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Urease from Cotton (*Gossypium hirsutum*) Seeds: Isolation, Physicochemical Characterization, and Antifungal Properties of the Protein

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Ureases (EC 3.5.1.5) are metalloenzymes that hydrolyze urea to produce ammonia and carbon dioxide These enzymes, which are found in fungi, bacteria, and plants, show very similar structures. Despite an abundance of urease in vegetal tissues, the physiological role of this enzyme in plants is still poorly understood. It has been previously described that ureases from the legumes jackbean (*Canavalia ensiformis*) and soybean (*Glycine max*) have insecticidal activity and antifungal properties. This work presents the physicochemical purification and characterization of a urease from cotton (*Gossypium hirsutum*) seeds, the first description of this enzyme in Malvaceae. The urease content varied among different cotton cultivars. Cotton seed urease (98.3 kDa) displayed low ureolytic activity but exhibited potent antifungal properties at sub-micromolar concentrations against different phytopathogenic fungi. As described for other ureases, the antifungal effect of cotton urease persisted after treatment with an irreversible inhibitor of its enzyme activity. The data suggest an important role of these proteins in plant defense.

KEYWORDS: Cotton seed; *Gossypium hirsutum*; purification; urease; antifungal protein

INTRODUCTION

Ureases (EC 3.5.1.5, urea amidohydrolase) are nickel-dependent metalloenzymes that catalyze the hydrolysis of urea to ammonia and carbon dioxide (*1*), enhancing the rate of the uncatalyzed reaction by a factor of 8×10^{17} (*2*). Ureases are widespread in plants, fungi, and bacteria (*3–5*). Whereas fungal and plant (e.g jackbean and soybean) ureases are homo-hexameric proteins of 90 kDa subunits, bacterial ureases are multimers of two or three subunit complexes. The high sequence similarity of all ureases indicates that they are variants of the same ancestral protein and are likely to possess highly conserved tertiary structures and similar catalytic mechanisms (*3–5*). Some bacterial ureases such as those from *Proteus mirabilis* and *Helicobacter pylori* (*6, 7*) play an important role in the pathogenesis of human and animal diseases. Urease production is also probably related in humans to the pathogenesis of diseases caused by fungal infection with *Coccidioides posadasii* (*8*), *Coccidioides immitis* (*9*), *Paracoccidioides brasiliensis* (*10*), and *Cryptococcus neoformans* (*11, 12*).

Despite the abundance in some plant tissues, mainly in seeds of some members of the families Fabaceae, Leguminosae, and Curcubitaceae, little has been revealed about their biological roles (*5, 13*). Urease has been proposed to function coordinately with arginase in the utilization of seed protein reserves during germination (*14*). A second role is the assimilation of urea derived from ureide metabolism (*15*) or imported from the environment because urea is an effective foliar fertilizer (*16*).

Our group has shown that plant and microbial ureases display several biological properties that are independent of their ureolytic activity, such as the activation of blood platelets, interaction with glycoconjugates, insecticidal (*6, 17*), and antifungal properties (*18*). Besides these activities, jackbean canatoxin, an isoform of urease, is also lethal to rats and mice by an intraperitoneal route (*17, 19*). The entomotoxic effect and the antifungal property of jackbean and soybean ureases persisted after treatment of the enzymes with an irreversible inhibitor of ureolytic activity, demonstrating that protein domain(s) other than the active site might be involved (*6, 18, 20*). Actually an internal entomotoxic peptide released upon digestion by insect cathepsins accounts for the insecticidal activity of jackbean ureases (*21–23*). These findings reinforce the hypothesis that ureases might be involved in plant defense mechanisms (*24*).

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Table 1. Seed Protein and Urease Content of Different Cultivars of Cotton Plants

cultivar	mg of protein/g of seed meal[a]	mU of urease/g of seed meal[b]	mU of urease/mg of protein
IAC 24	41.4 ± 1.2 a[c]	41.1 ± 4.9 ah[d]	3.40
MT 506	34.5 ± 3.5 abc	108.0 ± 3.5 bi	3.13
Cedro	38.3 ± 0.1 d	147.1 ± 7.0 ch	3.84
Glanless 2003	43.7 ± 2.3 b	104.0 ± 0.7 d	2.38
Coker 312	38.1 ± 1.8 e	20.3 ± 0.2 e	0.53
MT 537	46.3 ± 1.4 cdef	77.7 ± 1.2 f	1.68
Embrapa BRS Cedro 2004	39.1 ± 0.2 f	118.0 ± 0.9 gbi	3.02

[a] Protein concentration of crude extracts of cotton seeds was measured by the dye-binding method of Spector et al. (*30*). [b] Urease activity of the extracts was assayed by the ammonia released and quantified colorimetrically by the method of Weatherburn (*33*). [c] Two means within a column followed by the same letter are statistically different ($p < 0.01$) by ANOVA. [d] Two means within a column followed one same letter are not statistically different ($p < 0.05$) by ANOVA. All other means differ from each other with $p < 0.001$.

