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Purification, primary structure and potential functions of a novel lectin from *Bauhinia forficata* seeds

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ABSTRACT

A new lectin, BfL, was purified from *Bauhinia forficata* seeds by ammonium sulfate fractionation, DEAE-Sephadex ion exchange chromatography, Sepharose-4B and chitin affinity chromatographies and Superdex 75 size exclusion chromatography. The molecular homogeneity and purity of BfL were assessed by reversed-phase HPLC. BfL appeared as a single band of approximately 27.0 kDa on SDS-PAGE under non-reducing and reducing conditions, and its molecular weight was determined to be 27,850 Da by LC/ESI-MS. BfL is a glycoprotein with a carbohydrate content of 6.24% determined by the phenol–sulfuric acid method. Fetuin, asialofetuin, thyroglobulin and azocasein inhibited the hemagglutinating activity of BfL, whereas saccharides did not. BfL hemagglutinating activity was stable at 100 °C for 30 min, pH-dependent, with the highest activity at pH 6.0, and metal-independent. The primary structure of BfL shows similarity with other lectins from the genus *Bauhinia*. Deconvolution of the BfL circular dichroism (CD) spectrum indicated the presence of α-helix and β structures. BfL increases coagulation time, but this effect is not related to human plasma kallikrein or human factor Xa inhibition. BfL also inhibits ADP- and epinephrine-induced platelet aggregation in a dose-dependent manner and is the only currently described lectin from *Bauhinia* that exhibits anticoagulant and antiplatelet aggregating properties.

1. Introduction

Lectins are proteins or glycoproteins of non-immunogenic origin that specifically and reversibly bind to different types of carbohydrates or glycoproteins [1]. These proteins, which serve as recognition molecules within a cell, between cells or between organisms, play important physiological roles [2]. Lectins exhibit many biological activities, including anti-insect [3], antifungal [4], antiviral [5], antibacterial [2,6], antiproliferative [7], antiplatelet aggregating [8], anti-inflammatory and analgesic activities [9].

Plant lectins are divided into different groups according to their carbohydrate binding specificity [7] and twelve different families based on their molecular structures and evolutionary relationships [10].

Leguminosae plants contain large amounts of lectin proteins [11]. This family is morphologically divided into the subfamilies Caesalpinoideae, Mimosoideae and Papilionoideae [11]. In contrast to the large numbers of lectins reported from the Papilionoideae subfamily, only a few lectins from Caesalpinoideae and Mimosoideae have been reported [11,12].

The genus *Bauhinia* (Caesalpinoideae) is commonly known as 'cow's hoof' because of the shape of its leaves. It is widely distributed in most tropical regions, including Africa, Asia and South America [13]. Plants from this genus have been reported to act as anti-diabetic agents [14] and contain Kunitz-type inhibitors, which have been shown to decrease inflammation and prolong blood clotting [15]. The *Bauhinia* lectins characterized thus far have been isolated from *Bauhinia purpurea* [16], *Bauhinia monandra* [17], *Bauhinia pentandra* [18], *Bauhinia variegata* [7,11,12] and *Bauhinia bauhinioides* [19]. Faria et al. [20] were the first to report the presence of lectins in the seeds of *Bauhinia forficata*. However, to the best of our knowledge, these lectins have not been characterized.

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Furthermore, their activity on human platelet aggregation has not been reported.

Platelets play a central role in hemostatic processes and consequently are involved in the formation of thrombus, which are responsible for vessel occlusion and ischemic injury to the brain and heart [21]. In this article, we report the purification, primary structure and biochemical characterization of a new lectin from *B. forficata* and its effect on blood clotting parameters and platelet aggregation, which may be important for antithrombotic and anticoagulant drug development.

2. Materials and methods

2.1. Materials

A Superdex 75 10/300 GL column was purchased from GE Healthcare (New Jersey, USA), and chitin was acquired from Sigma (Saint Louis, USA). Sugar and glycoproteins were obtained from Sigma (Saint Louis, USA). Phenol was purchased from Labsynth (São Paulo, Brazil). Electrophoretic reagents were obtained from Bio-Rad (Richmond, USA), and standard molecular weight markers were purchased from Fermentas (Ontario, Canada). PT (Prothrombin Time), TT (Thrombin Time) and aPTT (Activated Partial Thromboplastin Time) reagents (Thromborel S, thrombin and Dade Actin Activated Cephaloplastin) were purchased from Dade Behring (Marburg, Germany) and stored at 4 °C. Agonist platelets, arachidonic acid, collagen and epinephrine were obtained from Helena Laboratories (Texas, USA). ADP was purchased from Chrono-log Corporation (Pennsylvania, USA). Thrombin was acquired from RDGmBH (Dieβen, Germany), and fibrinogen was purchased from Sigma (Saint Louis, USA). All the other reagents used were of analytical grade.

2.2. Hemagglutination activity (HA)

HA was determined with glutaraldehyde-treated erythrocytes using the method reported by Correia and Coelho [22]. Two-fold serial dilutions of lectin (50 μl) in microtiter V-plates containing 50 μl of 0.15 M NaCl were mixed with 50 μl of either a 2.5% (v/v) suspension of human red blood cells of each blood type (A, B, AB, O) or rabbit red blood cells at 20 °C. The results were recorded after 45 min, when the blank containing only red blood cells fully precipitated and appeared as a dot at the bottom of the well. HA (inverse of the titer) was defined as the lowest sample dilution showing full hemagglutination.

2.3. Purification of the BfL lectin

B. forficata seeds were powdered and homogenized (10% w/v) overnight at 4 °C in 0.1 M sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl (PBS). The homogenate was filtered and centrifuged at 5071 × *g* for 15 min at 4 °C. The proteins of the supernatant (extract) were precipitated using 0–40% and 40–80% ammonium sulfate saturation (F0-40 and F40-80). The F40-80 fraction was solubilized in 0.1 M Tris–HCl buffer, pH 8.0 (THB) and dialyzed against water for 24 h. The fraction (50 mg) was then applied to a DEAE-Sephadex (5 ml) column equilibrated in THB. The unadsorbed proteins (fraction D1) were eluted with the same equilibration buffer, whereas the adsorbed proteins (fraction D2) were eluted with THB containing 0.15 M NaCl. The lectin fraction D1 (16 mg) was applied to a 10 ml Sepharose-4B column in the same buffer. Following the elution of the unadsorbed proteins (fraction S1), the adsorbed proteins (fraction S2) were eluted with THB containing 0.3 M NaCl. The lectin fraction S1 (11 mg) was subjected to affinity chromatography using a 5-ml chitin column equilibrated with THB. Following the removal of the unadsorbed proteins (fraction Q1), the column was eluted with 1.0 M acetic acid (fraction Q2). This lectin-containing fraction Q2 was lyophilized, resuspended in water, filtered and subjected to size exclusion chromatography with a Superdex 75 10/300 GL column (GE Healthcare) equilibrated in 0.15 M NaCl using an ÄKTA Purifier (GE Healthcare). Protein was eluted with the same buffer at a flow rate of 0.5 ml/min, and the absorbance was monitored at 215 nm. The first eluted absorbance peak (Sd1) contained purified lectin (BfL). HA and protein concentration were determined for all the chromatographic fractions monitored at 280 and 215 nm.

