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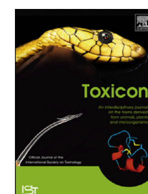

Role of lysine and acidic amino acid residues on the insecticidal activity of Jackbean urease

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A B S T R A C T

Canavalia ensiformis has three isoforms of urease: Jackbean urease (JBU), Jackbean urease II and canatoxin. These isoforms present several biological activities, independent from the enzymatic property, such as entomotoxicity and antifungal properties. The entomotoxic activity is a property of the whole protein, as well as of a 10 kDa peptide released by insect digestive enzymes. Here we have used chemical modification to observe the influence of lysines and acidic residues on JBU enzymatic and insecticidal activities. Chemical modification of lysine residues was performed with dimethylamine–borane complex and formaldehyde, and acidic residues were modified by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and ethylenediamine. Derivatized ureases, called JBU-Lys (lysine-modified) and JBU-Ac (acidic residues-modified), were assayed for their biochemical and insecticidal properties. Neither modification altered significantly the kinetic parameters analyzed, indicating that no residue critical for the enzyme activity was affected and that the modifications did not incur in any significant structural alteration. On the other hand, both modifications reduced the toxic activity of the native protein fed to *Dysdercus peruvianus*. The changes observed in the entomotoxic property of the derivatized proteins reflect alterations in different steps of JBU's toxicity towards insects. JBU-Ac is not susceptible to hydrolysis by insect digestive enzymes, hence impairing the release of toxic peptide(s), while JBU-Lys is processed as the native protein. On the other hand, the antidiuretic effect of JBU on *Rhodnius prolixus* is altered in JBU-Lys, but not in JBU-Ac. Altogether, these data emphasize the role of lysine and acidic residues on the insecticidal properties of ureases.

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1. Introduction

Ureases (urea amidohydrolase; EC 3.5.1.5) are nickel-dependent enzymes that catalyze the hydrolysis of urea to ammonia and carbon dioxide (Dixon et al., 1975). Ureases have been isolated from a wide variety of organisms including plants, fungi and bacteria. In plants, ureases are homotrimers or homohexamers of a ~90 kDa subunit and supposedly participate in the use of urea as nitrogen source (Carlini and Polacco, 2008). Evidences pointing to a possible involvement of ureases in the plant defense against some insect pests and phytopathogens have been

Abbreviations: CNTX, canatoxin; JBUre-I, jackbean urease; JBUre-II, jackbean urease isoform II.

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documented (Carlini and Grossi-de-Sa, 2002; Carlini and Polacco, 2008; Staniscuaski and Carlini, 2012). Thus, newly described properties of plant and microbial ureases, such as entomotoxic and fungitoxic activities, have widened the proposed physiological roles of ureases (Real-Guerra et al., 2013).

In *Canavalia ensiformis* (Leguminosae) three urease isoforms were identified: Jackbean urease (JBU), Jackbean urease II (JBUre-II) and canatoxin (CNTX). These proteins were shown to present several biological effects, including toxicity to insects and fungi (Becker-Ritt et al., 2007; Follmer et al., 2004; Mulinari et al., 2011; Postal et al., 2012; Staniscuaski et al., 2005, 2009, 2010). These biological activities are completely independent from the ureolytic activity (Follmer et al., 2004; Mulinari et al., 2011; Postal et al., 2012). Elucidation of which domain is related to each biological activity could lead to the development of several urease-based biotechnological tools. One of the biologically active domains of Jackbean ureases, the one responsible for its insecticidal activity, has been identified. It is a ∼10 kDa fragment released by cleavage promoted by insect digestive enzymes (Carlini et al., 1997; Ferreira-DaSilva et al., 2000). This peptide, called pepcanatox, was isolated following *in vitro* hydrolysis of CNTX by a *Callosobruchus maculatus* (bruchid beetle) gut homogenate (Ferreira-DaSilva et al., 2000). A recombinant peptide equivalent to pepcanatox was developed from the JBUre-II corresponding sequence, and named Jaburetox (Jackbean urease toxin) (Mulinari et al., 2007). This peptide was lethal to several insects, such as *Dysdercus peruvianus*, *Spodoptera frugiperda*, *Blattella germanica*, *Rhodnius prolixus* and *Triatoma infestans*, but it was innocuous when injected or ingested by mice and neonate rats (Mulinari et al., 2007; Tomazetto et al., 2007). For simplicity reasons, the term Jaburetox will be used here as synonymous of *C. ensiformis* urease entomotoxic peptides, regardless of their origin (JBU or JBUre-II). It is worth mention that, within the entomotoxic peptide region, JBU and JBUre-II present 74 and 82% of sequence identity and similarity, respectively. The mode of action of Jaburetox, as well as that of urease, is not fully understood. JBU and Jaburetox are capable of altering the serotonin-induced secretion of insects Malpighian tubules, indicating an effect on the osmotic balance of the insects (Staniscuaski et al., 2009), both *ex vivo* and *in vivo* (Carlini et al., 1997). JBU also can alter the secretion and contraction patterns of the anterior midgut in *R. prolixus* (Staniscuaski et al., 2010).

