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Soybean ubiquitous urease with purification facilitator: An addition to the moonlighting studies toolbox

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ABSTRACT

Ureases are nickel-dependent enzymes that catalyze the hydrolysis of urea to ammonia and carbon dioxide. In soybean (*Glycine max*), the embryo-specific urease (eSBU), the ubiquitous urease (uSBU), and a third isoform (SBU-III) are synthesized. Our group has previously demonstrated that eSBU, purified from seeds, has antifungal properties against phytopathogenic fungi, entomotoxicity against *Dysdercus peruvianus*, the ability to induce blood platelet aggregation, and these properties are independent of its enzymatic activity. Here we describe the biological properties of apo-uSBU fused to glutathione S-transferase (GST) produced in *Escherichia coli*. Removal of GST affected apo-uSBU stability. We performed a Response Surface Methodology to optimize GST-uSBU production to 5 mg per liter and then bioassays were carried out. The recombinant protein exhibited inhibitory effects on filamentous fungi and affected fungal secondary metabolism. *Candida albicans* and *C. tropicalis* were also susceptible to GST-uSBU and formed pseudo-hyphae. The fusion protein was toxic against *Rhodnius prolixus,* with the toxicity being accompanied by *in vivo* and *in vitro* hemocyte aggregation. Rabbit platelet also aggregated in the presence of GST-uSBU. Thus, uSBU displayed similar biological properties as previously described for eSBU even when fused to GST, reinforcing the proposed role of ureases in plant defense.

1. Introduction

Ureases (EC 3.5.1.5, urea amidohydrolase), metalloenzymes that catalyze the hydrolysis of urea to produce ammonia and carbon dioxide, are produced by plants, fungi and bacteria but not by animals [1,2]. Ureases contain two catalytically important atoms of nickel [3] that require a set of accessory proteins to place them in the active site of the apoenzymes [4,5]. In plants, ureases are abundant mainly in seeds of some members of the Fabaceae and Curcubitacea families [1]. It has been postulated that these enzymes are not only involved in nitrogen bioavailability, but also in plant defense processes [6]. Over the last two decades our group has demonstrated that ureases from different organisms are multifunctional proteins, displaying a number of biological properties unrelated to their enzyme nature, including platelet aggregation, insecticidal, and antifungal activities [6–10].

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Soybean (*Glycine max*) produces three urease isoenzymes: the embryo-specific urease (eSBU) encoded by the *Eu1* gene (GenBank accession AY230157, Phytozome accession Glyma05g27840.1), the ubiquitous urease (uSBU) encoded by the *Eu4* gene (GenBank accession AY230156, Phytozome accession Glyma11g37250.1), and a third isoform recently characterized, the putative SBU-III urease, encoded by the *Eu5* gene (Glyma08g103000) [11]. Whereas eSBU is synthesized only in the developing embryo, uSBU is present in small amounts in all plant tissues [12,13]. SBU-III transcripts were found in low levels in seeds one day after dormancy break, roots of young plants, and developing seeds [11]. In addition, while uSBU is supposedly responsible for recycling metabolically-derived urea, the role of eSBU remains elusive. It was suggested that the new soybean urease SBU-III is not involved in nitrogen availability, due to accumulated mutagenesis and deletion in important residues close to the active site [11]. Mutants lacking eSBU do not accumulate urea in any tissue and have no impairment on the use of urea as their sole nitrogen source [14–17]. eSBU and uSBU isoforms share 87% identity (92% similar coding sequences) [18]. As described in [11], the new putative urease *Eu*5 cDNA sequence share 79.6% identity with *Eu*4 (uSBU) and 91.8% identity with *Eu*1 (eSBU) cDNA.

Soybeans are susceptible to many predators and pathogens including insects, nematodes, fungi, and viruses. These pathogens and pests, usually tissue-specific, cause serious damages to seeds, roots, leaves, stems, and pods. Despite control measures, these organisms determine losses of soybean production by almost 28% worldwide [19]. Development of new technologies to control plant pathogens and pests is quite urgent, and the exploration of plant natural compounds represents a major strategy to this end [20]. We have previously demonstrated that eSBU, purified from soybean seeds, displays several biological properties that are independent of its ureolytic activity [13] such as the activation of rabbit blood platelets, insecticidal activity against *Dysdercus peruvianus* [21] and also *in vitro* growth inhibition of the phytopathogenic fungi *Colletotrichum musae*, *Curvularia lunata*, *Fusarium oxysporum*, *F. solani*, *Penicillium herquei*, *Trichoderma* sp., *T. viride,* and *T. pseudokoningii* [22].

In contrast, uSBU, which occurs in such low concentration in plant tissues that conventional purification is very difficult [23], was less explored in the literature. In a recent study, transgenic soybean plants were obtained in which silencing of the uSBU gene resulted in co-suppression of all urease genes [24]. These urease-null plants were more susceptible to *P. herquei* (maize pathogen), *Phomopsis* sp. and *Rhizoctonia solani* (soybean pathogens), and to the biotrophic fungus *Phakospora pachyrhizi*, the agent of the Asian rust disease [24]. These observations reinforced the hypothesis that uSBU could also be involved in plant defense [25].

Aiming a better understanding of the role of ureases in plants and their potential biotech uses in agriculture, here we have optimized the production of a glutathione-S-transferase-(apo) uSBU fusion protein in *Escherichia coli* using a Response Surface Methodology and report on the characterization of important biological properties of this recombinant protein.

2. Material and methods

2.1. PCR amplification and cloning of ubiquitous urease cDNA

The plasmid pGPTV-JIT containing soybean ubiquitous urease cDNA was kindly provided by Dr. Mark Taylor (Scottish Crop Research Institute, Dundee, Scotland). The cDNA was excised with *Eco*RI (Fermentas), ligated into a pBluescript KS (+) plasmid (Stratagene) previously digested with *Eco*RI (Fermentas) and dephosphorylated. The plasmid pBluescript KS-Urease was sequenced on an ABI PRISM 3100 automated sequencer (Applied Biosystems, Foster city, CA). BLAST alignment was performed to compare our sequence with the sequence available at GenBank (acession no. AJ276866). In order to clone urease in the expression vector, its cDNA was reamplified by polymerase chain reaction (PCR) using the pBluescript KS-Urease as template. The primers used were: forward primer 5′ TTA AAA ATG AAA CTG AGT CC 3′ and reverse primer 5′ TTA AAA GAG GAA GTA ATT TCG 3′. The PCR conditions were 35 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 45 s and extension at 72 °C for 10 min. The final product was purified using GFX PCR DNA and gel band purification kit (GE Healthcare), digested with *Sma*I (Fermentas), phosphorylated using T4 polynucleotide kinase and cloned into a pGEX-4T-2 glutathione S-transferase fusion vector (GE Healthcare) that had been digested and dephosphorylated with *Sma*I (Fermentas) and thermosensitive alkaline phosphatase (Fermentas), respectively. The correct ORF was confirmed by restriction analysis and sequencing.

2.2. GST-uSBU expression and purification

Escherichia coli BL21-CodonPlus (DE3)-RIL cells (Stratagene) were transformed by heat shock with pGEX-4T-2-*GST-uSBU* plasmid that encodes the GST-uSBU fusion protein. Cells were cultured overnight at 37 °C and 200 rpm in 50 mL conical tubes containing 15 mL of 2-XYT medium (16 g L^{-1} tryptone, 10 g L^{-1} yeast extract, 5 g L^{-1} NaCl, pH 7.0), ampicillin (100 mg L^{-1}) and chloramphenicol (40 mg L^{-1}). Aliquots of 2 mL were inoculated in 1 L Erlenmeyer flasks containing 200 mL of 2-XYT medium and incubated at 37 °C, 200 rpm until absorbance at 600 nm reached 0.7. Then, IPTG (isopropyl-β-D-thiogalactoside) was added to the culture (1 mM) and temperature was cooled down to 28 °C for additional 16 h at 200 rpm incubation. Cell suspensions were centrifuged at 10,000 x *g* for 10 min and pellets were suspended in 20 mL PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3) and this cell suspension was sonicated (20 cycles of 1 min, 20 kHz frequency). The final cell lysate was centrifuged at 14,000 × *g* for 40 min and supernatant was submitted to affinity chromatography as follows.

A column with 2 mL of Glutathione Sepharose 4B resin (GE Healthcare) was equilibrated with 40 mL PBS and the purification was performed as manufacturerís instructions. Unbound proteins were washed out with 10 mL PBS. The GST-uSBU fusion protein was eluted in 10 mL of 50 mM Tris-HCl, pH 8.0, containing 10 mM reduced glutathione (Fig. S1). Samples were dialyzed against 50 mM sodium phosphate, pH 7.5, containing 1 mM EDTA and 5 mM β-mercaptoethanol to remove reduced glutathione. For bioassays, samples were concentrated in 30 kDa Amicon devices by centrifugation for 15 min at 4000 × *g*. For some experiments, samples were centrifuged using Amicon Ultra centrifugal filter units (Ultra-15, MWCO 50 kDa, Millipore) for 30 min at 2500 × g, to yield two fractions (>50 kDa and <50 kDa).

Protein concentrations were determined by Bradford assay [26] and purity was checked *via* 10% SDS-PAGE.

