

Risk assessment of the antifungal and insecticidal peptide Jaburetox and its parental protein the Jack bean (*Canavalia ensiformis*) urease

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ABSTRACT

Jaburetox (JBTX) is an insecticidal and antifungal peptide derived from jack bean (*Canavalia ensiformis*) urease that has been considered a candidate for developing genetically modified crops. This study aimed to perform the risk assessment of the peptide JBTX following the general recommendations of the two-tiered, weight-of-evidence approach proposed by International Life Sciences Institute. The urease of *C. ensiformis* (JBU) and its isoform JBURE IIb (the JBTX parental protein) were assessed. The history of safe use revealed no hazard reports for the studied proteins. The available information shows that JBTX possesses selective activity against insects and fungi. JBTX and JBU primary amino acids sequences showed no relevant similarity to toxic, antinutritional or allergenic proteins. Additionally, JBTX and JBU were susceptible to *in vitro* digestibility, and JBU was also susceptible to heat treatment. The results did not identify potential risks of adverse effects and reactions associated to JBTX. However, further allergen (e.g. serum IgE binding test) and toxicity (e.g. rodent toxicity tests) experimentation can be done to gather additional safety information on JBTX, and to meet regulatory inquiries for commercial approval of transgenic cultivars expressing this peptide.

1. Introduction

Jack bean (*Canavalia ensiformis*) has historically been a source of promising proteins for biochemistry research (Carlini; Ligabue-Braun, 2016). The jack bean urease (JBU) which was the first enzyme to be crystallized (Sumner, 1926) and the widely studied lectin Concanavalin

A (Sridhar; Seena, 2006) are notable examples of the importance of this plant species. In the early 80's, a toxic protein called Canatoxin (CNTX) was isolated from *C. ensiformis* seeds which is lethal to mice when injected intraperitoneally (LD₅₀ = 2 mg/kg) as well as to insects (Carlini; Guimarães, 1981; Carlini et al., 1997; Follmer et al., 2001). The insecticidal activity of CNTX, selective to insects with cathepsin-based

Abbreviations: AA, amino acids; CNTX, Canatoxin; CTNBio, Brazilian National Technical Commission on Biosafety; HOSU, History of safe use; IDP, Intrinsically disordered protein; JBU, Jack bean urease; JBURE II, Jack bean urease isoform II; JBTX, Jaburetox; SGF, Simulated Gastric Fluid; SIF, Simulated Intestinal Fluid; SDAP, Structural Database of Allergenic Proteins

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digestion (Carlini et al., 1997), has elicited discussions about its role in plant defense (Stanisçuaski; Carlini, 2012). Twenty years after this protein was first isolated, it was discovered that CNTX is actually one of the isoforms of urease found in *C. ensiformis* (Follmer et al., 2001). JBURE-II, later renamed as JBURE-IIb, was identified as a third isoform of *C. ensiformis* urease (Pires-Alves et al., 2003; Mulinari et al., 2011).

The entomotoxic effect of CNTX partly depends on the release of a 10 kDa internal peptide (Pepcanatox) by cathepsin-like enzymes in the digestive tract of insects, which has been experimentally demonstrated using digestive contents from *Callosobruchus maculatus*, *Dysdercus peruvianus* and *Oncopeltus fasciatus* (Ferreira Dasilva et al., 2000; Stanisçuaski et al., 2005; Defferrari et al., 2011). From the N-terminal sequence of Pepcanatox and using the cDNA of the JBU isoform II (JBURE-IIb) as template, the recombinant peptide called Jaburetox-2Ec was obtained by heterologous expression in *Escherichia coli* containing in its sequence a V5 viral epitope and 6 His-tag. This peptide presented a broad spectrum of insecticidal activity including to insects not susceptible to CNTX such as *Spodoptera frugiperda* (Mulinari et al., 2007). Thereafter, a variant of the Jaburetox-2Ec polypeptide was cloned and expressed in *E. coli* without the V5 viral epitope. This peptide with 93 amino acids residues was named Jaburetox (JBTX) (Postal et al., 2012).

JBTX revealed similar insecticidal activity as its V5-containing version and the list of insects affected by the recombinant peptide is continuously increasing, to include other hemipterans such as *O. fasciatus* (Defferrari et al., 2011; Martinelli et al., 2014) and also cockroaches, such as *Blattella germanica*, *Phoetalia pallidum* and *Nauphoeta cinerea* (Martinelli et al., 2014; unpublished results). JBTX shows also remarkable deterrent effects against yeasts and filamentous fungi (Postal et al., 2012; Carlini; Ligabue-Braun, 2016). Because of these features, JBTX has been seen as an alternative for the biological control of agricultural pests through the development of genetically modified (GM) crops.

The development of a transgenic plant follows a sequence of stages ranging from the selection of the biocidal genes to be used in the plant transformation to the risk assessment of the new cultivar for human and environmental health. Often, the safety assessment of the exogenous proteins and the whole plant has been performed only in the later stages. Certainly, this is not an effective approach, because it can lead to the production of plants containing proteins that may potentially present risks to non-target organisms (Farias et al., 2015a). In recent years, our group has proposed that the safety evaluation of recombinant proteins could be done even before obtaining the first transgenic plants in greenhouse, through a process we named early risk assessment of candidate proteins. This approach has been shown to be quite effective in identifying undesirable risks (e.g., allergenic potential) of these molecules prior to their use for the development of biotech products (Farias et al., 2015b; Pinto et al., 2015). Besides, the early risk assessment approach can prevent time and effort consuming activities, as it can help to envisage changes to the protein structure in order to reduce its potential risks (Pinto et al., 2015).

The current strategies for assessing the risk of consumption of recombinant proteins are essentially carried out based on FAO (Food and Agriculture Organization) and WHO (World Health Organization) guidelines presented in the 2nd edition of Codex Alimentarius - Foods Derived from Modern Biotechnology (Codex, 2009). Alternatively, the International Life Sciences Institute (ILSI) proposed a two-tiered (Tier I and II) approach based on weight-of-evidence that is well accepted (Delaney et al., 2008). This approach stands out from the others by taking into account all data obtained in a holistic way. The first step (Tier I) includes the history of safe use (HOSU) of the protein and the gene-donor species, comparisons of amino acids sequence in protein databases, descriptions of the mode of action and specificity, *in vitro* digestibility and temperature stability tests, as well as the expression level and dietary intake of the recombinant proteins. When data from Tier I are not conclusive, it is recommended to perform tests in Tier II, which comprise acute and repeated dose toxicological studies and/or

hypothesis-based testing (Delaney et al., 2008).

The ILSI two-tiered approach has been initially proposed for recombinant proteins in the late stages of GM crop development. However, this approach has proven to be suitable for early risk assessment of candidate proteins and has been useful to support the safe use of these molecules for developing biotech products (Farias et al., 2015b; Pinto et al., 2015). In this context, this study aimed to perform an early risk assessment of JBTX insecticidal peptide and of *C. ensiformis* ureases, JBU and JBURE-IIb (from which JBTX derived), since this peptide is a candidate for developing transgenic crops. For that, we followed the recommendations of ILSI tiered weight-of-evidence approach, except for temperature stability testing for JBTX and acute toxicity in rodents for both JBTX and JBU, because this information readily available in the literature. Additionally, expression levels of the recombinant protein were not measured because this analysis will be performed after obtaining the first transgenic cultivars expressing this peptide. On the other hand, the inclusion of additional bioinformatics analyses was favored. This study allowed us to collect information about JBTX risks that will be crucial to decision-making on the use of this peptide for plant transformation.

