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Characterization of JBURE-IIb isoform of *Canavalia ensiformis* (L.) DC urease

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ABSTRACT

Ureases, nickel-dependent enzymes that catalyze the hydrolysis of urea into ammonia and bicarbonate, are widespread in plants, bacteria, and fungi. Previously, we cloned a cDNA encoding a *Canavalia ensiformis* urease isoform named JBURE-II, corresponding to a putative smaller urease protein (78 kDa) when compared to other plant ureases. Aiming to produce the recombinant protein, we obtained *jbure-IIb*, with different 3′ and 5′ ends, encoding a 90 kDa urease. Three peptides unique to the JBURE-II/-IIb protein were detected by mass spectrometry in seed extracts, indicating that *jbure-II/-IIb* is a functional gene. Comparative modeling indicates that JBURE-IIb urease has an overall shape almost identical to *C. ensiformis* major urease JBURE-I with all residues critical for urease activity. The cDNA was cloned into the pET101 vector and the recombinant protein was produced in *Escherichia coli*. The JBURE-IIb protein, although enzymatically inactive presumably due to the absence of Ni atoms in its active site, impaired the growth of a phytopathogenic fungus and showed entomotoxic properties, inhibiting diuresis of *Rhodnius prolixus* isolated Malpighian tubules, in concentrations similar to those reported for JBURE-I and canatoxin. The antifungal and entomotoxic properties of the recombinant JBURE-IIb apourease are consistent with a protective role of ureases in plants.

1. Introduction

Urease (EC 3.5.1.5, urea amidohydrolase), an enzyme found in many plants [1,2], catalyzes the hydrolysis of urea to form ammonia and bicarbonate [3,4]. The main function of urease in plants is to recycle nitrogen (N) from external or internally generated urea, as demonstrated under a number of experimental conditions [2,5–7].

Urease is also found in bacteria, fungi and in some invertebrates [3]. Plant and fungal ureases are homo-oligomeric proteins (formed by identical ~90 kDa subunits), while bacterial enzymes are multimers, formed by a complex of two (e.g., *Helicobacter pylori*) or three (e.g., *B. pasteurii* and most other bacterial ureases) subunits [1,4,8]. The single subunit of plant or fungal ureases aligns with the primary sequence of the small subunits of most bacterial enzymes (in the order $\gamma-\beta-\alpha$). For example, the N-terminal portion of plant or fungal urease has domains that closely resemble the β and γ chains of *Bacillus pasteurii* urease or the A subunit of *Helicobacter pylori* urease, while the C-terminal portions of plant and fungal chains resemble the large subunits of bacterial ureases (i. e. the α chain of *B. pasteurii* urease and the B subunit of *H. pylori* enzyme). The high sequence similarity among ureases indicates that they are variants of the same ancestral enzyme and are likely to possess similar tertiary structures and catalytic mechanisms [1,2,4,8].

In soybean (*Glycine max* L. [Merrill]), there are two distinct, non-allelic urease isoenzymes, differing in both structural and biochemical properties. The ubiquitous urease is a constitutive enzyme expressed in all organs [9]. The embryo-specific urease is synthesized exclusively in developing embryos [10] being retained in the mature seeds, where its activity is 1,000-fold greater than that of the ubiquitous enzyme [11,12]. The absence of ubiquitous urease activity in soybeans results in necrotic leaf tips due to urea accumulation [13], indicating

Abbreviations: ABA, abscisic acid; CNTX, canatoxin; DTT, dithiothreitol; JBURE-I, jack bean major urease; JBURE-II, jack bean urease isoform II; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcription and polymerase chain reaction

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an important role in recycling metabolic urea. On the other hand, mutants lacking the embryo-specific urease do not exhibit any of the abnormalities associated with the loss of ubiquitous urease, suggesting that this isoform plays no essential metabolic role in the plant.

Canavalia ensiformis also presents multiple urease isoforms [14,15]. Mamiya and co-workers [16] determined the primary structure of the 840-amino acid subunit of the major jack bean urease (herein named JBURE-I) and Riddles et al. [17] reported the nucleotide sequence of its cDNA. Later, our group showed that canatoxin (CNTX), a neurotoxic protein (dimer of 95 kDa subunits) from *C. ensiformis*, is a urease isoform [14]. CNTX displays insecticidal [18,19] and fungitoxic [20] properties, suggesting that ureases may be involved in plant defense [21,22]. JBURE-I and embryo-specific soybean urease, but not the bacterial urease from *Bacillus pasteurii*, also show insecticidal properties when tested against the cotton pest *Dysdercus peruvianus*[23]. On the other hand, jackbean, soybean [20], and cotton [24] ureases as well as the dichain enzyme of the bacterium *H. pylori*[20] possess antifungal activity at sub-micromolar concentrations, inhibiting spore germination and/or mycelial growth of a number of filamentous fungi. Neither the entomotoxic nor the fungitoxic effects of ureases are due to the release of ammonia since treatment of ureases with p-hydroxymercurybenzoate, an irreversible inhibitor of ureolytic activity, did not interfere with these activities [20,23]. The entomotoxicity relies mostly on an internal 10 kDa peptide (pepcanatox) released by hydrolysis of CNTX/JBURE-I by cathepsins in the digestive system of susceptible insects [18,19,25]. The entomotoxic effect of jaburetox-2Ec, a recombinant protein based on the pepcanatox sequence [26], reinforced the potential biotechnological use of these proteins and derived peptides as bioinsecticides.

Pires-Alves et al. [15] reported the cloning from ABA (abscisic acid)-treated leaves of a cDNA encoding an isoform of *C. ensiformis* urease named JBURE-II. This putative urease was described as a third urease isoform, with a shorter polypeptide chain (78 kDa), a more basic theoretical isoelectric point and an amino acid sequence not matching that available for CNTX [14] or JBURE-I [16]. All plant and fungal ureases reported to date have a conserved ~90 kDa subunit. Aiming to investigate if the smaller size of JBURE-II was unique among plant ureases, we succeeded in cloning a new cDNA encoding full-length JBURE-II, but with the size expected for a urease, which differed at the 5′ end and lacked the premature translational termination suggested in the original *jbure-II* cDNA sequence [15]. The new full length cDNA, called *jbure-IIb*, was cloned into the pET101 vector and the recombinant JBURE-IIb protein was successfully produced in *Escherichia coli* cells. Although devoid of ureolytic activity, the purified protein impaired growth of a phytopathogenic fungus and showed entomotoxic activity inhibiting diuresis in Malpighian tubules isolated from *Rhodnius prolixus*. Three peptides unique to JBURE-II/-IIb were detected in a proteomic analysis of germinated seeds confirming that *jbure-IIb* gene is functional. Phylogenetic analysis and molecular modeling of this urease are also presented.

