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IN FOCUS

Bothrops protease A, a unique highly glycosylated serine proteinase, is a potent, specific fibrinogenolytic agent

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Summary. *Background:* The hemostatic system is the major target of snake venom serine proteinases (SVSPs) that act on substrates of the coagulation, fibrinolytic and kallikrein–kinin systems. *Bothrops* protease A (BPA), the most glycosylated SVSP, is a non-coagulant, thermostable enzyme. A cDNA encoding BPA showed that the protein has a calculated molecular mass of 25 409 Da, implying that ~ 62% of its molecular mass as assessed by sodium dodecylsulfate polyacrylamide gel electrophoresis (67 kDa) is due to carbohydrate moieties. *Results:* Here we show that BPA is a potent fibrinogenolytic agent *in vitro*, as it readily degraded human and rat fibrinogen at a very low enzyme concentration. Partially N-deglycosylated BPA (p-*N*-d-BPA) generated similar fibrinogen products, but with enhanced fibrinogenolytic activity. *In vivo*, injection of 0.75 nmoles of BPA in rats completely avoided thrombus formation induced by stasis in the vena cava, or by endothelium injury in the jugular vein. Moreover, it decreased the fibrinogen plasma level and prolonged the recalcification time. Cleavage of fibrinogen in human and rat plasma was observed with native BPA and p-*N*-d-BPA by electrophoresis followed by western blot using an anti-fibrinogen antibody. BPA did not cause unspecific degradation of plasma proteins and did not cleave isolated albumin, vitronectin and fibronectin at the same concentration used with fibrinogen. Serine proteinase inhibitors failed to inhibit BPA, probably due to steric hindrance caused by its huge carbohydrate moieties. *Conclusions:* To the best of our knowledge, this investigation underscores a new, thermostable, specific defibrinogenating agent that may have an application in the prevention of thrombus formation.

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Introduction

Proteolytic enzymes of the coagulation cascade belong to the S1 (chymotrypsin) family of serine proteinases. These enzymes are composed of a single polypeptide that folds into two globular domains connected by a sequence called the autolysis loop. The catalytic site contains a highly reactive serine residue that plays a key role in the formation of a transient acylenzyme complex, which is stabilized by the presence of histidine and aspartic acid residues within the active site [1]. Snake venom glands synthesize a variety of serine proteinases [snake venom serine proteinases (SVSPs)] capable of affecting hemostasis. They act on macromolecular substrates of the coagulation, fibrinolytic and kallikrein–kinin systems, and on platelets to cause an imbalance of the prey hemostatic system [2]. The structure of SVSPs shows the typical fold of chymotrypsin-like enzymes, in which the active site cleft is located at the junction of the two six-stranded β-barrels. Most SVSPs are glycoproteins showing a variable number of glycosylation sites; however, little is known about the role of the sugar moiety in their structure and function. *Bothrops* protease A (BPA) is a thermostable SVSP isolated from the venom of *Bothrops jararaca* that, unlike most venom proteins, is stable at pH values between 3 and 9 [3]. A cDNA encoding BPA showed that the mature protein is composed of 234 residues with a calculated molecular mass of 25 409 Da, implying that approximately 62% of its molecular mass as assessed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) (67 kDa) is due to carbohydrate moieties [4]. Eight N-glycosylation and two O-glycosylation putative sites were found in the BPA sequence [4]. Various lines of evidence indicated that glycosylation has a stabilizing effect on BPA. Partial N-deglycosylation of BPA turned it into a protein that was more susceptible to hydrolysis at acidic and alkaline pH values. Fluorescence emission spectroscopy indicated that in partially N-deglycosylated BPA (p-*N*-d-BPA), tryptophan

residues are more exposed to a polar environment than in the fully glycosylated protein. The kinetic parameters of p-*N*-d-BPA upon the substrate Benzoil-Arg-4-nitroanilide (Bz-Arg-*p*NA) were similar to those of the native form, indicating that carbohydrate moieties did not play a role in the interaction of BPA with small substrates [4].

Among several snake venom proteins that target the hemostatic system, procoagulant and anticoagulant proteinases have contributed to our understanding of the mechanisms of blood coagulation and have provided potential new leads for the development of drugs to treat or to prevent unwanted clot formation [5]. In this work, we show that BPA is a potent fibrinogenolytic agent and that its carbohydrate moieties play a role in its interaction with fibrinogen and plasma inhibitors.

Materials and methods

BPA

BPA was isolated as described elsewhere [3]. Briefly, a *B. jararaca* venom solution (500 mg in 10 mL 0.15 M NaCl) was heated at 86 °C for 10 min, and centrifuged at 10 000 × *g*; the supernatant was then subjected to saturation with ammonium sulfate. The fraction precipitated at 70–80% was equilibrated in 0.05 M Tris–HCl, pH 7.6, and chromatographed on a DEAE–cellulose column (1.5 × 20 cm); proteins were eluted stepwise with 0.1, 0.2 and 0.3 M Tris–HCl, pH 7.6. The protein peak eluted with 0.3 M Tris–HCl showed the highest amidolytic activity, and was rechromatographed to obtain homogeneous BPA. Partial N-deglycosylation of BPA was performed as described previously [4].