Chemical modification of amino acids residues can provide essential information about protein structure and functions. For JBU, this approach has been used to demonstrate the influence of histidine residues in the copper-induced oligomerization of JBU, and how this affected its ureolytic and insecticidal activities (Follmer and Carlini, 2005). In this work, we have performed chemical modification of lysine, aspartic and glutamic acid residues of JBU aiming to characterize the influence of these residues on its enzymatic and insecticidal activities. The data gathered deepened our knowledge on ureases and will help in the future development of biotechnological applications using these proteins (or their isolated domains) for plant protection against pests and pathogens.

2. Material and methods

2.1. JBU

C. ensiformis urease Type III was purchased from Sigma–Aldrich. An additional step of gel-filtration in a Superdex 200 Column (GE Healthcare), equilibrated in 50 mM HEPES, 250 mM NaCl, pH 7.5, was used to obtain the protein in homogeneity conditions. The purity of JBU was checked by SDS-PAGE electrophoresis. Protein content of samples was determined by their absorbance at 280 nm (A_{280}). The extinction coefficient value ($\varepsilon_{280} = 54{,}780\ M^{-1}\ cm^{-1}$) was calculated using the ProtParam tool (http://au.expasy.org/tools/protparam.html).

2.2. Chemical modification of carboxylic groups

The methods of Hoare and Koshland (1966) and Pho et al. (1977), were followed with few adaptations. Urease (1 mg/mL), in 200 mM phosphate buffer, pH 7.0, was mixed with a solution of ethylenediamine, in phosphate buffer, with the pH previously adjusted to 7.0 using phosphoric acid. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) was added to the solution in two steps, separated by 12 h, and the reaction proceeded under constant stirring at 4 °C, for 24 h. The experimental condition for JBU modification was a molar ratio of 1:100:500 (protein acidic residues:EDC:ethylenediamine). The protein solution was then exhaustively dialyzed against 20 mM sodium phosphate, 150 mM NaCl, pH 7.5, for removal of the excess of reagents. After dialysis, the homogeneity of the derivatized protein was verified by gel-filtration in a Superdex 200 Column (GE Healthcare), equilibrated in 20 mM Tris–HCl, 200 mM NaCl, pH 7.5. The modified protein, herein called JBU-Ac, was stored at 4 °C until use in the subsequent assays.

2.3. Chemical modification of lysine residues

The methylation of lysine residues was performed according to Walter et al. (2006). Briefly, the reaction was carried out in 50 mM HEPES (pH 7.5), 250 mM NaCl at protein concentration of 1 mg/mL. Twenty microliters of freshly prepared 1 M dimethylamine–borane complex (ABC; Sigma–Aldrich) and 40 μL of 1 M formaldehyde were added per mL of protein solution. The reaction was incubated at 4 °C for 2 h. The addition of ABC and formaldehyde was repeated and the incubation proceeded for another 2 h. After a final addition of 20 μL of ABC, the reaction was incubated overnight at 4 °C, under constant stirring. At the end of the reaction, the derivatized protein was submitted to gel-filtration in a Superdex 200 Column (GE Healthcare), equilibrated in 20 mM Tris–HCl, 200 mM NaCl, pH 7.5, to remove the excess of the modifying reagents and to verify the homogeneity of the protein. The modified protein, herein called JBU-Lys, was stored at 4 °C until use in the subsequent assays.

2.4. Analysis of the chemical modifications

The extension of chemical modification of lysine and acidic residues was monitored by the analysis of free

amines content in the protein samples, according to Pradel and Kassab (1968). Quantification was performed using a glycine standard curve (as reported by Harkouss et al. (2012)). Briefly, 5 μL of 5 mM fluorescamine (Sigma–Aldrich) in methanol was added to JBU samples (diluted to 0.1 mg/mL, in 20 mM NaPB pH 8.0, final volume of 100 μL). One hour after the reaction started, the fluorescence was monitored in a Spectra-Max microplate reader (Molecular Devices), with excitation wavelength at 390 nm and emission at 465 nm. The non-specific fluorescence of corresponding fluorescamine-untreated samples was subtracted.

