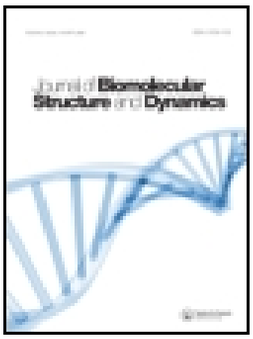


Taylor & Francis
Taylor & Francis Group

Journal of Biomolecular Structure and Dynamics

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/tbsd20

One enzyme, many faces: urease is also canatoxin

Carlo Frederico Moro, Fábio C. S. Nogueira, Carlos Gabriel Moreira Almeida, Rafael Real-Guerra, Pedro Ferrari Dalberto, Cristiano V. Bizarro, Rodrigo Ligabue-Braun & Celia R. Carlini

To cite this article: Carlo Frederico Moro, Fábio C. S. Nogueira, Carlos Gabriel Moreira Almeida, Rafael Real-Guerra, Pedro Ferrari Dalberto, Cristiano V. Bizarro, Rodrigo Ligabue-Braun & Celia R. Carlini (2022): One enzyme, many faces: urease is also canatoxin, Journal of Biomolecular Structure and Dynamics, DOI: 10.1080/07391102.2022.2158938

To link to this article: https://doi.org/10.1080/07391102.2022.2158938

JOURNAL OF BIOMOLECULAR STRUCTURE AND DYNAMICS
https://doi.org/10.1080/07391102.2022.2158938

One enzyme, many faces: urease is also canatoxin

Carlo Frederico Moro[a], Fábio C. S. Nogueira[b], Carlos Gabriel Moreira Almeida[a], Rafael Real-Guerra[c], Pedro Ferrari Dalberto[d], Cristiano V. Bizarro[d,e], Rodrigo Ligabue-Braun[f]* and Celia R. Carlini[g,h]*

[a]Graduate Program in Medicine and Health Sciences, Pontificia Universidade Católica do Rio Grande do Sul (PUCRS), Porto Alegre, RS, Brazil; [b]Proteomics Unit, Institute of Chemistry, Federal University of Rio de Janeiro, Rio de Janeiro, RJ, Brazil; [c]Interdisciplinary Department, Universidade Federal do Rio Grande do Sul, Tramandaí, RS, Brazil; [d]Graduate Program in Cellular and Molecular Biology, Pontificia Universidade Católica do Rio Grande do Sul (PUCRS), Porto Alegre, RS, Brazil; [e]National Institute of Science and Technology in Tuberculosis (INCT-TB), Centro de Pesquisas em Biologia Molecular e Funcional (CPBMF), Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS), Porto Alegre, RS, Brazil; [f]Department of Pharmacosciences, Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA), Porto Alegre, RS, Brazil; [g]Brain Institute—Instituto do Cérebro do Rio Grande do Sul (INSCER), Porto Alegre, RS, Brazil; [h]National Institute of Science and Technology in Brain Diseases, Excitotoxity and Neuroprotection (INCT-EN), Porto Alegre, RS, Brazil

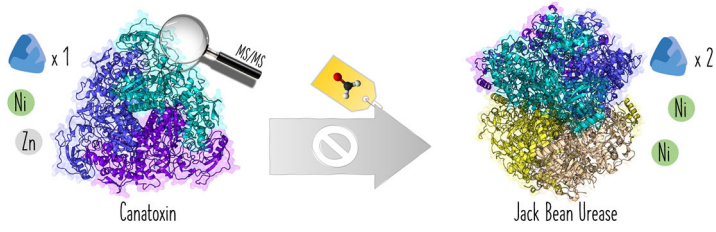
Communicated by Ramaswamy H. Sarma

ABSTRACT

Ureases catalyze the hydrolysis of urea into carbamate and ammonia. Well-conserved proteins, most plant ureases are hexamers of a single chain subunit, like the most abundant isoform of the jack bean (*Canavalia ensiformis*) urease (JBU). Canatoxin (CNTX) was originally isolated from these seeds as a neurotoxic protein, and later characterized as an isoform of JBU with lower molecular mass and enzyme activity. Inactive CNTX oligomers form upon storage and stabilization of CNTX was achieved by treatment with low concentration of formaldehyde, avoiding its oligomerization. Here, nano-LC-MS/MS-based peptide analysis of CNTX revealed 804 amino acids identical to those of JBU's sequence (840 amino acids). *De novo* sequencing of CNTX revealed 15 different peptides containing substitution of amino acid residues, denoting CNTX as a product of a paralog gene of JBU. The MS/MS analysis of formaldehyde-treated CNTX showed that amino acid residues located at the trimer–trimer interface of JBU's hexamer were modified. The data confirmed that CNTX is an isoform of JBU and elucidated that stabilization by formaldehyde treatment occurs by modification of amino acids at the protein's surface that prevents the formation of the hexamer and of higher molecular mass inactive aggregates.

HIGHLIGHTS

- Canatoxin (CNTX) is an isoform of jack bean urease (JBU, hexamer of 90 kDa chains)
- MS/MS sequencing of CNTX showed 804 amino acids identical in JBU (840 residues)
- Formaldehyde treatment of CNTX stabilizes its toxicity and avoids oligomerization
- Modified amino acid residues in CNTX are at the trimer–trimer interface of JBU



ARTICLE HISTORY
Received 11 August 2022
Accepted 9 December 2022

KEYWORDS
Formaldehyde; tandem mass spectrometry; amino acid modification; protein surface; oligomerization; stabilization

1. Introduction

Ureases, metalloenzymes that catalyze the hydrolysis of urea into ammonia and carbamate, are well-conserved proteins although differing in their quaternary structures. While plant and fungal ureases are hexamers of a single chain subunit, bacterial ureases can be trimers, hexamers, or dodecamers, whose 'monomers' are composed of two or three subunits (Kappaun et al., 2018; Ligabue-Braun et al., 2013). The ammonia-independent toxicity of ureases was first described for

CONTACT Celia R. Carlini ccarlini@ufrgs.br; celia.carlini@pq.cnpq.br Brain Institute—Instituto do Cérebro do Rio Grande do Sul (INSCER), Av. Ipiranga 6690, Bldg 63, Porto Alegre, RS, Brazil.
*These authors share the senior authorship

Supplemental data for this article can be accessed online at https://doi.org/10.1080/07391102.2022.2158938

canatoxin (CNTX), originally isolated as a neurotoxic protein from jack bean (*Canavalia ensiformis*) seeds (Carlini & Guimaraes, 1981), and later characterized as an isoform of the most abundant urease, JBU, found in the same seeds (Follmer et al., 2001). At submicromolar concentrations, CNTX promotes exocytosis in several cell models, such as platelets and synaptosomes, and possesses a potent pro-inflammatory activity, both *in vivo* and *in vitro* (reviewed in Carlini & Ligabue-Braun, 2016; Kappaun et al., 2018). Its convulsant activity and effects on the central nervous system of rodents were recently characterized (Almeida et al., 2021). Moreover, the neurotoxin possesses insecticidal and fungitoxic properties (for reviews see Carlini & Ligabue-Braun, 2016; Staniscuaski & Carlini, 2012). Further studies have shown that the other two isoforms of urease of *C. ensiformis*, namely JBU and JBURE-II, also display non-enzymatic properties (Follmer et al., 2001; Follmer et al., 2004; Mulinari et al., 2011). To demonstrate that CNTX and JBU induce ureolysis-independent effects, these proteins were treated with the irreversible inhibitor *p*-hydroxymercuribenzoate (Follmer et al., 2001). The enzymatically inactive CNTX and JBU maintained the same EC_{50} for the platelet exocytosis-inducing effect and similar LD_{50} (ev route) in mice, when compared to the untreated proteins (Follmer et al., 2001). Most of the non-enzymatic activities of CNTX were also observed for other plant (soybean, pigeon pea, cotton) ureases and for the bacterial enzymes from *Helicobacter pylori*, *Sporosarcina (Bacillus) pasteurii* (reviewed in Carlini & Ligabue-Braun, 2016; Kappaun et al., 2018) and *Proteus mirabilis* (Broll et al., 2021; Grahl et al., 2021).