2.3. Recombinant glutathione-S-transferase production and purification

The recombinant glutathione-S-transferase (rGST), used as control in biological tests, was produced by culturing *E. coli* BL21-CodonPlus (DE3)-RIL cells (Stratagene) carrying the empty pGEX-4T-2 vector (GE Healthcare). Conditions of culture and induction were the same as described above with the exception that flasks were incubated for 3 h after IPTG addition to a final 1 mM concentration and 37 °C. Purification of rGST was performed using the same protocol described above. In all experiments the concentration of GST-uSBU or rGST was expressed in mg mL^{-1}. To calculate the concentration of rGST (control), we used a ratio (uSBU/rGST)

considering the molecular weight of each monomer (uSBU–90 kDa and rGST–26 kDa). In this case, we used the ratio of 3.5.

2.4. Western blot

Western blot analyses were performed essentially according to [27]. Protein bands were transferred by gravity from 10% SDS-PAGE gels to PVDF membranes (Hybond-P, GE Healthcare). Membranes were blocked with 5% casein in Tris buffered saline (TBS), washed, incubated with rabbit anti-Jaburetox-2Ec (Jaburetox 2Ec is a recombinant peptide derived from *Canavalia ensiformis* urease; [28]) polyclonal antibody (1:7500) or mouse anti-GST monoclonal antibody (1:2000; donated by Dr. Henrique Bunselmeyer Ferreira, Centre for Biotechnology, UFRGS) for 2 h at room temperature. After TBS washings, membranes were exposed to anti-rabbit IgG (1:20,000) alkaline phosphatase conjugate (Sigma) or anti-mouse IgG (1:20,000) alkaline phosphatase conjugate (Sigma), respectively. Colorimetric detection was carried out by using 30 mM 5-bromo-4-chloro-3-indolyl-phosphate *p*-toluidine (BCIP; Sigma) and 0.75 mM nitro-blue tetrazolium chloride (NBT; Sigma). Jack bean (*Canavalia ensiformis*) urease was used as positive control (Urease type C-III; Sigma Aldrich) and bovine serum albumin (BSA; Sigma Aldrich) as a negative control.

2.5. Mass spectrometry

The SDS-PAGE-resulting GST-uSBU band (~120 kDa) was excised and gel fragments were minced in small pieces. Protein digestion was performed according to [29]. Peptides generated were subjected to reversed phase chromatography (NanoAcquity UltraPerformance LC-UPLC; Waters) in a Nanoease C18, 75 mm ID at 35 °C. The column was equilibrated with 0.1% trifluoroacetic acid (TFA) and peptides were eluted in 20 min *via* a gradient ramping from 0 to 60% acetonitrile 0.1% TFA at 0.6 nL/min constant flow. Eluted peptides were subjected to electro spray ionization and analyzed by mass spectrometry using a Q-TOF Micro spectrometer (Micromass, Waters). The voltage applied to the cone for ionization was 35 V. The three most intense ions in the range of 200–2000 *m*/*z* and +2 or +3 charges were selected for fragmentation. The acquired MS/MS spectra were processed using Proteinlynx v. 2.0 software (Waters) and generated.mgf files were used to perform database searches using MASCOT software v. 2.4.00 (Matrix Science) against NCBI database, restricting the organism to "*Glycine max* (taxid:3847), november 2012. Tripsin/P was selected as enzyme. Search parameters allowed a maximum of one missed cleavage, carbamidomethylation of cysteine, possible oxidation of methionine, peptide tolerance of 1.2 Da, and MS/MS tolerance of 1.2 Da. A minimum of two unique non overlapping peptides was defined as criterion for protein match.

2.6. Urease activity

Urease activity was evaluated using Urea segregation agar method. *E. coli BL21*-CodonPlus (DE3)-RIL cells (Stratagene) harboring the pGEX-4T-2-*GST-uSBU* vector were used and urease activity was evaluated visually as a reddish color around colonies due to local medium alkalization [30].

2.7. Optimization of GST-uSBU production

In order to determine the best conditions of GST-uSBU production, effects of temperature, IPTG concentration, and time of induction on protein yield were evaluated using a 2^3 experimental model with 5 replicates, resulting in 19 experiments (Table 1). Culture conditions and protein preparations were performed exactly as described above, except for the variable being evaluated. In the statistical model, coded (independent) variables corresponded to temperature (X_1), IPTG concentration (X_2), and time (X_3), while protein concentration (Y) was taken as the dependent variable [31]. The software Statistica v. 6.0 (Statsoft) was used for regression analysis of experimental results. The quality of fit of the first-order model equation was expressed by R^2, coefficient of determination, and its statistical significance were determined by Fischer's F Test.

2.8. GST-uSBU quantification by enzyme-linked immunosorbent assay (ELISA)

The concentration of GST-uSBU in culture supernatants was determined by ELISA. Aliquots of cell lysate supernatants (50 μL) were adsorbed onto ELISA plates overnight at 4 °C. Wells were blocked with 5% casein in TBS for 2 h, 3 times washed with TBS, then incubated with primary antibody anti-Jaburetox 2Ec (1:500) in 2% casein-TBS. After 2 h, wells were washed and incubated for 2 h with secondary anti-rabbit IgG conjugated with alkaline phosphatase (1:10,000; Sigma) in 2% casein-TBS. The color reaction was developed with 50 μL of 1 mM *p*-nitrophenylphosphate, 10 mM sodium borate, 0.25 mM magnesium chloride, pH 9.8. Absorbance was determined on a SpectraMax M3 (Molecular Devices) plate reader at 405 nm. The concentration of the fusion protein was calculated using a standard curve prepared with the purified fusion protein (0.01–1 μg). Results were expressed in mg L^{-1}.

2.9. Antifungal assays

Strains of the filamentous fungi *Colletotrichum musae*, *Curvularia lunata*, *Fusarium oxysporum*, *Rhizoctonia solani*, and *Trichoderma viride* were donated by Dr. José Tadeu Abreu de Oliveira (Universidade Federal do Ceará) and Dr. Lidia Mariana Fiuza (Universidade do Vale do Rio dos Sinos). *Penicillium expansum* M-02 was donated by Dr. Isa Beatriz Noll (Universidade Federal do Rio Grande do Sul). *Cercospora chevalier*, *Fusarium lateritium*, *Mucor* sp., *Penicillium herquei*, *Phomopsis* sp., *Pythium oligandrum*, and the yeasts *Candida parapsilosis* (CE002), *Candida tropicalis* (CE017), *Candida albicans* (CE022), *Kluyveromyces marxiannus* (CE025), *Pichia membranifaciens* (CE015), and *Saccharomyces cerevisiae* (1038) were kindly provided by Dr. Valdirene Gomes (Universidade Estadual do Norte Fluminense).

Antifungal activity against filamentous fungi and yeasts was assayed according to [32] with minor modifications. Filamentous fungi were cultured on Potato Dextrose Agar (Oxoid) plates at 28 °C until sporulation. Spores were washed out with sterile distilled water, quantified in a Neubauer chamber, and 10^6 spores mL^{-1} were inoculated in 96 wells flat bottom plates containing GST-uSBU (0.24 mg mL^{-1}) or rGST (0.069 mg mL^{-1}) final concentration in Potato Dextrose Broth. GST-uSBU and rGST were dialyzed previously against 10 mM Tris-HCl buffer, pH 7.0. An aliquot of the last change of dialysis buffer was used as negative control. As a positive control, 0.1% hydrogen peroxide was used. Plates were incubated at 28 °C and monitored turbidimetrically at 620 nm after 24 and 48 h.

Yeasts were cultured in Sabouraud Agar (Accumedia) plates for 24 h at 28 °C, then washed with saline solution and quantified in a Neubauer chamber. Samples of 0.24 mg mL^{-1} of GST-uSBU or 0.069 mg mL^{-1} rGST (ratio of uSBU/GST = 3.5) were incubated with 10^4 cells.mL^{-1} of each yeast type in U-bottom microplates, in Sabouraud Broth at 28 °C for 24 h. As negative control, was used saline and a fused protein GST-enolase 1 from *Echinococcus granulosus* expressed in *Escherichia coli* BL21 (DE3) RIL (ratio of Enolase/GST = 2), kindly provided by Dr. Henrique Ferreira [33]. After 24 h of incubation, 20 μL of each well were 10-fold serially diluted in saline solution and plated in Sabouraud agar (drop plate method) to determine the number of Colony Forming Units (CFU) after 24 h at 28 °C. Three independent bioassays with triplicated groups were

Table 1
Experimental design and results of the 2^3 factorial design.

Run	X_1 Temperature (°C)	X_2 IPTG (mM)	X_3 Time of induction (h)	Y_1 GST-uSBU concentration (mg.L^{-1})
1	(−1) 20	(−1) 0.28	(−1) 5.20	0.921 ± 0.12
2	(1) 28	(−1) 0.28	(−1) 5.20	0.000
3	(−1) 20	(1) 0.82	(−1) 5.20	0.021 ± 0.0046
4	(1) 28	(1) 0.82	(−1) 5.20	0.000
5	(−1) 20	(−1) 0.28	(1) 14.76	0.000
6	(1) 28	(−1) 0.28	(1) 14.76	1.066 ± 0.12
7	(−1) 20	(1) 0.82	(1) 14.76	0.000
8	(1) 28	(1) 0.82	(1) 14.76	2.653 ± 0.31
9	(−1.68) 18	(0) 0.55	(0) 9.98	3.564 ± 0.16
10	(1.68) 30	(0) 0.55	(0) 9.98	0.099 ± 0.0072
11	(0) 24	(−1.68) 0.10	(0) 9.98	4.254 ± 0.42
12	(0) 24	(1.68)1.00	(0) 9.98	3.061 ± 0.49
13	(0) 24	(0) 0.55	(−1.68) 2.00	2.927 ± 0.44
14	(0) 24	(0) 0.55	(1.68) 18.00	3.477 ± 0.62
15	(0) 24	(0) 0.55	(0) 9.98	5.362 ± 0.60
16	(0) 24	(0) 0.55	(0) 9.98	5.522 ± 0.22
17	(0) 24	(0) 0.55	(0) 9.98	5.056 ± 1.22
18	(0) 24	(0) 0.55	(0) 9.98	5.367 ± 0.55
19	(0) 24	(0) 0.55	(0) 9.98	5.267 ± 0.48

carried out. Results were presented as means ± standard deviations (SD).