2.4. Reversed-phase HPLC

BfL (2 ml to 0.2 mg/ml) was concentrated by ultrafiltration (Amicon MILLIPORE Ultra-15, Mr 10,000 cut-off), filtered, centrifuged and injected into the HPLC system (Shimadzu LC-10AD-Kyoto, Japan). The column (μ-Bondapak C_{18}) was equilibrated with 0.1% trifluoroacetic acid (TFA) in Milli-Q water, and the bound protein was eluted with a non-linear gradient of 90% acetonitrile and 0.1% TFA in Milli-Q water (solvent B) (B = 5% at *t* = 5 min; B = 70% at *t* = 27 min; B = 80% at *t* = 60 min; and B = 100% at *t* = 65 min) for 65 min with a flow rate of 0.7 ml/min. The elution profile was monitored at 215 and 280 nm.

2.5. Protein concentration assay

The total protein contents of the crude extract, chromatographic fractions and the purified lectin were measured according to the method of Lowry et al. [23] with a bovine serum albumin (BSA) standard curve over the range of 0–500 μg/ml.

2.6. Carbohydrate-binding specificity

To investigate the specificity of BfL for carbohydrates, HA inhibition (HAI) tests were performed in a manner analogous to those described by Correia and Coelho [22]. A two-fold serial dilution sample (50 μl) of BfL (0.045 mg/ml) was mixed with 50 μl of several carbohydrate solutions (initial concentration of 200 mM) in 0.15 M NaCl and incubated for 20 min. Subsequently, a 2.5% glutaraldehyde-treated erythrocyte suspension from blood type B was added to each mixture. The carbohydrate and glycoprotein concentrations were 200 mM and 15.62–500 μg/ml, respectively.

2.7. Carbohydrate content

The total neutral sugar concentration of BfL (0.465 mg/ml) was measured in a microplate by the phenol-sulfuric acid method according to Masuko et al. [24] using D(+)-mannose as a standard. To summarize, 150 μl of concentrated sulfuric acid was rapidly added to 50 μl of mannose (0–10 μg) in a well of a 96-well microplate followed by the addition of 30 μl of 5% phenol in water. The mixture was heated for 5 min at 90 °C in a static water bath (floating). After cooling to room temperature for 5 min in another water bath, the microplate was wiped dry, and the absorbance at 490 nm was measured by a microplate reader (Spectra Count™).

2.8. Effects of pH, temperature and metal ions

The effect of pH on HA was evaluated by incubating 50 μl of BfL (0.1 mg/ml) at different pH values for 1 h at room temperature in 50 μl of selected buffers (10 mM citrate phosphate buffer, pH 4–6.5 and 10 mM Tris–HCl buffer, pH 7–9). The heat stability of BfL was determined by incubating the protein at different temperatures (30–100 °C) for 30 min. The effects of Ba^{2+}, Ca^{2+}, Mg^{2+} and Mn^{2+} ions were determined by incubating BfL, previously dialyzed with 0.2 M EDTA, in 50 μl of each metal ion (5, 10, 20 and 40 mM) in 0.15 M NaCl. HA assays were performed in all the experiments.

2.9. SDS-PAGE

Electrophoresis under denaturing, non-reducing and reducing (dithiothreitol, 200 mg/ml) conditions was performed according to the procedure of Laemmli [25] using 5% (w/v) stacking and 10% (w/v) separating gels. The gels were stained for 30 min with 0.25% (w/v) Coomassie Brilliant Blue R-250 and destained in 10% (v/v) acetic acid.

2.10. Mass spectrometry

LC/ESI-MS data were acquired using a Waters instrument (model 3100) coupled with a Waters Alliance separation module (model e2695) and a detector (model 2489) controlled by a Dell workstation. A Waters Nova-Pak C_{18} column (2.1 mm × 150 mm, 60 Å, 3.5 μm) was equilibrated with 0.1% trifluoroacetic acid (TFA) in Milli-Q water (solvent A). The bound protein was eluted with a linear gradient (5–95%) of 90% acetonitrile with 0.1% TFA in Milli-Q water (solvent B) for 30 min (λ = 214 and 220 nm) at a flow rate of 0.4 ml/min. Mass measurements were performed in positive mode (ES^+) using the following conditions: a mass range between 200 and 2000 *m/z*; nitrogen gas flow, 6.0 l/h; capillary, 4.0 kV; cone voltage, 40 V; extractor, 3.0 V; source heater, 120 °C; solvent heater, 400 °C; ion energy, 1.0 V; and multiplier, 500.85 V.

2.11. Primary sequence determination

Edman degradation [26] was performed with an automatic gas-phase sequencer (492cLC; Applied Biosystems) using the conditions recommended by the manufacturer. The samples for sequencing were reduced and alkylated with iodacetamide. Proteins were digested with trypsin, chymotrypsin and Asp-N and Lys-C endopeptidases (sequencing grade, Roche, Germany) using an enzyme/protein ratio of 1:50 [27]. Enzymatic deglycosylation (N-linked oligosaccharides) was performed using N-glycosidase F (PNGase F) from *Flavobacterium meningosepticum* (Roche, Germany). One unit of the enzyme was added to 10 μg of BfL, followed by incubation for 4 h at 37 °C. The peptides were separated on a 1 mm × 150 mm Jupiter Proteo 90A column (Phenomenex, Aschaffenburg, Germany) using an acetonitrile gradient (0–60%) with 0.1% (v/v) trifluoroacetic acid for 40 min at a flow rate of 0.1 ml/min. The molecular weights of the glycosylated and deglycosylated peptides were determined by mass spectrometry. The similarity of the sequences was searched using the BLAST protein sequence database [28], and the sequences were aligned with the MULTALIN program [29]. The protein sequence data reported in this paper appear in the UniProt Knowledgebase under the accession number P86993.

