Biochimica et Biophysica Acta xxx (2013) xxx–xxx

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen

Structure–function studies on jaburetox, a recombinant insecticidal and antifungal peptide derived from jack bean (*Canavalia ensiformis*) urease

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ARTICLE INFO

Article history:
Received 12 May 2013
Received in revised form 2 November 2013
Accepted 6 November 2013
Available online xxxx

Keywords:
Site-directed mutagenesis
β-hairpin
Urease-derived peptide
Molecular modeling
Membrane-disturbing
Insect

ABSTRACT

Background: Ureases are metalloenzymes involved in defense mechanisms in plants. The insecticidal activity of *Canavalia ensiformis* (jack bean) ureases relies partially on an internal 10 kDa peptide generated by enzymatic hydrolysis of the protein within susceptible insects. A recombinant version of this peptide, jaburetox, exhibits insecticidal, antifungal and membrane-disruptive properties. Molecular modeling of jaburetox revealed a prominent β-hairpin motif consistent with either neurotoxicity or pore formation.
Methods: Aiming to identify structural motifs involved in its effects, mutated versions of jaburetox were built: 1) a peptide lacking the β-hairpin motif (residues 61–74), JbtxΔ-β; 2) a peptide corresponding the N-terminal half (residues 1–44), Jbtx N-ter, and 3) a peptide corresponding the C-terminal half (residues 45–93), Jbtx C-ter.
Results: 1) JbtxΔ-β disrupts liposomes, and exhibited entomotoxic effects similar to the whole peptide, suggesting that the β-hairpin motif is not a determinant of these biological activities; 2) both Jbtx C-ter and Jbtx N-ter disrupted liposomes, the C-terminal peptide being the most active; and 3) while Jbtx N-ter persisted to be biologically active, Jbtx C-ter was less active when tested on different insect preparations. Molecular modeling and dynamics were applied to the urease-derived peptides to complement the structure–function analysis.
Major conclusions: The N-terminal portion of the Jbtx carries the most important entomotoxic domain which is fully active in the absence of the β-hairpin motif. Although the β-hairpin contributes to some extent, probably by interaction with insect membranes, it is not essential for the entomotoxic properties of Jbtx.
General significance: Jbtx represents a new type of insecticidal and membrane-active peptide.

1. Introduction

Ureases (EC 3.5.1.5, urea amidohydrolase), are nickel dependent enzymes that catalyze urea hydrolysis into ammonia and carbon dioxide. Evolutionarily conserved [1], these proteins have been isolated from a wide variety of organisms including plants, fungi and bacteria. In plants, ureases contribute to the bioavailability of nitrogen and in defense mechanisms [2,3]. Ureases represent an unexplored group of plant proteins with potential use for insect control [3,4] and as antifungal agents [5]. Studies have shown that ureases from *Canavalia ensiformis* (jack bean) and *Glycine max* (soybean) display insecticidal activity (reviewed in [6]) and antifungal properties, inhibiting growth and affecting membrane integrity of filamentous fungi [7] as well as of yeasts [8] in the 10^{-7} M range. The urease from pigeon pea (*Cajanus cajan*) was recently described to exhibit insecticidal and antifungal properties at similar dose ranges [9].

The molecular basis of the insecticidal mechanism of action of plant ureases is not yet completely understood [6]. It has been demonstrated that the entomotoxic effect of canatoxin [10], an isoform of *C. ensiformis* (jack bean) urease [11], is partially due to an internal 10 kDa peptide

Abbreviations: Jbtx, jaburetox; JbtxΔ-β, β-hairpin deleted version of Jbtx; Jbtx N-ter, N-terminal domain of Jbtx; Jbtx C-ter, C-terminal domain of Jbtx; Jbtx-2Ec, a version of Jbtx containing a V5 epitope; LUV, large unilamellar vesicle; MD, molecular dynamics; RMSD, root mean square deviation; CD, circular dichroism

