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Jaburetox-2Ec: An insecticidal peptide derived from an isoform of urease from the plant *Canavalia ensiformis*

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ABSTRACT

Canatoxin, a urease isoform from *Canavalia ensiformis* seeds, shows insecticidal activity against different insect species. Its toxicity relies on an internal 10 kDa peptide (pepcanatox), released by hydrolysis of Canatoxin by cathepsins in the digestive system of susceptible insects. In the present work, based on the N-terminal sequence of pepcanatox, we have designed primers to amplify by PCR a 270-bp fragment corresponding to pepcanatox using JBURE-II cDNA (one of the urease isoforms cloned from *C. ensiformis*, with high identity to JBURE-I, the classical urease) as a template. This amplicon named jaburetox-2 was cloned into pET 101 vector to obtain heterologous expression in *Escherichia coli* of the recombinant protein in C-terminal fusion with V-5 epitope and 6-His tag. Jaburetox-2Ec was purified on Nickel-NTA resin and bioassayed in insect models. *Dysdercus peruvianus* larvae were fed on cotton seed meal diets containing 0.01% (w/w) Jaburetox-2Ec and, after 11 days, all individuals were dead. Jaburetox-2Ec was also tested against *Spodoptera frugiperda* larvae and caused 100% mortality. In contrast, high doses of Jaburetox-2Ec were innocuous when injected or ingested by mice and neonate rats. Modeling of Jaburetox-2Ec, in comparison with other peptide structures, revealed a prominent β-hairpin motif consistent with an insecticidal activity based on either neurotoxicity or cell permeation.

1. Introduction

Canatoxin, a toxic protein isolated from *Canavalia ensiformis* seeds [5], and more recently identified as an isoform of urease [13], displays insecticidal properties when fed to insects that relay on cathepsins as their main digestive enzymes as the kissing bug *Rhodnius prolixus*, the cowpea weevil *Callosobruchus maculatus*, the Southern green soybean stinkbug *Nezara viridula* and the cotton stainer bug *Dysdercus peruvianus* [4,7,31]. The major form of *C. ensiformis* urease and the soybean seed urease

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Abbreviations: CNTX, canatoxin; IPTG, isopropyl-beta-D-thiogalactopyranoside; JBURE-I, jack bean urease I (classical urease); JBURE-II, jack bean urease isoform II; LB, Luria-Bertani medium.

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also have entomotoxic activity suggesting that this property may be common to all plant ureases [14].

The entomotoxic effect of urease is independent of its ureolytic activity [14] and requires proteolytic processing of the protein by insect enzymes [7,12]. Previous studies have shown that the entomotoxicity of canatoxin relies on an internal peptide (pepcanatox), which could be produced in vitro by hydrolyzing the protein with cathepsins obtained from susceptible insects [12,17].

To fully understand the biological activity and mode of action of urease-derived peptides would require large amounts of pepcanatox, which would be difficult to accomplish given the low yield of the production methods available [6].

In this report, we describe a method for production of a recombinant peptide equivalent to pepcanatox. For that, we have amplified a pepcanatox-like cDNA fragment from JBURE-II, a gene encoding one of the urease isoforms from *C. ensiformis* [26]. This amplicon, named *Jaburetox-2* (Jack bean urease toxin), was subcloned into an expression vector to produce recombinant Jaburetox-2Ec in *Escherichia coli* [25]. The entomotoxic effect of this recombinant peptide was demonstrated using the cotton stainer bug *D. peruvianus* (Hemiptera: Pyrrhocoridae), a model insect that utilizes cathepsins as the main digestive enzymes and features an acidic gut [28], as well as the fall armyworm *Spodoptera frugiperda* (Lepidoptera: Noctuidae), a model insect displaying serine proteinases and an alkaline digestive system [11,13,28].

The mode of insecticidal action of plant ureases or derived peptides has not been studied in detail so far. In *Rhodnius prolixus* impairment of diuresis and symptoms suggestive of neurotoxicity, such as incoordinated movements of limbs and antenna and reversible paralysis in sub-lethal doses [17], are seen following a meal of urease or pepcanatox. In this work, the molecular modeling of Jaburetox-2Ec was proposed to aid the identification of possible motifs, which could be involved in entomotoxic activity.