Cleavage of proteins by BPA

Two hundred micrograms of human fibrinogen (Kabi Diagnostica, Mölndal, Sweden) or rat fibrinogen (prepared by M. L. Santoro, Instituto Butantan, according to [6]) was incubated with 0.4 μg of native BPA or p-*N*-d-BPA for 0 min, 5 min, 10 min, 15 min, 30 min or 24 h at 37 °C in 0.1 M Tris–HCl, pH 7.5. Human fibrin was prepared using fibrinogen (Kabi Diagnostica, Basel, Switzerland) and thrombin (Roche, Switzerland) [7]. One hundred micrograms of fibrin was incubated with 0.2 μg of native BPA for 15 min, 30 min, 60 min or 24 h at 37 °C in 0.1 M Tris–HCl, pH 7.5. Bovine albumin (2.5 μg), human fibronectin or human vitronectin (Sigma) was incubated with 0.25 μg or with 5 ng of BPA in 0.1 M Tris–HCl, pH 7.5, for 60 min or 24 h at 37 °C. Reactions were stopped by adding Laemmli sample buffer and then subjected to SDS-PAGE [8]. For protein identification, protein bands were excised, destained, and in-gel trypsin digested [9]. The tryptic peptide mixture was lyophilized, dissolved in 0.1% trifluoroacetic acid, subjected to ZipTip C18 (Millipore Co., Bedford, MA, USA), and spotted onto the sample plate of an Ettan matrix-assisted laser-desorption ionization time of flight (MALDI-TOF)/Pro mass spectrometer (GE Healthcare, Uppsala, Sweden) mixed with the same volume of a saturated solution of α-cyano-4-hydroxycinnamic acid (Sigma) in 50% acetonitrile/0.1% trifluoroacetic acid, and analyzed using $P_{14}R$ [$(M+H)^+$ 1533.8582] and angiotensin II [$(M + H)^+$ 1046.5423] (Sigma, Saint Louis, MO, USA) as external calibrants.

Fibrinogenolytic activity of BPA in plasma

Seven hundred micrograms of plasma proteins (citrated human or rat plasma) was incubated with either 0.42 or 1.35 μg of BPA for 1 h at 37 °C, and then subjected to SDS-PAGE [8] followed by electroblotting onto nitrocellulose membrane using a Hoefer Mini VE blot module (GE Healthcare). Seven hundred micrograms of albumin-depleted human plasma [10] was incubated with 0.42 μg of native BPA or p-*N*-d-BPA for 2 or 4 h at 37 °C, and then subjected to SDS-PAGE [8] followed by electroblotting. Western blot analysis was performed according to Burnette [11], using as primary antibody a 1 : 500 dilution of rabbit anti-mouse fibrinogen (Biodesign International, Saco, ME, USA), and as second antibody a 1 : 1000 dilution of goat anti-rabbit IgG–peroxidase conjugate (Calbiochem, Darmstadt, Germany). Blots were developed using 2.9 mM 4-chloro-1-naphthol in 0.05 M Tris–HCl, pH 7.5, 0.15 M NaCl, 0.3% methanol and 0.5% H_2O_2 as substrate.

For analysis by two-dimensional electrophoresis, 700 μg of albumin-depleted human plasma [10] was incubated with 1 μg of BPA for 2 and 24 h at 37 °C and then subjected to isoelectric focusing using immobilized pH gradient (IPG) strips (18 cm, pH 3–10, GE Healthcare) for the first dimension and 8–18% SDS–polyacrylamide gels for the second dimension [12]. Western blotting was performed as described above.

In vivo *prevention of thrombus formation in rats*

Procedures involving animals were conducted according to the Guidelines for Use of Animals in Biochemical Research [13]. Male Wistar rats (180–220 g) were anesthetized by intraperitoneal injection of 60 mg kg^{-1} sodium pentobarbital (Abbott, São Paulo, Brazil) and allocated in groups of five animals that received 0.15 M NaCl, or 300 IU kg^{-1} heparin or 250 μg kg^{-1} BPA in 0.15 M NaCl as 1 mL per 30 min infusions into the left jugular vein. Thirty minutes later, prevention of thrombus formation was tested. For stasis-induced thrombus formation in the vena cava, we strictly followed the method of Reyers *et al.* [14]. The method of Maffei *et al.* [15] was used for injury-induced thrombus formation in the jugular vein. In both models, when the thrombus was present, it was removed, blotted onto filter paper, and immediately weighed. The incidence of thrombi in the BPA-treated and heparin-treated groups was compared to that in control group by the Fisher exact test. Differences were considered to be significant when $P < 0.05$. Alternatively, male rats were injected with 0.15 M NaCl or 250 μg kg^{-1} BPA in 0.15 M NaCl as 1 mL per 30 min infusions into the left jugular vein, and 60, 120 and 180 min later blood was collected from the carotid artery and mixed with sodium citrate to a final concentration of 0.38%. Blood

was also collected from the animals of the control group; this was not treated. Plasma was obtained by blood centrifugation and used for fibrinogen determination [16]. Fibrinogen was precipitated by mixing 100 μL of plasma with 200 μL of 2.33 M glycine and incubating for 1 h at room temperature. Precipitated fibrinogen was solubilized by adding 100 μL of 6.66 M urea in 0.2 M NaOH, and the absorbance at 280 nm was used for determination of concentration [16,17]. Plasma samples were also subjected to recalcification assays as described below.

Plasma recalcification assay

To 50 μL of rat plasma, 25 μL of a 50 μg mL^{-1} BPA solution or 0.15 M NaCl was added and incubated for 1 h at 37 °C. The clot formation was initiated by adding 5 μL of 0.25 M $CaCl_2$. Alternatively, to 50 μL of plasma from rats injected with BPA (see above), 5 μL of 0.25 M $CaCl_2$ was added. The time taken for visible clot to appear from the time of addition of $CaCl_2$ was recorded.

Fibrin dissolution assay

Clots were obtained by incubation of 250 μL of human citrated plasma with 20 μL of 0.25 M $CaCl_2$ at 37 °C. BPA (12 μg per 30 μL) or 30 μL of 0.02 M Tris–HCl, pH 7.5, was added, and incubation was prolonged for 2 h; clots were then blotted onto filter paper and weighed.

Amidolytic activity

The amidolytic activity of BPA towards peptide *p*-nitroanilides H-D-Phe-Pip-Arg-*p*NA, H-D-Val-Leu-Arg-*p*NA, H-D-Val-Leu-Lys-*p*NA (Kabi Vitrum, Stockholm, Sweden) and Tos-Gly-Pro-Arg-*p*NA (Boehringer, Mannheim, Germany) was measured using the Vitor[3] (PerkinElmer – 1420 multilabel counter) and the software Wallac 1420 (PerkinElmer, Turku, Finland). Release of *p*-nitroaniline was followed at 405 nm at 37 °C in a mixture of 45 μL of 0.1 M Tris–HC1, pH 8.0, 50 μL of substrate solution, and 5 μL of BPA. For determination of K_m and V_{max}, six different substrate concentrations (0.1–2.0 mM) were used, and a molar absorbance of 10 200 for *p*-nitroaniline was considered for calculation of amidolytic activity [18], and data were analyzed using GraFit 5.0 (Erithacus Software).

