Purification, Characterization, and Amino Acid Sequence of a Serine Proteinase, PA-BJ, with Platelet-Aggregating Activity from the Venom of *Bothrops jararaca*†

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ABSTRACT: A platelet-aggregating enzyme, PA-BJ, was isolated from the venom of the snake *Bothrops jararaca*. PA-BJ in a concentration of 3.2×10^{-8} M promoted 95% platelet aggregation in platelet-rich plasma. SDS−polyacrylamide gel electrophoresis under reducing conditions showed a single protein band with an M_r of 30 000. PA-BJ catalyzed the hydrolysis of several *p*-nitroanilide peptide substrates containing Arg or Lys at the scissile bond; among these the most sensitive were the thrombin substrates D-Phe-Pip-Arg-*p*NA and Tos-Gly-Pro-Arg-*p*NA. Both the platelet-aggregating and amidolytic activities of PA-BJ were abolished by reaction with phenylmethanesulfonyl fluoride. Several benzamidine derivatives, which are competitive inhibitors of trypsin-like serine proteinases, also inhibited the amidolytic activity of PA-BJ. Among the compounds tested, the thrombin inhibitor NAPAP [N^{α}-[(2-naphthylsulfonyl)-glycyl]-4-amidinophenylalanine piperidide] showed the strongest inhibitor activity on PA-BJ. The complete amino acid sequence of PA-BJ, which, to the best of our knowledge, is the first of a platelet-aggregating enzyme from snake venom, was deduced from the N-terminal sequencing of overlapping fragments cleaved from the reduced and S-pyridylethylated protein by chemical and enzymatic methods. PA-BJ is composed of 232 amino acid residues and contains one N- and one O-glycosidically linked carbohydrate moiety at residues Asn^{20} and Ser^{23}. Sequence comparison to other venom serine proteinases revealed significant homology, mainly in regions around the catalytic triad and conserved cysteine residues.

Snake venoms of the crotalid and viperid species contain an abundance of serine and metalloproteases. Some of the serine proteinases resemble thrombin in their ability to trigger the clotting of fibrinogen through fibrinopeptide release; others lack fibrinogen-clotting activity, but can directly aggregate platelets in platelet-rich plasma and washed platelet suspensions. Kirby *et al.* (1979) isolated thrombocytin from the venom of *Bothrops atrox*, a serine proteinase that induces the aggregation and secretion of platelets as well as clot retraction (Niewiarowski *et al.*, 1979). Crotalocytin, another platelet-aggregating enzyme, was isolated from the venom of *Crotalus horridus horridus* (Schmaier *et al.*, 1980; Schmaier & Colman, 1980). From the venom of *Bothrops jararacussu*, Hill-Eubanks *et al.* (1989) isolated an F VIII activator, BJV-$VIII_{CP}$, which also causes the aggregation of human washed platelets. Recently, another basic serine proteinase, MSP 1, was isolated from the venom of *Bothrops moojeni* (Serrano *et al.*, 1993). MSP 1 exhibits direct platelet-aggregating activity and enhances ADP-induced platelet aggregation. All of these snake venom platelet aggregation inducers were found to be classical DFP-sensitive serine proteinases with amidolytic activity on synthetic thrombin substrates. Similar to the venom of *B. atrox*, which contains two thrombin-like enzymes with complementary functions, namely, thrombocytin and the fibrinogen-clotting enzyme batroxobin (Stocker & Barlow, 1976; Kirby *et al.*, 1979), the venom of the pit viper *Bothrops jararaca* (Jararaca) also contains several components that may affect hemostasis. Recently, Nishida *et al.* (1994) reported the isolation of a fibrinogen-clotting serine proteinase named bothrombin from this venom. The amino acid sequence of bothrombin is homologous to that of batroxobin. In this work, we describe the purification and characterization of PA-BJ, a basic serine proteinase from *B. jararaca* venom with platelet-aggregating activity. In addition, the complete amino acid sequence of PA-BJ was elucidated, which is the first example of the primary structure of a snake venom enzyme with platelet-aggregating activity.

EXPERIMENTAL PROCEDURES

Purification of PA-BJ. Crude, dried *B. jararaca* venom (520 mg) (Institute Butantan, São Paulo, Brazil) was dissolved in 52 mL of 0.15 M NaCl, and the turbid solution was clarified by centrifugation (1600*g*, 20 min). Ammonium sulfate was added to the supernatant; the fraction of 202 mg of protein precipitating between 313 and 662 g/L ammonium sulfate was dissolved in 10 mL of water, dialyzed against 0.05 M Tris-HCl buffer (pH 7.5), and chromatographed on a DEAE-Sephacel (Pharmacia) column (1.6 × 16 cm) previously equilibrated with the dialysis buffer. The eluate with nonadsorbed protein was lyophilized and dialyzed against 0.05 M Tris-HCl buffer (pH 7.5). The resulting

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solution, containing 48 mg of protein in 14 mL, was applied to a column (2.4 × 16 cm) of SP-Sephadex C-50 (Pharmacia) equilibrated with the same buffer. After it was washed with 250 mL of starting buffer at a flow rate of 10 mL/h, the column was developed with a linear gradient from 0 to 0.6 M NaCl in 0.05 M Tris-HCl (pH 7.5) (total volume 700 mL) at a flow rate of 30 mL/h (Figure 1A). Fractions containing PA-BJ (158−169), as monitored by platelet-aggregating activity or D-Phe-Pip-Arg-*p*NA[1] hydrolysis, were combined, lyophilized, and dialyzed against 0.05 M sodium phosphate buffer (pH 8.0). Samples of 1 mL containing 1.25 mg of protein were applied separately to a Mono S column (HR 5/5, Pharamacia) equilibrated with the same buffer. For the elution of PA-BJ, a linear gradient from 0 to 0.375 M NaCl was applied over 40 min at a flow rate of 1 mL/min using a Pharmacia FPLC system (Figure 1B). Fractions eluted in peak 2 were rechromatographed under identical conditions (Figure 1C).