2. Material and methods

2.1. JBTX and JBU samples

The recombinant peptide Jaburetox (JBTX) containing the 6 His-tag and molecular mass of 11.03 kDa was produced following the protocol described by Broll et al. (2017), with some modifications. *Escherichia coli* BL21(DE3)-RIL cells containing pET23a-Jaburetox plasmid were cultured in 15 mL LB (Luria Bertani) broth, 100 µg mL⁻¹ ampicillin e 40 µg mL⁻¹ chloramphenicol for 16 h, 37 °C and 200 rpm. All the content was inoculated in 1 L of autoinduction medium (tryptone 10 g.L⁻¹, yeast extract 5 g.L⁻¹, glycerol 5 g.L⁻¹, (NH₄)₂SO₄ 3,3 g.L⁻¹, KH₂PO₄ 6,8 g.L⁻¹, Na₂HPO₄ 7,1 g.L⁻¹, glucose 0,5 g.L⁻¹, lactose 2 g.L⁻¹ and MgSO₄ 0.12 g.L⁻¹), with 100 µg mL⁻¹ ampicillin and 40 µg mL⁻¹ chloramphenicol and cultured at 37 °C, 200 rpm, until A_{600nm} reaches 0.7. The induction conditions were 16 h, 20 °C and 200 rpm. After, the culture was centrifuged at 8,000 × g for 10 min at 4 °C and the pellet was resuspended in 100 mL of buffer A (tris-HCl 50 mM, NaCl 500 mM and imidazole 20 mM, pH 7.5) and the cell suspension was sonicated (20 cycles of 1 min, 20 kHz frequency). The supernatant was separated by centrifugation at 15,000 × g for 40 min at 4 °C and purified in a Ni²⁺-loaded Chelating Sepharose (GE Healthcare) previously equilibrated with buffer A. Unbound proteins were washed with buffer A. We performed a second washing with buffer B (tris-HCl 50 mM, NaCl 500 mM and imidazole 70 mM, pH 7.5) and Jaburetox elution with buffer C (tris-HCl 50 mM, NaCl 500 mM and imidazole 500 mM, pH 7.5). The eluted fraction was loaded in a Hiload Superdex 200 26/60 prep grade, equilibrated with buffer D (sodium phosphate 50 mM, EDTA 1 mM and tris(2-carboxyethyl)phosphine) – TCEP 1 mM, pH 7.5) mounted in an Äkta Purifier system (GE Life Technologies). Urease type C-III from Jack bean (JBU) (hexameric form of Mr 540 kDa) was commercially acquired from Sigma Aldrich Co. (St. Louis, USA). The quality of both samples was assessed by SDS-PAGE (Laemmli, 1970). The protein samples presented purity > 90%.

2.2. History of safe use of *C. ensiformis* and derived proteins

A literature review on the history of safe use (HOSU) of *C. ensiformis* species, its ureases and JBTX peptide was performed as described by Constable et al. (2007). For the search, the PubMed database (<http://www.ncbi.nlm.nih.gov/pubmed/>) was accessed in the period from June 6 to 10, 2019 using the following keywords and their combinations: (a) “Jaburetox”; (b) “Jack bean urease”; (c) “*Canavalia ensiformis*”; and “food use”; (d) *Canavalia ensiformis* and “antinutrient”; (e) “*Canavalia ensiformis*” and “allergenicity”; (f) “*Canavalia ensiformis*” and

“toxicity”; (g) “*Canavalia ensiformis*” and “review”.

2.3. Bioinformatics analysis

2.3.1. Amino acid sequences of JBTX and *C. ensiformis* ureases

The amino acids sequences of JBTX and *C. ensiformis* ureases, JBU and JBURE IIB, are deposited at GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>) with accession numbers of 2MM8A, AAA83831.1 and ACL14297.1 respectively, and presented in Supplementary Fig. 1. The sequences were analyzed in FASTA format, and for sequence 2MM8A the histidine tag was removed.

2.3.2. Similarity searches in NCBI non-redundant database

The NCBI non-redundant (NR) general protein database (<http://www.ncbi.nlm.nih.gov/BLAST/>) was used to search similarities of the full-length amino acid sequences of JBTX and *C. ensiformis* ureases (JBU and JBURE IIB) with those of proteins described as allergenic, toxic and antinutritional. The algorithm used was BLASTP 2.2.31 + and the scoring matrix was BLOSUM62. Specific details of the alignments were carefully checked [E-value < 0.01, shared identity > 50%, alignment size and gap frequency ≤ 6] to indicate the relevance of any similarity found, since there is no defined method for the investigation of toxic and antinutritional proteins (Herouet-Guicheney et al., 2009). The protein identifiers (ID) of relevant matches were then submitted to the UniProt database (<https://www.uniprot.org/>) in order to find the protein name. Then, the protein was screened in NCBI for reports of adverse effects that could be associated with the consumption of this protein. The NR database was also assessed for comparisons of JBTX, JBU and JBURE IIB sequences by using the limiting keywords “allergen”, “antinutrient” and “toxin” for each sequence tested. All comparisons were run in the period from March 10 to 18, 2019.

2.3.3. Similarity searches in allergen databases

The full-length amino acid sequences of JBTX, JBU and JBURE IIB were analyzed for checking similarities with known allergenic proteins following the general recommendations of the Codex Alimentarius (Codex, 2009). The sequences were compared to those of allergens deposited in the databases Structural Database of Allergenic Proteins (SDAP) (<http://fermi.utmb.edu/SDAP/>), updated on February 25, 2013; and AllergenOnline, version 19 database (<http://www.allergenonline.com/>), updated on February 10, 2019.

The parameters analyzed were: (1) Full FASTA comparisons using as filter E-value cut-off of 0.01 for detection of identity > 50% (Aalberse, 2000); (2) Comparisons using an 80 aa window for detection of identity > 35% (FAO/WHO, 2001); and (3) Search of short sequences of eight, seven or six contiguous amino acids identical to those of allergenic proteins.

Additionally, the database of the Subcommittee on Allergen Nomenclature of the WHO and International Union of Societies of Immunology (www.allergen.org) was used in order to verify the presence of allergens related to *C. ensiformis* species. All research was carried out on March 10, 2019.

2.3.4. Searches of N-glycosylation sites

The full-length amino acid sequences of the JBTX, JBU and JBURE IIB were analyzed for the presence of N-glycosylation sites by accessing the NetNGlyc 1.0 Server program (<http://www.cbs.dtu.dk/services/NetNGlyc/>) as described by Xu et al. (2009). The search was run on March 10, 2019.

2.3.5. Searches of allergen motifs

The sequences in FASTA format were also submitted to the AlgPred server, (<http://www.imtech.res.in/raghava/algpred/>) (Saha; Raghava, 2006). This server allows the prediction of allergens based on five approaches. Here, the MEME/MAST approach was employed for JBTX, JBU and JBURE II analysis, where MEME identifies the motifs in a

sequence using a position-dependent probability matrix while MAST uses as input a file which shows the descriptions of one or more motifs, in the output format of MEME, and searches in a database of sequences for those that correspond to the allergenic motifs. The search was run on March 10, 2019.

2.4. Mode of action and specificity

A literature review was carried out to gather informations on the mode of action and specificity of JBTX and *C. ensiformis* ureases against insects. For this purpose, the Pubmed database (<http://www.ncbi.nlm.nih.gov/pubmed/>) was consulted on June 9, 2019 using selected keywords and their combinations, as follows: (1) “Jaburetox”; (2) “mode of action” and “Jaburetox”; (3) “mode of action” and “Jaburetox” and “specificity”; (4) “mode of action” and “Jack bean urease”; (5) “mode of action” and “Jack bean urease” and “specificity”; (6) “mode of action” and “Jack bean urease isoform II”; (7) “mode of action” and “Jack bean urease isoform II” and “specificity”.

2.5. Susceptibility to *in vitro* digestion

The susceptibility to *in vitro* digestion of JBTX and JBU was tested as described by Farias et al. (2015b), with minor modifications. The samples were individually incubated in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) at a concentration of 1.2 mg/mL in a sequential manner. Briefly, JBTX and JBU were incubated in SGF [34 mM NaCl, 0.07% HCl, pH 2.0 and 1 mg/mL of pepsin (Sigma P7012)] at 37 °C under stirring. Aliquots of 100 µL were collected at different incubation times and mixed with the stopping buffer (2% SDS, 10% Glycerol, 6% β-mercaptoethanol, 0.01% bromophenol blue, 200 mM DTT and 500 mM Tris-HCl pH 2.0) in ratio of 1:1 (v/v). Then, the remaining solution was adjusted to pH 8.0 with 1 M Tris-HCl and SIF (50 mM potassium phosphate, pH 8.0 and 1 mg/mL pancreatin - Sigma P7545) was added. The mixture was incubated again at 37 °C under stirring, and aliquots of 100 µL were collected and mixed with the stopping buffer (3% SDS, 17% glycerol, 8.5% β-mercaptoethanol, 0.01% bromophenol blue, 170 mM DTT, 6 mM PMSF, 200 mM Tris-HCl pH 7.2). Aliquots of JBTX and JBU digestions were collected at 1, 5, 10, 30, 120 min of incubation in SGF and SIF. The digestibility of both proteins was monitored by Tricine-SDS-PAGE (15%) (Schagger; Von Jagow, 1987) stained using Coomassie Blue R-250 following the Blue silver method (Candiano et al., 2004). The digestions were also checked through Western blot analysis using an anti-JBTX rabbit polyclonal antibody (1:7,500) (Towbin et al., 1979). The same antibody was used for both proteins because JBTX is a peptide derived from a JBU isoform.