Effect of serine proteinase inhibitors upon BPA

Forty micrograms of soybean trypsin inhibitor (SBTI; Worthington Biochemical Corp., Lakewood, NJ, USA) was preincubated with 0.25 μg of native BPA or p-*N*-d-BPA for 15 min at 25 °C, and the mixtures were tested for amidolytic activity towards 0.2 mM H-D-Val-Leu-Arg-*p*NA (Bachem, Heidelberg, Germany) in 0.1 M Tris–HCl, pH 8.0, at 37 °C. Anti-thrombin III (1 U) (Sigma) and heparin (3 U) (General Biochemicals, Chagrin Falls, OH, USA) were combined, and then 0.25 μg of BPA or p-*N*-d-BPA was added and the mixtures were incubated for 30 min at 25 °C. Bovine pancreatic trypsin inhibitor (500 U) (BPTI; Bayer, São Paulo, Brazil) was preincubated with 0.25 μg of BPA or p-*N*-d-BPA for 30 min at 25 °C. Benzamidine (20 mM) (Sigma) was incubated with 0.25 μg of BPA or p-*N*-d-BPA for 5 min at 25 °C. The mixtures were subjected to amidolytic activity assay and SDS-PAGE as described above.

Results

BPA in vitro *fibrinogenolytic activity*

BPA was described as a serine proteinase possessing esterolytic and amidolytic activities and devoid of coagulant (thrombin-like) activity. It was shown to digest protamine and gelatine [3], and on the insulin B-chain, BPA cleaved Arg22–Gly23, Phe25–Tyr26 and Tyr26–Thr27 bonds [19]; however, these are not natural substrates for SVSPs. Fibrinogen is the main target of snake venom proteolytic enzymes in plasma, so we initially tested the ability of BPA to hydrolyze fibrinogen *in vitro*. Incubation of BPA with human fibrinogen at a 1 : 500 (w/w) enzyme/substrate ratio led to a rapid degradation of fibrinogen chains. After 30 min of incubation, the α-chain had disappeared, whereas the γ-chain remained untouched (Fig. 1A). Two fibrinogen fragments of 46 and 14 kDa were identified by MALDI-TOF mass spectrometry as pieces of the α-chain (Fig. 1I; supplementary Table S1). BPA was also active upon rat fibrinogen. After 30 min, most of the α- and β-chains had been degraded, whereas the γ-chain was not affected (Fig. 1D). Complete N- and O-deglycosylation of BPA is only possible under denaturing conditions, and generates enzymatically inactive products. Therefore, to test the role of the sugar moiety of BPA in its interaction with fibrinogen, we used the partially N-deglycosylated enzyme [4]. Figure 1B,E shows that removal of part of the carbohydrates enhanced the proteolytic activity of BPA towards human and rat fibrinogen. The α- and β-chains of human and rat fibrinogen completely disappeared after 30 min of incubation with p-*N*-d-BPA. Four cleavage products were identified as fragments from the human fibrinogen α-chain (Fig. 1I; supplementary Table S1). With a longer incubation time (24 h), BPA and p-*N*-d-BPA had similar effects, showing that the γ-chain was resistant to cleavage (Fig. 1C,F).

The action of BPA towards fibrin was compared at the same enzyme/substrate ratio used for fibrinogen. Fibrin chains were apparently not affected, but after 15 min of incubation, a fragment of ~ 44 kDa appeared and was stable until 60 min of incubation (Fig. 1G). At the same time, a faint band of ~ 65 kDa that was present in fibrin disappeared. This band corresponds to residual fibrinogen α-chain that was not converted into α-polymer during fibrin formation as identified by mass spectrometry analysis (not shown), indicating that the 44-kDa fragment originated from fibrinogen α-chain and not from fibrin. However, after 24 h of incubation with BPA, four main bands corresponding to cleavage products were observed that apparently originated from the α-polymer and from the

