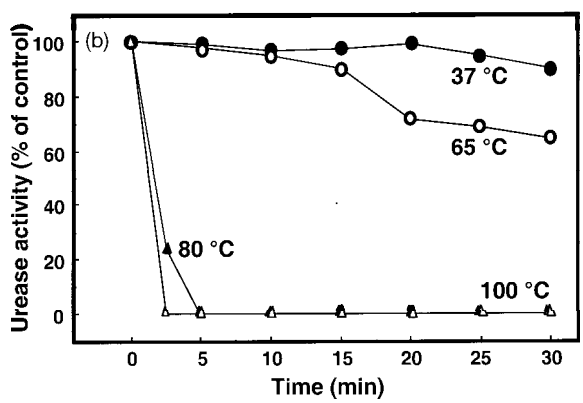


Fig. 5 a, Effect of pH on urease activity. Enzyme assays were conducted in citrate buffer (pH 3·0–7·0) and Hepes buffer (pH 7·0–10·0) as described in the Methods section. Data, expressed as percentages of maximum enzyme activity in enzyme assay buffer (pH 7·75) at 37 °C, and are means±SD values from three separate determinations. b, Thermal stability of *C. immitis* urease. The purified urease (50 ng) was incubated at the indicated temperatures (37 °C [●] normal assay condition, 65 °C, [○], 80 °C [▲], and 100 °C [△]), then cooled to 4 °C and assayed for enzyme activity. Data are expressed as percentages of maximum enzyme activity at 37 °C, and represent six enzyme activity determinations using two separately purified urease preparations. SD values (not shown) were smaller than data symbols.

Table 4 Effect of acetohydroxamic acid on urease activity in the mycelial cytosol isolated from 4-day-old mycelial cultures of *C. immitis*

	Urease activity (%)*					
Supplement to growth medium	0 mM	1 mM	5 mM	10 mM	20 mM	50 mM
Acetohydroxamic acid (AHA)	100	62±2	34±3	0	0	0

*Percentage activity of urease in the control sample (100%) was conducted in the absence of AHA. The data for the test cytosolic fractions showed less than 10% variation for the mean of three assays using each concentration of AHA.

during the 48 h incubation period (Fig. 6a). As expected, the presence of 10 mM AHA in the culture medium resulted in a sharp decrease in urease activity (Fig. 6b). Since AHA is a reversible inhibitor, the urease activity gradually recovered during the incubation period. However, it is evident that the inhibitory effect of AHA on urease activity was not accompanied by a major decrease in detectable ammonium in the growth medium compared to the control culture (Fig. 6b).

Discussion

In vitro growth of the saprobic and parasitic phases of *C. immitis* results in release of ammonium and a concomitant increase in pH of the culture medium [4]. During colonization of host lung tissue following infection by *C. immitis*, abscesses are formed with distinctly alkaline pH [21]. Such alkalinization of the microenvironment at sites of *in vivo* growth of the fungus may compromise host defenses and contribute to the pathogenicity of *C. immitis*. *In vivo* transcription of the *C. immitis* urease gene is elevated in abscesses which contain endosporulating spherules [4], and the peak of production of the urease protein *in vitro* as measured by immunoblots of parasitic cell homogenates was correlated with the endosporulation phase of the parasitic cycle [21]. Urease activity may be at least partly responsible for ammonium release by *C. immitis* and for the alkalinization of its microenvironment. The urease of *C. immitis* is also of interest because the recombinant form of the protein has been shown to be immunogenic in mice and capable of providing immunoprotection to the animals against a lethal challenge of the pathogen [22]. In addition, we have reported that immunization of mice with DNA that encodes the urease protein provides even better immunoprotection against the disseminated form of the fungal disease [22]. While these earlier studies have focused on isolation and expression of the *C. immitis* urease gene [4,5], this is the first report of the purification of the native enzyme.

Although urease is a cytosolic enzyme [6], the protein is detected in the culture filtrates of both the saprobic and parasitic phases of *C. immitis* after *in vitro* growth for 5 and 6 days, respectively [21]. The urease may be released from stationary saprobic phase cells that have undergone partial autolysis, but it could also be released with the contents of mature spherules at the time of rupture and endospore release [4,8]. Extracellular urease activity has been reported in *H. pylori* and suggested to

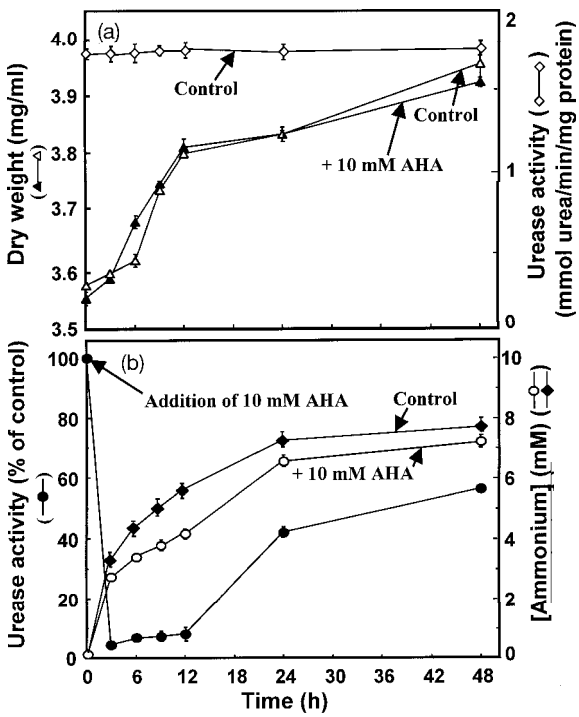


Fig. 6 Effect of acetohydroxamic acid on urease activity, ammonium release into the growth medium, and change in dry weight of the saprobic phase of *C. immitis*. a, Changes in urease activity during the 48-h period of incubation in the absence of AHA (control ◇), and changes in dry weight of mycelia in the presence of 10 mM AHA (▲) or absence of AHA (control; △) are shown in (a). The effects of addition of 10 mM AHA on urease activity (●) and ammonium release into the medium (○), compared with amount of ammonium released in the absence of AHA (control; ◆). Percentage urease activity in cytosolic fractions (b) at time of transfer of mycelia to the medium supplemented with 10 mM AHA represented 100%. Data points represent means±SD of three separate analyses.