2.6. Urease enzymatic activity

To determine urease activity, samples (10 µg) were incubated with urea 20 mM in PBS at pH 7.2, for 30 min at 37 °C. The ammonia released was measured colorimetrically by the Phenol-Hypochlorite method (Weatherburn, 1967).

2.7. JBU thermal stability

JBU samples were prepared by solubilization of JBU type C3 (Sigma-Aldrich) in sodium phosphate 20 mM, EDTA 1 mM, 2-mercaptoethanol 5 mM, pH 7.4, and immediately subjected to the thermal stability assay. JBU samples were incubated for 60 min at temperatures ranging from 30 to 100 °C in a Veriti® Thermal Cycler (Thermo Fisher Scientific). After this 60 min period all samples were cooled down and allowed to rest for 24 h at 4 °C, as to allow any possible renaturation to take place. To measure the urease activity, all samples were pre-incubated for 5 min at the assay temperature before the reactions were set up. Data are presented as percentage of activity retained after the heat treatment in comparison to the untreated enzyme. All

Table 1
Literature review on the history of safe use of Jack bean (*Canavalia ensiformis*), its urease and derived proteins.

Species or protein/ peptide studied	Protocol of administration	Concentration/administration route	Exposure time	Observed effects	Organism tested	Reference
<i>Canavalia ensiformis</i>	Seed flour added to soy-based diet	30% of seed flour/oral diet	42 days	No adverse effects	Cornish rock chicken, <i>Gallus gallus domesticus</i>	Sasipriya; Siddhuruju (2013). Akpapunam; Sefa-Dedeh (1997)
	Source of starch; P, Ca, Mg, Zn, Cu, Ni; Source of essential amino acids, except methionin and cysteine	- *	-	-	-	-
	Synthetic liquid diet containing Concanavalin A	50, 500, 1,000 and 1,500 µg/ml/oral diet	15 days	Increase of the pre-productive period, mortality and average time of development of generation (T) and decrease of the fecundity and the intrinsic rate of natural increase (rm)	The wheat aphid, <i>Rhopalosiphum padi</i> L.	Sprawka et al. (2014)
Canatoxin (CNTX)	Concanavalin A purified lectin	5, 10 and 15 µg/in vitro	0, 0.5, 1 and 2 h	Reduction of sperm motility, production of reactive oxygen species (ROS)	Bull nelore, <i>Bos taurus</i>	Kaefer et al. (2013)
	Seed crude extract	100–200 mg/kg/Intraperitoneal injection	0.5 and 24 h	dyspnea, hypothermia, coma and death	Rat, <i>Rattus norvegicus</i>	Carlini; Guimarães (1981)
	Artificial diet containing CNTX	0.25% (w/w) CNTX/oral diet	72 h	Weight loss in 3rd instar nymphs and mortality of 80–100%	Kissing bug, <i>Rhodnius prolixus</i>	Carlini et al. (1997)
	Artificial diet containing CNTX	2.5 µg/mg/body weight/oral diet	48 h	100% mortality in 3rd instar nymphs	<i>R. prolixus</i>	Ferreira-Da Silva et al., 2000
	Artificial diet containing CNTX	0.01% oral diet	10 days	Reduction of weight gain and delay in the development of 3rd instar nymphs and mortality of 90–100%	Cotton stainer bug, <i>Dysdercus peruvianus</i>	Stanisquaski et al. (2005)
Jack bean urease (JBU)	Purified CNTX	Single dose of CNTX (3 mg/kg)/orally	5 days	Adults and newborns showed no signs of toxicity 5 days after ingestion	Adult and newborn Wistar rats, <i>R. norvegicus</i>	Mulinari et al. (2007)
	Urease (Sigma)	6 µg/injection into the hemocoele	6 h	Aggregation of hemocytes	<i>R. prolixus</i>	Defferrari et al. (2014a)
	Cotton seed meal-based diet containing urease (Sigma)	0.01–0.04% (w/w) of urease/oral diet	11 days	100% mortality of 3rd instar nymphs	<i>D. peruvianus</i>	Piovesan et al. (2008)
	Urease (Sigma)	0.36 µM/in vitro	24 h	Inhibition of proliferation, morphological changes with formation of pseudohyphas, changes in the transport of H(+), carbohydrate metabolism and permeabilization of membranes	Filamentous fungi and yeasts	Postal et al. (2012)
	Cotton seed meal-based diet containing urease (Sigma)	0.01% and 0.2% (w/w) of urease/oral diet	14 days	Mortality of nymphs > 80%	Large milkweed bug, <i>Oncopeltus fasciatus</i>	Defferrari et al. (2011)
Jaburetox-2Ec	Urease (Sigma)	5×10^{-10} M/in vitro dissected whole tubules	20 min	No changes in the transepithelial potential of the Malpighi tubules 5th instar nymphs	<i>R. prolixus</i>	Stanisquaski et al. (2009)
	Urease (Sigma)	0.25 µg/mg body weight/injection into the hemocoele	24 h	96% mortality	<i>R. prolixus</i>	Stanisquaski et al., 2010
	Cotton seed meal-based diet containing Jaburetox-2Ec; solution containing Jaburetox-2Ec	0.01% (w/w) of Jaburetox-2Ec; 20 µL of a solution containing 16.3 µg of Jaburetox-2Ec; single dose of Jaburetox-2Ec (10 mg/kg)/orally	15 days; 8 days; 5 days	100% mortality of 3rd instar nymphs; reduction of 30% in the weight of 3rd instar larvae; adults and newborns showed no signs of toxicity 5 days after ingestion	<i>D. peruvianus</i> ; <i>Spodoptera frugiperda</i> ; adult and newborn Wistar rats, <i>R. norvegicus</i>	Mulinari et al. (2007)
Jaburetox (JBTX)	Purified JBTX	JBTX injected in the proportion of 2 µg/inset/injection into the hemocoele	18 h	Increase in gene expression of UDP-N-acetylglucosamine pyrophosphorylase (UAP), chitin synthase and nitric oxide synthase (NOS)	<i>R. prolixus</i>	Moyetta et al. (2017)
	Purified JBTX	23 µM/in vitro	1 h	Jbtx-FITC interacts with the membrane debris obtained after cell lysis of <i>S. cerevisiae</i> and tissue homogenates of nerve cord	<i>Saccharomyces cerevisiae</i> ; lobster cockroach, <i>Nauphoeta cinerea</i>	Broll et al. (2017).
	<i>Escherichia coli</i> cells expressing JBTX	Fish diet soaked with a suspension of lyophilized <i>E. coli</i> cells containing 100 µg JBTX/oral diet	6 days	97.5% mortality of larvae	Yellow fever mosquito, <i>Aedes aegypti</i>	Becker-Ritt et al. (2017).

(continued on next page)

Table 1 (continued)

Species or protein/ peptide studied	Protocol of administration	Concentration/administration route	Exposure time	Observed effects	Organism tested	Reference
Purified JBTX		0.05 µg/mg b.w./injection into the hemocoele; 0.1 µg/mg b.w./oral diet; 1×10^{-15} M/ <i>in vitro</i> ; 0.015 µg/mg b.w./injection into the hemocoele; 32 µg/g b.w./ <i>in vivo</i> nerve-muscle preparation	96 h; 24 h; 1 min; 96 h; 120 min	> 90% (injection) and > 40% (oral diet) mortality of fifth instar of <i>R. prolixus</i> ; inhibition of fluid secretion in Malpighian tubules of <i>R. prolixus</i> ; > 60% mortality of fifth instar of <i>O. fasciatus</i> ; neuromuscular paralysis in <i>Phaenicia pallida</i> Altered the locomotive behavior inducing reduction in distance traveled and increase in the standing time of cockroach species (<i>Nauphoeta cinerea</i>), induced a maximal neuromuscular blockade of 80.72% (n = 6, p < 0.05) and was cardiotoxic, decreasing the heart rate of the cockroach. Voltage clamping analyzes of Jbtx (200 nM) applied on heterogeneously expressed <i>Xenopus laevis</i> oocytes with Nav 1.1 channels evidenced an expressive increase in sodium currents	<i>R. prolixus</i> ; <i>Oncopectus fasciatus</i> ; pallid cockroach, <i>P. pallida</i>	Martinelli et al. (2014)
Purified JBTX		32 µg/g during 20 min of shooting; 200 nM in oocytes from 1 to 7 days			<i>N. cinerea</i> and <i>Xenopus laevis</i>	Dos Santos et al. (2019)

* (–): information not available.

measurements are means of at least four independent measurements.