2.10. Evaluation of yeast cell permeability

The permeabilization of yeast plasma membrane promoted by GST-uSBU or rGST was assessed with the fluorescent dye SYTOX Green (Invitrogen) as described by [32]. Yeast cell suspensions were incubated with GST-uSBU or rGST for 24 h and then exposed to 0.2 M SYTOX Green for 30 min at room temperature. Cells were observed under an Axioskop 40 microscope (Zeiss) equipped with filter for fluorescence detection (excitation wavelength 450–490 nm and emission 500 nm).

2.11. Antibacterial activity

Bacillus cereus ATCC 14579 was kindly donated by Dr. Adriano Brandelli (Universidade Federal do Rio Grande do Sul). *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853 were kindly provided by Pharmacist Elaine Staatlander (Hospital Presidente Vargas). Bacteria were inoculated in culture tubes containing 5 mL Luria Bertani (LB-Accumedia) medium and incubated overnight at 37 °C and 120 rpm shaking. Bacterial cultures were diluted in LB to reach 0.5 in the McFarland scale and distributed in 96 wells flat plates containing 0.24 mg mL^{-1} of GST-uSBU or 0.069 mg mL^{-1} rGST incubated at 37 °C for 24 h without shaking. Dialysis buffer was used as negative control and 0.1% hydrogen peroxide as positive control. After 24 h of incubation, 20 μL of each well were 10-fold serially diluted in saline solution and plated on LB agar (drop plate method) to determine the number of CFU after 24 h at 37 °C [34], with modifications). Three independent bioassays with triplicated groups were carried out. Results were presented as means ± SD.

2.12. Insecticidal activity and hemocyte aggregation in Rhodnius prolixus

Fifth instar *Rhodnius prolixus* (insect mean body weight ~50 mg) were kindly provided by Dr. Hatisaburo Masuda and Dr. Pedro Oliveira (Universidade Federal do Rio de Janeiro) and by Dr. Denise Feder (Universidade Federal Fluminense). GST-uSBU or rGST (0.05 μg mg^{-1} of insect body weight), prepared in *R. prolixus* saline solution [35], were injected into the hemocoel of insects organized in groups of 5 insects each, using a Hamilton syringe. In the control group, 5 insects were injected with *R. prolixus* saline solution. Insects were observed for 96 h [29]. Two independent bioassays were carried out.

Hemocyte aggregation was performed according to [36]. For *in vivo* experiments, unfed insects were injected into the hemocoel with GST-uSBU or rGST diluted in *R. prolixus* saline (dose of 6 μg protein per insect). Control insects were injected solely with saline. Six hours after injection, insects were surface sterilized by immersion in 70% ethanol and the hemolymph was collected from a cut in one of the legs. Hemolymph samples were immediately diluted in cold anticoagulant solution (10 mM EDTA,100 mM glucose, 62 mM NaCl, 30 mM sodium citrate, 26 mM citric acid, pH 4.6) at a ratio of 1:5 v/v (anticoagulant:hemolymph). The number of cells and aggregates (defined as a cluster of nine or more cells) in samples was then determined by counting them in a Neubauer chamber. Three independent bioassays were carried out with three insects in each group.

For *in vitro* experiments, insects were surface sterilized as described above; the hemolymph was collected and immediately diluted with cold *R. prolixus* saline (1:1 v/v). Thereafter, GST-uSBU (0.120 μg mL^{-1}) or rGST (0.034 mg mL^{-1}) were added to the tubes and incubated for 1 h at room temperature with gentle agitation. Controls were also carried out incubating the preparation with equivalent volumes of saline alone. The number of cells and aggregates was counted as above; four independent assays were performed.

2.13. Rabbit platelet aggregation

Aggregation of rabbit platelets promoted by GST-uSBU or rGST was assayed according to [21]. Platelet-rich plasma (PRP) was prepared from rabbit blood collected from the ear central artery. Platelet aggregation was monitored turbidimetrically using a Lummi-Aggregometer (Chrono-Log). PRP (300 μL) was pre-incubated for 2 min at 37 °C with constant agitation and then test samples (30 μL) were added: 0.033 mg mL^{-1} and 0,096 mg mL^{-1} GST-uSBU or 0,027 mg mL^{-1} rGST. Positive PRP controls were carried out with 20 μM ADP and 20 μg mL^{-1} collagen (Sigma).

Fluorescein isothiocyanate (FITC)-labeled GST-uSBU was prepared according to [37]. Rabbit PRP was incubated with 800 nM FITC-GST-uSBU under vortex stirring for 5 min at room temperature. Platelet aggregates were recovered after centrifugation. Pellets were smeared on glass slides and observed under Axioskop 40 (Zeiss) fluorescence microscopy.

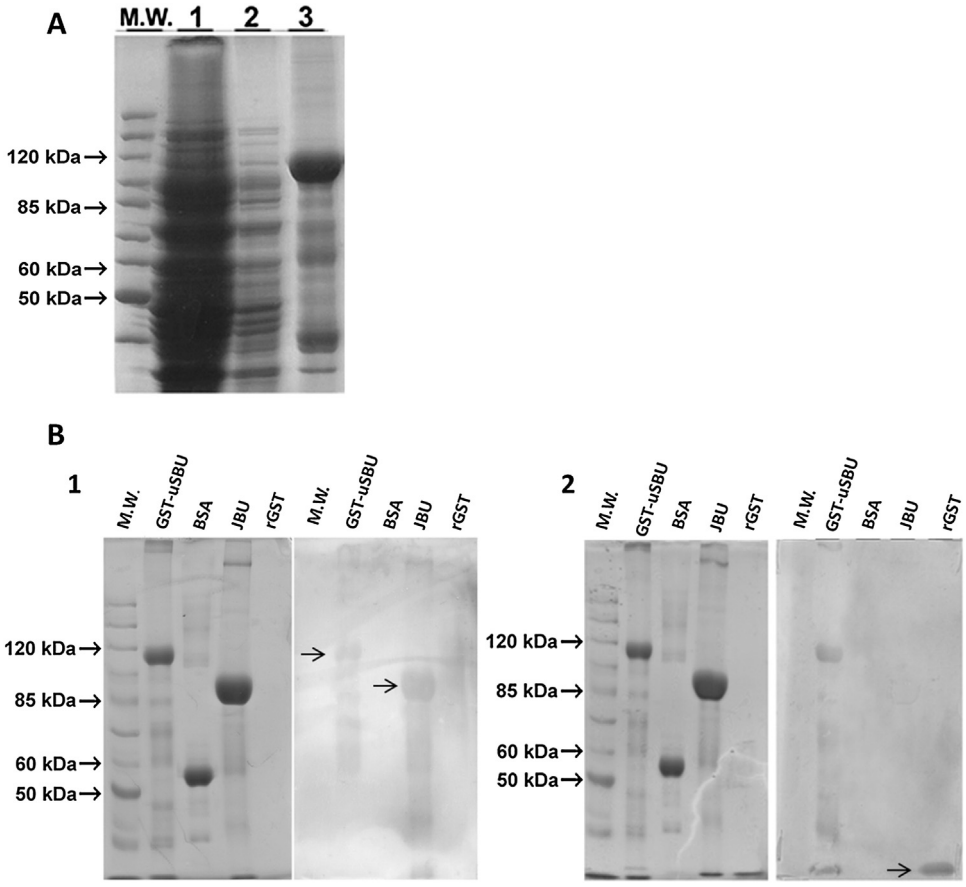

Fig. 1. (A) 10% SDS-PAGE of purification steps of GST-uSBU using Glutathione Sepharose resin. M.W. Molecular ladder marker; Lane 1: crude extract; Lane 2: Unbound fraction; Lane 3: Eluted fraction (B) 10% SDS – PAGE (left) and Western blots (Right). M.W. Molecular ladder marker, Lane 1: GST-uSBU; Lane 2: negative control (BSA); Lane 3: positive control (JBU), Lane 4: rGST. Samples of 12 μg of each protein were applied to the gels. Western blot were developed with anti-Jaburetox-2Ec (in 1) or anti-GST (in 2) antibodies, respectively.

2.14. Statistical analysis

All results were submitted to analysis of variance (ANOVA) and the significance of differences among means was determined by Tukey test, with $p \leq 0.05$ considered statistically significant. Analyses were performed with the GraphPad Prism 5.0.1. software for Windows (GraphPad Software).

2.15. Molecular modelling

GST-uSBU structural models were built with Modeller 9.15 [38] using as templates the structures of *Schistosoma japonicum* GST (PDB id 1M99, [39]) and *C. ensiformis* urease (PDB id 3LA4, [40]). The putative dimer of GST-uSBU was built based on dimerization data from the GST crystal [39] and the putative hexamer of GST-uSBU was built based on symmetry data from the *C. ensiformis* urease crystal [40]. The models were stereochemically evaluated with Procheck [41].