2. Materials and methods

2.1. Plant material and total RNA isolation

Canavalia ensiformis (seeds obtained locally or from Sigma Chem. CO, St Louis, MO, USA) plants were grown in vermiculite with Murashige and Skoog medium. Leaves from 15 day-old seedlings were cut at the petiole and immersed in MS medium containing 100 μM abscisic acid (100 mM stock solution in ethanol, Life Technologies), as previously described [15]. After keeping the leaves for 18 h in the dark, total RNA was isolated from the ABA-treated leaves using the plant protocol from the RNeasy kit (QIAGEN), according to manufacturer's instructions.

For the mass spectrometry assays, mature seeds were washed with 1% sodium hypochlorite, 70% ethanol and imbibed in distilled water at 56 °C for 5 min. Seeds were grown in regular soil for 3 days (3 D), under 18 h light/8 h dark cycle, 23 °C, and watered once a day.

2.2. RT-PCR

The internal region (I) of the *jbure-II* cDNA was obtained by cDNA cloning. Reverse transcription of total RNA was done using an oligo d (T)-anchor primer and MMLV-RT enzyme (Invitrogen). RT-PCR was performed in 50 mM Tris–HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 6.8 mM DTT, 0.34 mM dNTPs, 1.36 μM oligo d(T)-anchor primer and 68 U MMLV-RT enzyme. The RT reaction was incubated for 1 h at 37 °C and used as template for PCR amplification with primers based on the *jbure-II* (AF468788) cDNA sequence (JBII-1F 5′ CGGAATTCATGAACCATTTTAACAGG 3′ and JB-II-1R 5′ CGTGAGCAATGTGAGAAGCGGCCGCAA 3′). The PCR system contained 2.5 U Taq Polymerase High Fidelity (Invitrogen), 200 μM dNTPs, 2.0 mM $MgCl_2$ and 400 nM each primer in a 50 μL final volume. The PCR protocol consisted of a denaturation step at 94 °C for 2 min, 30 cycles at 94 °C for 1 min, 50 °C for 45 s and 72 °C for 3 min, followed by a final elongation step at 72 °C for 5 min.

2.3. 5′RACE

Two micrograms of total RNA was used as template for 5′RACE, using the Ure-8 (5′ GTGTGCAGAAGGGAA 3′) primer in a reverse transcription reaction to obtain the first cDNA strand. The reverse transcription reaction was performed as described earlier. The PCR was carried out using Ure-9 (5′ GTAAAGCAGCTATGGAGAT 3′) and dT-AP (5′ GACCACGCGTATCGATGTCGACTTTTTTTTTTTTTTT 3′) primers and the second round used Ure-14 (5′ GTCAACCATGGAGACAGGCCAATC 3′) and AP (5′ GACCACGCGTATCGAATGTCGAC 3′) primers.

2.4. PCR-overlap and full length jbure-IIb cDNA

To obtain the full length *jbure-IIb* cDNA three fragments of cDNA corresponding to the 5′ region, an internal fragment and the 3′ region were ligated by PCR-overlap as described ahead. To produce enough mass of the fragments, PCR was performed in a final volume of 50 μL containing 2.5 U of *Pfu*, 200 μM dNTPs, 2.0 mM $MgCl_2$ and 400 nM of each oligonucleotide:

Internal fragment (I):
Ure-4: 5′ AGTGACAGTTCATGATCCGATT 3′
Ure-3R: 5′CCATTTACTATGGCTGGATT 3′
Fragment 3′:
Ure-7: 5′ AAGTTCAAGGGTAAGAGAAC 3′
JBURE-2Rev 5′TCACACTTTGACTCAATATAAACAA 5′
Fragment 5′:
CNTX-2: 5′ ATGAAGCTGAGTCCTCG 3′
Ure-14R: 5′ GATTGGCCTGTCTCCATGGTTGAC 3′.

PCR conditions were: denaturation at 94 °C for 2 min, 30 cycles of 1 min at 94 °C, 50 °C for 45 s, 72 °C for 3 min and a final elongation step at 72 °C for 5 min. The reaction products were separated in agarose gel, the corresponding bands were excised and the cDNAs were purified with a Gene Clean kit according to the manufacturer's instructions.

To ligate fragments I and 3′, *Pfu* and primers Ure-4 and JBURE-2R were used for the PCR (94 °C for 1 min, 80 °C for 1 min, 72 °C for 1 min, 50 °C for 1 min, 30 cycles of 1 min at 94 °C, 72 °C for 45 s, 55 °C for 45 s, 72 °C for 2 min, and a final elongation step of 5 min at 72 °C. The PCR product (fragment I + 3′) was separated in an agarose gel, the band was excised and purified. The cDNA fragment I + 3′ was used as template for the second overlap reaction by adding fragment 5′ and primers CNTX-2 and JBURE-2R. PCR conditions were the same as before using DNA polymerase *Taq* Platinum (Invitrogen).

The reaction product corresponding to the full length *jbure-IIb* cDNA was separated in an agarose gel, and the band was excised, purified and cloned into the pGEMT (Invitrogen) vector.

2.5. Expression of jbure-IIb in E. coli

The full length *jbure-IIb* cDNA was first amplified by PCR in a 25 μL reaction containing 200 μM dNTPs, *Pfu* 1× buffer, 2.5 U *Pfu* DNA polymerase (Stratagene), 400 nM of the oligonucleotides JBURE-101F: 5′ CACCATGAAGCTGAGTCCTCG 3′ and JBURE-101R: 5′ AAAGATGAAGTAATTTTGAGAAAGTGG 3′, and 500 ng of *jbure-IIb*/pGEMT (Invitrogen) vector described earlier. The PCR protocol consisted of denaturation at 94 °C for 1.5 min; 29 cycles of 30 s at 94 °C, annealing at 52 °C for 45 s, extension for 2 min at 72 °C, and final extension at 72 °C for 5 min. The PCR product was separated in an agarose gel, the band was excised and purified. Five nanograms of the PCR product was ligated into a pET101/D-TOPO (Invitrogen) vector in a reaction containing 1 μL each of 200 mM NaCl, 10 mM $MgCl_2$ and pET101 vector, for 20 min at room temperature. Competent *E. coli* TOP 10 cells were transformed by heat shock with 10 ng (in 3 μL) of pET/*jbure-IIb* by incubating 200 μL of cells for 30 min in an ice-bath followed by 30 s at 42 °C. The cells were ice-chilled again and 250 μL of SOC culture medium (2% Tryptone, 0.5% Yeast Extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) was added at room temperature. After 30 min at 37 °C while agitating at 200 rpm, the cells were plated in LB agar, containing 100 μg/mL ampicilin. Colonies resistant to ampicilin were selected and used as template for PCR in the same conditions as before to confirm transformation.

Competent *E. coli* BL-21 Star (DE3) (200 μL) were then transformed with 10 ng pET101/*jbure-IIb* expression vector by incubation in an ice-bath for 30 min, followed by 30 s at 42 °C, ice-chilled again and then 250 μL of SOC medium at room temperature was added.