2.5. Ureolytic activity assay

To determine urease activity, samples (10 μg) were incubated with urea (0.01–55 mM) for 10 min at 37 °C, in 50 mM sodium phosphate buffer (pH 7.5). The ammonia released from the hydrolysis of urea was measured colorimetrically using the phenol-hypochlorite method (Weatherburn, 1967). One unit of urease was defined as the quantity of protein that releases 1 μmoL of ammonia per minute, at 37 °C, pH 7.5. Kinetic parameters (K_m, V_{max} and K_{cat}) were calculated as in Cleland (1979) from three independent measurements. The hexameric form of JBU with a molecular mass of 540.000 Da was considered for K_{cat} calculations.

2.6. Insecticidal activity

The insecticidal activity of JBU and the derivative ureases was evaluated in feeding trials with the cotton stainer bug *D. peruvianus* (Hemiptera), as described in Staniscuaski et al. (2005). Briefly, JBU and its derivatives were fed to the insects by adding the freeze-dried protein (at final concentration of 0.1% w/w) to their cotton seed meal diet. The toxicity was expressed as daily survival rate during a period of 17 days.

2.7. JBU *in vitro* hydrolysis

For the *in vitro* hydrolysis of JBU, a homogenate of *D. peruvianus* intestines was used as source of proteolytic enzymes as described by Staniscuaski et al. (2005). Briefly, whole intestines of fourth instars nymphs were removed, homogenized, and centrifuged at 4 °C at 12,000 × *g* for 5 min. The supernatant was kept frozen at −20 °C until the enzymatic assays. To determine the enzymatic activity, the homogenate (protein final concentration of 1.0 unit of absorbance at 280 nm) was incubated with azocasein (final concentration of 0.5%). One unit of enzymatic activity was defined as the amount of enzyme releasing 1.0 unit of absorbance at 420 nm (A_{420}) of acid-soluble peptides per hour at 37 °C, at pH 5.6.

Digestion of JBU with *D. peruvianus* proteinases was performed as described by Piovesan et al. (2008), using a ratio of 0.5 mU of homogenate to 1.0 μg of urease, incubated in 5 mM ammonium formate, pH 5.6, at 37 °C, under continuous stirring. The enzyme preparation was added to the urease solution in two aliquots, separated by a 12 h interval. The reaction was stopped by freeze-drying the samples. The hydrolysis was analyzed by SDS-PAGE on gradient gels (8–20%).

2.8. Structural analysis

The 3D structure of JBU (PDB ID: 3LA4; Balasubramanian and Ponnuraj, 2010) was downloaded from the Protein Data Bank (http://www.rcsb.org). The PyMOL Molecular Graphics System (Schrödinger, LLC) was used to visualize the structure of JBU, to localize specific amino acids residues and domains within the protein and to generate the figures.

2.9. Antidiuretic activity

The effect of the chemical modifications on JBU activities on weight loss and Malpighian tubules secretion were assessed using *R. prolixus* as a model. The insects were kindly provided by Dr. Hatisaburo Masuda and Dr. Pedro L. Oliveira, Institute of Medical Biochemistry, Universidade Federal do Rio de Janeiro, RJ, Brazil. Insects (4th instars) were fed on saline solution containing 1 mM ATP, supplemented with buffer or the test proteins (dose of 2 μg/mg of insect), for 15 min and weighted right after. Weight loss was assessed at 0, 1.5, 3, 20, 24 and 48 h after feeding.

The Ramsay assay with Malpighian tubules was used to evaluate the fluid secretion rate, performed as described by Staniscuaski et al. (2009).

2.10. Statistical analysis

Results are expressed as mean ± standard error. Significance of differences between means was determined using ANOVA followed by Dunnett test (GraphPad Instat software). Data were considered statistically different when $p < 0.05$. Detailed information for each assay is given in the figures captions.

3. Results

3.1. Chemical modification of lysine and acidic amino acid residues

After the derivatization reaction, more than 90% of JBU-Lys or JBU-Ac was recovered. The extension of the modifications in the derivatized proteins was evaluated from the analysis of their free amine content, which was expected to be increased in JBU-Ac and decreased in JBU-Lys. The fluorescence of the fluorescamine-treated proteins (Fig. 1) indicated the modification of 14 lysines in JBU-Lys, out of a total of 49 found in JBU, and of 22 acidic residues in JBU-Ac, from a total of 99 found in the native protein. Similar numbers of modified residues were detected after two independent modification assays for each derivatized protein.

