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Characterization of a new coagulant enzyme isolated from *Trimeresurus okinavensis* (Himehabu snake) venom

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(Chief: Prof. Yoshito MATSUMOTO)

Abstract

A potent, proteinaceous inducer of coagulation factors, has been purified to homogeneity from *Trimeresurus okinavensis* (Himehabu snake) by ion exchange chromatography and molecular sieving. It has an apparent molecular weight of 28,000 and it specifically converts fibrinogen to fibrin through an enzymatic reaction. This activity was confirmed by a method using synthetic substrate S-2238, though the fraction from a DEAE sephacel column chromatography shows amidolytic activity of S-2251 and S-2366, which are commonly used to measure plasmin and activated protein C activity, respectively, as well as S-2238. This thrombin-like activity of the final product was not affect by the addition of heparin and AT-III. This enzyme also aggregates normal platelets. It is considered that this purified enzyme would be useful as a hemostatic agent or research reagent.

Key words: Snake venom, Thrombin like enzyme, Platelet aggregation.

Introduction

Humans may suffer from lethal snake bites in many areas of the world. Many enzymes contained in snake venom can also be used for therapeutic and research purposes. Some snake venom constituents affect normal hemostasis by adversely altering blood coagulation and platelet aggregation. With regard to blood coagulation, the activities of these components include: (i) a thrombin-like enzyme, (ii) a prothrombin activator, (iii) an activator for

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coagulation factor V, (iv) an activator for factor X, (v) a protein C activator, and (vi) an activator for kininogen[1].

A number of coagulant enzymes with thrombin-like specificity have been purified from the venoms of snakes of the viperid family[2]. The CG-B1P fraction was partially isolated from *Trimeresurus okinavensis* with a heat-treatment procedure by Katoh et al., and it was found to have thrombin-like activity and was able to convert fibrinogen to fibrin[3]. However, the coagulant activity of this snake venom was not adequately established.

The present study used different methods for purification than previous reports, and purified a new proteinase from *Trimeresurus okinavensis*, which is appears to act by a mechanism similar to thrombin.

Anchord, which is established from *Agkistrodon rhodostoma* venom, is famous for a thrombin like enzyme. We investigated the characteristics of the proteinase from *Trimeresurus okinavensis* as compared with anchord.

Material and Methods

Measurement of coagulant activity

Coagulant activity of samples was measured by the activated partial thromboplastin time (aPTT) with Ci-trol (DADE, Puerto Rico, USA) and aPTT reagents (Kokusai-Shiyaku, Koube, Japan). Here 50 μl of sample was mixed with 100 μl of Ci-trol, and after incubation at 37°C for 30 sec, 100 μl of aPTT reagent was added to the mixture. The reaction mixture was then incubated at 37 °C for 1 min and the clotting time was measured by the addition of 100 μl 50 mM $CaCl_2$ with Coag-stat (Kokusai-shiyaku).

Purification of coagulant fraction from snake venom

Snake venoms from *Trimeresurus okinavensis* (Himehabu snake) and *Agkistrodon ralys blomhoffii* (Mamushi snake) were obtained from Wako (Osaka, Japan). Purification of fractions which have coagulant activity was performed with three step column chromatography. First, 500 μl of the original snake venom (0.2 mg/ml) from *Trimeresurus okinavensis* was applied to a DEAE sephacel (Pharmacia Biotech, Uppsala, Sweden) column (7 ×100mm), and eluated with 0.5 M NaCl. The coagulant activity of each fraction was measured by aPTT assay. Next, 500 μl of the fraction with coagulant activity was applied to a superose 6X gel filtration column (Pharmacia Biotech). The coagulant fraction determined by the aPTT assay was dialysed with tris buffer (0.02 M tris HCl buffer, pH 7.3). Finally,2 ml of the coagulant fraction was applied to a Mono Q column (Pharmacia Biotech) in a FPLC system (GP-250; Pharmacia Biotech), and eluated by 0.5 M NaCl. The final product was obtained by measuring aPTT.