Analytical Procedures. Protein concentrations were determined (Lowry *et al.*, 1951) by using bovine serum albumin as a standard. Blood-clotting activity was measured on human fibrinogen (Henriques *et al.*, 1960). Kinin-releasing activity was determined by using dog kininogen as substrate (Fink *et al.*, 1985). Hemorrhagic activity was estimated similar to the method of Kondo *et al.* (1960), as described by Mandelbaum *et al.* (1982). Caseinolytic activity was assayed by a modification of the Kunitz method (1947), as described by Mandelbaum *et al.* (1982). Molar concentrations of PA-BJ were determined by active site titration with *p*-nitrophenyl *p'*-guanidinobenzoate according to Chase and Shaw (1970).

Platelet-Aggregating Activity. Citrated human blood (0.38% sodium citrate) was centrifuged at 250*g* for 4 min at room temperature, and the platelet-rich plasma (PRP) was removed. The remaining blood was centrifuged for an additional 10 min at 900*g*, and platelet-poor plasma (PPP) was obtained. Washed platelet suspensions were prepared essentially as described by Mustard *et al.* (1972) from blood collected in acid citrate dextrose (ACD) (Aster & Jandl, 1964). Following centrifugation of PRP at 900*g* for 10 min, the supernatant was discarded and the pellet was washed twice in Tyrode-ACD solution; finally, the pellet was resuspended in Tyrode solution (pH 7.4) containing 5 mM HEPES. The platelet count was 3×10^8 mL^{-1}. Platelet aggregation was determined by measuring the decrease in turbidity over 4 min (Born & Cross, 1963) using a Minigator II aggregometer (Payton). The aggregometer was calibrated by setting PRP to 0% and PPP to 100% aggregation, or (when experiments were done with washed platelets) by setting washed platelet suspension to 0% and saline to 100% aggregation. Aliquots of PRP or platelet suspension (0.4 mL) were kept at 37 °C for 3 min before the addition of 0.1 mL of sample or saline. Activity was expressed as a percentage of aggregation.

Amidolytic Activity. The amidolytic activity of PA-BJ on peptide *p*-nitroanilides D-Phe-Pip-Arg-*p*NA, D-Val-Leu-Arg-*p*NA, Bz-Ile-Glu-Gly-Arg-*p*NA, D-Val-Leu-Lys-*p*NA, Bz-Phe-Val-Arg-*p*NA (Kabi Vitrum, Sweden), Tos-Gly-Pro-Arg-*p*NA (Boehringer Mannheim, Germany), Z-Val-Gly-Arg-*p*NA (Novabiochem, Germany), and Bz-Pro-Phe-Arg-*p*NA (Pentapharm, Switzerland) was measured photometrically. The release of *p*-nitroaniline was followed continuously at 405 nm at 25 °C in a mixture of 780 μL of 0.1 M Tris-HCl buffer (pH 8.0), 10 μL of substrate solution, and 10 μL of PA-BJ. Activities were calculated by using a molar absorbance of 10 200 for *p*-nitroaniline (Fiedler *et al.*, 1978). For determining K_m and V_{max}, at least six different substrate concentrations (0.05−1.0 mM) were used; the data were fitted to the Michaelis−Menten equation with a nonlinear regression analysis program (Enzfitter, Biosoft, Cambridge).

K_i values for the inhibition of PA-BJ by benzamidine derivatives were determined according to Stürzebecher *et al.* (1986) at three substrate concentrations without inhibitor and at two different inhibitor concentrations. Tos-Gly-Pro-Arg-*p*NA (0.025 mL; 2.0, 1.0, or 0.5 mM) was added to a mixture of 0.2 mL of 0.1 M Tris-HCl buffer (pH 8.0), 5% ethanol, inhibitor, and 0.05 mL of PA-BJ solution (15 μg/mL) in microplate wells (Dynatech). After 10 min at 30 °C, the reaction was stopped with 0.025 mL of 100% acetic acid, and the released *p*-nitroaniline was measured at 405 nm in a Microplate Reader MR700 (Dynatech). The K_i values were calculated (Dixon, 1953) by linear regression using the software Fig.P (Biosoft, Cambridge).

SDS−polyacrylamide gel electrophoresis was carried out according to Laemmli (1970) with gradient gels (10−20% polyacrylamide) under reducing conditions. Protein bands were detected by silver staining (Heukeshoven & Dernick, 1985). The molecular mass markers were myosin Hchain (195 000), phosphorylase B (100 000), bovine serum albumin (71 000), ovalbumin (44 000), carbonic anhydrase (28 000), β-lactoglobulin (18 400), and lysozyme (15 000).

Amino Acid Composition. Samples of native PA-BJ were hydrolyzed under vacuum in 5.7 M hydrochloric acid at 110 °C for 20 h. Amino acid analyses were performed on a Beckman high-performance analyzer system 6300.

Amino acid sequences were determined by Edman degradation with a gas-phase sequencer (Hunkapiller *et al.*, 1983) (Model 473 A, Applied Biosystems) according to the manufacturer's instructions. This system uses reversed-phase HPLC for identification of the phenylthiohydantoin derivatives of the amino acids (Hunkapiller & Hood, 1983).

Reduction and Pyridylethylation. PA-BJ (2 nmol) was incubated in 100 μL of 0.25 M Tris-HCl buffer (pH 8.5), 6 M guanidine hydrochloride, 1 mM EDTA, and 5% (v/v) β-mercaptoethanol at room temperature; after 16 h, 5 μL of 4-vinylpyridine (Sigma) was added and the incubation was continued for 90 min (Friedman *et al.*, 1970). After acidification with formic acid, S-pyridylethylated protein was desalted by HPLC using an Aquapore RP 300 column (2.1 × 30 mm, Applied Biosystems). Elution was performed with 0.1% trifluoroacetic acid for 10 min, followed by a gradient of 0−80% acetonitrile (containing 0.1% trifluoroacetic acid) over 10 min at a flow rate of 0.3 mL/min. The effluent was monitored continuously at 206, 254, and 280 nm.