2. Materials and methods

2.1. Primer design and polymerase chain reaction

The JBURE-II fragment corresponding to pepcanatox was identified by alignment of the pepcanatox N-terminal sequence with the deduced amino acid sequence of JBURE-II (Genbank Acession number AF468788). The CCAC sequence and an ATG start codon were added to the 5′-end of the forward primer (5′-CACCATGGGT CCAGTTAATGAAGCC-3′). The reverse primer was designed based on JBURE-II sequence and the predicted size of the entomotoxic peptide (10 kDa), corresponding to 81 amino acid residues (Fig. 1, 5′-ATAACTTTTCCACCTCC-3′). PCR amplification of Jaburetox-2Ec fragment from JBURE-II gene was performed in a 25 μl reaction, containing 500 ng of pGEMT-easy/JBURE-II as the template DNA, 400 nM of each primer, 200 μM of each deoxynucleoside triphosphate (dNTP), and 2.5 U Pfu DNA polymerase (Stratagene) in buffer. The reaction was carried out in a programmable thermocycler using the following reaction cycles: initial denaturation at 94 °C for 1.5 min followed by 30 consecutive cycles of denaturation at 94 °C for 30 s, annealing for 45 s at 54° C, and extension at 72 °C for 1 min, and a final extension at 72 °C for 5 min. The size (270 bp) and amount of the Jaburetox-2Ec amplicon was monitored on 1.5% agarose gel.

2.2. Jaburetox-2Ec amplicon cloning

The PCR product was purified from agarose gel using the GenClean kit (BIO 101) and cloned into the pET 101 D/TOPO vector (Invitrogen), using 5 ng of the PCR product in a reaction containing 200 mM NaCl, 10 mM $MgCl_2$ and pET 101 vector. Chemically competent *E. coli* (TOP 10) cells were transformed with 3 μl of the ligation reaction and grown overnight in LB medium containing 100 μg/ml ampicillin. The positive transformants were analyzed by PCR, using whole colonies as a source for DNA template. The amplification of *Jaburetox-2Ec*

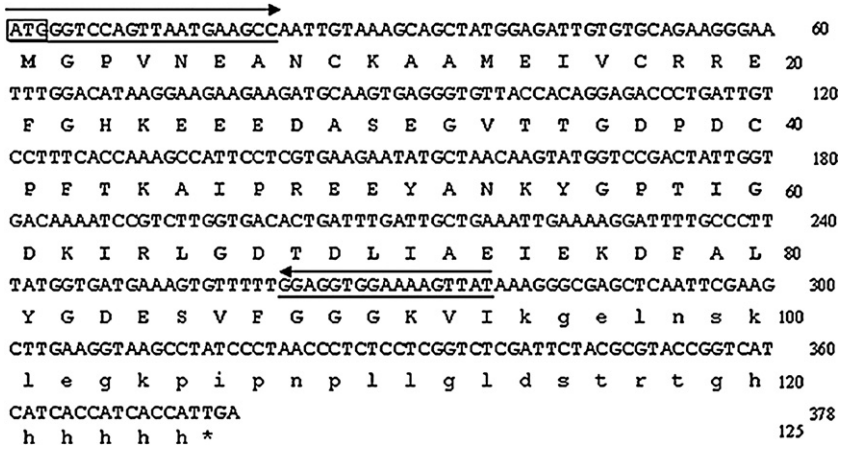

Fig. 1 – Nucleotide and its deduced amino acid sequence of a cDNA for the Jaburetox-2Ec. The deduced amino acid sequence is shown below their respective codons. Nucleotide and amino acid numbers of Jaburetox sequence are indicated on the right side. The box indicates an additional start codon. The primers regions are underlined. The Jaburetox sequence is shown in uppercase letters and fusion V-5 epitope and polyhistidine vector sequence is shown in lowercase letters.

was monitored by agarose gel electrophoresis with DNA stained with ethidium bromide (0.5 μg/ml). Three recombinant plasmids containing insert of expected size were purified from recombinant colonies (mini-preparations) and sequenced on ABI 3700 automated sequence analyzer (Applied Biosystems, Perkin-Elmer), using T7 forward primer to confirm the presence, correct DNA sequence and insert orientation.

2.3. Expression of recombinant Jaburetox-2Ec and polyacrylamide gel electrophoresis

A pET 101/Jaburetox-2Ec recombinant plasmid was transformed into chemically competent *E. coli* BL21 Star (DE3) and inoculated into 10 ml of LB containing 100 μg/ml ampicillin. Cultures were incubated at 37 °C until OD_{600} reached 0.6–0.8. IPTG was added to a final concentration of 0.75 mM and 0.5 ml of culture was sampled after 1 h after induction. Cultures without induction served as control. Cells were centrifuged (5000 × *g*, 10 min), resuspended into SDS-PAGE sample buffer and analyzed by Sodium dodecyl sulfate polyacrylamide gel 12% (SDS-PAGE) according to Laemmli [20] and stained with Coomassie Blue R-250.