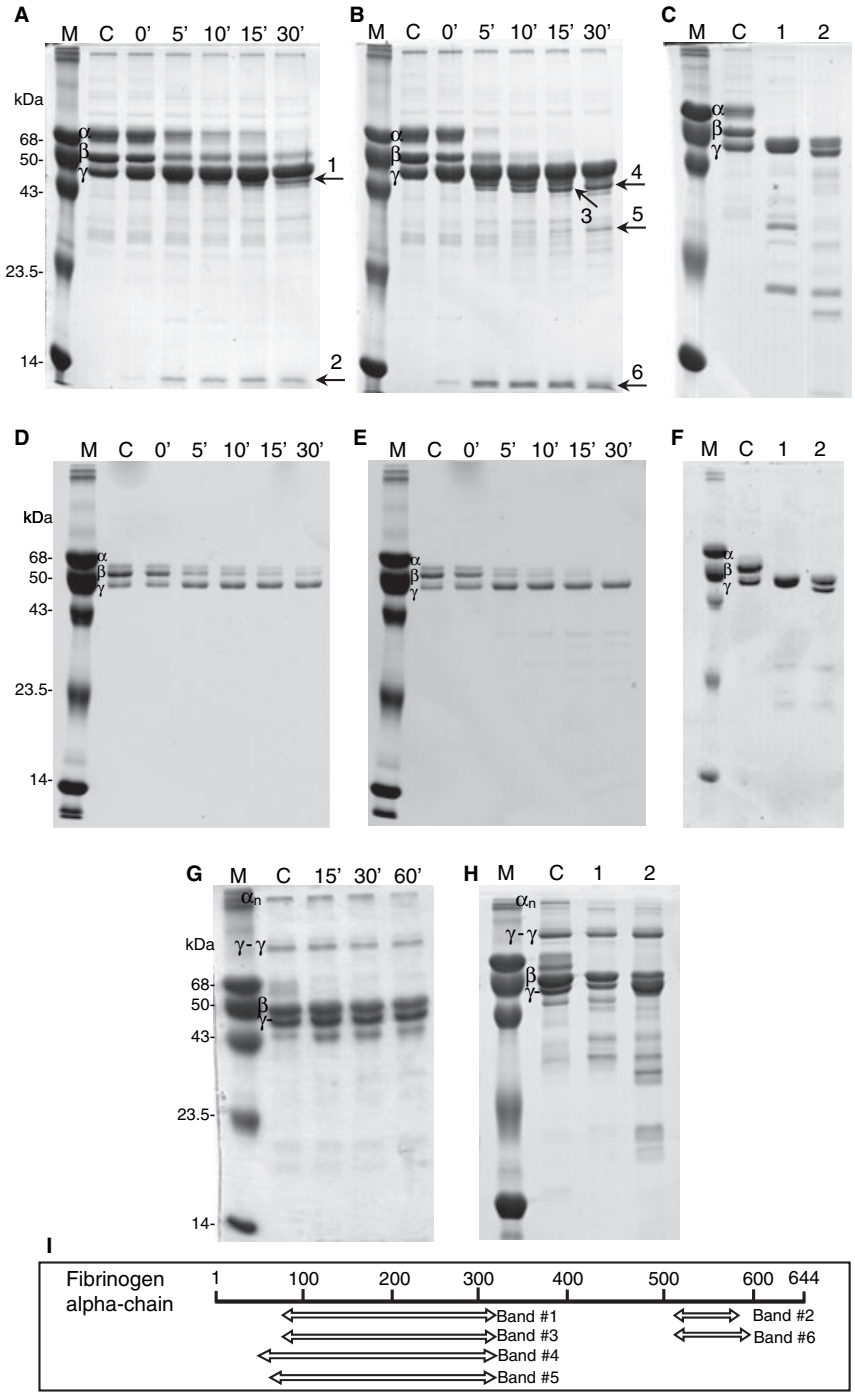

Fig. 1. *Bothrops* protease A (BPA) activity towards fibrinogen and fibrin. (A, D) Human (A) and rat (D) fibrinogen cleavage by BPA. Lane M: molecular mass markers. Lane C: fibrinogen incubated without BPA for 30 min. Lanes 3–7: fibrinogen incubated with BPA at a 1 : 500 enzyme/substrate ratio. (B, E) Human (B) and rat (E) fibrinogen cleavage by partially N-deglycosylated BPA (p-*N*-d-BPA). Lane C: fibrinogen incubated without p-*N*-d-BPA for 30 min. Lanes 3–7: fibrinogen incubated with p-*N*-d-BPA at a 1 : 500 enzyme/substrate ratio. (C, F) Human (C) and rat (F) fibrinogen incubation with BPA for 24 h. Lane C: fibrinogen incubated without BPA for 24 h. Lane 1: fibrinogen incubated with BPA at a 1 : 500 enzyme/substrate ratio. Lane 2: fibrinogen incubated with p-*N*-d-BPA at a 1 : 500 enzyme/substrate ratio. (G) Human fibrin incubation with BPA. Lane C: fibrin incubated without BPA for 60 min. Lanes 3–5: fibrin incubated with BPA at a 1 : 500 enzyme/substrate ratio. (H) Human fibrin incubation with BPA for 24 h. Lane C: fibrin incubated without BPA for 24 h. Lane 1: fibrin incubated with BPA at a 1 : 500 enzyme/substrate ratio. Lane 2: fibrin incubated with p-*N*-d-BPA at a 1 : 500 enzyme/substrate ratio. Fibrinogen α-, β- and γ-chains, and fibrin α-polymer (α_n), γ-dimer (γ–γ) and β- and γ-chains are indicated. (I) Identification of fibrinogen cleavage products. Bands indicated in (A) and (B) were identified as pieces of fibrinogen α-chain by mass spectrometry. Double-ended arrows indicate the regions to which the identified peptides belong.

β-chain (Fig. 1H). Similarly, p-*N*-d-BPA showed hydrolytic activity towards fibrin after 24 h of incubation, and generated main fragments from 18 to 44 kDa. On the other hand, the enzyme was not able to dissolve fibrin in plasma clots. Incubation of 12 μg of BPA with 250 μL of human plasma clots for 2 h did not cause changes in the weighed fibrin clots, which varied between 2.0 and 2.2 mg ($n = 6$), similarly to the controls incubated with buffer. Taken together, these results showed that BPA was not active towards the fibrin structure with incubation times up to 2 h, but was rather specific against fibrinogen.

BPA in vivo *fibrinogenolytic activity*

BPA fibrinogenolytic activity *in vivo* was illustrated by its ability to prevent thrombus formation using two different models of experimental thrombosis in rats. After 30 min of injection of 0.75 nmoles BPA per animal, it completely abolished thrombus formation induced by stasis in the vena cava, or by endothelium injury in the jugular vein (Table 1). Confirming the functional significance of the activity of BPA towards fibrinogen *in vivo*, the determination of fibrinogen level in the plasma of rats injected with BPA showed an increasing reduction in fibrinogen concentration up to 180 min (Table 2). The reduction in fibrinogen level was accompanied by prolongation of recalcification time that peaked at 60 min after BPA injection and was detected in all periods of time tested (Table 2).

TIn parallel, we tested the effect of BPA on the plasma recalcification time *in vitro*. Incubation of 50 μL of rat plasma with 20 pmoles of BPA for 1 h at 37 °C caused incoagulability of the plasma within 16 h, whereas the control plasma showed a recalcification time of 100 ± 10 s (not shown). Taken together, these findings indicate that the prevention of thrombus formation by BPA is related, at least in part, to its activity towards fibrinogen.

