Characterization of the Prothrombin Activator from the Venom of *Oxyuranus scutellatus scutellatus* (Taipan Venom)†

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ABSTRACT: The prothrombin activator from the venom of *Oxyuranus scutellatus scutellatus* has been partially purified by gel filtration and ion-exchange chromatography. The activator is a large protein. The partially purified activator hydrolyzes the synthetic substrate Bz-Ile-Glu-Gly-Arg-*p*-nitroanilide (S2222) and activates prothrombin, prethrombin 1, prethrombin 2, and meizothrombin (des fragment 1). S2222 hydrolysis is inhibited by benzamidine, soybean trypsin inhibitor, and bovine pancreas trypsin inhibitor. N^{α}-*p*-Toluenesulfonyl-L-arginine methyl ester also inhibits S2222 hydrolysis, but it is not hydrolyzed by the venom activator. The activator is also inhibited by an inhibitor present in the venom. S2222 hydrolysis is insensitive to diisopropyl fluorophosphate, antithrombin III, chicken ovomucoid trypsin inhibitor, EDTA, *o*-phenanthroline, and activated protein C. In addition, the enzyme does not hydrolyze the synthetic substrates Bz-Phe-Arg-*p*-nitroanilide (S2160) or D-Val-Leu-Lys-*p*-nitroanilide (S2251). The pH optimum for S2222 hydrolysis is between 7.5 and 8.0. Phospholipid enhanced the rates of activation of both prothrombin and prethrombin 2. However, the activation of prethrombin 1 is insensitive to the presence of phospholipid. The activation of prethrombin 2 by the venom activator is stimulated by prothrombin fragment 2. Treatment of the enzyme with high concentrations of NaCl stimulates the rate of S2222 hydrolysis. This treatment, however, eliminates the procoagulant activity. NaCl-treated activator is unable to activate prethrombin 2 but retains activity toward meizothrombin (des fragment 1). These results indicate that the prothrombin activator from taipan snake may be comprised of either two enzymes or a single enzyme with two active sites.

Numerous snake venoms contain procoagulants. The venom of Russell's viper contains activators of both factor X (Kisiel et al., 1976) and factor V (Schiffman et al., 1969), and activators of prothrombin have been observed in the venoms of *Echis carinatus* (Morita et al., 1976; Franza et al., 1975), *Notechis scutatus scutatus* (Jobin & Esnouf, 1966), *Dispholidus typus* (Guillin et al., 1978), and *Oxyuranus scutellatus scutellatus* (Owen & Jackson, 1973). Thrombin-like enzymes have been isolated from the venoms of *Crotalus adamanteus* (Markland & Damus, 1971) and *Bothrops atrox* (Holleman & Weiss, 1976).

The mechanisms by which the snake venom proteins activate a particular coagulation factor vary for each species and the proteins often differ from mammalian enzymes in activation pathways and mechanisms. The prothrombin activators from *E. carinatus* and *D. typus* appear to function by similar mechanisms, in which the first cleavage in prothrombin activation is in the thrombin portion of the molecule and results in the formation of meizothrombin[1] (Morita et al., 1976; Guillin et al., 1978). In contrast, activated factor X (Xa) and the prothrombin activator from the venom of *O. scutellatus scutellatus* appear to work through similar mechanisms. The fragment region of prothrombin is first removed and then prethrombin 2 is converted to thrombin (Owen & Jackson, 1973; Jackson et al., 1975).

Since the apparent molecular size of the activator is quite large compared to other prothrombin activators and since it is insensitive to serine protease inhibitors, we have undertaken the characterization of the enzymatic properties of the partially purified prothrombin activator, which are reported in this paper.