Snake venom from *Agkistrodon ralys blomhoffii* was applied to a DEAE sephacel column, and eluated by 0.5 M NaCl. 500 μl of the total elution fraction was applied to the gel filtration column (superose 6X), similar to the purification method described above. Each fraction was

tested with an aPTT assay.

The affinity of the final product to heparin

To investigate the affinity to heparin, the final tris buffer (2 ml) dialysed product was applied to a Heparin sepharose CL-6B (Pharmacia Biotech) column (7 × 80 mn) and washed with tris buffer. Each fraction eluated by tris buffer containing 0.5 M NaCl and the pass through fraction were tested wiht aPTT.

Amidolytic activity of the final product on synthetic substrates

The amidolytic activity of the final product was investigated. A hundred and sixty μl of the sample (final product 〔64 μg/ml〕, fraction 11 from the gel filtration, and fraction 14 from the DEAE column chromatography) diluted at 1:4 with tris buffer was mixed with 50 μl of S-2238 (1.8 mM), S-2376 (1 mM), S-2366 (1.2 mM), and S-2251 (2.2 mM) (Chromogrnix AB, Mölndal, Sweden), all synthetic substrates for thrombin activity, factor Xa activity, protein C activity, and plasmin activity, respectively. After incubation at 37°C for 15 min, 100 μl of 50% acetic acid solution was added to the mixture to stop a chromogenic reaction, and the mixture was measured at 405 nm by an autoreader (Microplate reader MTP-120 ; Corona electro, Katsuta, Japan).

Effect of the final product on platelet aggregation

The effect of the final product on platelets was investigated by an aggregometer (Hema Tracer 601; Niko Bioscience, Tokyo, Japan). The aggregation reaction of 200 μl of platelet rich plasma (PRP : $40 \times 40^4/\mu$l) from normal subjects was initiated by the addition of 25 μl of sample (final product 〔64 μg/ml〕, fraction 14 from the DEAE column chromatography, and original snake venom).

Effect of the final product on fibrin formation in the presence or absence of antithrombin III

The effect of the final product on fibrin formation was investigated. A hundred μl of purified human fibrinogen solution (3 mg/ml) was mixed with 100 μl of the final product (32, 64 μg/ml) or 5 μg/ml thrombin (DADE) solution at 37°C , and the fibrinogen clotting time was measured. Further, to investigate the influence of antithrombin III (AT-III ; BioPor ag, Bubendorf, Switzerland), 50 μl of 20μg/ml AT-III was simultaneously mixed in the presence of 500u/ml heparin sodium (20μl : Mochida, Osaka, Japan).

Analysis of molecular weight

The molecular weight of the samples (final product, fraction 11 from the gel filtration column, and fraction 14 from the DEAE column chromatography) was investigated by SDS-PAGE (3-10% polyacrylamide).

Results

Purification of coagulant fraction from original snake venom

Original snake venom was applied to a DEAE sephacel column and the fraction eluated by 0.5 M NaCl was tested by aPTT assay. There was a shortening of aPTT in fractions 13 to

15, as shown in Fig. 1. The coagulant fraction was applied to a gel filtration column, and coagulant activity was determined in fraction 11. The gel filtration fraction obtained from *Agkistrodon ralys blomhoffii* did not show coagulant activity, but demonstrated a prolongation of aPTT (Fig. 2). A shortening of aPTT was determined in fraction 9 obtained from elution fractions of Mono Q column chromatography (Fig. 3).

The affinity of the final product to heparin

The final product was applied to a Heparin sepharose CL-6B column. Almost all of the sample was collected in the pass through fraction. The pass through fraction and the elution fraction were tested by aPTT, and shortening of aPTT was determined only in the pass through fraction (data not shown).