2.6. Production and purification of recombinant JBURE-IIb

E. coli BL21 Star (DE3) cells carrying the pET 101/*jbure-IIb* plasmid were inoculated into 5 mL of LB containing 100 μg/mL ampicillin, and after 16 h at 37 °C, transferred to 50 mL of LB medium containing 100 μg/mL ampicillin. The cells were grown at 37 °C under shaking until OD600 reached 0.6–0.8 and then 0.1 mM IPTG was added. After 16 h induction, the cells were harvested by centrifugation and resuspended in 10 mL of lysis buffer (50 mM sodium phosphate pH 7.0, 400 mM NaCl, 100 mM KCl, 10% v/v glycerol, 0.5% v/v Triton X-100 and 10 mM imidazole), sonicated, and centrifuged (14,000 *g*, 20 min). The supernatant was loaded onto a 2 mL Ni affinity column (Ni-NTA–QIAGEN), previously equilibrated in 50 mM sodium phosphate buffer pH 8.0, 300 mM NaCl and 10 mM imidazole. After 30 min, the column was washed with 20 mL of the same buffer, containing 20 mM imidazole. The His-tagged protein was eluted in a high purity state with 200 mM imidazole in the equilibration buffer and quantified by the Bradford method. Cultures without IPTG induction and cells carrying the β-galactosidase gene served as control. All samples were analyzed by sodium dodecyl sulfate polyacrylamide gel 12% (SDS-PAGE) stained with Coomassie Blue R-250.

2.7. DNA sequencing and analysis

The amplification products were gel-purified using a Gene-Clean kit (BIO 101) according to supplier's recommendations and cloned into pGEMT-easy (Promega) vector. XL-1Blue *E. coli* cells were transformed and white recombinant colonies were chosen for DNA minipreparations. The plasmids obtained were sequenced on an automated ABI PRISM ® DNA sequencer, using T7 or SP6 primers.

Sequence comparisons were performed using BLASTx software (http://www.ncbi.nlm.nih.gov). Alignments were obtained using CLUSTALW software (http://www.ebi.ac.uk/clustalw). The translation of DNA to protein was obtained at Expasy (http://ca.expasy.org/tools/#translate). Predicted molecular size and pI of the putative protein was obtained by submitting the sequence to the ProtParam tool available at the Expasy site (http://ca.expasy.org/tools/#primary).

2.8. Phylogenetic analysis

Phylogenetic analysis was performed with an initial blast of the JBURE-IIb amino acid sequence over a non-redundant database by BLASTp (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_blast.html) [27]. A total of 23 fungal and plant ureases were selected. The retrieved sequences were then multiple aligned with ClustalW [28] and the multiple-sequence alignments were saved in FASTA format. BioEdit software, version 7.0.8.0 [29], was used to manipulate the amino acid sequences when necessary. The estimation of the phylogenies were conducted by neighbor-joining (NJ) [30], using the p distance and the Poisson-corrected amino acid distances to analyze the amino acid sequences, available in the MEGA software version 4.1 (Molecular Evolutionary Genetics Analysis) [31]. A total of 10,000 repetitions were performed using the bootstrapping method [32] to determine the reliability of each node of the tree.

2.9. Comparative modeling

The construction of a tridimensional model for JBURE-IIb was performed using homology modeling techniques, employing MODELLER9v9 [33]. The JBURE-IIb peptide sequence was submitted to BLASTp [27] analysis against a database of PDB structures. Based on this analysis, all four urease structures available were chosen as templates, i.e. *Bacillus pasteurii* urease (PDB id 2UBP, [34]), *Klebsiella aerogenes* urease (PDB id 1FWJ, [35]), *Helicobacter pylori* urease (PDB id 1E9Z, [36]), and *Canavalia ensiformis* urease (PDB id 3LA4 [37]. The modeling was carried out by combining all templates. Fifty models were built and had their stereochemical evaluation and theoretical validation of 3D profile assessed with PROCHECK [38] and Verify3D [39], respectively. The best model was selected based on these assessments. The nickel atoms were placed on the active site taking template coordinates as reference.

2.10. Mass spectrometry analysis of C. ensiformis urease-like proteins

In a previous study, Demartini and co-workers [40] analyzed *Canavalia ensiformis* plants using immunoprecipitation and LC-MS^2 [40]. The raw data collected in that study regarding the immunoprecipitation step performed with anti-JBURE-I monoclonal antibodies were re-mined as described ahead. Output files from LC-MS^2 analyses were uploaded into BioWorks 3.3.1 SP1 ™ (Thermo Fisher, San Jose, CA, USA) for SEQUEST™ search, using the soybean sequences protein database Gmax.main_genome.scaffolds.fasta.gz, avaliable at ftp://ftp.jgi-sf.org/pub/JGI_data/phytozome/v4.0/Gmax/assembly/sequences.

Canavalia ensiformis entries available at www.pubmed.gov were manually included in the soybean database prior to indexing and searching the data. High confidence protein identification was performed by applying additional criteria to the SEQUEST™ search parameters such as: 1) a minimum of two unique and no overlapping peptides for protein assignment; 2) minimum peptide probability of 0.05% for each peptide match; 3) minimum cross correlation values (*Xcorr*) for single, double and triple charged peptides were 1.5, 2.0 and 2.5, respectively.

Output files from BioWorks were exported into .xls format and were submitted to manual analyses.

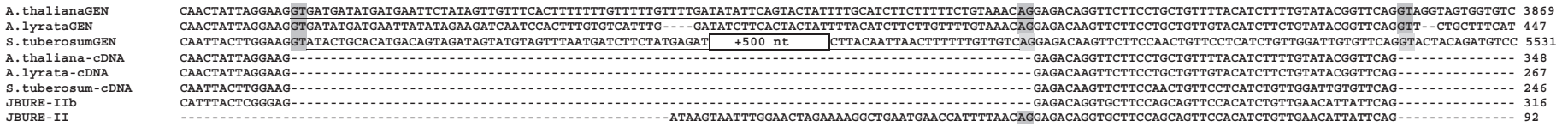


Fig. 1. Alignment of 5′*jbure-II* and 5′*JBURE-IIb* with genomic *Arabidopsis thaliana* urease coding region (*A.thaliana*GEN, AC011020), genomic *Arabidopsis lyrata* (*A. lyrata*GEN, NW_003302554.1), *Solanum tuberosum* genomic sequence (*S.tuberosum*GEN, AJ276864.1), *Arabidopsis thaliana* urease cDNA (*A.thaliana*-cDNA, NM_105422.3), *Arabidopsis lyrata* cDNA (*A.lyrata*-cDNA, XM_002887083.1) and *Solanum tuberosum* urease cDNA (*S.tuberosum*-cDNA, AJ308544.1. The box indicates the number of nucleotides in the *S. tuberosum* urease intron not included in the figure. Introns are underlined and potential splicing signals are shaded.