Cyanogen Bromide Cleavage. Reduced, S-pyridylethylated PA-BJ was dissolved in 70% (v/v) formic acid containing 10% cyanogen bromide (w/v) (Merck) and incubated in the dark at room temperature for 14 h (Gross & Witkop, 1961). After lyophilization, the CNBr peptides were redissolved in 20% formic acid for HPLC separation.

[1] Abbreviations: DFP, diisopropyl phosphorofluoridate; NAPAP, N^{α}-[(2-naphthylsulfonyl)glycyl]-4-amidinophenylalanine piperidide; Pip, pipecolyl; *p*NA, *p*-nitroanilide; PMSF, phenylmethanesulfonyl fluoride; PRP, platelet-rich plasma.

Table 1: Isolation of PA-BJ from the Venom of *Bothrops jararaca*

step	total protein mg	total protein %	specific activity[a] (munits/mg)	total activity[a] (munits)	yield (%)	platelet aggregation[b]
crude venom	520	100	2.5	1300	100	nd[c]
50−90% ammonium sulfate saturation	202	39	5.9	1192	92	nd[c]
DEAE-Sephacel	48	9.2	9.6	461	35	2.4
SP-Sephadex C-50	27	5.2	16	432	33	1.0
Mono S chroamtography	17	3.3	19	323	25	1.0
Mono S rechromatography	14	2.7	20	280	22	0.9

[a] Enzymatic activity was determined using the substrate D-Phe-Pip-Arg-*p*NA; 1 munit = hydrolysis of 1 nmol/min. [b] The values represent the concentrations of protein, μg/mL, that caused 70% platelet aggregation in PRP. [c] Not determined.

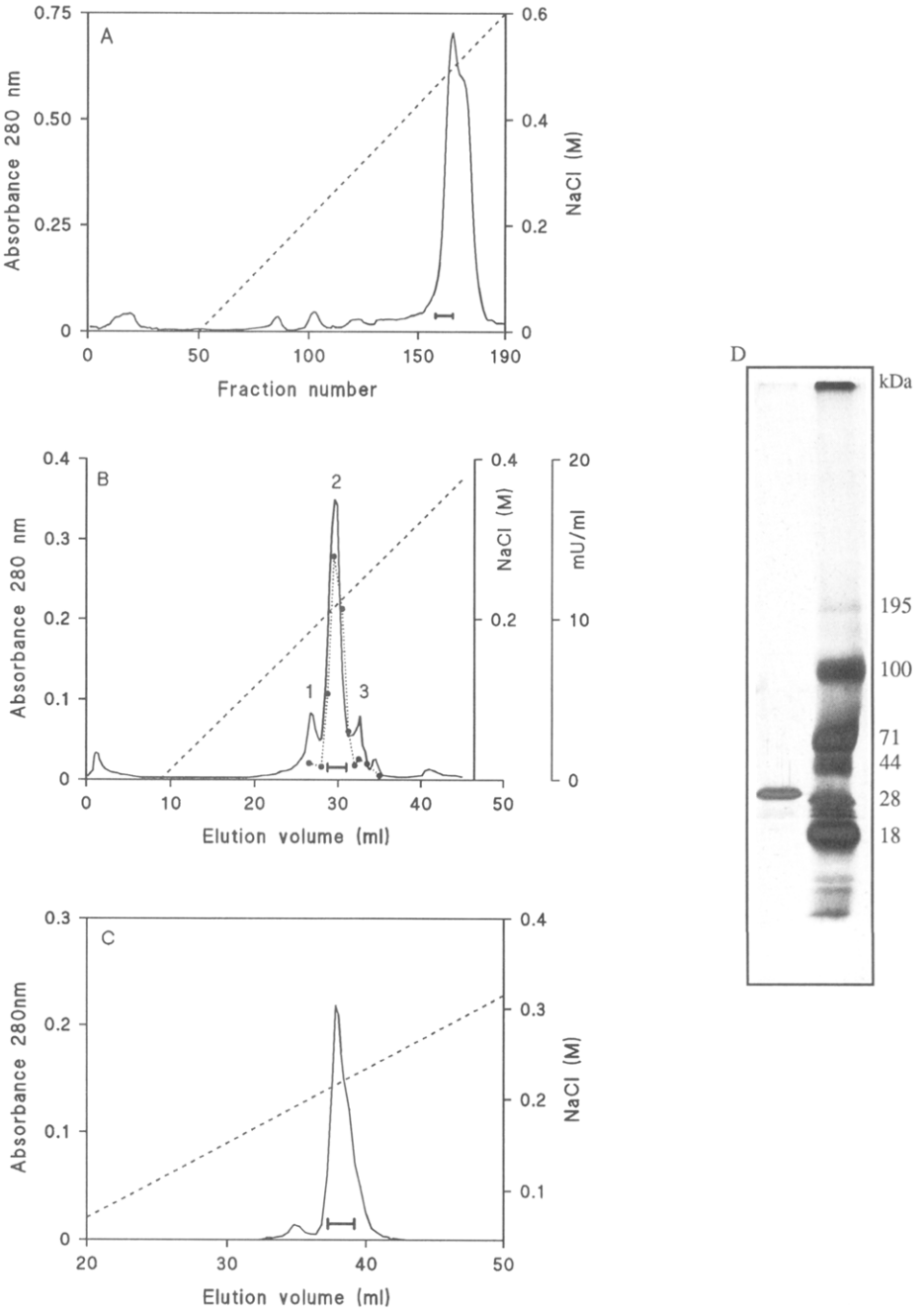

FIGURE 1: Purification of PA-BJ from *B. jararaca* venom: (A) SP-Sephadex C-50 chromatography; (B) Mono S chromatography; (C) Mono S rechromatography. The elution profiles were monitored at 280 nm (—). The dashed lines indicate the salt gradient; the dotted line with solid circles (Figure 1B) indicates enzymatic activity with substrate D-Phe-Pip-Arg-*p*NA (1 munit = hydrolysis of 1 nmol/min). The bars indicate the fractions that contained PA-BJ. (D) SDS−PAGE of PA-BJ and molecular mass markers.

