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Biological activities of a lectin from *Bothrops jararacussu* snake venom

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Abstract

Snake venoms contain saccharide-binding lectins. In this work, we examined the biological activities of a lectin (BjcuL) purified from *Bothrops jararacussu* snake venom by chromatography on non-derivatized Sepharose 4B and Sephacryl S-200 HR. The protein, a homodimer with subunits of 14.5 kDa, gave a single immunoprecipitin line in immunoelectrophoresis and cross-reacted in ELISA with antivenoms raised against *Bothrops* spp. (lanceheads), *Micrurus* spp. (coral snakes), *Crotalus durissus terrificus* (South American rattlesnake), and arthropod (*Loxosceles gaucho, Phoneutria nigriventer* and *Tityus serrulatus*) venoms. BjcuL agglutinated human formaldehyde-fixed erythrocytes at $\geq$100 ng/ml and was inhibited by lactose and EDTA ($\geq$2 mM) and high concentrations ($>$100 mM) of glucose and sucrose, but not by *N*-acetylglucosamine. BjcuL had no direct hemolytic activity and was devoid of esterase, PLA_2 and proteolytic activities. The lectin (up to 200 μg/ml) did not aggregate human platelet-rich plasma (PRP) or washed platelets (WP), nor did it alter the aggregation induced by ADP in PRP or by thrombin in WP. When injected into mouse hind paws, BjcuL (10–100 μg/paw) caused edema and increased vascular permeability, with a maximum effect after 1 h that persisted for up to 6 h (edema) or gradually decreased after the peak interval (vascular permeability). No hemorrhage was observed in BjcuL-injected paws. In anesthetized rats, *B. jararacussu* venom (200 μg/kg, i.v.) produced sustained hypotension (maximum decrease of ~60%) whereas a similar dose of BjcuL decreased the blood pressure by ~15%, with a rapid return to the resting level.

Keywords: Edema; Hemagglutination; Hypotension; Lectin; Platelet aggregation; Vascular permeability; Venom

1. Introduction

Snake venom lectins (SVL) are C-type (Ca^{2+}-dependent), saccharide-binding [mostly galactose, but also mannose (Zha et al., 2001)] proteins that were initially described in the 1980s (Gartner et al., 1980; Gartner and Ogilvie, 1984; Ogilvie and Gartner, 1984; Ogilvie et al., 1986). Since their initial description, SVL have been isolated from several genera, including *Agkistrodon* (Gartner and Ogilvie, 1984; Komori et al., 1999), *Bitis* (Nikai et al., 1995), *Bothrops* (Gartner et al., 1980; Lomonte et al., 1990; Ozeki et al., 1994; Carvalho et al., 1998; Guimarães-Gomes et al., 2004; Havt et al., 2005), *Bungarus* (Zha et al., 2001), *Crotalus* (Gartner and Ogilvie, 1984), *Dendroaspis* (Ogilvie et al., 1986), *Lachesis* (Gomes-Leiva and Aragón-Ortiz, 1986; Ogilvie et al., 1986; Aragón-Ortiz et al., 1989, 1990), and *Trimeresurus* (Liang and Wang,

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1993; Nikai et al., 2000). Several of these proteins have been partially or wholly sequenced (Hirabayashi et al., 1991; Aragón-Ortiz et al., 1996; Komori et al., 1999; Nikai et al., 2000; Carvalho et al., 2002) and, in some cases, their crystal structure determined (Walker et al., 2004) and their genes cloned (Xu et al., 1999; Zha et al., 2001; Guimarães-Gomes et al., 2004; Kassab et al., 2004).

SVL generally account for $\leq 1\%$ of the dry weight of venom, with most of them occurring as homodimers with a molecular mass of ~ 28 kDa (Lomonte et al., 1990; Hirabayashi et al., 1991; Ozeki et al., 1994). SVL characteristically cause hemagglutination, although other actions such as mitogenic activity in lymphocytes (Djaldetti et al., 1980; Hembold et al., 1985, 1986; Mastro et al., 1986), platelet aggregation (Gartner et al., 1980; Gomes-Leiva and Aragón-Ortiz, 1986; Ogilvie et al., 1986, 1989; Ozeki et al., 1994), induction of paw edema in mice (Lomonte et al., 1990), the modulation of Ca^{2+} release from skeletal muscle sarcoplasmic reticulum (Ohkura et al., 1996; Hirata et al., 1999) and renal effects (Havt et al., 2005) have also been reported. In addition to true lectins, snake venoms also contain various proteins with C-type lectin-like motifs but which generally lack the ability to cause hemagglutination. Many of the latter proteins have important hemostatic actions, including the modulation of platelet aggregation (Wisner et al., 2002; Andrews et al., 2003; Morita, 2004; Lu et al., 2005; Ogawa et al., 2005).

The venom of the South American pitviper, *Bothrops jararacussu* (jararacuçu) contains a lectin (BjcuL) (Carvalho et al., 1998, 2002). Studies with cultured cells have shown that BjcuL can inhibit the proliferation of various tumoral cell lines (Pereira-Bittencourt et al., 1999; Carvalho et al., 2001). However, little is known of the immunological properties and other biological activities of this protein. In this report, we describe some additional properties of BjcuL.