3. Results and discussion

3.1. History of safe use

Table 1 shows the main results found in the scientific literature for the construction of the history of safe use for *C. ensiformis* species as well as the urease-related proteins (JBU, JBURE-IIb and CNTX) and the derived peptide JBTX. This species is among the 71 most important legume plants in human diets, being consumed locally by traditional communities in Africa, Asia, West India, Latin America and India (Akpapunam; Sefa-Dedeh, 1997). The main adverse effects reported for this species were related to the ingestion of Concanavalin A lectin (Con A) which was detected in feeding trials with mammals (Kaefer et al., 2013). Indeed, lectins are hemagglutinins, known to be classical anti-nutritional and toxic proteins, that when ingested cause harmful local and systemic reactions in the consumers (Vasconcelos; Oliveira, 2004). Despite the common origin, *C. ensiformis* ureases and JBTX are distinct proteins/peptide from Con A, and they do not present lectin activity. Several studies with the ureases and the peptide were also gathered. CNTX is lethal to mice but only if administered by intraperitoneal route (Carlini; Guimarães, 1981; Carlini et al., 1984). JBU and CNTX are toxic to insects partly due to the release of a 10 kDa peptide from the native proteins by cathepsin-like enzymes present in the insect midgut (Piovesan et al., 2008; Defferrari et al., 2011; Stanisçuaski et al., 2010; Stanisçuaski; Carlini, 2012), which potentially contribute to increase the specificity of their toxic activity against insects. It is important to mention that the JBTX peptide did not cause acute toxicity in rodents, either when administered intraperitoneally in mice or orally in neonatal rats (Mulinari et al., 2007). Thus, the HOSU of *C. ensiformis* ureases and JBTX do not show greater risks associated to these molecules.

3.2. Bioinformatics results

In silico analysis of the amino acid sequences of JBTX peptide and the JBU and JBURE IIb proteins carried out in the NCBI nr database did not identify relevant match to any known allergenic, toxic and/or anti-nutritional proteins (Table 2). In detail, JBTX, JBU and JBURE IIb showed similarity to several hypothetical proteins with > 50% identity, but these proteins are mostly ureases and no adverse effects were found related to them. Likewise, when the limiting keyword “toxin” was used many results were found with > 50% identity with alpha-, beta- and/or gamma subunits of ureases from different bacteria species and strains. When the keywords “Allergen” and “Antinutrient” were used, no significant results were obtained.

The prediction of allergenicity performed in allergen protein databases (AllergenOnline and SDAP) showed no identity > 50% for JBTX peptide, JBU and JBURE IIb proteins to any allergenic protein by using the Full-FASTA search parameter (Table 3). According to Aalberse (2000) it is likely that a protein will cross-react or share the same epitopes only if they have between 50 and 70% similarity throughout their amino acid sequence. Using the FAO/WHO identity criterion > 35% in a 80 aa sliding window, the peptide and the JBU did not show identity with any known allergenic proteins (Table 1), differently from the JBURE IIb isoform which showed similarity with a protein in the SDAP database, where 6 alignments corresponded to the Ory s 33 kDa allergen of the *Oryza sativa* species, and in AllergenOnline, where an alignment corresponded to the allergen Enolase 2, allergen of the *Hevea brasiliensis*.

The similarity with allergenic proteins was also analyzed using as a parameter searches of six, seven and eight identical contiguous amino acids (Table 3). The six and seven searches were carried out only in SDAP. Using as a parameter the identity of six contiguous amino acids, JBU revealed similarity to seven known allergens being: Per a 3.0201 (*Periplaneta americana* partial allergen), Har a 2.0101 (*Harmonia*

Table 2

Protein matches for the full-length amino acids sequences of Jaburetox (JBTX) peptide, jack bean urease (JBU) and JBU isoform IIB (JBURE IIB) in the NCBI non-redundant (nr) protein sequences database^a.

Protein/peptide	Protein matched; ID; [Species]	Protein name ^b	Adverse effects reported ^c
JBTX	Hypothetical protein CR513.30655; ID: RDX87828.1; [<i>Mucuna pruriens</i>]	Uncharacterized protein	–
	Hypothetical protein TSU_325140; ID: GAU41300.1; [<i>Trifolium subterraneum</i>]	NA ^d	NP ^e
	Hypothetical protein GLYMA_11G248700; ID: KHR31453.1; [<i>Glycine max</i>]	NA	NP
	Hypothetical protein TanjilG_25242; ID: OIW01134.1; [<i>Lupinus angustifolius</i>]	Urease	– ^f
	Hypothetical protein Ahy_A10g050566 isoform B; ID: RYR35419.1; [<i>Arachis hypogea</i>]	NA	NP
	Hypothetical protein Ahy_A10g050566 isoform A; ID: RYR35418.1; [<i>Arachis hypogea</i>]	NA	NP
	Hypothetical protein LR48_Vigan03g314300; ID: KOM39761.1; [<i>Vigna angularis</i>]	Urease	–
	Hypothetical protein PHAVU_001G260000g; ID: XP_007163737.1; [<i>Phaseolus vulgaris</i>]	Urease	–
	Hypothetical protein CR513.22907, partial; ID: RDX94687.1; [<i>Mucuna pruriens</i>]	Uncharacterized protein	–
	Hypothetical protein Ahy_B10g101599 isoform A; ID: RYQ82990.1; [<i>Arachis hypogea</i>]	NA	NP
	Hypothetical protein Ahy_B10g101599 isoform B; ID: RYQ82988.1; [<i>Arachis hypogea</i>]	NA	NP
	Hypothetical protein Ahy_B10g101599 isoform C; ID: RYQ82989.1; [<i>Arachis hypogea</i>]	NA	NP
	Hypothetical protein DVH24_041023; ID: RXH79876.1; [<i>Malus domestica</i>]	NA	NP
	Hypothetical protein PHAVU_001G260000g; ID: XP_007163737.1; [<i>Phaseolus vulgaris</i>]	Urease	–
	BnaA02g13220D; ID: CDY12654.1; [<i>Brassica napus</i>]	Urease	–
	Unamed protein product; ID: VDD22196.1; [<i>Brassica oleracea</i>]	Uncharacterized protein	–
	BnaC02g45820D; ID: CDY51608.1; [<i>Brassica napus</i>]	Urease	–
	Unamed protein product; ID: VDC87778.1; [<i>Brassica rapa</i>]	Uncharacterized protein	–
	Hypothetical protein BRARA_B017; ID: RID74641.1; [<i>Brassica rapa</i>]	Urease	–
	Hypothetical protein C5167_033175; ID: RZC70029.1; [<i>Papaver somniferum</i>]	NA	NP
	Hypothetical protein POPTR_0086177900; ID: PNT25284.2; [<i>Populus trichocarpa</i>]	NA	NP
	Hypothetical protein GLYMA_08G103000; ID: KRH42647.1; [<i>Glycine max</i>]	Uncharacterized protein	–
	Hypothetical protein AALP_AA2G093800; ID: KFK41162.1; [<i>Arabis alpina</i>]	Urease	–
	Hypothetical protein AQUCO_01200284v1; ID: PIA50886.1; [<i>Aquilegia coerulea</i>]	Urease	–
	Hypothetical protein EUGRSUZ_B03469; ID: KCW86884.1; [<i>Eucalyptus grandis</i>]	Uncharacterized protein	–
	Hypothetical protein AQUCO_01200284v1; ID: PIA50887.1; [<i>Aquilegia coerulea</i>]	Urease	–
JBU	Hypothetical protein LR48_Vigan03g314300; ID: KOM39761.1; [<i>Vigna angularis</i>]	Urease	–
	Hypothetical protein PHAVU_001G260000g; ID: XP_007163737.1; [<i>Phaseolus vulgaris</i>]	Urease	–
	Hypothetical protein CRG98_001272; ID: PKI78329.1; [<i>Punica granatum</i>]	Urease	–
JBURE IIB	Hypothetical protein LR48_Vigan03g314300; ID: KOM39761.1; [<i>Vigna angularis</i>]	Urease	–
	Hypothetical protein PHAVU_001G260000g; ID: XP_007163737.1; [<i>Phaseolus vulgaris</i>]	Urease	–
	Hypothetical protein CRG98_001272; ID: PKI78329.1; [<i>Punica granatum</i>]	Urease	–

^a NCBI nr protein database (<https://www.ncbi.nlm.nih.gov/>) results that met the search criteria: E-value < 0.01, shared identity > 50%, alignment size and gap frequency ≤ 6. No limiting keywords were used in the search.

