

# Interaction of viper venom serine peptidases with thrombin receptors on human platelets

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**Abstract** The serine peptidases, thrombocytin and PA-BJ, isolated from the venom of *Bothrops atrox* and *Bothrops jararaca*, respectively, induce platelet aggregation and granule secretion without clotting fibrinogen. The specific platelet aggregation activity of each enzyme was about 15 times lower than that of thrombin. This activity was blocked by monoclonal antibodies recognizing protease activated receptor 1 (PAR1) and by heparin, but not by hirudin nor thrombomodulin. Both enzymes induced calcium mobilization in platelets and desensitized platelets to the action of thrombin and the SFLLRN peptide. We compared the effect of thrombin, PA-BJ, and thrombocytin on the degradation of the soluble N-terminal domain of the PAR1 receptor. The major cleavage site by thrombin and both viper enzymes was Arg41–Ser42. In addition, a rapid cleavage of the peptide bond at Arg46–Asn47 by the viper enzymes was observed, resulting in the inactivation of the tethered ligand. PA-BJ and thrombocytin both cleaved at 41–42 and 46–47 peptide bonds, and fragment 42–103 disappeared rapidly. Both viper enzymes caused calcium mobilization in fibroblasts transfected with PAR4 and desensitized these cells to the thrombin action. In conclusion, both PAR1 and PAR4 mediate the effect of viper venom serine peptidases on platelets. © 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

**Key words:** Thrombin receptor; Protease activated receptor; Platelet activation; Viper venom protease; Calcium mobilization; Heparin; Hirudin; Thrombomodulin

## 1. Introduction

Thrombin action on human platelets is mediated by two protease activated receptors: PAR1 [1] and PAR4 [2,3]. Cleavage of PAR1 occurs at Arg41–Ser42 located within the long exodomain of the receptor [4]. SFLLRN is a new amino-terminus and functions as a tethered peptide ligand binding to another site on the thrombin receptor and induces an intracellular signaling [4]. SFLLRN mimics the new N-terminus and acts as an agonist of platelet aggregation and secretion [4]. Other enzymes, such as cathepsin G [5,6] and plasmin [7,8], also cleave PAR1 at 41–42, causing its activation. In addition, these enzymes cleave other peptide bonds in the receptor, leading to its progressive inactivation.

Two viper venom enzymes, thrombocytin, isolated from the venom of *Bothrops atrox* [9,10], and PA-BJ, isolated from

*Bothrops jararaca* [11], cause platelet aggregation and a release reaction without clotting fibrinogen. Both enzymes were purified into homogeneity and have a molecular weight of about 30 kDa. They are typical serine peptidases as established by studying the hydrolysis of synthetic peptides and the effect of the inhibitors of serine and histidine active binding sites. The full amino acid sequence of PA-BJ has been reported [11]; this enzyme is 25 residues shorter than thrombin and shows a 31% amino acid identity to thrombin. The comparison of the amino acid sequences of PA-BJ with the sequences of other serine peptidases allowed the putative identification of the catalytic triad of His41, Asp86, and Ser180 in this enzyme.

The purpose of our study was to investigate the effect of these enzymes on PAR1 expressed on platelets, on the recombinant N-terminal domain of PAR1, and on cells transfected with PAR4.

## 2. Materials and methods

### 2.1. Enzymes

Highly purified human thrombin was generously provided by Dr. J. Fenton, Albany, NY, USA. PA-BJ was purified at the Butantan Institute, Sao Paulo, Brazil, according to Serrano et al. [11]. Thrombocytin was purified according to the modified method of Kirby et al. [9] and provided by Pentapharm, Basel, Switzerland. All three preparations appeared to be homogeneous as determined by SDS-PAGE.

### 2.2. Monoclonal antibodies (mAb) against the thrombin receptor

mAb IIaR was provided by Biodesign, Kennebunk, ME, USA and mAb SPAN-12 and WEDE-15 were purchased from Immunotech, Westbrook, ME, USA. The IIaR and SPAN-12 bound to peptide <sup>35</sup>NATLDPRSFLLR<sup>46</sup> which contains thrombin cleavage sites between R41 and S42. The WEDE-15 antibody binds to the <sup>51</sup>KYEPFWEDEEKNES<sup>64</sup> motif, a hirudin binding domain in PAR1, and is retained by PAR1 after limited proteolysis with thrombin [12].