Trypsin, Chymotrypsin, and Arg-C Endoproteinase Cleavages. Reduced, pyridylethylated PA-BJ or peptide from a previous cleavage (2 nmol) was incubated with trypsin, chymotrypsin, or *Clostridium histolyticum* Arg-C endoproteinase (all of sequencing grade, Boehringer Mannheim, Germany) in 100 μL of 0.1 M ammonium hydrogen

carbonate buffer (pH 8.0) for 14 h at 37 °C using an enzyme: protein ratio of 1:30 (w/w). The reaction was stopped by adjusting the pH to 2.0 with formic acid.

Asp-N Endoproteinase Cleavage. X−Asp bonds were selectively cleaved by incubating 2 nmol of S-pyridylethylated PA-BJ with Asp-N endoproteinase (sequencing grade, Boehringer Mannheim) in 100 μL of 0.05 M sodium phosphate buffer (pH 8.0) at 37 °C for 10 h by using an enzyme:protein ratio of 1:40 (w/w). The reaction was stopped as described earlier. For the cleavage of X−Cys-(SO_3H) bonds (Tetaz *et al.*, 1990), 2 nmol of PA-BJ was oxidized by incubation in 50 μL of performic acid for 1 h at 4 °C (Hirs, 1967), diluted with water, lyophilized, and incubated with Asp-N endoproteinase as described earlier.

Pepsin Cleavage. Peptide (1 nmol) was incubated with 1 μg of pepsin (sequencing grade, Boehringer Mannheim) in 5% (v/v) formic acid for 2.5 h at room temperature.

N- and O-Deglycosylation. Peptide CB3TR (1 nmol) was incubated in 100 μL of 0.02 M sodium phosphate buffer containing 0.08% sodium azide, 0.01 M EDTA, and 2% Nonidet P-40 at 95 °C for 2 min. After cooling, 1 unit of *N*-glycosidase F or 2 munits of *O*-glycosidase (Boehringer Mannheim) was added, and the mixture was incubated for 18 h at 37 °C. The reaction was stopped by acidification with 10 μL of formic acid, and the products were submitted to Edman degradation.

Peptide separations were achieved by HPLC (Pharmacia) on a LiChrospher 100 RP18 column (125 × 4 mm, Merck), which was eluted with a linear gradient from 100% A (=0.1% trifluoroacetic acid in water (v/v)) to 40% A/60% B (=0.1% trifluoroacetic acid in acetonitrile (v/v)). The effluent was monitored continuously at 206, 254, and 280 nm.

Peptide nomenclature is according to the reagent/method used for cleavage: CB, cyanogen bromide; TR, trypsin; AN, endoproteinase Asp-N; Ox-AN, endoproteinase Asp-N of oxidized peptide. Arabic numerals indicate the peak elution orders in chromatograms. Peptides resulting from the digestion of a fragment have the designation of the parent fragment followed by the indicator of the second cleavage method: TR, trypsin; RC, Arg-C endoproteinase; CT, chymotrypsin; PP, pepsin.

RESULTS

Purification of PA-BJ. The isolation of the platelet-aggregating enzyme, PA-BJ, from the venom of *B. jararaca* was achieved by using a five-step procedure summarized in Table 1. On DEAE-Sephacel ion-exchange chromatography, the platelet-aggregating activity was in the fraction of nonadsorbed material that also contained 35% of the total D-Phe-Pip-Arg-*p*NA-hydrolyzing activity present in the crude venom. By this second purification step, the caseinolytic, blood-clotting, kinin-releasing, and hemorrhagic activities were completely removed. No hemorrhagic activity was detectable with doses of up to 20 μg of protein of this fraction, whereas for crude venom the minimum hemorrhagic dose was 5 μg (Mandelbaum & Assakura, 1988). The absence of coagulant activity was demonstrated by the fact that no clot formation could be observed for up to 20 min when 37 μg of protein was incubated with 0.8 mg of human fibrinogen in a total volume of 0.25 mL at 37 °C; for comparison, under identical conditions 5 μg of the crude

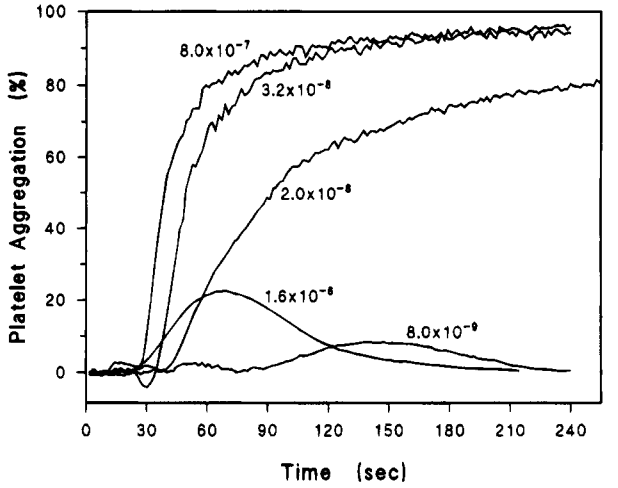

FIGURE 2: Aggregation of platelets in platelet-rich plasma promoted by various doses of PA-BJ. The experiments were carried out as described in Experimental Procedures. The numbers indicate the molar concentrations of PA-BJ that caused the respective platelet aggregation curve.

venom caused clot formation within 60 s. Similarly, no kininogenase activity could be detected when 5 μg of protein of this fraction was incubated with dog kininogen, whereas 5 μg of crude venom released kinin at a rate of 28 fmol/min. Enzymes with fibrinogen-clotting and kinin-releasing activities could be eluted from the DEAE-Sephacel column and were further purified (data not shown).