^b Accessed at UniProt database (<https://www.uniprot.org/>) using the ID number.

^c Searched through the combination of the species name source of the protein and the protein name in PubMed (<https://www.ncbi.nlm.nih.gov/pubmed/>).

^d Not Available.

^e Not Performed.

^f Not found.

axyridis allergen), Gly m 6.05101 (allergen subunit of glycinin A3B4 from *Glycine max*), Pas n 10101 (allergen precursor of *Paspalum notatum*), Zea m 1 (*Zea mays* pollen allergen), Ory s 1 (*Oryza sativa* beta-expansin allergen). The JBURE IIB isoform showed similarity with nine allergens, including: Pen ch 35.0101 (*Penicillium chrysogenum* allergen), Tri a 25.0101 (*Triticum aestivum* allergen), Per a 3.0201 (*Periplaneta americana* allergen), Har a 2.0101, Gly m 6.0501, Gly m 6.0401 (Glycine A5A4B3 allergen from *G. max*), Blo t 2.0104 (*Blomia tropicalis* allergen), Ole a 1.0101 (*Olea europaea* allergen), Tri r 4.0101 (*Trichophyton rubrum* allergen) (Table 1). Using as a parameter the identity of seven contiguous amino acids, only JBU and JBURE IIB showed similarity with the allergen Har a 2.0101 from the lady beetle *H. axyridis*.

Using the search criterium of eight contiguous amino acids, for JBTX, JBU and JBURE IIB no match in the searched databases was found (Table 3). Likewise, when the database of the Subcommittee on Allergens Nomenclature of the WHO and International Union of Societies of Immunology was accessed, no allergen report was found concerning *C. ensiformis* species.

In the search of allergen linear motifs, the MEME/MAST analysis tool of Algpred was accessed, and no match to allergen was identified. For Rathinam et al. (2017) linear motifs are characteristic features of allergenic proteins that are difficult to find in non-allergenic proteins.

According to Garrido-Arandia et al. (2014), proteins, carbohydrates and lipids constitute a complex group that aid in the recognition of allergens by the human immune system. The physical, biochemical and immunological attributes of plant allergens have been increasingly

studied; however, no definitive conclusion has been generated about what makes a protein an allergen. In this perspective, the N-glycosylation pattern is a unique attribute of plant allergens that are not found in mammals and may be related to allergenic sensitization (Garrido-Arandia et al., 2014). The search for N-glycosylation sites using the NetNGlyc 1.0 Server program showed the presence of potential N-glycosylation sites for the JBU isoform at positions 31, 233, 572 and 735 and for the JBURE IIB isoform at position 31, 89, 524, 572, 735 and 801. However, no potential N-glycosylation sites for the JBTX peptide were found.

3.3. Entomotoxic mode of action and specificity

For a better understanding of the mode of action of Jaburetox, molecular modeling studies have been performed and they suggested the presence of a β -hairpin motif in peptide's the C-terminal portion, subsequently observed in the JBU's crystallographic structure, suggesting that this motif could be implicated in the JBTX's insecticidal and membrane disrupting activities (Mulinari et al., 2007; Barros et al., 2009). However, site-directed mutagenesis performed on JBTX for deletion of this β -hairpin motif indicated that it is not necessary for the entomotoxic activity of the peptide (Martinelli et al., 2014). Furthermore, NMR studies revealed that this β -hairpin motif disassembles when JBTX is in aqueous medium, where it behaves as an intrinsically disordered protein (IDP) (Lopes et al., 2015). Concerning the entomotoxic mode of action of JBTX, Galvani et al. (2015) were the first

Table 3

Protein/ peptide	Full length FASTA search ^a			80mer sliding window search ^b			Short contiguous amino acids sequences search ^c			Allergen motifs search ^d	N-glycosylation sites ^e Number (position in the aa sequence)
	Identity %			Identity %			Allergen name (Accession number)				
	Allergen name (Accession number) Species			Allergen name (Accession number) Species			Allergen name (Accession number) Species				
	Allergen Online ^g	SDAP ^h	Allergen Online	SDAP	Allergen Online ^f	SDAP	8 aa	6 aa	7 aa	8aa	
JBTX JBU	No matches No matches	No matches No matches	No matches No matches	No matches No matches	No matches No matches	No matches Per a 3.0201 (AAB09632) <i>Periplaneta americana</i> Har a 2.0101 (17291858) <i>Harmonia axyridis</i>	No matches No matches	No matches No matches	No matches Har a 2.0101 (17291858) <i>Harmonia axyridis</i>	No matches No matches	No matches 4 (31, 233, 572 and 735)

(continued on next page)

Table 3 (continued)

Protein/ peptide	Full length FASTA search ^a		80mer sliding window search ^b		Short contiguous amino acids sequences search ^c		Allergen motifs search ^d	N-glycosylation sites ^e Number (position in the aa sequence)
	Identity % Allergen name (Accession number) <i>Species</i>	SDAP ^b	Identity % Allergen name (Accession number) <i>Species</i>	SDAP	Allergen Online ^f	SDAP		
JBURE IIb	No matches	No matches	35.30% Enolase 2 (2-phosphoglycerate) (14423687) <i>Hevea brasiliensis</i>	36.25% Ory s 33 Kd (BAB71741) <i>Oryza sativa</i> 36.25% Ory s 33 Kd (BAB71741) <i>Oryza sativa</i> 37.50% Ory s 33 Kd (BAB71741) <i>Oryza sativa</i> 37.50% Ory s 33 Kd (BAB71741) <i>Oryza sativa</i> 37.50% Ory s 33 Kd (BAB71741) <i>Oryza sativa</i> 36.25% Ory s 33 Kd (BAB71741) <i>Oryza sativa</i> 36.25% Ory s 33 Kd (BAB71741) <i>Oryza sativa</i> 36.25% Ory s 33 Kd (BAB71741) <i>Oryza sativa</i> 36.25% Ory s 33 Kd (BAB71741) <i>Oryza sativa</i>	8 aa	6 aa	7 aa	8aa
	No matches	No matches	35.30% Enolase 2 (2-phosphoglycerate) (14423687) <i>Hevea brasiliensis</i>	36.25% Ory s 33 Kd (BAB71741) <i>Oryza sativa</i> 36.25% Ory s 33 Kd (BAB71741) <i>Oryza sativa</i> 37.50% Ory s 33 Kd (BAB71741) <i>Oryza sativa</i> 37.50% Ory s 33 Kd (BAB71741) <i>Oryza sativa</i> 37.50% Ory s 33 Kd (BAB71741) <i>Oryza sativa</i> 36.25% Ory s 33 Kd (BAB71741) <i>Oryza sativa</i> 36.25% Ory s 33 Kd (BAB71741) <i>Oryza sativa</i> 36.25% Ory s 33 Kd (BAB71741) <i>Oryza sativa</i>	No matches	Pen c35.0101 (ADK47483) <i>Penicillium chrysogenum</i> Tri a 25.0101 (G9LDX4) <i>Triticum aestivum</i> Per a 3.0201 (AAB09632) <i>Periplaneta americana</i> Har a 2.0101 (17291858) <i>Harmonia axyridis</i> Gly m 6.0501 (Q7GC77) <i>Glycine max</i> Gly m 6.0401 (Q9GB11) <i>Glycine max</i> Blot t 2.0104 (A6XEP6) <i>Blomia tropicalis</i> Ole e 1.0101 (S75766) <i>Oleo europea</i> Tri 4.0101 (AAD52012) <i>Trichophyton rubrum</i>	No matches	6 (31, 89, 524, 572, 735 and 801)

^a Full FASTA comparisons using as filter E-value < 0.01 for detection of identity > 50% (Aalberse, 2000).

^b Comparisons using a sliding window of 80 aa (amino acids) for detection of identity > 35% (CODEX, 2009).

^c Search of short sequences of eight, seven or six contiguous aa identical to those of allergenic proteins.