3.2. Measurement of kinetic parameters

In order to analyze the effect of lysine and acidic residues modification on the ureolytic activity of JBU, the kinetic parameters (K_m, V_{max} and K_{cat}) of native and derivatized JBU were calculated (Supplementary Table 1). No significant

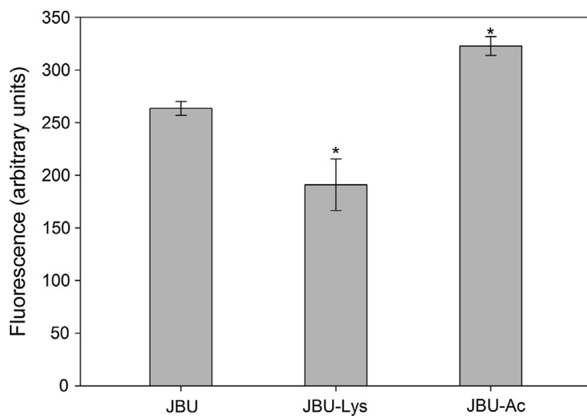

Fig. 1. Free amine content of proteins after chemical modifications. Samples were incubated with fluorescamine and fluorescence was monitored (excitation at 390 nm and emission at 465 nm). JBU, Jackbean urease; JBU-Lys, lysine-modified JBU; JBU-Ac, acidic residues-modified JBU. Data are mean ± standard errors of two independent experiments, performed in triplicates ($n = 6$). *Indicates values statistically different ($p \leq 0.05$) from the native protein JBU.

alterations of these parameters were observed for both modified proteins, in comparison to the native JBU.

3.3. Entomotoxic activity

As previously described (Follmer et al., 2004), JBU is highly toxic to the cotton stainer bug *D. peruvianus*, with a LD_{50} value of 0.017% (w/w) of protein added to the cotton meal, when administrated in feeding trials. Here, we have used both native and the two derivatized JBU to verify the effect of the modifications upon the insecticidal activity. Both chemical modifications affected the entomotoxic activity of JBU, drastically reducing this effect (Fig. 2). After 17 days, the survival rate for JBU-fed groups was reduced to 18% of the control group, while JBU-Lys and JBU-Ac-fed groups survival rates were 46% and 58%, respectively (Fig. 2, inset). There was no statistical difference between the lethalities observed for JBU-Ac and JBU-Lys when compared to each other.

3.4. *JBU* in vitro *digestion*

It was previously demonstrated that an essential step for the entomotoxic effects of plant ureases is their hydrolysis by insects' digestive enzymes, releasing toxic peptides (Carlini et al., 1997; Defferrari et al., 2011; Ferreira-DaSilva et al., 2000; Piovesan et al., 2008). The *in vitro* digestion of JBU with *D. peruvianus* enzymes resulted in the release of several fragments from the protein, including peptide(s) in the 10 kDa range, as expected (Fig. 3, lane 2). When the derivatized proteins were subjected to the same digestion process, JBU-Lys showed no alteration in the pattern of the released fragments (Fig. 3, lane 4) when compared to the native protein. In contrast, JBU-Ac was resistant to hydrolysis by the gut homogenate, thus preventing the release of the toxic peptide(s) (Fig. 3, lane 6).

Analysis of the location of the entomotoxic peptide (Jaburetox) within JBU sequence showed two aspartic acid residues flanking this region (Fig. 4). The three dimensional structure of the trimeric JBU revealed that Asp-229 (at the N-terminal of Jaburetox) is localized at the protein surface and therefore is potentially susceptible to chemical modification (Supplementary Fig. 1). On the other hand, Asp-323 (flanking the C-terminal region of Jaburetox) seems to be buried into the interface between two monomers and therefore may not be accessible to modification.

As no alteration in the fragmentation pattern of JBU-Lys by insect digestive enzymes was seen, the reduction caused by this type of chemical modification in its insecticidal

