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

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Canatoxin is a toxic protein from *Canavalia ensiformis* seeds, lethal to mice (LD_{50} = 2 mg/kg) and insects. Further characterization of canatoxin showed that its main native form (184 kDa) is a non-covalently linked dimer of a 95 kDa polypeptide containing zinc and nickel. Partial sequencing of internal peptides indicated homology with urease (EC 3.5.1.5) from the same seed. Canatoxin has approx. 30% of urease's activity for urea, and K_m of 2–7 mM. The proteins differ in their affinities for metal ions and were separated by affinity chromatography on a Zn^{2+} matrix. Similar to canatoxin, urease activates blood platelets and interacts with glycoconjugates. In contrast with canatoxin, no lethality was seen in mice injected with urease (10 mg/kg). Pretreatment with *p*-hydroxymercuribenzoate irreversibly abolished the ureolytic activity of both proteins. On the other hand, *p*-hydroxymercuribenzoate-treated canatoxin was still lethal to mice, and both treated proteins were fully active in promoting platelet aggregation and binding to glycoconjugates. Taken together, our data indicate that canatoxin is a variant form of urease. Moreover, we show for the first time that these proteins display several biological effects that are unrelated to their enzymic activity for urea.

Key words: isoenzyme, multidomain, nickel, metalloprotein, zinc.

INTRODUCTION

The jack bean *Canavalia ensiformis* is the source of a variety of interesting proteins which have contributed as milestones in modern biochemistry. One of these is urease, the first protein to be crystallized by Sumner [1], and the first nickel-containing enzyme described [2]. The lectin concanavalin A was also isolated from jack beans by Sumner and Howell [3].

In 1981, we isolated [4] a toxic protein from jack beans named canatoxin, which accounts for 0.5% of seed dry weight. Canatoxin is lethal to rats and mice (LD_{50} = 2 mg/kg) by intraperitoneal injection, but it is inactive given orally [4,5]. Canatoxin induces exocytosis in several cell types, including platelets, synaptosomes, pancreatic islets, macrophages, neutrophils and mast cells [6–9]. Canatoxin-treated rats showed increased plasma levels of gonadotropins and insulin [10,11]. Lipoxygenase metabolites are involved in most pharmacological effects of canatoxin [6–8,11–13] and the protein disrupts Ca^{2+}-transport across membranes [14,15]. More recently, the insecticidal properties of canatoxin were described, suggesting it may play a defensive role in the plant [16,17].

In the present study we now report the partial amino acid sequence and physicochemical properties of this protein demonstrating that canatoxin is a variant form of the jack bean urease (EC 3.5.1.5). The original isolation method [4] was modified to yield a highly purified toxic protein, free from urease contamination.

MATERIALS AND METHODS

Materials

C. ensiformis seeds were purchased from Casa Agrodora (São Paulo, Brazil). Dipipecolylamine (DPA)–Sepharose 6B was a generous gift from Dr Jerker Porath (Department of Biochemistry, University of Arizona, Tucson, AZ, U.S.A.). All other chemicals and reagents were obtained from Sigma Chemical Co. (St Louis, MO, U.S.A.) unless otherwise stated.

Protein determination

The protein content of samples was determined by absorbance at 280 nm. Alternatively, the methods of Spector [18] or Lowry et al. [19] were used.

Purification of canatoxin

The isolation of canatoxin was as previously reported [4], but with the inclusion of a new purification step as described below. Briefly, *C. ensiformis* seeds (20%, w/v) were homogenized in

Abbreviations used: AHA, acetohydroxamic acid; DPA, dipipecolylamine; *p*-HMB, *p*-hydroxymercuribenzoate; PIXE, particle-induced X-ray emission.

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25 mM Tris/HCl, pH 7.5, and the extract, referred to as PREP-A, was mixed with 30 % (v/v) ethanol. The mixture was centrifuged (8500 ***g*** for 30 min at 4 °C) and the resulting supernatant (PREP-B) dialysed against 25 mM Tris/HCl, pH 7.5 (2 × 8 l). Ammonium sulphate was added to PREP-B to give a 0–35 % saturated solution and, after stirring for 2 h, the mixture was centrifuged (8500 ***g*** for 30 min at 4 °C) and the resulting supernatant was precipitated with 35–55 % saturated ammonium sulphate. After standing at 4 °C overnight, the precipitate was collected, resuspended in 25 mM Tris/HCl/100 mM NaCl (pH 7.5), and dialysed against the same buffer to give PREP-C. PREP-C was mixed with DEAE-cellulose equilibrated in 25 mM Tris/HCl/100 mM NaCl (pH 7.5). After stirring for 2 h, the resin was filtered, washed with the same buffer and then eluted with 25 mM Tris/HCl/200 mM NaCl (pH 7.6). The eluent was collected and referred to as PREP-D. Next, aliquots of PREP-D were concentrated 10-fold by ultrafiltration and applied on to a BioGel A 0.5 m column (2.2 cm × 120 cm; Bio-Rad, Richmond, CA, U.S.A.) equilibrated in 50 mM sodium phosphate, pH 7.5. Two peaks were obtained and the fractions corresponding to these peaks were collected to give PREP-F-I and PREP-F-II, respectively. To further increase the purity of canatoxin, a new step was used. A Zn^{2+}-chelated form of DPA–Sepharose 6B was prepared by washing the resin with 50 mM $ZnCl_2$, pH 6.2, and then with 20 mM sodium phosphate/1 M NaCl (pH 7.5). Samples of PREP-F-II (10 mg of protein) were applied on to a 5 ml column packed with the resin. After removal of the unbound material (PREP-G-I) with 20 mM sodium phosphate/1 M NaCl (pH 7.5), the first fraction (PREP-G-II) was eluted with 100 mM imidazole, pH 7.5. The second elution was carried out with 10 mM EDTA in 20 mM sodium phosphate, pH 7.5. The toxic protein (PREP-G-III) eluted in this step represented approx. 80 % of the initial material.

