Thrombocytin, a Serine Protease from *Bothrops atrox* Venom. 1. Purification and Characterization of the Enzyme†

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ABSTRACT: Thrombocytin, a platelet-activating enzyme from *Bothrops atrox* venom, has been purified to homogeneity by precipitation with sodium salicylate and chromatography on heparin–agarose. Thrombocytin is a single-chain glycoprotein with a molecular weight of 36 000 which contains 5.6% carbohydrate. It causes platelet aggregation, release of platelet serotonin, and activation of factor XIII. The most sensitive substrate for the amidolytic activity of thrombocytin was Tos-Gly-Pro-Arg-*p*-nitroanilide hydrochloride. The activity of thrombocytin on this substrate and on platelets was inhibited by diisopropyl fluorophosphate (DFP), soybean trypsin inhibitor, and several arginine chloromethyl ketones. Active site titration with nitrophenyl guanidinobenzoate demonstrated that approximately 86% of the preparation was in the active form. These experiments demonstrate the presence of serine and histidine in the active site of thrombocytin and suggest that thrombocytin is a classical serine protease with a platelet-activating activity similar to thrombin.

The crude venom of *Bothrops atrox* causes the clotting of fibrinogen and also aggregates human platelets. One enzyme, called batroxobin or reptilase, has been purified from this venom (Stocker & Barlow, 1976; Holleman & Weiss, 1976). Batroxobin converts fibrinogen to fibrin but does not activate platelets (Niewiarowski et al., 1972). We have reported (Niewiarowski et al., 1977a) the preliminary characterization of the complementary enzyme, called thrombocytin, which activates platelets but has minimal fibrinogen-clotting activity.

The purpose of this communication is to report the further purification of thrombocytin to apparent homogeneity and to describe some of its properties. We have demonstrated that the platelet-activating and amidolytic activities of thrombocytin are both related to the serine active center of the molecule. Both activities are blocked by DFP[1] and by chloromethyl ketones.

Materials and Methods

Reagents. Phosphorylase A was from Worthington Biochemicals (Freehold, NJ), and *p*-nitrophenyl guanidinobenzoate (NPGB) was from Cyclo Chemicals (Los Angeles, CA). Bovine serum albumin, soybean trypsin inhibitor, ovalbumin, myoglobin, and trypsin (grade A) were from Sigma (St. Louis, MO). Lysine hydrochloride was obtained from Senn Chemicals (Dielsdorf, Switzerland). Piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (Pipes) was obtained from Calbiochem and Ellman's reagent [5,5′-dithiobis(2-nitrobenzoic acid)] was obtained from Aldrich Chemical Co. (Milwaukee, WI). Batroxobin was from Pentapharm Laboratories. Human thrombin, prepared by the method of Fenton et al. (1977), was kindly supplied by Dr. John Fenton (Albany, NY). Fibrinogen free of factor XIII was prepared from human fibrinogen (Kabi, Stockholm, Sweden) by ammonium sulfate precipitation and gel filtration on Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ) as described by Niewiarowski et al. (1977b). Factor XIII (fibrin-stabilizing factor), prepared by the method of Lorand & Gotoh (1970), was kindly supplied by Dr. L. Lorand (Evanston, IL). Heparin sodium (Pentapharm Laboratories) was obtained from beef lung and had an anticoagulant activity greater than 120 units/mg.

Chromozym TH (Tos-Gly-Pro-Arg-pNA·HCl), Chromozym UK (Bz-Val-Gly-Arg-pNA), and Chromozym PK (Bz-Pro-Phe-Arg-pNA) were from Pentapharm Laboratories. S-2222 (Bz-Ile-Glu-Gly-Arg-pNA) and S-2160 (Bz-Phe-Val-Arg-pNA) were from Ortho Diagnostics, Raritan, NJ. Bz-Arg-pNA was from Sigma. Thiobenzyl carbobenzoxylysinate (Z-Lys-SBzl) was prepared by the procedure of Green & Shaw (1979).

The chloromethyl ketone derivatives of Phe-Ala-Arg and Phe-Ala-Lys were prepared as described by Kettner et al. (1978) and Kettner & Shaw (1978), respectively. D-Phe-Pro-Arg-CH_2Cl was prepared by the general procedure described by Kettner & Shaw (1978).

Buffers. Tris–saline buffer contained 0.01 M Trizma base, 0.15 M NaCl, and 0.1% sodium azide and was adjusted to pH 7.4 with HCl. Tris–imidazole–saline buffer contained 0.1 M Trizma base, 0.1 M recrystallized imidazole, and 0.15 M NaCl and was adjusted to pH 8.4 with NaOH.

Preparation of Heparin–Agarose. CNBr–Sepharose (Pharmacia, Uppsala, Sweden) (5 g) was allowed to swell in 500 mL of 0.001 N HCl, collected on a sintered glass filter, and washed with an additional 500 mL of 0.001 N HCl. Heparin (1 g), dissolved in 200 mL of bicarbonate buffer (0.1 M $NaHCO_3$–0.15 M NaCl, pH 8.3), was added to the swollen beads and stirred slowly for 2 h at room temperature. The beads were collected on a sintered glass funnel and washed

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[1] Abbreviations used: NPGB, nitrophenyl guanidinobenzoate; DFP, diisopropyl fluorophosphate; SBTI, soybean trypsin inhibitor; pNA, *p*-nitroaniline; Z-Lys-SBzl, thiobenzyl carbobenzoxylysinate; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); Ellman's reagent, 5,5′-dithiobis(2-nitrobenzoic acid); LTU, light transmission units; PRP, platelet-rich plasma; $NaDodSO_4$, sodium dodecyl sulfate; 5-HT, 5-hydroxytryptamine (serotonin).