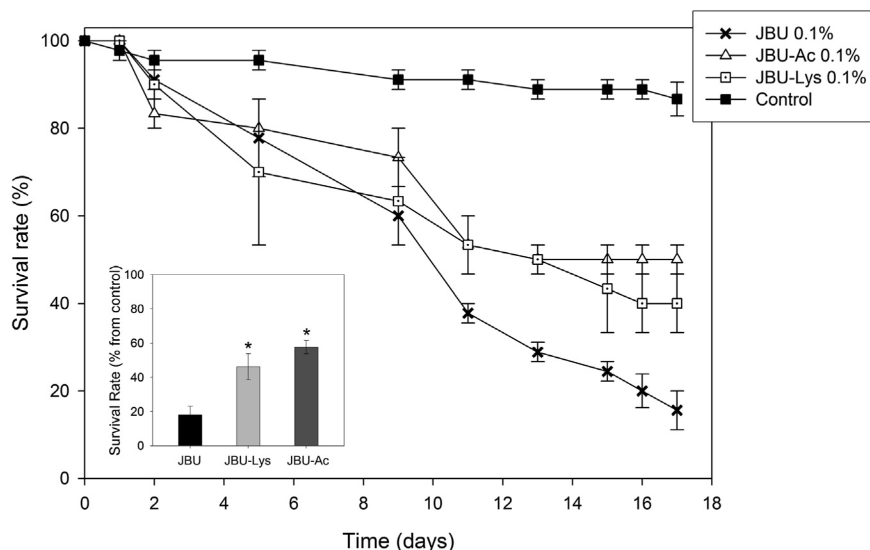

Fig. 2. Insecticidal Activity of JBU and derivatized proteins on *Dysdercus peruvianus*. Native JBU and derivatives were assayed for toxicity to *D. peruvianus* (third instar nymphs). Proteins were added to cotton flour at 0.10% (w/w). The mortality was followed during 17 days. Results are the average of two independent assays, in triplicates (15 nymphs each). Inset: survival rate at day 17, expressed as percentage from the control group. *Statistically different from the JBU-fed group, $p \leq 0.05$.

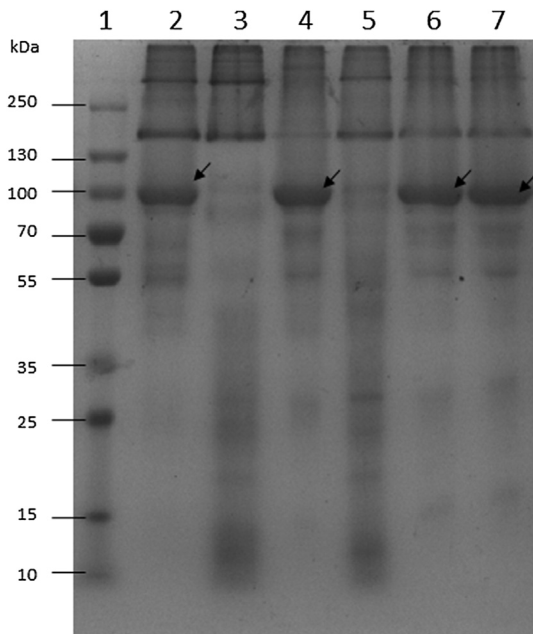

Fig. 3. *In vitro* hydrolysis of JBU. Native JBU and derivatives were submitted to proteolytic hydrolysis by *Dysdercus peruvianus* midgut homogenates. The proteolytic activity of the homogenate was determined using azocasein as substrate and expressed in mU (see Material and methods). Urease hydrolysis was then performed using a ratio of 0.5 azocaseinolytic mU of homogenate per μg of urease, during 24 h at 37 °C, pH 5.6. Controls consisted of the proteins subjected to the same incubation treatment in the absence of homogenate. Hydrolysis was analyzed by SDS-PAGE, in a gradient (8–20%) gel, Coomassie blue Staining. Lines (1): molecular mass standard; (2): control Native JBU; (3): JBU + homogenate; (4): control JBU-Lys; (5): JBU-Lys + homogenate; (6): control JBU-Ac; (7): JBU-Ac + homogenate. The arrows indicate the intact urease.

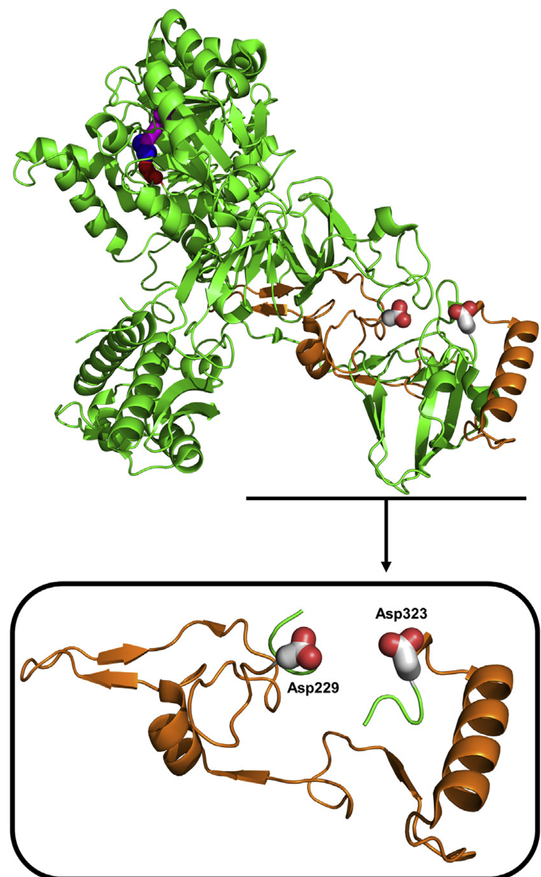

Fig. 4. Location of Jaburetox within JBU. The toxic peptide Jaburetox (orange) is flanked by two aspartic acids (Asp229 and Asp323, shown as gray/red). The two nickel ions (blue spheres) as well as Lys-490 (magenta) and Asp-633 (red) are represented within the active site of the protein. In the bottom, the sequence of JBU is shown, between residues 225 and 327, where the Jaburetox sequence is in bold and the aspartic residues, in the peptide cleavage site, are highlighted. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

effect is clearly related to interference(s) in a later step of the entomotoxic action.