Although many biological properties of CNTX have been characterized since its first description, its physicochemical nature has not been fully elucidated. Genes encoding JBU and JBURE-II were characterized but attempts at cloning a gene for CNTX were not successful so far. Partial sequence of CNTX and immunological cross-reactivity suggested an extensive similarity to JBU's primary structure (Follmer et al., 2001). However, several physicochemical and biochemical characteristics distinguish CNTX from JBU. Only CNTX induces convulsions given by intraperitoneal route while both ureases are convulsive given endovenously (Follmer et al., 2001). The proteins can be efficiently separated by metal-affinity chromatography (Follmer et al., 2004). In neutral solution, JBU occurs mostly in a hexameric form with Mr 540 kDa, while native CNTX was characterized as a non-covalently linked dimer of 90 kDa chains. CNTX has a lower ureolytic activity, equivalent to about 1/3 of that of JBU, and a different sensitivity pattern to inhibitors (Follmer et al., 2001). Interestingly, JBU and CNTX differ in their metal content, the latter containing one mol of Zn^{2+} and one mol of Ni^{2+} per mol of 90 kDa subunit, instead of the two moles of Ni^{2+} per subunit reported for the JBU monomer (Follmer et al., 2002).

CNTX is unstable in solution, quickly loses its toxic activity and precipitates after a few days of storage. It was hypothesized that the inactivation of canatoxin in solution occurs as a consequence of its tendency to form aggregates. As the instability of the semipurified CNTX hampered attempts of further purification steps, several conditions were tested aiming to increase CNTX's stability. Most notably, a treatment with formaldehyde stabilized the biological activity of CNTX for at least 60 days, avoiding its oligomerization and enabling the obtention of the purified protein.

Formaldehyde is a potent electrophile used in protein chemistry since 1920s, mainly to produce cross-linked proteins, or to cross-link proteins to nucleic acids. One of the most common uses of formaldehyde is to produce inactivated toxoids and viruses as antigens for vaccination and for tissue fixation in histology, which require high concentrations of formaldehyde (>1% v/v) and long reaction times (hours-days) (Kamps et al., 2019; Metz et al., 2013; Sutherland et al., 2008; Yamanushi et al., 2015). More recently, formaldehyde has been proven useful to study protein-protein interactions *in vivo*, since it can permeate the cell membrane and cross-link interacting proteins in their natural medium. For this purpose, more gentle formaldehyde treatment is used, like 0.05%, and shorter incubation times, up to 20 minutes (Sutherland et al., 2008; Toews et al., 2008).

The precise mechanism by which formaldehyde promotes stabilization of CNTX has not been elucidated so far, though it was assumed that it did so by preventing aggregation of the protein. In this study, we aimed to further characterize CNTX and investigate the mechanism by which the treatment with formaldehyde stabilizes the protein and avoids its oligomerization. Our data confirm that CNTX is an isoform of JBU, and show that the proteins share extensive similarity of their amino acid sequences. Biologically active CNTX exists in a dimeric or trimeric state, which can be stabilized, as demonstrated here, by formaldehyde-induced modifications of amino acid residues involved in the oligomerization process.

2. Materials and methods

2.1. Canatoxin

CNTX was obtained following the procedure described in Follmer et al., 2001, with minor modifications. Briefly, mature seeds of jack bean (*C. ensiformis*; Casa Agrodora, São Paulo, Brazil) previously soaked in 20 mM sodium phosphate pH 7.5 (NaPB 7.5) at 4 °C, were dehusked and homogenized in the same buffer, at a 20% dry seed weight/80% buffer ratio. After centrifuging the homogenate, the pH of supernatant (extract) was adjusted to 7.5 with 1 M NaOH and then absolute ethanol, previously chilled at −20 °C, was slowly added under agitation to give a final 30% ethanol/70% extract ratio. The mixture was promptly centrifuged at 10,000 g at 4 °C for 30 min. The precipitate was discarded, and the supernatant was immediately (within 2 hours after addition of ethanol) put under dialysis (10 kDa cut-off membrane) against NaPB 7.5. After dialysis, the material was fractionated with ammonium sulfate. The precipitate obtained at 35–55% saturation was resuspended and dialyzed against NaPB 7.5 to remove the excess of salt. At this point, samples were treated with 0.02% v/v formaldehyde (final concentration), and kept at 4 °C to ensure stability (see next section, 2.2.). The formaldehyde treatment was repeated after each further purification step. The dialyzed material was then submitted to ion exchange chromatography. After adding 3 M NaCl to the sample to give 100 mM NaCl final

concentration in NaPB 7.5 (equilibrium buffer), the material was mixed with Q-Sepharose (GE Health Sciences) resin equilibrated in the same buffer, and kept under gentle agitation on ice for 90 min. The resin/extract mixture was filtered on a sintered glass funnel, extensively washed with the equilibrium buffer and then eluted with NaPB 7.5 containing 200 mM NaCl. The eluted fraction was ultrafiltered in Amicon devices (Millipore) with 100 kDa cut-off, to give a final protein concentration of 1–2 mg/mL and then applied to a Superdex 200 HiLoad 26/600 (GE Health Sciences) size-exclusion column, previously calibrated with high molecular mass standards (GE Health Sciences). Protein concentration of fractions was determined by absorbance at 280 nm. Purity of canatoxin after the gel-filtration step was checked by electrophoresis in 7.5% polyacrylamide −0.1% sodium dodecyl sulfate gels (Figure S1, Supplementary material).

2.2. Stability of canatoxin

To establish the best conditions for storage of CNTX, freshly prepared samples of CNTX after the ammonium sulfate precipitation step, at 8 to 10 mg/mL protein concentration, in NaPB pH 7.5, were stored at 25 °C, 4 °C or −20 °C, containing or not the following additives: 3 M NaCl; 0.1% bovine serum albumin; 0.02% v/v formaldehyde; 1 mM *p*-hydroxymercuribenzoate; 5 mM dithiothreitol; 0.05% Triton X-100. After storage for 5, 10, 25, 40 and 60 days, the convulsant (seizure-inducing) activity of samples was evaluated in mice as described in 2.3.

2.3. Convulsant activity in mice

Adult Swiss mice (males, 28–35 g) were obtained from the Centre for Biological Experimental Models (Pontifical Catholic University of Rio Grande do Sul). The animals were housed at 25 °C with access *ad libitum* to food and water and 12 hours light:dark cycles. All animal protocols in this work were approved by local animal care committee, under the authorization number 7907/2018 and followed the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978).

To test the effect of different storage conditions (see Section 2.2) on CNTX stability, samples of the toxin were tested in mice (N = 8) by intraperitoneal injection, to give doses of 6.6 mg protein/kg body weight (LD_{50} at this purification step is ∼6.1 mg protein/kg body weight (Follmer et al., 2001).

The toxic activity of formaldehyde-treated CNTX (CH_2O-CNTX) after the gel-filtration step was also evaluated. Groups of 8 mice were injected intraperitoneally with 0.1 mL of the protein solution in NaPB 7.5, in doses varying from 1.5 to 3.5 mg protein/kg body weight.

After the injections, the animals were returned to their cages and observed for neurotoxic symptoms (convulsions) and death. Most frequently, the animals died after convulsions within the 12 hours afterwards. Animals that survived 12 hours after CNTX administration were anaesthetized with thiopental and sacrificed by decapitation. Toxicity of freshly prepared CNTX was considered as 100%.

2.4. Formaldehyde treatment

Formaldehyde 38 - 40% (Merck) was added to all CNTX samples after the ammonium sulfate fractionation, and after all subsequent purification steps, to give 0.02% v/v (0.0158–0.0166 M) or 0.1% v/v (0.079–0.083 M) final concentration, whereas part of the protein solution was left without any treatment. Reaction with formaldehyde proceeded for at least 8 hours (overnight) at 4 °C before any analysis. After the reaction, the excess of formaldehyde was not removed as it is volatile and the reaction is reversible at lower concentrations of the compound (Metz et al., 2004). For determining the effect of formaldehyde treatment on the convulsant activity of CNTX, protein samples treated with 0.02% formaldehyde were tested in mice by intraperitoneal route as described in 2.3. Buffer solution (NaPB 7.5) containing 0.02% formaldehyde was injected into mice to serve as negative control.