Assay of biological activities of canatoxin

The toxicity of canatoxin, expressed as LD_{50}, was determined by intraperitoneal injection of Swiss mice with various doses of canatoxin ($n = 6$ animals per dose) and lethality within 24 h was recorded [4].

Aggregation of rabbit blood platelets was assayed as in [6] with simultaneous detection of ATP secretion via a dual channel Lumi-aggregometer (Chrono-Log, Haverton, PA, U.S.A.).

Indirect haemagglutination was performed as in [5]. Briefly, samples (25 μl) were incubated for 1 h at 25 °C with rabbit erythrocytes (4 %, v/v) and then transferred to microplate wells containing 25 μl of anti-canatoxin IgM-rich antibodies, with or without inhibitory compounds. After incubation for 2 h at 25 °C, the minimal amount of protein inducing cell agglutination was recorded.

Antibodies

Rabbits were immunized with canatoxin as in [20]. The IgM-enriched serum obtained after 21 days was used for agglutination assays. After two booster immunizations, the anti-canatoxin IgG fraction was purified [20] and adsorbed with canavalin [21], jack bean urease (type C3) and concanavalin A by incubating the IgG preparation with nitrocellulose membranes containing each of the proteins. A monoclonal anti-urease mouse IgG was purified in the same way.

Immunoassays

Double-immunodiffusion assays were carried out as in [22]. For dot blots [23], samples (50 ng–5 μg) were spotted on to nitrocellulose membranes (Schleicher-Schuel, Keene, NH, U.S.A.) and incubated with rabbit anti-canatoxin IgG (1:30000 dilution) or mouse anti-urease IgG (1:5000 dilution) overnight at 4 °C. Goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, U.S.A.) or anti-mouse IgG conjugated to alkaline phosphatase were used as secondary antibodies. Colour reactions were developed with Nitro Blue Tetrazolium and 5-bromo-4-chloroindol-3-yl phosphate ('BCIP'), pH 9.6.

Urease activity

To determine urease activity, samples were incubated with 0.014–5.35 mM urea for 10 min at 37 °C. The ammonia released was measured colorimetrically [24]; 1 unit of urease releases 1 μmol ammonia/min at 37 °C and pH 7.5. Kinetic parameters were calculated as in [25]. For inhibitory studies, the proteins were incubated with *p*-hydroxymercuribenzoate (*p*-HMB; 0.01–100 μM final concentration), acetohydroxamic acid (AHA; 0.05–500 μM final concentration) or the corresponding diluents for 18–24 h at 4 °C.

FPLC

Gel filtration was performed on Superose 6 or 12 HR 10/30 columns, equilibrated in 50 mM sodium phosphate buffer (pH 7.5). Reversed phase chromatography was performed on C2–C18 columns (PepRPC; Amersham Pharmacia Biotech, Uppsala, Sweden) using acetonitrile gradients. FPLC apparatus used for gel filtration and reversed phase chromatography was obtained from Amersham Pharmacia Biotech.

SDS/PAGE

Samples were resolved by SDS/PAGE on 10 % (w/v) polyacrylamide gels containing 0.1 % SDS run for 2–3 h at 20 mA [26]. Gels were stained with Coomassie Brilliant Blue R-250.

Molecular mass-determinations

The molecular mass of canatoxin was estimated by gel filtration [27] after SDS/PAGE [26], and by ultracentrifugation [28] using glycerol gradients (5–30 %, v/v). The proteins used as molecular mass-standards were thyreoglobulin (660 kDa), apoferritin (440 kDa), β-amylase (200 kDa), bovine γ-globulin (160 kDa), β-galactosidase (116 kDa), phosphorylase B (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), soya bean trypsin inhibitor (20 kDa), α-lactalbumin (14.2 kDa) and cytochrome *c* (12.8 kDa).

Amino acid composition of canatoxin

Amino acid composition of 2 mg samples of dried protein were determined after hydrolysis in 6 M HCl for 22 h at 110 °C, as described in [29]. The analyses were performed at the Interdepartmental Center for Protein Chemistry, Universidade de São Paulo (São Paulo, Brazil) and at the Arizona Research Laboratories (Tucson, AZ, U.S.A.).