^d Search of allergens motifs based on the MEME/MAST tool using the AlgPred server (<http://www.imtech.res.in/raghava/algpred/>)(Saha and Raghava, 2006).

^e Search of N-glycosylation sites in the primary aa sequence by accessing the NetNGlyc1.0 Server program (<http://www.cbs.dtu.dk/services/NetNGlyc/>).

^f This database does not allow the searching 6 or 7 contiguous amino acid sequences.

^g <http://www.allergenonline.org/>.

^h <http://fermi.utmb.edu/>.

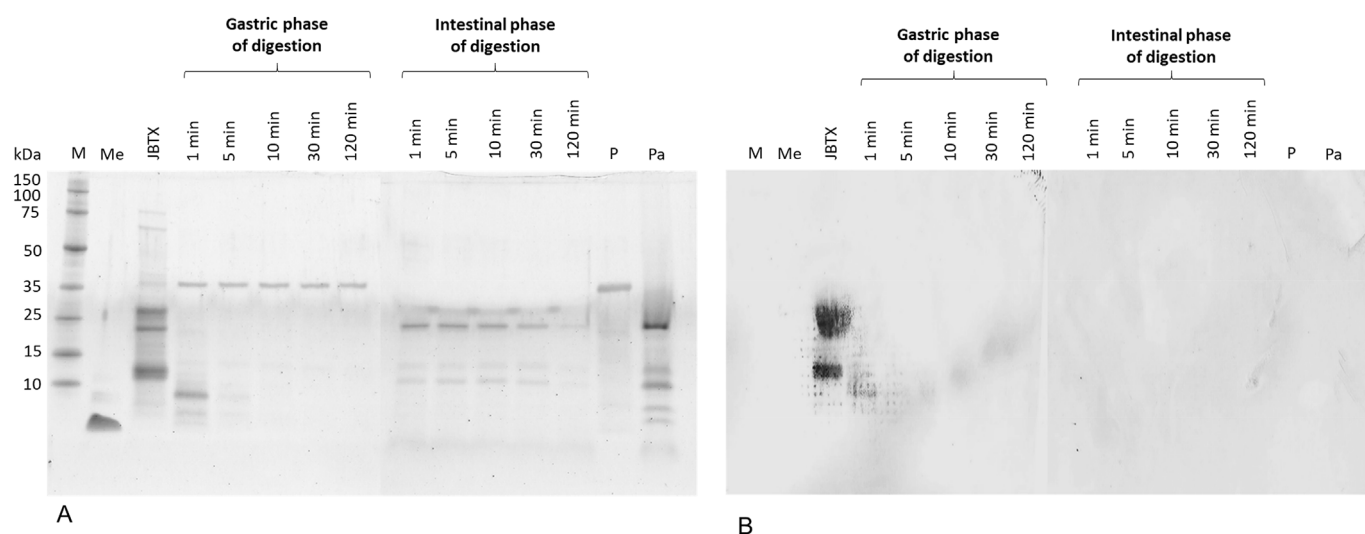


Fig. 1. Tricine-SDS-PAGE (15%) (A) and Western blot (B) of Jaburetox (JBTX) peptide (11.03 kDa) at an initial concentration of 1.2 mg/mL after increasingly high periods of incubation in simulated gastric fluid (gastric phase of digestion) followed by simulated intestinal fluid (intestinal phase of digestion). 24 μ g of JBTX or digested material were loaded into each well. From left to right: M - molecular mass markers (V8491, Promega); Me - Melittin from honey bee venom, 2.85 kDa (M2272, Sigma Aldrich); 1, 5, 10, 30 and 120 min of incubation in simulated gastric fluid and simulated intestinal fluid; P - pepsin; Pa - pancreatin. Within the JBTX lane the protein bands with molecular weights above 11.03 kDa are JBTX aggregates (dimers).

to describe the neurotoxicity of the peptide in the kissing bug *Triatoma infestans*. After feeding on a JBTX-containing diet, the peptide was immunolocalized in the insect's brain, where it caused a pronounced inhibition of NOS (nitric oxide synthase) activity. Furthermore, using immunoprecipitation followed by proteomic analysis, JBTX was found to bind to UAP (UDP-N-acetylglucosamine pyrophosphorylase) in the insect's brain. Later Fruttero et al. (2017) demonstrated that JBTX also targeted the central nervous system of *R. prolixus*, both *in vivo* or *in vitro*, inducing changes of the enzymatic activities of NOS and of UAP, and promoting changes in the levels of UAP and chitin synthase genes expression. The peptide also affected *R. prolixus*' immune system causing hemocyte aggregation and prophenoloxidase activation in a prostaglandin-mediated fashion, thereby rendering the insect more susceptible to bacterial infection (Fruttero et al., 2016; Moyetta et al., 2017). JBTX was able to cause mortality of *Aedes aegypti* larvae (100 μ g JBTX/oral diet, for 6 days) in a feeding assay whereas in a dose as low as of 0.1 μ g it provoked death of *T. infestans* bugs (Becker-Ritt et al., 2017). Finally, JBTX (32 μ g/g) altered the locomotive behavior in cockroach species (*Nauphoeta cinerea*), reducing distance traveled and increasing the standing time; it also induced a maximal neuromuscular blockade of 80.72% ($n = 6$, $p < 0.05$) and showed cardiotoxic effects by decreasing its heart rate. Voltage clamping analyzes of JBTX (200 nM) applied on heterogeneously expressed *Xenopus laevis* oocytes with Nav 1.1 channels evidenced an expressive increase in sodium currents. Thus, it was demonstrated that JBTX acts on complex behavioral alterations starting from the initial activation of the voltage-dependent sodium channels (dos Santos et al., 2019).

In the case of *C. ensiformis* ureases, besides canatoxin, JBU was also found to be toxic to insects (Follmer et al., 2004). Several studies were conducted to characterize the insect's proteinase(s) involved in the proteolytic activation of the ureases that lead to the release of entomotoxic peptides in different insects (Carlini et al., 1997; Ferreira-Da Silva et al., 2000; Stanisquaski et al., 2005; Piovesan et al., 2008; Defferrari et al., 2011; Real-Guerra et al., 2013). Using *R. prolixus* as model, the ureases were shown to disrupt water transport leading to inhibition of diuresis (Stanisquaski et al., 2009), to alter the muscle contractility of the crop (Stanisquaski et al., 2010), to induce hemocyte aggregation (Defferrari et al., 2014a) and to cause the death of the insect through a phospholipase A2-mediated mode of action (Defferrari et al., 2014b). The alterations promoted by JBU in cockroaches

(*Nauphoeta cinerea*) lowered the heart rate and caused behavioral changes, probably by interfering with cholinergic, octopaminergic and GABAergic neurotransmission at the insect's central nervous system (Carrazoni et al., 2016). Finally JBU was shown to modulate neurotransmitter release at insect neuromuscular junctions, probably by disrupting on the Ca^{2+} machinery at the pre-synaptic region of the neurons (Carrazoni et al., 2018). The activity of JBU was also evaluated in six different yeast species: *Candida parapsilosis*, *C. tropicalis*, *C. albicans*, *Kluyveromyces marxianus*, *Pichia membranifaciens* and *Saccharomyces cerevisiae*. JBU inhibited the growth of *C. tropicalis* and *P. membranifaciens* at the lowest dose tested - 0.18 μ M. For the other yeasts, such as *K. marxianus*, the cell culture became more turbid than the control culture in the presence of JBU up to 0.72 μ M, suggesting an increase in growth and absence of antifungal effect. In comparison, the determination of colony forming units from the treated yeasts indicated a fungicidal effect on all species after 24 h of exposure to 0.36 μ M JBU (Postal et al., 2012).

Overall, evidence from literature indicates specific effects of *C. ensiformis* ureases and JBTX towards insects and certain fungi species. Entomotoxic effects seem to be related to interference with synaptic activity. Although studies have been performed in mammals, it did not show signs of toxicity, indicating the specificity of the mode of action towards target species.