The effect of formaldehyde treatment on the oligomerization state of CNTX was evaluated after the ion exchange chromatography step, with protein samples kept in the absence or in the presence of 0.02% formaldehyde. On days 1 and 60 after ion exchange chromatography and addition (or not) of formaldehyde, samples were applied to a Superdex 200 HiLoad 26/600 (GE Health Sciences) size-exclusion column, previously calibrated with high molecular mass standards (GE Health Sciences), with a calibration curve described by following the equation: $y = 48471e^{-0.033x}$ ($R^2 = 0.991$), where y is the molecular mass in Daltons and x is the elution volume. Eluted fractions were analyzed for convulsant activity in mice by intraperitoneal route, as described in 2.3.

2.5. Mass spectrometry analysis

CNTX samples after the Q-Sepharose purification step were either left untreated or treated with formaldehyde (0.02% or 0.1% v/v), overnight at 4 °C. The samples were then subjected to the gel filtration chromatography, and the formaldehyde treatment was repeated for the previously treated CNTX samples.

Samples of gel-filtered 1-day old CNTX (non-treated) and CH_2O-CNTX (formaldehyde-treated) were separated by denaturing SDS-PAGE (∼30 μg per gel lane, Figure 1, Supplementary material). The 90 kDa bands of two independent CNTX batches (either treated with 0.02% formaldehyde or the corresponding non-treated samples) were excised and gel fragments from two lanes of each sample were pooled together. The gel fragments were subjected to in-gel trypsin digestion and peptide extraction (Demartini et al., 2011), and then the peptides were analyzed by nanoLC-MS/MS at the Centro de Pesquisas em Biologia Molecular e Funcional (CPBMF), Pontifical Catholic University of Rio Grande do Sul. Chromatographic separations were performed on an Eksigent nanoLC Ultra 1 D plus autosampler (currently part of AB Sciex) connected to an LTQ-XL Orbitrap Discovery hybrid

instrument (Thermo Fisher Scientific), through a nanoelectrospray ion source (Thermo Fisher Scientific). Data were collected with one FT full-scan (400–1600 m/z range; 30,000 resolution) followed by data dependent CID MS/MS spectra of the 8 most intense ions in the ion trap, with dynamic exclusion (1 repeat count, 30 s repeat duration, 100 exclusion list size, and 30 s exclusion duration). The mass spectrometer and HPLC were controlled by the Xcalibur data system (Thermo Fisher Scientific).

A third sample of CNTX was treated with 0.1% formaldehyde after the Q-Sepharose chromatography, and the treatment was repeated after the gel-filtration step. Formaldehyde-treated and the corresponding non-treated CNTX bands were excised from SDS-PAGE gels, and sent for MS/MS analysis carried out at the Laboratory of Proteomics, Laboratório de Apoio ao Desenvolvimento Tecnológico (LabProt/LADETEC), Federal University of Rio de Janeiro, Brazil. The nanoLC-MS/MS apparatus consisted of an Easy-nLC 1000 (Thermo Fisher Scientific) with a stationary C18 trap-column (~2 cm length, 100 μm internal diameter, 5 μm particle diameter, ReproSil-Pur C18-AQ resin, Dr. Maisch GmbH; Ammerbuch, Germany) and column (20 cm length, 75 μm internal diameter, 3 μm particle diameter, ReproSil-Pur C18-AQ resin, Dr. Maisch GmbH; Ammerbuch, Germany) coupled to an nano-electrospray ionization Q-Exactive Plus (Thermo Fisher Scientific) mass spectrometer. Data were collected during a nLC gradient of 60 min and acquisition with alternating full-scan MS (resolution 70,000 at m/z 400 m/z range; m/z range 350 to 2,000; Maximum IT of 100 ms; AGC of 3e6) followed by data dependent HCD MS/MS spectra of the 20 most intense ions (with $z \geq +2$; resolution of 17,500 at m/z 400; normalized collision energy of 30; Maximum IT of 50 ms; AGC of 1e6; dynamic exclusion of 45 s). The mass spectrometer and nLC were controlled by the Xcalibur 3.0 data system (Thermo Fisher Scientific).

Tandem mass spectra were searched from RAW files with the software Comet (Eng et al., 2013) in the PatternLab for Proteomics (Santos et al., 2022). The database contained the non-redundant *Glycine max* proteomes and the 20 sequences available for *Canavalia ensiformis* urease-related proteins in the Uniprot database (accessed: July 01, 2022) as well as reverse sequences. Search space included all full and semi-tryptic peptide candidates. Methylolation (+30.021480 Da), iminization (+12.010700 Da), and oxidation (+15.994900 Da) were considered as modifications. The validity of the peptide spectrum matches (PSMs) was assessed using the module Search Engine Processor (SEPro, PatternLab for Proteomics), with a false discovery rate of 1%.

De novo sequencing and database search was performed using novor cloud (https://app.novor.cloud/). Parameters used were variable PTMs of Methylol, Thiazolidine, Oxidation (FHMW), precursor and fragment error tolerance of 15 ppm and 0.02 Da, respectively, enzyme trypsin and 1% FDR. For the search, the database cited previously was used.

The MS/MS data shown here represent the combined results of three independent CNTX samples (different purification batches), two of which were submitted to 0.02% and one to 0.1% formaldehyde treatment before analysis.

Alignment of sequences of JBU and JBURE-II was performed with Clustal Omega (EMBL-EBI) (Sievers et al., 2011) software and manually for the peptides identified by the *de novo* analysis.

2.6. Molecular structure and visualization

The JBU structure in its hexameric form was built using biological assembly information from Protein Data Bank entry 3LA4 (Balasubramanian & Ponnuraj, 2010), and Modeller 9.22 (Marti-Renom et al., 2000) was applied for the completion of missing regions. Additional references for the native JBU hexamer were taken from the cryo-EM structure under PDB entry 7KNS (Feathers et al., 2021). All structural visualizations were performed with PyMOL (The PyMOL Molecular Graphics System, Version 1.8 Schrödinger, LLC).

Putative dimeric structures for *C. ensiformis* ureases were built using SymmDock, a docking algorithm designed for predicting cyclically symmetric homomultimers (Schneidman-Duhovny et al., 2005a, 2005b). The structure under PDB ID 3LA4 (Balasubramanian & Ponnuraj, 2010) was used as reference and missing regions were completed with Modeller 9.23 (Marti-Renom et al., 2000). The best docking solutions (top ranking based on score) were selected as the most likely to resemble the putative urease dimer. The interactions between protein chains were inspected with PBDsum, through the 'Generate' option (Laskowski et al., 2018).

2.7. Statistical analysis

The results of convulsant activity of protein samples are presented as mean ± standard error of the mean (SEM). Prism GraphPad (version 6.0, GraphPad Software Inc., San Diego, California) was used for the analysis. Statistical differences were considered significant when $p < 0.05$.

3. Results and discussion

3.1. Effect of formaldehyde on the stability and oligomerization of CNTX

CNTX rapidly loses its biological activity, not allowing storage of purified samples. Aiming to increase the protein's stability, different conditions of storage of semipurified CNTX (after the ammonium sulfate step; 8 to 10 mg/mL) were tested following for its intraperitoneal toxicity in mice. CNTX was more stable if kept at 4 °C in neutral diluted solutions (below 1.0 A_{280}/mL) (Figure 1). It precipitated if subjected to freezing/thawing in any of the tested conditions. Addition of either 3 M NaCl, 0.1% bovine serum albumin, 1 mM p-hydroxymercuribenzoate, 5 mM dithiothreitol or 0.05% Triton X-100 did not improve its stability (not shown). In contrast, treatment of CNTX (< 1.0 mg/mL, pH 7.5, kept at 4 °C) with 0.02% formaldehyde for at least 8 h significantly improved its stability, maintaining the toxin fully active for up to 60 days (Figure 1).