Microsequencing studies

Internal peptides obtained by hydrolysing canatoxin with *N*-tosyl-L-phenylalanine chloromethyl ketone ('TPCK')-treated trypsin (1:20 mass ratio) or endoproteinase Lys-C (1:40 mass ratio), were isolated by reversed phase chromatography. Sequence analyses were performed at the Protein Sequencing Unit,

Department of Biochemistry, Universidade Federal do Rio de Janeiro (Rio de Janeiro, Brazil) and the Microchemistry Facility, Harvard University (Boston, MA, U.S.A.).

Carbohydrate content

Total neutral carbohydrate was determined as in [30].

Metal determinations

Protein solutions were dialysed against 5 mM EDTA and analysed for atomic absorption on a Varian AA-1475 spectrophotometer at the Institute of Biophysics "Carlos Chagas Filho", Universidade Federal do Rio de Janeiro (Rio de Janeiro, Brazil). Alternatively, multielemental determination of metals was performed by particle-induced X-ray emission (PIXE) [31,32]. Briefly, acetone (70 %, v/v) was added to the proteins in solution and the precipitates were collected on cellulose filters (0.45 μm; Sartorius, São Paulo, Brazil). The mass of protein retained on the filter was estimated by calculating the difference in the dry weight. The filters were irradiated with a proton beam (5 nA current) for 3600 s on a 3 MeV Tandetron machine at the Institute of Physics, Universidade Federal do Rio Grande do Sul (Rio Grande do Sul, Brazil). Results were analysed by the GUPIX software [33].

RESULTS

Table 1 summarizes the purification of canatoxin. Figure 1(A) illustrates a typical gel filtration trace, with two peaks of toxic fractions, PREP-F-I and PREP-F-II, containing approx. 20 and 80 % of the total protein, respectively. Both PREP-F-I and PREP-F-II were lethal at similar doses (2–3 mg/kg) when injected into mice and induced platelet aggregation at concentrations of 10–20 μg of protein/ml (results not shown).

Table 1 Purification of canatoxin

Samples were removed during the purification steps of canatoxin and assayed for urease specific activity (spec. act.) and LD_{50} as described in the Materials and methods section. The protein recovered, purification index and purity were also assessed for each purification step. The data for PREP A–PREP-D were obtained from [4]. Data for the LD_{50} (mg/kg of body weight required to kill 50% of mice within 24 h) and protein recovered (g of protein/kg of seed) are means ± S.E.M. of seven to nine preparations. Data for the urease specific activity (m-units/μg of protein) are means of at least two runs. The purification index and yield (%) were calculated by comparison with the LD_{50} of crude extracts. Nt; no toxicity was detected.

Purification step	Urease spec.act.	LD_{50}	Protein recovered	Purification index	Yield
Crude extract					
PREP-A	16.7	122 ± 30	340 ± 26	1	100
Ethanol treatment					
PREP-B	–	25.3 ± 5.8	27.0 ± 3.1	4.8	38
Ammonium sulphate					
0–35%	28.1	Nt			
35–55% (PREP-C)	12.8	6.1 ± 1.2	5.7 ± 0.6	20.0	34
DEAE-cellulose					
Non-retained	12.9	Nt			
Eluted PREP-D	8.2	3.3 ± 1.1	5.2 ± 0.9	37.0	57
Gel filtration					
PREP-F-II	12.2	2.3 ± 0.7	1.1 ± 0.4	53.0	17
Zn^{2+}-DPA column					
PREP-G-III	5.9	2.0 ± 0.5	0.9 ± 0.2	61.0	16

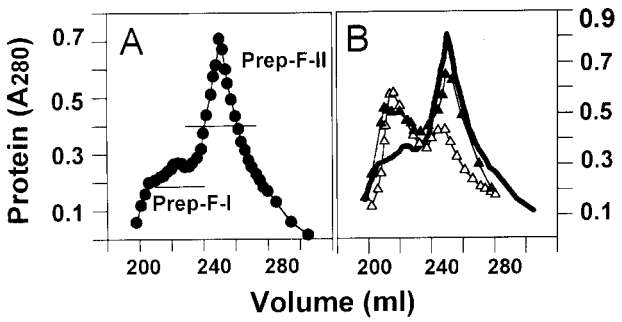

Figure 1 Gel filtration and effect of storage of canatoxin

(**A**) PREP-D (50 mg in 5 ml) was applied into a BioGel A column (2.2 cm × 120 cm) at 8.0 ml/h flux and the absorbance of the fractions determined at 280 nm. The toxic fractions (above the bars) were pooled to give PREP-F-I and PREP-F-II. (**B**) Gel filtration of the same batch of PREP-D was performed as in (**A**) on day zero (solid line) and after storage at 4 °C for 45 (▲) or 70 days (△).

Characterization of canatoxin

SDS/PAGE of PREP-F-II (Figure 2) resulted in two protein bands; band A, which did not enter the separating gel, and band C with a molecular mass of 95–100 kDa. In some gels a very faint band (band B), between bands A and C, was observed. The same bands appeared in non-denaturing gels or under reducing conditions. PREP-F-I consisted mainly of band A after SDS/PAGE. Storage at 4 °C led to the spontaneous formation of PREP-F-I, an aggregated form of canatoxin (Figure 1B).