Table 1 Prevention of thrombus formation by *Bothrops* protease A (BPA) in rats

Group	Number of animals	Thrombi	Wet weight (mg)
Model – Injury-induced thrombus formation in the jugular vein			
0.15 M NaCl	5	5	6.4 ± 1.4
Heparin 300 IU kg^{-1}	5	0	NA
BPA 250 μg kg^{-1}	5	0	NA
Model – Stasis-induced thrombus formation in the vena cava			
0.15 M NaCl	5	5	28.6 ± 11.2
Heparin 300 IU kg^{-1}	5	0	NA
BPA 250 μg kg^{-1}	5	0	NA

NA, not applicable. Data are means ± SE.

BPA fibrinogenolytic activity in plasma

We first analyzed the effect of BPA towards plasma proteins by two-dimensional electrophoresis. Figure 2A,B shows the two-dimensional electrophoresis images of albumin-depleted human plasma incubated with BPA at a 1 : 700 (w/w) enzyme/substrate ratio or with buffer. Visual inspection of the gels indicated no notable differences, suggesting that BPA had no general proteolytic activity towards plasma proteins. Likewise, incubation of plasma with BPA for 24 h showed no extensive degradation of proteins (Fig. 2C,D). On the other hand, a specific assessment of fibrinogen cleavage in human plasma by BPA using an anti-fibrinogen antibody showed areas of clear differences between the two images (Fig. 2F, rectangular outlines) that indicated the cleavage of fibrinogen. We next evaluated the ability of BPA to cleave fibrinogen in fresh plasma. Figure 2G,H shows the western blot analysis using anti-fibrinogen antibody of human and rat plasma incubated with BPA. The presence of albumin in plasma masked the fibrinogen bands recognized by the antibody in the control experiment, but cleavage products of ~ 30–45 kDa were clearly observed in both plasma species.

Finally, Fig. 2I shows the western blot analysis using anti-fibrinogen antibody of albumin-depleted plasma incubated with BPA or p-*N*-d-BPA for 2 and 4 h. In both cases, α- and β-chains were degraded, but p-*N*-d-BPA seemed to be more active towards fibrinogen, as degradation products were further degraded by p-*N*-d-BPA. These data are in agreement with our finding that partial deglycosylation of BPA positively affected its ability to cleave fibrinogen.

BPA activity towards other plasma proteins

To further check the specificity of BPA for fibrinogen, we tested its ability to cleave other plasma proteins at a high concentra-

Table 2 Effect of *Bothrops* protease A (BPA) on rat fibrinogen plasma level and plasma recalcification time

Group	Time (min)	Fibrinogen (g L^{-1})	Reduction rate as compared to control group 1 (%)	Reduction rate as compared to 0.15 M NaCl group (%)	Recalcification time (min)	Prolongation of recalcification time as compared to control group 1 (%)	Prolongation of recalcification time as compared to 0.15 M NaCl group (%)
Control 1*	NA	1.94 ± 0.22	–	–	1.50 ± 0.10	–	–
Control 2	60	1.34 ± 0.15	31.0	–	7.42 ± 0.72	395	–
(0.15 M NaCl)	120	1.18 ± 0.05	39.1	–	4.45 ± 0.65	197	–
	180	1.20 ± 0.24	38.1	–	3.15 ± 0.40	110	–
BPA	60	1.00 ± 0.17	48.4	25.3	26.19 ± 14.06	1646	253
(250 μg kg^{-1})	120	0.89 ± 0.25	54.1	24.5	6.86 ± 3.10	357	54
	180	0.82 ± 0.18	57.7	31.6	10.82 ± 4.25	621	243

*No treatment. NA, not applicable. Data are means ± SE ($n = 3$).