Purification of CNTX, its instability and tendency to aggregate upon storage have been previously described (Follmer

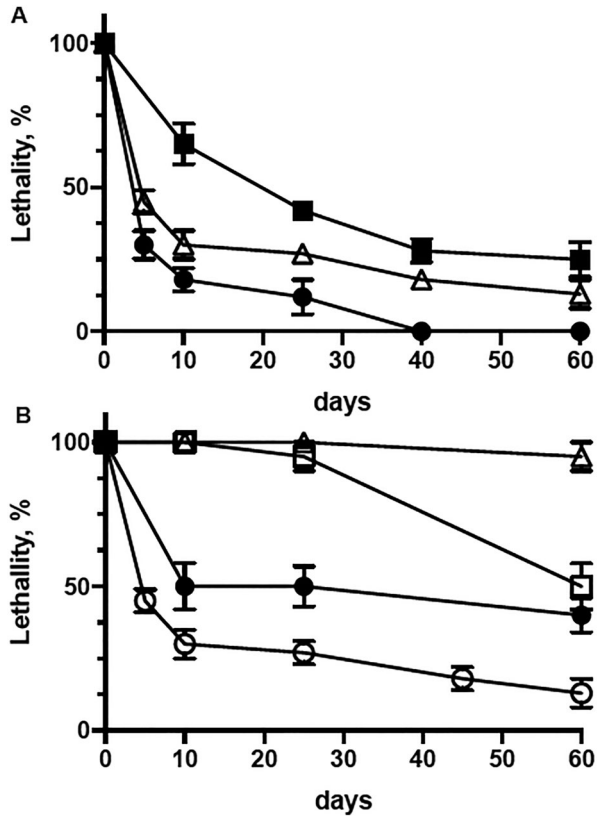


Figure 1. Storage conditions and stabilization of CNTX. Protein samples after the ammonium sulfate fractionation step, at 8 to 10 mg/mL protein concentration, in NaPB pH 7.5, were stored at 25 °C, 4 °C or -20 °C, containing or not 0.02% v/v formaldehyde. After 5, 10, 25, 40 and 60 days, the convulsant-lethal activity of samples in mice was evaluated. Storage conditions were: (A) -20 °C, filled squares; 4 °C, open triangles; 25 °C, filled circles. (B) 4 °C, diluted 1:10, open squares; 4 °C, diluted 1:10, with 0.02% formaldehyde, open triangles; 4 °C, with 3 M NaCl, filled circles; 4 °C with 0.05% Triton X-100, open circles. Data shown are median ± SD of at least 3 experimental points run with different CNTX's batches.

et al., 2001). Here, the effect of formaldehyde treatment on the molecular mass of CNTX was monitored by size exclusion chromatography comparing the elution profiles of CNTX and CH_2O-CNTX after the last step of purification (gel-filtration). Initially, both samples presented an identical elution profile showing two protein peaks, corresponding to a Mr of ~540 kDa and ~270 kDa, in the Superdex 200 HiLoad 26/600 column (Figure 2). After storage at 4 °C for 40 days, both CNTX samples (kept at 4 °C at 1.0 mg/mL protein concentration, in NaPB 7.5) were analyzed again by gel-filtration. Similar to what we reported in Follmer et al., 2001, the elution pattern of the non-treated 40-days old CNTX sample showed a decrease in the peak of the ~270 kDa protein, with broadening of the Mr ~540 kDa peak, as well as the appearance of a peak in the dead volume of the column, corresponding to high Mr aggregates (> 1,400 kDa)(Figure 2B).This would be consistent with CNTX trimers oligomerizing into hexamers, and aggregates of higher molecular mass. In contrast, for the 40-days old CH_2O-CNTX sample (Figure 2C), the ~270 kDa peak is still present despite a reduction in area, and the increase in the ~540 kDa peak is less pronounced than that seen for the nontreated sample.

After the gel filtration step (Figure 2), the intraperitoneal toxicity (convulsions followed by death) of the formaldehyde-treated CNTX (CH_2O-CNTX; ~270 kDa peak) was in the same range (100% lethality with 2.9 ± 0.6 mg protein/Kg mouse body weight, N = 12; 50% lethality, with 2.0 ± 0.6 mg protein/Kg, N = 8) as that reported for the non-treated CNTX in the same step of purification (Follmer et al., 2001). In contrast to non-treated gel-filtered CNTX, which loses its convulsant activity after a couple of days, gel-filtered CH_2O-CNTX maintains similar convulsant and lethal activity in mice for up to 60 days. The protein peaks with molecular mass higher than 270 kDa (Figure 2) were innocuous to mice.

In Follmer et al., 2001, we reported that CNTX had an apparent Mr of 183.7 kDa, which would indicate a dimeric state of the urease. In contrast, here a Mr of 263 kDa was estimated for CNTX, consistent with a trimeric form of urease. Dimeric plant ureases have been described (reviewed in Kappaun et al., 2018). An equilibrium between dimeric and trimeric states of CNTX shifting towards one of the conformers according to the experimental setting could explain these contrasting data. Another possible explanation for these findings could rely on the source, the jack bean seeds, from which CNTX was isolated in the two instances. It has been reported for the lectin concanavalin A, also present in the same seeds, that the time elapsed after flowering and the degree of desiccation of the seeds affected the subunit composition and oligomeric organization of this protein (Bowles

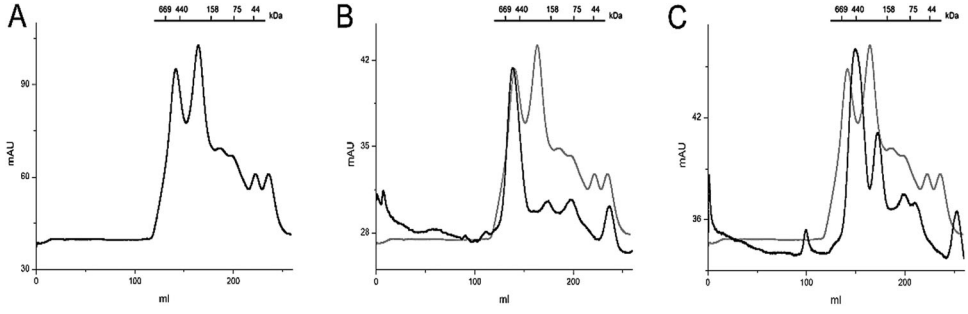


Figure 2. Effect of formaldehyde treatment on the oligomerization of CNTX. Formaldehyde at 0.02% v/v was added to part of the CNTX sample during the purification protocol after the ion-exchange chromatography step, as described in the Methods section. The panels show gel-filtration patterns of formaldehyde-treated and non-treated CNTX samples in a Superdex 200 HiLoad 26/600 column of CNTX after different storage times, as determined by absorbance at 280 nm. Panel (A). Gel-filtration of a CNTX sample 1 day after ion-exchange chromatography; Panel (B). Gel-filtration of the same CNTX sample as in A, after 40 days of storage at a ~1mg/mL, 4 °C; Panel (C). Gel-filtration of the formaldehyde-treated CNTX sample, after 40 days of storage at a ~1mg/mL, 4 °C. In both panels (B) and (C), the initial chromatogram (A) is shown superimposed as a gray line. The lines on the top of the graphics illustrate the calibration of the Superdex 200 column, with the Mr of the standard proteins shown at their respective elution volumes.

Figure 3. Peptides obtained by *de novo* sequencing of CNTX, aligned with the sequences of JBURE-II (1st line) and JBU (2nd line). In red letters: amino acid residues identified by *de novo* analysis that differ from the JBU sequence; yellow highlight indicates multiple counts in the MS/MS analysis. Underlined: amino acids modified by formaldehyde in CNTX. Green highlight: amino acid residues identified by *de novo* analysis that are potential sites for formaldehyde modification in CNTX.

et al., 1986). It can then be hypothesized that differences in the degree of desiccation of the seeds could also influence the oligomeric state of CNTX, or that of JBU. Although we did not investigate in this work the conditions affecting the dimer-trimer equilibrium of the protein, our findings clearly demonstrated that CNTX is a lower molecular mass isoform of *C. ensiformis* urease. This difference in oligomeric state may explain, for instance, the distinct physicochemical

Table 1. MS/MS analysis of modified amino acids residues found in CNTX treated with 0.1% formaldehyde.