Materials and Methods

Materials. *O. scutellatus scutellatus* snake venom, soybean trypsin inhibitor, bovine pancreas trypsin inhibitor, chicken ovomucoid trypsin inhibitor, QAE-Sephadex-Q50, and tosyl-L-arginine methyl ester (Tos-Arg-OMe)[2] were purchased from Sigma Chemical Co. Benzamidine, *o*-phenanthroline, and *m*-aminobenzamidine were purchased from Aldrich Chemical Co. The synthetic substrates Bz-Ile-Glu-Gly-Arg-*p*-nitroanilide (S2222), Bz-Phe-Val-Arg-*p*-nitroanilide (S2160), and D-Val-Leu-Lys-*p*-nitroanilide (S2251) were purchased from Ortho. Bovine blood was the generous gift of the Wilson Foods Corp. Heparin was the generous gift of the Upjohn Co. All other reagents were the highest grade available.

Proteins. Bovine prothrombin, prethrombin 1, and thrombin were purified as described previously (Owen et al., 1974). Prethrombin 2 was prepared by reacting prothrombin with factor Xa and calcium chloride. Prethrombin 2 was subsequently purified by ion-exchange chromatography on sulfopropyl-Sephadex C-50 and *m*-aminobenzamidine–agarose as described elsewhere (Esmon & Jackson, 1974). Meizo-

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[1] The nomenclature used for the polypeptide fragments derived from prothrombin activation [prethrombin 1, prethrombin 2, meizothrombin (des fragment 1) and fragment 2] is that developed by the International Committee on Thrombosis and Hemostasis (Paris, July 1975).

[2] Abbreviations used: S2222, Bz-Ile-Glu-Gly-Arg-*p*-nitroanilide; S2160, Bz-Phe-Arg-*p*-nitroanilide; S2251, D-Val-Leu-Lys-*p*-nitroanilide; Tos-Arg-OMe, N^{α}-*p*-toluenesulfonyl-L-arginine methyl ester; DIP-meizothrombin (des fragment 1), diisopropylphosphorylmeizothrombin (des fragment 1).

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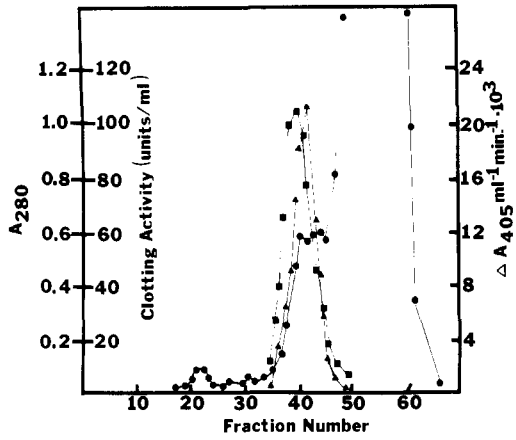

FIGURE 1: Chromatography of the crude taipan venom on Ultrogel-22. 100 mg of venom was dissolved in 3 mL of 0.15 M NaCl and 0.05 M NaOAc, pH 5.8. The venom was chromatographed at 22 °C on a column (1.5 × 100 cm) of Ultrogel-22 in the same buffer. 2.5-mL fractions were collected and assayed for protein (●), clotting activity (■), and S2222 hydrolase activity (▲). Samples removed from fractions 50–58 inhibited plasma clotting initiated by either the venom activator or factor Xa. They did not inhibit thrombin-initiated clotting.

thrombin (des fragment 1) was prepared by digesting prethrombin 1 with the purified prothrombin activator from *E. carinatus* venom and isolated by ion-exchange chromatography as described elsewhere (Walker & Esmon, 1979). Activated protein C was purified from a barium eluate as described elsewhere (Walker et al., unpublished experiments). Fibrinogen was purified from barium-adsorbed plasma (Straughn & Wagner, 1966). Antithrombin III was purified from barium-adsorbed plasma as previously described (Walker & Esmon, 1979).

Phospholipid was prepared from acetone-dried bovine brain homogenate by the method of Bligh & Dyer (1959). Phospholipid vesicles were prepared by mixing the phospholipid in chloroform and then drying under nitrogen onto the walls of a glass tube. The lipid was dispersed into buffer (0.1 M NaCl and 0.02 M Tris-HCl, pH 7.5) by sonicating the tube with a bath sonicator.

