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Antifungal properties of *Canavalia ensiformis* urease and derived peptides

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ABSTRACT

Ureases (EC 3.5.1.5) are metalloenzymes that hydrolyze urea into ammonia and CO_2. These proteins have insecticidal and fungicidal effects not related to their enzymatic activity. The insecticidal activity of urease is mostly dependent on the release of internal peptides after hydrolysis by insect digestive cathepsins. Jaburetox is a recombinant version of one of these peptides, expressed in *Escherichia coli*. The antifungal activity of ureases in filamentous fungi occurs at submicromolar doses, with damage to the cell membranes. Here we evaluated the toxic effect of *Canavalia ensiformis* urease (JBU) on different yeast species and carried out studies aiming to identify antifungal domain(s) of JBU. Data showed that toxicity of JBU varied according to the genus and species of yeasts, causing inhibition of proliferation, induction of morphological alterations with formation of pseudohyphae, changes in the transport of H^+ and carbohydrate metabolism, and permeabilization of membranes, which eventually lead to cell death. Hydrolysis of JBU with papain resulted in fungitoxic peptides (~10 kDa), which analyzed by mass spectrometry, revealed the presence of a fragment containing the N-terminal sequence of the entomotoxic peptide Jaburetox. Tests with Jaburetox on yeasts and filamentous fungi indicated a fungitoxic activity similar to ureases. Plant ureases, such as JBU, and its derived peptides, may represent a new alternative to control medically important mycoses as well as phytopathogenic fungi, especially considering their potent activity in the range of 10^{-6}–10^{-7} M.

1. Introduction

Ureases (EC 3.5.1.5) are nickel-dependent enzymes that catalyze urea hydrolysis into ammonia and carbon dioxide, and are synthesized by plants, fungi and bacteria [13,20]. Urease of jackbean (*Canavalia ensiformis*) seeds was the first enzyme ever to be crystallized [41], consisting of a hexamer of a single chain of 840 amino acid residues, with a molecular mass of 97 kDa [16,20,38]. It has been postulated that in plants these proteins contribute to the bioavailability of nitrogen and participate in defense mechanisms [12,16].

C. ensiformis produces several urease isoforms: the more abundant jackbean urease (JBURE-I), and two less abundant proteins, canatoxin (CNTX) [17] and JBURE-IIB [26]. CNTX-like proteins and urease accumulate in the mature seed, consistent with the proposed defense role associated with both insecticidal [40] and fungicidal properties [7,26].

Insecticidal activity of Jackbean urease depends mostly on the release of an entomotoxic peptide formed by proteolytic enzymes upon ingestion by the insect [15]. This peptide, Pepcanatox, was characterized and based on its sequence, a recombinant peptide named Jaburetox-2EC was produced using the corresponding sequence of the urease isoform JBURE-II as template [27]. This peptide has 93 amino acids and its toxicity to several insects, including some species that were not affected by the native urease, has been demonstrated [40].

CNTX was the first urease shown to inhibit the radial growth of several filamentous fungi [29]. In 2007, Becker-Ritt et al. [7] reported the fungicidal activity of the embryo specific urease from *Glycine max* (soybean), the major urease from *C. ensiformis* and of a bacterial urease from *Helicobacter pylori*, regardless of their ureolytic activity, toward different phytopathogenic fungi. Urease from other sources also display fungicidal activity, such as the cotton (*Gossypium hirsutum*) seed urease [23] and the

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recombinant JBURE-IIb apourease from *C. ensiformis* [26]. Recently, it was demonstrated that soybean plants lacking urease due to gene silencing were more susceptible to fungal infection, reinforcing the hypothesis of participation of these proteins in plant defense [44]. The fungitoxic activity of ureases occurs at submicromolar doses, making these proteins 2–3 orders of magnitude more potent than any other known antifungal proteins of plant origin, producing injuries to the cell wall and/or cell membrane and plasmolysis [6,7].

Infectious diseases, mainly candidiasis and aspergillosis, caused by yeasts and filamentous fungi are a serious problem worldwide, especially in tropical and subtropical countries where the number of immunosuppressed patients (who often develop these diseases), has increased over the last decade. The drugs available for treating these mycoses have low efficiency, low solubility and high toxicity, causing severe collateral effects. Besides these problems, the emergence of strains resistant to current therapeutic agents makes essential and urgent the identification of new antifungal compounds [35]. Despite numerous reports on the occurrence and activity of proteins and antimicrobial peptides originated from plant, some have already been successfully tested as transgenes to confer resistance to plants against fungi and/or insects [6], only a few have been evaluated for therapeutic potential in human mycoses [3]. The search for new antifungal compounds from plants became extremely urgent considering the spread of invasive mycoses, particularly in immunocompromised patients, caused by pathogenic fungi or in plants by soil fungi (e.g., *Alternaria*, *Curvularia* and *Rhizopus*), before considered as fungi of low virulence, and which are currently being considered as emerging pathogens [14].

Plants are an excellent source of compounds having antifungal activity, since they are continuously exposed to a broad range of phytopathogenic fungi in the environment. Plant antifungal peptides include defensins, lipid transport proteins, chitinases, lectins, thionins, cyclopeptide alkaloids and other less common types [6,14,28].

In this work we describe the toxic activity of JBU and of Jaburetox in pathogenic yeast. Studies on the mechanisms of their antifungal action have shown interference on energy metabolism and proton transport, morphological changes and permeabilization of the fungal membrane. Fungitoxic urease-derived peptides were obtained by enzymatic hydrolysis and provided clues to the location of antifungal domain(s) of the protein.