3.5. Effect on the antidiuretic activity of JBU

We also evaluated the effects of the chemical modifications on the antidiuretic property displayed by plant ureases on *R. prolixus*, seen *in vivo* as a reduction of *R. prolixus* weight loss after feeding (Carlini et al., 1997) and *ex vivo* as the inhibition of serotonin-induced secretion by isolated Malpighian tubules (Mulinari et al., 2011; Staniscuaski et al., 2009). During feeding, *R. prolixus* can ingest a blood meal up to 10 times its own weight. This great increase in volume is rapidly reduced within the first 3 h after feeding, during which the insect actively excretes close to 40% of the weight gained (Orchard, 2006). As previously seen for CNTX (Carlini et al., 1997), ingestion of JBU also caused a decrease in the rate of weight loss in *R. prolixus* (Fig. 5A). While insects fed on saline lost over 65% of the post-feeding weight in 48 h, JBU-fed insects reduced their weight in less than 45%. The rate of weight loss in JBU-Ac-fed insects was the same of that seen for the native protein. In contrast, the antidiuretic effect of JBU-Lys was completely abolished. In isolated *R. prolixus* Malpighian tubules, the antidiuretic activity of JBU reduces the rates of serotonin-induced fluid secretion (Staniscuaski et al., 2009). Here, the lack of effect of JBU-Lys in reducing the rate of insect weight loss after feeding was accompanied by a significant decrease of its antidiuretic effect on Malpighian tubules (Fig. 5B). While both JBU and JBU-Ac decreased the Malpighian tubules secretion by ca. 75%, the inhibition caused by JBU-Lys was only about 30%.

4. Discussion

Here we have chemically modified lysine and acidic residues in Jackbean urease aiming to identify their contribution to the enzymatic and insecticidal properties of the protein.

Although both a lysine and an aspartic acid residue are present in the active site of the enzyme and are essential for its activity, after either modification, we observed no significant change in the ureolytic property, as reflected by the

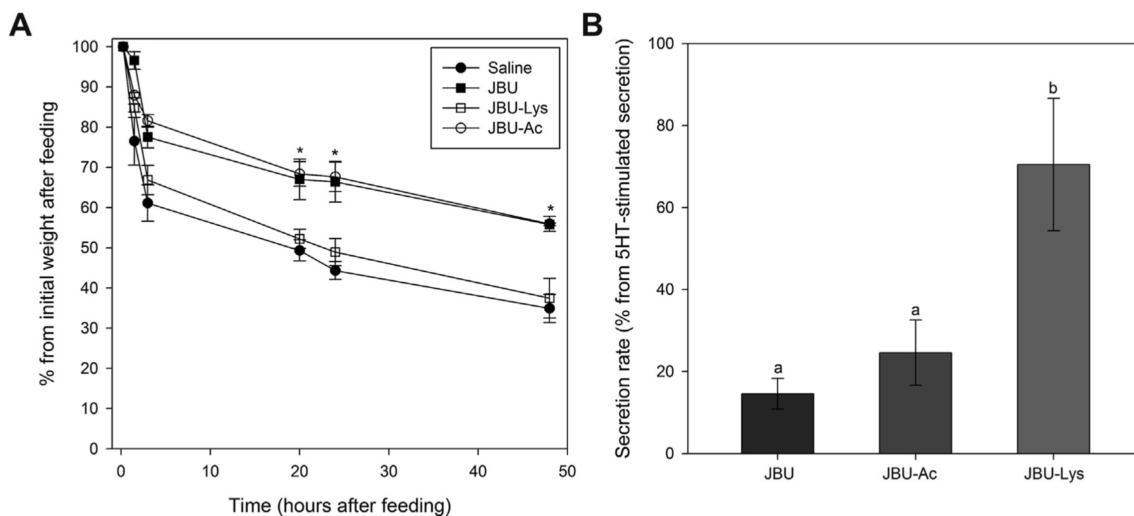

Fig. 5. Activity of JBU and derivatives on *R. prolixus* diuresis. (A) Weight loss after feeding. *Statistically different from the control (saline-fed) group, $p \leq 0.05$. (B) Malpighian tubules secretion assay. Columns with distinct letters are statistically different ($p \leq 0.05$).