Assays. Thrombin was assayed by either a fibrinogen clotting assay or the rate of hydrolysis of the synthetic substrate S2160. Standard curves were constructed with purified thrombin. The prothrombin activator was assayed by diluting a sample of activator into 0.300 mL of buffer (0.1 M NaCl and 0.02 M Tris-HCl, pH 7.5, containing 1 mg/mL bovine serum albumin). Clotting was initiated by the addition of 0.100 mL of oxalated bovine plasma. A standard curve of arbitrary units was constructed with partially purified taipan venom activator. Hydrolysis of S2222 by the activator was determined by adding a sample of venom (0.010 mL) to a reaction mixture with a final volume of 0.600 mL containing 0.1 M NaCl, 0.02 M Tris-HCl, pH 7.5, 0.005 M $CaCl_2$, and 0.1 mM S2222. The rate of hydrolysis at 25 °C was determined as the change in absorbance at 405 nm. Protein was determined by measuring absorbance at 280 nm. The molecular weights and extinction coefficients used for calculating substrate concentrations were as follows: thrombin, 37 000 and $E_{1\%}^{1cm}$ = 21.4; prethrombin 2, 37 000 and $E_{1\%}^{1cm}$ = 21.4; prethrombin 1 and meizothrombin (des fragment 1), 50 000 and $E_{1\%}^{1cm}$ = 19.2; prothrombin, 72 000 and $E_{1\%}^{1cm}$ = 15.5; fragment 2, 12 900 and $E_{1\%}^{1cm}$ = 12.3 (Owen et al., 1974).

Results

Purification of the Prothrombin Activator from Taipan Snake Venom. The crude venom was chromatographed on Ultrogel-22 (Figure 1). Procoagulant and S2222 hydrolase activities were eluted from the column at the front of the first major protein peak. The recovery of coagulant activity was over 100-fold in excess of the starting activity in the crude venom, apparently due to the removal of an inhibitor which was eluted later. There was a 30-fold excess recovery of S2222 hydrolase activity (Table I).

Table I: Purification of Taipan Venom Prothrombin Activator on Ultrogel-22[a]

	starting material, 3.0 mL	Ultrogel-22 pool
total A_{280}	148	24
total S2222 hydrolase (A_{405}/min)	1.35	39.5
total clotting act. (units)	1400	148 500
S2222 sp act. [A_{405}/(min A_{280})]	0.0091	1.64
clotting sp act. (units/A_{280})	9.37	6190

[a] The units are in arbitrary units. The assay is described under Materials and Methods.

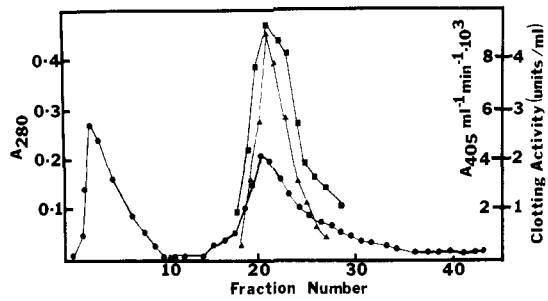

FIGURE 2: Chromatography of taipan venom activator on QAE-Sephadex-Q50. Fractions 35–45 from the Ultrogel column (Figure 1) were pooled and chromatographed at 22 °C on a column (0.9 × 30 cm) of QAE-Sephadex-Q50. The column was equilibrated in 0.15 M NaCl and 0.05 M NaOAc, pH 5.8, and developed with a linear gradient (50 mL/chamber) from 0.15 to 0.6 M NaCl in the acetate buffer. Clotting activity (■); protein (●); S2222 hydrolase (▲). Fraction volume was 2 mL.