The effect of the final product on platelet aggregation

Platelets obtained from a healthy subject were aggregated strongly by the addition of the final product. The original solution of snake venom and fraction 14 from the DEAE column chromatography also induced aggregation of the normal PRP (Fig. 4).

The effect of the final product on synthetic substrates

The enzymatic characteristics of the final product was investigated by mixing it with synthetic substrates of several proteases. As shown in Fig. 5, fraction 14 from the DEAE column chromatography had a strong amydolytic activity with substrates S-2238, S-2366, and S-2251, but the final product mainly hydrolyzed substrate S-2238.

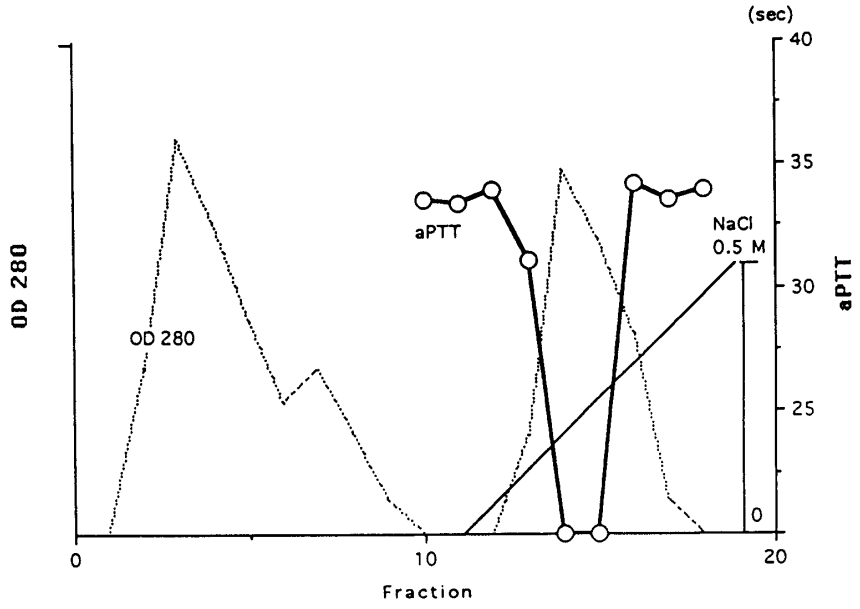


Fig. 1 DEAE sephacel column chromatography for purification of coagulant enzyme from *Trimeresurus okinavensis*. Five hundred μl original snake venom dissolved in tris buffer was applied to a DEAE column equilibrated with tris buffer. After washing with more than three times the volume of tris buffer, the fraction was eluated by tris buffer containing 0-0.5 M NaCl. Flow rate 0.5 ml/min, fraction size 2 ml/fraction.