The cotton plant *Gossypium hirsutum* L.(Malvaceae) accounts for 90% of the world production of cotton fibers. According to the U.S. Department of Agriculture, the world production of cotton seeds will be 25.24 million tons in 2007/2008. Brazil is today the fifth major cotton producer, with 2.28 million tons of seeds harvested in 2006, increasing to 3.76 million tons in 2007. The cotton plant is attacked by a wide range of economically important fungal pathogens. The seedling disease complex, fungal wilt pathogens and boll rots, are the major cotton diseases worldwide. Among the most destructive cotton diseases are *Fusarium* wilt and *Verticillium* wilt, which are caused by *Fusarium oxysporum* f. sp. *vasinfectum* and *Verticillium dahliae* Kleb, respectively (*25*).

Whole cottonseeds are a very popular feed for dairy cattle and are uniquely high in fiber, energy (from fat), and protein (*26*). It has been demonstrated previously that the wilt fungus can remain viable after passing through the gut of a cow. Therefore, feeding cattle on seed husks from a crop infected with *Fusarium* wilt is a potential means of spreading the disease (*27*).

Cottonseed is also attacked by the saprophytic fungus *Aspergillus flavus*, which produces aflatoxin, one of the deadliest mycotoxins known (*28*). The presence of aflatoxin in cottonseed endangers the health of livestock consuming cottonseed meal used in animal feeds and the health of humans consuming milk products from the affected livestock (*29, 30*).

Differences in the content of secondary metabolites and defense proteins among cultivars of commercially available cotton plants as well as in wild varieties may offer a source of resistance genes against insect attack and fungus-induced diseases. In the present work we have analyzed the content of seed urease of different cotton cultivars and purified *G. hirsutum* seed urease (GHU), the first Malvaceae for which this enzyme has been described, and characterized some of its enzymatic parameters. In addition, we describe the inhibitory activity of the cotton seed urease upon mycelial growth and/or spore germination of filamentous fungi that are important plant pathogens.

MATERIALS AND METHODS

Cotton Seeds. Cotton seeds of seven cultivars planted in Brazil were kindly supplied by Dr. Thales Lima Rocha and Maria Fátima Grossi de Sá, from the Centro Nacional de Recursos Genéticos e Biotecnologia (Cenargen), Brasília, DF. The following cotton cultivars were tested: IAC24, Mato Grosso MT 506, Cedro, Glandless 2003, Coker 312, Mato Grosso MT 537, and EMPRABA BRS Cedro 2004. For the assays,

Table 2. Purification of *Gossypium hirsutum* Urease

purification step[a]	total A_{280}[b]	UA_{280}^{-1}	total U[c]	purification index	yield (%)
crude extract	4425	0.012	52.66	1	100
0−0.4 $(NH_4)_2SO_4$ fraction	689.4	0.015	10.341	1.26	20
Source 15Q 360 mM fraction	1.992	5.3	10.56	445.38	20.4
gel filtration (Superose-6)	0.024	29.23	0.695	2456.3	1.34

[a] See details of the purification steps in the text. [b] Protein content of fractions as indicated by absorbance at 280 nm. After the Source 15 column, analyses of fractions by the dye-binding method (*31*) gave very similar results of protein content. [c] Urease activity was determined colorimetrically according to the method of Weatherburn (*33*). Typical results. Similar data were obtained for at least three preparations.