2. Material and methods

2.1. Materials

N-Acetylglucosamine, acrylamide, agarose, ammonium persulfate, bovine casein, bromophenol blue, 4-chloro-1-naphthol, dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), lactose, β-mercaptoethanol, *N*,*N*′-methylene-bis-acrylamide, molecular weight markers for polyacrylamide gel electrophoresis, 4-nitro-3-(octanoyloxy)benzoic acid, *o*-phenylenediamine, rabbit anti-horse IgG-peroxidase conjugate, sodium dodecyl sulfate (SDS), *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED), Nα-*p*-tosyl-L-arginine methyl ester (TAME), Tris, trypsin and Tween-20 were obtained from Sigma Chemical Co. (St Louis, MO, USA). Sepharose 4B, Sephacryl S-200 HR and chromatographic columns were from Amersham Biosciences (Piscataway, NJ, USA). Iloprost was from Schering-Plough (Welwyn Garden City, UK). Sodium pentobarbital (Hypnol®) was from Cristália (Itapira, SP, Brazil). All other reagents were of the highest grade available. Multi-well plates (high protein binding) for ELISA were from Corning (Corning, NY, USA) and microtiter V-well plates for hemagglutination were from Greiner (Frickenhausen, Germany).

2.2. Venom and antivenoms

Bothrops jararacussu venom obtained from adult snakes of both sexes was from the Instituto Butantan (São Paulo, SP, Brazil) or CETA (Centro de Extração de Toxinas Animais, Morungaba, SP, Brazil). Commercial equine antivenoms raised against *Bothrops* species (*B. alternatus, B. jararaca, B. jararacussu, B. moojeni*, and *B. neuwiedi*), against *Micrurus* (coral snakes) species (*M. frontalis* and *M. corallinus*), against *Crotalus durissus terrificus* (South American rattlesnake), and against arachnids (spiders—*Loxosceles gaucho* and *Phoneutria nigriventer* and scorpion—*Tityus serrulatus*) (Cardoso and Yamaguchi, 2003) were obtained from the Instituto Butantan.

2.3. Animals

Male Wistar rats (200–250 g) and male Swiss white mice (25–30 g) were obtained from the university's central breeding colony and housed on a 12 h light/dark cycle at 22 °C with free access to water and standard rodent chow (Purina®, Campinas, SP, Brazil). The experiments described here were done in accordance with the guidelines of the Brazilian College for Animal Experimentation (COBEA).

2.4. Purification of *B. jararacussu* venom lectin (BjcuL)

B. jararacussu venom (100 mg batches) was dissolved in 5 ml of Tyrode solution (composition, in mM: NaCl 137, Na_2PO_4 0.42, $NaHCO_3$ 11.9, $CaCl_2$ 1.8, $MgCl_2$ 0.49 and KCl 2.7) and centrifuged (2000*g*, 10 min, 25 °C) to remove insoluble material. The resulting supernatant was applied to a column (1 cm × 10 cm) of Sepharose 4B equilibrated with Tyrode solution at 20 °C and the column then washed with the same solution at a flow rate of 1 ml/min until the absorbance at 280 nm had returned to baseline. The lectin bound to the column was eluted with Tyrode solution containing 200 mM lactose and fractions of 1 ml were collected. The elution profile was monitored by reading the absorbances of the fractions in a Uvikon 810 spectrophotometer (Kontron Instruments, Milan, Italy). The lectin obtained in the preceding step was dialyzed against distilled water, lyophilized, and resuspended in 0.1 M Tris–HCl, pH 7.5, before being applied to a column (1 cm × 30 cm) of Sephacryl S-200 HR equilibrated with this same buffer. The protein was eluted at a flow rate of 0.5 ml/min using the same buffer and 1 ml fractions were collected. The elution profile was monitored as described above. Reverse phase high performance liquid chromatography (RP-HPLC) was done with an ÄKTA purifier 10 chromatographic system

using a Sephasil Peptide C18 reverse phase column (12 μm, ST 4.6×250 mm; Amersham Biosciences) equilibrated with 0.1% trifluoroacetic acid (TFA) containing 5% acetonitrile. The lectin was eluted with a linear gradient (5–90%) of acetonitrile in TFA at a flow rate of 1 ml/min and the elution profile was monitored at 280 nm.

2.5. Protein concentration

The protein concentrations of venom and purified BjcuL solutions were estimated by the absorbance at 280 nm, assuming that an $A_{280_{nm}}$ nanometer of 1.0 = 1.0 mg of protein/ml.

2.6. Electrophoresis

SDS-PAGE (Laemmli, 1970) was done in mini-gels (8 cm×10 cm, 10% acrylamide) using a Mighty Small II SE260 apparatus (Hoefer-Pharmacia, San Francisco, CA, USA). *Bothrops jararacussu* venom and BjcuL were diluted in 0.063 M Tris–HCl buffer, pH 6.8, containing 2% SDS, 5% glycerol and 0.001% bromophenol blue and then boiled for 4 min before electrophoresis at 100 V (constant). In some experiments, β-mercaptoethanol (5 mM) was included in the sample preparation. At the end of the run, the gels were silver stained, dried and documented. Molecular mass markers were included in the runs.