2.4. Purification of Jaburetox-2Ec using Ni-NTA agarose

For isolation and purification of Jaburetox-2Ec, 250 ml of LB medium containing 100 μg/ml ampicillin were inoculated with 5 ml of the above culture. The cells were grown 2 h at 37 °C under shaking (OD_{600} = 0.7) and then IPTG was added to 1 mM. After 2 h, the cells were harvested by centrifugation and resuspended in 10 ml of lysis buffer (50 mM sodium phosphate buffer, 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, centrifuged (14,000 × *g*, 20 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), which was previously equilibrated with the equilibration buffer (50 mM sodium phosphate buffer pH 8.0, 300 mM NaCl, 10 mM imidazole). After 30 min, the column was washed with 20 ml of the same buffer, containing 20 mM imidazole. The protein was eluted with the equilibration buffer containing 200 mM imidazole and quantified by the Bradford method [2]. The samples were dialyzed against deionized water.

2.5. Western blot analysis

This was done according to the Towbin method [32]. Purified Jaburetox-2Ec was electrophoresed and transferred to a polyvinylidene fluoride (PVDF) membrane, then immersed in blocking buffer, consisting of 5% nonfat dry milk in phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl and 4.3 mM $Na_2HPO_4{\cdot}7H_2O$, pH 7.3), and 0.05% Tween. After washing, the membrane was incubated with a rabbit IgG anti-canatoxin antibody [13], diluted 1:5000 in blocking buffer, with gentle shaking for 3 h at room temperature, followed by a 2 h incubation with anti-rabbit IgG alkaline phosphatase conjugate (Sigma Chem. Co., 1:5000 dilution) as secondary antibody. The colorimetric detection was carried out by using BCIP (5-bromo-4-chloro-3-indolyl-phosphate *p*-toluidine salt) and NBT (nitro-blue tetrazolium chloride).

2.6. Insect bioassays

2.6.1. Insecticidal effect of Jaburetox-2Ec in D. peruvianus

The bioassay was carried out according to Stanisçuaski et al. [31]. Third instars *D. peruvianus* were fed on artificial cotton seeds, consisting of gelatin capsules (size 2 or 3, Elli Lilly Co.) containing cotton seed flour and 0.01% (w/w) freeze-dried Jaburetox-2Ec or canatoxin (purified according Follmer et al. [15]). The purified Jaburetox-2Ec was exhaustively dialyzed against 5 mM sodium phosphate pH 7.5 buffer and the last change of dialysis buffer was used as control in the bioassay. Groups of 30 insects were tested in triplicates and monitored for survival during 15 days.

2.6.2. Insecticidal effect of Jaburetox-2Ec in S. frugiperda

Three groups of six third instar *S. frugiperda* were reared on *Phaseolus vulgaris* leaves. The insects were kept in individual plastic cups closed with silkscreen tissue at 26 °C, 85% humidity, 12-h dark–12-h light cycles. At days 0, 2 and 4, drops (20 μl) of a phosphate buffered solution containing 16.3 μg Jaburetox-2Ec were air-dried onto the surface of foliar discs (30.5 mm^2) and fed to each larva. Control larvae received foliar discs containing 20 μl air-dried dialysis buffer. The mortality in the group and individual weight gain were measured daily.

2.7. Toxicity of Jaburetox-2Ec in mammalian models

Adult Swiss mice (males, 20 g) or neonate Wistar rats from the central animal facility of Universidade Federal do Rio Grande do Sul were used for these experiments. Groups of six animals each were injected by intraperitoneal route or received orally (intragastric tubing) a single dose of canatoxin [15] (3 mg/kg, equivalent to 1.5 LD_{50} [13]) or Jaburetox-2Ec (10 mg/kg) in phosphate buffered saline. The animals were kept in individual cages, except neonate rats which were returned to their mother's, and observed daily during the next 5 days. The experimental protocols were designed according to approved institutional protocols for animal experimentation.

2.8. Statistical analyis of bioassays

Unpaired Student *t*-test was applied to the bioassay data and $p < 0.05$ was considered significant.

2.9. Ab initio modeling of Jaburetox-2Ec

ROSETTA was used for *ab initio* model building using default protocols: 2000 individual models were constructed from 3- and 9-residue segments using Monte Carlo substitution and optimization protocols [29,30]. These were clustered based on RMSD calculations [27] and visualized and compared with PyMOL (http://pymol.sourceforge.net).