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with five 100-mL portions of bicarbonate buffer. The nonreacted CNBr groups were blocked by stirring the beads in 200 mL of 0.08 M ethanolamine for 2 h at room temperature. The heparin–agarose was then washed in a funnel with bicarbonate buffer, followed by 0.1 M sodium acetate buffer, pH 4.0, until the pH of the filtrate was 4.0. This material was poured into a chromatography column and equilibrated with 0.1 M lysine hydrochloride buffer, pH 7.4, before use.

Fractionation of B. atrox (marajoensis) Venom. *B. atrox* venom was obtained by Pentapharm Laboratories from snakes kept in captivity and bred from *B. atrox* specimens captured in the state of Maranhao, Brazil. The species and subspecies were identified from the classification described by Hoge (1965). Thrombocytin was prepared from this crude venom by sodium salicylate precipitation and chromatography on heparin–agarose. Crude venom (15 g) was dissolved in 300 mL of 0.15 M NaCl–acetic acid (pH 3.0), and 4.5 g of sodium salicylate dissolved in 30 mL of distilled water was added. The precipitate was separated by centrifugation and then extracted with 300 mL of ethanol, containing 1% acetic acid, for 2 h. The precipitate was centrifuged, washed with 20 mL of ethanol, and dried under vacuum. The dried material was dissolved in 300 mL of 1% ammonium formate, pH 3.0. The solution was stirred with 30 g of Amberlite IR-45 and filtered, and the pH of the filtrate was adjusted to 8.0. This solution was desalted and concentrated by ultrafiltration using a Diaflo UM-2 membrane (Amicon, Lexington, MA). This material was referred to as fraction S.

The concentrated fraction S was applied to a column (26 × 450 mm) of heparin–agarose which had been equilibrated with 0.1 M lysine hydrochloride (adjusted to pH 7.4 with NaOH). Fractions were eluted by a stepwise increase of the lysine concentration. The fibrinogen-clotting enzyme (batroxobin) was eluted with 0.1 M lysine hydrochloride while thrombocytin appeared as the very last peak with 1 M lysine. The thrombocytin-containing fractions (100 mL) were pooled, partially desalted by ultrafiltration using a Diaflo UM-2 membrane, dialyzed against 0.5% acetic acid, concentrated to a volume of 10 mL, and lyophilized.

Protein concentration in solutions of thrombocytin was determined from the absorption at 280 nm, using an $E_{280}^{1\%}$ of 6.35. The extinction coefficient of thrombocytin was determined after concentration by ultrafiltration and desalting by diafiltration against 0.5% acetic acid. The thrombocytin was freeze-dried and dried over P_2O_5. Weighed samples were dissolved in 0.15 M NaCl, and the absorbance was determined. These results were confirmed by dialyzing separate preparations of thrombocytin against 0.05 M ammonium bicarbonate, determining their absorption spectra on a Cary 15 recording spectrophotometer, and then lyophilizing the samples and determining the total protein content by amino acid analysis. Concentrations of other protein solutions were determined by the method of Lowry et al. (1951), using bovine serum albumin as a standard.

Amidolytic and Thioesterase Activity of Thrombocytin. The amidolytic activity of thrombocytin and other enzymes on peptide *p*-nitroanilides was measured by photometric determination of the released *p*-nitroaniline (Svendsen et al., 1972). Unless otherwise indicated, the measurements were performed at 37 °C in a system containing 1.7 mL of Tris–imidazole–saline buffer (pH 8.4), 0.1 mL of thrombocytin, and 0.2 mL of substrate (dissolved in deionized water). Final concentration of the substrate was 0.15 mM. Thrombocytin was dissolved in Tris–imidazole–saline buffer, pH 8.4, containing 1 mg/mL albumin. Released *p*-nitroaniline was measured at 405 nm by continuous recording in a Gilford spectrophotometer. An extinction coefficient of 10 600 was assumed for *p*-nitroaniline at this wavelength and pH. The thioesterase activity of thrombin was determined by the procedure of Green & Shaw (1979). The assay contained 1.75 mL of 0.2 M Tris–0.2 M NaCl buffer, pH 7.0, 50 μL of Z-Lys-SBzl (4×10^{-3} M), 5 μL of Ellman's reagent (52 mg/mL in dimethylformamide), and 0.2 mL of the test sample. Esterase activity was monitored at 412 nm on a Beckman 5230 spectrophotometer, and the change in absorbance was measured on a recorder scale of 0–0.01 and a chart speed of 1.0 in./min.

Factor XIII Activation. This was tested by a modified assay of clot solubility in 2% acetic acid according to Loewy et al. (1960). The incubation mixture contained 0.1 mL of sample, 0.1 mL of factor XIII (15 μg), 0.2 mL of 0.05 M Tris–0.15 M NaCl buffer (pH 7.4), 0.2 mL of 0.25 M $CaCl_2$, 0.1 mL of 0.5% fibrinogen, and 0.1 mL of batroxobin (40 units/mL). Clots formed in this system were incubated for 15 min at 37 °C. Then 2 mL of 2% acetic acid was added to each clot. All clots formed in the absence of thrombin or thrombocytin dissolved in 2–5 min.