3.4. *In vitro* digestibility and thermal stability

Fig. 1A shows the high susceptibility of the JBTX peptide to *in vitro* digestion, with complete degradation of its corresponding peptide band of 11.03 kDa occurring within 10 min of SGF incubation. Bands with molecular weights above 11.03 kDa correspond to the spontaneous formation of JBTX aggregates (dimers) which form even under reducing conditions (Lopes et al., 2015). In addition, minor contaminants from the heterologous peptide expression process may also appear. The protein band corresponding to JBU (around 90 kDa) was also susceptible to pepsin digestion, with complete degradation occurring in less than 5 min of incubation (Fig. 2A). In the JBU lane, some bands with molecular weights below 90 kDa are visible. These are likely urease degradation products, which can either occur naturally in *C. ensiformis* seeds or form during the purification process, being therefore recognized by urease specific antibodies (Fig. 2B). Such products have

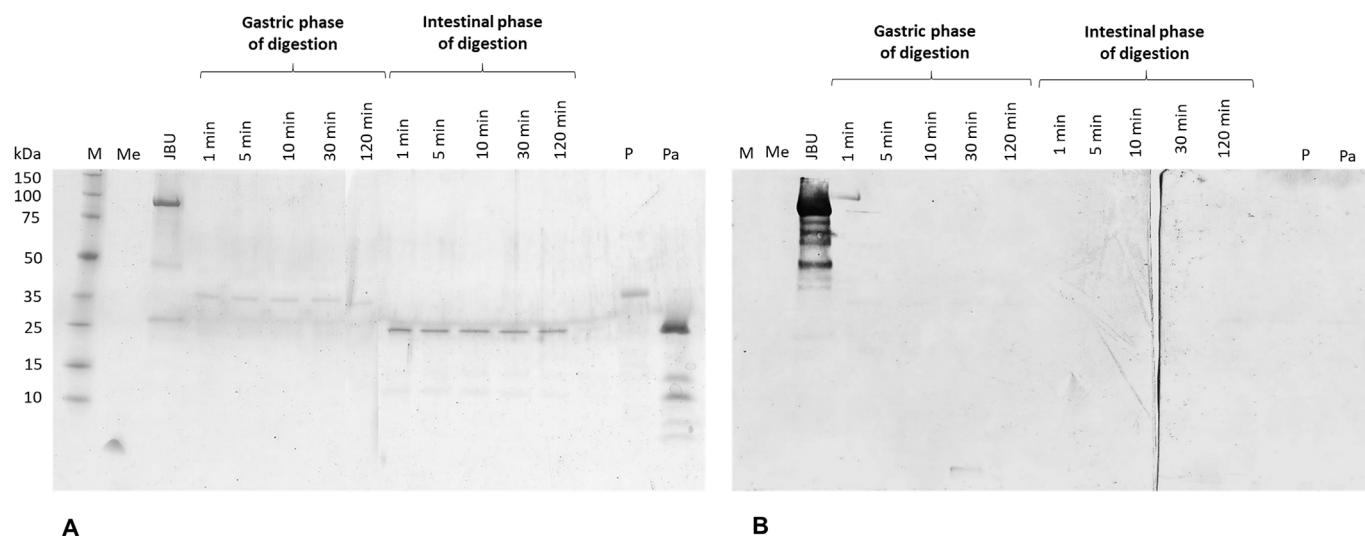


Fig. 2. Tricine-SDS-PAGE (15%) (A) and Western blot (B) of Jack bean (*Canavalia ensiformis*) urease (JBU) (around 90 kDa) at an initial concentration of 1.2 mg/mL after increasingly high periods of incubation in simulated gastric fluid (gastric phase of digestion) followed by simulated intestinal fluid (intestinal phase of digestion). 24 μ g of JBU or digested material were loaded into each well. From left to right: M - molecular mass markers (V8491, Promega); Me - Melittin from honey bee venom, 2.85 kDa (M2272, Sigma Aldrich); 1, 5, 10, 30 and 120 min of incubation in simulated gastric fluid and simulated intestinal fluid; P - pepsin; Pa - pancreatin. Within the JBU lane the protein bands below 90 kDa are urease degradation products.

been previously reported (Dixon et al., 1980; Takishima et al., 1988).

The blotting of digested JBTX and JBU samples with anti-JBTX antibody (Figs. 1B and 2B) confirmed that no protein band could be recognized after 10 and 5 min of pepsin incubation, respectively.

Collectively, digestibility studies do not comprise a direct correlation between allergenicity and resistance to digestion (Bøgh; Madsen, 2016). However, when this test is associated with other characteristics of the protein, such as amino acid sequence similarities, it has been pointed out as suitable for assessing the allergenicity potential of a new protein (Codex, 2009). One particularity shared by many food allergens is the ability to resist to gastric and intestinal digestion. Proteins that are resistant to this process have a high probability of reaching the intestinal mucosa where the absorption occurs. Thus, the ability to remain intact in the gastrointestinal tract of monogastric animals is considered a condition for allergenicity (Do et al., 2016).

Most proteins, including the toxic ones, are sensitive to degradation by digestive enzymes, losing their biological activity. After digestion, their amino acids are used for nutritional purposes, similar to dietary proteins (Delaney, 2017).

We have accessed the thermal stability of JBU by evaluating its capacity to retain its urease activity after incubation at different temperatures for 60 min. Fig. 3 shows that JBU is able to remain almost fully active when incubated at up to 30 °C. It starts to lose some activity between the temperatures of 40–60 °C (around 15%) which indicates some degree of denaturation. Above 60 °C the loss of activity is more prominent, retaining only about 50% of its activity at the temperature of 70 °C, dropping to less than 2% at 80 °C and reaching zero at temperatures above 90 °C. Urease is considered an undesirable enzyme for many crops, including soybean (*Glycine max*), since its presence can lead to intoxication by ammonia when urea is used as an additive in animal feed (Zverev; Sesikashvili, 2018). Fortunately, ureases as well as other antinutritional components of these crops, such as protease inhibitors, lipoxygenases and lectins, are thermolabile molecules and heat treatment is an accessible and relatively cheap way to attain their inactivation. The urease from the Syrian mesquite (*Prosopis farcta*) has been shown to be stable when incubated for 40 min at temperatures ranging from 20 to 35 °C, reaching 50% of inactivation at temperatures near 60 °C and being completely inactivated at temperatures above 80 °C (Hamzah, 2014). A urease from the pigeon pea (*Cajanus cajan*) has also been tested for its thermal stability, showing a 50%

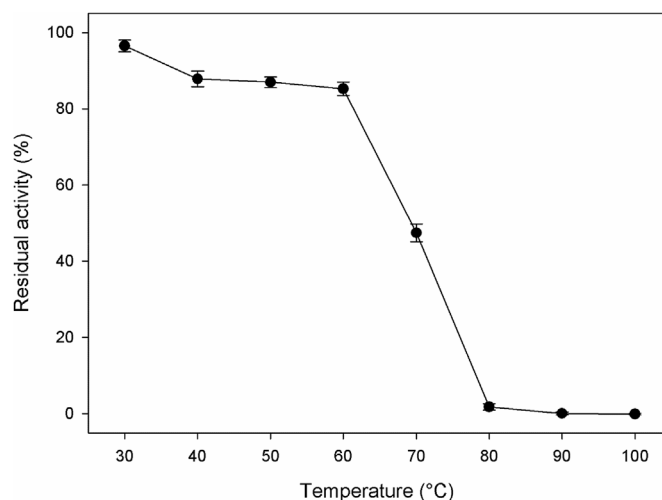


Fig. 3. Thermal stability of Jack bean (*Canavalia ensiformis*) urease (JBU). Urease activity was tested after JBU (10 μ g) was incubated for 60 min at temperatures ranging from 30 to 100 °C. Data is shown as mean $\pm$ standard error from four independent assays.

inactivation after 12 min of incubation at 75 °C (Arya, 2013). At last, the urease from soybean (*G. max*), which is a very closely related homologous from the *C. ensiformis* urease, has also been analyzed for its thermal stability, being completely inactivated after incubation for 15 min at 90 °C (Zverev; Sesikashvili, 2018), 60 min at 75 °C (Reshetnik, 2007), and 45 min at 100 °C (Savage et al., 1995), in a very similar fashion as we have observed here for the jack bean urease. Therefore, the data suggests that in spite of a relative thermal resistance JBU and its counterparts from plant sources, can be completely inactivated by heat treatment.