Gel filtration on a Superose 6 column of PREP-F-II revealed a main peak with an apparent molecular mass of 183.7 ± 17.8 kDa ($n = 16$; Figure 3B). By analytical ultracentrifugation, the estimated mass of PREP-F-II was 170 kDa (results not shown). Thus the main native form of canatoxin was a non-covalently linked dimer consisting of a single 95–100 kDa polypeptide. PREP-F-I gave a main peak of about 1400 kDa as estimated by extrapolating the calibration curve of the Superose 6 column (Figure 3A).

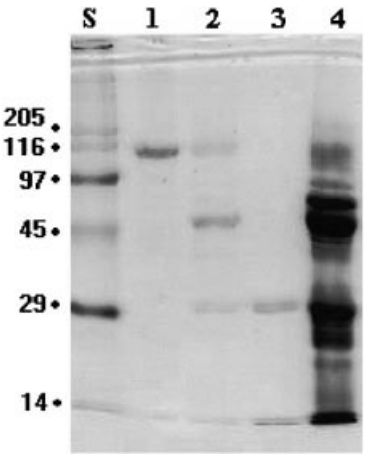

Figure 2 SDS/PAGE analysis of canatoxin

SDS/PAGE analysis was performed using a 10% (w/v) polyacrylamide gel and the gel was stained with Coomassie Blue. Lane 1, canatoxin (PREP-F-II, 5 μg); lane 2, urease (8 μg); lane 3, concanavalin A (5 μg) and lane 4, *C. ensiformis* crude extract (PREP-A, 100 μg). Lane S, molecular mass standards are shown (in kDa) on the left.

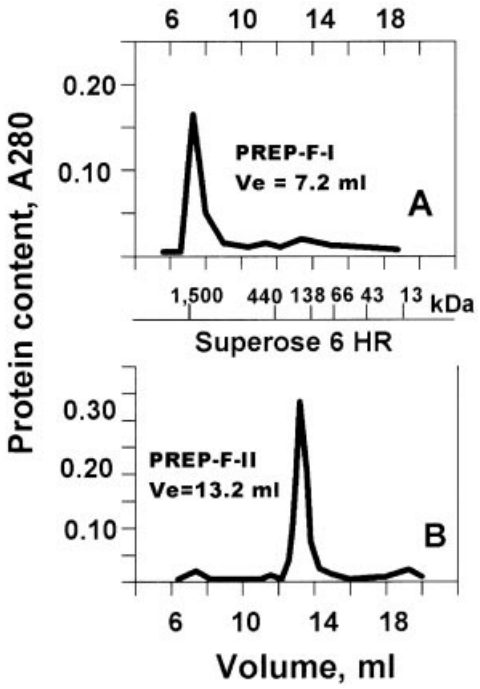

Figure 3 Gel filtration patterns of two forms of canatoxin

Gel filtration of 100 μg of PREP-F-I (**A**) or PREP-F-II (**B**), respectively, using an FPLC Superose 6 HR 10/30 column, equilibrated with 50 mM sodium phosphate, pH 7.5, at a flow rate of 0.3 ml/min. The absorbance was determined at 280 nm. The column was calibrated with molecular mass standards (in kDa) as shown between (**A**) and (**B**). Elution volume (Ve) is shown.

A 0.1% (w/v) solution of salt-free lyophilized canatoxin (PREP-F-II), dissolved in 25 mM Tris/HCl, pH 7.5, gave an absorption coefficient of 1.0 at 280 nm. Similar values were obtained by the methods of Spector [18] or Lowry et al. [19]. Canatoxin contained less than 0.5% (w/w) neutral carbohydrates (results not shown).

Stabilization of canatoxin

In our first description of canatoxin [4], the protein was increasingly unstable during purification. Canatoxin is inactivated upon freezing/thawing or in conditions below its isoelectric point (pH 4.7–4.8). Storage with proteinase inhibitors, glycerol, gelatin, detergents, lyophilized or as a precipitate in saturated ammonium sulphate did not protect the protein. Dithiothreitol (5 mM) or *p*-HMB (200 μM) neither interfered with the initial toxicity nor decreased its inactivation rate. Stabilization of canatoxin's lethal activity for up to 30 days was achieved by keeping the protein at 4 °C, at a concentration of less than 1.0 mg/ml in solutions with pH 7.5 and with merthiolate as preservative.

Amino acid composition and partial sequence of canatoxin

Table 2 shows the amino acid composition of canatoxin. A mass of 97180 Da was calculated for the monomer, which is in good agreement with 95000 Da as estimated by SDS/PAGE. Partial sequences were determined for 13 internal peptides of canatoxin (PREP-G-III, see below), obtained by trypsin or endoproteinase Lys-C digestions. The data indicated a high degree of homology with jack bean urease (EC 3.5.1.5). Figure 4 shows canatoxin-derived peptides aligning with urease [34–36]. Although a blocked N-terminal residue was found in canatoxin, the homology was apparently more pronounced at the N-terminal region of urease. When exposed to pH 3.0 and 2 mM EDTA, canatoxin formed a soluble fragment of approx. 10 kDa, whereas the remaining polypeptide chain precipitated irreversibly. Peptides derived from this fragment aligned with the C-terminal region of urease, but contained several amino acid substitutions. No such fragment was seen when urease was submitted to the same treatment.