Platelet Activation by Thrombocytin. Human platelet-rich plasma (PRP) was prepared by collecting blood in 0.1 volume of 0.1 M sodium citrate and centrifuging at 150*g* for 15 min. Washed platelet suspensions were prepared by the method of Mustard et al. (1972) from blood collected in acid citrate dextrose. The final platelet count in the suspension was (0.5–1) × 10^9. Platelet aggregation was studied in a Payton aggregometer (Payton Associates, Inc., Scarborough, Ontario), and results were expressed in arbitrary light transmission units (LTU) as described previously (Niewiarowski et al., 1972). Platelets were labeled by incubating for 10 min with [^{14}C]-5-hydroxytryptamine (0.3 μCi/10^9 cells). Release of [^{14}C]-5-HT was measured as described previously (Niewiarowski et al., 1972). [^{14}C]-5-HT had a specific activity of 30–50 mCi/mmol and was supplied by Amersham/Searle Corp. (Arlington Heights, IL).

NaDodSO$_4$–polyacrylamide gel electrophoresis was carried out by the method of Weber & Osborn (1975) on 7% polyacrylamide gels. The gels were stained with Coomassie Brilliant Blue by the method of Fairbanks et al. (1971). Phosphorylase A (M_r 100 000), bovine serum albumin (M_r 69 000), fibrinogen (M_r α chain 70 000; M_r β chain 58 000; M_r γ chain 48 000), ovalbumin (M_r 43 500), soybean trypsin inhibitor (M_r 21 500), and myoglobin (M_r 17 000) were used as standards of known molecular weight. Staining for glycoproteins was performed by using the periodate–Schiff technique (Zacharius et al., 1969).

Immunoelectrophoresis of fraction S and thrombocytin was performed on plates of 1% agarose in 0.1 M barbital buffer, pH 8.6, according to the method of Scheidegger (1955). Samples were applied and run at 5.3 V/cm (5 mA/plate) for 40 min at room temperature. Polyvalent antiserum (raised by multiple injections of crude *Bothrops atrox* venom into a horse) was obtained from Behringwerke (Mahrburg, Germany). This antiserum was placed in the center trough, and diffusion was allowed to take place for 15 h. The plates were soaked in saline for 4 h, then washed in water, dried, and stained with Amidoschwarz (1 mg/mL) in 10% acetic acid in ethanol.

Analysis of Thrombocytin on Acid Gels. Samples of thrombocytin (50 μg) were run on polyacrylamide gels at pH 4.3 according to the method of Reisfeld et al. (1962). One gel was stained with Coomassie Brilliant Blue, and three

identical gels were sliced into 3-mm segments. The corresponding segments were combined, crushed, and extracted with 0.3 mL of 0.01 M phosphate–0.15 M NaCl buffer overnight at 4 °C. The gel particles were sedimented, and 50-μL aliquots of the supernatants were added to 1 mL of PRP in the aggregometer for estimation of platelet-aggregating activity. Aliquots (0.1 mL) were also assayed with Chromozym TH (0.18 mM) in Tris–imidazole–saline buffer (pH 8.4) for amidolytic activity. Factor XIII activating activity was estimated on aliquots of the supernatants as described above.

Amino Acid and Carbohydrate Analysis. Protein samples for amino acid analysis were hydrolyzed with 6 N HCl in sealed, evacuated vials at 110 °C for 24, 52, and 95 h. Cysteine was determined by hydrolysis of a separate sample after prior oxidation with performic acid (Hirs, 1967). The amino acids were analyzed on a single column with a Beckman Model 119 automatic amino acid analyzer (Beckman Instruments, Inc., Spinco Division, Palo Alto, CA) equipped with an Infotronics integrator (Columbia Scientific Industries Corp., Austin, TX). The tryptophan content was estimated spectrophotometrically (Bencze & Schmid, 1957). The sialic acid content was determined by the method of Warren (1959) after hydrolysis in 0.1 N H_2SO_4 for 1 h at 80 °C. *N*-Acetylneuraminic acid (Sigma, St. Louis, MO) was used as a standard. Neutral hexoses were determined by the phenol–sulfuric acid method (Dubois et al., 1956) using mannose as a standard. Amino sugars were determined by a modification (Boas, 1953) of the Elson–Morgan reaction after hydrolysis in 6 N HCl for 8 h at 100 °C. Glucosamine was used as a standard.

Labeling of Thrombocytin with [^{14}C]*DFP.* Thrombocytin (1.7 mg/mL) and trypsin (0.83 mg/mL) were each incubated with [^{14}C]diisopropyl fluorophosphate (sp act. 103 mCi/mmol; New England Nuclear, Boston, MA) at a concentration of 0.3 mM for 30 min at 37 °C. Bovine serum albumin (0.1 mg/mL) was then added, and protein was precipitated with cold 10% trichloroacetic acid for 30 min at 0 °C. The precipitates were centrifuged and the pellets washed with cold 10% trichloroacetic acid. The pellets were solubilized with NCS (Amersham, Arlington Heights, IL), and radioactivity was counted in a Triton–toluene scintillant. Background incorporation into albumin represented only 0.5% of the incorporation into thrombocytin.