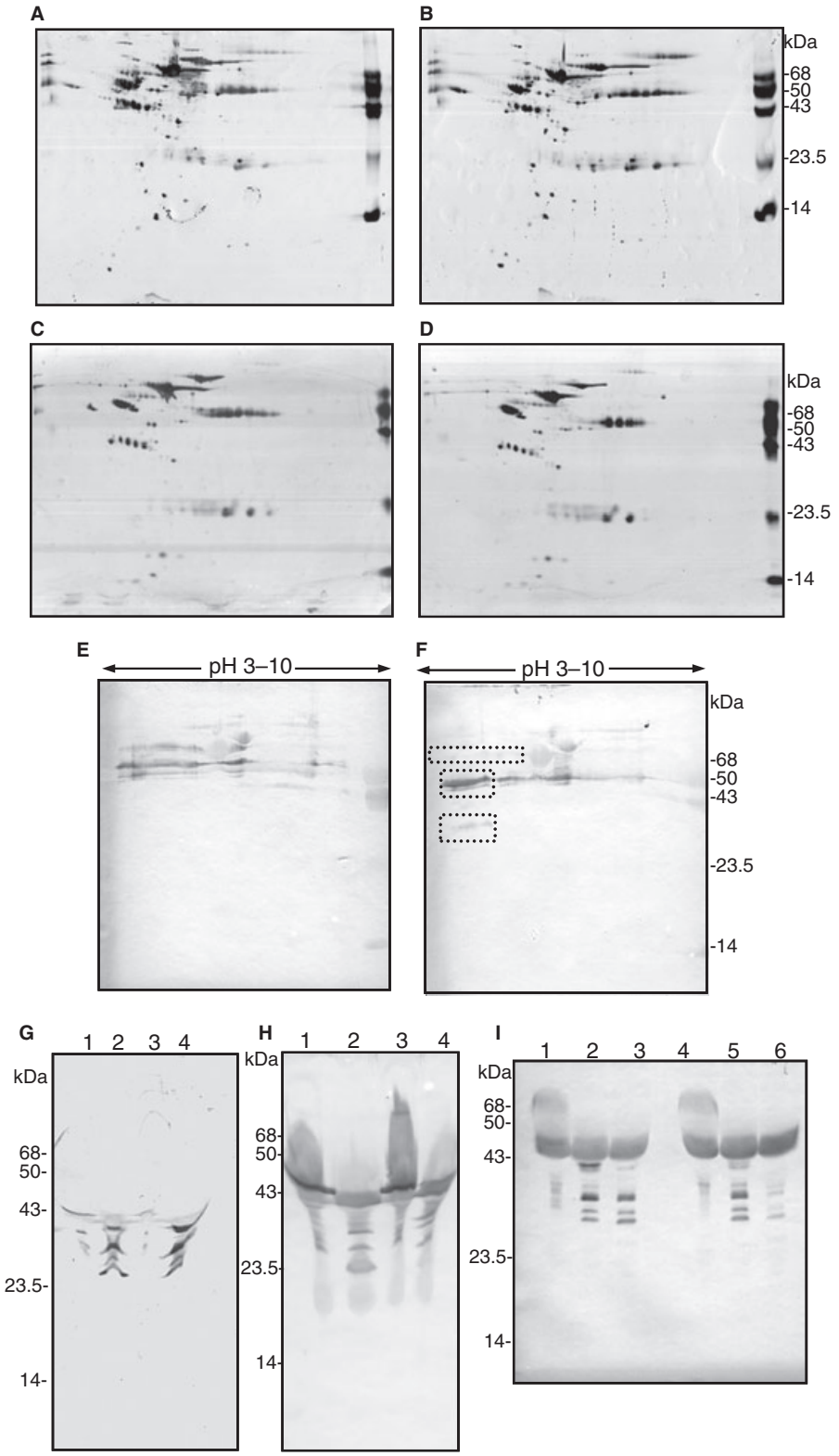

Fig. 2. *Bothrops* protease A (BPA) activity towards plasma proteins. (A–D) Two-dimensional electrophoresis of albumin-depleted human plasma proteins (700 μg) incubated for 2 h (A, B) and 24 h (C, D) without BPA (A, C) or with 1 μg of BPA (B, D). (E, F) Immunostaining using an anti-fibrinogen antibody after two-dimensional electrophoresis of albumin-depleted human plasma proteins (700 μg) incubated for 1 h without BPA (E) or with 1 μg of BPA (F). Rectangular outlines indicate gel regions that showed differences between (E) and (F). (G, H) Immunostaining after one-dimensional electrophoresis of human (G) and rat (H) plasma proteins incubated for 1 h without BPA (lanes 1 and 3), with 1.35 μg of BPA (lane 2), or with 0.42 μg of BPA (lane 4). (I) Immunostaining after one-dimensional electrophoresis of albumin-depleted human plasma proteins (700 μg) incubated for 2 h (lanes 1–3) or 4 h (lanes 4–6) without BPA (lanes 1 and 4) or with 0.42 μg of BPA (lanes 2 and 5) or partially N-deglycosylated BPA (lanes 3 and 6).