2.16. Ethics statement

The use of rabbits to collect blood were approved by the Ethics Committee for the Use of Animals of the Federal University of Rio Grande do Sul, concerning housing and care of laboratory animals (CEUA – protocol number 23078.00605212014-03).

3. Results

3.1. GST-uSBU production and purification

Initially 2 mg of the fusion protein per one liter of culture were obtained. Fractions of the purification steps are shown in Fig. 1A. Together with the intense band of fusion protein of ~120 kDa (uSBU: 90 kDa and rGST: 26 kDa), other bands of smaller masses could be seen in the gel, representing GST fused to truncated (incompletely translated) uSBU (the construct has GST fused on the N-terminal region of urease). Part of the truncated GST-uSBU was successfully removed by ultrafiltration in 30 kDa membranes.

3.2. Expression optimization – experimental design

Analysis of the effects of three independent variables (temperature, IPTG concentration, and time after induction) on the expression yield was performed. Conditions used and resulting concentrations of GST-uSBU in the 19 experiments are shown in Table 1. Central point conditions were the most favorable: 24 °C, 0.55 mM of IPTG and approximately 10 h, increasing yield of GST-uSBU to more than 5 mg per liter of culture (Fig. 2 and Table 1). For convenience, we chose induction for 14 h (overnight) which also fitted in the region of optimal production of the surface response curve (Fig. 2B and C).

Eq. (1) describes the yield (protein concentration) of GST-uSBU according to the independent variables.

$$Y\ (\text{protein concentration}) = 5.40 - 0.22X_1 - 1,70X_1^2 - 1.05X_2^2$$

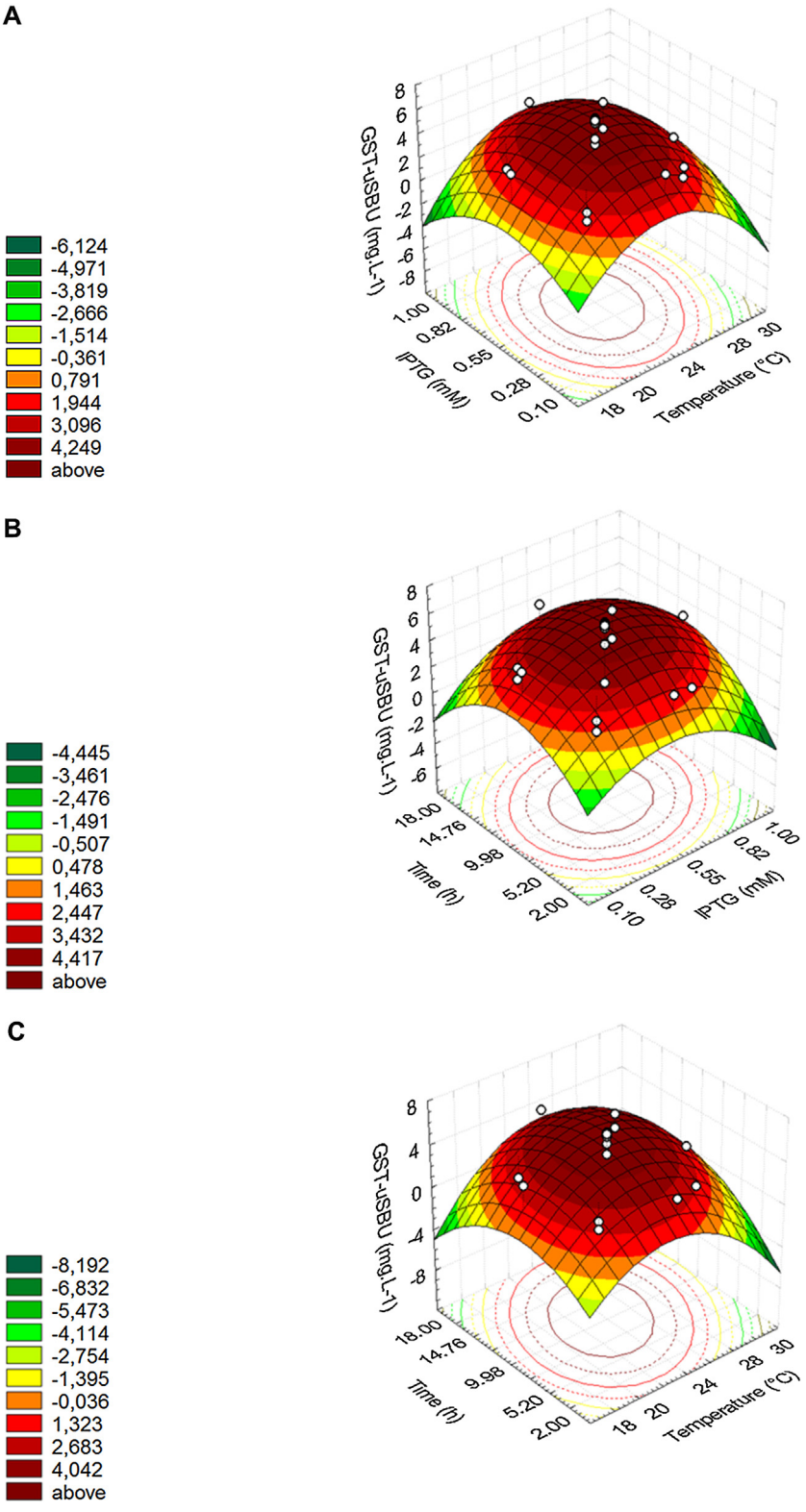

Fig. 2. Response surfaces for GST-uSBU production as a function of (A) IPTG concentration and temperature, (B) time and IPTG concentration, (C) time and temperature.

$$+0.27X_3 - 1.21X_3^2 + 0.31X_1X_2 + 0.58X_1X_3 + 0.32X_2X_3 \quad (1)$$

Table 2 shows effects of the independent variables on GST-uSBU production. The linear effect of time of induction, within the period of 5.20 h to 14.76 h, was the most expressive variable, increasing in 0.54 mg L^{-1} the concentration of GST-uSBU. Increase of temperature from 18 °C to 30 °C (quadratic) or from 20 °C to 28 °C (linear) decreased the protein expression in 3.41 mg L^{-1} and 0.45 mg L^{-1}, respectively. Increase in the concentration of IPTG from 0.10 mM to 1.0 mM (quadratic) also decreased protein expression in 2.11 mg L^{-1}.

Table 2
Main effects and interaction analysis for GST-uSBU production.

Factors	Effect (GST-uSBU concentration)	Standard error	*t* value	*p* value
Mean	5.42238	0.076022	71.3266	0.000000*
Temperature (L)	−0.44621	0.092155	−4.8419	0.008388*
Temperature (Q)	−3.40822	0.092279	−36.9338	0.000003*
IPTG (L)	−0.19308	0.092155	−2.0951	0.104226
IPTG (Q)	−2.11428	0.092279	−22.9118	0.000021*
Time (L)	0.54248	0.092155	5.8866	0.004163*
Time (Q)	−2.43706	0.092279	−26.4097	0.000012*
Temperature × IPTG	0.62175	0.120353	5.1661	0.006670*
Temperature × Time	1.16525	0.120353	9.6819	0.000637*
IPTG × Temperature	0.62175	0.120353	5.1661	0.006670*

Software Statistica, L: Linear, Q: Quadratic, confident level of 95% and R^2 of 0.75.
* Significant factors, $p < 0.05$.