Active site titration with NPGB was performed by the method of Chase & Shaw (1970).

Inactivation Studies with Chloromethyl Ketones. Kinetics of the inactivation of thrombocytin were studied by the procedure described previously for thrombin (Kettner & Shaw, 1977). Thrombocytin (1.6 μg) was incubated at 25 °C with the affinity labels in 1.00 mL of 0.05 M Pipes–0.2 M NaCl buffer, pH 7.0. Four 0.2-mL aliquots were removed at 5-min intervals and were assayed for remaining thioesterase activity. The pseudo-first-order rate constants for inactivation were determined from the slopes of semilogarithmic plots of remaining esterase activity plotted against time.

Results

Purification of Thrombocytin. Thrombocytin was purified from the venom of *B. atrox* by precipitation with sodium salicylate and chromatography on heparin–agarose. In a typical experiment, precipitation of crude venom with sodium salicylate yielded 74 mg of partially purified fraction S per gram of crude venom. This fraction contained both fibrinogen-clotting and platelet-aggregating activity.

Figure 1 shows the elution pattern after application of 3 g of fraction S onto heparin–agarose. The first peak which was eluted with 0.1 M lysine contained approximately 85% of the fibrinogen-clotting activity, but it had no effect on platelets. This was mostly batroxobin. Thrombocytin was eluted as the last peak after application of 1 M lysine. It had potent platelet-aggregating activity and it promoted clot retraction, but it only contained traces (less than 0.3%) of the total fibrinogen-clotting activity present in fraction S.

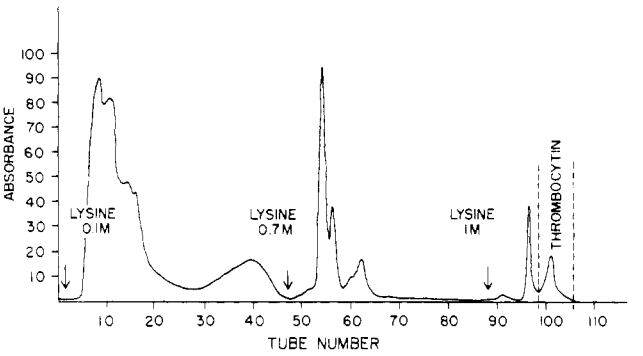

FIGURE 1: Purification of thrombocytin by chromatography on heparin–agarose. Fraction S (3 g) was applied to a 26 × 450 mm column. Each tube corresponds to 20 mL of eluate. Absorbance was measured at 280 nm and expressed in arbitrary units corresponding to the chart divisions.

Table I: Recovery and Increase of Specific Activities during Purification of Thrombocytin from *B. atrox* (*marajoensis*) Venom

		amidolytic act.[a]		platelet-activating act.[b]	
fraction	mg of protein	units/mg of protein	recovery of act. (%)	units/mg of protein	recovery of act. (%)
crude venom	11 610	0.3	100	0.2	100
thrombocytin	7.2	37.7	8	667.7	207

[a] One unit of thrombocytin acting on Chromozym TH releases 1 μmol of *p*-nitroaniline per min at 37 °C. [b] One unit of thrombocytin is that amount which releases 25% of the total [^{14}C]-5-HT from a suspension of washed human platelets under our assay conditions. The incubation mixture was composed of 1.0 mL of platelet suspension (6 × 10^8 platelets) and 0.1 mL of the tested sample. After stirring for 3 min in an aggregometer, we centrifuged the samples (2 min; 7500*g*), and [^{14}C]-5-HT radioactivity was counted in the supernatant.

It was difficult to evaluate the recovery and increase of specific activity during purification of thrombocytin. The specific activity measured by an amidolytic assay increased 125 times, and the recovery of this activity was 8.0% (Table I). On the other hand, specific platelet-activating activity (evaluated on the basis of the release of [^{14}C]serotonin) increased 3300 times, and the recovery of this activity was 207%. However, measurement of neither of these activities adequately reflected the purification of thrombocytin. Recovery of amidolytic activity was underestimated since the batroxobin which eluted with the first peak from the heparin–agarose column also possessed amidolytic activity. It was difficult to estimate the specific platelet-aggregating activity during purification of thrombocytin since crude venom and fraction S also contained inhibitors of platelet aggregation. Incubation of platelets with crude venom abolished their sensitivity to thrombin. For this reason, the recovery of specific platelet-activating activity during purification of thrombocytin was probably overestimated in this assay system.

Electrophoretic Studies. Purified thrombocytin showed only a single protein band on $NaDodSO_4$–polyacrylamide gel electrophoresis (Figure 2). The mobility was not significantly affected by prior reduction with dithiothreitol. These data

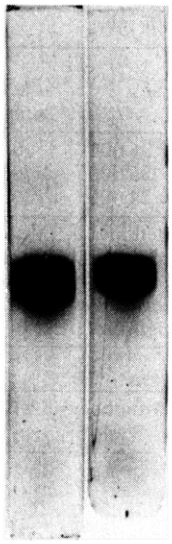

FIGURE 2: $NaDodSO_4$–polyacrylamide gel electrophoresis of thrombocytin. Samples (30 μg) of thrombocytin were incubated in $NaDodSO_4$–urea buffer either without (A) or with (B) dithiothreitol (5 mg/mL) for 3 min at 100 °C. Samples were applied to 7% polyacrylamide gels and run for 18 h at 3 mA/gel.