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0304-4165/$ – see front matter © 2013 Published by Elsevier B.V.
http://dx.doi.org/10.1016/j.bbagen.2013.11.010

(pepcanatox), that is released from the protein upon hydrolysis by insect cathepsin-like digestive enzymes [12–16]. Jaburetox-2Ec (Jbtx-2Ec), a recombinant peptide analog to pepcanatox, exhibited a potent insecticidal effect on two economically important crop pests: *Spodoptera frugiperda* (fall armyworm) and *Dysdercus peruvianus* (cotton stainer bug) [17,18]. Jbtx-2Ec was also shown to both permeabilize large unilamellar liposomes (LUVs) [19] and to affect transmembrane potential of insect Malpighian tubules, causing inhibition of diuresis [20]. A β-hairpin motif in the modeled structure of Jbtx-2Ec has been proposed [17,19] and its presence has been confirmed in the crystallographic structures of jack bean [21] and pigeon pea [9] ureases. This motif is present also in one class of pore-forming peptides and neurotoxic peptides [22] such as charybdotoxin, which affect ion channels [23]. A variant form of Jbtx-2Ec lacking the fused V5-antigen, here called simply Jbtx, also exhibited antifungal activity [8].

Aiming to identify motifs possibly involved in the different biological activities of Jbtx, here we described the cloning and expression of mutated versions of the Jbtx-encoding cDNA. Truncated versions of the peptide, with deletions of the regions of the β-hairpin motif, the N-terminal or the C-terminal halves of the molecule, were tested on LUV permeabilization, for insecticidal and other entomotoxic effects. Structural analyses of the truncated peptides were also carried out.

2. Materials and methods

2.1. Jbtx cDNA constructs

Jaburetox-2Ec, the first version of the recombinant urease-derived peptide cloned in [17], harbored a V5-antigen with 18 amino acids derived from the pET101/D-TOPO plasmid. In order to eliminate this foreign sequence, the jack bean urease truncated cDNA encoding 93 amino acids, called simply jaburetox (Jbtx), was cloned and expressed in *Escherichia coli* via pET-23a vector (Novagen), as described in [8]. This sequence was used as template for site-directed mutagenesis and PCR amplifications of the mutant forms as described below.

2.2. Jbtx lacking the internal β-hairpin (JbtxΔ-β)

In order to delete the β-hairpin motif (residues 61–74) of the Jbtx peptide, site-directed mutagenesis was performed using the QuickChange Site-directed Mutagenesis Kit (Stratagene). As this method is often used to generate a few nucleotide deletions, some modifications in the primers' design were made, as described by [24]. Pairs of complementary primers were designed (Table 1), and site-directed mutagenesis was performed according to the kit manufacturer's instructions. The deleted gene version was confirmed by sequencing on an ABI Prism 3100 automated sequencer (Applied Biosystems) platform (ACTGene Ltd, Center of Biotechnology, UFRGS). Sequence comparisons were performed using the BLASTx software [25], available at (http://www.ncbi.nlm.nih.gov). The resulting peptide was called JbtxΔ-β.

Table 1
Primers used in this study.

Primer	Size	Sequence
5′ Del_β-hairpin	40-Mer	AGTATGGTCCGACTATTGGTGAAAAGGATTTTGCCCT TTA
3′ Del_ β-hairpin	40-Mer	TAAAGGGCAAAATCCTTTTCACCAATAGTCGGACCAT ACT
5′ Del_ α-helix	40-Mer	CTTTCACCAAAGCCATTCCTTATGGTCCGACTATTGGTGA
3′ Del_ α-helix	40-Mer	TCACCAATAGTCGGACCATAAGGAATGGCTTTGGTGA AAG
5′ N-terminal	25-Mer	CCAACATATGGGTCCAGTTAAATGA
3′ N-terminal	25-Mer	CCCCCTCGAGGGTGAAAGGACAATC
5′ C-terminal	25-Mer	CCAACATATGAAAGCCATTCCTCGT
3′ C-terminal	25-Mer	CCCCCTCGAGTATAACTTTTCCACC