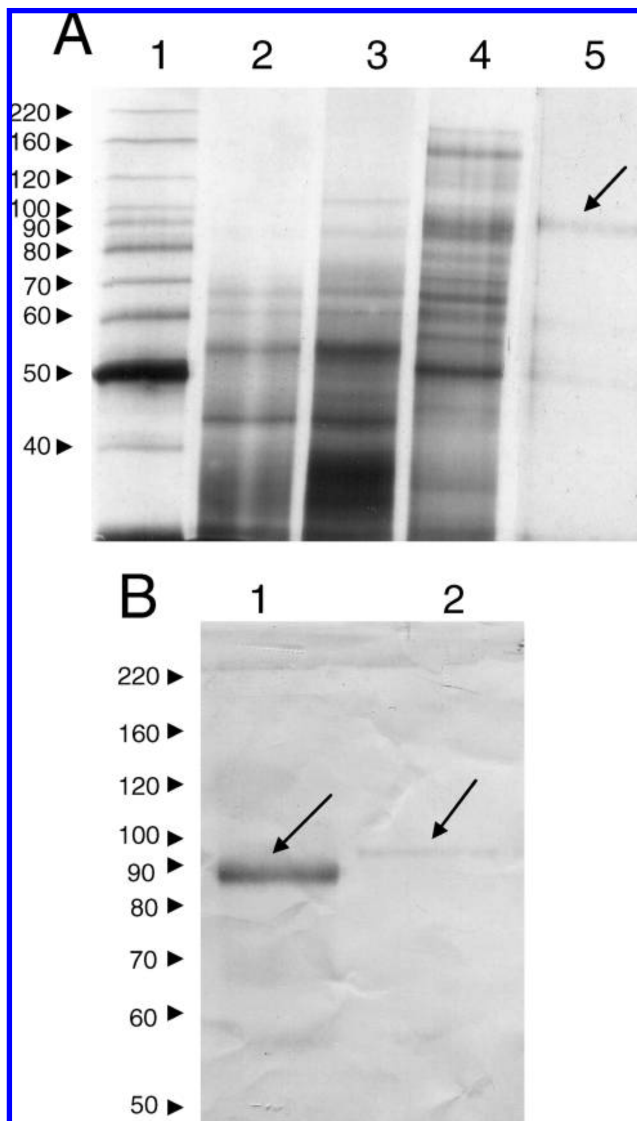

Figure 1. Eletrophoresis and Western blot of purified *Gossypium hirsutum* urease (GHU). (**A**) SDS-PAGE (10% gel): lane 1, molecular mass standards; lane 2, prep D (Source 15Q), 3 μg; lane 3, prep C (Q-Sepharose), 8 μg; lane 4, crude extract, 20 μg; lane 5, purified GHU, 0.9 μg. (**B**) Western blot using anti-canatoxin IgG as the primary antibody: lane 1, *Canavalia ensiformis* major urease, 4 μg; lane 2, purified GHU, 9 μg. The arrows indicate the bands corresponding to the ureases.

the seeds were cut in halves, and the cotyledons were removed and ground in an analytical mill (Ika, Germany). Crude extract at 15% w/v of the seeds meals was prepared by suspending 1.5 g of meal in 10 mL of 20 mM sodium phosphate buffer (NaPB), 1 mM EDTA, and 2 mM 2-mercaptoethanol, pH 7.5. The mixture was stirred for 2.5 h in