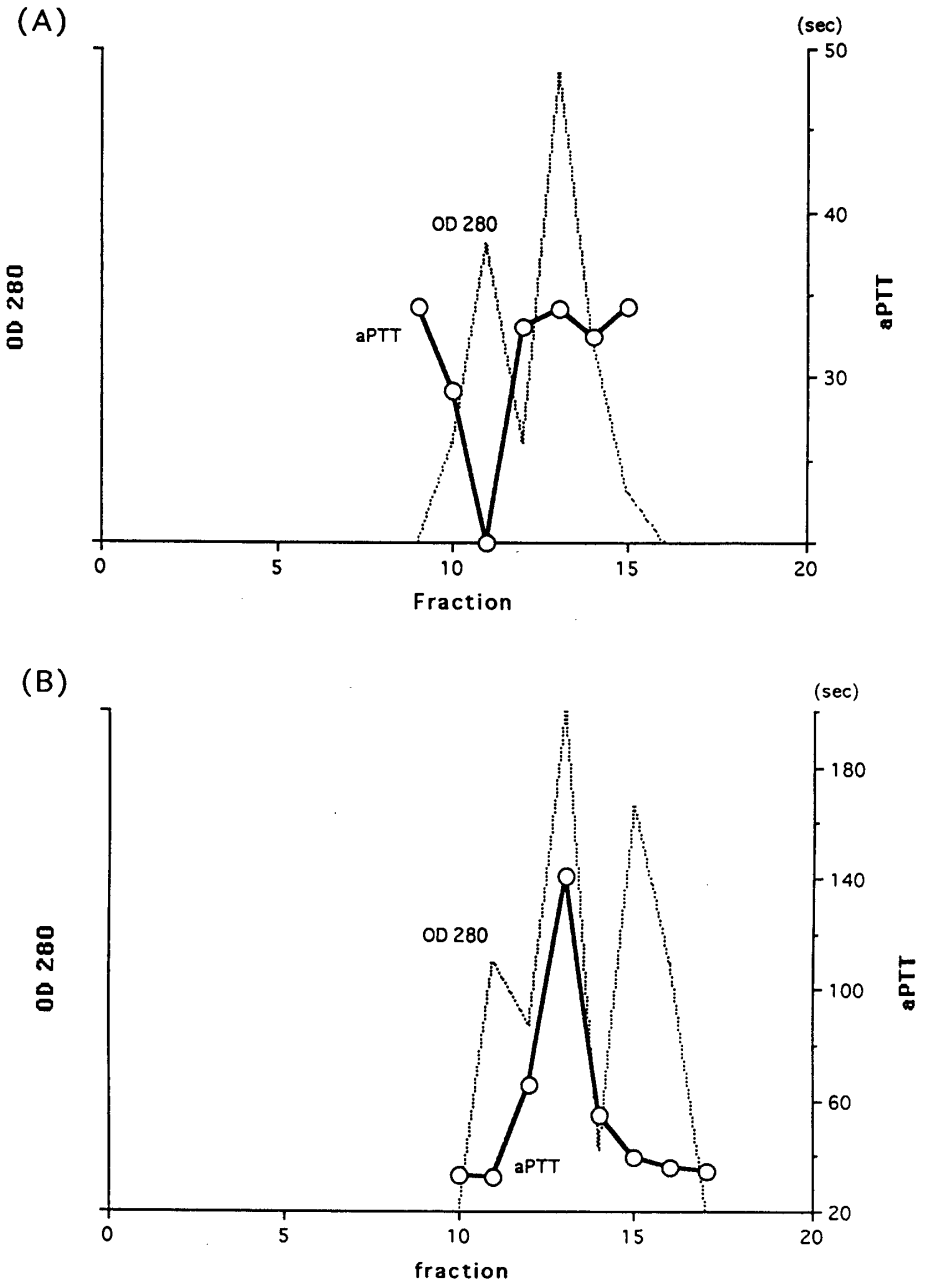


Fig. 2 Gel filtration with superose 6X column. (A) Gel filtration of the sample from *Trimeresurus okinavensis* venom. (B) Gel filtration of the sample from *Agkistrodon ralys blomhoffii* venom. Five hundred μl of the sample from the DEAE column was applied to the column. Tris buffer containing 0.15 M NaCl as running buffer. Flow rate 0.5 ml/min, fraction size 2 ml/fraction.

Effect of the final product on fibrin formation in the presence or absence of AT-III

Fibrin formation into a very loose clot was induced by 64 μg/ml of the final product. The addition of AT-III did not affect the fibrinogen clotting time (Table 1).

Molecular analysis of the final product

The molecular weight of the final product was determined by SDS-PAGE, and the main band was detected at approximately 28,000 and a small band at 21,000 (Fig. 6).