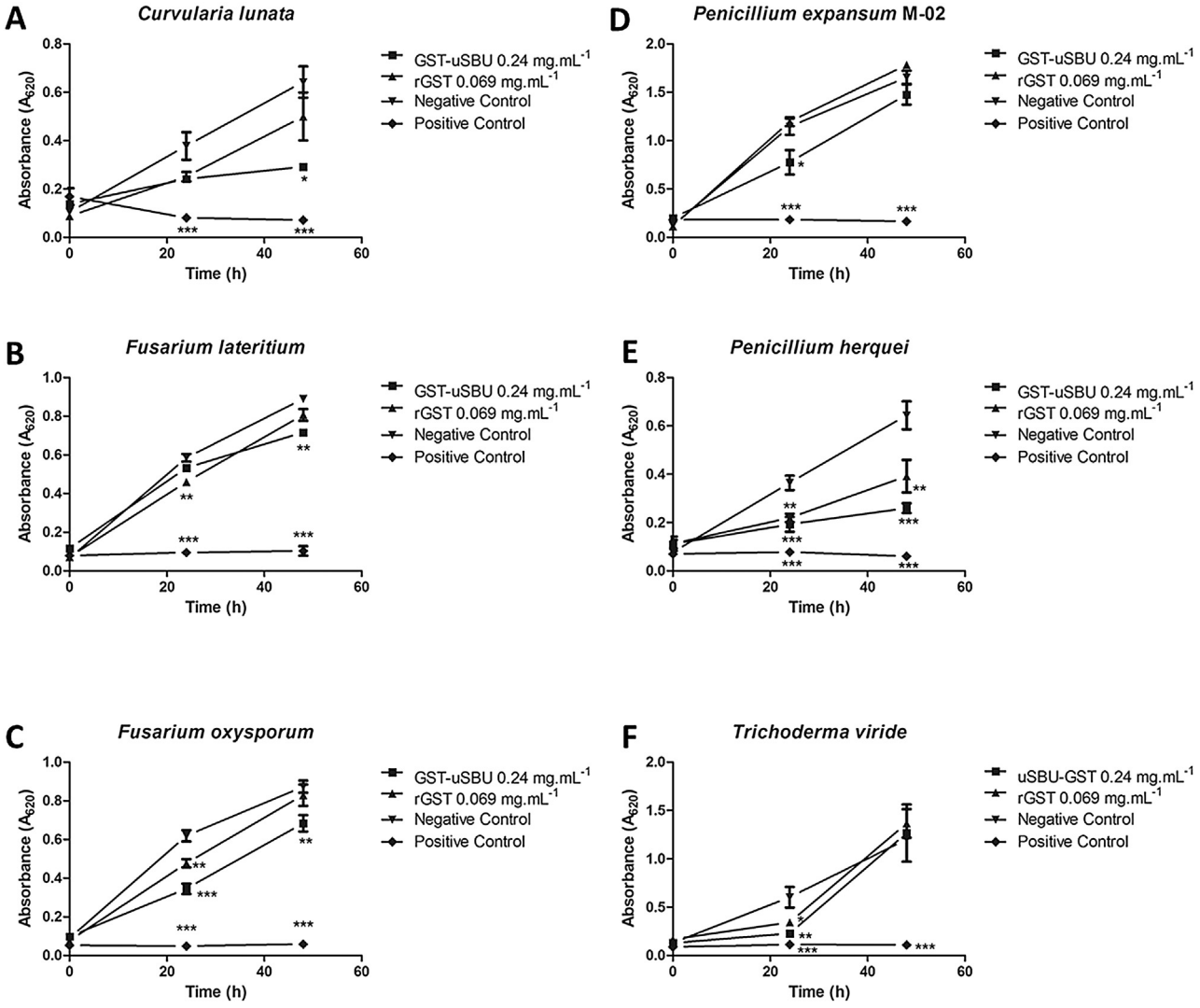

Fig. 3. Effect of GST-uSBU on fungal growth. Spores (10^6 spores mL^{-1}) were incubated on 96-wells plates containing Potato Dextrose Broth, with 0.24 mg mL^{-1} GST-uSBU; 0.069 mg mL^{-1} rGST (negative control) or 0.1% hydrogen peroxide (positive control). The incubation was performed for 48 h, at 28 °C and fungal growth was estimated at 24 h intervals (absorbance at 620 nm (A_{620nm}). N = 3, $^*p \leq 0.050$, $^{**}p \leq 0.020$, $^{***}p \leq 0.0001$.

The ANOVA of the regression model gave an F value of 4.13, higher than the tabulated F value 3.23, for a significance level of 95%. Therefore, the model was considered significant.

3.3. Western blot and mass spectrometry analyses

In order to ascertain the identity of the optimized protein and analyze the lower molecular mass proteins eluted from the affin-

ity column, we performed Western Blot analyses (Fig. 1B). Smaller mass bands reacted with both anti-Jaburetox 2Ec and anti-GST antibodies, thus confirming they are uSBU fragments (products of incomplete translation).

Additional confirmation of the fused protein identity was obtained by mass spectrometry of the major band (~120 kDa band) seen in the gel. The data showed 20% of coverage of soybean ubiquitous urease sequence (Fig. S2).

3.4. Biological characterization of GST-uSBU

3.4.1. Urease activity

The fusion protein GST-uSBU is not competent for urea hydrolysis. As expected, no enzymatic activity of purified GST-uSBU was detected using the Phenol – Hypochlorite method. Accordingly, no change in coloration of the urea segregation agar was seen after a 24 h culture of GST-uSBU expressing *E. coli*. Such lack of enzymatic activity reflects the fact that only the structural gene was cloned in the pGEX-4T-2-*GST-uSBU* construct *i.e.* genes for the accessory proteins were not included in this construct.

3.4.2. Antifungal activity

Effect of GST-uSBU on the fungal growth was evaluated. *C. lunata*, *F. lateritium*, *F. oxysporum*, *P. expansum*, *P. herquei,* and *T. viride* were susceptible to the fusion protein at 0.24 mg mL^{-1} (Fig. 3). rGST alone also showed some antifungal effect after short incubation times but much less pronounced than that observed for the fusion protein. *P. herquei* was one of the most sensitive to GST-uSBU antifungal effect, also affecting mycelial growth and fungal viability (Fig. S3). Besides the inhibition, changes in pigment production by the fungi *P. herquei* and *F. oxysporum* were also observed (Fig. 4). Usual production by *P. herquei* of high amounts of red pigments was inhibited in the presence of GST-uSBU. On the other hand, *F. oxysporum* was induced to produce purple pigments.

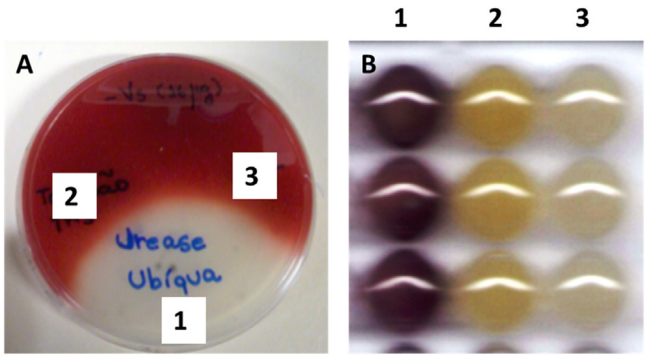

Fig. 4. Effect of GST-uSBU on the pigmentation of (A) *Penicillium herquei* and (B) *Fusarium oxysporum*. (1) GST-uSBU; (2) Negative Control (Tris-HCl buffer 10 mM, pH 7.0); (3) rGST. (A is a Petri dish, B is the bottom-up view of a microplate).

The inhibition of red pigment production was also observed for *P. herquei* treated with eSBU purified from seeds [10]. No changes in pigmentation were seen for the negative control or rGST-treated fungi.

The effect of GST-uSBU on yeast growth was also evaluated. Susceptible yeasts were *Candida albicans* and *C. tropicalis* (Fig. 5). In order to eliminate the possibility that GST is acting as an enhancer of antifungal activity during the fusion with uSBU, a second control was used. A hybrid GST-Enolase was tested against *C. albicans* and *C. tropicalis*. These experiments showed that enolase fused to GST do not present antifungal activity against these two yeasts, reinforcing our results that the main antifungal protein was uSBU and not rGST (Fig. S4).

Fig. 6 illustrates macroscopic changes in the phenotype of *C. albicans* after exposition to the fusion protein. According to [42], the regular extreme-jagged shaped colonies observed for GST-uSBU-

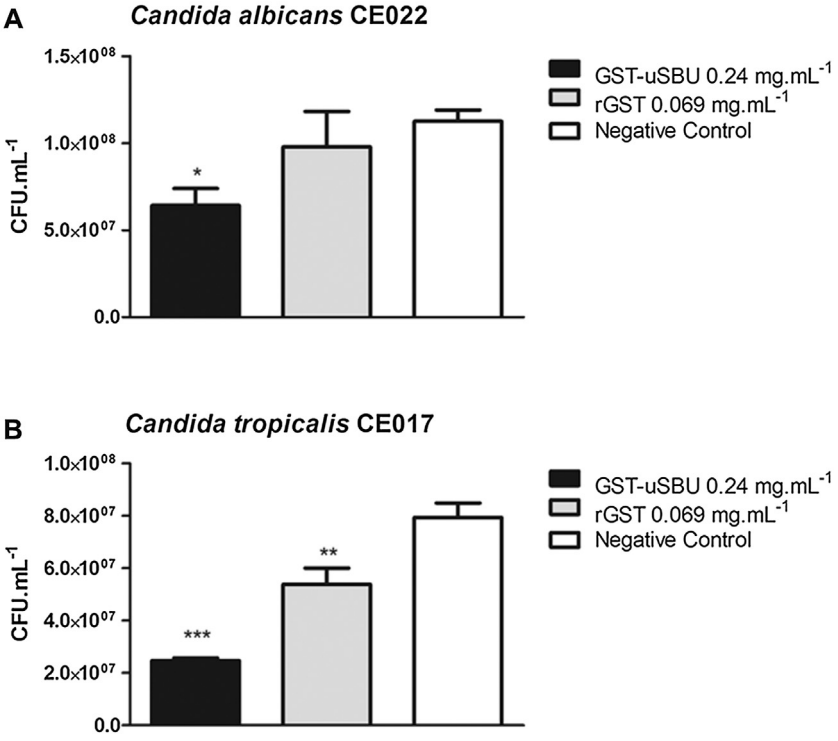

Fig. 5. Colony Forming Unit (CFU) assay of yeasts. 10^4 cells mL^{-1} were incubated on U-shaped 96-wells plates containing Sabouraud Broth with: 0.24 mg mL^{-1} GST-uSBU, 2 0.069 mg mL^{-1} rGST and Tris-HCl buffer 10 mM, pH 7.0 (negative control). The incubation was performed for 24 h, at 28 °C and after that, the CFU was determined. N = 3, $^{*}p \leq 0.0475$, $^{**}p \leq 0.0121$, $^{***}p \leq 0.0001$.