3. Results

3.1. Jaburetox-2Ec cloning

The Jaburetox-2Ec fragment was amplified by PCR from the JBURE-IIB gene, previously cloned in pGEMT-easy by Pires-

Fig. 2 – Alignment of Jaburetox-2Ec with other ureases. Aligment of Jaburetox-2Ec with plant, fungal and bacterial ureases using CLUSTAL W. The sequences are from *Canavalia ensiformis* JBURE-I (GenBank Accession no. AAA83831) C.eI, *Canavalia ensiformis* JBURE-II (AAN08919) C.eII, *Glycine max* embryo-specific (AAO85884) G.mE, *Glycine max* ubiquitous (AAO8583) G.mU, *Arabidopsis thaliana* (AAG52306) A.t, *Solanum tuberosum* (CAC43860) S.t, *Oriza sativa* (BAB78715) O.s, *Schizosaccharomyces pombe* (CAB52575) S.p, *Helicobacter pylori* chain B (AAL86896) H.pB, *Bacillus pasteurii* chain C (4UBPC) B.pC and *Klebsiella aerogenes* chain C (AAA25149) K.aC. The box indicates the region between beta and alpha domains (bacterial UreB and UreC).

Alves et al. [26] and introduced into pET 101 vector using primers that inserted an initiation codon (Fig. 1). The sequence was cloned in frame to V-5 epitope and a poly-Histidine tag. The Jaburetox-2Ec sequence was compared with other sequences from Databanks using BLASTp and showed identity ranging from 53 to 73% to plant ureases (*Oriza sativa* and JBURE-I isoform from *C. ensiformis*, respectively). The alignments in Fig. 2 show that Jaburetox-2Ec is located between the beta and alpha domains being partially absent in all microbial ureases sequenced so far.

3.2. *Expression of V5-6His tagged recombinant Jaburetox-2Ec in E. coli and purification*

The pET/Jaburetox-2Ec vector was transformed into *E. coli* strain BL21 Star (DE3). The resulting *E. coli* BL21 (pET101/Jaburetox-2Ec) strain produced V5-His6 tagged recombinant Jaburetox-2Ec, driven by the IPTG-inducible T7 promoter. A differential band, corresponding to the expected 13 kDa (10 kDa of Jaburetox-2 and 3 kDa of V-5 epitope and 6 His tag) recombinant protein was observed in SDS-PAGE for the *E. coli* BL21 (pET101/Jaburetox-2Ec) total lysate after IPTG induction (Fig. 3A). This band was absent from the control (not induced) total lysate. The expression conditions were optimized to increase the yield of Jaburetox-2Ec and the best results were obtained after induction with 1 mM IPTG at 37 °C for 2 h. Using this condition, Jaburetox-2Ec was purified from 250 ml *E. coli* (pET 101/Jaburetox-2Ec) culture. The bulk of recombinant protein remained soluble after sonication of the cells, allowing the purification of the native protein by Ni^{2+} affinity chromatography (Fig. 3B). The final yield of purified Jaburetox-2Ec was estimated as 6.3 mg/L of *E. coli* culture. The recombinant Jaburetox-2Ec was recognized by anti-canatoxin polyclonal antibodies (Fig. 3B).

3.3. *Insecticidal effect of Jaburetox-2Ec*

The entomotoxic effect of recombinant Jaburetox-2Ec was tested against third instar *D. peruvianus*, fed on artificial cotton seeds containing 0.01% (w/w) freeze-dried purified Jaburetox-