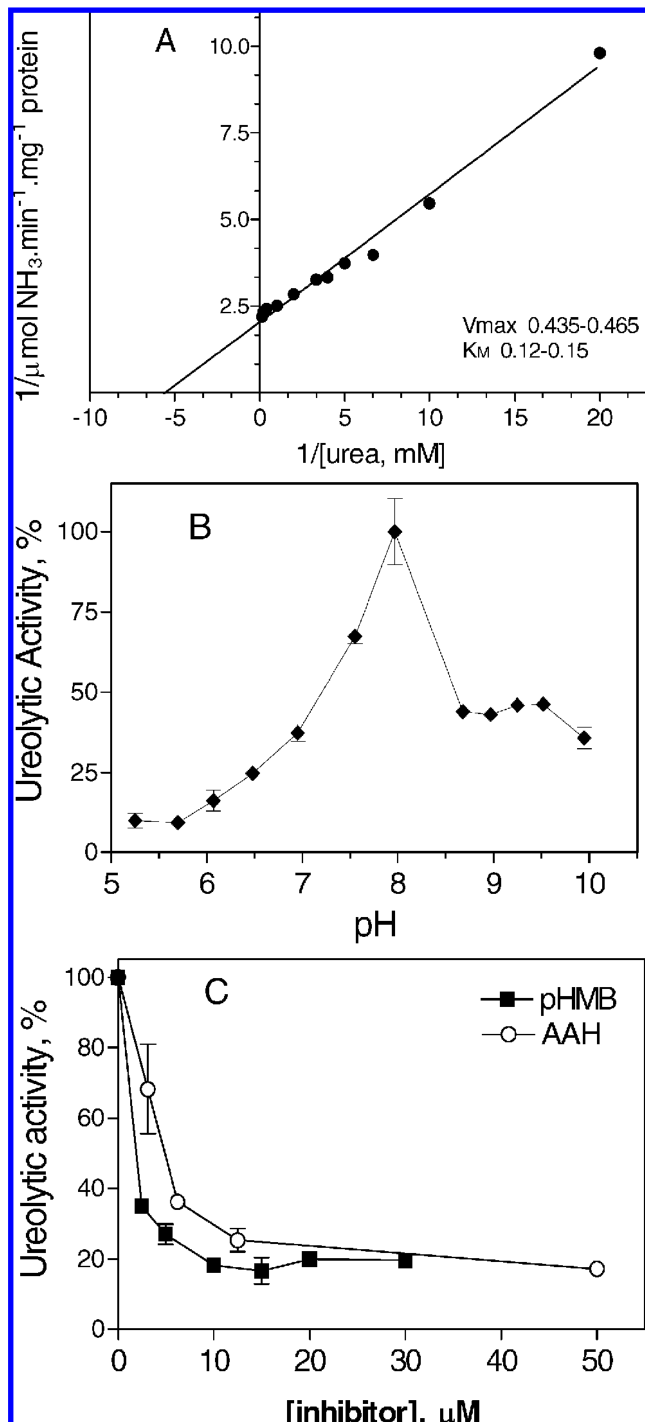

Figure 2. Kinetic parameters and inhibitors of *Gossypium hirsutum* urease. (**A**) Double-reciprocal plot of GHU enzymatic activity The enzyme activity of GHU in 20 mM sodium phosphate buffer at 37 °C was measured in the presence of concentrations of urea varying in the 0.05−10 mM range. Values of K_m and V_{max} were calculated using Instat GraphPad software version 3.05. Typical experiment.(**B**) Determination of optimum pH. The enzyme activity upon 10 mM urea was measured in different pH-buffered media: 50 mM sodium citrate−sodium phosphate (pH 3.0−7.0), 50 mM sodium phosphate (pH 7.5−8.0), and 50 mM sodium borate (pH 8.5−10). Data represent means and standard deviation of triplicated points. (**C**) Determination of 50% inhibitory concentrations of *p*-hydroxymercuribenzoate (*p*-HMB) or acetohydroxamic acid (AHA) upon GHU. For these experiments, samples of GHU were incubated with the indicated concentrations of the inhibitors at 4 °C for 18−24 h, and then the residual ureolytic activity was compared to that of the enzyme incubated with the corresponding diluent (100% activity).

Table 3. Comparative Data on Plant Ureases from *Canavalia ensiformis* (JBU, Major Isoform), *Glycine max* (SBU, Seed Specific), and *Gossypium hirsutum* (GHU)

	JBU[a]	SBU[b]	GHU[c]
Physicochemical Properties			
molecular mass (kDa), SDS/PAGE	90.7	93.6	98.3
native form	hexamer	hexamer	hexamer
Urease Activity			
K_m (urea, mM)	2−3.6	0.2−0.6	0.12−0.15
V_{max} (U/mg)	13700	1300−1600	29
IC_{50}[d]			
p-hydroxymercuribenzoate	70	38	59
acetohydroxamic acid	42	216	152

[a] Data on JBU were taken from Follmer et al. (*17*) and Follmer et al. (*6*). [b] Data on SBU were taken from Follmer et al. (*6*) and Polacco et al. (*44*). [c] This paper. [d] Fifty percent inhibitory concentrations expressed as moles of inhibitor per mole of urease.

an ice bath and filtered on cheesecloth; after standing overnight at 4 °C, the filtrate was spun 3three times at 16000*g*. The final supernatant was designated crude extract and used for determination of protein content and urease activity.

Purification of GHU. Cotton seeds (cultivar IAC24) were kindly provided by Antônio Poletti, Núcleo de Sementes e Mudas de Aguaí, São Paulo, Brazil. After the seeds had been cut into halves, the cotyledons were removed and ground in an analytical mill. The cotyledon meal was extracted five times in chloroform at a 50% w/v under stirring, 30 min at room temperature each. The defatted meal was extracted at a 20% w/v proportion of 20 mM sodium phosphate, 1 mM EDTA, and 2 mM 2-mercaptoethanol, pH 7.5 (buffer A), for 3 h at 4 °C under stirring. After filtration and several cycles of centrifugation (30000*g*, 20 min, 4 °C), the precipitate was discarded and the colored supernatant was dialyzed against extraction buffer. This crude extract (prep A) was fractionated by $(NH_4)_2SO_4$ precipitation, and the 0−0.4 fraction containing the ureolytic activity was extensively dialyzed against buffer A adjusted to pH 7.0 until a negative reaction with Nessler reagent was obtained, yielding an almost colorless prep B. The next step consisted of an ion exchange chromatography in Q-Sepharose (Amersham-Biotech Pharmacia). Samples of prep B were mixed with the resin ($5.0A_{280}$ of protein per 1 mL of resin) equilibrated in the same buffer and left under stirring overnight at 4 °C. After washing the resin with equilibrating buffer to remove the nonretained proteins, elution of urease-enriched fractions was achieved by adding 300 mM NaCl to the buffer. The eluted fractions were pooled to make prep C. After dialysis against buffer A, pH 7.5, prep C was applied into a Source 15Q column mounted on a FPLC system, equilibrated in the same buffer. The retained proteins were eluted with a three-step NaCl gradient (0−300 mM in 5 mL, 300−400 mM in 10 mL, 400−1000 mM in 5 mL). The active fractions, eluted around 360 mM NaCl, were pooled and concentrated 10-fold in a Centricon cartridge (30000 cutoff, Millipore). The urease-enriched material (prep D) was then applied into a Superose 6 HR 10/30 gel filtration column (Amersham-Biotech Pharmacia) equilibrated in buffer A, mounted in a FPLC system. The protein peak containing the urease activity was denoted purified GHU, and its purity was analyzed by SDS-PAGE.

Protein Content Determination. The protein content of samples was determined by their absorbance at 280 nm in a quartz cuvette with a 1 cm light path. Alternatively, dye-binding methods (*31, 32*) were applied to protein fractions using bovine serum albumin as standard.