Figure 1A shows the elution profile of ion-exchange chromatography on a SP-Sephadex C-50 column of the material not bound to DEAE-Sephacel. Fractions containing platelet-aggregating activity were eluted at a NaCl concentration of 0.5 M. The material was subsequently chromatographed on a Mono S column (Figure 1B). PA-BJ was eluted at a NaCl concentration of 0.22 M. Peak 2 (Figure 1B) was rechromatographed on the same column (Figure 1C). By using this method, 14 mg of purified PA-BJ could be obtained from 520 mg of crude venom (Table 1). SDS−PAGE under reducing conditions showed that the enzyme consisted of a single polypeptide chain with an apparent relative molecular weight of 30 000 (Figure 1D). The specific activity of PA-BJ on D-Phe-Pip-Arg-*p*NA was 20 nmol/min/mg. The purified PA-BJ caused extensive platelet aggregation. Depending on the PA-BJ concentration in platelet-rich plasma, aggregation of up to 95% was induced (Figure 2). On washed platelet suspensions it promoted 40% aggregation at a concentration of 10^{-7} M; in comparison, with thrombin about one-hundredth of this concentration is necessary to cause a similar effect (Niewarowski *et al.*, 1979).

Substrate Specificity of PA-BJ. PA-BJ cleaves *p*-nitroaniline from several synthetic peptide substrates. As shown in Table 2, D-Phe-Pip-Arg-*p*NA and Tos-Gly-Pro-Arg-*p*NA (commonly used substrates for thrombin) were the most susceptible to hydrolysis by PA-BJ; however, it also hydrolyzed D-Val-Leu-Arg-*p*NA (substrate for glandular kallikrein) and Bz-Phe-Val-Arg-*p*NA (substrate for thrombin). To a lower extent, PA-BJ also hydrolyzes D-Val-Leu-Lys-*p*NA, Bz-Ile-Glu-Gly-Arg-*p*NA, and Z-Val-Gly-Arg-*p*NA, whereas Bz-Pro-Phe-Arg-*p*NA (substrate for plasma kallikrein) is a rather poor substrate. These results indicate that the enzyme has a preference for Arg in the P_1 position [nomenclature of

Table 2: Kinetic Parameters for the Hydrolysis of Peptide Chromogenic Substrates by PA-BJ

substrate	K_m (mM)		k_{cat} (s^{-1})		k_{cat}/K_m (mM^{-1} s^{-1})	
	PA-BJ[a]	thrombin	PA-BJ[a]	thrombin	PA-BJ	thrombin
D-Phe-Pip-Arg-*p*NA	0.32 ± 0.03	0.0016	27.2 ± 1.05	95	85	59000
Tos-Gly-Pro-Arg-*p*NA	0.25 ± 0.02	0.004	7.86 ± 0.52	100	31	25000
D-Val-Leu-Arg-*p*NA	0.74 ± 0.08	0.35	17.8 ± 0.94	19	24	54
Bz-Phe-Val-Arg-*p*NA	0.14 ± 0.03	0.018	2.74 ± 0.24	38	20	2100
D-Val-Leu-Lys-*p*NA	1.07 ± 0.15	1.40[b]	9.63 ± 0.93	1.2[b]	9	0.9[b]
Bz-Ile-Glu-Gly-Arg-*p*NA	0.55 ± 0.04	0.057	3.21 ± 0.28	0.94	6	17
Z-Val-Gly-Arg-*p*NA	0.52 ± 0.04	0.073	0.73 ± 0.15	3.2	1.4	45
Bz-Pro-Phe-Arg-*p*NA	0.70 ± 0.08	0.49[b]	0.04 ± 0.014	0.11[b]	0.06	0.22[b]

[a] Data are given as mean ± SD, $n = 3$. [b] Data are for human thrombin. Kinetic data for PA-BJ were determined at pH 8.0. The data presented for thrombin are from Lottenberg *et al.* (1981). They were determined with bovine thrombin (all data except as indicated) at pH 7.8 or with human thrombin at pH 8.0.

Table 3: Inhibition of PA-BJ by Benzamidine Derivatives

inhibitor	K_i (μM)		
	PA-BJ[a]	batroxobin[b]	thrombin[b]
N^{α}-[(2-naphthylsulfonyl)glycyl]-4-amidinophenylalanine piperidide	15 ± 4.6	1.7	0.006
N^{α}-tosyl-3-amidinophenylalanine piperidide	75 ± 17	320	0.34
N^{α}-tosyl-4-amidinophenylbutyric acid anilide	110 ± 29	13	40
N^{α}-tosyl-4-amidinophenylalanine piperidide	170 ± 30	130	2.3
3-amidinobenzyl phenyl ketone	723 ± 102	224	69
4-amidinobenzyl phenyl ketone	>1000	98	7.5
(4-amidinophenyl)pyruvic acid	>1000	350	6.5
N^{α}-(tosylglycyl)-3-amidinophenylalanine methyl ester	>1000	290	66

[a] Data are given as mean ± SD, $n = 3$. [b] Data from Stürzebecher *et al.* (1986). K_i values given were determined at pH 8.0.

Schechter and Berger (1967)] and that the residues in P_2 and P_3 markedly influence the activity of PA-BJ.

Inhibition Studies. Incubation of PA-BJ for 15 min at room temperature with 1 mM phenylmethanesulfonyl fluoride abolished its amidolytic as well as its platelet-aggregating activity, indicating the involvement of the active site serine residue for both activities. The inhibition of the amidolytic activity of PA-BJ was also studied by using benzamidine derivatives, which are competitive inhibitors of trypsin-like serine proteinases. Table 3 shows the K_i values for the inhibition of PA-BJ in comparison with batroxobin, the thrombin-like enzyme from the venom of *B. atrox*, and thrombin (Stürzebecher *et al.*, 1986). The most pronounced inhibition of PA-BJ was observed for the relatively selective thrombin inhibitor NAPAP, but even for this best inhibitor the K_i value for PA-BJ was higher than that for batroxobin and thrombin by 1 and 3 orders of magnitude, respectively. In general, the affinity of most of the benzamidine derivatives tested is higher for thrombin than for PA-BJ and batroxobin, but for all inhibitors the inhibition constants for PA-BJ increased in exactly the same order as for thrombin.