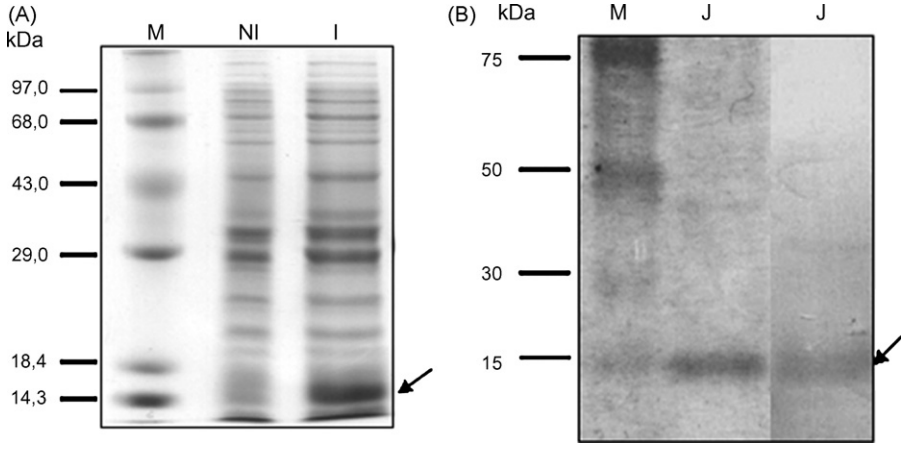

Fig. 3 – Production of recombinant Jaburetox-2Ec. (A) SDS-PAGE analysis of the recombinant Jaburetox-2Ec in cell lysates. Lane NI, without induction. Lane I, 1-h induction with 0.75 mM IPTG. (B) SDS-PAGE analysis of purified Jaburetox-2Ec. Lane J: 10 μg protein eluted from the Ni^{2+} affinity column chromatography. Right side: Western blot analysis of recombinant Jaburetox-2Ec with anti-canatoxin antibodies and anti-rabbit IgG alkaline phosphate conjugates. In all panels: lane M, molecular markers. The numbers on the left indicates molecular mass of markers in kDa. The gels were stained with Coomassie blue.

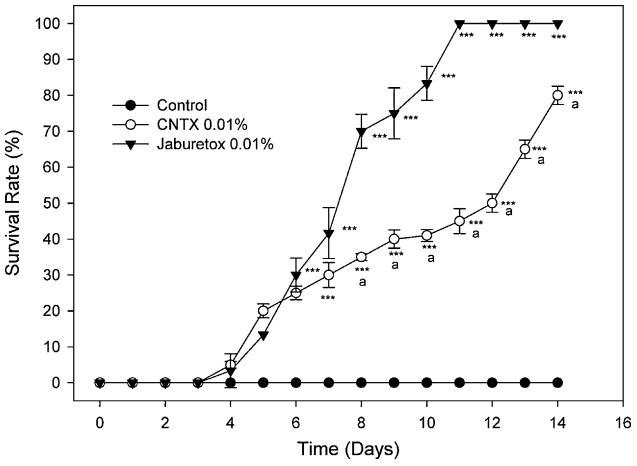

Fig. 4 – Insecticidal effect of Jaburetox-2Ec on *D. peruvianus*. Third instar nymphs fed on artificial seeds containing 0.01% (w/w) Jaburetox-2Ec. Surviving insects were counted daily. Results are expressed as mean mortality and S.E.M. of three independent experiments in triplicates. Level of significance in the *t*-test in comparison to control insects ($^{***}p < 0.001$) or between the experimental groups ($^{a}p < 0.001$) are indicated.

2Ec. For comparison, canatoxin was fed to insects at the same dose. The insects were observed for mortality during 15 days. As shown in Fig. 4, the lethal effect of Jaburetox-2Ec was time dependent, with a lag phase of 3–4 days and 100% mortality being reached after 11 days. Mortality of canatoxin-fed insects showed a slower rate with 20% insects still alive at the end of the experiments.

In order to test the insecticidal activity against insects with trypsin-based digestion and alkaline midguts, which are insensitive to intact ureases [7], third instars *S. frugiperda* received a diet of *P. vulgaris* foliar discs containing air-dried Jaburetox-2Ec. The larvae were given 16.3 μg of Jaburetox-2Ec on days 0, 2 and 4. On the second day, the mean weight of larvae feeding Jaburetox-2Ec was approximately 30% smaller than the controls (Fig. 5A). A lag phase of 2 days was observed before lethality of the insects and on sixth day, after ingesting a total of 47 μg jaburetox-2Ec, all larvae were dead (Fig. 5B).

3.4. *Toxicity of Jaburetox-2Ec to mice and neonate rats*

Mice and neonate rats injected with a single dose of 10 mg/kg of Jaburetox-2Ec were alive and showed no signs of toxicity 5 days after the injection, contrasting to animals that received intraperitoneally 3 mg/kg of canatoxin (equivalent to 1.5 LD_{50} [13]), which died within 12 h after injection. The toxic effects of canatoxin in mice and rats have been previously described [5,8]. When Jaburetox-2Ec or canatoxin were given by oral route at the same dose, all animals survived with no symptoms of intoxication.

3.5. *Modeling of Jaburetox-2Ec*

Modeling was carried out in order to see if the structural properties of Jaburetox-2Ec offered any clues as to its mode of action. As shown in Fig. 2, the N- and C-terminal portions of the peptide are homologous to regions of different chains of bacterial ureases, potentially providing templates for modeling of Jaburetox-2Ec. For example, the Jaburetox-2Ec sequence could be aligned with residues 204 onwards of chain A of the *H. pylori* urease crystal structure [18] (PDB code 1e9y) and the first 51 residues of chain B of the same structure. However, these regions have few contacts in the crystal structure, within themselves and between the chains—evidently once the Jaburetox-2Ec sequence is cleaved from its parent enzyme it must undergo significant structural reorganization.