Table 2 Amino acid composition of canatoxin and urease

The percentage contribution of a given amino acid to the total number of amino acids (100%) is shown for both canatoxin and urease. Data are means ± S.E.M. of four preparations of canatoxin (two PREP-F-II and two PREP-G-III). Only one determination of tryptophan was performed. The number of amino acid residues was calculated considering a mass of 95 kDa for the canatoxin monomer. Data for urease were deduced from its cDNA (GenBank® accession number M65260).

	Canatoxin		Urease
Amino acid	%	No. residues	%
Lys	5.55 ± 0.48	41	5.8
His	2.17 ± 0.37	15	2.9
Arg	4.10 ± 0.24	25	4.4
Trp	0.5	2	0.5
Asp/x	10.72 ± 0.61	89	10.6
Thr	6.25 ± 0.54	59	6.5
Ser	7.32 ± 0.80	80	5.6
Glu/x	11.55 ± 1.17	85	8.0
Pro	6.20 ± 0.39	61	5.0
Gly	8.85 ± 0.55	147	9.4
Ala	8.25 ± 0.47	110	8.8
Cys	1.45 ± 0.45	13	1.8
Val	6.42 ± 0.60	62	6.7
Met	2.35 ± 0.05	17	2.5
Ile	5.60 ± 0.61	47	7.8
Leu	7.77 ± 0.58	65	8.3
Tyr	2.35 ± 0.36	17	2.6
Phe	2.90 ± 0.17	19	2.7

Double immunodiffusion assays showed an identity reaction between canatoxin (PREP-G-III, see below) and urease. The proteins had the same titre in dot blots either with anti-canatoxin or anti-urease antibodies (results not shown).

Urease activity and intraperitoneal toxicity in mice were not co-purified (Table 1), indicating that any urease contaminating the canatoxin preparation could be removed. Therefore our method of isolating canatoxin [4] was modified to remove any contaminating urease by using a Zn^{2+}-DPA–Sepharose 6B column [37]. PREP-F-II samples applied into the Zn^{2+} column (Figure 5) gave three fractions: (1) non-retained material without lethal or urease activity (PREP-G-I); (2) a minor fraction with high urease activity (PREP-G-II) eluted with imidazole; and (3) a main toxic fraction (PREP-G-III; 70–80% of the total protein) with low urease activity. Rechromatography did not decrease the urease activity associated with canatoxin in PREP-G-III any further.

Commercial preparations of highly purified urease (type C3) gave the same three fractions in the zinc column, the relative proportion of which varied according to the lot number, with 60–70% of the protein always being eluted in the imidazole fraction (results not shown).

Metal content of canatoxin

Canatoxin (PREP-F-II) contains zinc (1.33 ± 0.38 mol/mol monomer) and nickel (1.17 ± 0.20 mol/mol monomer) as de-

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001 MKLSPREVEK LGLHNAGYLA QKRLARGVRL NYTEAVALIA SQIMEYARDG EKTVAQLMCL GQHLLGRRQV LPAVPHLLNA VQVEATFPDG TKLVTVHDPI
                                                                                                       ||||||||
                                                                                                       LVTVHDPI
101 SRENGELQEA LFGSLLPVPS LDKFAETKED NRIPGEILCE DECLTLNIGR KAVILKVTSK GDRPIQVGSH YHFIEVNPYL TFDRRKAYGM RLNIAAGTAV
    ||||||||||                         |||||| |   |                  |||||||||| |||||||||| |||||
    SRENGELQEA                         IPGEILXE XXXL                 GDRPIQVGSH YHFIEVNPYL TFDRR
201 RFEPGDCKSV TLVSIEGNKV IRGGNAIADG PVNETNLEAA MHAVRSKGFG HEEEKDASEG FTKEDPNCPF NTFIHRKEYA NKYGPTTGDK IRLGDTNLLA
                                 | |  | || | |     |    |||||                           ||||||
                                 G PPKE-NLXYA XH    AFT  HEEEK                           YGPTTGD
301 EIEKDYALYG DECVFGGGKV IRDGMGQSCG HPPAISLDTV ITNAVIIDYT  GIIKADIGIK DGLIASIGKA GNPDIMNGVF SNMIIGANTE VIAGEGLIVT
                                                              ||||
                                                              ADIG
401 AGAIDCHVHY ICPQLVYEAI SSGITTLVGG GTGPAAGTRA TTCTPSPTQM RLMLQSTDYL PLNFGFTGKG SSSKPDELHE IIKAGAMGLK LHEDWGSTPA
                                 ||| |||||||||
                                 VGG GTGPAAGTR
501 AIDNCLTIAE HHDIQINIHT DTLNEAGFVE HSIAAFKGRT IHTYHSEGAG GGHAPDIIKV CGIKNVLPSS TNPTRPLTSN TIDEHLDMLM VCHHLDREIP
                                                                          | ||  ||                        Ni-Ni
                                                                      LVEPSE  TNXVXL
601 EDLAFAHSRI RKKTIAAEDV LNDIGAISII SSDSQAMGRV GEVISRTWQT ADPMKAQTGP LKCDSSDNDN FRIRRYIAKY TINPAIANGF SQYVGSVEVG
                                                                                           | |||||||||| ||||||||||
                                                                                           Y TINPAIANGF SQYVGSVEVG
701 KLADLVMWKP SFFGTKPEMV IKGGMVAWAD IGDPNASIPT PEPVKMRPMY GTLGKAGGAL SIAFVSKAAL DQRVNVLYGL NKRVEAVSNV RKLTKLDMKL
    |||| ||  |               | |||  |||| |                                                                 |
    KLADXVMYTP VQX        VMPAWAN IGDPXA                                                                   L
801 NDALPEITVD PESYTVKADG KLLCVSEATT VPLSRNYFLF
    |||| ||  | |
    NDALQEIXVM PXALYA
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Figure 4 Partial amino acid sequence of canatoxin: homology with urease