2.7. Immunoelectrophoresis

Samples of *B. jararacussu* venom and BjcuL were run in 1% agarose gels in 0.1 M Tris–HCl, pH 8.0, at room temperature and a fixed voltage (120 V) for 90 min. The gels were prepared on microscope slides. After electrophoresis, commercial bothropic antivenom was added to a trough cut in the agarose and the slides then incubated in a humidified chamber for 48 h. After this period, the slides were washed in 0.15 M NaCl for 12 h, then dried at 37 °C for 36 h and stained with 0.4% amido black in 10% acetic acid for 10 min. After destaining in 5% acetic acid, the slides were dried and documented.

2.8. Enzyme-linked immunosorbent assay (ELISA)

The reactivity of *B. jararacussu* venom and purified BjcuL with commercial equine antirenum raised against a pool of venoms from *Bothrops* species (*B. alternatus, B. jararaca, B. jararacussu, B. moojeni* and *B. neuwiedi*) was assessed essentially as described by Valério et al. (2002). Ninety-six-well plates were coated overnight at 4 °C with 100 μl of bothropic antivenom (diluted 1:1000 in 0.1 M sodium carbonate, pH 9.6) and then washed with 0.9% NaCl containing 0.05% Tween-20 followed by incubation with varying amounts of venom or BjcuL diluted in incubation buffer (phosphate-buffered saline, PBS, containing 0.05% Tween-20 and 0.25% bovine casein) for 1 h at room temperature. The plates were subsequently washed with 0.9% NaCl containing 0.05% Tween-20 and incubated with an affinity (protein G-Sepharose)-purified IgG-peroxidase conjugate (1:1000, in incubation buffer) against *Bothrops* venom components. After further washes, the plates were incubated with substrate (100 μl of 0.2 mg of *o*-phenylenediamine/ml and 0.05% H_2O_2 in 0.15 M citrate buffer, pH 5.0) for up to 30 min in the dark at room temperature. The reactions were stopped by adding 50 μl of 5% H_2SO_4 and the final absorbances were read at 492 nm in a SpectraMax 340 multiwell plate reader (Molecular Devices, Sunnyvale, CA, USA).

To examine the cross-reactivity of BjcuL with commercial antivenoms, 96-well plates were coated overnight with BjcuL (5 μg/well) diluted in sodium carbonate then washed and incubated for 1 h at room temperature with serial dilutions of antivenoms prepared in incubation buffer. After washing, the plates were incubated with a rabbit anti-horse IgG-peroxidase conjugate (diluted 1:1000 in incubation buffer) and then processed as described above.

2.9. Enzymatic activities

Phospholipase A activity was assayed by the method of Holzer and Mackessy (1996) modified for 96-well plates (Beghini et al., 2000). Proteolytic and esterase activities were determined using casein (Delpierre, 1968) and TAME (Viljoen et al., 1979) as substrates, respectively.

2.10. Hemagglutination

Hemagglutinating activity was determined by the method of Nowak et al. (1976) using microtiter V-well plates and serial two-fold dilutions of *B. jararacussu* venom and BjcuL. Each well contained 50 μl of a 10% suspension of human formaldehyde-fixed, trypsinized erythrocytes in phosphate-buffered saline (PBS, pH 7.4), and varying amounts of venom or BjcuL in 100 μl of PBS. The negative control contained 50 μl of cell suspension and 100 μl of PBS. Following the addition of erythrocytes, the plates were shaken briefly and incubated at room temperature (∼25 °C) for 2 h. Unagglutinated erythrocytes formed a button at the bottom of the wells, whereas agglutinated erythrocytes formed a diffuse coat or mantle. To examine the requirement for Ca^{2+} and the inhibition by sugars, BjcuL (10 μg/well) was incubated for 2 h at 25 °C with different concentrations of sugars or EDTA diluted in PBS and the hemagglutinating activity then determined as described above.

2.11. Platelet aggregation

Blood from individuals who had not been on any medication for the previous 10 days was collected in 3.8% sodium citrate (9:1, v/v) and then centrifuged (200*g*, 15

min, 25 °C) to obtain platelet-rich plasma (PRP). The cell pellet was centrifuged again (2000*g*, 15 min) and the resulting supernatant (platelet-poor plasma, PPP) was used to calibrate the aggregometer. Washed platelets (WP) were prepared as described by Radomski and Moncada (1983). For each test, 0.5 ml of platelet suspension was incubated with stirring for 3 min at 37 °C in a Payton two-channel aggregometer. Subsequently, varying amounts of BjcuL were added and the platelet response then monitored. The ability of BjcuL to inhibit aggregation was examined by incubating platelets for 1–3 min with different quantities of BjcuL prior to stimulation with ADP (0.5–10 μM, PRP) or thrombin (50–100 IU/ml, WP). The aggregation was monitored for up to 5 min and the responses were compared with those obtained for these agonists in the absence of BjcuL.

2.12. Paw edema and vascular permeability

Male Swiss mice were injected intravenously with Evans blue (50 μl of a 0.25% (w/v) solution/g of body weight) and 30 min later received an intraplantar injection (50 μl, in 0.9% saline) of venom (10, 30 or 100 μg/paw) or BjcuL (2.5, 5, 10, 30 or 100 μg/paw) in the left hind paw. The contralateral paw was injected with saline (50 μl/paw) and served as the control. After 0.5, 1, 2, 4 and 6 h, the mice were killed with an overdose of anesthetic and the paw edema (Levy, 1969) and dye exudate (Gamsé et al., 1980) then measured. For this, the paws were amputated at the tarsocrural joint and weighed on an analytical balance. The edema (expressed in milligrams) was calculated as the difference in weight between the left (treated) and right (untreated) paws. The paws were subsequently minced into small pieces, placed in a test tube with formamide (3 ml) and incubated in a water bath at 57 °C for 24 h. At the end of this period, the absorbance of the supernatants was measured at 619 nm (Uvikon 810 spectrophotometer) and the amount of Evans blue present in the extracts was determined from a standard curve of the dye prepared in formamide. The results were expressed as microgram of dye/milligram of paw weight.