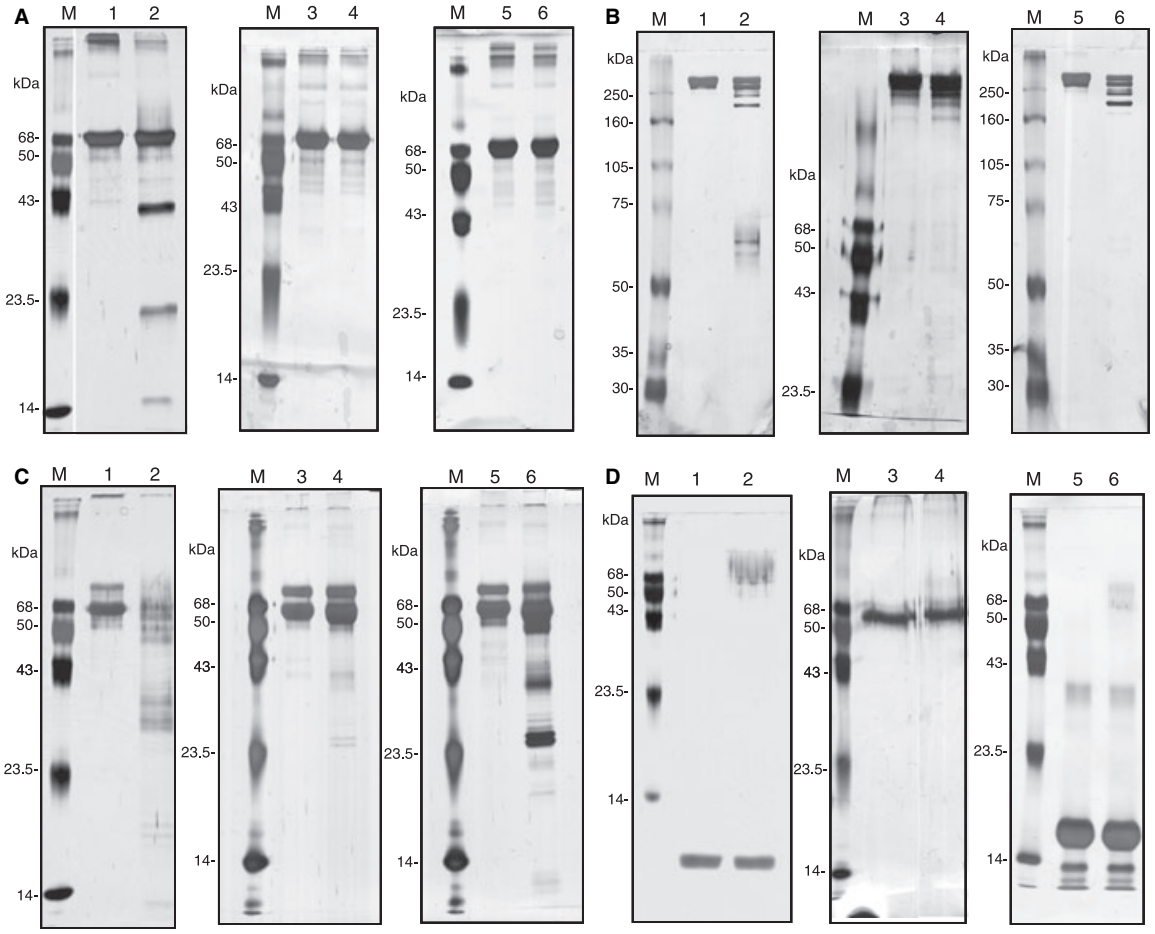

Fig. 3. *Bothrops* protease A (BPA) activity towards isolated plasma proteins and serine proteinase inhibitors. Plasma proteins incubated at a 1 : 10 enzyme/substrate ratio with BPA for 1 h: (A) albumin alone (lane 1) or incubated with BPA (lane 2); (B) fibronectin alone (lane 1) or incubated with BPA (lane 2); (C) vitronectin alone (lane 1) or incubated with BPA (lane 2). Proteins incubated at a 1 : 500 enzyme/substrate ratio with BPA for 1 h (lanes 3 and 4) and 24 h (lanes 5 and 6): (A) albumin alone (lanes 3 and 5) or incubated with BPA (lanes 4 and 6); (B) fibronectin alone (lanes 3 and 5) or incubated with BPA (lanes 4 and 6); (C) vitronectin alone (lanes 3 and 5) or incubated with BPA (lanes 4 and 6). Incubation of serine proteinase inhibitors with BPA: (D) bovine pancreatic trypsin inhibitor alone (lane 1) or with BPA (lane 2); anti-thrombin III alone (lane 3) or with BPA (lane 4); soybean trypsin inhibitor alone (lane 5) or with BPA (lane 6). Lane M: molecular mass markers.

tion. The incubation of albumin, fibronectin and vitronectin with BPA at a 1 : 10 enzyme/substrate ratio for 1 h showed only partial degradation (Fig. 3A–C). Incubation for 1 h at a 1 : 500 enzyme/substrate ratio showed no significant hydrolytic activity of BPA towards the proteins. Moreover, a prolonged incubation of 24 h showed only partial cleavage of fibronectin and vitronectin (Fig. 3B,C). These data corroborated our findings that fibrinogen is the main protein susceptible to BPA cleavage in plasma.

Effect of serine proteinase inhibitors upon BPA

In order to test the functional significance of the carbohydrate moiety of BPA in the protein interaction with serine proteinase inhibitors, we tested the susceptibility of BPA and p-*N*-d-BPA to inhibition by anti-thrombin III/heparin, SBTI and BPTI. Table 3 shows that the activity of BPA and p-*N*-d-BPA towards D-Val-Leu-Arg-*p*NA was not affected by these inhibitors; however, benzamidine, a small, reversible inhibitor, caused ~ 90% inhibition of both enzymes. Moreover, to evaluate the possibility that the lack of activity of these inhibitors towards BPA could be caused by their cleavage, we

Table 3 Effect of serine proteinase inhibitors on *Bothrops* protease A (BPA) activity towards D-Val-Leu-Arg-*p*NA

	V_{max} (nmol min^{-1})	Inhibition (%)
BPA	2.0	–
BPA + benzamidine	0.19	90.5
p-*N*-d-BPA	2.12	–
p-*N*-d-BPA + benzamidine	0.15	92.8
BPA + SBTI	2.3	0
p-*N*-d-BPA + SBTI	2.4	0
BPA + Anti-thrombin III/heparin	2.19	0
p-*N*-d-BPA + Anti-thrombin III/heparin	2.5	0
BPA + BPTI	2.12	0
p-*N*-d-BPA + BPTI	2.86	0

p-*N*-d-BPA, partially N-deglycosylated BPA; SBTI, soybean trypsin inhibitor; BPTI, bovine pancreatic trypsin inhibitor.

Table 4 Hydrolysis of peptide substrates by *Bothrops* protease A

Substrate	K_m (mM)	V_{max} (nmol min^{-1})
D-Phe-Pip-Arg-*p*NA	0.29 ± 0.08	0.690 ± 0.168
D-Val-Leu-Arg-*p*NA	0.31 ± 0.03	0.134 ± 0.006
Tos-Gly-Pro-Arg-*p*NA	0.53 ± 0.14	0.044 ± 0.0075
D-Val-Leu-Lys-*p*NA	NH	NH

NH, no hydrolysis. Data are means ± SE (n = 3).

examined the incubation mixtures by SDS-PAGE and observed that the proteins were not affected by BPA.

Amidolytic activity

The substrate specificity of BPA was tested using peptide substrates. As shown in Table 4, D-Phe-Pip-Arg-*p*NA and D-Val-Leu-Arg-*p*NA were the most susceptible to hydrolysis by BPA. BPA showed a clear preference for Arg at the P1 position [20], as no hydrolysis by BPA was seen when Arg of D-Val-Leu-Arg-*p*NA was substituted with Lys in D-Val-Leu-Lys-*p*NA. Moreover, comparison of V_{max} values showed that residues at P2 and P3 influenced BPA activity towards these substrates.

Discussion

Generally, SVSPs affect pathways of the coagulation cascade by limited proteolysis or by unspecific degradation of substrates. Despite their primary substrate specificity, similar to trypsin, their stringent macromolecular substrate specificity contrasts with the less specific activity of trypsin. To illustrate this fact, besides BPA, *B. jararaca* venom contains four different serine proteinases that show ~ 70% primary structure identity and display specific biological activities: bothrombin is a coagulant enzyme [21], PA-BJ is a platelet-aggregating enzyme [22], KN-BJ displays coagulant and bradykinin-releasing properties [23], and TL-BJ is a coagulant enzyme [24].

Venom fibrin(ogen)olytic metalloproteinases preferentially degraded fibrinogen α-chain [25]. Serine proteinases usually have activity towards fibrinogen β-chain. However, there are exceptions, and specificity for α- or β-chains is not absolute, as there may be further degradation of the alternative chain with time.