2.11. Protein content determination

The protein content of fractions was determined according to [41], using bovine serum albumin as standard.

2.12. Urease activity

Urease-catalyzed ammonia release from urea was determined colorimetrically [42]. One unit of urease activity was defined as the amount of enzyme required to release 1 μmol NH_3 per min at 37 °C and pH 7.5 under the conditions described. Attempts at in vitro activation of purified recombinant JBURE-IIb were done according to Park and Hausinger, 1995 [43]. Briefly, aliquots of 18 μg of purified JBURE-IIb were incubated at pH 7.5 or 8.3, up to 16 h at room temperature, with varying sodium bicarbonate (1–30 mM) and nickel chloride (1–500 μM) concentrations, and then assayed for ureolytic activity in the presence of 100 mM urea, pH 7.5.

Production of JBURE-IIb by transformed *E. coli* was detected in the urea segregation medium as described in [44]. Briefly, cells were grown 24 h at 37 °C in LB agar plates containing 100 μg/mL ampicilin, 1 mM IPTG, 100 μM $NiCl_2$, 0.35 mg/mL cresol red and 60 mg/mL urea. Alkalinization due to urease activity results in a reddish color around the colonies.

2.13. Antifungal activity

Penicillium herguei (a urease-susceptible phytopathogenic fungus [20,24] was kindly provided by Dr. Valdirene Gomes, Universidade Estadual do Norte Fluminense, Campos dos Goytacazes, RJ, Brazil. For turbidimetric evaluation of fungal growth, spores (10^3 in 10 μL) were inoculated onto 96-well plates containing 110 μL potato dextrose broth (Becton Dickenson Co.) buffered to pH 7.0 with 10 mM sodium phosphate (to avoid precipitation of urease), containing 1 mM 2-mercaptoethanol, incubated at 28 °C for 16 h, followed by addition of 50 μL of protein solutions (0.28, 0.57 and 1.15 μM final concentration) in the same buffer (time zero). As controls, 50 μL buffer alone or 9.5% v/v H_2O_2 were 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 60 h [20,24].

2.14. Entomotoxic activity — inhibition of fluid secretion in Malpighian tubules

JBURE-I (jackbean urease, type C-3, Sigma Chem Co., St. Louis, USA) and purified recombinant JBURE-IIb were assayed for inhibitory activity upon serotonin-induced diuresis in isolated *Rhodnius prolixus* Malpighian tubules using a modified Ramsay assay as described in [45]. Briefly, whole tubules from fifth instars (kindly provided by Dr. Hatisaburo Masuda and Pedro Lagerblad, Inst. Medical Biochemistry, Universidade Federal do Rio de Janeiro, Brazil) were dissected under saline and transferred to a Sylgard-coated Petri dish containing 20 μL drops of saline overlaid with water-saturated mineral oil. The proximal end of the tubule was pulled out of the saline droplet and wrapped around a minute pin. Secretion from tubules into the oil was measured for saline plus 2.5×10^{-8} M serotonin (control), which was then replaced with the proteins plus serotonin (test). Secretion rate was expressed as nanoliters per minute or the percentage of fluid secretion measured after the addition of the protein relative to control. Seven replicates were done for each experiment.

3. Results

3.1. Cloning of JBURE-IIb

Detached young leaves of *C. ensiformis* were treated with 100 μM ABA. Total leaf RNA was the template for RT with anchored primers. cDNA was used as template for PCR amplification with primers based on the *jbure-II* (AF468788) cDNA sequence (JBII-1F 5′ and JB-II-1R; see Materials and methods). The purified RT-PCR product was ligated into the pGEM-T-Easy vector (Promega). Nucleotide sequence analysis indicated that the fragment matched to the previously characterized *jbure-II* sequence [15], with a nucleotide insertion (G) at position 2191, resulting in a distinct extended 3′ end.

5′RACE was performed on total RNA from ABA-treated leaves producing a 550 bp fragment that encoded a new 5′ sequence for the *jbure-II* cDNA. Finally, a ~3 kbp cDNA was obtained by PCR-overlapping the new 5′ and 3′ regions with the previously cloned cDNA internal region. This cDNA, designated as *jbure-IIb*, has the start codon in the expected position, similar to all other plant ureases, showing a typical urease N-terminal gamma domain, exhibiting 86% identity to JBURE-I. We suggest possible alternative splicing of *jbureIIb* mRNA due to the presence of splicing signal motifs as shown in Fig. 1.

The complete cDNA sequence of *jbure-IIb* was deposited in GenBank, under accession number EU938655. Fig. 2 shows the predicted sequence of JBURE-IIb containing 840 amino acids, with a molecular mass of 90,059.6 Da and a theoretical pI of 5.60. The deduced amino acid sequence of the ORF of *jbure-IIb* cDNA was compared to the sequence of JBURE-I [16,17] and partial sequence of canatoxin, as well as to that of other ureases (Supplementary Fig. 1). The sequence of JBURE-IIb exhibited 71 to 82% amino acid identity to plant ureases: 82% — Jack bean JBURE-I (accession number AAA83831), 81% — soybean (*G. max*) embryo-specific urease (AAO85884), 79% — soybean ubiquitous urease (AAO85883), 71% — *Arabidopsis thaliana* urease (AAG52306), 71% —*Solanum tuberosum* urease (CAC43859) and 71% —*Oryza sativa* urease (BAB78715).