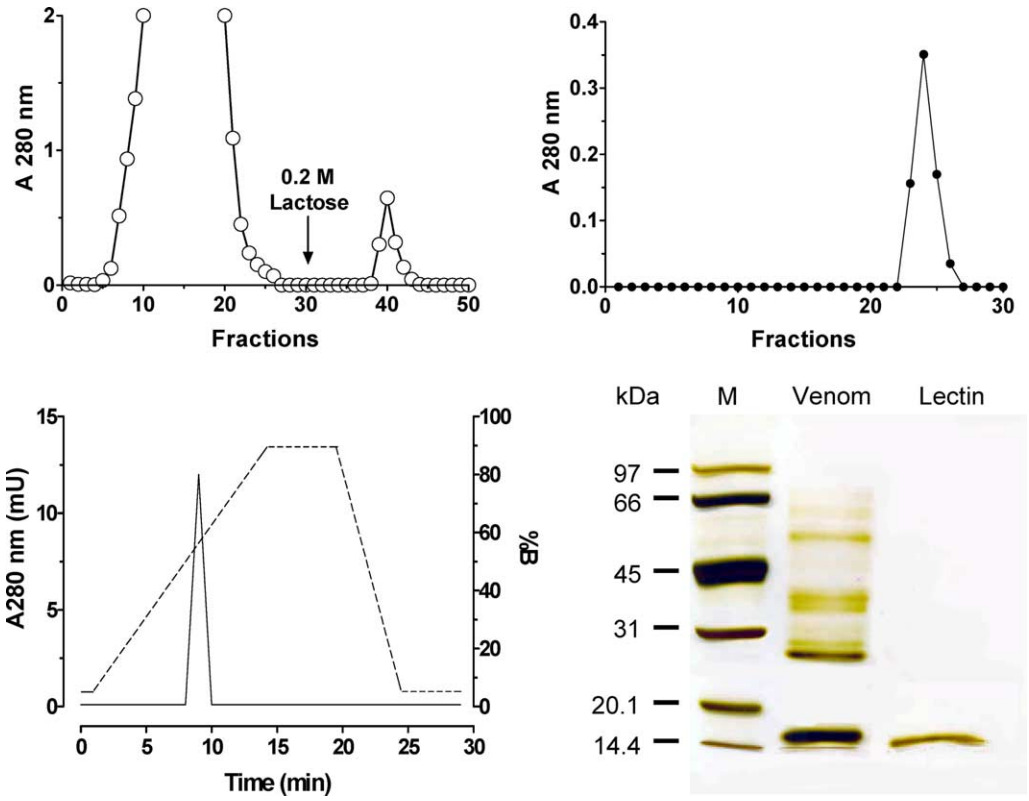

Fig. 1. Purification of BjcuL on non-derivatized Sepharose 4B (A) and elution profile following gel filtration on Sephacryl S-200 HR (B) and RP-HPLC (C). Panel D shows the SDS-PAGE profiles of *B. jararacussu* venom (without β-mercaptoethanol) and BjcuL (with β-mercaptoethanol); the non-reduced protein had a molecular mass of ~29 kDa (not shown). BjcuL was purified as described in Section 2. For electrophoresis, 7 μg of venom and 25 μg of BjcuL were applied to a 10% gel that was subsequently silver-stained. The molecular mass markers (in kDa) were lysozyme (14.4), trypsin inhibitor (20.1), carbonic anhydrase (31), ovalbumin (45), BSA (66), and phosphorylase b (97). M, molecular mass markers.

2.13. Arterial blood pressure measurements

Male Wistar rats were anesthetized with sodium pentobarbital (>60 mg/kg, i.p.) and a tracheostomy was done to allow the animals to breath room air. The rats were cannulated for the measurement of arterial blood pressure (via a carotid artery) and the administration of anesthetic, venom, BjcuL or saline (0.9%, w/v, NaCl; control injection of vehicle solution) (via a femoral vein). The injections were given in a volume of 0.1 ml and washed in with 0.1 ml of 0.9% NaCl. Changes in blood pressure were recorded continuously via a transducer (Abbott, Chicago, IL, USA) connected to a computer data acquisition system (Transonic Systems Inc., Ithaca, NY, USA). After allowing 15 min for stabilization, *B. jararacussu* venom or BjcuL was injected and the changes in blood pressure were monitored for 20 min and expressed as the percent change relative to the values obtained immediately before venom or BjcuL injection.

2.14. Statistical analysis

The results were expressed as the mean ± SEM for the number of experiments or animals indicated. Statistical comparisons were done using Student's unpaired *t*-test or analysis of variance (ANOVA) followed by the Bonferroni test, as appropriate. A value of $p < 0.05$ indicated significance.