### 2.3. Other reagents

SFLLRN peptide (TRAP) was purchased from Bachem (Torrance, CA, USA) and thrombomodulin from American Diagnostica, Inc. (Greenwich, CT, USA). All other reagents, including heparin, hirudin and Fura-2-AM, were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

### 2.4. Human platelet suspension

Human platelet suspension was prepared by the differential centrifugation method according to Mustard et al. [13] or by gel filtration on a Sepharose 2B column. Platelet aggregation was measured in a Payton aggregometer. In order to study intracellular calcium mobilization, platelet-rich plasma was incubated with Fura-2-acetoxymethyl ester and separated from the non-incorporated label by differential centrifugation or gel filtration. Platelet suspensions (10<sup>8</sup>/ml) treated with serine peptidases or with SFLLRN were maintained in a Perkin Elmer LS-5 spectrofluorometer in a water-jacketed cuvette with stir-

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ring at 37°C. Fura-2-fluorescence was monitored continuously, using a setting of 340 nm (excitation) and 510 nm (emission). Total calcium was estimated after cell lysis with digitonin and compared with the calcium standard. The fluorescence was quenched by EGTA.

### 2.5. Recombinant soluble PAR1 domain

The human PAR1 exodomain, TR78 (residues 26–103,  $M_r$  8925 Da) was produced in *Escherichia coli* at the C-terminal portion of a fusion protein, ketosteroid isomerase, as described previously [8]. It was purified by reverse-phase HPLC using a C-18 column and was eluted at 35% acetonitrile. The circular dichroism and NMR spectroscopy of TR78 suggested appropriate folding of this molecule. TR78 was solubilized at pH 7.2 in the phosphate-buffered saline before proteolytic digestion.

### 2.6. Digestion of PAR1 by proteolytic enzymes and separation of its degradation products

Aliquots of TR78 at 100 µg were incubated at room temperature with 80 ng of thrombin or 168 ng of PA-BJ or 168 ng thrombocytin for 2 min or 2 h. The cleavage products were then applied into a C-18 column and eluted with the acetonitrile gradient. Molecular weight of the eluted fractions was determined by mass spectrometry (Maldi) at the Wistar Institute, Philadelphia, PA, USA.

### 2.7. Fibroblasts transfected with PAR4

KF:hP4-S12 cells originated from the University of California, San Francisco, CA, USA. The cell line was created by stably transfecting KOLF cells with the human PAR4 gene containing a FLAG epitope tag at its amino-terminus. KOLF cells are lung fibroblasts from a PAR1 knockout mouse. KF:hP4-S12 cells were grown in DME with 10% bovine calf serum (containing 250 g hygromycin per ml) at 37°C in a CO<sub>2</sub> incubator. They were labeled with Fura-2-acetoxymethyl ester on the cell culture plates before they reached confluency. After detachment of cells with a Gibco dissociation buffer in the absence of proteolytic enzymes, non-bound dye Fura-2-AM eliminated by gentle centrifugation and the cells were suspended in DMEM medium [14].

## 3. Results and discussion

We confirmed that viper venom enzymes aggregated human platelets in plasma and plasma-free suspension, and, in contrast to thrombin, the viper venom proteases did not clot fibrinogen. Thrombin was the most potent platelet aggregating agent. The EC<sub>50</sub> for thrombin amounted to 0.76 nM; for PA-BJ, it amounted to 12.5 nM, and for thrombocytin, to 4.3 nM. SFLLRN aggregated platelets with EC<sub>50</sub> of 120 M (data not shown). Heparin inhibited platelet aggregation induced by all three enzymes, whereas only thrombomodulin and hirudin inhibited thrombin's effect (Table 1). The monoclonal antibody IIaR at 0.2 µg/ml and 2.5 µg/ml completely inhibited the effect of thrombin and venom proteases, respectively (Table 1). Monoclonal antibodies SPAN-12 and WEDE-15 caused a weaker inhibitory effect (not shown). Table 2 shows the effect of the three enzymes on calcium mobilization in platelets and their inhibition by IIaR, SPAN-12, and WEDE-15 antibodies. Fura labeled platelets incubated with