The procoagulant activity was pooled and subjected to ion-exchange chromatography on QAE-Sephadex (Figure 2). The procoagulant activity was eluted near the start of the NaCl gradient. The peak fraction contained a protein which appeared as two bands on gel electrophoresis in dodecyl sulfate with molecular weights corresponding to 220 000 and 160 000. Upon reduction this material was separated into two bands with molecular weights of 105 000 and 76 000. Both of the bands reacted with the Schiff–periodate stain for carbohydrate.

Enzymatic Properties of the Prothrombin Activator. Prothrombin, prethrombin 1, and prethrombin 2 were substrates for the venom activator. The respective Michaelis constants differed and are listed in Table II. The venom preparation hydrolyzed the synthetic factor Xa substrate S2222 (K_m = 0.6 mM) but would not hydrolyze S2160, the thrombin substrate, S2251, the plasmin substrate, nor Tos-Arg-OMe, which was, however, a competitive inhibitor of S2222 hydrolysis (K_i = 1.1 mM). Hydrolysis of S2222 was also sensitive to benzamidine and soybean trysin inhibitor (Table I). Bovine pancreatic trypsin inhibitor was a progressive inhibitor. Hydrolysis of S2222 was not inhibited by diisopropyl fluorophosphate, antithrombin III, chicken ovomucoid trypsin inhibitor, or the synthetic substrates S2160 or S2251. It was also insensitive to EDTA, *o*-phenanthroline, and activated protein C.

Prothrombin activation was stimulated fourfold by 8 μg/mL crude phospholipid, and prethrombin 2 activation was stimulated threefold by 170 μg/mL phospholipid; prethrombin 1 activation was not influenced by phospholipid. Addition of

Table II: Substrates and Inhibitors of Taipan Venom Prothrombin Activator[a]

substrates	K_m (mM)
prothrombin	0.00076
prethrombin 1	0.001
prethrombin 2	0.01
S2222	0.6
inhibitors	**K_i (mM)**
Tos-Arg-OMe	1.1
benzamidine	0.065
soybean trypsin inhibitor	0.013[b]
	0.0027[c]
bovine pancrease trypsin inhibitor	ND[d]
DIP-meizothrombin (des fragment 1)	0.006
noninhibitors	**highest concn tested (mM)**
diisopropyl fluorophosphate	10
S2160	1.0
S2251	1.0
antithrombin III	0.01
o-phenanthroline	10
chicken ovomucoid trypsin inhibitor	0.01

[a] Inhibitors were tested by the effect on S2222 hydrolysis. The reactions were carried out at room temperature in 5 mM $CaCl_2$, 0.02 M Tris-HCl, pH 7.5, 0.1 M NaCl, and 0.2 mM S2222. The reactions were carried out in a final volume of 0.600 mL. [b] 0.1 M NaCl. [c] 2.0 M NaCl. [d] Bovine pancrease trypsin inhibitor was a progressive inhibitor of S2222 hydrolysis by taipan venom activator. K_i was not determined.

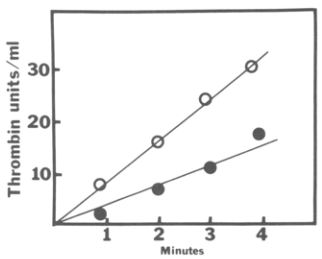

FIGURE 3: Effect of prothrombin fragment 2 on the rate of activation of prethrombin 2 by the taipan venom prothrombin activator. Prethrombin 2 (0.5 μM) was activated at 37 °C with the taipan venom activator (2.5 μg/mL) in the presence (○) or absence (●) of prothrombin fragment 2 (10 μM) in 0.1 M NaCl, 0.02 M Tris-HCl, 0.005 M $CaCl_2$, pH 7.5, and 1 mg/mL bovine serum albumin. The reaction volume was 0.60 mL. Thrombin was determined at the indicated time by a fibrinogen clotting assay. Fragment 2 did not activate prethrombin 2 in the absence of the venom activator.

an excess of prothrombin fragment 2 stimulated prethrombin 2 activation 2.5-fold (Figure 3).