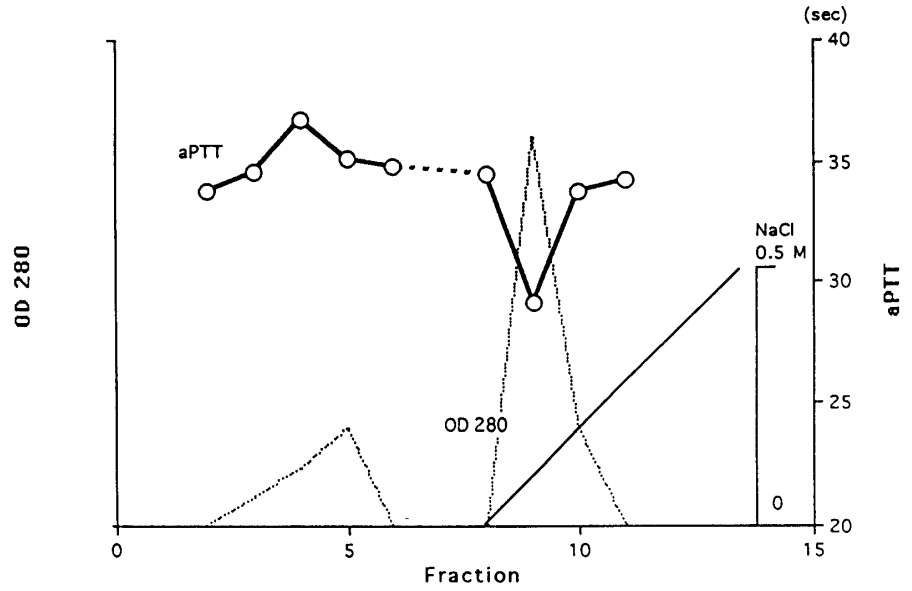


Fig. 3 Purification of the final product from *Trimeresurus okinavensis* with Mono Q column chromatography. Two ml of the sample the from gel filtration was applied to the Mono Q column equilibrated with tris buffer, and eluated by tris buffer containing 0-0.5 M NaCl. Flow rate 1ml/min, fraction size 2 ml/fraction.