Alignment of the 840 amino acids of urease (EC 3.5.1.5), deduced from its cDNA (GenBank® accession number M65260), and the internal peptides of canatoxin. The two nickel (Ni) atoms in the active site of urease are indicated.

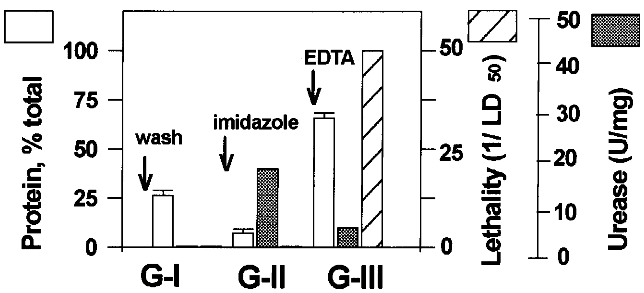

Figure 5 Zn^{2+}-DPA–Sepharose affinity chromatography

PREP-F-II (10 mg of protein) in 20 mM sodium phosphate/1 M NaCl (pH 7.5) was applied on to a 5 ml column of Zn^{2+}-DPA–Sepharose 6B, equilibrated with the same buffer. After removing the non-retained proteins (PREP-G-I; G-I), the first fraction was eluted with 100 mM imidazole, pH 7.5 (PREP-G-II; G-II). The column was then equilibrated in 20 mM sodium phosphate, pH 7.5, and a second elution was performed using 10 mM EDTA to yield PREP-G-III (G-III). The fractions were analysed for protein, urease (units/mg) and toxic activity ($1/LD_{50}$). Data are means ± S.E.M of 15 runs.

termined by atomic absorption spectrophotometry. Calcium (1.88 ± 0.68 mol/mol) and iron were also detected. It should be noted that these analyses were done with canatoxin before the Zn^{2+}-chelating column and that the metals could not be removed from the protein by dialysis against EDTA. PIXE analysis confirmed the presence of zinc and nickel in canatoxin, but not in urease, which showed no detectable zinc besides the well known stoichiometry of two nickel atoms per subunit [38]. Higher contents of calcium (approx. 10-fold) and iron were found in the aggregated form of canatoxin (PREP-F-I).

Comparison of biological properties of canatoxin and urease

Kinetics and inhibitors of urea hydrolysis

Canatoxin has a lower ureolytic activity, corresponding to approx. 30–40 % of urease's activity (Table 3). Two inhibitors of urease, *p*-HMB [39] and AHA [40,41], inhibited its ureolytic activity (Figure 6). AHA, a chelator of Ni^{2+} and Zn^{2+} ions [42],

Table 3 Kinetic parameters of ureolytic activity of canatoxin and urease

Kinetic parameters were determined at 37 °C in 20 mM sodium phosphate with 0.014–5.35 mM urea. Molecular masses of 184 and 270 kDa were taken as the minimal molecular form expressing enzymic activity for canatoxin and urease, respectively [38]. Data are means ± S.E.M. of three to four determinations.

Protein	K_m (mM)	V_{max} (μmol NH_4^+/mg per min)	K_{cat} (s^{-1})	K_{cat}/K_m ($M^{-1} \cdot s^{-1}$)
Canatoxin:				
pH 6.5	1.5 ± 0.2	3000 ± 200	9200	6.1×10^6
pH 7.5	1.8 ± 0.1	5900 ± 400	18093	1.0×10^7
pH 8.5	7.7 ± 1.5	14100 ± 2600	43240	5.6×10^6
Urease:				
pH 6.5	1.5 ± 0.	6000 ± 1400	9700	1.2×10^6
pH 7.5	2.9 ± 0.6	13700 ± 3300	61650	2.1×10^7
pH 8.5	7.1 ± 1.7	26700 ± 6900	120150	1.7×10^7

gave the same IC_{50} for both proteins. In contrast, urease is 10-fold more susceptible to *p*-HMB ($IC_{50} = 0.5$ μM) than canatoxin.

For the following studies, commercial preparations of urease (type C3), consisting of at least 70 % of the total protein equivalent of PREP-G-II, were compared with urease-free canatoxin (PREP-G-III).