Direct evidence that substrate proteolysis occurred during generation of thrombin activity was obtained by activating meizothrombin (des fragment 1) and diisopropylphosphorylmeizothrombin (des fragment 1) (DIP-meizothrombin) and assessing activation with gel electrophoresis in dodecyl sulfate (Figure 4). Both zymogen forms proved to be equivalent substrates for the activator. DIP-meizothrombin was next used as an inhibitor of S2222 hydrolysis in order to see if the hydrolysis of the synthetic substrate was by the same enzyme that cleaved the fragment 2 region from meizothrombin (des fragment 1). DIP-meizothrombin (des fragment 1) was found to be an inhibitor of S2222 hydrolysis (K_i = 0.006 mM).

The pH dependence of S2222 hydrolysis was evaluated. The pH optimum for the enzyme was between pH 7.5 and pH 8.0.

The rate of S2222 hydrolysis was sensitive to the NaCl concentration. High salt concentrations increased the maximum velocity of hydrolysis and had no effect on the K_m (Table II). At high NaCl concentrations, soybean trypsin inhibitor

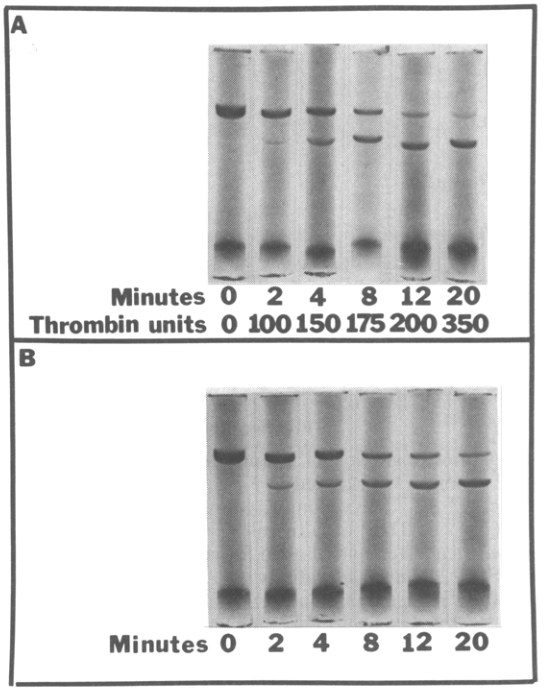

FIGURE 4: Activation of meizothrombin (des fragment 1) and diisopropylphosphorylmeizothrombin (des fragment 1). Meizothrombin (des fragment 1) (4.6 μM) or the diisopropylphosphorylmeizothrombin (des fragment 1) was activated at 37 °C with the partially purified venom prothrombin activator (4 μg/mL) in 0.1 M NaCl, 0.02 M Tris-HCl, and 5 mM $CaCl_2$, pH 7.5. At the times indicated, samples (20 μg) were removed, made 1% in sodium dodecyl sulfate, and placed in a boiling water bath for 1 min. At the same time samples were removed and assayed for thrombin formation. The cleavage of meizothrombin was monitored by disc gel electrophoresis in sodium dodecyl sulfate on gels containing 11% acrylamide. The samples were not reduced prior to electrophoresis. (A) The activation time course of meizothrombin (des fragment 1). (B) The activation time course of diisopropylphosphorylmeizothrombin (des fragment 1).

was a more effective inhibitor, with the K_i decreased from 0.013 mM at 0.1 M NaCl to 0.0027 mM at 2 M NaCl.

Treatment of the venom activator with 2 M NaCl resulted in loss of procoagulant activity but not S2222 hydrolase activity. Since two bonds are cleaved during prothrombin activation, loss of the ability to cleave either bond might block prothrombin activation. Since, of the two taipan venom sensitive bonds, meizothrombin (des fragment 1) contains only the Arg_{274}–Thr_{275} bond and prethrombin 2 contains only the Arg_{323}–Ile_{324} bond, these two substrates were used to examine the possibility that high salt concentration selectively inhibits the activity toward one of these bonds. Salt-modified taipan activator was unable to activate prethrombin 2 but retained full activity in the activation of meizothrombin (des fragment 1) (Figure 5).