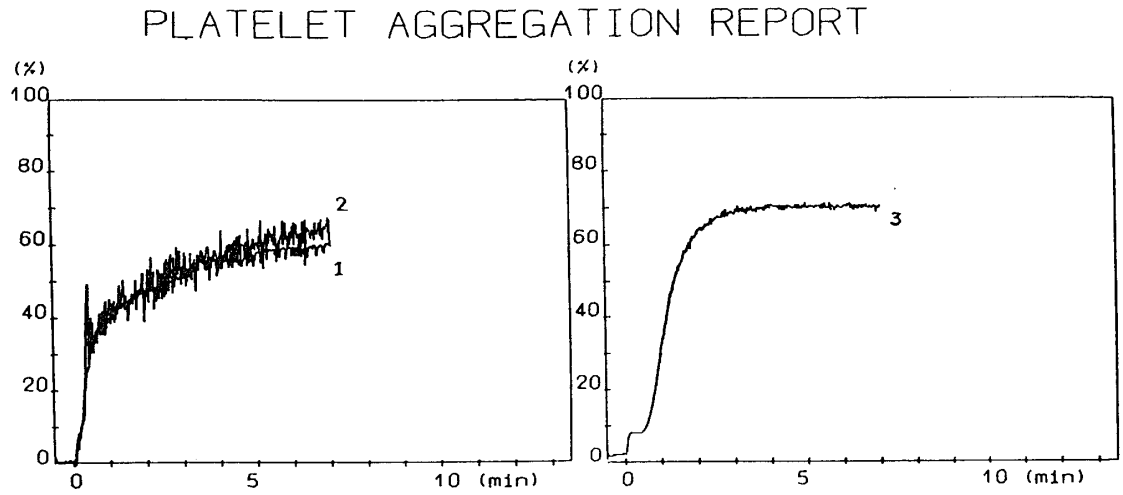
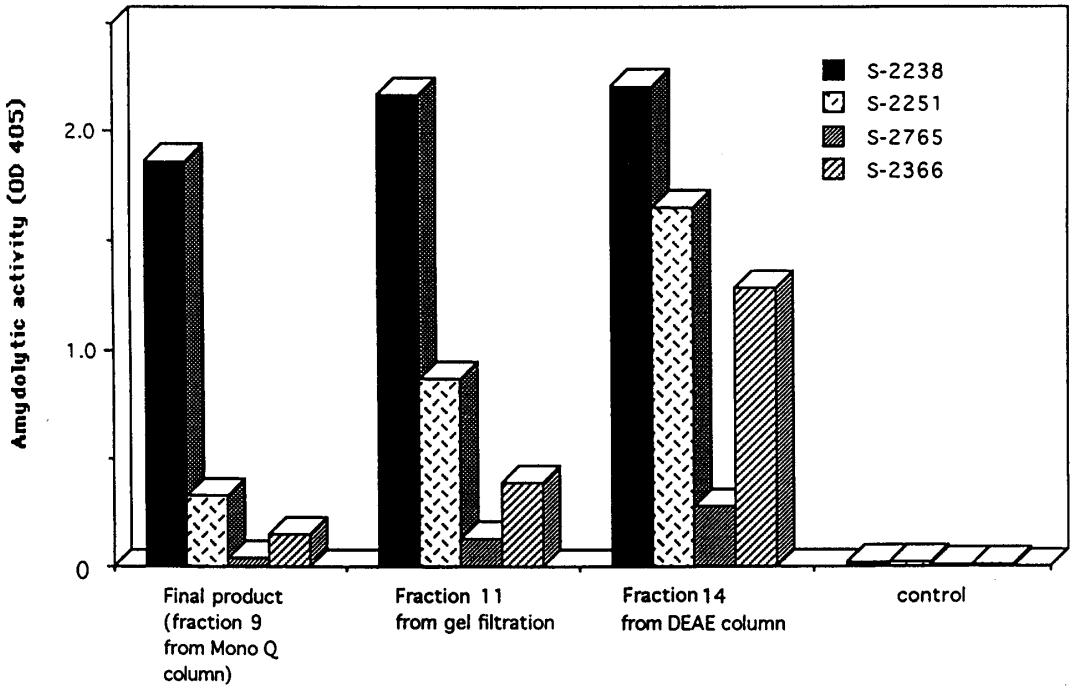


Fig. 4 Effect of the final product on aggregation of normal platelets. 1: Twenty five μl of original snake venom. 2: Fifty μl of fraction 14 from the DEAE column chromatography. 3: Fifty μl of the final product obtained from Mono Q column chromatography. All samples induced strong aggregation of normal platelets.



Feg. 5 Effect of samples on amidolytic activity. Samples (fraction 14 from the DEAE column chromatography, fraction 11 from the gel filtration, and the final product) were able to hydrolyze S-2238, which is a synthetic substrate for thrombin. Amidolytic activity of the final product to substrate S-2251 and S-2366 was very weak, however the fractions from DEAE column chromatography and gel filtration had a strong amidolytic activity on these substrates.

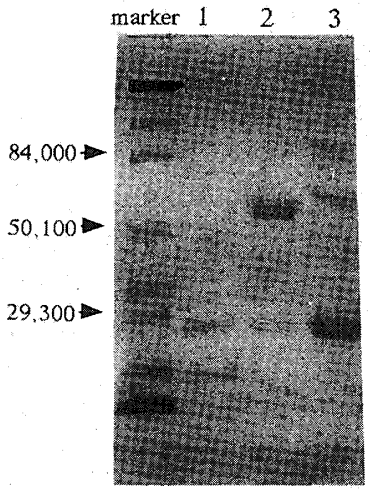


Fig. 6. Molecular weight of the final product by SDS-PAGE. 1 : the final product; 2 : fraction 11 from the gel filtration; 3 : fraction 14 from DEAE column chromatography. Samples were not reduced.