Few SVSPs are described as being capable of causing extensive degradation of fibrinogen, and they do not require any other factors for this activity. This characteristic feature of fibrin(ogen)olytic SVSPs makes them potential clinical agents with which to treat occlusive thrombi. Brevinase, from *Agkistrodon blomhoffii brevicaudus*, cleaved fibrinogen α- and β-chains and degraded fibrin [26]. Mucrosobin [27] and TM-5 [28,29], from *Trimeresurus mucrosquamatus*, were described as β-fibrinogenases but their activity towards fibrin was not reported. Recombinant harobin, from *Lapemis hardwickii*, was demonstrated to cleave both fibrinogen and fibrin [30].

The findings about BPA fibrinogenolytic activity described here point to a unique enzyme, in the sense that it showed specific human and rat fibrinogen degradation with a short incubation time and at a low enzyme/substrate ratio (1 : 500) but did not cleave fibrin or dissolve plasma clots *in vitro*. Even after 24 h of incubation with BPA, fibrinogen γ-chains showed resistance to proteolysis, and only a discrete activity of BPA on fibrin was observed. Moreover, *in vivo*, the enzyme at a very low dose and in a short period of time (30 min) was able to prevent thrombus formation. The susceptibility of fibrinogen to proteolysis by BPA was also shown by the decrease in its concentration in plasma from rats treated with the enzyme, and by the prolongation of plasma clotting time upon recalcification.

The specificity of BPA for fibrinogen in plasma was shown by an overall analysis of protein profile after incubation with BPA that showed no extensive degradation. The cleavage of fibrinogen by BPA in plasma was observed using fresh and albumin-depleted plasma and an anti-fibrinogen antibody that revealed cleavage products among plasma proteins. Cleavage of fibrinogen isolated or in plasma was observed with native BPA and p-*N*-d-BPA, and interestingly, the partially N-deglycosylated enzyme showed enhanced fibrinogenolytic activity, suggesting that removal of part of the carbohydrates of BPA may have facilitated its interaction with fibrinogen.

The assessment of BPA's ability to cleave other plasma proteins showed that albumin, fibronectin and vitronectin were only partially hydrolyzed at a high enzyme/substrate ratio (1 : 10), whereas in a 1 : 500 incubation these proteins were not affected by BPA, except for fibronectin and vitronectin, which were only partially hydrolyzed after a 24-h incubation period. These results confirmed the narrow substrate specificity of BPA.

The proteolytic steps of the coagulation cascade are regulated by feedback loops, local cofactor molecules, and the serpins. Considering the high fibrinogenolytic activity of BPA in plasma, it is plausible to speculate that it escapes inhibition by serpins. When we checked the susceptibility of BPA and p-*N*-d-BPA to anti-thrombin III/heparin, no inhibition of its amidolytic activity towards a peptide substrate was detected. Moreover, SBTI and BPTI were also ineffective in inhibiting BPA and p-*N*-d-BPA. None of these proteins was degraded by BPA, indicating that the lack of inhibition is probably due to the inability of these inhibitors to interact with the polypeptide core of the enzyme, which is protected by its huge carbohydrate moiety. The recently determined crystal structure of protein C activator ACC-C (Protac®) indicated the strategic positioning and role of three carbohydrate moieties of ACC-C in protein C recognition, binding, and activation [31]. Moreover, crystal structure comparison of *Agkistrodon acutus* serine proteinases I and II (AaV-SP-I and AaV-SP-II) revealed that Asn35-linked oligosaccharides in both proteinases cause spatial hindrance to the binding of some natural inhibitors, and might be involved in the enzyme–inhibitor interactions [32]. BPA has a significantly

larger carbohydrate moiety than ACC-C and AaV-SPs, and p-*N*-d-BPA still contained enough carbohydrates to restrict the interaction with proteinase inhibitors.

SVSPs share extensive sequence homology with other serine proteinases, but subtle differences determine their substrate specificities. The analysis of BPA activity towards peptide substrates showed a clear preference for arginine over lysine at the P1 position, indicating that the arginine residue interacts with Asp189 in the enzyme S1 site via the formation of salt-bridges between the arginine guanidinium group and the aspartate carboxylate group [33].

Fibrinogen-depleting agents reduce fibrinogen in blood plasma, and as a consequence blood viscosity is reduced and blood flow increases. This effect may be helpful, for instance, to remove a blood clot blocking the artery and re-establish blood flow to the affected area of the brain after an ischemic stroke [34]. Defibrinogenating agents derived from snake venoms in use as therapeutics or in clinical trials such as the SVSPs batroxobin (Defibrase®) and ancrod (Viprinex™) act by releasing fibrinopeptide A from fibrinogen α-chain, leading to the formation of desAA-fibrin monomer and resulting in an anticoagulant effect due to fibrinogen depletion [35–37]. In the case of ancrod, it has been shown that the therapy results in the formation of large amounts of soluble fibrin, which in turn displays a catalytic effect in the tissue-type plasminogen activator-induced activation of plasminogen [36]. BPA does not form fibrin. Rather, it prevents the formation of fibrin by degrading fibrinogen α- and β-chains, and several lines of experimental evidence in this study suggest that it could be used in low doses as an agent to prevent thrombus formation in pathologic conditions where specific degradation of fibrinogen is desirable. The thermostability of BPA due to its high carbohydrate content is a convenient feature in the optimization of a production process, because of its enhanced stability. Moreover, considering the role of the large carbohydrate moiety of BPA in its resistance to proteinase inhibitors, one could also envision an enhanced resistance of BPA to degradation by proteinases in plasma. Taken together, these findings identify BPA as a powerful defibrinogenating agent and provide a rational foundation for the exploration of its therapeutic potential.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

Supplementary material

The following supplementary material can be found at http://www.blackwell-synergy.com/loi/jth :

Table S1. Identification of products from fibrinogen α-chain. Protein bands were identified by *in gel* trypsin digestion and matrix-assisted laser-desorption ionization time of flight mass spectrometry.

This material is available as part of the online article from http://www.blackwell-synergy.com/doi/abs/10.1111/j.1538-7836.2008.02995.x.

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