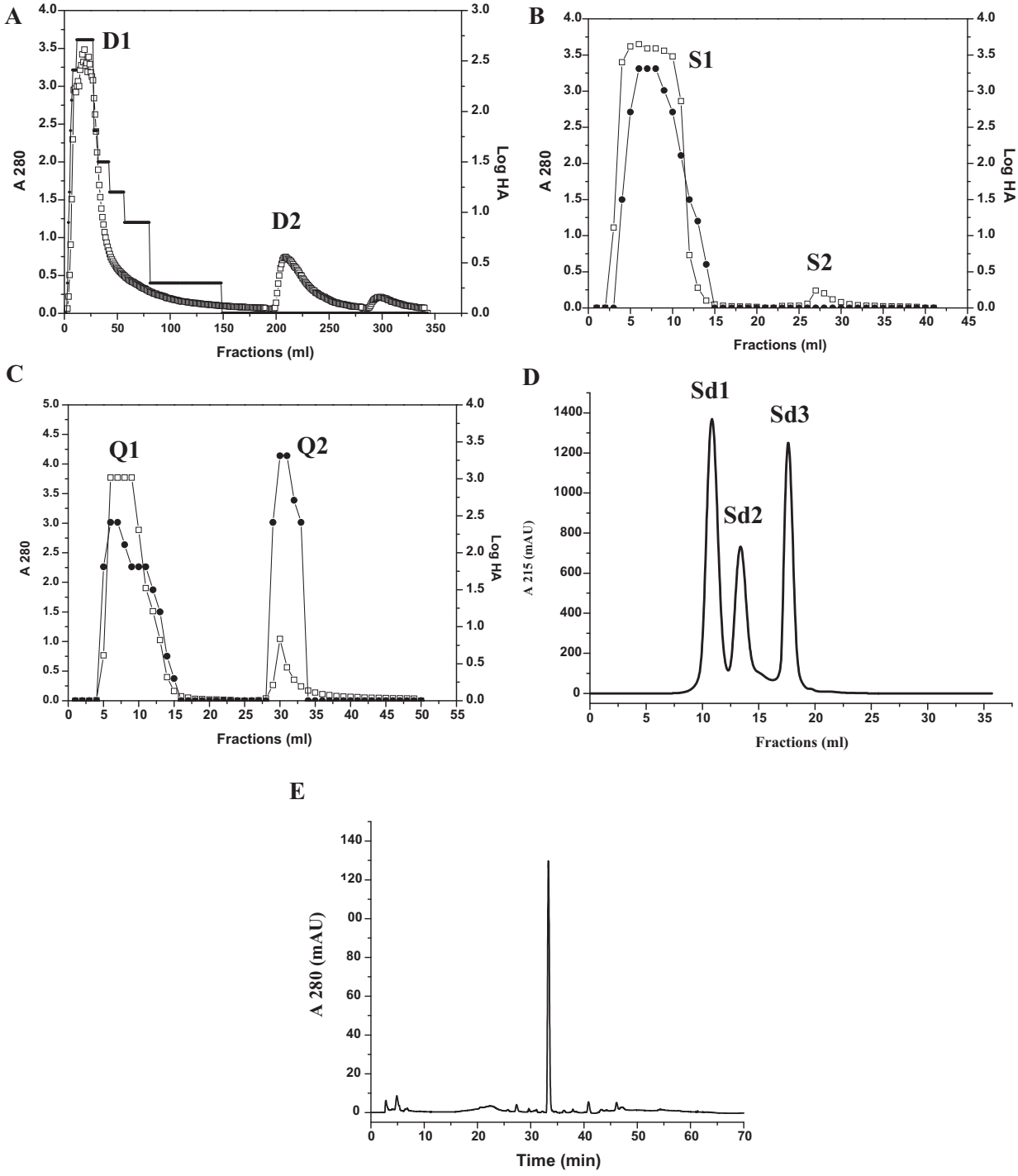

Fig. 1. Purification of *B. forficata* lectin by chromatography *via* (A) DEAE-Sephadex, (B) Sepharose-4B, (C) chitin, (D) Superdex 75 and reversed-phase HPLC (E). F40-80 was applied to a DEAE-Sephadex column (5 ml) (A) equilibrated with 0.1 M Tris–HCl buffer, pH 8.0. Non-adsorbed lectin (fraction D1) was applied to a 10 ml Sepharose-4B column (B), and the unbound lectin (fraction S1) was applied to a 5 ml chitin column (C). Absorbance at 280 nm (□) and log of HA (●). Adsorbed proteins (Q2) were eluted with 1.0 M acetic acid, lyophilized, solubilized in water and gel filtrated on a Superdex 75 10/300 GL column (D) with 0.15 M NaCl using an ÄKTA Purifier. Hemagglutinating activity was detected in fraction Sd1. BfL homogeneity was analyzed by HPLC using a Bondapak C_{18} reversed-phase column (E). The elution profile was monitored at 280 nm.

2.12. Circular dichroism (CD)

CD data were acquired on a JASCO J-810 spectropolarimeter (Jasco Corporation, Japan) in the 195–250 nm region using a circular quartz cuvette with a 1 mm path length at 25 °C under constant nitrogen purging. Measurements of the protein solution (0.114 mg/ml, in water) were recorded as an average of 16 scans. Secondary structure percentages were calculated by deconvoluting the CD spectra using the CDPro software package, which contains three popular CD analysis programs, CONTIN, SELCON3 and CDSSTR. One or all of the three programs were used with a reference protein set consisting of 48 proteins [30,31]. The CD data are expressed in terms of the mean residue ellipticity [θ] (deg cm^2 dmol^{-1}), assuming the protein contains 233 residues.

2.13. Determination of coagulation times (activated partial thromboplastin time, aPTT; prothrombin time, PT; and thrombin time, TT)

PT, TT and aPTT were determined using a semi-automated BFT II coagulometer (Dade Behring) according to the method of Silva et al. [32]. The total plasma was obtained by the centrifugation of several human blood samples at 1726 × *g* for 15 min (25 °C). The controls for the PT and TT assays were made with 50 μl of saline