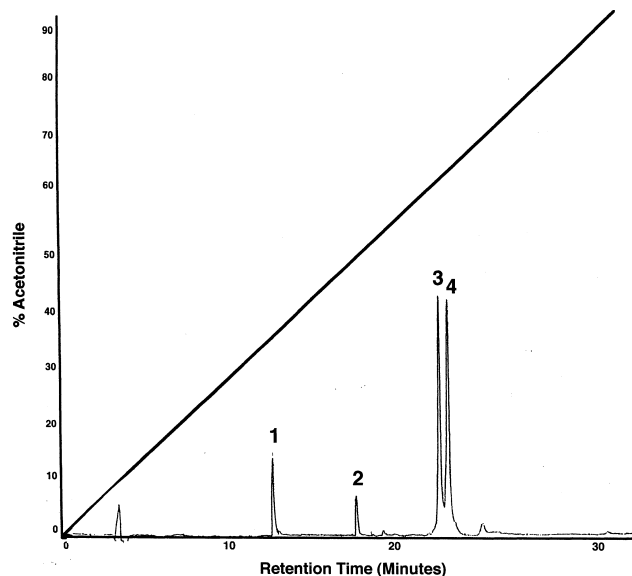


Fig. 1. HPLC elution pattern of TR78 (100 µg) digested with PA-BJ (1.7 µg) for 2 h at 37°C. Fraction 1 corresponds to fragment Ala26–Arg41, fragment 3 to Asn47–Leu103, and fragment 4 to Ser42–Leu103.

thrombin (0.76 nM), PA-BJ (12.5 nM), thrombocytin (4.3 nM), and SFLLRN (120 µM) rapidly responded with calcium mobilization and, following further incubation for 5 min, became desensitized to all agonists (not shown).

In the next series of experiments the PAR1 extracellular domain, TR78, was digested by thrombin, PA-BJ, and thrombocytin for different time periods, and the incubation mixtures were applied on HPLC column. Fig. 1 shows the elution pattern of TR78 degraded by PA-BJ in an acetonitrile gradient. Molecular weights of the eluted fragments were determined by mass spectrometry. This permitted us to deduce the amino acid sequences of the isolated fragments and to identify peptide bonds cleaved during the digestion of the recombinant receptor. In agreement with the previous study [8], thrombin exclusively cleaved at the peptide bond R41–S42 up to 2 h of incubation time, releasing two degradation products having the molecular weights of 1783 and 7170 Da (not shown). On the other hand, the cleavage of the R41–S42 and R46–N47 bonds took place simultaneously after 2 min of incubation of TR78 with PA-BJ (Fig. 1). Three degradation products separated on HPLC had molecular weights of 1783, 7170, and 6553 Da. The last fragment indicates cleavage at the site R46–N47; this is known to inactivate the tethered thrombin receptor ligand [8]. Fragment 7170 with Ser at N-terminus greatly decreased or disappeared after 2 h incubation, being converted to fragment 6553 (not shown). The incubation of

Table 1  
Inhibition of thrombin and viper venom enzymes induced platelet aggregation by thrombin and viper venom serine peptidases

Agonist	Inhibition %				
	Heparin	Hirudin	Thrombomodulin	IIaR	
	100 U	12.5 U	5 nM	0.1 µg	2.5 µg
Thrombin 300 ng/ml	100	100	100	100	100
PA-BJ 500 ng/ml	100	0	0	10	100
Thrombocytin 500 ng/ml	100	0	0	10	100

Representative of three similar experiments.

Table 2

Ca<sup>2+</sup> release from platelet intracellular stores and the inhibitory effect of monoclonal antibodies blocking PAR1

Enzyme	Ca <sup>2+</sup> release			
	Control (no antibody)	IIaRA (3 µg/ml)	SPAN-12 (5.0 µg/ml)	WEDE-15 (5.0 µg/ml)
Thrombin, 0.4 µg/ml	33.7%	4.7%	4.7%	0%
PA-BJ, 3.4 µg/ml	57.1%	12.8%	4.7%	0%
Thrombocytin, 3.4 µg/ml	46.6%	4.7%	0	0%

Representative of three similar experiments. The amount of calcium released by digitonin (61 µg/ml) was accepted as 100%.

TR78 with thrombocytin resulted in the same degradation pattern (not shown). The degradation of the tethered ligand in the receptor by PA-BJ and by thrombocytin may explain why these enzymes are less potent than thrombin in causing platelet aggregation and release reaction.