measured kinetic parameters. In the case of JBU-Lys, this result was expected, since during urease maturation process in bacteria and plants the active site lysine residue undergoes a post translational carbamylation (Zambelli et al., 2011). On the other hand, the result observed for JBU-Ac suggests that this residue is probably not accessible to the modifying reagents, as they were not capable of affecting the enzyme activity. As depicted in the crystallographic structure of JBU (Balasubramanian and Ponnuraj, 2010), the enzyme active site is buried into a deep cavity, with both Lys-490 and Asp-633 being involved in the coordination of the nickel ions, and therefore not accessible to the modifying reagents (Fig. 4). Furthermore, the unaltered enzymatic activity is a strong indication that there were no major alterations in the protein structure due to the chemical modifications.

Despite their highly conserved structures and catalytic mechanisms, little is known about the physiological role of ureases in the source organisms, especially in plants (Carlini and Polacco, 2008). The widespread distribution of ureases in leguminous seeds as well as the accumulation pattern of the protein during seed maturation is suggestive of an important physiological role (Carlini and Polacco, 2008). Canatoxin, first isolated as a highly toxic protein (Carlini and Guimaraes, 1981) and later identified as an isoform of JBU (Follmer et al., 2001), displays insecticidal activity against insects of different orders (Carlini et al., 1997; Staniscuaski and Carlini, 2012; Staniscuaski et al., 2005). The entomotoxic property of CNTX is independent of its enzymatic activity and involves both the intact protein and peptides released by the insect's digestive enzymes, with a 10 kDa peptide representing the most toxic fragment (Ferreira-DaSilva et al., 2000). The more abundant isoform of urease, JBU, was as lethal as CNTX in feeding trials either with the cotton stainer bug *D. peruvianus* (Follmer et al., 2004), the kissing bug *R. prolixus* (Staniscuaski et al., 2009), or the milkweed bug *Oncopeltus fasciatus* (Defferrari et al., 2011). The insecticidal activity towards *D. peruvianus* was partially affected for both JBU-Lys and JBU-Ac, as compared to the native protein. It is known that one essential step in ureases insecticidal activity is their hydrolysis by the insects' digestive enzymes (Carlini et al., 1997; Defferrari et al., 2011; Ferreira-DaSilva et al., 2000; Piovesan et al., 2008). The results obtained showed that the modification of acidic residues affected the toxic property by blocking the release of the entomotoxic peptide(s) from the urease molecule. Analysis of the localization of the toxic peptide, Jaburetox, within JBU structure shows two aspartic acid residues flanking up- and down-stream the peptide sequence. It has been previously demonstrated that JBU is hydrolyzed by *D. peruvianus* digestive enzymes preferentially between the residues Ala-228 and Asp-229, at the N-terminal region of Jaburetox, and between Arg-322 and Asp-323, at the C-terminal region (Piovesan et al., 2008). Even though one of these residues (Asp-323) may not be accessible, the modification of a single Asp residue flanking the entomotoxic peptide could impair its release. It is also important to note that, according to the results presented here, JBU-Ac seems not to be hydrolyzed at all by the insect digestive enzymes. This result is consistent with previous observations that the main class(es) of *D. peruvianus* digestive enzymes hydrolyze bonds at the N- or C-terminal sides of aspartic acid residues (Piovesan et al., 2008). The release of the toxic peptide(s) from JBU is probably a sequential process, where bigger fragments are formed first and then processed further as the digestion proceeds. The effect of modified acidic amino acid residues of JBU in hampering the release of its internal toxic peptide(s) is probably a consequence of steric hindrance that prevents the insect digestive enzymes from hydrolyzing the protein, hence decreasing its toxic activity. The remaining toxicity observed for JBU-Ac is probably due to the activity of the intact protein. It is clear now that the toxicity of plant ureases to insects is a complex event, with different physiological processes being affect by the action of toxic peptides as well as by the whole

protein (Staniscuaski and Carlini, 2012). It has been previously shown that, upon feeding in *R. prolixus*, the intact molecule of JBU is able to cross the gut epithelia, being detected in the insect hemolymph, from where it can reach target tissues (Staniscuaski et al., 2010). Therefore, even though JBU-Ac is not hydrolyzed by the insects' digestive enzymes, the intact protein is probably still active on its target tissues, leading to a lower lethality of the derivatized protein.