Lethality in mice

Highly purified canatoxin (PREP-G-III) had an LD_{50} of 2.0 ± 0.5 mg/kg (see Table 1). In contrast, the commercial urease showed no signs of toxicity at doses of 10 mg/kg when injected intraperitoneally into mice. Canatoxin (PREP-G-III) and urease were treated with 200 μM *p*-HMB overnight at 4 °C and then dialysed. Although both treated proteins were devoid of ureolytic activity, canatoxin remained toxic in mice with no change in its LD_{50}, whereas the thiol-derivatized urease remained non-lethal.

Platelet aggregation and ATP secretion

Similar to canatoxin [6], urease induced aggregation of rabbit platelets (Figure 7), accompanied by the secretion of platelet

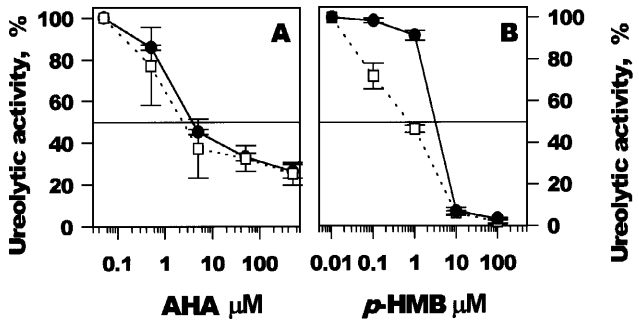

Figure 6 Inhibition of ureolytic activity by AHA and *p*-HMB

Aliquots (0.1 mg/ml) of canatoxin (●; PREP-G-III) or urease (□) were incubated for 18–24 h at 4 °C with AHA (**A**) or *p*-HMB (**B**) and then assayed for residual ureolytic activity. Data are means ± S.E.M. of at least five independent experiments.

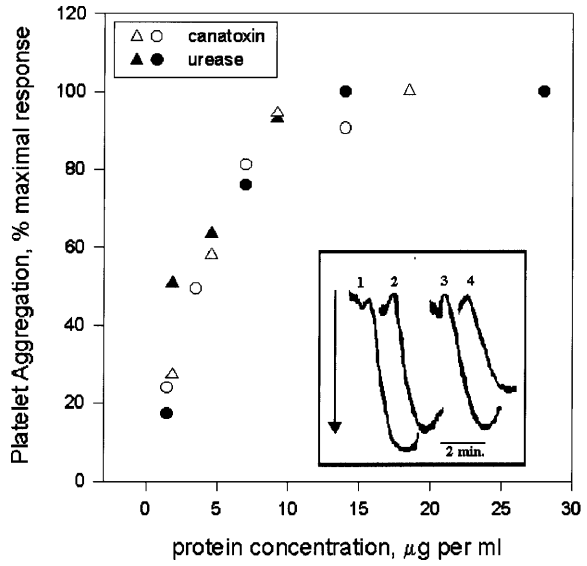

Figure 7 Platelet aggregation induced by canatoxin and urease

Platelets suspensions (300 μl) were challenged with canatoxin (○, △) or urease (●,▲) and aggregation was measured turbidimetrically [6]. The points represent the percentage of maximal response obtained in two experiments for each protein. Inset. The arrow indicates an increase in light transmittance during platelet aggregation as registered by the Lumi-aggregometer: (1) canatoxin (PREP-G-III, 15 μg/ml); (2) *p*-HMB-treated canatoxin (PREP-G-III, 15 μg/ml); (3) urease (15 μg/ml) and (4) *p*-HMB-treated urease (15 μg/ml).

granules and release of ATP into the medium (results not shown). A similar ED_{50} (approx. 2.0 μM) for platelet aggregation was estimated for the proteins. After treatment with *p*-HMB, both canatoxin and urease were still able to activate platelets (Figure 7).

Indirect haemagglutination

The indirect haemagglutination induced by canatoxin and its inhibition by gangliosides has been previously described [5]. Urease also promoted indirect agglutination of rabbit erythrocytes as 2 μg of either protein induced the agglutination of 10^6 cells. Potential inhibitors of urease- or canatoxin-induced indirect haemagglutination were tested [including 50 mM glucose, galactose, mannose, fucose, glucosamine, *N*-acetyl-glucosamine, mannosamine, *N*-acetyl-mannosamine, 100 mM of arabinose, ribose, xylose, fructose, threalose, lactose, maltose and 1 % (w/v) of chitin, mucin, ovomucoid and thyreoglobulin], but no effects were seen. In contrast, in the presence of 0.05 % (w/v) bovine total gangliosides or 0.12 % (w/v) fetuin, the indirect hemagglutination produced by either protein was completely inhibited. Again, both proteins retained their ability to bind to the cell surface after pretreatment with *p*-HMB.

DISCUSSION

We have shown in the present study using partial sequence data, immunological studies and kinetic parameters of its ureolytic activity that canatoxin, a neurotoxic protein from *C. ensiformis* seeds, is an isoform of the 'classical' jack bean urease (EC 3.5.1.5). Modification of the isolation procedure resulted in a protein free from urease contamination, with a LD_{50} of 2.0 mg/kg, a 61-fold purification index and 16 % yield. The separation of canatoxin and urease can also be achieved in iminodiacetic-based resins (C. Follmer, G. E. Wassermann, C. R. Carlini, unpublished work).