Primary Structure of PA-BJ. Amino acid sequence determination of overlapping peptides by automated Edman degradation allowed the elucidation of the entire amino acid sequence of PA-BJ (Figure 3). The sequence obtained is consistent with the amino acid analysis data (not shown) of the full-length protein. By sequence analysis of nonreduced PA-BJ, 52 residues could be determined with blanks at those positions corresponding to cysteine residues and glycosylation sites.

Four peptides were obtained by cyanogen bromide treatment of the pyridylethylated PA-BJ and sequenced as far as possible. One of these fragments, CB3, corresponded to the N-terminal sequence of PA-BJ. To obtain additional information, the cyanogen bromide fragments were subsequently submitted to limited proteolysis with trypsin, Arg-C endoproteinase, chymotrypsin, or pepsin.

Fourteen main fragments were obtained from the digestion of pyridylethylated PA-BJ with trypsin; two of them, TR10 and TR13, were hydrolyzed further with chymotrypsin. Digestion and sequencing of the tryptic fragment TR14 revealed the C-terminal portion of PA-BJ. To generate peptides overlapping with trypsin fragments, pyridylethylated PA-BJ was cleaved N-terminal to aspartic acid residues, and oxidized PA-BJ was cleaved N-terminal to cysteic acid residues with Asp-N endoproteinase.

The N-glycosylation site Asn^{20} and the *O*-glycosylation site Ser^{23} were determined by sequencing the fragment CB3TR after incubation with *N*-glycosidase F and *O*-glycosidase, respectively.

Sequence Homology. Figure 4 shows the alignment of the amino acid sequence of PA-BJ, composed of 232 residues, with those of other known snake venom serine proteinases and bovine thrombin. Comparison of these sequences reveals striking homology among the snake venom enzymes, but a low degree of similarity to thrombin.

DISCUSSION

A new serine proteinase was purified from the venom of *B. jararaca* by a combination of ammonium sulfate precipitation and ion-exchange chromatographic steps. The enzyme was named PA-BJ, indicating its platelet-aggregating activity. PA-BJ is a basic glycoprotein with a p*I* higher than 9.0 according to isoelectric focusing experiments (data not shown). Purified PA-BJ is free of fibrinogen coagulant activity and, therefore, clearly is different from bothrombin, an acidic fibrinogen-clotting serine proteinase from the same venom (Nishida *et al.*, 1994). PA-BJ resembles, in many aspects, thrombocytin and MSP 1 isolated from the venoms of *B. atrox* (Kirby *et al.*, 1979) and *B. moojeni* (Serrano *et*