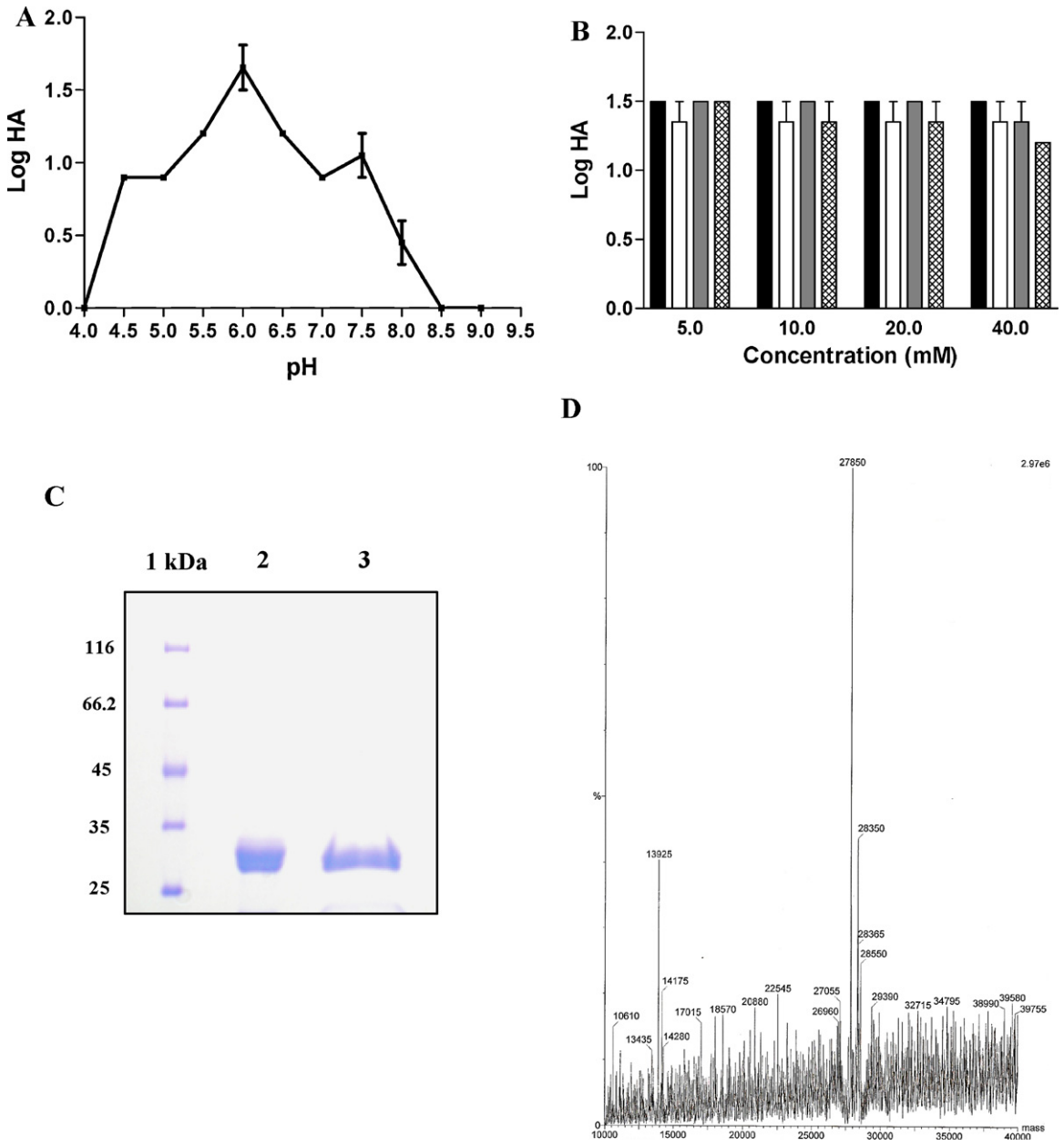

Fig. 2. (A) Effects of pH on the HA of BfL (initial HA, 32 U). (B) The effects of Mn^{2+} (■), Mg^{2+} (□), Ba^{2+} (▩) and Ca^{2+} (⊠) on the HA of BfL were determined (initial HA, 32 U). (C) SDS-PAGE of BfL using a 10% polyacrylamide gel. Lane 1 represents the molecular weight markers (kDa): β-galactosidase (116); bovine serum albumin (66.2); ovalbumin (45); lactate dehydrogenase (35); and REase Bsp98I (25). Lanes 2 and 3 represent BfL (30 μg) heated at 100 °C for 7 min in the absence and presence of DTT, respectively. (D) Mass spectra of BfL by LC/ESI-MS.

Table 1
Purification of *Bauhinia forficata* lectin.

Purification steps	Volume (ml)	Protein (mg/ml)	Total protein (mg)	HA[a]	THA[b]	SHA[c]	Yield (%)	Purif.
Crude extract	750	3.03	2272.5	256	192,000	84.5	100	1
F40-80	125	1.59	198.7	2048	256,000	1288	133.3	15.2
D1 (DEAE-Sephadex elution)	110	0.59	64.9	512	56,320	867.8	29.3	10.3
S1 (Sepharose-4B elution)	100	0.45	45	512	51,200	1137.8	26.7	13.5
Q2 (Chitin elution)	44	0.4	17.6	1024	45,056	2560	23.5	30.3
Sd1 (Superdex 75 elution)	44	0.13	5.7	1024	45,056	7876.9	23.5	93.2

Purif.: fold purification.
[a] Hemagglutinating activity was performed with 2.5% (v/v) suspension of glutaraldeyde-treated erythrocytes.
[b] Total hemagglutinating activity = HA × volume (ml).
[c] Specific hemagglutinating activity = HA/protein (mg/ml).

Table 2
Carbohydrate and glycoprotein specificity of *B. forficata* lectin.

Carbohydrates	Minimal concentration (mM) needed for inhibition of 4 U of HA	Carbohydrates	Minimal concentration (mM) needed for inhibition of 4 U of HA	Glycoproteins	Minimal concentration (mg/ml) needed for inhibition of 4 U of HA
Glucose	–	Maltose	–	Fetuin	0.5
Mannose	–	Sucrose	–	Asialofetuin	0.25
Fructose	–	Lactose	–	Thyroglobulin	0.0625
Galactose	–	Methyl-α-D-glucopyranoside	–	Azocasein	0.01562
Xylose	–	Methyl-α-D-mannopyranoside	–		
Arabinose	–	Methyl-β-D-galactopyranoside	–		
Fucose	–	N-acetyl-D-glucosamine	–		
Ribose	–	N-acetyl-D-galactosamine	–		
Rhamnose	–	Glucosamine hydrochloride	–		
Trehalose	–	Galactosamine hydrochloride	–		
Raffinose	–	Glucuronic acid	–		

The effect of carbohydrates and glycoproteins on BfL activity was determined after preincubation for 20 min at room temperature. Initial hemagglutinating activity was 128 hemagglutinating units. Dashes indicate no inhibitory activity at a concentration of 200 mM for carbohydrates and 0.5 mg/ml for the glycoproteins. The experiment was repeated twice and the results were reproducible.

and 50 μl of plasma. After incubation for 60 s, 100 μl of reagent (Thromborel S and 1.5 U/ml of thrombin for the PT and TT assays, respectively) were added to the control assays. Different concentrations of BfL (50 μl of 1.5, 3.0 and 4.0 μM) were incubated for 60 s with 50 μl of plasma, followed by the subsequent addition of 100 μl of the PT or TT reagent. The aPTT control assay contained 50 μl of saline, 50 μl of plasma and 50 μl of aPTT reagent (Dade actin-activated cephaloplastin). After incubation for 120 s, 50 μl of 0.025 M calcium chloride were added to the control assay. BfL (1.5, 3.0 and 4.0 μM) was incubated for 120 s with 50 μl of plasma and 50 μl of the aPTT reagent, followed by the addition of 50 μl of 0.025 M calcium chloride. The tests were performed in duplicate, and the results are expressed as the averages of each sample measurement.

2.14. Effect of lectin on human factor Xa and human plasma kallikrein (HuPK) activity

Lectin inhibitory activity of proteinase was determined by pre-incubating the enzyme for 10 min at 37 °C with increasing concentrations of lectin (0.03–7.0 μM) in a final volume of 250 μl. Factor Xa (21 nM) activity was determined by measuring the hydrolysis of the substrate Boc-Ile-Glu-Gly-Arg-AMC (0.57 mM, solubilized in DMSO) by factor Xa in 0.02 M Tris–HCl buffer, pH 7.4 containing 140 mM NaCl, 5.0 mM $CaCl_2$, and 0.1% bovine serum albumin. For HuPK (14.7 nM), the substrate HD-Pro-Phe-Arg-pNa (0.4 mM) was used in 0.1 M Tris–HCl buffer, pH 8.0 containing 0.5 M NaCl. The reactions were followed for approximately 30 min and stopped by the addition of 50 μl 10% (v/v) acetic acid. Using a Hitachi F-2000 spectrofluorometer, the residual enzyme activity was calculated from the absorbance of HuPK at 405 nm and the excitation and emission of factor Xa at 380 and 460 nm, respectively. The interference of DMSO on enzyme activity was carefully controlled.

2.15. Platelet aggregation

Venous blood from healthy volunteers was collected into plastic tubes containing 3.8% trisodium citrate. Platelet rich plasma (PRP) was obtained by centrifugation at 141 × *g* for 12 min at room temperature. Washed platelets (WPs) were obtained by centrifugation of PRP added to 2% EDTA (EDTA/PRP 1:20) at 880 × *g* for 15 min, followed by two washes with 0.9 mM Na_2HPO_4, 10 mM $NaHCO_3$, 2.5 mM KCl, 2.1 mM $MgCl_2$, 22 mM $C_6H_5Na_3O_7$, 140 mM NaCl, 0.055 mM glucose and 0.053 mM BSA, pH 6.5. The pellet was suspended in 2 ml of Tyrode's buffer (10 mM Hepes, 134 mM NaCl, 1 mM $CaCl_2$, 12 mM $NaHCO_3$, 2.9 mM KCl, 0.34 mM Na_2HPO_4, 1 mM $MgCl_2$ and 0.055 mM glucose, pH 7.4). The platelets were counted with a KX-21 N counter and adjusted to a final concentration of 3×10^8/ml. The aggregation was measured by the Born and Cross turbidimetric method [33] and monitored at 37 °C for 6 min using a Chrono-log aggregometer with 500 μl of the platelet suspension and ADP (10 μM), arachidonic acid (500 μg/ml), collagen (4 μg/ml), epinephrine (60 μM), fibrinogen (500 μg/ml) or thrombin (1.0 U) as an agonist with continuous stirring. The concentrations of dialyzed and concentrated BfL were 1.5 μM and 3.0 μM, respectively. Controls were made at the beginning and end of each experiment. All the experiments were approved by the Ethics Committee of the Federal University of São Paulo, number CEP 1543/11, according to Brazilian federal law.