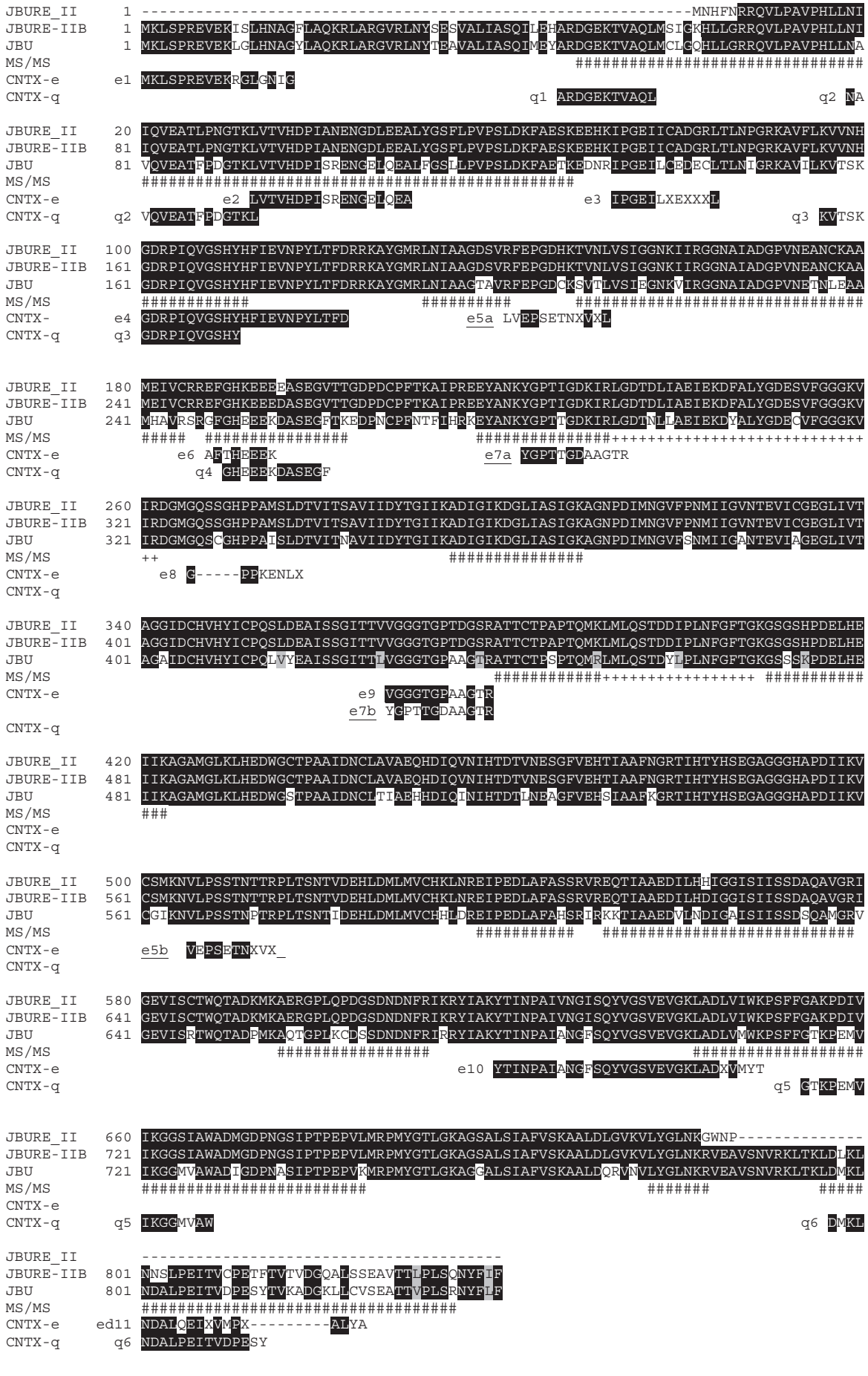
3.2. Detection of JBURE-II/-IIb protein in seeds by mass spectrometry

In contrast to JBURE-I and canatoxin, we were unable to purify JBURE-II/-IIb from extracts of quiescent seeds by conventional chromatographic methods. On the other hand, a preliminary proteomic analysis [40] of urease-like immunoreactive proteins in seeds and in early stages of embryo development (Fig. 3), identified three peptides unique to JBURE-II/-IIb, not found in JBURE-I, corresponding to amino acids 293 to 304 (D297N and I299L-first residue JBURE-II/-IIb, position, second residue JBURE-I), 305 to 322 (F306Y and S313C) and 452 to 469 (D459Y and I460L). Peptides matching JBURE-I/canatoxin were also detected in the same assay and represented more abundant molecular species than their JBURE-II/-IIb counterparts (Fig. 3).

3.3. Expression and purification of recombinant 6-His tagged JBURE-IIb

E. coli BL21 Star (DE3) carrying the pET101/*jbure-IIb* vector expressed the recombinant urease and its ureolytic activity (indicated by a reddish color around the colonies) could be observed after a 24 h growth in a urea segregation agar supplemented with urea and $NiCl_2$. In order to purify the recombinant protein, the bacterial cells were grown in LB medium at 37 °C with 16 h induction in the presence of

Fig. 2. Deduced amino acid sequence of JBURE-IIB and comparison with other *C. ensiformis* urease isoforms. *Canavalia ensiformis* JBURE-II: *Canavalia ensiformis* isoform II (AAN08919); *Canavalia ensiformis* JBURE-IIB: this paper; JBURE-I: *Canavalia ensiformis* (Jack bean) urease isoform I (AAA83831); CNTX-e and CNTX-q: Canatoxin's peptides sequenced either by EDMAN degradation (CNTX-e) or by mass spectrometry (chymotryptic fragments — CNTX-q; unpublished data). Peptide e5 and e7 aligned in two different regions (indicated as a and b). The figure also shows the 18 unique (some overlapping) peptides matching to JBURE-I (indicated in the MS/MS row by "#") and the 3 unique peptides of JBURE-II/-IIb (indicated in the MS/MS row by the sign " + "), identified in Demartini et al., [40] by mass spectrometry. Identical amino acids are shaded in black and similar amino acids are shaded in gray. The alignment was carried out using ClustalW (http://www.ebi.ac.uk/clustalw/) and presented graphically by BOXSHADE 3.21 (http://www.ch.embnet.org/software/BOX_form.html).


JBURE_II
JBURE-IIB
JBU
MS/MS
CNTX-e
CNTX-q

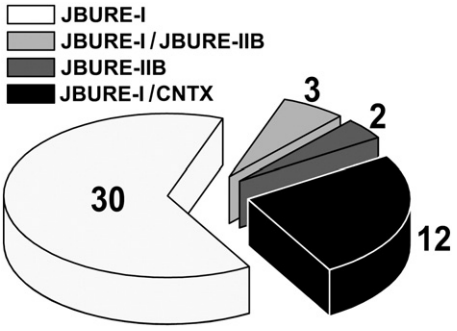


Fig. 3. The graphs illustrate the number of peptides unique to JBURE-I, JBURE-II/-IIb, or common to more than one isoform of urease as detected by mass spectrometry of the proteins "pulled-down" with anti-JBURE-I monoclonal antibodies from seed extracts [data were taken from 40]. The coverage of the JBURE-I protein in these assays was 57% (484 amino acids residues identified out of 840). The peptides unique to JBURE-II/-IIb (shown in Fig. 2) were 301–322, 293–304 and 452–469.