2. Materials and methods

2.1. C. ensiformis urease (JBU)

Urease type C-III from Jack bean (Sigma Aldrich) was used in all experiments. The protein (hexameric form, Mr 540 kDa) was solubilized in 50 mM Tris buffer, pH 7.0, and quantified by absorbance at 280 nm (0.604 A280 was considered equivalent to a 1.0 mg/mL protein solution). Enzyme-inactivated JBU was obtained by treating the protein with the active site inhibitor p-hydroxy-mercurybenzoate (Sigma Aldrich) as described in [17]. Excess of the inhibitor was removed by extensive dialysis against Tris buffer.

2.2. PCR amplification and cloning of Jaburetox

Jaburetox-2Ec, the recombinant peptide obtained by Mulinari et al., 2007 [27], contained 93 urease-derived amino acids, plus a V5 antigen and a C-terminal His-tag. The vector pET 101-D-TOPO containing Jaburetox-2Ec coding sequence was used as template in a polymerase chain reaction. In order to obtain a recombinant peptide containing the His-tag and lacking the V5 antigen, a set of primers were designed, the cDNA was amplified by PCR, cloned into pET 23-a vector and expressed in BL21-CodonPlus (DE3)-RIL (Stratagene). This new peptide was called Jaburetox.

The forward primer sequence was Jaburetox 5′ CCAACATATGGGTCCAGTTAA TGAAGCCAAT 3′ (the underline shows the NdeI site) and the reverse primer sequence was Jaburetox 5′ CCCCCTCGAGTATAACTTTTCCACCTCCAAAAACA 3′ (the underline shows the XhoI site). The PCR reaction was carried out in the following conditions: denaturation at 95 °C for 3 min, annealing at 55 °C for 30 s and elongation at 72 °C for 2 min. A total of 35 cycles were used and the final product was then digested with NdeI (Fermentas, Eugene, OR, USA) and XhoI (Fermentas, Eugene, OR, USA), dephosphorylated with thermosensitive alkaline phosphatase (Promega, Madison, WI, USA). The plasmid pET 23a::Jaburetox was sequenced using a ABI PRISM 3100 automated sequencer (Applied Biosystems, Foster city, CA).

2.3. Expression and purification of recombinant Jaburetox

For isolation and purification of Jaburetox, 200 mL of Luria broth medium containing 100 μg/mL ampicillin and 40 μg/mL chloramphenicol were inoculated with 2 mL of the overnight culture. The cells were grown 2 h at 37 °C under shaking (OD600 = 0.7) and then 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added. After 3 h, the cells were harvested by centrifugation and suspended in 10 mL of lysis buffer (50 mM tris buffer, pH 7.5, 500 mM NaCl, 5 mM imidazole), sonicated, centrifuged (14,000 × *g*, 30 min) and 10 μL of supernatant or 5 μL of the pellet sample were analyzed by SDS-PAGE. The supernatant was loaded onto a 2 mL Ni affinity column (Ni-NTA, QIAGEN, Hilden, Germany), which was previously equilibrated with the equilibration buffer (50 mM Tris buffer, pH 7.5, 500 mM NaCl, 5 mM imidazole). After 30 min, the column was washed with 20 mL of the same buffer, containing 50 mM imidazole. The recombinant peptide was eluted with the equilibration buffer containing 200 mM imidazole and quantified by the Bradford method [9]. The samples were dialyzed against the 50 mM phosphate buffer, pH 7.5, 1 mM EDTA, 5 mM β-mercaptoethanol. A molecular mass of 10,128.2 Da (ExPASY ProtParam tool) was considered for Jaburetox.

2.4. Fungi and yeast

The yeasts *Candida parapsilosis* (CE002), *Candida tropicalis* (CE017), *Candida albicans* (CE022), *Kluyveromyces marxiannus* (CE025), *Pichia membranifaciens* (CE015), and *Saccharomyces cerevisiae* (1038) and filamentous fungi *Colletotrichum lindemuthianum*, *Colletotrichum musae*, *Colletotrichum gloeoporioides*, *Fusarium laterithium*, *Fusarium solani*, *Fusarium oxysporum*, *Phomopsis* sp., *Mucor* sp., *Trichoderma viridae*, *Pythium oligandrum*, *Lasiodiplodia theomobrae*, *Cercospora chevalier* and *Rhizoctonia solani* were kindly provided by Dr. Valdirene Gomes from the Laboratory of Physiology and Biochemistry of Microorganisms, Center of Bioscience and Biotechnology, Universidade Estadual do Norte Fluminense, Campos dos Goytacazes, RJ, Brazil or by Dr. José Tadeu Abreu de Oliveira from the Department of Biochemistry, Universidade Federal of Ceará, Fortaleza, Ceará, Brazil. The yeasts were maintained on Sabouraud agar (1% peptone, 2% glucose and 1.7% agar). The fungi were maintained on potato agar (PDA) at 4 °C.

2.5. Hydrolysis of JBU

JBU was hydrolyzed using different commercial enzyme: trypsin (EC 3.4.21.4 – Sigma–Aldrich, St. Louis, MO, USA), chymotrypsin (EC 3.4.21.1 – Sigma–Aldrich, St. Louis, MO, USA) papain (Merck, Darmstadt, Alemanha), pepsin (EC 3.4.23.1 – Sigma, St. Louis, MO, USA). Different conditions of hydrolysis were tested, varying pH, incubation time and enzyme:substrate ratio. The reaction mixture after