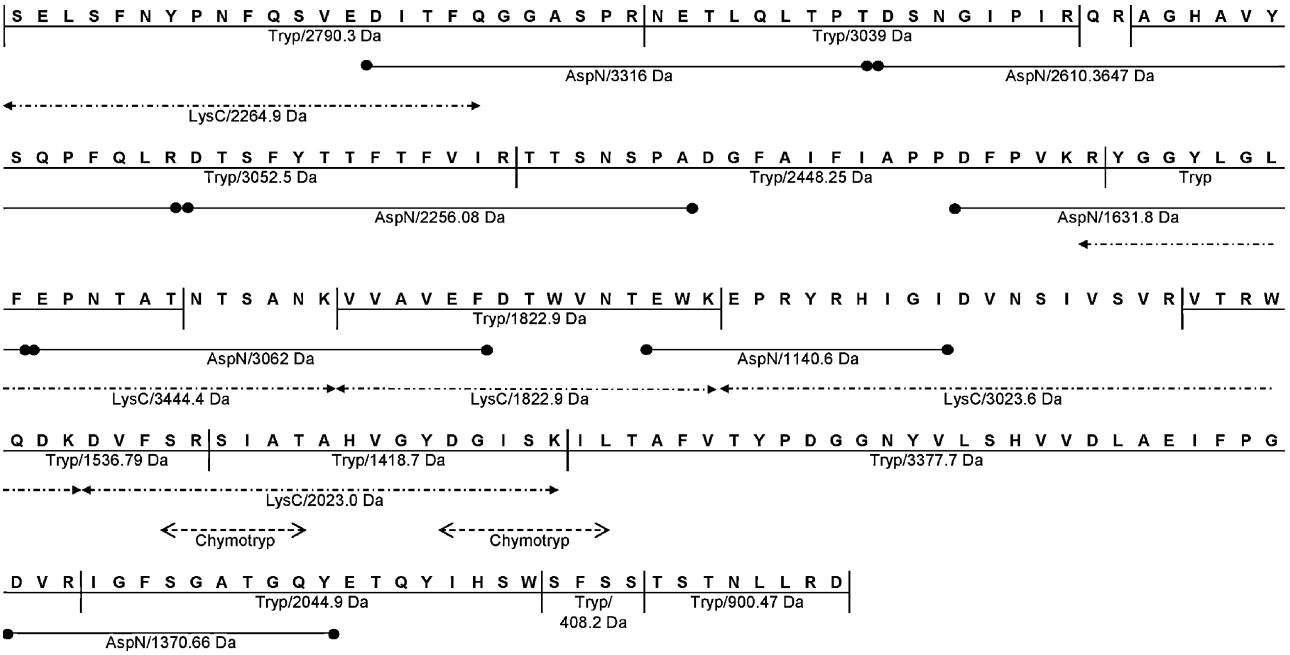

Fig. 3. Primary sequence of BfL constructed from the analysis of peptide fragments and their corresponding molecular masses resulting from hydrolysis by specific enzymes. Tryp: trypsin; Chymotryp: chymotrypsin; AspN: Asp-N; LysC: Lys-C.

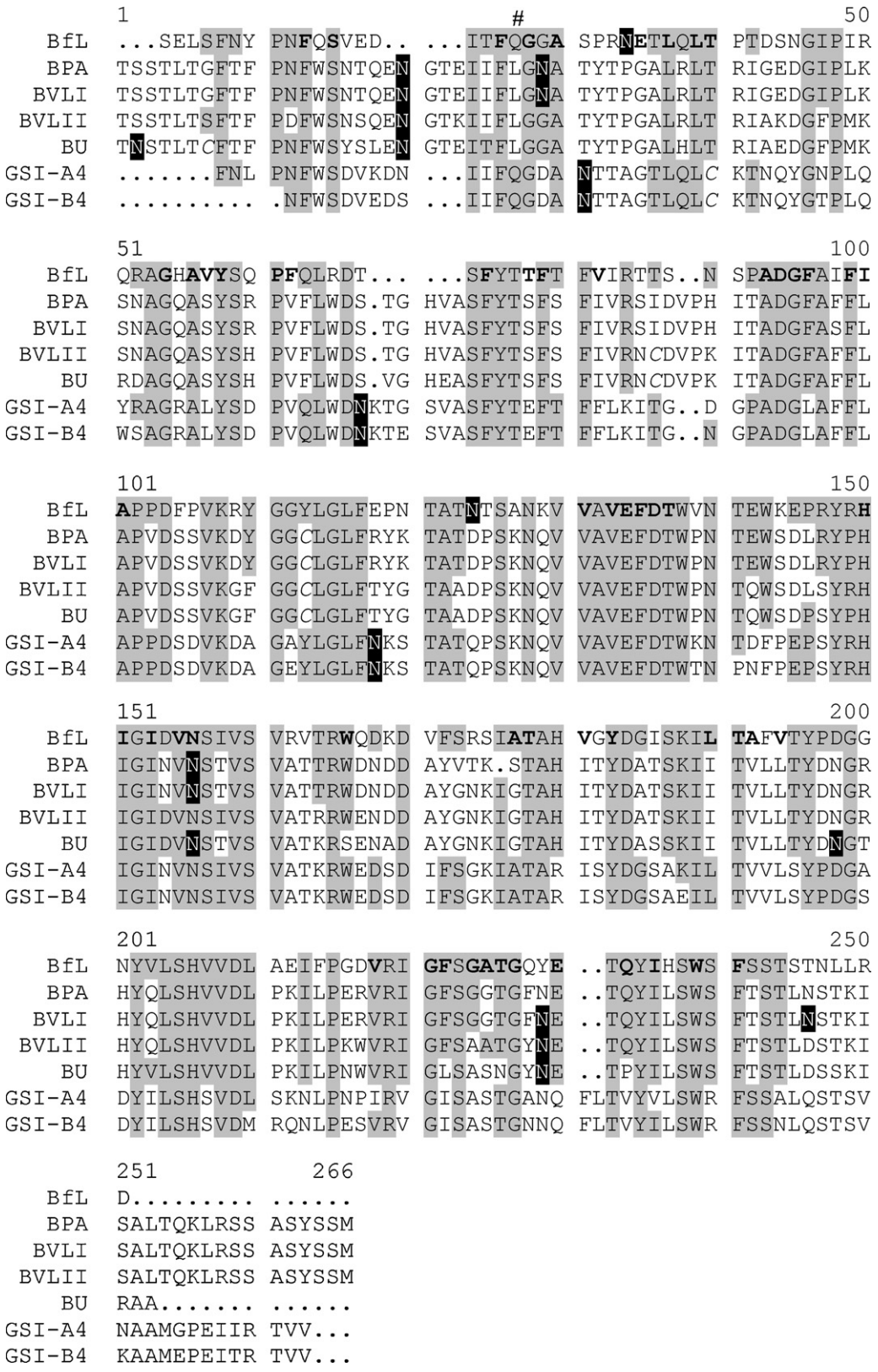

Fig. 4. Similarities between the amino acid sequences of *B. forficata* lectin (**BfL**), *B. purpurea* lectin (**BPA-P16030.2**) [16], *B. variegata* BVL isolectins (**BVL I-ABQ45362.1** and **BVL II-ACB87491.1**) [12], *B. ungulata* lectin (**BU-ABD19775.1**) [12] and *G. simplicifolia* GSI isolectins (**GSI-A4-AAL65146.1** and **GSI-B4-AAL65147.1**) [12,16]. The dashes indicate the gaps that were introduced for optimal alignment and maximum similarity for the MULTALIN program. Residues identical to BfL are displayed in gray boxes, and glycosylated amino acids are shown in black boxes. The cysteine residues are indicated in italics. Q predominates in position 19 (#); K is present in this position to a lesser extent. The conserved domains that are characteristic of legume lectins are indicated in bold.