To further evaluate the mechanism by which these enzymes activate platelets, we investigated their effect on calcium mobilization in the suspension of lung fibroblasts transfected with PAR4 (cell line KF:hP4-S12). These fibroblasts were isolated from the lung of the mouse deficient in PAR1. Fig. 2 shows that the challenge of Fura-2 labeled PAR4 transfected cells with thrombin (30 nM), thrombocytin (300 nM), and PA-BJ (300 nM) resulted in a similar intracellular calcium flux. In addition, KF:hP4-S12 cells preincubated with venom enzymes were desensitized to thrombin. These data indicate that PA-BJ and thrombocytin may activate the PAR4 receptor in a similar fashion to thrombin.

We conclude that both PA-BJ and thrombocytin activate

PAR1 by splitting the Arg41–Ser42 peptide bond. The lower activity of PA-BJ and thrombocytin, as compared to thrombin, may result from the subsequent inactivation of PAR1 by the venom enzymes. The venom enzymes both activate and inactivate PAR1; human plasmin has been shown to have a similar effect [8]. Matthews et al. [15] presented evidence that thrombin binds to both Arg41–Ser42 and the hirudin domain of PAR1 and that the binding to the hirudin domain potentiates the proteolytic activity of thrombin. It is known that thrombin binding to hirudin correlates with its binding to thrombomodulin and the thrombin receptor's hirudin domain. Although monoclonal antibody WEDE-15, recognizing the hirudin binding domain in PAR1, inhibits the effect of PA-BJ and thrombocytin on the platelet, this effect is not inhibited by hirudin and thrombomodulin. Thrombomodulin–hirudin binding sites [16] are located in the exosite domain of thrombin, and thrombomodulin competes for the same binding sites on the thrombin molecule with hirudin [17]. This exosite domain of thrombin has little homology with corresponding regions of PA-BJ [11]. It can be hypothesized that the PA-BJ exosite is not able to bind hirudin, thrombomodulin, and it binds with a lower affinity to thrombin receptor hirudin's domain. Further studies are needed to verify this hypothesis.

Both venom enzymes can be useful tools in studies on the structure and function of thrombin receptors. They seem to be more selective than thrombin since they do not proteolyse and clot fibrinogen. Viper venom enzymes could have the advantage over thrombin in studies on thrombin receptors expressed on endothelial cells [5,18]. Thrombomodulin is a component of endothelial cell membranes and a potent inhibitor of thrombin effects on clotting and platelet aggregation [19]; however, it has no effect on platelet aggregation induced by venom enzymes, suggesting that it does not interfere with their effect on PAR1 and PAR4.

In conclusion, we propose that the effect of viper venom serine peptidases PA-BJ and thrombocytin on platelets is mediated both by PAR1 and by PAR4, the same receptors which mediate the thrombin effect on platelets. In contrast to thrombin, viper venom enzymes both activate and inactivate the receptor by removing the receptor-anchored ligand.

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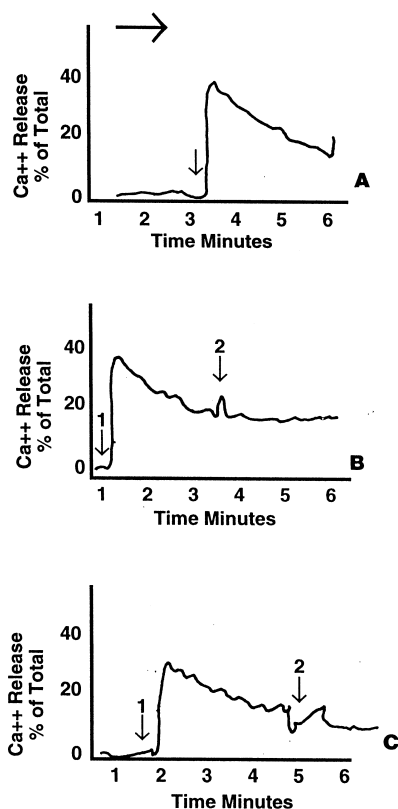


Fig. 2. Tracing of calcium release from intracellular stores of KF:hP4-S12 cells labeled with Fura-2. A: Effect of thrombin (30 nM) indicated by an arrow. B: Effect of PA-BJ (300 nM), arrow 1, followed by the effect of thrombin 30 nM, arrow 2. C: Effect of thrombocytin (300 nM), arrow 1, followed by thrombin (30 nM), arrow 2.

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