3. Results

3.1. Purification of BjcuL

Fig. 1(A) shows the elution profile of BjcuL from a non-derivatized Sepharose 4B column in the presence of 200 mM lactose. Of the four lots of venom screened, one did not contain lectin; the remaining three lots gave the elution profile shown here. Chromatography of BjcuL on Sephacryl S-200 HR yielded only one major peak that, in the absence of lactose, was retarded on the column and eluted with the void volume (24 ml) (Fig. 1(B)). This peak was homogenous by RP-HPLC (Fig. 1(C)). The amount of protein recovered after the Sepharose 4B step accounted for <1% of the venom applied based on the absorbance at 280 nm. SDS-PAGE revealed a principal band of ~29 kDa which, in the presence of β-mercaptoethanol, reduced to a single band of ~14.5 kDa (Fig. 1(D)), indicating that BjcuL occurred as a homodimer. The purified protein was devoid of esterolytic, phospholipase A_2 and proteolytic activities.

3.2. Immunological properties of BjcuL

Immunoelectrophoresis of BjcuL against commercial bothropic antivenom gave only one precipitin line compared to the venom (Fig. 2), and this finding was confirmed by immunodiffusion (results not shown). ELISA showed that BjcuL reacted with bothropic antivenom, but to a lesser extent than the venom (Fig. 3(A)). When tested against different antivenoms, BjcuL reacted most with antivenom raised against the venoms of South American lanceheads (*Bothrops* spp.) followed by coral snakes (*Micrurus* spp.), South American rattlesnake (*C. d. terrificus*) and arachnids (spiders—*L. gaucho* and *P. nigriventer* and scorpion—*T. serrulatus*) (Fig. 3(B)).

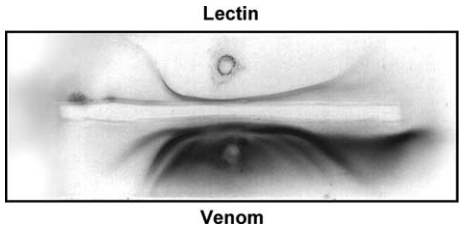

Fig. 2. Immunoelectrophoresis of *B. jararacussu* venom and BjcuL. Forty micrograms of venom and BjcuL were applied to wells cut in a 1% agarose gel and electrophoresed at 120 V for 90 min. After electrophoresis, a central trough was cut in the gel and filled with commercial bothropic antivenom. The slide was then placed in a humidified chamber for 48 h to allow for diffusion to occur. Washing and staining were then done as described in Section 2.

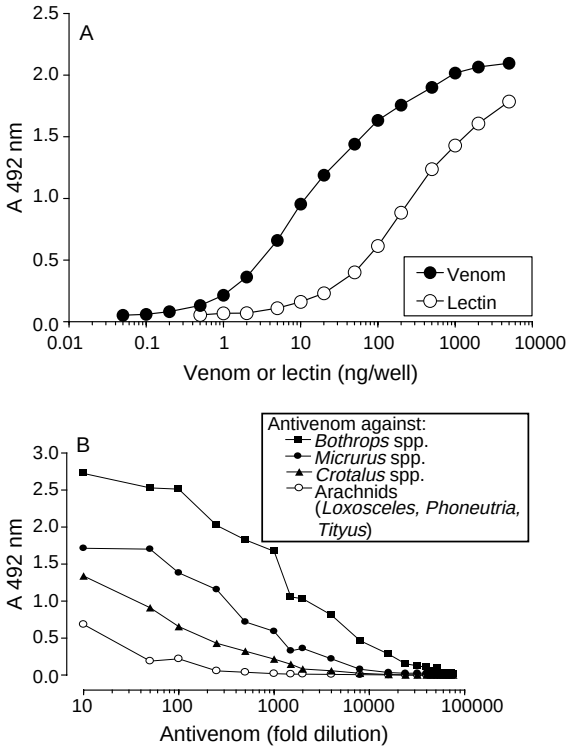

Fig. 3. ELISA immunoreactivity of *B. jararacussu* venom and BjcuL with commercial bothropic antivenom (A) and cross-reactivity of BjcuL with various commercial antivenoms (B). The ELISAs were done as described in Section 2.

3.3. Hemagglutination by BjcuL

BjcuL agglutinated human formaldehyde-fixed erythrocytes at concentrations ≥ 0.1 μg/ml ($n=7$) whereas *B. jararacussu* venom produced hemagglutination at ≥ 1.5 μg/ml ($n=3$). Both lactose and EDTA (≥ 2 mM; $n=7$ each) prevented hemagglutination by BjcuL. At high concentrations (>100 mM), glucose and sucrose, but not *N*-acetylglucosamine (200 mM), inhibited BjcuL-induced hemagglutination ($n=3$ each). BjcuL had no direct hemolytic activity based on examination of the hemagglutination plates at the end of the assay.

3.4. Platelet aggregation

At concentrations up to 200 μg/ml, BjcuL did not aggregate human platelets in PRP or in WP ($n=3$ each). Similarly, the lectin did not inhibit the aggregation induced by ADP (3 μM, $n=3$, PRP) or thrombin (100 IU/ml, $n=3$, WP) (Fig. 4).