Contrasting with the results observed for JBU-Ac, the lysine modification of JBU caused no interference on the hydrolysis by insects' enzymes, as observed in the *in vitro* digestion. Analyzing the sequence of JBU, it can be noted that there are no lysine residues close to the cleavage sites. This suggests that the modification of lysine residues affected the toxicity of the whole protein, rather than the release of the toxic peptide(s). Other studies have shown that lysine residues are necessary for the toxicity of the mosquito-active *Bacillus thuringiensis* toxin, since lysine modification led to a dramatic drop in toxicity (Pfannenstiel et al., 1985). Hassani et al. (1999) reported that acetylation of lysine residues reduced the toxicity of scorpion toxin VII by affecting the binding capability of this toxin to sodium channels from cockroaches, being α-type scorpion toxins also affected in a similar manner (Darbon et al., 1983; Sampieri and Habersetzer-Rochat, 1978). Lysine residues were also shown important for the toxicity of Ts1, a neurotoxin isolated from *Tityus serrulatus* (Polikarpov et al., 1999). The results showed here may indicate that lysine residues are important in the binding of JBU to the insect target tissues. Interestingly, only JBU-Lys lost its activity upon *R. prolixus* diuresis. JBU probably interacts with the membrane of the Malpighian tubules (through an unknown membrane protein/receptor) and triggers a signaling pathway, involving eicosanoids metabolites, that leads to antidiuresis (Staniscuaski et al., 2009). It is possible that altering lysine residues at the urease surface impairs its interaction with the membrane, abolishing the antidiuretic effect. It is also possible that the step being affected for the lysines-modified JBU is its absorption into the hemolymph, from where it could reach the targeted tissues. As shown before, the intact protein can be found in *Rhodnius* hemolymph 30 min after its ingestion by the insects (Staniscuaski et al., 2010). It is not known yet how and where this absorption occurs. In *Bombyx mori*, it has been demonstrated that the dietary mulberry urease is absorbed from the insect gut into the hemolymph and the presence of urease binding molecule(s) in the gut brush border membrane that would mediate this process was postulated (Kurahashi et al., 2005). The identity of such molecule(s) has not been investigated so far.

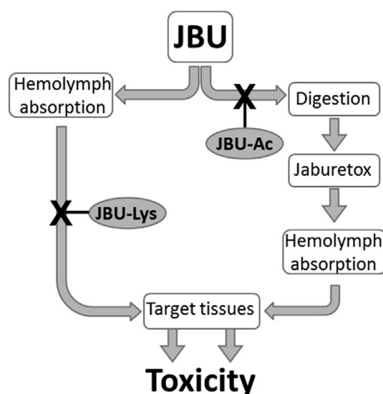

Fig. 6. Effect of chemical modifications on JBU action in insects. JBU is ingested by the insects and it is absorbed into the hemolymph or transported to the insect gut, where it is digested, generating toxic peptide(s), including Jaburetox. JBU-Lys does not alter the digestion of the protein, but instead impairs JBU action in the target tissues in the hemolymph. JBU-Ac, on the other hand, blocks the release of the toxic peptide(s), reducing the toxicity of the protein.

Fig. 6 summarizes the possible effects of chemical modifications on the JBU biological properties investigated here. JBU fed to the insects can follow two pathways: 1) be absorbed into the hemolymph and/or 2) be transported to the insect posterior midgut, where it is digested, generating toxic peptide(s), one of which is Jaburetox (Staniscuaski and Carlini, 2012). Since JBU-Lys is hydrolyzed similarly to the native protein, we postulate that the modification of lysines probably impairs JBU absorption into the hemolymph and/or its action on target tissues, including the Malpighian tubules. In JBU-Ac, on the other hand, the release of toxic peptide(s) upon hydrolysis by insect's enzymes is blocked, reducing the toxicity of the protein. Since the intact protein can still be absorbed into the hemolymph, a residual toxicity is observed. There was no significant difference in the lethality between the two derivatives forms of JBU, corroborating the idea that a combinatory effect of both, peptides and intact protein, is relevant to its entomotoxic property.

5. Conclusions

C. ensiformis ureases are complex proteins with several biological activities. The entomotoxic activity is of great interest, since the search for natural insecticides, with none or reduced threat to the environment or to human health is an attractive alternative to synthetic chemical insecticides for pest management (Isman, 2006). Altogether, the data herein contributed to our understanding of structure/function of the urease entomotoxic activity and represent an advance on the possible use of ureases and/or their derived-peptides as biological tools in pest management.

Ethical statement

All the authors declare that: the paper has not been previously published in whole or in part and is not currently being considered for publication elsewhere; all authors have contributed significantly to the execution, analysis and writing of the paper.

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Conflict of interest

The authors declare that there are no conflicts of interest.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.toxicon.2013.05.008.

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