Urease Activity and Kinetic Parameters. To determine urease activity, samples were incubated with 10 mM urea in 20 mM sodium phosphate, pH 7.5, for 20 min at 37 °C, and the ammonia released was measured colorimetrically (*33*). One unit of urease releases 1 μmol of ammonia per minute, at 37 °C, pH 7.5. The same buffer was used for determining kinetic parameters and studies with inhibitors. Kinetic parameters (K_m and V_{max}) were calculated using Instat GraphPad software version 3.05, measuring the enzyme activity over a substrate concentration ranging from 0.05 to 10 mM urea. For inhibitory studies, the proteins were incubated with acetohydroxamic acid (AHA; 3.1−75

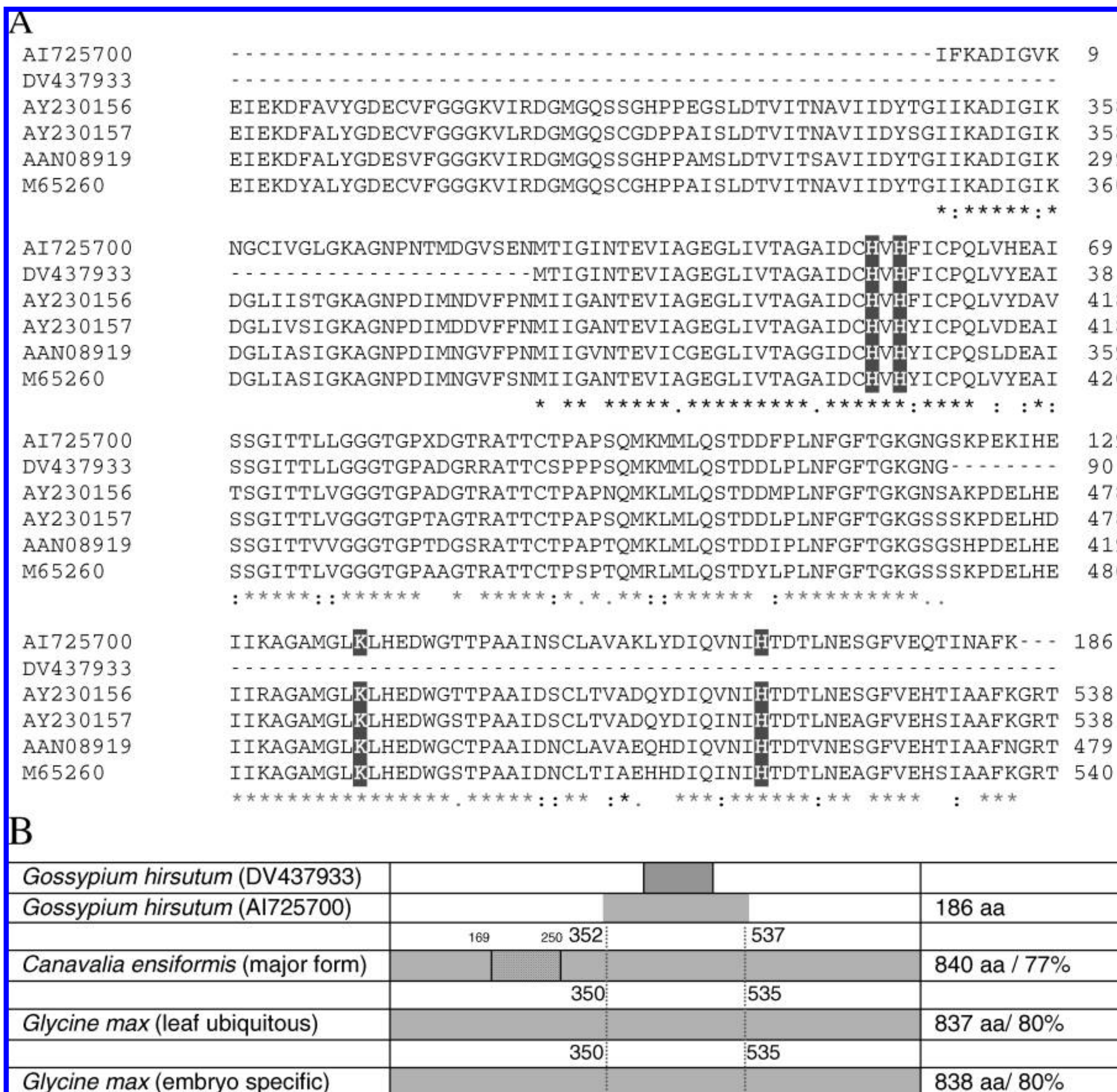

Figure 3. Comparison of primary sequences of jackbean and soybean ureases and the deduced amino acid sequences from two ESTs of *Gossypium hirsutum*. (**A**) ClustalW analysis of the partial amino acid sequences (translated from cDNA at the ExPASy Proteomic Server) of ureases from *Canavalia ensiformis* (M65260 major urease; AAN08919, isoform JBURE-II) and *Glycine max* (AY230156, leaf ubiquitous urease; AY230157 embryo-specific urease) compared with the deduced sequences of *G. hirsutum* (AI725700, from cotton fiber; DV437933, cotton senescent cotyledons). Four of six amino acids present in the active site of ureases (highlighted) are also present in the two ESTs from *G. hirsutum* urease. (∗) Identical amino acids; (:) amino acids with similar chemical characteristics of the side chains; (.) less conserved amino acids. (**B**) Schematic representation of the complete primary sequences of jackbean and soybean ureases and the aligments of the two *G. hirsutum* ESTs. The number of amino acid residues of the polypeptides chains and the percentage of identity of the cotton EST compared to the other plant ureases are indicated. The entomotoxic peptide of jackbean urease comprises amino acids 169−250, indicated by the hatched area.