3.5. Edema and vascular permeability

Bothrops jararacussu venom and BjcuL produced edema and increased the vascular permeability in mouse hind paws, with both effects being maximal after 1 h (Fig. 5(A) and (B)). However, whereas the edema was generally maintained for up to 6 h after venom or lectin injection (Fig. 5(A)), vascular permeability tended to return to normal with time in both cases ($\sim 50\%$ return to basal values after 6 h) (Fig. 5(B)). The edema and increase in vascular permeability caused by BjcuL were dose-dependent when measured at the peak response (1 h) (Fig. 6(A) and (B)). In the case of *B. jararacussu* venom, no dose-dependence was seen for edema formation at the doses tested (maximal response was already observed at 10 μg/paw), whereas the increase in vascular permeability was greater with the highest dose (100 μg/paw) compared to the two lower doses (10 and 30 μg/paw). At all doses, the responses to BjcuL were significantly smaller than those to venom (Fig. 6), and BjcuL doses <10 μg/paw were without effect. No macroscopic hemorrhage was observed in mouse

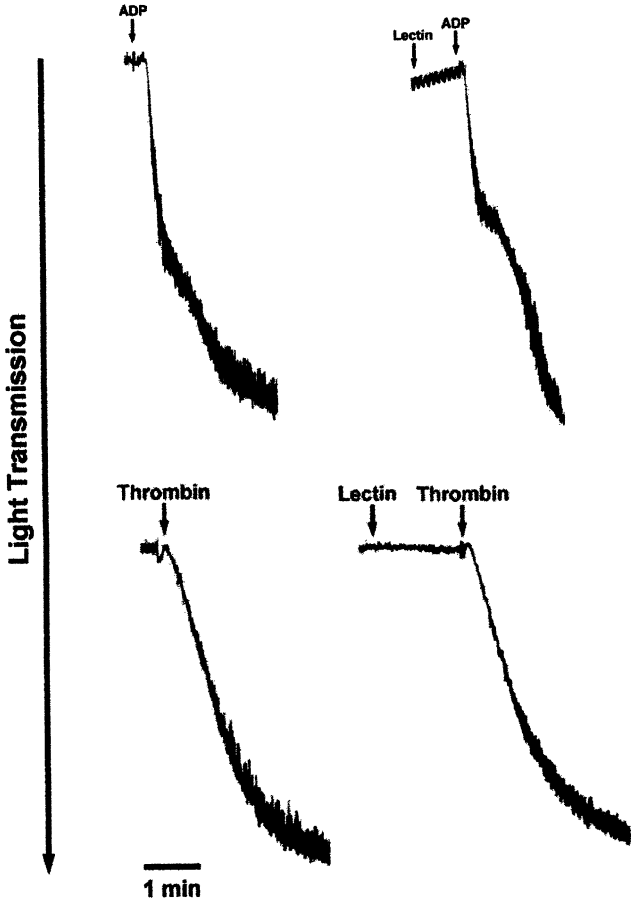

Fig. 4. Failure of BjcuL (200 μg/ml) to induce platelet aggregation in PRP (upper traces) and washed platelets (lower traces) and lack of effect on aggregation induced by ADP (3 μM, in platelet-rich plasma, PRP) and thrombin (100 IU/ml, in washed platelets, WP). BjcuL was incubated with PRP or WP prior to stimulating the platelets with the respective agonists. The tracings are representative of three experiments each.

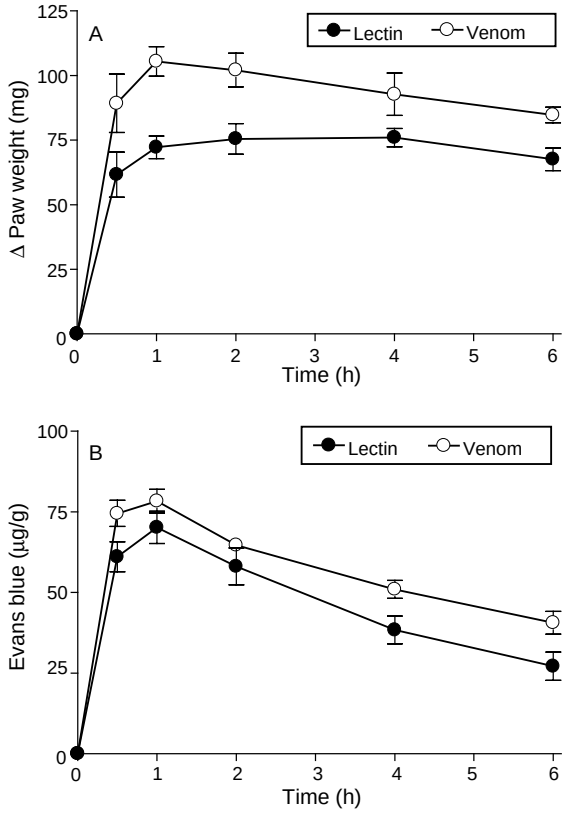

Fig. 5. Time-dependent changes in the edema (A) and vascular permeability (B) of mouse hind paws following the injection of *B. jararacussu* venom (30 μg/paw) and BjcuL (30 μg/paw). Edema was expressed as the change (Δ) in paw weight (mg) and vascular permeability as the extravasation of Evans blue (μg/g). The points represent the mean ± SEM of 4–5 mice.