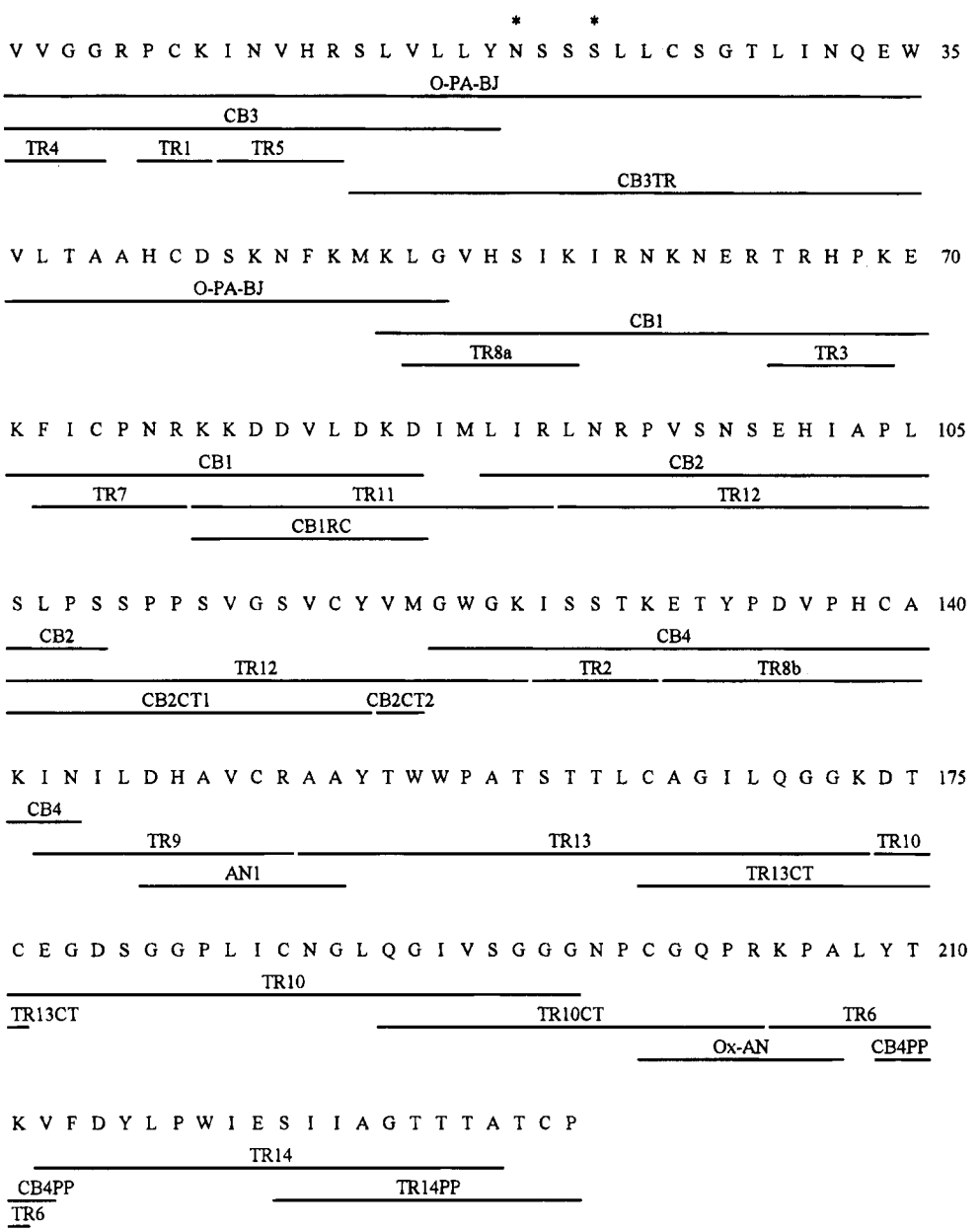

FIGURE 3: Complete amino acid sequence of PA-BJ. Determined peptide sequences are indicated by bars. For details on peptide preparation and nomenclature, see Experimental Procedures. The sequence designated O-PA-BJ was obtained by sequencing intact PA-BJ. Asterisks indicate glycosylation sites.

al., 1993). All three enzymes are basic glycoproteins with similar molecular masses. Moreover, they aggregate platelets directly in platelet-rich plasma. All three enzymes also cause the aggregation of washed platelets, demonstrating that they possess platelet-activating activity that is, like that of thrombin, independent of exogenous fibrinogen and different from the fibrinogen-dependent activity of bothrombin. Their platelet-aggregating activity is dependent on the integrity of the catalytic site since it is inhibited (along with the amidolytic activity) by DFP or PMSF.

The hydrolytic activity toward various peptide nitroanilides with Arg or Lys in position P_1 (Schechter & Berger, 1967) is strongly influenced by the peptide moiety adjacent to the scissile bond (Table 2). The catalytic efficiency, expressed by the k_{cat}/K_m ratio, is highest for D-Phe-Pip-Arg-*p*NA, which has been reported to be the most susceptible synthetic substrate for various snake venom proteinases (Teng *et al.*, 1989). D-Phe-Pip-Arg-*p*NA and Tos-Gly-Pro-Arg-*p*NA are the best substrates for both PA-BJ and thrombin, although for PA-BJ, k_{cat}/K_m is about 3 orders of magnitude lower, mainly due to the high K_m value. From the preference for such substrates and inhibitors, for which thrombin also has a high affinity (Tables 2 and 3), it seems reasonable to assume that the peptide bond of the thrombin receptor that is cleaved by thrombin, Asp-Pro-Arg||Ser-Phe (Vu *et al.*, 1991), is also accessible to PA-BJ, and one might speculate that platelet activation is triggered by PA-BJ and thrombin in the same way. However, more detailed studies on substrate specificity and the platelet-aggregating mechanism will be necessary to clarify this point.

The relative molecular weight of 25 218 calculated from the amino acid sequence of PA-BJ is lower than the value of 30 000 estimated by SDS−PAGE, probably due to the carbohydrate moieties. The glycosylation sites in PA-BJ were directly identified by performing Edman degradations before and after deglycosylation with *N*- and *O*-glycosidases.