μM final concentration), *p*-hydroxymercuribenzoate (*p*-HMB; 2.5−30 μM final concentration), or the corresponding volume of the inhibitor diluents, for 18−24 h at 4 °C.

For determination of the optimal pH, aliquots of the enzyme were assayed with 10 mM urea at 37 °C in the following buffers and pH: 50 mM citrate−phosphate, pH 3.0−7.0; 50 mM sodium phosphate, pH 7.5−8.0; and 50 mM sodium borate, pH 8.5−10.

SDS−Polyacrylamide Gel Electrophoresis (SDS-PAGE). Electrophoresis in 10% polyacrylamide gels containing 0.1% sodium dodecyl sulfate (*34*) was run at 20 mA for 2−3 h. Molecular mass standards were as follows: band 1, 220 kDa; band 2, 160 kDa; band 3, 120 kDa; band 4, 100 kDa; band 5, 90 kDa; band 6, 80 kDa; band 7, 70 kDa; band 8, 60 kDa; band 9, 50 kDa; band 10, 40 kDa; BenchMarch Protein Ladder, catalog no. 10747-012 (Invitrogen). The gels were stained with silver nitrate (*35*) and digitalized, and the molecular mass of samples was determined using the software UN-SCAN-IT version 5.1.

Western Blot. The proteins previously separated by SDS-PAGE were transferred by diffusion to a PVDF membrane (0.45 μm pore, Millipore). The crossed immunoreactivity was detected according to the method of Towbin et al. (*36*), using anti-canatoxin polyclonal IgG developed in rabbits (*17*) as primary antibody (1:5000 dilution) and anti-rabbit IGg coupled to alkaline phosphatase (Sigma Chemical Co.) as the secondary antibody. The color reaction was developed with 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) *p*-toluidine salt and nitroblue tetrazolium chloride (NBT).

Comparison of Urease Sequences. Amino acid sequences of ureases from *C. ensiformis* (M65260 major urease; AAN08919, isoform JBURE-II) and *G. max* (AY230156, leaf ubiquitous urease; AY230157 embryo-specific urease) were compared with the deduced sequences (translated from cDNA at the ExPASy Proteomic Server) of *G. hirsutum* (AI725700, from cotton fiber; DV437933, cotton senescent cotyledons, clone F5F9) using BLAST and ClustalW.