We therefore employed the emerging technology of *ab initio* protein modeling to the Jaburetox-2Ec sequence. We used the Rosetta program [27,29,30] which assembles many different models from protein fragments and ranks them according to how many times similar models emerge from independent trials and are clustered together. In this case, no particular model emerged as appearing favorable with the top 10 models

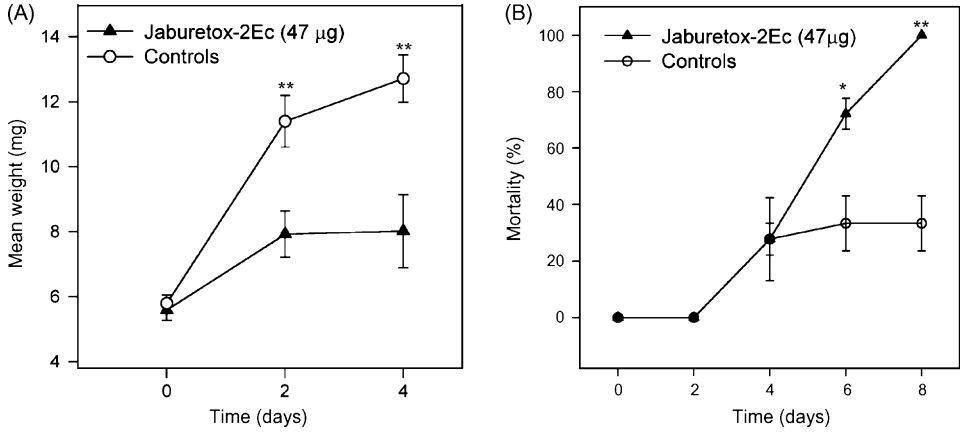

Fig. 5 – Insecticidal effect of Jaburetox-2Ec in *S. frugiperda*. Three groups of six third instars *S. frugiperda* received at days 0, 2 and 4, a foliar (*P. vulgaris*) disc containing 16.3 μg air-dried Jaburetox-2Ec. (A) Weight gain of individual insects at days 0, 2 and 4. (B) Mortality (%) of larvae in each group was registered on days 0, 2, 4, 6 and 8. Data are expressed as mean and S.E.M. of one experiment out of three. Asterisks indicate level of significance in the *t*-test ($^{*}p < 0.05$; $^{**}p < 0.005$) in comparison to control insects.

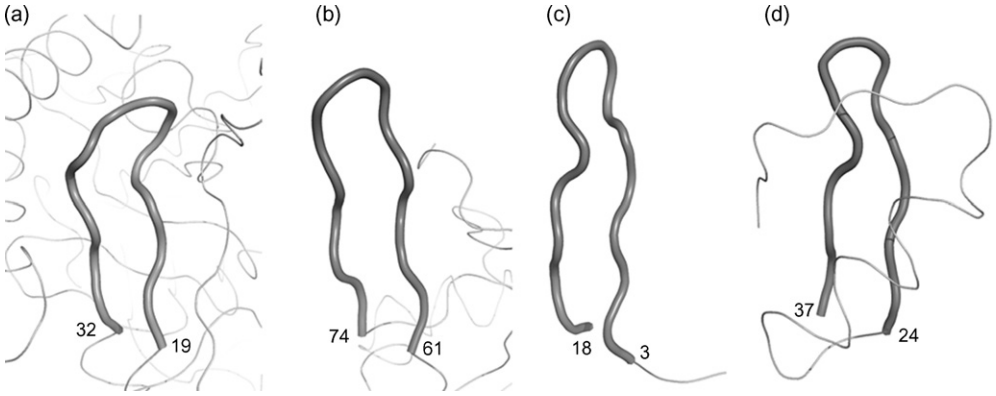

Fig. 6 – *Ab Initio* modeling of Jaburetox-2Ec and comparison to other β-hairpin motifs. β-Hairpin motifs (shown as cartoon representation in darker color) in (a) the intact *H. pylori* urease [24] (PDB code 1e9y), (b) one of the top 10 *ab initio* models of Jaburetox-2Ec, (c) protegrin [1] (PDB code 1pg1) and (d) charybdotoxin [21] (PDB code 2crd). The termini of the motifs are labeled with residue numbers.

all representing clusters of similar size. Nevertheless, although differing significantly in the packing of α-helices, with a single exception, they contained a large, generally exposed, β-hairpin structure (Fig. 6b). Since this feature was near-ubiquitous among the model set, and also present in the known crystal structures of urease (Fig. 6a), it was viewed as a reliable predictor with possible implications for function as discussed below.

4. Discussion

Plant genetic transformation with exogenous genes encoding factors of resistance to phytophagous insects is an interesting strategy to reduce crop losses due to insect attack. Efforts have been focused on studies of different insecticidal proteins [4].