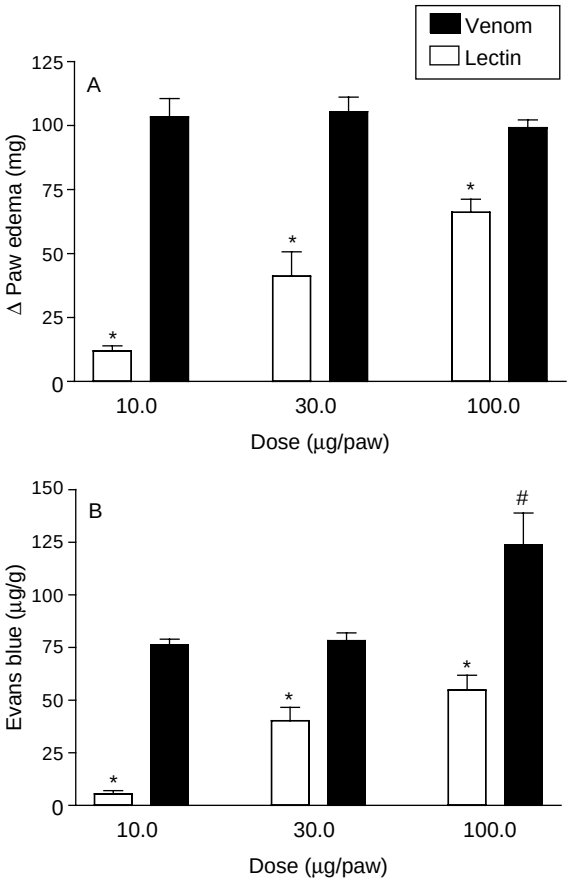

Fig. 6. Dose-dependence of the edema (A) and the increase in vascular permeability (B) produced by BjcuL in mouse hind paws. Edema and vascular permeability were expressed as defined in Fig. 5. Lower doses of BjcuL (≤5 μg/paw) did not cause edema or alter vascular permeability. Note that at the doses tested, the changes observed with *B. jararacussu* venom showed little dose-dependence. The columns are the mean ± SEM of 4–5 mice. *$p < 0.05$ compared to venom. #$p < 0.05$ compared to other venom doses. The responses to the three doses of BjcuL in each panel were also significantly different ($p < 0.05$) among themselves.

paws injected with BjcuL, in contrast to the venom, which caused marked hemorrhage within minutes of injection, even at the lowest dose.

3.6. Arterial blood pressure

In anesthetized rats, the intravenous injection of *B. jararacussu* venom (200 μg/kg) produced immediate, sustained hypotension (~60% decrease in blood pressure) that lasted for 15 min followed by a gradual recovery after 20 min (Fig. 7). In constrast, a similar dose of BjcuL (200 μg/kg) caused only a slight decrease (~15%) in blood pressure with a return to basal values after 5 min. A lower dose of BjcuL (100 μg/kg) produced no significant change in blood pressure (data not shown).

4. Discussion

Various SVL have been isolated from venoms of the genus *Bothrops*, including *B. atrox* (Gartner et al., 1980), *B. godmani* (Lomonte et al., 1990), *B. insularis* (Guimarães-Gomes et al., 2004), *B. jararaca* (Ozeki et al., 1994), *B. jararacussu* (Carvalho et al., 1998) and *B. pirajai* (Havt et al., 2005). In the case of *B. insularis*, a survey of gene expression in the venom glands using expressed sequence tags (ESTs) identified gene clusters for at least two SVL—one related to puff adder (*B. arietans*) lectin (PAL) and another to *T. stegnejeri* lectin (TSL) (Junqueira-de-Azevedo and Ho, 2002).

Based on its properties (molecular mass of ~29 kDa with identical monomer subunits, ability to cause hemagglutination, inhibition by lactose and EDTA), the BjcuL isolated here is apparently the same as that purified by chromatography of *B. jararacussu* venom on an immobilized D-galactose column (Carvalho et al., 1998)

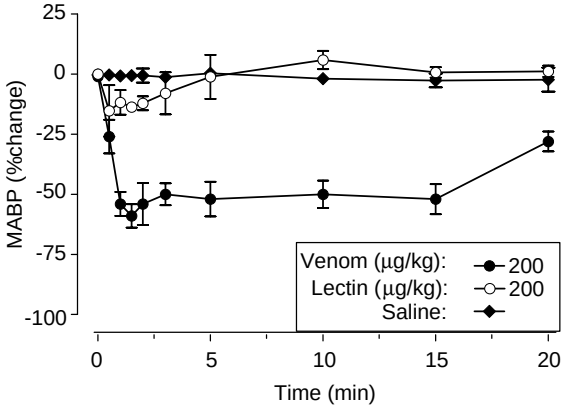

Fig. 7. Blood pressure changes in anesthetized rats following the intravenous injection of *B. jararacussu* venom and BjcuL (200 μg/kg each). The decrease in blood pressure from 1.5 to 2 min for BjcuL and for all time points from 0.5 min onwards for venom was significantly different ($p < 0.05$) from the pre-injection (resting) values and saline controls. The points are the mean ± SEM of five rats. MABP, mean arterial blood pressure.

and recently cloned (Kassab et al., 2004). These properties are also very similar to those of Ca^{2+}-dependent (C-type) lectins isolated from the venoms of other species of *Bothrops* and *Crotalus*. The venom content of BjcuL (< 1%) agreed with that for several other SVL (generally ≤1%) (Ogilvie et al., 1986; Ozeki et al., 1994; Nikai et al., 2000).