Discussion

Early studies (Owen & Jackson, 1973) on the activation of prothrombin by the taipan snake venom demonstrated that the venom did not hydrolyze Tos-Arg-OMe nor was it inhibited by the active-site inhibitors DFP, phenylmethanesulfonyl fluoride, *p*-(chloromercuri)benzoate, or EDTA. As most proteinases are inhibited by one or more of these reagents, these results left open the possibility that the activator in taipan venom was not a protease and utilized the active site of prothrombin to catalyze thrombin formation. We have now shown that the venom contains a specific hydrolase which can hydrolyze the factor Xa substrate S2222 while not hydrolyzing the thrombin (S2160) or plasmin (S2251) substrates. Further

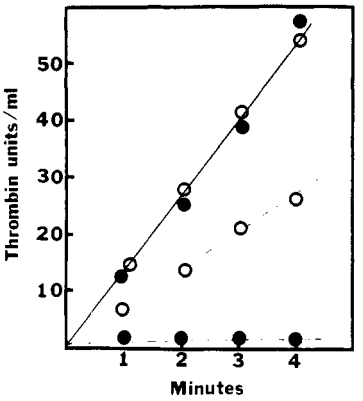

FIGURE 5: Activation of prethrombin 2 and meizothrombin (des fragment 1) by the taipan venom activator treated with 2 M NaCl. Prethrombin 2 (0.5 μM) was activated with the venom prothrombin activator (2.5 μg/mL). Prior to the activation study, the activator either was (●---●) or was not (O---O) brought to 2 M in NaCl. The activation was performed at 37 °C in 0.1 M NaCl, 1 mg/mL bovine serum albumin, 5 mM $CaCl_2$, and 0.02 M Tris-HCl, pH 7.5. The NaCl concentration in the final reaction mixture was identical regardless of whether the venom activator had been treated with NaCl. Meizothrombin (2 μM) was activated with the taipan activator either before (●—●) or after (O—O) salt treatment as described above. The reaction conditions and activator concentration were identical with those described above. Thrombin activity was measured by the fibrinogen clotting assay. The thrombin activity of the meizothrombin (des fragment 1) was subtracted from the thrombin activity following activation.

evidence that a protease is directly involved in prothrombin activation comes from the ability of the activator to cleave diisopropylphosphorylmeizothrombin (des fragment 1), in which the active site of the substrate is blocked. Loss of procoagulant, but not S2222 hydrolase activity, after exposure of the activator to 2 M NaCl suggests either that S2222 hydrolase is a contaminant of the preparation or that an additional activity is required to convert prothrombin to thrombin. This latter hypothesis is supported by the observation that the 2 M NaCl treated activator retains the ability to cleave fragment 2 from meizothrombin but is not able to catalyze the activation of prethrombin 2.

The nature of the second activity remains unknown but appears to be distinct from that of S2222 hydrolase. Several features of the preparation suggest that the two activities are physically associated. Although the S2222 hydrolase and prothrombin activator have apparent molecular weights which are unusually high for proteinases, no prothrombin activator is resolved completely by gel chromatography from S2222 hydrolase. The S2222 hydrolase reproducibly was eluted slightly later than procoagulant activity. Second, the two activities are coeluted by a salt gradient from QAE-Sephadex. The observation that the procoagulant may be composed of two chains may suggest that each activity is associated with a particular chain. Our observation that the procoagulant activity has an apparent molecular weight around 400 000 is consistent with a two-chain structure of 220 000 and 160 000, yielding a protein of molecular weight 380 000. However, the exact relationship of the activities and subunit structure will require the isolation and physical characterization of the activator on a larger scale than is currently feasible. The ultimate proof that two distinct activities are required for the complete prothrombin activator will require separation and subsequent recombination of the isolated chains.

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