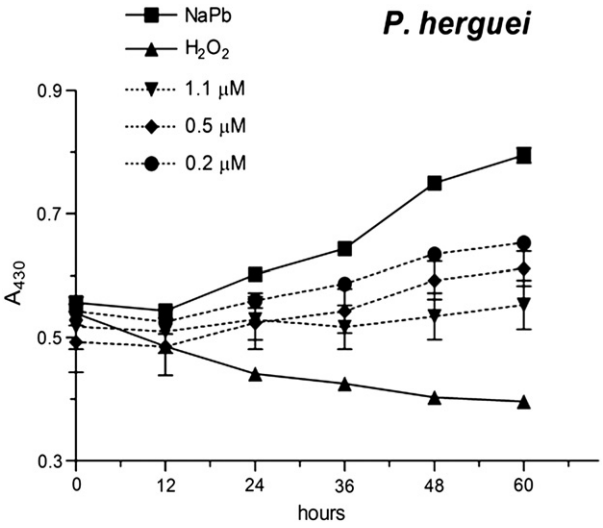


Fig. 4. Antifungal activity of purified recombinant JBURE-IIb. Spores (10^3/10 μL) of *Penicillium herguei* were inoculated onto 96-well plates containing 110 μL of potato dextrose buffer buffered to pH 7.0 with 10 mM NaPB, 1 mM β-mercaptoethanol, incubated at 28 °C for 16 h, and then 50 μL of a JBURE-IIb solution (final concentrations of 0.2 to 1.1 μM) in the same buffer were 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 60 h. Fungal growth was measured as an increase in the medium turbidity (mean ± SD of triplicate points). Turbidity in the presence of 9% H_2O_2 was taken as negative control. One experiment out of at least three with similar results is shown.

0.1 mM IPTG. Under these conditions the recombinant protein was recovered in the soluble fraction. For purification, the supernatant of lysed cells was loaded onto a Ni affinity column and the His-tagged protein was eluted in a high purity state in the 200 mM imidazole fraction. SDS-PAGE analysis (not shown) confirmed the purity and the expected molecular mass ~90 kDa for the recombinant JBURE-IIb.

3.4. Biological properties of the recombinant His-tagged JBURE-IIb

3.4.1. Urease activity

In this work our purpose was to produce the apoenzyme and test the recombinant protein for biological properties not related to urea hydrolysis. As expected, the recombinant purified protein showed no detectable hydrolysis of urea. Attempts for in vitro activation of purified recombinant JBURE-IIb according to the protocols described by Park and Hausinger, 1995 [43] for *Klebsiella aerogenes* apourease failed to render recombinant JBURE-IIb able to hydrolyze urea. However, recombinant *E. coli* BL21 Star (DE3) grown in the presence of Ni and IPTG tested positive for urease activity in the urea segregation agar assay indicating production of vestigial amounts of a properly folded and enzymatically active JBURE-IIb.

3.4.2. Antifungal activity of recombinant JBURE-IIb

Purified recombinant JBURE-IIb inhibited micelial growth of *Penicillium herguei*, a phytopathogenic fungus known to be susceptible to urease antifungal effects (Fig. 4). The antifungal effect of JBURE-IIb occurred in concentrations of 0.27 to 1.15 μM (assuming a hexameric form in solution), the same dose range reported for the other ureases [20,24].

3.4.3. Entomotoxic activity of recombinant JBURE-IIb

As described for JBURE-I [45] recombinant JBURE-IIb was fully active in the inhibition of serotonin-induced fluid secretion in isolated Malpighian tubules of the kissing bug *Rhodnius prolixus* (Fig. 5). Diuresis was reduced to 24.36% ± 6.84 or 16.37% ± 8.83 of that measured in control tubules (N = 7), in the presence of 5×10^{-10} M JBURE-I or JBURE-IIb, respectively.

3.5. Sequence and phylogenetic analyses

In this work, we focused on sequence comparisons only of plant and fungal ureases, which are structurally formed by a single large subunit. Comparisons of the JBURE-IIb amino acid sequence were done over a non-redundant database by BLASTp [27], leading to a selection of 24 fungal and plant urease sequences. The retrieved sequences were then aligned to build phylogenetic trees, revealing that all selected fungal ureases have diverged from a common sequence, clustering together in the phylogenetic tree (Fig. 6). Plant ureases are also clustered together, hinting at a possible common ancestor of all Viridiplantae ureases. As expected, the JBURE-IIb urease is closely related to the *C. ensiformis* major urease, JBURE-I, and to the *G. max* ubiquitous and embryo-specific ureases. Since the complete sequence of canatoxin is yet not available, it was not included in the tree.

3.6. Comparative modeling

Until very recently only bacterial ureases had their 3D structure successfully resolved [34–36]. The crystal structure of JBURE-I, the

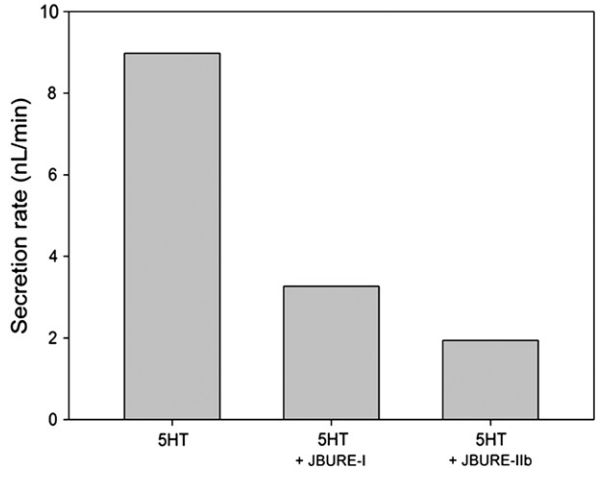


Fig. 5. Effect of purified recombinant JBURE-IIb on *Rhodnius prolixus* isolated Malpighian tubules. Tubules were incubated with serotonin (2.5×10^{-8} M) for 20 min and then washed with saline and incubated with the same dose of serotonin (5-HT, 5-hydroxytryptamine) plus JBURE-I or JBURE-IIb, at 5×10^{-10} M, for another 20 min. The secretion rate was expressed as nanoliter of fluid excreted per minute. A typical result of three experiments is shown.