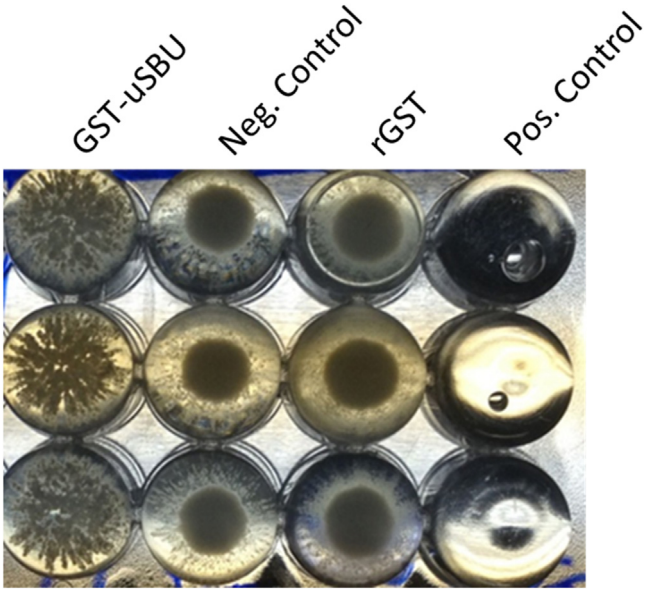

Fig. 6. Changes in phenotype of GST-uSBU treated *C. albicans* after 24 h incubation with 0.24 mg mL^{-1} GST-uSBU. This change was not observed in: Negative Control, Tris-HCl buffer 10 mM, pH 7.0), 0.069 mg mL^{-1} rGST and Positive Control (H_2O_2). Bottom-up view of a microplate, typical results are shown.

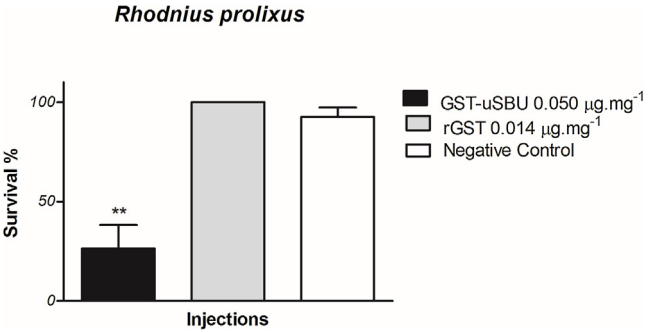

Fig. 7. Insecticidal effect of GST-uSBU on fifth instar *R. prolixus* 96 h after injections into the hemocoel. No lethal effect was observed in insects injected with rGST. A group of insects was injected with *R. prolixus* saline as negative control. N = 5 per group, $^{**}p \leq 0.0014$.

treated *C. albicans* are indicative of a nearly pure culture of pseudohyphae yeasts.

In order to investigate whether GST truncated proteins observed in SDS-PAGE (Fig. 1B) could also be active against fungi, samples were ultrafiltrated in a 50 kDa cutoff device. Only the fraction with molecules of >50 kDa retained antifungal effect against *C. tropicalis*, demonstrating that fragments smaller than 50 kDa are not active (Fig. S5).

3.4.3. Antibacterial activity

No antibacterial activity of GST-uSBU against *P. aeruginosa*, *E. coli*, and *S. aureus* was observed at dose of 0.24 mg mL^{-1}. On the other hand, *B. cereus* was susceptible to GST-uSBU and also to rGST. This fact suggests that the antibacterial molecule, in this case, is rGST and not uSBU (Fig. S6).

3.4.4. Insecticidal activity

A survival rate of only 26% was obtained for *R. prolixus* 96 h after injection of a dose of 0.05 µg GST-uSBU per mg of insect body weight (Fig. 7). rGST (0.014 µg mg^{-1}) showed no deterrent effect when injected into the insects. This result indicates that GST-uSBU shares the entomotoxic properties observed for eSBU and jack bean ureases [8].

3.4.5. Hemocytes and rabbit platelet aggregation

The presence of aggregated hemocytes in *R. prolixus* hemolymph *in vivo* was evaluated 6 h after hemocoel injection of 0.12 µg mg^{-1} of GST-uSBU, 0.034 µg mg^{-1} rGST or saline. Aggregates were observed only in the hemolymph of GST-uSBU-treated insects (more than 200 aggregates per µL of hemolymph) (Fig. 8A), indicating that the fusion protein displays this same entomotoxic effect as described for JBU [36]. Again, rGST was inactive. Similarly, the *in vitro* experiments showed that only GST-uSBU induced significant hemocyte aggregation when compared to rGST alone or saline (Fig. 8B–C).

Rabbit platelets were also activated by GST-uSBU in the concentrations of 0.033 mg mL^{-1} and 0.096 mg mL^{-1} (Fig. 9A). rGST at 0.027 mg mL^{-1} did not cause platelet aggregation. FITC-labeled GST-uSBU was used to visualize platelet aggregates induced by the fusion protein (Fig. 9B). Residual erythrocytes (indicated by an arrow in the bright field (Fig. 9B1) present in platelet-rich plasma preparation were not fluorescent in the dark field (Fig. 9B2), indicating that GST-uSBU interacted only with the platelets.

3.4.6. GST-uSBU modelling

Molecular modeling of GST-uSBU in dimeric and hexameric states are shown in Fig. 10A and B, highlighting putative binding poses for these macromolecular complexes. The dimerization of GST, as observed in its crystallographic form, could be a driving force for oligomerization of the GST-uSBU fused protein. This dimerization could even act crosslinking different urease oligomers (monomers, trimers or hexamers), forming larger protein networks.

4. Discussion

Here we have optimized production and performed biological characterization of the fusion protein GST-uSBU. The fusion protein is not enzymatically active and this was an advantage that allowed us to avoid using the generally toxic urease inhibitors. rGST was employed as a control in all assays, to ensure that the biological effects seen were indeed restricted to uSBU.

It has been previously reported that fusion of a protein to a large affinity tag, such as GST, can be advantageous in terms of increased expression, enhanced solubility (the hydrophilic surface of GST makes the fusion protein soluble), protection from proteolysis, improved folding, and stability of recombinant proteins in the soluble form, besides enabling protein purification *via* affinity chromatography [43–45]. We have attempted to cleave off GST from the fused protein by using thrombin, which resulted in an incomplete cleavage of GST-uSBU (data not shown). This could be due to steric hindrance caused by GST blocking the thrombin cleavage site. In addition, short-term precipitation of the cleaved protein was observed. As depicted in Fig. 10, molecular models of GST-uSBU in dimeric or hexameric states suggest that the GST moiety could preclude the access of thrombin to its cleavage site on the fused protein.

In the literature, works using proteins fused to GST were reported. It is important to highlight that these fused proteins maintained their biological activities. Spiezia and co-workers [46] expressed conotoxin V2, from *Conus ventricosus*, fused to GST and this protein showed high insecticidal activity at a dose of 100 pM per body weight of *Galleria mellonella* larvae. The authors reported that due to high hydrophobicity of conotoxin, it was not possible to cleave the GST tag, so the fused protein was used in the biological assays. Della-Casa and collaborators [47] reported the isolation of a disintegrin from *Bothrops insularis* venom, called Insularin. In this