Formaldehyde-treated				Non-treated			
Imine (+12)		Methylol (+30)		Imine (+12)		Methylol (+30)	
Residue*	Counts**	Residue	Counts	Residue	Counts	Residue	Counts
Lys304	**10**	**Cys59**	**14**	**Lys263**	**4**	**Tryp728**	**10**
Lys263	**9**	**Tryp728**	**12**	**Tryp728**	**3**	**Cys268**	**3**
His251	**6**	**Cys268**	**10**	His170	2	**Cys329**	**3**
His76	**6**	**His76**	**5**	His331	2	**Cys59**	**3**
Tryp728	**6**	**Lys613**	**5**	Lys662	2	Arg68	2
Lys745	**5**	**Arg575**	**4**	Lys716	2	Cys313	2
Lys22	**4**	**Cys139**	**4**	Cys59	1	Cys663	2
Lys282	**4**	**Cys329**	**3**	His14	1	Cys821	2
Lys613	**4**	**Cys433**	**3**	His585	1	His76	2
Lys662	**4**	**Cys592**	**3**	His76	1	Cys443	1
His331	**3**	**Cys663**	**3**			His172	1
His585	**3**	**Cys821**	**3**			His242	1
Lys52	**3**	Cys313	2			His251	1
Lys679	**3**	His585	2			His331	1
Tryp708	**3**	Lys612	2			Lys255	1
His479	2	Tryp708	2			Lys613	1
His545	2	Arg68	2			Lys818	1
Lys10	2	Arg185	1				
Lys474	2	Arg747	1				
Lys612	2	Cys207	1				
Lys716	2	His242	1				
Lys755	2	Lys255	1				
His14	1	Lys282	1				
His170	1	Lys662	1				
His172	1	Lys709	1				
Lys02	1						
Lys128	1						
Lys255	1						
Lys277	1						
Lys483	1						
Lys782	1						
Lys817	1						
Lys818	1						

*Residues are numbered according to JBU sequence (PDB 3LA4).
**Residues with high confidence of modification (3 or more spectral counts) are shown in bold letters.

properties of CNTX when compared to JBU, such as the elution pattern in ion exchange or metal-affinity chromatographies (Follmer et al., 2001; Follmer et al., 2004), despite the extensive identity between the proteins.

3.2. MS analysis of CNTX: peptide mapping and de novo sequencing by nano-LC-MS/MS analysis

For both the CNTX and CH_2O-CNTX samples, the largest spectrum counts were obtained for peptides matching to JBU (urease OS=*Canavalia ensiformis*, OX = 3823 PE = 1 SV = 3: locus P07374). The identified peptides across all analyses provided a combined 95.7% sequence coverage of JBU (804/840 amino acids).

On the other hand, *de novo* sequencing of CNTX revealed several peptides containing residues dissimilar to those present in JBU, of which 15 residues were found in repeated counts (Figure 3). The data confirmed that CNTX is either the product of a paralogous gene or an alternative splicing of the JBU gene. The dissimilar peptides were also compared against the JBURE-II urease (Uniprot Q8H6V8) sequence (Mulinari et al., 2011), which although less abundant than CNTX or JBU, has also been detected in *C. ensiformis* extracts (Demartini et al., 2011). However, all *de novo* peptides matching the JBURE-II sequence were in regions with sequences identical to that of the JBU, indicating that there was no significant contamination of JBURE-II in the MS sample.

3.3. MS analysis of CNTX after formaldehyde treatment

A considerable number of peptides containing modifications previously described as resulting from reaction with formaldehyde (Kamps et al., 2019; Metz et al., 2004) were detected in the MS/MS analysis of CH_2O-CNTX samples (Table 1). Iminization (+ 12 Da, 97 counts) was found for 33 different residues, and methylolation (+ 30 Da, 87 counts) was found in 25 different residues across the tested CH_2O-CNTX samples (biological triplicate).

Of the imine-modified residues, 15 were of high-confidence (3+ spectral counts for modified peptides), 6 medium-confidence (2 spectral counts) and 12 low-confidence (1 spectral count). Of the methylol-modified ones, 12 were high-confidence, 5 medium-confidence and 9 low confidence (Table 1).

In the non-treated CNTX samples, some possible iminization (10) and methylolation (17) sites were found. However, those were largely low-confidence hits, with only 2 imine and 4 methylol sites scoring more than 3 spectral counts across all samples. The same residues were found modified in the formaldehyde-treated protein (CH_2O-CNTX), in all cases with a much higher number of spectral counts. These modifications

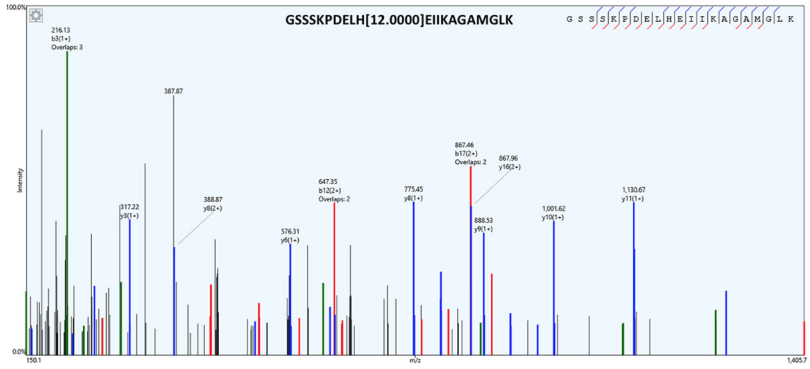


Figure 4. LC-MS/MS spectrum of the GSSSKPDELH[+12.0000] EIIKAGAMGLK peptide, containing the imine-modified His479 residue, that yielded fragmented ions y11/y12 and b9/b10.

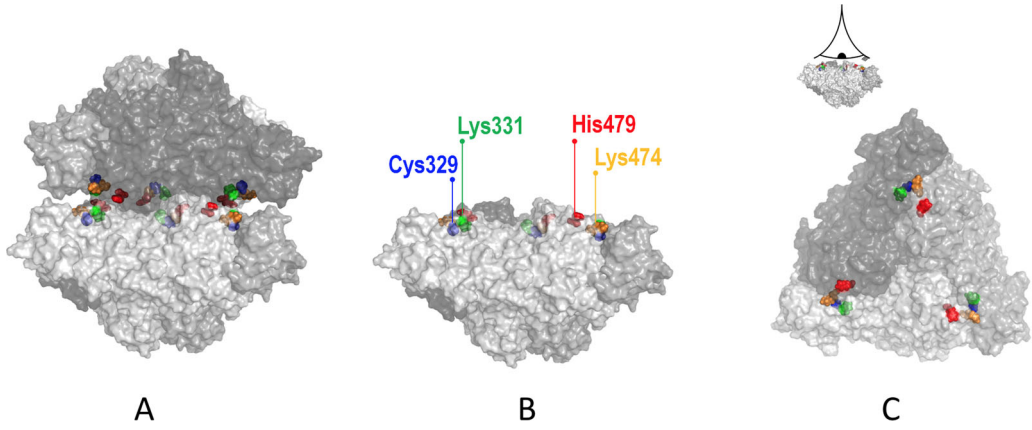


Figure 5. 3 D structures of JBU showing the amino acid residues modified by formaldehyde treatment in CNTX and their location at the trimer–trimer interface. Panel (A) shows the hexamer of JBU (PDB entry 3LA4) and the trimer appears in panels (B, lateral view) and (C, top view, showing the inter-trimer surface of one trimer). Each 90 kDa subunit (monomer) is shown in a different shade of gray. Imine-modified His331, His479 and Lys474 residues and the methylolated Cys329 residue are depicted in each of the three JBU monomers, at the surface of the trimer–trimer interface (B)(C). As a consequence of this location, these residues would be more exposed to react with formaldehyde in the trimeric form of JBU, as compared to the hexamer.

in the non-treated CNTX could likely be artifacts from the analysis algorithm or, in the case of the more numerous hits, may represent peptides differing between the sequences of CNTX and JBU, the latter one used as search base (Table 1).

An example of MS spectrum showing a peptide containing the modified amino acid residue His479 is shown in Figure 4. Additional spectra for relevant modified peptides can be found in the Supplementary Online Material.

Formaldehyde can modify many amino acid residues in several ways, reacting with their N-terminal or side-chain amino groups, forming methylol adducts which may react further to form different compounds (Metz et al., 2004). Lysine, tryptophan, and cysteine side chains are prone to methylolation by low concentrations of formaldehyde. Although a methylol group is the first modification introduced by formaldehyde, identified by an addition of 30 mass units to the modified residue, it is reversible and generally not the most abundantly found. Dehydration of the methylol group yields a more stable imine adduct, or Schiff base, resulting in the addition of 12 mass units to the modified residue. In the case of cysteine, methylolation of the thiol group can lead to the formation of hemithioacetal and thiazolidine products (Metz et al., 2004). The imine group can further react by cross-linking to other amino acid residues forming intramolecular or intermolecular methylene bridges, or become methylated, resulting in a N^{ε}-methyllysine [Lys(Me)] or $N^{\varepsilon},N^{\varepsilon}$-dimethyllysine [$Lys(Me)_2$] residues (Kamps et al., 2019). These cross-linking reactions usually require higher formaldehyde concentrations and longer periods of incubation (Sutherland et al., 2008; Toews et al., 2008).