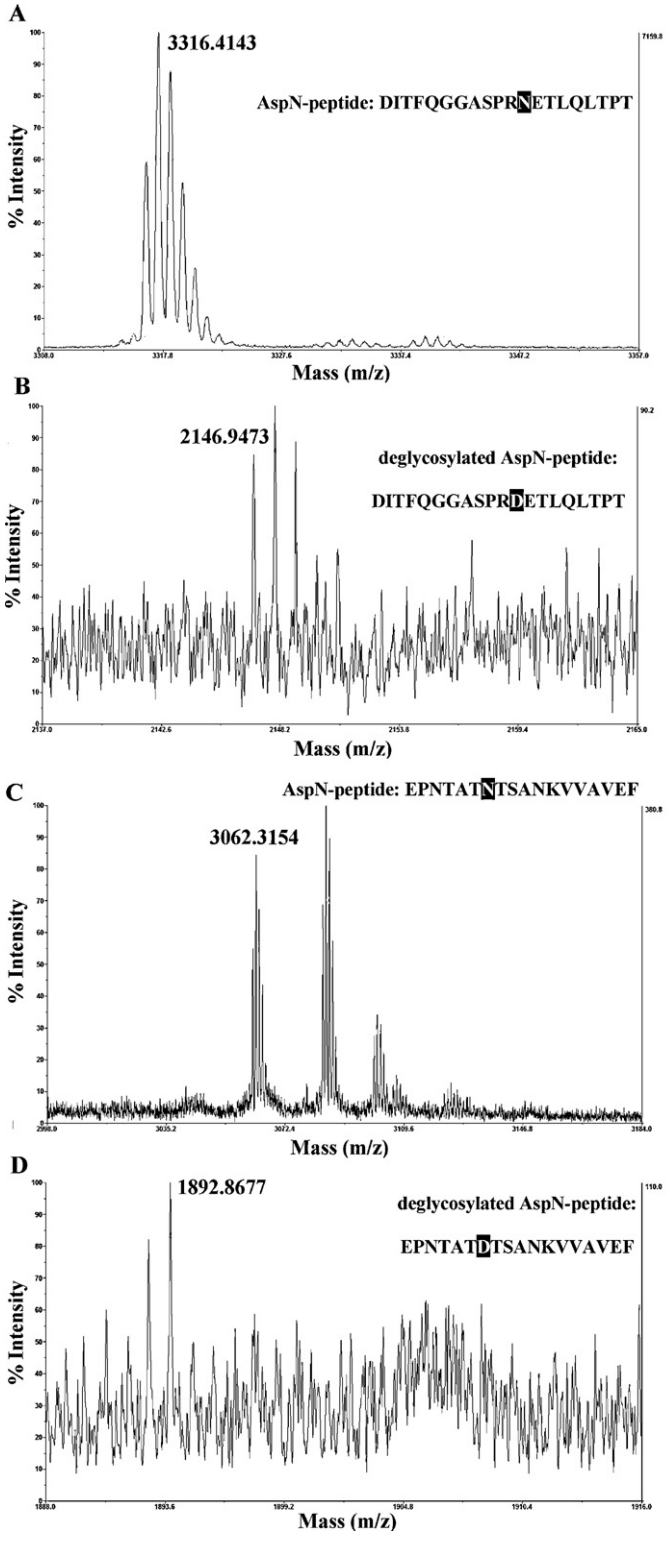

Fig. 5. Mass spectra of glycosylated and deglycosylated AspN-peptides. The AspN-peptide DITFQGGASPRNETLQLTPT (15–34 amino acids) (A) with a molecular mass of 3316.4 Da and its deglycosylated form (B), resulting from N-glycosidase F hydrolysis, with a molecular mass of 2146.9 Da. The AspN-peptide EPNTATNTSANKVVAVEF (102–119 amino acids) (C) with a molecular mass of 3062.3 Da and its deglycosylated form (D), resulting from N-glycosidase F hydrolysis, with a molecular mass of 1892.8 Da.

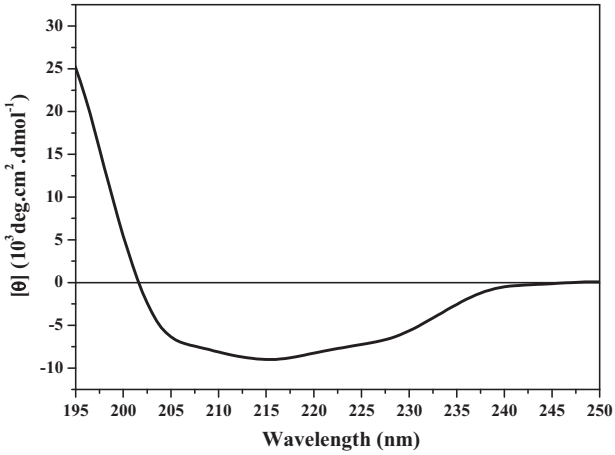

Fig. 6. Circular dichroism (CD) spectrum of BfL.

2.16. Statistical analysis

Differences between mean values were analyzed using a one-way ANOVA followed by Tukey's test. Values were considered to be significant when $p < 0.05$.

3. Results and discussion

3.1. Purification

The purification of *B. forficata* seed lectin involved extraction in 0.1 M sodium phosphate buffer, pH 7.4 containing 0.15 M NaCl and four chromatographic steps, including ion exchange chromatography with a DEAE-Sephadex column, affinity chromatography with Sepharose-4B and chitin columns and size exclusion chromatography with a Superdex 75 column. The unadsorbed fraction (D1) containing the hemagglutinating activity from the ion exchange column (Fig. 1A) was subjected to affinity chromatography on a Sepharose-4B column, resulting in unadsorbed (S1) and adsorbed (S2) fractions. HA was determined for fraction S1 (Fig. 1B), which was subsequently subjected to a chitin column. A bioactive peak (Q2) presenting an HA of 1024 hemagglutinating units (Fig. 1C) was obtained. As shown in Fig. 1D, size exclusion chromatography on a Superdex 75 column resolved the proteins into three peaks, and only the first peak (Sd1 fraction) showed strong lectin activity (BfL). The yields of the chromatographic fractions with specific hemagglutinating activities and protein concentrations are presented in Table 1. The methodology established for lectin purification is efficient because a homogenous molecule was purified by *ca.* 93 fold and with a 23.5% yield. The homogeneity of BfL was confirmed by the elution of one peak from a C_{18} column (Fig. 1E) with 72.0% acetonitrile. This material was used as the starting material for sequencing.