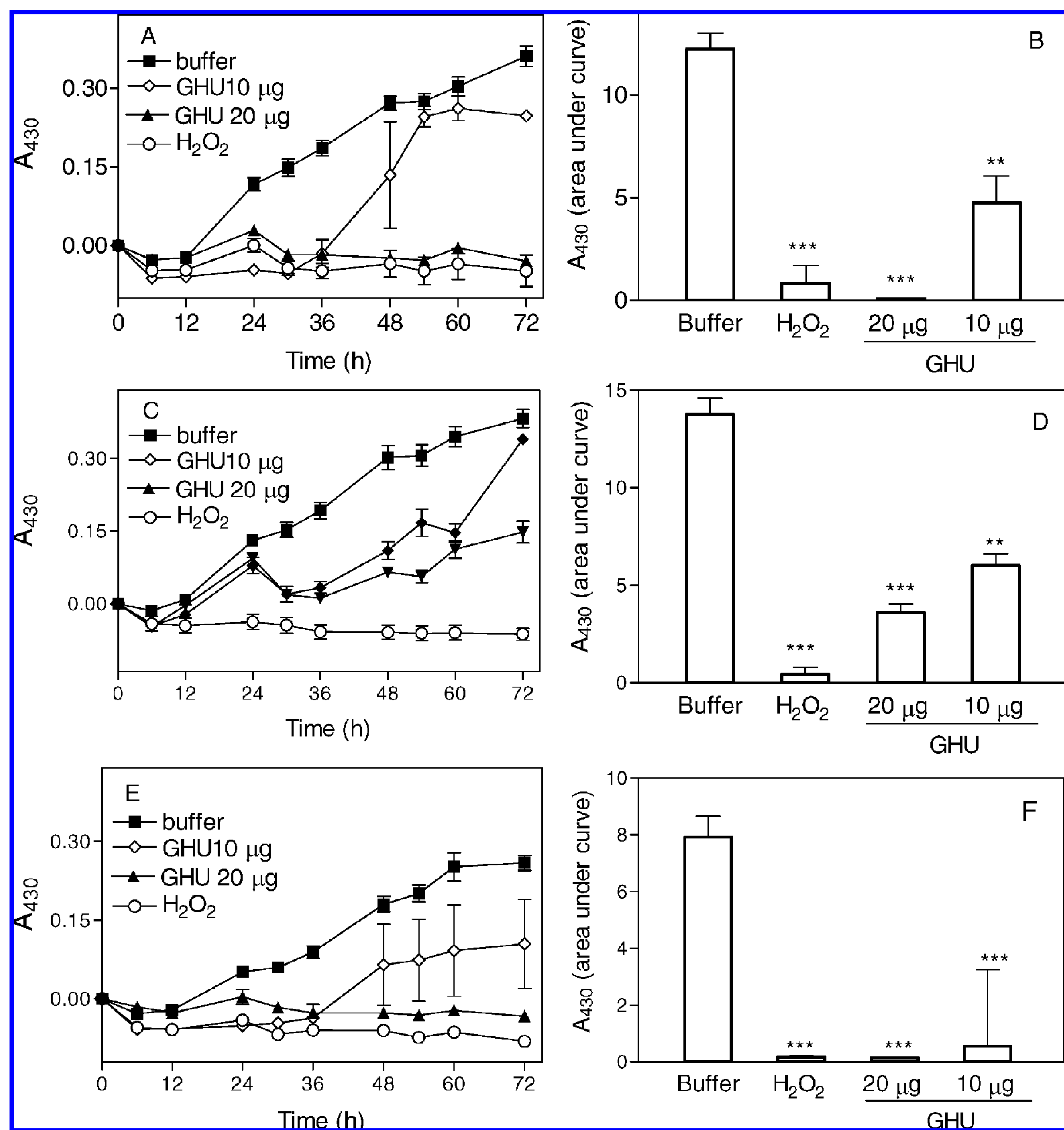

Figure 4. Antifungal activity of *Gossypium hirsutum* urease. Spores (3×10^3/ 10 μL) of the different fungi were inoculated onto 96-well plates containing 110 μL of PDB buffered to pH 7.0 with 10 mM NaPB and 1 mM β-mercaptoethanol, incubated at 28 °C for 16 h, and then 50 μL of a GHU solution containing 10 or 20 μg of protein (0.11 or 0.22 μM, final concentration, respectively) in the same buffer was added to the wells. The plates were incubated at 28 °C, and the absorbance at 430 nm (A_{430}) was recorded every 12 h until 72 h. (**A**, **B**)*Colletotrichum musae*; (**C**, **D**) *Penicillium herguei*; (**E**, **F**) *Curvularia lunata*. One experiment of at least three with similar results is shown. (**A**, **C**, **E**) Fungal growth as an increase in the medium turbidity (mean ± SD of triplicate points); (**B**, **D**, **F**) area under the growth curve corrected for the turbidity in the presence of 9% H_2O_2, taken as negative control. Data were analyzed by ANOVA followed by Student−Newman−Keuls test. $**$, $p < 0.01$; $***$, $p < 0.001$.

Fungi. *Colletotrichum musae* and *Curvularia lunata* were a gift from Dr. José Tadeu Abreu de Oliveira, Department of Biochemistry and Molecular Biology, Universidade Federal do Ceará, Fortaleza, CE, Brazil. *Penicillium herguei* was kindly provided by Dr. Valdirene Gomes, Universidade Estadual do Norte Fluminense, Campos dos Goytacazes, RJ, Brazil.

Antifungal Activity. Turbidimetric evaluation of fungal growth was done according to the method of Becker-Ritt et al. (*18*). Briefly, spores (3×10^3) in 10 μL were inoculated onto 96-well plates containing 110 μL of potato dextrose broth (PDB, Becton Dickenson Co.) buffered to pH 7.0 with 10 mM NaPB (to avoid precipitation of urease), containing 1 mM β-mercaptoethanol, and incubated at 28 °C for 16 h, followed by the addition of 50 μL of protein solutions in the same buffer (time 0). As controls, 50 μL of buffer alone or 9.5% v/v H_2O_2 was used. The plates were incubated at 28 °C without shaking, and the absorbance at 430 nm (A_{430}) was followed on a plate reader (Spectramax, Molecular Devices) at 12 h intervals up to 72 h.

Statistical Analysis. The results were subjected to analysis of variance (ANOVA), and the significance of differences among means was determined according to the Student−Newman−Keuls test using Instat GraphPad software, with $p \leq 0.05$ considered to be statistically significant.

RESULTS AND DISCUSSION

Crude extracts of cotton seeds have low urease activity, corresponding to about 1/1000 to 1/10000 of the urease activity of jackbean extracts (*17*). **Table 1** shows the protein content

and urease levels in the seeds of seven cultivars of cotton plants grown in Brazil. Although the protein content varied about 30% among the cultivars, the urease activity of the different seeds varied 7-fold, ranging from 20 mU to almost 150 mU per gram of seed meal. We have previously described variations also in the levels of soybean seed urease (*37*).