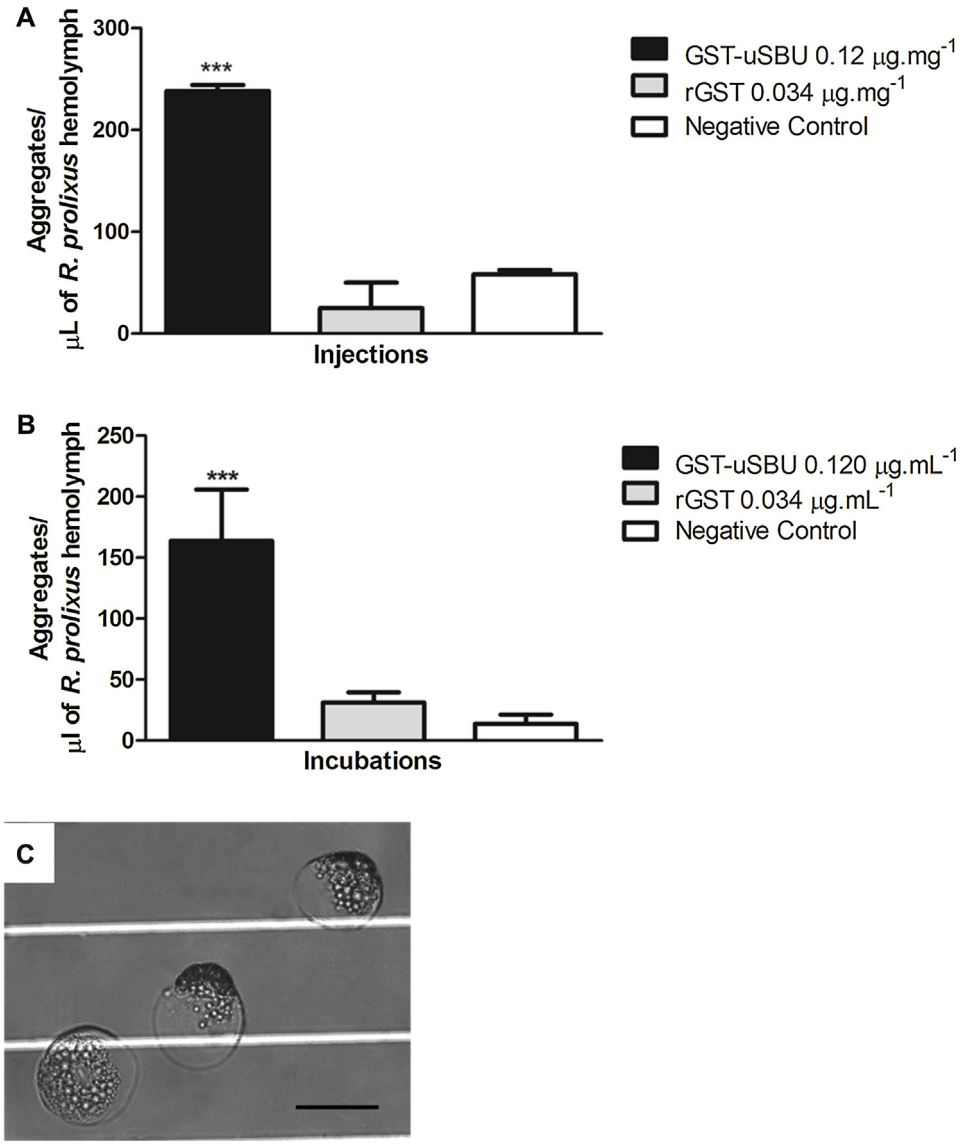

Fig. 8. Hemocyte aggregation induced by GST-uSBU. (A) For *in vivo* experiments *R. prolixus* were injected with GST-uSBU, rGST (0.12 μg mL^{-1} or 0.034 μg mL^{-1}, respectively) or saline as negative control. After 6 h, the hemolymph was collected and the aggregates were counted using a Neubauer chamber. N = 3, ***$p \leq 0.0001$. (B) For *in vitro* experiments, the hemolymph was collected and incubated with GST-uSBU, rGST or saline alone for 1 h at room temperature and the aggregates counted as above. N = 4, ***$p \leq 0.0001$. (C) Bright field image of representative aggregates induced by GST-uSBU *in vitro*. The bar correspond to 50 μm.

work the expression of recombinant Insularin fused to GST was also carried out. Both native and recombinant protein inhibited platelet aggregation induced by ADP and also caused inhibition of endothelial cell adhesion. Similarly to what we observed in our work, the removal of the GST tag was followed by precipitation of Insularin.

The GST tag helped to maintain the fusion protein GST-uSBU soluble and stable for at least 14 days at 4 °C. Even so, GST-uSBU had a tendency to aggregate upon more prolonged storage (data not shown), as also noted for native JBU [48] and recombinant JBURE-IIb [49]. Addition of EDTA and β-mercaptoethanol to the storage buffer decreased the rate of precipitation. Hence, the buffer exchange was performed one day prior to the biological assays.

The optimization of recombinant protein expression is often performed using a trial-and-error approach, with different expression variables being tested independently from each other. Therefore, variable interactions are lost, which makes the trial-and-error approach time-consuming [50]. Here, we performed the optimization using the variables temperature, IPTG concentration, and time. The best conditions of GST-uSBU production were defined as 24 °C, 0.55 mM IPTG and 14 h, yielding more than 5 mg of the fusion protein per liter of culture. Production of the recombinant protein PsaA from *Streptococcus pneumoniae* serotype 14 was optimized using the same variables chosen here. Low temperature (25 °C) and low IPTG concentration (0.1 mM) also favored the expression of PsaA, as well as long periods of cultivation (16 h) [51]. According to [50], high temperature promotes cell growth, but is detrimental to heterologous protein expression, because a higher growth rate could lead to a higher probability of plasmid loss. Hence, some proteins greatly benefit from a slower, longer induction period, which generally requires low temperature [50]. Besides, use of low temperature and low IPTG concentration optimizes costs of the bioprocess, making it more eco-friendly.

GST-uSBU showed all the biological properties previously reported by our group for plant ureases, such as antifungal and insecticidal effects, and aggregation-inducing activity upon insect hemocytes and rabbit platelets. The filamentous fungi *C. lunata, F. oxysporum, P. herquei*, and *T. viride* were susceptible to native eSBU (0.15 mg mL^{-1}) purified from soybean seeds [22] as well as

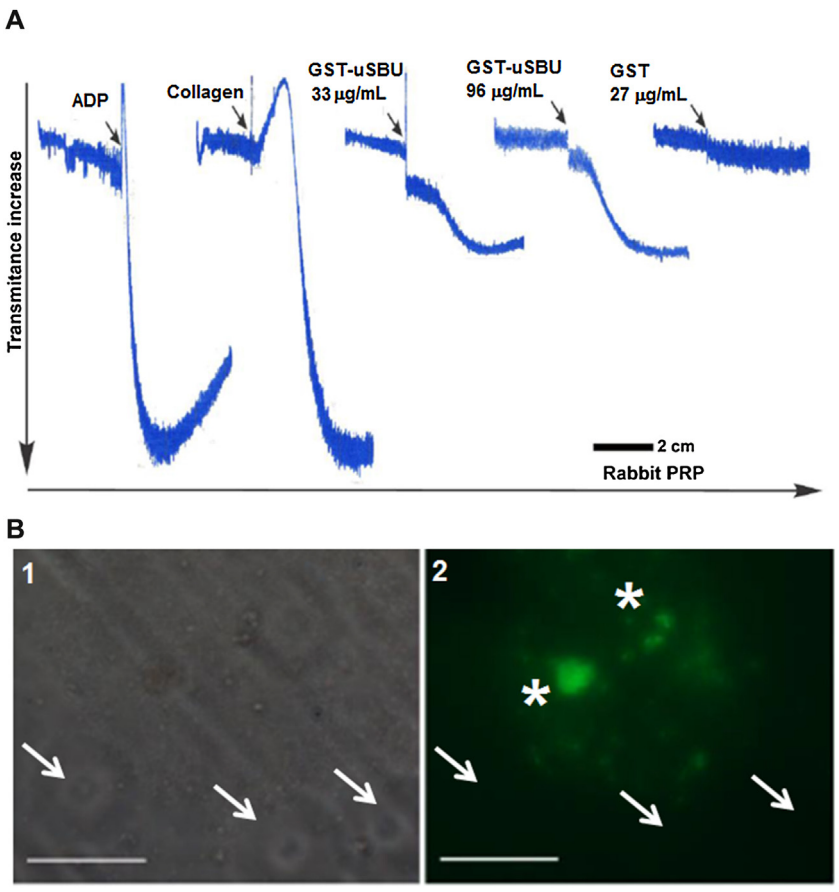

Fig. 9. GST-uSBU aggregates rabbit platelets. (A) Rabbit platelet aggregation promoted by 27 µg mL^{-1} and 96 µg.mL^{-1} GST-uSBU. Positive controls with ADP (20 µM) and Collagen (20 µg. mL^{-1}) are also shown. The negative control was 27 µg mL^{-1} rGST. (B) Panels demonstrating the formation of platelet aggregates induced by FITC-labeled GST-uSBU. B1 is the dark field and B2, the bright field. Arrows indicate erythrocytes and stars indicate platelet aggregates. Bars correspond to 20 µm.

Table 3
Comparative table of plant urease doses used in biological assays.

Plant Ureases							
	Canavalia ensiformis			*Glycine max*		*Gossypium hirsutum*	*Cajanus cajan*
Biological assays	JBU	CNTX	rJBURE-IIB	eSBU	rGST-uSBU	GHU	PPU
Antifungal activity							
Filamentous fungi	0.31 mg mL^{-1} [22]	1 mg [61]	0.15 mg mL^{-1} [49]	0.15 mg mL^{-1} [22]	0.24 mg mL^{-1}	10 µg [62]	10 µg [53]
Yeasts	0.10 mg mL^{-1} [32]	n.t.	n.t.	n.t.	0.24 mg mL^{-1}	n.t.	n.t.
Insecticidal activity							
R. prolixus injection	0.25 µg.mg^{-1} [60]	n.t.	n.t.	n.t.	0.05 µg.mg^{-1}	n.t.	n.t.
Aggregation							
Rabbit platelets	15.8 µg mL^{-1} [21]	54 µg mL^{-1} [63]	n.t.	22.2 µg mL^{-1} [21]	33.0 µg mL^{-1}	n.t.	n.t.
R. prolixus hemocytes	0.12 µg mg^{-1} [36]	n.t.	n.t.	n.t.	0.12 µg mg^{-1}	n.t.	n.t.

n.t. not tested, JBU: Jackbean urease, CNTX: Canatoxin, rJBURE-IIB: recombinant JBURE-IIB, eSBU: embryo-specific soybean urease, rGST-uSBU: recombinant GST-ubiquitous soybean urease, GHU: *Gossypium hirsutum* urease, PPU: Pigeon pea urease.

to GST-uSBU (0.24 mg mL^{-1}) as described in Table 3. This similar spectrum of inhibition agrees with the high amino acid identity between the two isoforms [18]. The oomycete *Pythium oligandrum* was not susceptible to GST-uSBU, nor was the agaricomycete *Rhizoctonia solani* (data not shown), similar to the lack of effect of JBU as reported by [32]. Under-expression of the ubiquitous form in silenced transgenic plants were tested against the pathogen biotrophic *P. pachyrhizi*, using detached soybean leaves, revealing that these plants were more susceptible to this pathogen [11]. Recently, Tezotto and co-workers [52] reported a study using paired near-isogenic lines (NILs) differing in the presence (Eu3) and absence (eu3-a) of the UreG protein. Both embryo specific and ubiquitous urease were synthesized, but inactive in the UreG null (eu3-a). A second paired NIL was employed in which one line (eu-1a) lacked the embryo-specific urease protein, with the ubiquitous intact. When comparing these paired NILs there was no difference