As to the thermal stability of JBTX, Lopes et al. (2015) were able to structurally characterize this peptide through several techniques, including CD and NMR spectroscopy. On the referred work, JBTX has been classified as an IDP and shown to present an overall low propensity to assume a stable secondary structure. Despite this fact it was possible to identify some secondary elements in its structure, such as an alpha-helical motif in its N-terminal portion, as well as two turn-like

structures, one located at the central portion, and one close to the C-terminal portion of the peptide. The presence of such structural elements is consistent with the observed structural transition suffered by JBTX in the thermal stability assays performed by the thermofluor technique. This assay revealed that JBTX undergoes unfolding process at an average melting temperature of approximately 55 °C, corroborating the hypothesis that JBTX is not a completely unfolded protein and indicating the existence of some degree of folding. This assay has also shown that the denaturing profile of JBTX is largely a pH-independent process. Some structural transition upon heating was also observed on the CD spectroscopy analysis under similar conditions, however this structural loss suffered by JBTX seemed to be reversed if the protein was properly cooled down. The same renaturation process was not tested with the thermofluor technique. It is worth mentioning that despite the observation of a denaturing process under heating conditions, the contribution of these structured portions of JBTX for its toxicity to insects is not yet clear. Some computational predictions have suggested that JBTX could acquire a much more structured form, however the conditions under which it could happen are yet to be shown. It is also hypothesized that the polypeptide could change its structure under contact with membrane constituents, given all the properties observed for the peptide in these conditions (Barros et al., 2009; Martinelli et al., 2014; Piovesan et al., 2014), which has already been shown for other IDPs (Tompa, 2012). On the other hand, such structural rearrangements could not take place since, by definition, IDPs do not require folding to perform their activities. This illustrates that thermal stability of JBTX is not a simple matter and much more studies will be necessary to clearly explain this variable.

Except for the evaluation of thermal stability (performed for JBU), acute toxicity in rodents and measurements of expression levels of the recombinant protein in transgenic plants, the other tests suggested by the ILSI approach were performed. Although there is a recommendation from some regulatory agencies for performing oral acute toxicity test in rodents with insecticidal proteins, we did not perform it because there are already published data reporting that JBTX and JBU are not toxic to mammals when administered by this route (Mulinari et al., 2007). This was reinforced by the data obtained in the present work which showed that these proteins are digested by gastrointestinal proteases. It is important to highlight that the set of tests performed, mostly *in silico*, provide preliminary evidence about the safety of JBTX.

4. Conclusions

The data gathered from the risk assessment of the insecticidal peptide Jaburetox supported its safe use in the context of agricultural biotechnology, considering its great potential for the development of transgenic plants resistant to insect and fungal attack. The history of safe use revealed no hazard reports for the peptide and proteins studied. *C. ensiformis* ureases and JBTX possess selective activity against insects and fungi, and JBTX primary amino acid sequence showed no relevant similarity to any known toxic, antinutritional or allergenic proteins. Additionally, JBTX was susceptible to digestion in simulated gastric fluid, with complete degradation occurring within 10 min of incubation. The results did not identify potential risks of adverse effects and reactions (allergy, toxicity and antinutritional) associated to JBTX that might suggest potential health risks to humans. However, further allergen (e.g. serum IgE binding test) and toxicity (e.g. rodent toxicity tests) experimentation can be done to get additional safety information on JBTX, and to meet regulatory inquiries for commercial approval of transgenic cultivars expressing this peptide.

Author contributions

Chayenne Alves Sá: Investigation, Writing - Original Draft, Writing - Review & Editing. Leonardo Rogério Vieira: Investigation. Luiz Carlos Pereira Almeida Filho: Investigation. Rafael Real-Guerra: Investigation,

Writing - Review & Editing. Fernanda Cortez Lopes: Investigation, Data Curation, Writing - Review & Editing. Terezinha Maria Souza: Data Curation, Writing - Review & Editing. Ilka Maria Vasconcelos: Conceptualization, Writing - Review & Editing. Fernanda Staniscuaski: Conceptualization, Data Curation, Writing - Original Draft. Célia Regina Carlini: Conceptualization, Writing - Review & Editing. Ana Fontenele Urano Carvalho: Resources, Supervision, Writing - Review & Editing. Davi Felipe Farias: Resources, Supervision, Conceptualization, Writing - Original Draft, Visualization, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.fct.2019.110977>.

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Supplementary Material

- A** >pdb[2MM8]A Chain A, Structural And Biochemical Characterization Of Jaburetox
MGPVNEANCKAAMEIVCRREFGHKEEEDASEGVTTGDPDCPFTKAIPREEYA
NKYGPTIGDKIRLGDITDLIAEIEKDFALYGDESVEFGGGKVIHHHHHH
- B** >AAA83831.1 urease [Canavalia ensiformis]
MKLSPREVEKLGHLNAGYLAQKRLARGVRLNYTEAVALIASQIMEYARDGE
KTVAQLMCLGQHLLGRRQVLPAVPHLLNAVQVEATFPDGTIKLTVHDPISRE
NGELQEALFGSLLPVPSLDKFAETKEDNRIPGEILCEDECLTLNIGRKAVILKVT
SKGDRPIQVGSHYHFIEVNPYLTFDRRKAYGMRLNIAAGTAVRFEPGDCKSVT
LVSIEGNKIVIRGGNAIADGPVNEINLEAAMHAVRSKGFGEHEEKDASEGFTK
EDPNCNPFNTFIHRKEYANKYGPTTGDKIRLGDITNLLAEIEKD YALYGDECVEG
GGKVIRDMGQSCGHPPAISLDTVITNAVIDYTGIKADIGIKDGLIASIGKAG
NPDIMNGVFSNMIIGANTEVIAGEGLIVTAGAIDCHVHYICPQLVYEAISSGITT
LVGGGTGPAAGTRATTCTPSPTQMRLMLQSTDYLPNFGFTGKGSSSKPDELH
EIKAGAMGLKLHEDWGSTPAIDNCLTAEHHDQNIHTDITLNEAGFVEHSI
AAFGRITHTYHSEGAGGGHAPDIKVCCKNVLPSSNTNTRPLTSNTIDEHLD
MLMVCHHLDREIPEDLAEFHSRIRKKTAAEDVLNDIGAISSSDSQAMGRVG
EVISRTWQTADPMKAQTGPKCDSSDNDNFRIRRYIAKYTINPALANGFSQYV
GSVEVGKLADLV MWKPSFFGTPKPEMVIKGGMVAVADIGDPNASIPTPEPVKM
RPMYGTLGKAGGALSIAFVSKAALDQRVNVLYGLNKRVEAVSNVRKLTCLD
MKLNDALPEITVDPESYTVKADGKLLCVSEATTVPLSRNYFLF
- C** >ACL14297.1 urease JBURE-IIB [Canavalia ensiformis]
MKLSPREVEKISLHNAGFLAQKRLARGVRLNYSSEVALIASQILEHARDGEKTV
AQLMSIGKHLLGRRQVLPAVPHLLNIIQVEATLPNGTKLTVHDPIDIANENGDL
EALYGSFLPVPSLDKFAESKEEHKIPGEICADGRITLNPGRKAVFLKVVNHGDR
PIQVGSHYHFIEVNPYLTFDRRKAYGMRLNIAAGDSVRFEFGDHKTIVNLVSI
NKIIRGGNAIADGPVNEANCKAAMEIVCRREFGHKEEEDASEGVTTGDPDCP
TKAIPREEYANKYGPTIGDKIRLGDITDLIAEIEKDFALYGDESVEFGGGKVIR
DMGQSSGHPPAMSLDTVITSVIDYTGIKADIGIKDGLIASIGKAGNPDIMNGV
FPMIIGVNTFVICGEGGLIVTAGGIDCHVHYICPQSLDEAISSGITT VVGSGTGPT
DGSRATTCTPAPTQMKLMLQSTDIDPLNFGFTGKGSGSHPDDELHEIKAGAMGL
KLHEDWGSTPAIDNCLAVAEQHDQVNIHTDITVNESGFVEHTIAAFNGRITHT
YHSEGAGGGHAPDIKVCCKNVLPSSNTNTRPLTSNTVDEHLDMLMVCHKL
NREIPEDLAEASSRVREQTAAEDLHDIGGISIISDAQAVGRIGEVISCTWQTAD
KMKAEGRPLQPDGSDNDNFRIRRYIAKYTINPAIVNGISQYVGSVEVGKLADLV
IWKPSFFGAKPDIVIKGGSIAWADMGDPNPSIPTPEPVLMRPMYGTLGKAGSAL
SIAFVSKAALDLGVKVLYGLNKRVEAVSNVRKLTCLDLKLNNSLPEITVCPET
TVTVDGQALSSEAVTILPLSQNYFIF

Figure S1. Amino acids sequences of the (A) Jaburetox peptide, (B) Jack bean (*Canavalia ensiformis*) urease and (C) Jack bean (*C. ensiformis*) urease isoform Iib.