3.2. Characterization of lectin activity

BfL agglutinated erythrocytes from rabbits and humans of all blood types with a minimum concentration of 1 μg. The lectin from *B. variegata candida* did not agglutinate rabbit erythrocytes [11]. On the other hand, purified BVL [12] and the lectin from *B. monandra* [17] agglutinated erythrocytes from rabbits and humans. These differences demonstrate that each lectin is unique in its specificity to carbohydrates or glycoproteins. BfL is an acidic protein with a theoretical pI of 5.4, and its highest value of hemagglutinating activity was obtained at pH 6.0 (Fig. 2A). This activity was not dependent on metal ions (Fig. 2B). Similar results were obtained by Wong et al. [34] for the purified mannose/glucose-specific lectin (designated CCL) from Chinese evergreen chinkapin.

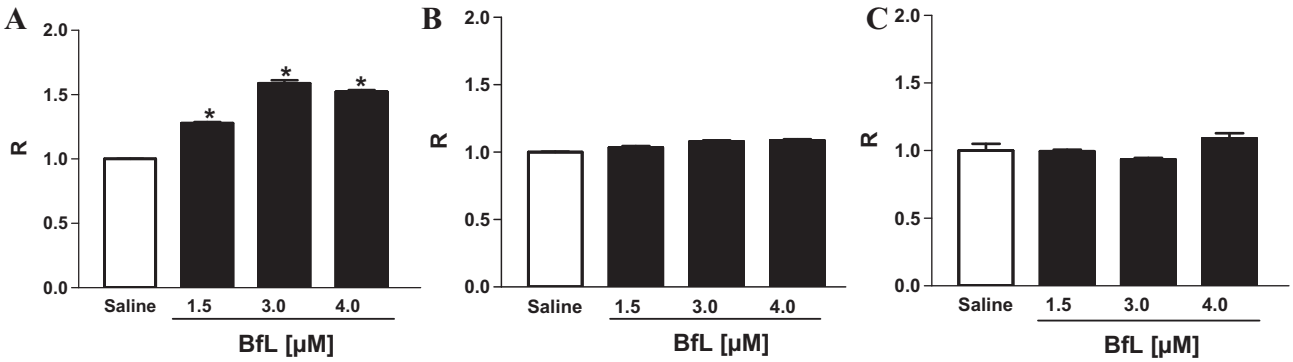

Fig. 7. The influence of BfL on blood coagulation. The results were determined by activated partial thromboplastin time (aPTT, A), prothrombin time (PT, B) and thrombin time (TT, C). R is the ratio of blood coagulation time in the presence of lectin to a normal (control) sample. *p*-Value <0.05 (*). The data represent means ± SDs, $n = 3$.

Additionally, Vaz et al. [4] obtained similar results for purified lectin from *Sebastiania jacobinensis*. The lectin from *B. pentandra* was shown to depend on divalent metal cations [18] while the lectin from *B. bauhinioides* (BBL) does not require metal ions for its activity [19] as well the lectin from *B. variegata candida* since it was not affected by EDTA [11]. Among the carbohydrates tested, no saccharide (with a maximum concentration of 200 mM) was capable of inhibiting BfL HA (Table 2). No saccharides were able to inhibit at least 4 U of HA [22,35]. The glycoproteins azocasein and thyroglobulin inhibited the HA of BfL at minimum concentrations of 15.6 and 62.5 μg/ml, respectively. The previously reported lectins from the genus *Bauhinia* show specificity for galactose and its derivatives. However, BfL is distinct, as a variety of sugars, including galactose, lactose, methyl-β-D-galactopyranoside, N-acetyl-D-galactosamine, galactosamine hydrochloride and raffinose, do not inhibit its activity. In the purification of BfL, lectin activity was detected in the unadsorbed fraction from the Sepharose-4B column, which is a polymer of agarose (galactose). However, the lectin activity of BfL was retained on the chitin column, which is a polymer of N-acetylglucosamine and not an efficient inhibitor, decreasing 1 U of HA. The interaction between lectin and carbohydrates is mediated by a combination of hydrogen bonds between sugar hydroxyl groups and the protein main-chain and side-chain groups, water-mediated contacts, van der Waals packing of the hydrophobic sugar ring facing against an aromatic residue and hydrophobic interactions [36].

3.3. Structural characterization

BfL appeared as a single band by SDS-PAGE with an apparent molecular mass of approximately 27.0 kDa under reducing and non-reducing conditions (Fig. 2C), suggesting that the native protein is not composed of disulfide-bonded subunits. Accordingly, only one peak was observed in the reversed-phase HPLC profile of the reduced and alkylated protein. The molecular mass of BfL was determined to be 27,850 ± 2.926 Da by mass spectrometry (Fig. 2D). The Edman degradation reaction [26] was used to deduce the complete primary structure of the BfL. The N-terminal segment was characterized by sequencing 25 amino acids. Moreover, the detection of the amino acid K at position 19 instead of Q indicated a variation of the protein form. Based on the peptides isolated by reversed-phase chromatography after proteolytic hydrolysis by specific enzymes (trypsin, chymotrypsin, Asp-N, Lys-C), BfL contains 233 amino acid residues (Fig. 3) and is similar to other lectins from plants, according to the Blast protein database [28]. A comparison of the sequences showed sequence conservation for *B. purpurea* lectin (54% identity), *B. variegata* isolectin I (BVLI, 54% identity), *B. variegata* isolectin II (BVLII, 53% identity), *Bauhinia ungulata* lectin (51.5% identity) and *Griffonia simplicifolia* isolectins (GSI-A4 and GSI-B4, 54% and 52% identity, respectively) (Fig. 4). BfL contains conserved domains that are characteristic of legume lectins (Fig. 4), the arcelin (also known as phytohemagglutinin-L) family of lectin-like defense proteins, the LecRK family of lectin-like receptor kinases, concanavalinA (ConA), and an alpha-amylase inhibitor, suggesting that this lectin belongs to the lectin_L-type superfamily [28]. Among the 233 amino acid residues in the BfL sequence, 23 are negatively charged (Asp and Glu) and 18 are basic (Arg and Lys) as evidenced by its low theoretical pI value (5.51). The theoretical molar extinction coefficient of BfL is 38.390 $M^{-1} \times cm^{-1}$ at 280 nm. Electrophoresis confirmed that if BfL contains cysteine residues, then they are not involved in disulfide-bond formation but are located in the primary structure. Distinct from *B. purpurea* lectin, *G. simplicifolia* isolectins (GSI-A4 and GSI-B4) and *B. variegata* isolectin I (BVLI), which contain a unique cysteine residue in their structures, and *B. ungulata* lectin, which contains three cysteine residues [12], BfL does not contain cysteine residues. Analysis of glycosylation by mass spectrometry and protein sequence determination identified an oligosaccharide N-linked to the plant protein. N-glycosylation is important due to its effects on folding, stability, protease resistance, solubility and interactions with other molecules [37]. The N-glycosylation sites in BfL are the asparagine residues at positions 26 and 108 within the conserved Asn-X-Ser/Thr sequence, where X is any amino acid with the exception of proline [37,38]. The glycosylated peptides were deglycosylated using N-glycosidase F, which converts the asparagine residue to an aspartate residue [39]. Each glycosylated asparagine had a molecular weight of 1170 Da as determined by the molecular mass differences between the glycosylated and deglycosylated peptide forms (Fig. 5). The carbohydrate content of BfL accounts for 8.4% of the mass of the lectin determined by mass spectrometry and 6.24% by the phenol-sulfuric acid method, which is slightly higher than the 4% sugar content of BvcL [11]. Based on CD data, the secondary structure content of BfL was estimated to be 19% α-helix, 27% β-sheet, 22% β-turn and 32% unordered structure (Fig. 6), indicating the presence of α-helices and β structures. To improve the reliability of protein CD analysis, three programs (CONTIN, SELCON3 and CDSSTR) were used, which resulted in a less than 2.0% deviation. The lectin of *B. monandra* (Bmoll) also has α-helices in its structure [40], although *B. purpurea* and *B. variegata candida* lectins structures are predominantly β-sheet. The lectin from *B. purpurea* contains 65% β-sheet, 19% β-turn and no α-helices [16], which is very similar to that described for the lectin (BvcL) from *B. variegata candida* [11]. BfL is a heat-stable lectin, similar to the protein