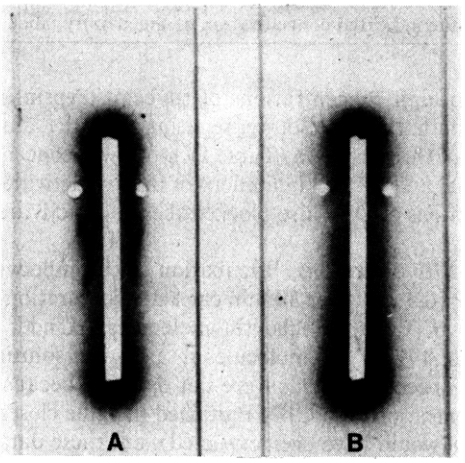

FIGURE 3: Immunoelectrophoresis of (A) fraction S (2 μg) and (B) purified thrombocytin (0.4 μg) using a polyvalent antiserum against crude *B. atrox* venom.

indicated that the enzyme consists of a single polypeptide chain. Its mobility relative to a series of proteins of known molecular weight suggested that it had an apparent molecular weight (reduced) of 36 000. Staining by the periodate–Schiff method indicated the presence of carbohydrate in thrombocytin. No other carbohydrate-staining bands were seen on the gels.

Immunoelectrophoresis of thrombocytin against polyvalent antiserum to *B. atrox* venom gave only one line, which migrated toward the cathode (Figure 3). In contrast, fraction S showed at least three other precipitation lines, in addition to the one attributable to thrombocytin.

Control experiments showed that thrombocytin can be kept at pH 4.0–4.3 for 3 h at 22 °C and for 2 days at 4 °C without any significant loss of its platelet-aggregating or amidolytic activities. Therefore, thrombocytin was electrophoresed on polyacrylamide gels at acid pH, and the activity was then eluted from the gels. Stained gels showed that the thrombocytin was somewhat heterogeneous under these conditions

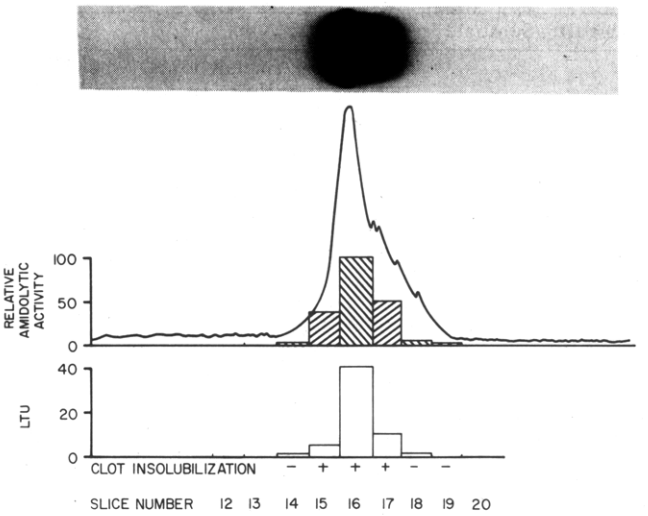

FIGURE 4: Elution of thrombocytin from pH 4.3 polyacrylamide gels. One gel was stained with Coomassie Brilliant Blue and scanned at 595 nm. Three identical gels were sliced into 3-mm sections and extracted with phosphate–saline buffer. The eluates were assayed for amidolytic activity (hatched bars), platelet-aggregating activity (open bars), and factor XIII activating activity, as measured by clot insolubilization.

Table II: Amino Acid and Carbohydrate Composition of Thrombocytin

amino acid	residues/100 amino acids	residues/molecule[a]
Asp	10.2	31.4
Thr	5.1	15.7
Ser	7.1	21.9
Glu	5.2	15.9
Pro	7.6	23.4
Gly	8.7	26.7
Ala	5.9	18.2
$^1/_2$-Cys	5.1	15.8
Val	6.3	19.5
Met	1.4	4.2
Ile	6.6	20.4
Leu	8.8	27.1
Tyr	1.7	5.1
Phe	1.5	4.7
His	2.4	7.3
Lys	8.0	24.5
Arg	5.2	16.0
Trp	3.3	10.3
total residues	100.1	308.3

carbohydrate	percent	residues/molecule[a]
neutral hexoses	1.8	3.6
hexosamine (as *N*-acetylhexosamine)	3.0	5.0
sialic acid (as *N*-acetylneuraminic acid)	0.8	1.0
total	5.6	9.6

[a] Calculated for a molecular weight of 36 000.

(Figure 4), but the amidolytic, platelet-aggregating, and factor XIII activating activities of the preparation all corresponded with the protein-staining components. This suggests that these activities are all functions of the major protein component and are not due to some trace enzyme in the preparation.

Amino Acid and Carbohydrate Composition. The amino acid and carbohydrate composition of thrombocytin is shown in Table II. The number of aromatic acid residues was rather low, which is in agreement with the low value of its absorbancy at 280 nm ($E_{1cm}^{1\%}$ at 280 nm = 6.35 ± 0.15) in 0.9% sodium chloride. Carbohydrate accounted for 5.6% of the total weight of the protein.