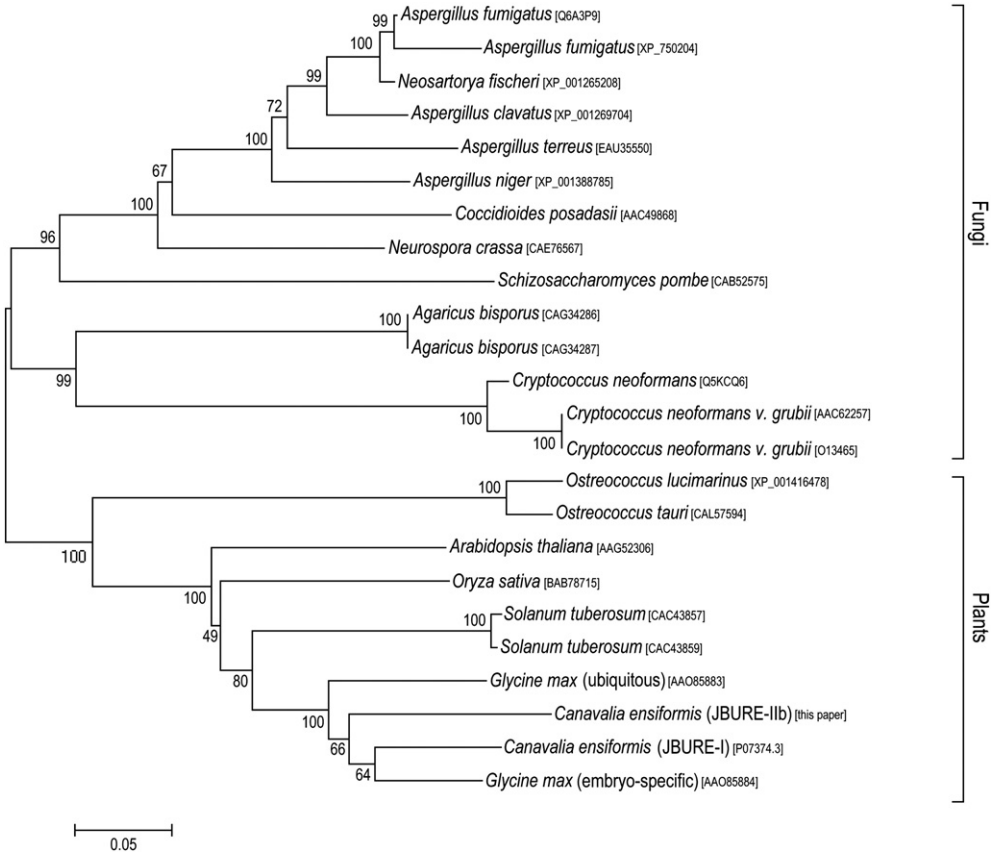


Fig. 6. Phylogenetic analysis of fungal and bacterial ureases. The percentage of replicate trees in which the associated sequences clustered together in the bootstrap test (10,000 replicates) is shown next to the branches. Reference codes for all sequences are given in square brackets.

major urease of *Canavalia ensiformis*, was finally solved in 2010 [37]. Based on these structures, a homology model of JBURE-IIb in its hexameric form is presented as Supplementary Fig. 2.

Compared to the 3D-crystallographic structure of *K. aerogenes* and *B. pasteurii* ureases, all residues experimentally reported, or structurally inferred, to be important for optimal enzyme activity are conserved in the JBURE-IIb structure (Supplementary Fig. 3). The amino acid residues involved in nickel coordination in *K. aerogenes* urease active site, H336, H338, H4448, H474 and D562, are present in the JBURE-IIb model corresponding to H407, H409, H519, H545, D633, respectively. The residue K419, which is carbamylated in *K. aerogenes* urease [46,47], corresponds to K490 in JBURE-IIb (Supplementary Fig. 3, panel A). All active site residues of *B. pasteurii* are present in JBURE-IIb in similar positions (H363, H365, K446, H475, H501, D633, Supplementary Fig. 3, panel B). However, when all residues within 7 Å of the putative catalytic site are considered, some of them in JBURE-IIb differed from residues strictly conserved in bacterial ureases. Q415S, P573T and M637V differ from bacterial and plant ureases (Supplementary Fig. 3, panel B). Positions P573 and M637 are strictly conserved in 127 analyzed sequences, and despite Q415 not being strictly conserved, JBURE-IIb is the only urease so far to have a serine residue in this position. Such unique alterations suggest possible differences in substrate specificity or enzyme efficiency of the JBURE-IIb protein. T406C, L467T, Q481I, and F544Y residues differ from bacterial ureases, but are conserved in plant ureases.

4. Discussion

We report here the cloning, molecular modeling and heterologous expression of full length JBURE-IIb urease from jack bean seedlings. The recombinant JBURE-IIb protein was obtained in *E. coli* cells, aiming to produce the apoenzyme, obtained by expression in a medium without supplementation of nickel and the holoenzyme, producing an ureolytically active enzyme by heterologous expression in the presence of high nickel concentration. The production of the apoenzyme allowed performing assays of biological properties of ureases known to be unrelated to urea hydrolysis, as fungitoxicity and diuresis inhibition in insect Malpighian tubules, an activity related to the entomotoxic effect of urease proteins.

The putative JBURE-II protein reported by Pires-Alves et al.[15] had a 78 kDa subunit and lacked 60 and 55 amino acid residues at the N- and C-termini, respectively, when compared to the ~90 kDa subunits of other plant ureases. Here, we described the complete cDNA encoding the full length urease isoform, with 840 amino acids.

The new *jbure-IIb* cDNA sequence contained a single nucleotide insertion, which modified the reading frame at the 3′ end. On the other hand, the difference observed between the 5′ end of *jbure-II* and *jbure-IIb* suggests a possible alternative splicing of the gene, generating two different mRNAs. The observation that urease genes from *A. thaliana*, *A. lyrata* and also *S. tuberosum*, have introns positioned immediately upstream of the first nucleotide common to both *jbure-II* and *jbure-IIb*, and a splicing motif signal (3′AG) occurs immediately upstream of this point in all three cases, goes along with this hypothesis (Fig. 1).

Alternative splicing of potato urease mRNA was previously reported [48]. This urease pre-mRNA was subject to extensive alternative splicing at different introns throughout the entire length of the sequence. The *C. ensiformis* urease mRNA was undetectable by Northern blot analysis using 40 μg of total leaf RNA (data not shown), suggesting mRNA instability or low transcription levels. Similar observations were made using 30 μg of total RNA from different tissues of potato plants [48]. Despite the low levels of urease pre-

mRNA found, urease activity in general appears not to be a limiting factor in plants [48].

Alternative splicing is an important cellular mechanism that increases the diversity of gene products [49]. The insecticidal and fungitoxic effects of plant ureases are not related to the ureolytic activity [14,20,24]. While the location of a putative fungitoxic "domain" of ureases is still unknown, the entomotoxicity is due to an internal peptide [18,19]. The previously reported sequence of *jbure-II* cDNA [15] served as base to produce a recombinant version of this entomotoxic peptide, designated jaburetox-2Ec [26]. Since the sequence encompassing the entomotoxic peptide is present in both forms (JBURE-II and JBURE-IIb), if alternative splicing occurs at the suggested site it does not compromise the urease insecticidal activity. It is important to mention that the JBURE-II isoform mRNA expression was induced by ABA treatment [15], a phytohormone that besides other functions also regulates expression of defense genes [50].

Small molecular size urease-like proteins were described previously [51] and can be routinely found in Western blots of *C. ensiformis* seeds extracts developed with polyclonal [52] or monoclonal [40] anti-urease antibodies. In the conditions of our assays, it is unlikely that these smaller urease-like proteins are artifacts due to manipulation of plant materials. Alternatively, they could represent products of proteolysis *in planta* and/or products of alternatively spliced mRNAs derived from the same gene giving rise to distinct proteins.

Additional reports on alternative splicing events in urease-producing eukaryotic organisms are needed to allow understanding of how this mechanism regulates the levels and isoforms of urease in plants.