Our results show at least two isoforms of urease in *C. ensiformis* seeds, with canatoxin being an isoform of lower ureolytic activity and higher toxicity in mice. Polyclonal anti-canatoxin or monoclonal anti-urease antibodies could not discriminate between the proteins, suggesting a high degree of identity. Our most recent results indicate a family of urease-related genes in *C. ensiformis*, with at least three members (M. Pires-Alves, M. F. Grossi-de-Sa, C. R. Carlini, M. G. Moraes, unpublished work). In that study, reverse transcriptase-PCR using jack bean total RNA as template and primers based on the urease sequence resulted in a cDNA clone with 86 % similarity to urease, besides urease cDNA itself. Previous reports [43,44] have also provided evidence for isoenzymes of jack bean urease with different amino acid sequences.

Canatoxin shows approx. 30–40 % of the ureolytic activity of the 'classical' urease at pH 7.5. The isoenzymes are inhibited by *p*-HMB or AHA, with urease being approx. 10-fold more susceptible to *p*-HMB than canatoxin.

Ureases may exist in different oligomerization states. The molecular mass of the jack bean 'classical' urease is 90 770, with a single subunit of 840 amino acids [34–36]. The native form is a hexamer [40], although other oligomeric forms may exist depending on pH and presence of salts [45,46]. Canatoxin is a non-covalently linked dimer consisting of a 95–100 kDa chain and, depending on the protein concentration, temperature, pH and presence of salts, is able to polymerize further during storage. Furthermore, canatoxin forms a soluble 10 kDa fragment at pH 3.0, which was first erroneously interpreted by us as being a subunit. The presence of EDTA increases the formation of the 10 kDa fragment, suggesting the involvement of metals in the stabilization of the protein structure. The exact nature of this process is presently unknown. It may indicate an acid-labile bond in the primary sequence of canatoxin, or an acid-labile cross-link to a small subunit. Urease also easily breaks down into fragments of 30–60 kDa, particularly in acidic media [41], but it precipitates without forming soluble fragments when submitted to the same conditions in which canatoxin releases its 10 kDa fragment.

The different oligomerization states of the proteins suggests distinct amino acids promoting the association of the monomers. A differential display of histidine residues at the surface of the proteins is expected from their behaviour on immobilized metal affinity chromatography [37].

Atomic absorption and PIXE analysis showed that there is one atom of zinc and one of nickel per monomer of canatoxin. PIXE analysis of urease confirmed the presence of nickel, but not

of zinc. Zerner [38] reported that jack bean urease partially depleted of nickel, and subsequently loaded with zinc, had approx. one-tenth of the ureolytic activity of the native enzyme. Some microbial ureases have mono-nickel-active sites [47]. Elucidation of the crystal structure of urease from *Klebsiella aerogenes* revealed a structural similarity to adenosine deaminase, a zinc metalloenzyme catalysing a mechanistically related reaction. The active site of the enzymes are three-dimensionally superimposable, with the zinc atom occupying a position equivalent to one of the nickel atoms in urease [48]. Thus its plausible that canatoxin could be a variant form of urease containing a hybrid 'zinc-nickel' active centre.

Urease was not lethal to mice when given intraperitoneally; a result confirming previous findings [49]. An extensive contamination of urease with canatoxin probably explains earlier conflicting results regarding the toxicity of jack bean urease [50]. On the other hand, urease displayed some, until now, unsuspected biological activities, such as the activation of blood platelets and binding to glycoconjugates. These properties may shed new light on the physiological roles of urease in plants and bacteria. The involvement of plant ureases in the bioavailability of N_2 is controversial [51]. Two ureases exist in soya bean plants, a highly active hexameric enzyme present only in the embryo and a ubiquitous, trimeric isoenzyme of much lower enzymic activity. No deleterious effect was seen in mutant plants lacking the embryo-specific urease, implying that its high ureolytic activity is not relevant for its physiological role [52–54].

The insecticidal property of canatoxin [16] is also unrelated to its ureolytic activity, since the entomotoxic effect depends on the protein being proteolytically 'activated' to give a toxic peptide [17]. Preliminary results have indicated that urease also shows insecticidal activity (C. T. Ferreira-DaSilva, F. Stanisçuaski, C. R. Carlini, unpublished work).

Microbial ureases consist of two or three subunits homologous to the jack bean single chain enzyme [47]. We are now investigating if microbial ureases display secretatogue properties and affinity for glycoconjugates. This may be relevant for the involvement of ureases in pathological conditions, such as the stomach ulcers produced by *Helicobacter pylori* [55] or urolithiasis due to *Proteus mirabilis* infection [56].

Taken together, our data suggest that canatoxin and urease belong to a novel group of multifunctional proteins with at least two distinct domains: a nickel- and thiol-dependent ureolytic active site and a thiol-independent domain(s), involved in the secretagogue and hemilectin properties of the proteins, as well as in the neurotoxic effect of canatoxin. Further elucidation of the three-dimensional structures of these molecules should provide new insights for correlating the structural features to their multiple biological effects.

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