Amidolytic Activity of Thrombocytin. Thrombocytin can

Table III: Substrate Specificities[a]

substrate	substrate concn ($\times 10^{-4}$ M)	amidolytic act. (μmol of pNA released per min per mg of enzyme) thrombocytin	thrombin	trypsin
Bz-Arg-pNA	1.3	0.0007	0.012	0.089
Bz-Pro-Phe-Arg-pNA (Chromozym PK)	3.0	0.029	0.013	1.7
Bz-Ile-Glu-Gly-Arg-pNA (S-2222)	1.1	1.4	0.39	99
Bz-Phe-Val-Arg-pNA (S-2160)	1.6	3.2	20.7	15.9
Tos-Gly-Pro-Arg-pNA (Chromozym TH)	0.8	6.1	158	102

[a] Enzymes were diluted in Tris–imidazole–saline buffer, and 100-μL aliquots were added to 400 μL of substrate which had also been diluted in Tris–imidazole–saline. Assays were conducted at 25 °C and absorbance was followed at 405 nm.

Table IV: Inhibition of Thrombocytin by SBTI and DFP[a]

inhibitor	platelet aggregation (LTU)	amidolytic act. (μmol of pNA released per min per mg of protein)
buffer, control	5.4	18.5
SBTI, 1000 μg/mL	0.0	1.9
SBTI, 50 μg/mL	2.9	9.0
SBTI, 12 μg/mL	4.1	14.0
DFP-treated thrombocytin	0.2	1.9

[a] Thrombocytin (20 μg/mL) was preincubated with SBTI at the indicated concentration for 10 min at 25 °C. Samples (0.2 mL) of these mixtures were added to 1 mL of PRP for estimation of platelet-aggregating activity. Other 0.2-mL samples were added to 0.2 mL of Tris–imidazole buffer (pH 7.8), and amidolytic activity was assayed by adding 0.1 mL of Chromozym TH to a final concentration of 0.2 mM. DFP-treated thrombocytin was prepared by incubating thrombocytin (92.7 μg/mL) with 10 mM DFP for 30 min and then dialyzing for 48 h against Tris–saline buffer. Aliquots (0.2 mL) of this were assayed for platelet-aggregating and amidolytic activity as described above.

cleave *p*-nitroaniline from several synthetic peptide substrates (Table III). It has a specificity similar to that of human α-thrombin but is not as active. It also had very high activity against some preparations of Chromozym UK (lot no. 125982) which were only about 60% pure, but highly purified preparations of this substrate were not readily cleaved by thrombocytin. This enzyme also had weak thioesterase activity against Z-Lys-SBzl. Chromozym TH was the best substrate for thrombocytin of those tested, so kinetic parameters for this substrate were examined more carefully, and it was the one used for most routine assays.

Thrombocytin activity on Chromozym TH was maximal at pH 8.0–8.4. At 27 °C, the K_m values for thrombocytin and human thrombin acting on chromozym TH were 91 and 25 μM, respectively. V_{max} for thrombin was 169 (μmol/min)/mg of enzyme and for thrombocytin was 12.3 (μmol/min)/mg of enzyme. These parameters were determined in the presence of albumin to prevent the denaturation and surface adsorption of the enzyme sometimes seen at very low concentrations. Albumin is not a substrate for thrombocytin [incubation of albumin (100 μg/mL) with thrombocytin (50 μg/mL) for 1 h at 37 °C caused no cleavage which was apparent on $NaDodSO_4$–polyacrylamide gels].

Inactivation of Thrombocytin by Soybean Trypsin Inhibitor and Diisopropyl Fluorophosphate. Incubation of thrombocytin with high concentrations of soybean trypsin inhibitor blocked both the platelet-aggregating and the amidolytic activities of thrombocytin (Table IV). Lower concentrations gave partial and parallel inhibitions of the two activities. High concentrations of DFP also blocked these two activities of the enzyme.

Table V: Titration with NPGB[a]

enzyme	M_r	active sites/mol	turnover [(μmol/min)/mg]
thrombocytin	36 000	0.86	0.0145
thrombin	36 500	0.97	0.0024
trypsin	24 000	0.59	0.0004

[a] The enzymes were diluted into 0.1 M sodium barbital buffer, pH 8.3, at 23 °C. NPGB in acetonitrile was added to give a final concentration of 1.1×10^{-4} M. Nitrophenol release was measured at 410 nm. An extinction coefficient of 16 595 was used for nitrophenol at this wavelength and pH.

Table VI: Reactivity of Thrombocytin with Peptides of Arginine Chloromethyl Ketone[a]

affinity label	concn (M)	$t_{1/2}$ (min)[b]	k_{app}/concn (M^{-1} $min^{-1} \times 10^{-4}$)[c]
D-Phe-Pro-Arg-CH_2Cl	5×10^{-8}	2	700
Phe-Ala-Arg-CH_2Cl	5×10^{-7}	29	4.8
Phe-Ala-Lys-CH_2Cl	1×10^{-3}	70	0.001

[a] Inactivations were conducted at 25 °C and pH 7.0. [b] $t_{1/2}$ is the half-time for the first-order inactivation of thrombocytin at the indicated concentration of the affinity label. [c] k_{app}/concn is the ratio of the apparent first-order rate constant for inactivation to the concentration of the affinity label. These values were calculated from the values of $t_{1/2}$ using the relationship $k_{app} = \ln 2/t_{1/2}$ and the indicated concentration of the affinity label.