occur as a result of bacterial cell autolysis followed by adsorption of the enzyme to the surface of intact bacteria [23]. Most of the active urease of *C. immitis*, however, is intracellular and the cytosol of the mycelial phase was used as the source of the enzyme isolated and purified to homogeneity in this study. The chromatographically purified urease enzyme exhibited a specific activity of 1750 μmol min^{-1} mg^{-1} protein, which is higher than the activity recorded for other fungal ureases obtained from *Aspergillus niger* (1341 U mg^{-1}) [20], *A. nidulans* (670 U mg^{-1}) [24], and *S. pombe* (700–800 U mg^{-1}) [17]. The specific activity of the *C. immitis* urease was more comparable to that reported for bacterial ureases (1000–5500 U mg^{-1}) [6]. The K_m of the purified enzyme (4·1 mM urea) is similar to the K_m reported for certain other fungi. The ureases of *A. niger* [20], *A. nidulans* [24], *Ustilago violacea* [25] and *S. pombe* [17] have K_m values for urea which range from 1·0 to 3·8 mM. In contrast, the K_m of bacterial ureases has a much greater range, from 0·1 mM to >100 mM [6].

The subunit stoichiometry of urease in this study, as in previous reports, was determined by comparing the native molecular mass of the enzyme to the subunit size estimated by SDS–PAGE [26]. Eukaryotic ureases have been reported to be multimeric and typically composed of identical subunits [6]. The urease subunit of *C. immitis* is characterized by a monomer of 101 kDa. The molecular size of the monomer of most other eukaryotic ureases is smaller; e.g. molecular mass of 40 kDa in the case of the *A. nidulans* urease [20], 80 kDa for the *U. violacea* urease [25], 83 kDa for *A. niger* [20], and 91 kDa for the Jack bean urease [27]. The native urease of *C. immitis* is estimated to be 450 kDa and, therefore, is probably composed of four subunits. The number of subunits of other eukaryotic ureases ranges from three in *A. niger* [20] to six in Jack bean [28] and eight in *Rhodosporidium paludigenum* [29]. The reported ureases with highest amino acid sequence homology to the *C. immitis* urease are of eukaryotic origin. As expected, we found closest homology to the ureases of *C. neoformans* [7] and *S. pombe* [17], the only two other fungal ureases which have so far been sequenced (58 and 61% identity, respectively). Only 55% sequence identity to the Jack bean urease was revealed, and little similarity exists between the *C. immitis* and bacterial ureases so far reported [5]. The *C. immitis* urease shares biochemical characteristics with other ureases, especially those of fungal origin. It showed maximum activity at pH 8·0, and the optima for other fungal ureases are typically in the pH range of 8·0–8·5 [20,24,30]. An exception is the urease of *U. violacea* which has a pH optimum of 7·0 [25]. Lower pH optima in the range of 7·0–7·8 have been reported for the majority of bacterial ureases [6]. The thermostability of the *C. immitis* urease is consistent with findings for other ureases, which have demonstrated that activity of the enzyme is stable up to 60–65 °C [26,31].

The effect of inhibitors on *C. immitis* urease activity described in this study was similar to those reported for other ureases. However, while the activity of the *A. niger* urease was inhibited by Mg^{2+} [20], our results showed that the presence of Mg^{2+} or Mn^{2+} slightly enhanced the activity of the *C. immitis* enzyme. The effect of other cations on *C. immitis* urease activity followed the general trends reported in the literature [6]. Na^+ and K^+ showed little to no effect, while Ni^{2+}, Zn^{2+}, and Cu^{2+} were inhibitory. We also showed that AHA, hydroxyurea, boric acid and thiourea, all of which are effective inhibitors of plant and bacterial ureases [6], showed some degree of inhibition on activity of the *C. immitis* enzyme. At a concentration of 10 mM, AHA is a potent inhibitor of urease activity in crude cytosolic preparations of *C. immitis*. We have presented evidence that the same concentration of this reversible urease inhibitor can block urease activity in mycelial cultures of *C. immitis,* but does not affect growth and has little effect on the amount of ammonium release into the medium. This latter observation was surprising, since we had speculated that disruption of the urease gene would result in loss of the ability of *C. immitis* to generate an alkaline microenvironment, and as a consequence show reduced virulence in the host. In support of this proposal, disruption of the urease gene in *C. neoformans* did result in reduced virulence of the pathogen in murine models of cryptococcosis [7].

Our results of *in vitro* studies of urease inhibition and ammonium release presented in this study suggest that other metabolic sources of ammonia exist in *C. immitis*, which may depend on the activity of such enzymes as glutaminase, asparaginase, serine dehydrotase, NAD^+- and NADPH-dependent glutamate dehydrogenase and aspartase [32]. Partial sequences of the genes that encode most of these enzymes of *C. immitis* have been obtained in our laboratory, and studies of differential gene expression during *in vitro* and *in vivo* growth of the pathogen are in progress. The enzyme(s) whose activity complements that of urease and results in release of ammonia from parasitic cells may represent a key virulence factor of *C. immitis*.

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