In this work, we have expressed in *E. coli* a peptide derived from JBURE-II, an isoform of urease from *C. ensiformis*, and demonstrated its insecticidal effect. The conditions for expression of the pET 101/Jaburetox-2Ec amplicon were optimized and a satisfactory yield was obtained. Jaburetox-2Ec was recognized by anti-canatoxin polyclonal antibodies (Fig. 2B) and the sequence of recombinant Jaburetox-2Ec was determined by mass spectrometry, confirming the correct translation of the recombinant peptide (data not shown).

The entomotoxicity observed for this recombinant peptide, derived from the JBURE-II urease isoform, corroborates the hypothesis that plant ureases are protoxins able to release entomotoxic peptide(s) upon limited proteolysis, as demonstrated for canatoxin [12]. As reported for canatoxin [7], the insecticidal activity of Jaburetox-2Ec occurs at very low doses at 0.01–0.1% (w/w), as compared to other plant-derived entomotoxic proteins [4]. The mortality observed against *D. peruvianus* after 10 days on diet containing Jaburetox-2Ec was two times higher than that observed for canatoxin under the same conditions. Calculations of doses effectively administered to the insects are difficult in the conditions of our bioassay (30 insects feeding on a single artificial seed during 15 days). Since the same w/w proportion of Jaburetox-2Ec (12.6 kDa) and canatoxin (monomer, 90 kDa) were present in the artificial seeds, the amount of processed/entomotoxic peptide released from canatoxin would be 7.5-fold lower, therefore the lower lethality could be related to a lower dose of active peptide in the insects. Additionally the increased lag phase observed for the entomotoxic effect of canatoxin could also reflect the need for proteolytic activation of the intact protein to release the active peptide. In a previous work, insects relying on serine-proteinases as their main digestive enzymes, including lepidopterans such as *S. frugiperda* [11,10], were shown to be resistant to canatoxin's insecticidal effect. This resistance was attributed to an extensive hydrolysis of canatoxin by this class of proteolytic enzymes [7]. Here we demonstrated that young forms of *S. frugiperda* are also susceptible to the entomotoxic activity of Jaburetox-2Ec. Other insects with trypsin-based digestion such as the soybean caterpillar *Anticarsia gemmatalis*, the German cockroach *Blatella germanica* and the termite *Cornitermis cumulans*, are also susceptible to Jaburetox-2Ec but not to canatoxin or urease (unpublished data). Thus, it appears that the species-specificity of plant ureases insecticidal activity is mainly related to the ability of the insect's digestive enzymes to adequately process the protein into an active entomotoxic peptide. If this step is not necessary, either by exposure to a preformed or a recombinant peptide, a broader range of insects is expected to be effectively controlled by this class of compounds.

Feeding trials have shown that the major isoform of *C. ensiformis* urease JBURE-I was as lethal as canatoxin against the kissing bug *R. prolixus* [4] and *D. peruvianus*, whereas both jackbean ureases were three-fold more potent than the soybean embryo specific urease [14]. The variation in entomotoxicity among plant ureases already studied is probably related to differences in the sequences corresponding to the Jaburetox peptide or inter-domain regions. In fact the N-terminal region of Jaburetox shares only 51% identity with the same region in soybean ureases, which is significantly lower than the overall identity of plant ureases, for instance, the beta (about 80% identity) or the alpha (about 83% identity) domains of JBURE-II as compared to soybean ureases (Fig. 2). Since the sequence corresponding to Jaburetox in ureases is not involved either in the enzymatic activity or in

subunit association, as shown by its absence in bacterial ureases, it probably diverged at a faster rate. Thus, variations of the biological properties of these sequences among plant ureases can be expected.

This internal sequence is also not responsible for the toxicity of canatoxin as Jaburetox-2Ec was innocuous at a 20-fold higher protein mass/body weight ratio than the LD_{50} for mice and rats [5,8], either by intraperitoneal injection or by oral route (neonatal rats were chosen because of the increased permeability of their digestive tract).

Examination of the bacterial urease structures makes clear that there must be significant rearrangement of the Jaburetox portion post-cleavage, ruling out conventional model building by homology. *Ab initio* models were therefore constructed with ROSETTA. These were used to provide clues as to the possible molecular mode(s) of insecticidal action of the peptide. It was already known that Jaburetox is not an inhibitor of insect digestive enzymes [7,17]. After Jaburetox ingestion, the insects were momentarily paralyzed and show uncoordinated movements of antennas preceding death, suggesting that it might act as a neurotoxin or lead to cell death by affecting membrane permeability.