Aiming to obtain abundant mRNA for the RT-PCR, we treated leaves with ABA since a previous study indicated that this phytohormone induces expression of urease and JBURE-II genes [15].

We have previously reported that CNTX is synthesized in immature embryos reaching peak levels (both protein and activity) at the end of the desiccation phase [52]; this same temporal pattern of expression was reported for urease (JBURE-I) in developing jack bean [53,54] and soybean [11] seeds. The initial aim for ABA treatment was to mimic desiccation during which urease and CNTX are maximally accumulated in the seeds, since this hormone is involved in the seed maturation, beside other functions, as mentioned earlier. The tissue distribution, temporal pattern and regulation of the urease gene family in *C. ensiformis* are currently being investigated.

So far we have not been able to isolate a urease isoform matching the properties of JBURE-II/-IIb either from mature jackbean seeds or leaves, indicating the very low levels of this protein present in the tissues. However, in a proteomic study of urease-like proteins during germination of *C. ensiformis*[40] three unique peptides matching JBURE-II/JBURE-IIb were detected in quiescent and in three days germinated seeds, contrasting to thirty unique peptides matching JBURE-I, which goes in agreement with the low abundance of JBURE-II/-IIb. Peptides matching JBURE-I and/or canatoxin's partial sequence were also detected in the same analysis (Figs. 2 and 3). The JBURE-IIb protein was apparently more abundant in the germinated seeds [40], probably being synthesized in the developing embryos. In this context it is noteworthy that phylogenetic analysis (Fig. 6) showed that JBURE-IIb and the soybean ubiquitous urease, to which a clear metabolic role was ascribed [9,55], are placed in different branches than the seed abundant urease JBURE-I or the embryo-specific soybean urease, suggesting divergent functions of these isoenzymes.

The high sequence identity of all known ureases and the conservation of catalytically important residues among eukaryotic and prokaryotic enzymes indicate that these proteins are likely variants of the same ancestral protein and possess similar tertiary structures and catalytic mechanisms [3,8,46]. The JBURE-IIb model reinforced the structural conservation that is characteristic of this class of proteins. All residues experimentally shown, or structurally inferred, to be important for optimal enzyme activity in JBURE-I, *K. aerogenes* and *B. pasteurii* are conserved in the JBURE-IIb sequence and structure (Supplementary Figs. 2 and 3). However, some differences were observed at residues near its active site, suggesting possible differences in substrate specificity or enzyme efficiency of JBURE-IIb protein.

From the JBURE-IIb 3D modeled structure (Supplementary Fig. 3), we postulate that this protein would be enzymatically active once its bi-metal center is correctly assembled. Nickel insertion into a urease is not a spontaneous process, requiring energy and several accessory proteins [56–59]. It is interesting to note, however, that the presence of metal on the active site seems to have little or no effect upon the conformation and dynamic behavior of JBURE-IIb (Ligabue-Braun, unpublished results). In this work our purpose was to produce the apoenzyme and test the recombinant protein for biological properties not related to urea hydrolysis. For that, transformed *E.coli* cells were grown in the absence of nickel supplementation in the medium. As we expected, the soluble recombinant JBURE-IIb was enzymatically inactive. Park and Hausinger, 1995 [43] successfully activated in vitro recombinant *Klebsiella aerogenes* apourease to up to ~30% of the expected specific activity by manipulating bicarbonate and nickel concentrations and pH of the medium. In contrast, all attempts we have made to activate *in vitro* the purified recombinant JBURE-IIb have failed. Because the recombinant protein has a 6 His tag, higher concentrations of nickel chloride were tested aiming to overcome any chelant effect without any success.

However, when transformed *E. coli* cells expressing recombinant JBURE-IIb were grown for 24 h in agar containing IPTG, 100 μM $NiCl_2$ and urea, the appearance of a reddish color around the colonies due to the alkalization by released ammonia clearly indicated the production of an active, properly folded enzyme. Thus at least a small amount of the recombinant protein was activated by *E. coli* under this condition. Although some *E. coli* strains are urease-positive [60,61], the available genome of *E. coli* BL21 Gold DE53 does not contain any urease-related sequence, thus the identity of *E. coli* proteins able to activate the heterologous urease is presently unknown. In this context, Olson and co-workers [62] reported that the hydrogenase accessory proteins HypA and HypB were also implicated in the activation of urease in *Helicobacter pylori*.

Witte and collaborators [63] were the first to describe heterologous expression of a plant urease in *E. coli*. The authors co-expressed the cDNA encoding *Arabidopsis thaliana* urease together with those encoding *A. thaliana* UreD, UreF and UreG. The recombinant *A.thaliana* urease in the bacterial extracts only showed measurable ureolytic activity in the presence of the three accessory proteins. The conditions they reported were similar to those we used here, except for the addition of nickel, with *E. coli* BL 21 (DE53) expressing *A.thaliana* urease upon induction with 1 mM IPTG and 100 μM $NiCl_2$ for 2 h, while we obtained optimal expression of JBURE-IIb with 0.1 mM IPTG for 16 h.

Fungitoxic properties of the cotton seed urease [24], the jackbean major urease (JBURE-1), the soybean embryo-specific urease and of the di-chain *Helicobacter pylori* urease [20] occurred at submicromolar concentrations and did not require ammonia release. Here we observed that the enzymatically inactive JBURE-IIb displayed fungitoxic effects in the same dose range of plant native ureases.

The insecticidal activity of plant ureases is not affected by treatment with the covalent inhibitor *p*-hydroxymercurybenzoate [23] and involves an internal 10 kDa peptide released from urease by cathepsins in the digestive system of susceptible insects [18,19,25]. More recently, it became clear that whole urease contributes to the entomotoxic activity, with effects and/or activation of signaling pathways not shared by the peptide [45,64]. Here, we demonstrated that the recombinant apoenzyme JBURE-IIb was fully active to inhibit diuresis in isolated *R. prolixus* Malpighian tubules in the same concentration (5×10^{-10} M) as JBURE-I.

In conclusion, in this paper we have characterized the cDNA encoding the full length JBURE-IIb urease isoform of *Canavalia ensiformis*.

The *jbure-II*/*-IIb* gene is functional in the plant and the recombinant JBURE-IIb apoprotein produced in *E. coli* displayed fungitoxic and entomotoxic activities, corroborating the hypothesis the plant ureases probably contribute to the plant arsenal of defense compounds.

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbapap.2011.07.022.

Author's contributions

FM cloned and expressed *jbureIIb* cDNA and drafted the manuscript; ABBR produced JBURE-IIb and tested antifungal activity; DRD worked on MS detection of ureases in plant material; RLB, HV and EKS worked on phylogeny and modeling of JBURE-IIb; FS conducted the assays with Malpighian tubules; RRF participated in the cloning experiments; CRC and MFGS conceived this study and coordinated all its phases.

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