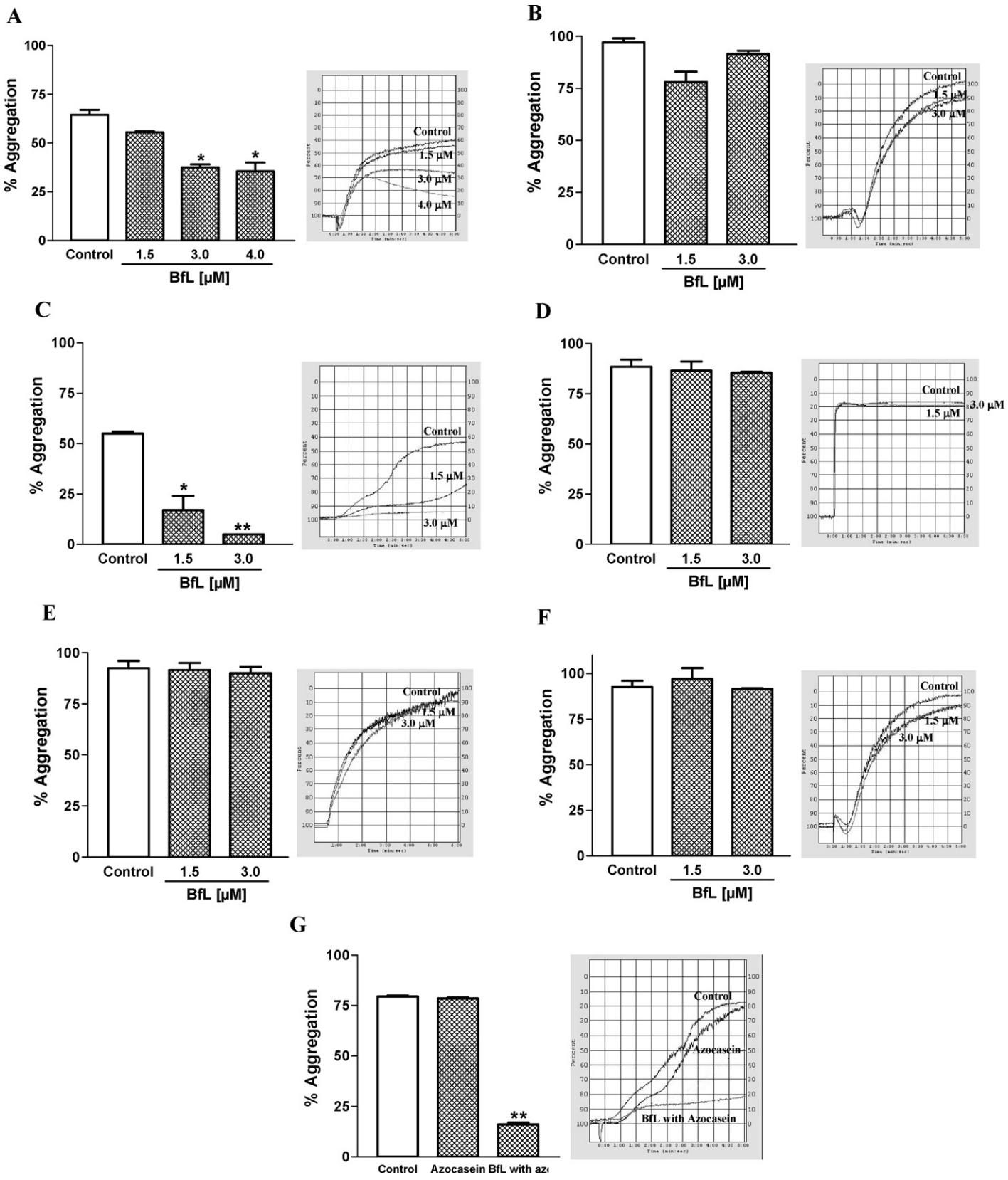

Fig. 8. The influence of BfL on platelet aggregation. Different concentrations of BfL were incubated with 2–3 × 10^8/ml of platelets for 5 min at 37 °C followed by the addition of the following platelet aggregation agonists: ADP (A), collagen (B), epinephrine (C), fibrinogen (D), thrombin (E) and arachidonic acid (F). (G) BfL (3.0 μM) was incubated with azocasein (0.125 mg/ml) for 30 min in the presence of the agonist epinephrine (BfL with azo). *p*-Value <0.05 (*) and *p*-value <0.001 (**). The data represent means ± SDs, *n* = 3.

reported by Ngai and Ng [41], Oliveira et al. [6] and Santos et al. [36], which maintained its HA after 100 °C treatment for 60 min (data not shown).

3.4. Effects of BfL on biological models of homeostasis in vitro

The aPTT coagulation time (Fig. 7A) was significantly prolonged at the tested BfL concentrations (1.5–4.0 μM). In contrast, the PT (Fig. 7B) and TT (Fig. 7C) coagulation times were not affected. These results suggest that BfL may interfere in coagulation factor activities, especially those that are involved in the intrinsic coagulation cascade pathway, except for human factor Xa and human plasma kallikrein (data not shown). Few studies have investigated the effect of lectins on coagulation parameters. Araújo et al. reported that a lectin from *Crataeva tapia* bark (CrataBL) showed anticoagulant activity *in vitro* and inhibitory activity against all blood coagulation factors of the intrinsic pathway [42]. However, most studies have been performed with proteinase inhibitors, such as the Kunitz-type inhibitor from *B. ungulata*, which inhibits activated factor X [43]. BfL has an inhibitory effect on platelet aggregation induced by ADP (Fig. 8A) and epinephrine (Fig. 8C). Almost total inhibition of platelet aggregation induced by epinephrine occurs at a concentration of 3.0 μM BfL. No interference of BfL in platelet aggregation was observed using collagen (Fig. 8B), fibrinogen (Fig. 8D), thrombin (Fig. 8E) or arachidonic acid (Fig. 8F) as agonists, suggesting that BfL does not interfere in these processes. Although some proteins have been shown to inhibit platelet aggregation [8,44], no inhibition of platelet aggregation has been reported for *Bauhinia* lectins. The proposed mechanism for lectin anti-aggregation activity involves the sugar moiety binding to the receptors on the platelet surface, especially integrins and leucine-rich repeated receptors (glycoprotein [GP] Ib/IX/V) [45]. In the case of BfL, the mechanism is not related to carbohydrate binding because treatment with azocasein did not interfere with platelet inhibitory properties (Fig. 8G). BfL may bind to ADP receptors ($P2Y_1$ and $P2Y_{12}$) or receptor α_{2A}, which is activated by epinephrine, all of which are G-protein coupled seven transmembrane receptors (GPCRs) [45].

In conclusion, we report a new glycoprotein (lectin) from the seeds of *B. forficata* and describe its primary structure and biological functions as an anticoagulant and platelet anti-aggregation protein.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.procbio.2012.03.008.

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