ELISA and immunoelectrophoresis showed that BjcuL was recognized by commercial antivenom raised against a pool of *Bothrops* venoms, which included *B. jararacussu* venom, thus indicating that this protein is antigenic. This finding agrees with the immunogenicity demonstrated for other SVL (Gartner and Ogilvie, 1984; Ogilvie et al., 1986; Lomonte et al., 1990; Hirata et al., 1999; Nikai et al., 2000). The lower ELISA cross-reactivity of BjcuL with crotalic antivenom compared to elapidic antivenom was unexpected since the genera *Bothrops* and *Crotalus* are phylogenetically more closely related to each other than to *Micrurus* (coral snakes), and also because elapid species are generally considered to be poor in lectins (Ogilvie and Gartner, 1984; but see Zha et al., 2001). The lower reactivity of BjcuL with antivenom raised against *C. d. terrificus* venom agrees with the low hemagglutinating activity reported for venom of this subspecies (Francischetti et al., 2000) and may indicate that the venom of this rattlesnake has a low content of true lectins. The lowest immunoreactivity with BjcuL was observed for antivenom raised against arthropod (spider and scorpion) venoms. This may indicate that these venoms are either poor in true lectins, or that there is little immunological identity between their lectins and SVL. In support of the latter possibility, the lectins identified so far in arthropodan venoms are smaller than SVL (<20 kDa) (Liang and Pan, 1995; Li and Liang, 1999; Lu et al., 1999; Khoang et al., 2001), and this could influence their antigenic properties and cross-reactivity with antibodies to SVL.

As with *B. jararaca* venom lectin (Ozeki et al., 1994), BjcuL did not aggregate human platelets. However, the ability to aggregate platelets apparently varies among SVL since Ogilvie et al. (1989) reported that lectins from the venoms of *A. p. leukostoma*, *C. atrox* and *L. muta* caused aggregation that was inhibitable by lactose, whereas a lectin from *B. atrox* venom caused only occasional platelet aggregation while *A. c. contortrix* venom lectin had no effect. The different abilities to aggregate platelets may reflect structural variations among these proteins. Physiologically, SVL with platelet aggregating activity may function synergistically with other venom proteins that aggregate platelets in vivo.

Bothrops venoms produce marked local effects such as edema, hemorrhage and necrosis (Gutiérrez and Lomonte, 2003) that are mediated principally by metalloproteinases (Gutiérrez and Rucavado, 2000) and myotoxic phospholipases A_2 (Gutiérrez and Lomonte, 1995). The contribution of SVL to these responses remains unclear. Lomonte et al. (1990) reported that a lectin from *B. goldmani* venom did not cause hemorrhage, myonecrosis, or lethality in mice, but did cause rapid (within 30 min), dose-dependent (12–50 μg/paw) edema that was sustained for up to 6 h. However, based on the low potency of this lectin and its low content in the venom, these authors concluded that this protein was probably not a major contributor to venom-induced edema. As shown here, BjcuL also produced dose-dependent edema and increased vascular permeability that persisted for up to 6 h, with time-dependent profiles that paralleled those of the venom. The doses of BjcuL required to produce edema were similar to those reported for various plant lectins in rat and mouse hind paws (Bento et al., 1993; Freire et al., 2003). Although we have not investigated the mechanism of BjcuL-induced edema, it may well involve histamine and/or serotonin since Lomonte et al. (1990) observed that pretreating mice with cyproheptadine (a histamine and serotonin receptor antagonist) significantly reduced the edema induced by lectin from *B. goldmani* venom. In contrast, Aragón-Ortiz et al. (1990) observed that the lectin from *L. muta* venom did not cause histamine release from isolated mast cells.

The cardiovascular actions of SVL have not been extensively investigated. As shown here, the i.v. administration of BjcuL to anesthetized rats caused only a small, transient hypotension at a dose of 200 μg/kg. In contrast, a similar dose of venom resulted in a marked and sustained decrease in blood pressure. This finding suggests that BjcuL probably contributed little to this venom-induced hypotension observed since a venom dose of 200 μg/kg would contain <2 μg of lectin/kg, a dose that had no effect on blood pressure (results not shown). Conversely, a BjcuL dose of 200 μg/kg would correspond to a venom dose of ~20 mg/kg, sufficient to kill rats within 5 min. Although the average yield of venom from *B. jararacussu* is ~150–

250 mg (Belluomini, 1963, 1968; Kaiser and Michl, 1971; Sanchez et al., 1992), which would correspond to <1.5–2.5 mg of BjcuL, the amount of venom injected in a bite and the subsequent concentrations of circulating BjcuL are unknown, so it is unclear what the real contribution of BjcuL to blood pressure changes would be. It is possible that a higher dose of BjcuL than that tested here may have had a greater effect on the cardiovascular system. In support of this suggestion, Aragón-Ortiz et al. (1989) showed that *L. muta* venom lectin caused marked hypotension in rats at a dose of 1.5 mg/kg, with double this dose being lethal to the animals.

In conclusion, the findings of this study have extended the biological activities of BjcuL and have shown that this lectin may be involved in the local effects (edema and increased vascular permeability) seen after envenoming by this species; the contribution of BjcuL to changes in blood pressure is less clear. This protein apparently has no significant effect on platelet aggregation, at least in vitro. Together, these findings suggest that the actions of BjcuL may be more local than systemic. The immunoreactivity of BjcuL with commercial bothropic antivenom indicates that there may be neutralization of the biological activity of this protein during treatment with antivenom.

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