Active Site Titrations. Incubation of thrombocytin with [^{14}C]DFP (0.3 mM) for 30 min caused incorporation of DFP into 79% of the thrombocytin molecules. Under similar conditions, 49% of the molecules in a trypsin solution were labeled. These conditions were not optimal, because of the low concentration of [^{14}C]DFP utilized (a value closer to 60% for trypsin would have been expected), but these data clearly indicate that the majority of the molecules in the thrombocytin preparation were DFP sensitive.

Active site titration of thrombocytin with NPGB indicated that approximately 86% of the thrombocytin molecules in the preparation were active (Table V). The turnover of NPGB by thrombocytin was much higher than that seen with thrombin or trypsin.

Inactivation of Thrombocytin by Chloromethyl Ketones. Thrombocytin was readily inactivated by peptides of arginine chloromethyl ketones (Table VI). Phe-Ala-Arg-CH_2Cl was a much more effective inhibitor then was Phe-Ala-Lys-CH_2Cl, suggesting that thrombocytin is highly specific for arginine residues. The tripeptide D-Phe-Pro-Arg-CH_2Cl was the most active of all the peptides tested. Actually, the values of $t_{1/2}$ and k_{app}/concentration which are given for this inhibitor are only approximations, since the reaction rate was too rapid to be determined accurately at the inhibitor concentrations tested. At lower concentrations of the affinity label, the sensitivity of the thioesterase assay became limiting.

At a higher concentration of thrombocytin and 1.2×10^{-6} M D-Phe-Pro-Arg-CH_2Cl, the activity of the enzyme was completely inactivated within a few minutes. The reaction of thrombocytin with less than stoichiometric amounts of affinity

Table VII: Comparison of the Inactivation of Esterolytic, Amidolytic, and Platelet-Aggregating Activities of Thrombocytin by D-Phe-Pro-Arg-CH_2Cl[a]

D-Phe-Pro-Arg-CH_2Cl concn ($\times 10^{-6}$ M)	% inhibition		
	thioesterase act.	amidolytic act.	platelet-aggregating act.
0	0	0	0
0.122	36	20	30
0.256	55	41	61
0.375	73	74	79
1.2	100	100	100

[a] Samples of thrombocytin (approximately 15 μg/mL) were incubated in 0.5 mL of 0.05 M Pipes buffer, pH 7.0, containing 0.2 M NaCl, bovine serum albumin (100 μg/mL), and the indicated concentration of inhibitor. Aliquots (20 μL) were removed at four intervals and assayed for thioesterase activity on Z-Lys-SBzl. After 20 min, enzyme activities had reached stable levels, and the remainder of each sample was dialyzed for 5 h at 4 °C against 0.05 M Tris buffer, pH 7.8. Approximately 0.5 mg of albumin was added to each sample, and they were lyophilized. Subsequently, the samples (containing approximately 7 μg of thrombocytin) were redissolved in 0.3 mL of 0.5 M Tris buffer, pH 7.8, and 0.1-mL aliquots were assayed at 37 °C for amidolytic activity on Chromozym TH and for platelet-aggregating activity in citrated PRP.

label went to completion, yielding samples of thrombocytin in which the thioesterase, amidolytic, and platelet-aggregating activities were each inactivated in proportion to the concentration of the affinity label (Table VII).

Discussion

Several lines of evidence suggest that the purified thrombocytin is homogeneous. (a) It gives a single protein band on $NaDodSO_4$–polyacrylamide gels, both in the presence and in the absence of reducing agents (Figure 2). (b) It gives a single precipitin line upon immunoelectrophoresis at pH 8.6 against a polyvalent antiserum (Figure 3). (c) It shows a single component, with only slight heterogeneity, on polyacrylamide gels at pH 4.3 (Figure 4). The amidolytic, platelet-aggregating, and factor XIII activating activities of the preparation are coincident with this protein band.

Experiments with inhibitors (Tables IV and VII) and with the elution of enzyme from the sliced gels (Figure 4) strongly suggest that the platelet-aggregating and amidolytic activities of thrombocytin depend on the same active site in the molecule. The experiment with extraction of thrombocytin from the sliced gels (Figure 4) also indicates the identity of thrombocytin with the enzyme which activated factor XIII.

Thrombocytin appears to be a typical serine protease, analogous to trypsin and thrombin, but with a different substrate specificity. It is much less active than trypsin and thrombin on a range of synthetic substrates (Table III). It is inhibited by DFP and can be titrated with NPGB. Its inhibition by soybean trypsin inhibitor and its activity on different arginine substrates also suggest its similarity to the classical serine proteases. Irreversible inhibition by arginine chloromethyl ketones further suggests that the active site of thrombocytin contains a histidine analogous to His-46 of trypsin.

The most effective inhibitor of thrombocytin tested was D-Phe-Pro-Arg-CH_2Cl. This reagent corresponds in sequence to an arginyl aldehyde which is a highly effective reversible inhibitor of thrombin (Bajusz et al., 1975). This chloromethyl ketone derivative was a very good inhibitor of thrombin (Kettner & Shaw, 1979) and reacted stoichiometrically with thrombocytin.