As expected for the mild condition (0.02% or 0.1%, 8–16 hours at 4 °C) of the formaldehyde treatment employed to stabilize CNTX, most of the modified peptides contained imine groups. Lysine, followed by tryptophan, histidine, and cysteine were the amino acid residues modified by formaldehyde in CNTX. Some residues were found possessing either an imine or methylol group in different spectra, which is consistent with the temporary character of the methylol modification. These modifications are reversible, explaining the need of multiple formaldehyde treatments of CNTX along its purification to keep it from oligomerizing and losing its biological activity (Metz et al., 2004). Some residues were found to be modified in all the samples, but in general the modifications varied along the protein sequence.

3.4. Oligomerization of CNTX and stabilization by formaldehyde

Considering its high similarity to JBU and the lack of the crystal structure of CNTX, localization of the amino acids modified by

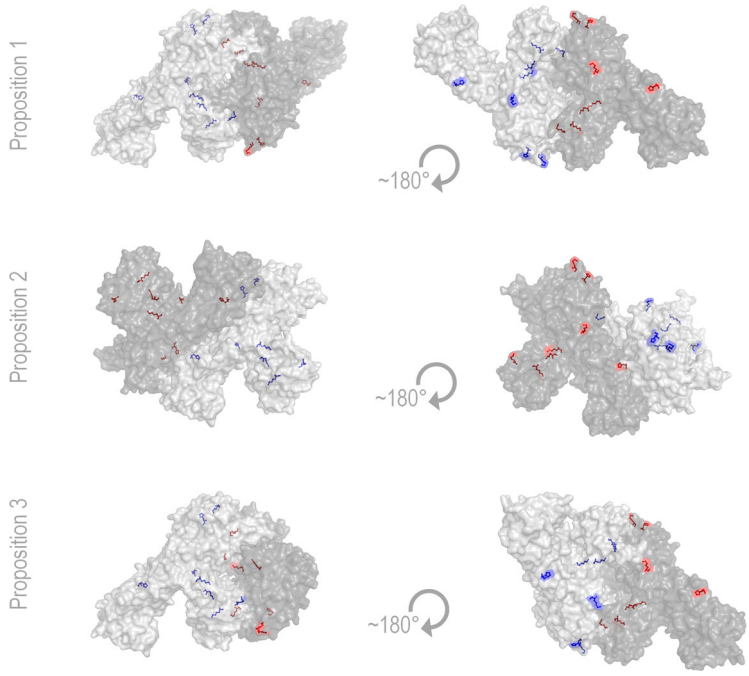


Figure 6. Top scoring solutions for the putative urease dimer. Residues affected by formaldehyde are color coded as Cys443 (blue), His331, His479 (green), Lys483, Lys 744, Lys 755 (red).

Table 2. JBU interfaces statistics (inspected with PDBsum).

Chains	Number of interface resdiues	Interface Area (Å2)	Number of salt bridges	Number of disulfide bonds	Number of hydrogen bonds	Number of non-bonded contacts
A}{B	102:113	4919:4834	16	–	69	803
& B}{C	102:113	4925:4826	16	–	67	800
& D}{F	101:112	4898:4807	16	–	67	792
A}{C	113:102	4834:4920	16	–	69	801
& D}{E	113:102	4834:4919	16	–	69	802
& E}{F	113:102	4834:4919	16	–	69	803
A}{E	8:8	586:586	–	–	2	38
& B}{F	8:8	586:586	–	–	2	38
& C}{D	8:8	586:586	–	–	2	38
A}{F	2:2	95:95	–	–	–	2
& B}{D	2:2	95:95	–	–	–	2
& C}{E	2:2	95:95	–	–	–	2

*All chains are equivalent, and all consecutive interfaces indicated with '&' are equivalent.

formaldehyde treatment was carried out using the JBU 3 D structure. Notably, the imine-modified residues Lys331, Lys474 and His479 are located at the interface between trimers, when considering the JBU hexamer (Figure 5). Other modified residues found at this surface were Lys483 and Lys782 in the treated sample, although with low spectral counts, while the methylol-modified Cys329 appeared in both, treated and non-treated CNTX.

By predicting the orientation of a putative urease dimer (Figure 6) via dedicated docking tools, it was possible to show that, even if dimeric, *C. ensiformis* ureases would have all the formaldehyde-affected residues exposed at the surface.

The presence of imine and methylol adducts at the interfaces could hinder or prevent the association of two trimers or three dimers to form hexamers of JBU. On the other hand, in contrast to a dimeric/trimeric CNTX, these amino acids would be less exposed at the surface of the hexameric JBU, and hence less or not susceptible to modification by formaldehyde. Formaldehyde has no effect on the stability of JBU (data not shown), which is also prone to oligomerization but at a much slower rate (Follmer et al., 2004). Inactive CNTX aggregates with >1,400 kDa were often found in untreated samples, after 45 days at 4 °C, as reported here (Figure 2B) and in Follmer et al., 2001. The data thus explain the stabilization of CNTX by the formaldehyde treatment, as it blocks, or at least delays, its tendency to oligomerize, thereby retaining its biological activity for a longer period.

Table 2 summarizes the interactions and/or contacts between the monomers and between trimers of JBU. The six monomers (ABCDEF) are considered equivalent, and are organized as two trimers, (ABC) and (DEF).

The JBU's trimers and hexamers are stabilized by salt bridges, hydrogen bonds and non-bonding contacts (Van der Waal interactions). There are no disulfide bonds in the JBU. The surface contact areas (~5000 Å^2) and the number and type of bonds between monomers in one trimer are very similar: 12–14 salt bridges, 58–60 H bonds, and 595–625 non-bonding interactions. In the ABC trimer, the A:B monomers have 92:104 amino acids on their surface, B:C have 93:104, and A:C have 101:94. Similar numbers of residues appear on the interface of the other trimer, DEF.

Table 3. Amino acids residues at the surface of interaction between monomers and trimers of JBU.

Monomers[1]		AF		AB		AC			
Contact position		Inter-trimer				Intra-trimer			
Bonded Amino acid[2]		Ala334	Ile335	Lys474	Glu477	Cys329	Gly320	Arg220	Gly330
Modified amino acid[3]									
Cys329	NB*			X					
	SB**								
His331	NB				X				
	SB				X				
Lys474	NB					X	X	X	X
	SB								
His479	NB	X	X						
	SB								

[1]monomers A, B and C form trimer ABC; monomers D, E and F form trimer DEF.
[2]amino acid residues at the inter-trimer or intra-trimer surface which make contact with formaldehyde-modified amino acids (numbered according to JBU sequence).
[3]modified amino acid residues in formaldehyde-treated CNTX (numbered according to JBU sequence).
*Non-bonded contact; ** salt bridge.

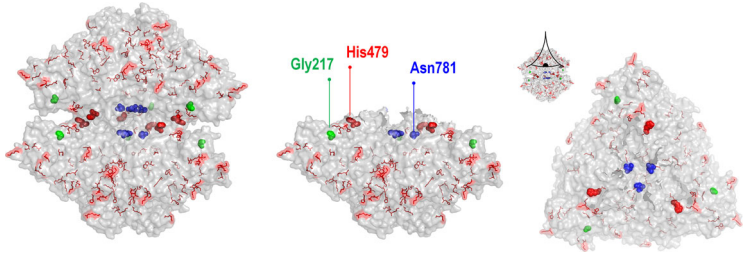


Figure 7. Localization of amino acid residues that differ between CNTX and JBU as identified by *de novo* sequencing. Left: Lateral view of the JBU hexamer, with the different amino acids found in CNTX indicated in red, while Gly217, His479 e Asn781 residues are shown in green, red and blue highlight, respectively. Center: Lateral view of the JBU trimer, depicting the Gly217, His479 e Asn781 residues involved in inter-trimer interactions. Amino acids in positions 217 and 781 in CNTX were both identified as arginine in the *de novo* analysis. Right: Top view of the JBU trimer, showing the inter-trimer surface of one trimer.