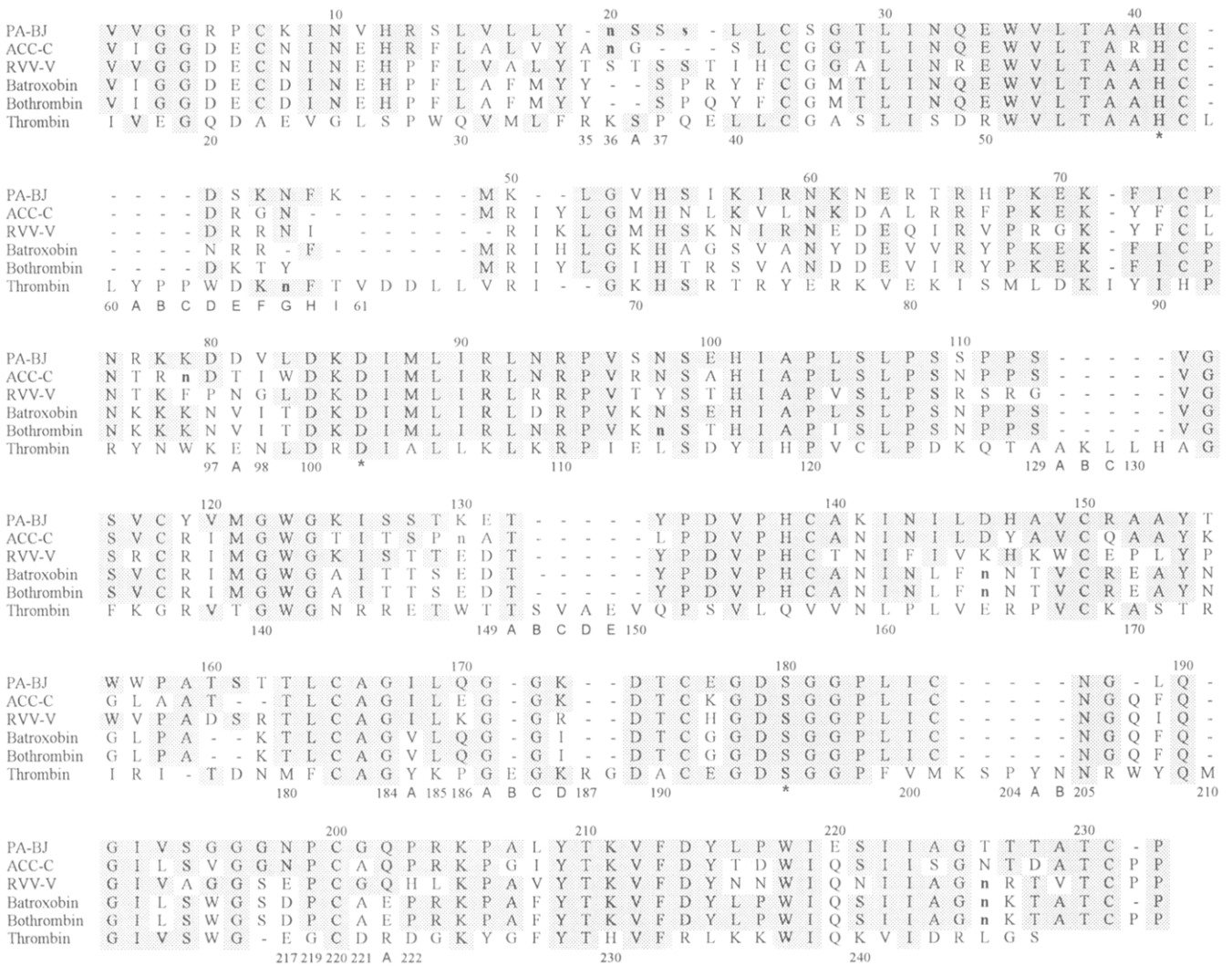

FIGURE 4: Sequence comparison of PA-BJ with the protein C activator (ACC-C) from *A. c. contortrix* venom (McMullen *et al*., 1989), the factor V-activating enzyme (RVV-V) from *V. russelli* venom (Tokunaga *et al*., 1988), the thrombin-like enzyme batroxobin from *B. atrox* venom (Itoh *et al*., 1987), and bovine thrombin B-chain (Magnusson *et al*., 1975). The numbers above refer to PA-BJ; for the numbering of thrombin the chymotrypsinogen nomenclature introduced by Bode *et al*. (1989) is used, which indicates inserted amino acids (inserted in comparison to chymotrypsinogen) by uppercase letters. Gaps are introduced to improve alignment. Residues identical to PA-BJ are shaded. Asterisks indicate the active triad of a serine proteinase. Potential glycosylation sites are shown in lowercase letters.

Two oligosaccharide chains were located close to each other in the N-terminal segment, one *N*-glycosidically linked to Asn[20] and the other *O*-glycosidically linked to Ser[23]. Snake venom serine proteinases usually vary in their glycosylation patterns; however, the *O*-glycosylation site identified in PA-BJ is unique in that for all other snake venom serine proteinases only N-linked oligosaccharide chains have been found.

Recently, the complete amino acid sequences of several snake venom serine proteinases have been reported: batroxobin (Itoh *et al*., 1987), flavoxobin (Shieh *et al*., 1988), RVV-V (Tokunaga *et al*., 1988), ACC-C (McMullen *et al*., 1989), ancrod (Burkhart *et al*., 1992), the thrombin-like enzyme from *Lachesis m. muta* venom (Magalhaes *et al*., 1993), and bothrombin (Nishida *et al*., 1994). These enzymes display different biological effects. Batroxobin, flavoxobin, ancrod, bothrombin, and the thrombin-like enzyme from *L. m. muta* have fibrinogen-clotting activities. RVV-V is an activator of the coagulation factor V, while ACC-C is a protein C activator. However, up to now no primary structure of a snake venom platelet-aggregating enzyme has been reported. In Figure 3 the sequences of PA-BJ, some snake venom proteinases, and bovine thrombin B-chain are shown. All of the venom enzymes comprise nearly the same number of residues, whereas thrombin is about 25 residues longer. PA-BJ has a higher similarity to the venom enzymes (to ACC-C, 68%; batroxobin, 65%; bothrombin, 65%; RVV-V, 63%) than to mammalian serine proteinases: bovine thrombin (31%), bovine trypsin (42%), and porcine glandular kallikrein (38%). The sequence comparisons allowed the putative identification of the catalytic triad His[41], Asp[86], and Ser[180] and of residues of the substrate-binding subsites. The Asp at position 174, six residues before the active site serine, corresponds to the primary specificity site; it is the same in thrombin and trypsin and is responsible for trypsin-like specificity (Stubs & Bode, 1993). PA-BJ contains 12 half-cystine residues which, on the basis of the homology with trypsin (Swift *et al*., 1982), probably form six intrachain disulfide bridges: Cys[7]−Cys[139], Cys[26]−Cys[42], Cys[74]−Cys[231], Cys[118]−Cys[186], Cys[150]−Cys[165], and Cys[176]−Cys[200] (PA-BJ numbering). Among these six disulfide bridges, Cys[74]−Cys[231] occurs only in the venom proteinases and might be a unique characteristic of these enzymes.

Like mammalian pancreatic serine proteases, PA-BJ lacks some characteristic structural features of thrombin to which special functional properties of thrombin have been assigned (Stubbs & Bode, 1993). As PA-BJ and thrombin have high platelet-aggregating activity in common, it is worthwhile to look for common structural features that might be involved in this activity. One candidate is the segment $Lys^{50}-Arg^{64}$ in PA-BJ, which corresponds to the anion-binding exosite $Arg^{67}-Glu^{80}$ in thrombin. Although the corresponding sequence segments have only 20% identity, they both contain four basic amino acids in excess of acidic ones (PA-BJ 2 Arg, 3 Lys, 1 Glu; thrombin 4 Arg, 2 Lys, 2 Glu), whereas for the other snake venom proteinases without platelet-aggregating activity this number is between −1 and 2. In thrombin, the anion-binding exosite is involved in the induction of platelet aggregation (Jakubowski & Maraganore, 1990), and it is tempting to speculate that the corresponding segment of PA-BJ is also involved in platelet-aggregating activity.

In conclusion, both catalytic properties and structural features of thrombin, PA-BJ, and other snake venom serine proteinases provide suggestions toward understanding similarities and differences in the distinct activities of the various enzymes. However, further studies including the elucidation of the three-dimensional structures will be necessary to understand fully which structural features of these enzymes are critical for their specific biological activities.

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