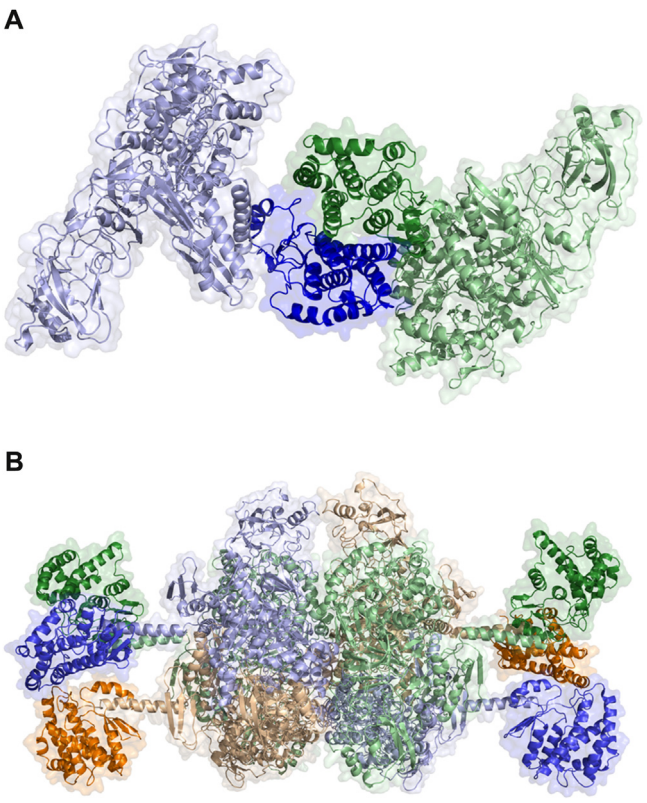

Fig. 10. Molecular models of GST-uSBU. (A) Molecular model of a putative dimer of GST-uSBU with monomers shown in blue (left) and green (right). Interacting GSTs (protruding from the N-terminal of the urease molecules) are shown in brighter colors than their urease counterparts. (B) Putative hexamer of GST-uSBU, built based on symmetry data from the *C. ensiformis* urease crystal. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

in susceptibility to *R. solani* within each NIL. Differences between NILs were ascribed to different genetic backgrounds between each pair, but alterations of embryo-specific urease activity or protein had no effect on susceptibility to *R. solani* within each NIL.

This genetic approach would profitably be repeated by examining sensitivity to *R. solani* in NIL lines containing or lacking the ubiquitous urease.

Cell walls of oomycetes are mainly composed of β-1,3-glucan polymers and cellulose, and unlike fungal cell walls, they contain little chitin [53]. These results suggest that the presence of chitin could be somehow related to the antifungal mode of action of ureases. Cellulolytic activity has been reported for a purified urease from pigeon pea (*Cajanus cajan*), but no chitinase activity was detected [54]. Thus, if there is any interaction between chitin and plant ureases, this interaction apparently does not cause the hydrolysis of chitin.

The fusion protein affected the secondary metabolism of filamentous fungi, increasing or decreasing the production of pigments in *F. oxysporum* and *P. herquei*, respectively. The pigments produced by *F. oxysporum*, characterized as naphthoquinones and anthraquinones [55,56], are active against bacteria, yeast, fungi, protozoa (*Leishmania brasiliensis*), and insect (*Calliphora erythrocephala*). Fungal naphthoquinones are synthesized under conditions of inhibition or total cessation of fungal growth [57]. Therefore, the increased pigmentation of *F. oxysporum* could be a defense mechanism of the fungus against the toxic GST-uSBU. On the other hand, the pigments produced by *P. herquei* belong to the class of phenalenones, which show diverse biological activities, including antimicrobial, anticancer and cytotoxic activities [58]. We hypothesize that due to the GST-uSBU interference on *P. herquei* pigment production, the fungus becomes more susceptible to the fusion protein. In fact, *P. herquei* is the fungus with most susceptibility to the effects of eSBU and JBU identified so far [22].

The antifungal activity of eSBU against yeasts has not been previously reported. On the other hand, JBU and Jaburetox were active in similar concentration ranges observed here for GST-uSBU, towards the same *Candida* species, and also against *K. marxiannus*, *P. membranifasciens,* and *S. cerevisiae* [32]. Formation of pseudo-hyphae was observed after treatment of *C. albicans*, *C. tropicalis,* and *P. membranifasciens* with the fusion protein. Pseudohyphal growth allows yeasts to escape from unfavorable growth conditions and to penetrate natural barriers. The elongated morphology provides more surface area, in comparison with oval shape, and allows pseudohyphae a more efficient absorption of nutrients [59]. Inhibition of β-(1,3)-glucan synthase, the mechanism of antifungal action of echinocandins, results in cytological and ultrastructural changes in fungi, characterized by growth as pseudo hyphae, thickened cell wall and buds failing to separate from mother cells [59]. The pseudo hyphae shape observed for GST-uSBU-treated yeasts could indicate an effect of uSBU directly on yeast cell wall and/or cell membrane, or a defense mechanism of the fungi against the fungitoxic protein, by assuming a more infective shape. More studies are necessary at this point to clarify the fungitoxic mode of action of ureases.

Jaburetox, a recombinant peptide consisting of a continuous, 92 amino acid segment derived from JBU, shows antifungal properties, but it is less active in comparison with the entire protein [32]. Here, no antifungal activity was detected for the GST-uSBU truncated fragments with masses ≤50 kDa, suggesting that uSBU's antifungal domain(s) is(are) not located in its first N-terminal ~25–30 kDa moiety.

Under the conditions described here, GST-uSBU did not affect *P. aeruginosa*, *E. coli* or *S. aureus* growth. Preliminary data with native eSBU purified from soybean seeds also indicated no effect on *Ralstonia solanacearum, Xanthomonas campestris* and *Xanthomonas axonopodis* (results not shown). Thus, it seems that ureases are devoid of bactericidal properties.

Regarding the insecticidal activity of soybean ureases, there are reports of feeding trials with eSBU in *Dysdercus peruvianus*. The insects were fed with diets containing 0.02-0.1% (w/w) of eSBU and an LD_{50} of 0.052% was determined [21]. Using *R. prolixus* as insect model, JBU was reported to cause 96% mortality after hemocoel injection into 5th instar at 0.25 μg mg^{-1} body weight [59] and 75% mortality at a 0.06 μg mg^{-1} dose (Defferrari, M.S., personal communication). Here we reported that hemocoel injection into *R. prolixus* of GST-uSBU at a 0.05 μg mg^{-1} dose also produced about 25% survival after 96 h. In addition, GST-uSBU induced aggregation of *R. prolixus* hemocytes, suggesting deterrent effect on the insect's immune system. Defferrari and co-workers reported for JBU, at the same dose, the presence of more than 400 aggregates per μL of *R. prolixus* hemolymph [36].

Aggregation of rabbit platelets has been described as a general property of ureases regardless of their quaternary organization, with reports of this property for the jack bean and soybean (eSBU) ureases (monomer), GST-uSBU (this paper), *Helicobacter pylori* (dimer) and *Bacillus pasteurii* (trimer) ureases [21,37]. Additionally, as previously reported for *H. pylori* urease [37], GST-uSBU was observed to bind only to platelets, not to erythrocytes. Aggregation of rabbit platelets induced by eSBU was reported by Follmer and co-workers, with an EC_{50} of 40 nM (22.2 μg mL^{1}) [21]. Since the level of identity between eSBU and uSBU is greater than that shared by plant and bacterial ureases, the less potent aggregating effect observed here for GST-uSBU could be due to some steric hindrance imposed by the GST moiety or to an 'incomplete' folding of uSBU, which could have affected its "platelet-aggregating domain".

Hence, all the biological properties of plant ureases screened for GST-uSBU are displayed by uSBU, even when fused to GST. The fusion of GST to the N-terminal amino acid of uSBU did not abolish or "conceal" the biologically active domains responsible for these properties.

5. Conclusions

Our results indicate GST-uSBU as an operational, usable model to study plant ureases. The fusion protein showed the same properties displayed by the native ureases, eSBU and JBU, isolated from seeds, with the advantages of high yield production and one-step purification protocol. Moreover, it was possible to explore more deeply the effects caused by ureases on fungi. GST-uSBU was capable to interfere in the secondary metabolism of filamentous fungi and caused morphology changes in yeasts. Altogether, these results further support the hypothesis of the role of ureases in plant defense.

Authorís contribution

AHSM cloned, expressed and characterized the fusion protein, performed the insecticidal assay and wrote the manuscript; FCL performed RSM and fusion protein characterization, antifungal and antibacterial assay and wrote the manuscript; VB performed rabbit platelet aggregation and fusion protein characterization; MSD performed *in vivo* hemocyte aggregation; LLF and NRM performed *in vitro* hemocyte aggregation; KK and DMT produced and purified fusion protein and helped in antifungal assays; RLB conducted molecular modeling; DRD performed Mass Spectrometry experiment; MP performed yeast permeabilization assay; MMS and ABBR contributed to the characterization of fusion protein; CRC and GP contributed significantly to the manuscript preparation, conceived and supervised all the work.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.procbio.2016.12.003.

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