The surface contact area between different trimers, ABC and DEF, is comparatively smaller, varying between ~70 to 770 Å^2, but there are contacts between all the monomers of one trimer (ABC) with all the monomers of the other trimer (DEF). Considering the triangular shape of the trimers, and that the JBU hexamer represents two superimposed triangles with their vertices aligned, the monomers from different juxtaposed trimers, such as A:F, B:E and C:D, have 11 amino acids from each monomer involved in contact between the trimers. There are no salt bridges between the trimers, the hexamers being stabilized by a few hydrogen bonds and non-bonding contacts.

Table 3 lists the amino acid residues modified by formaldehyde in CNTX that are located at the interaction surfaces between the JBU monomers in a trimer or between trimers.

It can be observed that among the amino acid residues modified by formaldehyde treatment of CNTX, most of them make intra-trimer contacts (e.g. while being in the A monomers, they just make contacts with B or C). Only His479 participates in inter-trimer contacts (AF). Therefore, this is the modification introduced by formaldehyde treatment that most directly hinders the formation of hexamers, thus resulting in the stabilization of CNTX (Figure 5). However, modifications of residues involved in intra-trimer contacts could alter the trimer's conformation and obstruct its oligomerization into a hexamer.

Moreover, results from the *de novo* sequencing analysis of CNTX indicated differences in amino acids residues that could participate in inter-trimer contacts. Some of the different amino acid residues found in CNTX introduce potential targets for additional formaldehyde modifications, namely (first AA from JBU-position-AA in CNTX): G61K, V210R, N217R, A240R, G294R, I424R, A435C, A436R, G437R, S608H and N781R. Figure 7 illustrates these distinct residues identified in CNTX positioned on the 3 D structure of the JBU hexamer. Noteworthy, two of these distinct residues, Gly217 and Asn781, which in JBU are involved in inter-trimer bonds, were identified in CNTX as arginine residues, which in addition to His479, could be susceptible to formaldehyde modification thereby blocking the protein oligomerization process.

4. Conclusions

In this work, we successfully characterized CNTX as a lower molecular mass urease with high similarity to the most abundant urease isoform, JBU, present in the jack bean seed. The native state of CNTX appears as a dimer or a trimer of the 90 kDa subunit, which could reflect differences in the amino acid sequence of residues lying at the surface of the monomers.

The observed differences in amino acid sequences for CNTX and JBU (and JBURE-II as well) point to paralog genes or alternative splicing events. The latter process accounts for urease isoforms in potatoes (Solanaceae) (Witte et al., 2005). Multiple urease genes, however, are found in at least one other legume, the soybean, from the same Fabaceae family as *C. ensiformis*. In the soybean there are two well-described ureases, the ubiquitous and the embryo-specific urease, along with a third non-catalytic, defense-only urease (Wiebke-Strohm et al., 2016). Considering the multiple toxin-like properties described for ureases, it is tempting to consider its co-option for defense in various legume plants.

The difference in oligomeric state of the *C. ensiformis* ureases impacts some of their physicochemical characteristics as well as biological properties, including their intraperitoneal toxicity to mice and possibly also their ureolytic activity.

The mechanism by which the formaldehyde treatment of CNTX stabilizes its biologically active conformation was elucidated and attributed to modifications introduced in the side chain of amino acid residues located at the interface between dimers or trimers, resulting in a protein less prone to oligomerization.

Acknowledgements

C.R.C is thankful to Prof. Jorge Almeida Guimarães for his guidance and contributions in the early stages of CNTX's studies.

Disclosure statement

The authors report there are no competing interests to declare.

ORCID

Carlo Frederico Moro http://orcid.org/0000-0002-8109-4883
Fábio C. S. Nogueira http://orcid.org/0000-0001-5507-7142
Carlos Gabriel Moreira Almeida http://orcid.org/0000-0002-4528-4233
Rafael Real-Guerra http://orcid.org/0000-0002-1346-3536
Pedro Ferrari Dalberto http://orcid.org/0000-0002-0929-6739
Cristiano V. Bizarro http://orcid.org/0000-0002-2609-8996
Rodrigo Ligabue-Braun http://orcid.org/0000-0002-2555-9754
Celia R. Carlini http://orcid.org/0000-0002-7027-2445

Data availability statement

The data that support the findings of this study are available from the corresponding author (C. R. C.) upon reasonable request.

Author contributions

CFM, CGMA and CRC conducted purification of CNTX and stability experiments, analyzed data and wrote the first draft of the manuscript, CFM and FCSN conducted MS experiments and analyzed data, RRG and PFD have conducted MS experiments, CVB analyzed MS data and reviewed the manuscript, RL-B conducted molecular modeling and wrote the manuscript, CRC conceptualized this work, interpreted data and wrote the manuscript.

References

Almeida, C. G. M., Costa-Higuchi, K., Piovesan, A. R., Moro, C. F., Venturin, G. T., Greggio, S., Costa-Ferro, Z. S., Salamoni, S. D., Peigneur, S., Tytgat, J., de Lima, M. E., Silva, C. N. D., Vinade, L., Rowan, E. G., DaCosta, J. C., Dal Belo, C. A., & Carlini, C. R. (2021). Neurotoxic and convulsant effects induced by jack bean ureases on the mammalian nervous system. *Toxicology*, *454*, 152737. https://doi.org/10.1016/j.tox.2021.152737

Balasubramanian, A., & Ponnuraj, K. (2010). Crystal structure of the first plant urease from jack bean: 83 years of journey from its first crystal to molecular structure. *Journal of Molecular Biology*, *400*(3), 274–283. https://doi.org/10.1016/j.jmb.2010.05.009

Bowles, D. J., Marcus, S. E., Pappin, D. J., Findlay, J. B., Eliopoulos, E., Maycox, P. R., & Burgess, J. (1986). Posttranslational processing of concanavalin A precursors in jackbean cotyledons. *The Journal of Cell Biology*, *102*(4), 1284–1297. https://doi.org/10.1083/jcb.102.4.1284

Broll, V., Perin, A. P. A., Lopes, F. C., Martinelli, A. H. S., Moyetta, N. R., Fruttero, L. L., Grahl, M. V. C., Uberti, A. F., Demartini, D. R., Ligabue-Braun, R., & Carlini, C. R. (2021). Non-enzymatic properties of *Proteus mirabilis* urease subunits. *Process Biochemistry*, *110*, 263–274. https://doi.org/10.1016/j.procbio.2021.08.023

Carlini, C. R., & Guimaraes, J. A. (1981). Isolation and characterization of a toxic protein from *Canavalia ensiformis* (jack bean) seeds, distinct from concanavalin A. *Toxicon*, *19*(5), 667–675. https://doi.org/10.1016/0041-0101(81)90104-5

Carlini, C. R., & Ligabue-Braun, R. (2016). Ureases as multifunctional toxic proteins: A review. *Toxicon: Official Journal of the International Society on Toxinology*, *110*, 90–109. https://doi.org/10.1016/j.toxicon.2015.11.020

Demartini, D. R., Carlini, C. R., & Thelen, J. J. (2011). Global and targeted proteomics in developing jack bean (*Canavalia ensiformis*) seedlings: An investigation of urease isoforms mobilization in early stages of development. *Plant Molecular Biology*, *75*(1–2), 53–65. https://doi.org/10.1007/s11103-010-9707-3

Eng, J., Jahan, T., & Hoopmann, M. (2013). Comet: An open-source MS/MS sequence database search tool. *Proteomics*, *13*(1), 22–24. https://doi.org/10.1002/pmic.201200439

Feathers, J. R., Spoth, K. A., & Fromme, J. C. (2021). Experimental evaluation of super-resolution imaging and magnification choice in single-particle cryo-EM. *Journal of Structural Biology: X*, *5*, 100047. https://doi.org/10.1016/j.yjsbx.2021.100047

Follmer, C., Barcellos, G. B., Zingali, R. B., Machado, O. L. T., Alves, E. W., Barja-Fidalgo, C., Guimaraes, J. A., & Carlini, C. R. (2001). Canatoxin, a toxic protein from jack beans (*Canavalia ensiformis*), is a variant form of urease (EC 3.5.1.5): Biological effects of urease independent of its ureolytic activity. *The Biochemical Journal*, *360*(Pt 1), 217–224. https://doi.org/10.1042/bj3600217