Peptides capable of forming pores in cell membranes are a very diverse group, divided into classes based on size and other characteristics [3]. Some insecticidal proteins, the Cry δ-endotoxins, form pores by insertion of an α-helical hairpin (two α-helices lying antiparallel) into the membrane [22]. In the set of Jaburetox models this motif was absent, ruling out this mode of action. Single amphiphilic helices such as the antibiotic peptide melittin [33] or peptides derived from the apoptosis regulator Bax can form membrane pores [16] but none of the predicted helices of Jaburetox are amphiphilic in nature. Equally, there is no sign in the Jaburetox sequence of five consecutive hydrophobic residues forming a β-turn, the motif responsible for membrane insertion of the toxin aerolysin [19].

In contrast, nine of the 10 proposed *ab initio* models of Jaburetox contained β-hairpin structures, formed from residues whose counterparts in bacterial urease structures also form this motif (Fig. 7). Intriguingly, this motif is common (Fig. 7) to both a class of pore-forming peptides [3] and to a type of neurotoxin [23], represented by charybdotoxin [1], whose toxicity arises from binding to and inhibition of membrane ion (K^+) channels. In this context it is noteworthy to mention that neurotoxic symptoms (such as paralysis and uncoordinated movements of limbs and antenna) are seen in *R. prolixus* intoxicated by Jaburetox-2Ec. In a study to be published somewhere else, we showed that very low concentrations (10^{-12} M) of Jaburetox-2Ec inhibit in a [K^+]-dependent manner the serotonin-stimulated diuresis of Malpighian tubules isolated from *R. prolixus* which probably is the cause of the impaired water excretion observed in intact insects after receiving a canatoxin meal [7].

In the above cases, a majority of natural peptides contain disulphide bridges but active cysteine-free analogues can be produced [21] and it is easy to imagine that the rest of the Jaburetox toxin folds so as to reproduce the role of the bridges in stabilizing the β-hairpin motif in the free peptide. The absence of the N-terminal part of the Jaburetox sequence in bacterial ureases would compromise this stabilization and no such structure would exist excised from the whole molecule, in agreement with our previous observation that *B. pasteurii* urease is not lethal to *D. peruvianus* [14].

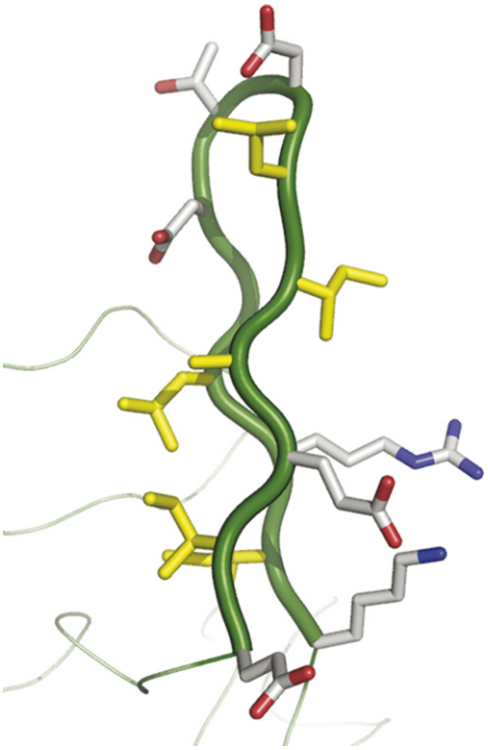

Fig. 7 – The amphiphilic character of the β-hairpin motif in one of the top 10 *ab initio* models of Jaburetox-2Ec. Hydrophobic residues (yellow carbon) predominate on the left face while hydrophilic residues (white carbon) form the majority of the other face. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

In the case of protegrins, a class of pore-forming antimicrobial peptides, the β-hairpin motif has a pronounced amphiphilic character [9]. *Ab initio* models of Jaburetox contain β-hairpins with similar characteristics (Fig. 7). In summary, while it was unrealistic to expect modeling alone to produce a definitive mode of action prediction, it was certainly capable of clearly ruling several out of consideration. As a working hypothesis the modeling suggests that a β-hairpin motif present in Jaburetox may be responsible for its insecticidal property through either ion channel inhibition or pore-forming activity.

5. Conclusions

In conclusion, in this paper we described the heterologous expression of a new insecticidal peptide, derived from an internal sequence of JBURE-II urease isoform. This result corroborates the hypothesis that plant ureases are protoxins, and can be related to plant arsenal of defenses against insects. The molecular modeling suggested activity based on

neurotoxicity or cell permeation. The insecticidal properties at low doses and the lack of acute toxicity to mammals emphasize the potential use of this protein in the control of insect pests.

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