Depending on the assay system, the platelet-activating activity of thrombocytin is 10–50 times lower than that of thrombin. Thrombocytin causes a very slow clotting of fibrinogen, and the fibrinogen-clotting activity of thrombin is at least 1600 times higher than that of thrombocytin (Niewiarowski et al., 1979).

It is interesting that *B. atrox* venom contains two thrombinlike enzymes with complementary functions: (a) batroxobin, which clots fibrinogen but has no effect on platelets, and (b) thrombocytin, which stimulates platelets but only has a minimal effect on the fibrinogen–fibrin conversion. The biological significance of the distribution of two essential functions of thrombin between two different protein molecules in the snake venom is not understood.

In summary, we have purified to homogeneity a novel thrombinlike enzyme, thrombocytin, and demonstrated that it is a classical serine protease. Since thrombocytin activates platelets and has a more limited specificity than thrombin, it may be helpful in establishing the mechanism of platelet activation induced by thrombin.

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Thrombocytin, a Serine Protease from *Bothrops atrox* Venom. 2. Interaction with Platelets and Plasma-Clotting Factors†

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ABSTRACT: Thrombocytin, a serine protease from *Bothrops atrox* venom, caused platelet aggregation and release of platelet constituents at a concentration of 10^{-7} M and clot retraction at a concentration of 2×10^{-9} M. Thrombocytin was slightly more active when tested on platelets in plasma than on washed platelets suspended in Tyrode–albumin solution. Thrombin was 5 times more active than thrombocytin when tested on platelets in plasma and 50 times more active when tested on washed platelets. The patterns of release induced by thrombocytin and thrombin were similar. Prostaglandin E_1 (10^{-5} M) produced complete inhibition of platelet release induced by thrombocytin and thrombin. Indomethacin (10^{-4} M) was without any effect. Antithrombin III, in the presence of heparin, inhibited the action of thrombocytin on platelets and on a synthetic peptide substrate (Tos-Gly-Pro-Arg-pNA·HCl). Formation of an antithrombin III–thrombocytin complex was demonstrated on $NaDodSO_4$–polyacrylamide gel electrophoresis. Hirudin and α_1-antitrypsin did not inactivate thrombocytin. Thrombocytin had a low fibrinogen-clotting activity (less than 0.06% that of thrombin). Thrombocytin also caused progressive degradation of the α chain of human fibrinogen, and it cleaved prothrombin, releasing products similar to intermediate 1 and fragment 1 produced by thrombin. Thrombocytin activated factor XIII by limited proteolysis and increased the procoagulant activity of factor VIII in a manner analogous to that of thrombin.

Thrombin is a serine protease which has several important physiological functions. It cleaves fibrinopeptides A and B from fibrinogen, leading to the formation of a fibrin clot (Blombäck et al., 1967), and it also interacts with platelets, causing platelet aggregation, clot retraction, and release of some platelet constituents (Niewiarowski & Thomas, 1966; Holmsen et al., 1969). In addition, thrombin activates factor XIII (Takagi & Doolittle, 1974), it modifies factors V (Colman, 1969) and VIII (Legaz et al., 1973) to increase their coagulant activities, it cleaves prothrombin to form fragment 1 and intermediate 1 (Magnusson et al., 1975; Walz et al., 1977), and it interacts with antithrombin III to form a stable complex (Rosenberg & Damus, 1973).

Bothrops atrox venom contains two distinct serine proteases which together mimic the activity of thrombin but individually have only a few of its activities. Batroxobin readily clots fibrinogen, but it does not affect platelets, does not activate factor XIII, and is not inhibited by heparin–antithrombin III (Stocker & Barlow, 1976). Thrombocytin, on the other hand, activates platelets and factor XIII but has very little clotting activity toward fibrinogen (Niewiarowski, et al., 1977a; Kirby et al., 1979).

The purpose of the present study was to examine the function and specificity of thrombocytin relative to thrombin. We have compared the effects of thrombocytin and thrombin on platelet aggregation, the platelet release reaction, and clot retraction. In addition we have studied the effects of thrombocytin on fibrinogen, on prothrombin, and on factors VIII, X, and XIII. Thrombocytin was readily inhibited by plasma antithrombin III in the presence of heparin, with the formation of a stable inactive complex.

Materials and Methods

Reagents. *Echis carinatus* venom, Russell's viper venom (RVV),[1] indomethacin, antimycin A, and deoxyglucose were from Sigma Chemical Co. (St. Louis, MO). Batroxobin was from Pentapharm (Basle, Switzerland). Prostaglandin E_1 was from Upjohn Co. (Kalamazoo, MI). Hirudin was obtained from Pentapharm and from Arzneimittel Werke, VEB, Dresden, East Germany. Fibrinogen used for electrophoretic

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[1] Abbreviations used: RVV, Russell's viper venom; $NaDodSO_4$, sodium dodecyl sulfate; DTT, dithiothreitol; Tcn, thrombocytin; AT III, antithrombin III; PRP, platelet-rich plasma; 5-HT, 5-hydroxytryptamine; LTU, light transmission units; pNA, *p*-nitroaniline; LA-PF_4, low-affinity platelet factor 4; PGE_1, prostaglandin E_1.

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