2.3. Jbtx N-terminal (Jbtx N-ter) and C-terminal (Jbtx C-ter) domain versions

The Jbtx gene regions corresponding to the N-terminal (residues 1–44) and C-terminal (residues 45–93) halves of the peptide were amplified by PCR with specifically designed primers (Table 1) and products were cloned into pET23a (Novagen). PCRs were performed in a final volume of 50 μL containing 50 ng of the template plasmid DNA, 200 ng of each primer, 200 μM each dNTPs, 2.5 U *Pfu* taq DNA polymerase (Fermentas) and 1× *Pfu* reaction buffer. Amplification was carried out under 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. After a total of 35 cycles, the final products were digested with *Nde*I and *Xho*I (Fermentas), dephosphorylated with thermosensitive alkaline phosphatase (Promega) and ligated into the expression vector pET23a (Novagen). The inserts of the recombinant plasmids were fully sequenced in order to confirm their sequences essentially as described above. The resulting peptides were called Jbtx N-terminal (Jbtx N-ter) and Jbtx C-terminal (Jbtx C-ter). A schematic representation of all Jbtx-related peptides is shown in Fig. 1.

2.4. Expression and purification of Jbtx recombinant peptides

Recombinant pET23a plasmids were transformed into *E. coli* BL21-CodonPlus (DE3)-RIL cells (Stratagene) for Jbtx gene expressions following the provider's instructions. For the purification of the original Jbtx peptide and its mutated forms, 200 mL of Luria B Bertrand medium containing 100 μg/mL ampicillin and 40 μg/mL chloramphenicol were separately inoculated with 2 mL overnight cultures of each *E. coli* strain. Cells were grown for approximately 2 h at 37 °C under shaking until an optical density of 0.7 was reached. At this point, IPTG was added to cultures to a final concentration of 0.5 mM. After 3 h of additional culture, 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 and centrifuged (14,000 *g*, 30 min). The supernatant was loaded onto a Ni^{+2} loaded Chelating Sepharose (GE Healthcare) column, previously equilibrated with the lysis buffer. After 30 min, the column was washed with 50 mL of the same buffer containing 50 mM imidazole. Bound protein was eluted with 200 mM imidazole in the lysis buffer. Samples were then dialyzed against buffer A (50 mM phosphate buffer, pH 7.5, 1 mM EDTA, 5 mM β-mercaptoethanol) in order to remove the imidazole. Protein concentration was measured by Bradford assay [26]. Predicted molecular mass of the peptides was obtained by submitting the deduced sequences to the ProtScale tool [27] available at the Expasy site (http://web.expasy.org/protscale).

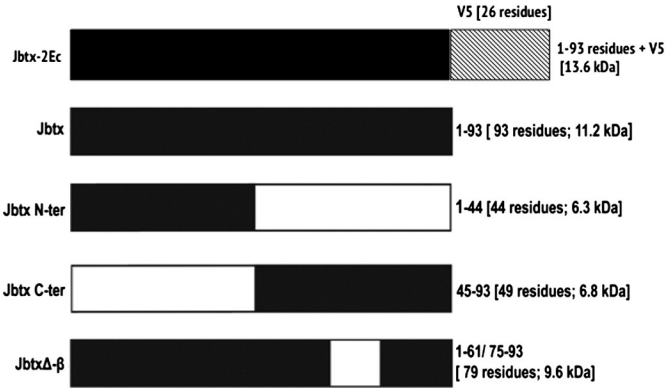

Fig. 1. Schematic representation of the sequences of jaburetox and mutants. The number of amino acid residues of each molecule (shown in black) is indicated on the right side.

Please cite this article as: A.H.S. Martinelli, et al., Structure–function studies on jaburetox, a recombinant insecticidal and antifungal peptide derived from jack bean (*Canavalia ensiformis*) urease, Biochim. Biophys. Acta (2013), http://dx.doi.org/10.1016/j.bbagen.2013.11.010