Because of its high enzyme content, cultivar IAC24 was chosen as the source for purification of the cotton seed urease. The ureolytic activity from defatted crude extracts precipitated at 0−0.4 saturated ammonium sulfate, and in pH 7.0 it was negatively charged, being retained in the Q-Sepharose chromatography and eluted with 300 mM NaCl. These behaviors are similar to those observed for the legume enzymes isolated from *C. ensiformis* and *G. max* ureases (*6, 17*). The purification protocol resulted in a 2400-fold purified GHU enzyme with a 1.3% yield (**Table 2**). **Figure 1A** shows the GHU purified enzyme as a major band with 98.3 kDa (a molecular mass slightly higher than that of the *C. ensiformis* major urease with 90.7 kDa) and the presence of two faint contaminant bands of lower masses. Western blot analysis shown in **Figure 1B** indicated that GHU shares antigenic determinants with *C. ensiformis* ureases, being recognized by anti-canatoxin polyclonal antibodies. The higher molecular mass of the GHU subunit is evident also in the blot assay. The molecular mass of native GHU determined in the gel filtration step was consistent with the enzyme being a hexamer (data not shown). **Figure 2** and **Table 3** show that the kinetic parameters of GHU are similar (V_{max} = 29 U/mg of protein, K_m for urea in the 10^{-4} M range, optimum pH 8.0) to those of ureases from Leguminosae and Curcubitacae (*4, 38*). As described for *C. ensiformis* urease (*17*), the enzymatic activity of GHU was inhibited by acetohydroxamic acid and *p*-hydroxymercuribenzoate in micromolar concentrations.

So far, there is little information available on the molecular structure of cotton urease, and a search in databanks revealed only two expressed sequence tags (ESTs) from cotton plant tissues with similarity to ureases (**Figure 3**). One of these ESTs encompasses four of the six highly conserved amino acids in the active site of well-known ureases (*5, 39, 40*). We are presently working on the primary sequence of GHU as well as attempting to clone its full-length cDNA.

We have recently shown (*18*) that ureases of leguminous plants (jackbean and soybean) as well as the dichain enzyme of the bacterium *Helicobacter pylori* possess antifungal activity in sub-micromolar concentrations, inhibiting spore germination and/or mycelial growth of a number of filamentous fungi. The antifungal property of ureases persisted after treatment with *p*-hydroxymercuribenzoate, indicating that it is not dependent on the ureolytic activity of the proteins. Scanning electron microscopy of urease-treated fungi suggested plasmolysis and cell wall injuries (*18*). Here we show (**Figure 4**) that purified GHU also displays antifungal effects against the phytopathogenic fungi *Colletotrichum musae*, *Curvularia lunata*, and *Penicillium herguei*. At 0.22 μM final concentration, GHU inhibited extensively the mycelial growth of *C. musae* and *C. lunata*, being more potent than soybean or jackbean ureases, which required 2−3-fold greater concentrations to produce similar effects (*18*). On the other hand, GHU was less effective against *P. herguei*, in contrast to our previous observation of the high sensitivity of this fungus to *C. ensiformis* and *G. max* ureases (*18*). It is not clear yet if the antifungal effect of GHU is fungicidal, actually killing the spores, or just fungistatic, inhibiting germination or vegetative growth. The data, however, confirm our previous observation (*18*) that the antifungal properties of ureases depend on the source of the protein as well as the type of fungus being examined.

We have no other clues to the location or characteristics of the antifungal domain of ureases, besides that it is not related to the ureolytic active site or to the insecticidal domain of plant enzymes (*18*). The antifungal domain of plant and bacterial ureases would probably show some divergence of the primary sequence to comply with the observed species specificity of different ureases. In this context it is noteworthy to mention that the region comprising the insecticidal domain of plant ureases diverged at a faster rate than other parts of the molecule (*23*).

Dysdercus peruvianus (Hemiptera: Pirrhocoridae), the cotton stainer bug, is one of the insect models we have used to characterize the insecticidal activity of plant ureases (*6, 41*). The ability of *D. peruvianus* to thrive on cotton seeds may be related to their low levels of GHU. Another alternative would be that during co-evolution *D. peruvianus* may have evolved mechanisms to escape GHU toxicity (*24*). We are presently working to produce sufficient quantities of GHU for bioassays with *D. peruvianus*.

With the increased use of transgenic cotton expressing *Bt Cry1* toxins to control lepidopteran and coleopteran pests (*42, 43*), it can be expected that secondary pests of this crop such as *D. peruvianus* will gain importance. It is important to mention that ureases and *Cry* proteins have a complementary spectrum of insecticidal activities depending on the type of digestive enzymes present in the target insects (*24*). If the cotton stainer bug is susceptible to increased levels of GHU, this fact would make this protein a suitable marker to select cotton plants with improved performance against this insect as well as more resistant to fungal diseases. As shown here, cotton cultivars differ in their urease contents. These cultivars were developed for increased yield and/or resistance to virus or abiotic stress such as drought. No information is available regarding their susceptibility to fungal disease or insect pests. Moreover, GHU and other plant ureases can be considered to be potential candidates for engineering resistance in plants against selected insects and fungal disease or to complement the protection provided by *Bt* toxins or other pesticide proteins.

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