Follmer, C., Carlini, C. R., Yoneama, M. L., & Dias, J. F. (2002). PIXE analysis of urease isoenzymes isolated from *Canavalia ensiformis* (jack bean) seeds. *Nuclear Instruments and Methods in Physics Research Section B: Beam Interactions with Materials and Atoms*, *189*(1–4), 482–486. https://doi.org/10.1016/S0168-583X(01)01128-4

Follmer, C., Pereira, F. V., Da Silveira, N. P., & Carlini, C. R. (2004). Jack bean urease (EC 3.5.1.5) aggregation monitored by dynamic and static light scattering. *Biophysical Chemistry*, *111*(1), 79–87. https://doi.org/10.1016/j.bpc.2004.03.009

Follmer, C., Real-Guerra, R., Wasserman, G. E., Olivera-Severo, D., & Carlini, C. R. (2004). Jackbean, soybean and *Bacillus pasteurii* ureases—Biological effects unrelated to ureolytic activity. *European Journal of Biochemistry*, *271*(7), 1357–1363. https://doi.org/10.1111/j.1432-1033.2004.04046.x

Follmer, C., Wassermann, G. E., & Carlini, C. R. (2004). Separation of jack bean (*Canavalia ensiformis*) urease isoforms by immobilized metal affinity chromatography and characterization of insecticidal properties unrelated to ureolytic activity. *Plant Science*, *167*(2), 241–246. https://doi.org/10.1016/j.plantsci.2004.03.019

Grahl, M. V. C., Uberti, A. F., Broll, V., Bacaicoa-Caruso, P., Meirelles, E. F., & Carlini, C. R. (2021). *Proteus mirabilis* urease: Unsuspected non-enzymatic properties relevant to pathogenicity. *International Journal of Molecular Sciences*, *22*(13), 7205. https://doi.org/10.3390/ijms22137205

Kamps, J., Hopkinson, R., Schofield, C., & Claridge, T. (2019). How formaldehyde reacts with amino acids. *Communications Chemistry*, *2*(1), 126. https://doi.org/10.1038/s42004-019-0224-2

Kappaun, K., Piovesan, A. R., Carlini, C. R., & Ligabue-Braun, R. (2018). Ureases: Historical aspects, catalytic, and non-catalytic properties—A review. *Journal of Advanced Research*, *13*, 3–17. https://doi.org/10.1016/j.jare.2018.05.010

Laskowski, R. A., Jabłońska, J., Pravda, L., Vařeková, R. S., & Thornton, J. M. (2018). PDBsum: Structural summaries of PDB entries. *Protein Science: A Publication of the Protein Society*, *27*(1), 129–134. https://doi.org/10.1002/pro.3289

Ligabue-Braun, R., Andreis, F. C., Verli, H., & Carlini, C. R. (2013). 3-to-1: Unraveling structural transitions in ureases. *Die Naturwissenschaften*, *100*(5), 459–467. https://doi.org/10.1007/s00114-013-1045-2

Marti-Renom, M. A., Stuart, A. C., Fiser, A., Sanchez, R., Melo, F., & Sali, A. (2000). Comparative protein structure modeling of genes and

genomes. *Annual Review of Biophysics and Biomolecular Structure*, *29*, 291–325. https://doi.org/10.1146/annurev.biophys.29.1.291

Metz, B., Kersten, G. F. A., Hoogerhout, P., Brugghe, H. F., Timmermans, H. A. M., de Jong, A., Meiring, H., ten Hove, J., Hennink, W. E., Crommelin, D. J. A., & Jiskoot, W. (2004). Identification of formaldehyde-induced modifications in proteins—Reactions with model peptides. *The Journal of Biological Chemistry*, *279*(8), 6235–6243. https://doi.org/10.1074/jbc.M310752200

Metz, B., Tilstra, W., van der Put, R., Spruit, N., van den IJssel, J., Robert, J., Hendriksen, C., & Kersten, G. (2013). Physicochemical and immunochemical assays for monitoring consistent production of tetanus toxoid. *Biologicals: Journal of the International Association of Biological Standardization*, *41*(4), 231–237. https://doi.org/10.1016/j.biologicals.2013.05.001

Mulinari, F., Becker-Ritt, A. B., Demartini, D. R., Ligabue-Braun, R., Staniscuaski, F., Verli, H., Fragoso, R. R., Schroeder, E. K., Carlini, C. R., & Grossi-de-Sa, M. F. (2011). Characterization of JBURE-IIb isoform of *Canavalia ensiformis* (L.) DC urease. *Biochimica et Biophysica Acta*, *1814*(12), 1758–1768. https://doi.org/10.1016/j.bbapap.2011.07.022

Santos, M. D. M., Lima, D. B., Fischer, J. S. G., Clasen, M. A., Kurt, L. U., Camillo-Andrade, A. C., Monteiro, L. C., de Aquino, P. F., Neves-Ferreira, A. G. C., Valente, R. H., Trugilho, M. R. O., Brunoro, G. V. F., Souza, T., Santos, R. M., Batista, M., Gozzo, F. C., Duran, R., Yates, J. R., 3rd, Barbosa, V. C., & Carvalho, P. C. (2022). Simple, efficient and thorough shotgun proteomic analysis with PatternLab V. *Nature Protocols*, *17*(7), 1553–1578. https://doi.org/10.1038/s41596-022-00690-x

Schneidman-Duhovny, D., Inbar, Y., Nussinov, R., & Wolfson, H. J. (2005a). Geometry-based flexible and symmetric protein docking. *Proteins*, *60*(2), 224–231. https://doi.org/10.1002/prot.20562

Schneidman-Duhovny, D., Inbar, Y., Nussinov, R., & Wolfson, H. J. (2005b). PatchDock and SymmDock: Servers for rigid and symmetric docking. *Nucleic Acids Research*, *33*(Web Server issue), W363–367. https://doi.org/10.1093/nar/gki481

Sievers, F., Wilm, A., Dineen, D., Gibson, T. J., Karplus, K., Li, W., Lopez, R., McWilliam, H., Remmert, M., Soding, J., Thompson, J. D., & Higgins, D. G. (2011). Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Molecular Systems Biology*, *7*, 539. https://doi.org/10.1038/msb.2011.75

Staniscuaski, F., & Carlini, C. (2012). Plant ureases and related peptides: Understanding their entomotoxic properties. *Toxins*, *4*(2), 55–67. https://doi.org/10.3390/toxins4020055

Sutherland, B. W., Toews, J., & Kast, J. (2008). Utility of formaldehyde cross-linking and mass spectrometry in the study of protein-protein interactions. *Journal of Mass Spectrometry*, *43*(6), 699–715. https://doi.org/10.1002/jms.1415

Toews, J., Rogalski, J. C., Clark, T. J., & Kast, J. (2008). Mass spectrometric identification of formaldehyde-induced peptide modifications under in vivo protein cross-linking conditions. *Analytica Chimica Acta*, *618*(2), 168–183. https://doi.org/10.1016/j.aca.2008.04.049

Wiebke-Strohm, B., Ligabue-Braun, R., Rechenmacher, C., De Oliveira-Busatto, L. A., Carlini, C. R., & Bodanese-Zanettini, M. H. (2016). Structural and transcriptional characterization of a novel member of the soybean urease gene family. *Plant Physiology and Biochemistry*, *101*, 96–104. https://doi.org/10.1016/j.plaphy.2016.01.023

Witte, C. P., Tiller, S., Isidore, E., Davies, H. V., & Taylor, M. A. (2005). Analysis of two alleles of the urease gene from potato: Polymorphisms, expression, and extensive alternative splicing of the corresponding mRNA. *Journal of Experimental Botany*, *56*(409), 91–99. https://doi.org/10.1093/jxb/eri014

Yamanushi, T. T., Boyett, M. R., Yamamoto, Y., Ohsaki, H., Hirakawa, E., & Dobrzynski, H. (2015). Comparison of formaldehyde and methanol fixatives used in the detection of ion channel proteins in isolated rat ventricular myocytes by immunofluorescence labelling and confocal microscopy. *Folia Morphologica*, *74*(2), 258–261. https://doi.org/10.5603/fm.2015.0041