Table 1 Fibrinogen clotting time

samples		clotting time (sec)	
		buffer	AT-III
thrombin	(5.0μg/ml)	10.8	n.c
final product	(32μg/ml)	2,270.0	2,346.1
	(64μg/ml)	469.2	481.8

(n. c : not clotted for 3600 sec. mean, n=2)

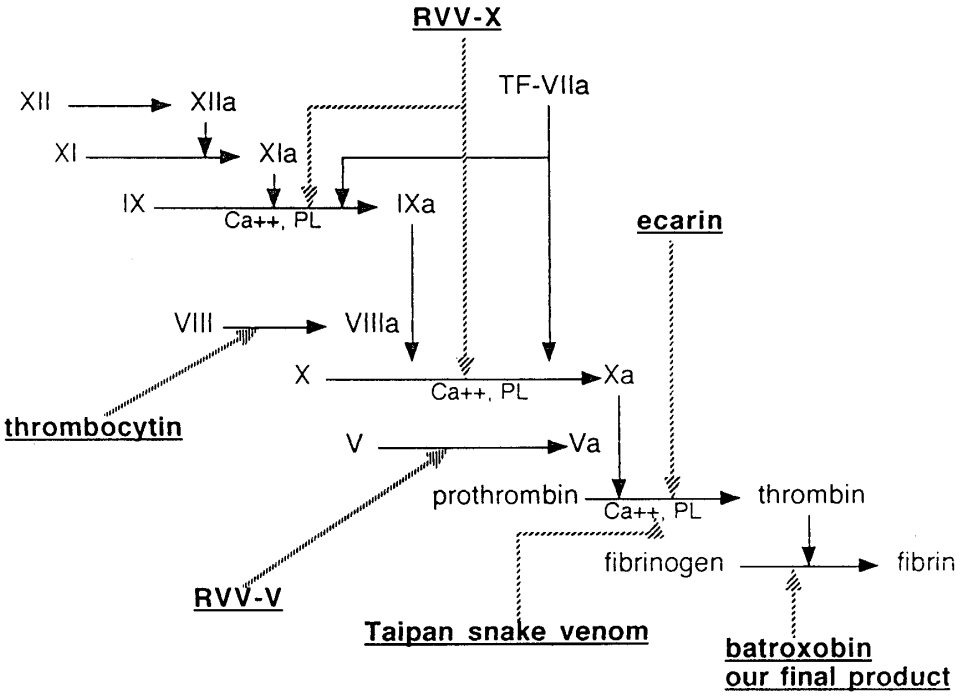


Fig. 7 Relationship between coagulant enzymes from snake venoms and coagulation reactions. TF : tissue factor, PL : phospholipids : RVV : *Russell's viper* venom.

Discussion

Snake venoms are known to contain many enzymes which affect normal coagulation and fibrinolysis. Among these, the enzyme that is associated with bleeding has largely been established. This report investigates the characteristics of coagulant enzymes with thrombin-like specificity in the venom of *Trimeresurus okinavensis*. Coagulant enzymes from *Trimeresurus okinavensis* were also reported by Shimura et al.[3]. However, the isolation of the enzyme used different methods from the procedures here, and this suggests that the final product reported here may be different from the enzyme described by Shimura et al. In the study here, the final product influenced normal coagulant activity, strongly converting fibrinogen to fibrin, and further aggregating normal platelets without other agonists. It was also confirmed that the final product hydrolyzes synthetic substrate S-2238, which is commonly used to measure

thrombin activity, showing that this product acts as a thrombin-like enzyme. At low concentrations during the isolation this enzyme made weak fibrin clots in the absence of calcium ions and phospholipids, but acted as a strong activator for fibrin formation when these cofactors were mixed with the enzyme. Therefore, the aPTT assay was used to determine coagulant activity. It is possible that this enzyme can also activate prothrombin in the presence of these cofactors, similar to thrombin activating other coagulation factors, for example factor X.

Coagulation enzymes isolated from snake venom are classified into two groups, platelet aggregation inducers and coagulation factor activators. Thrombocytin, purified from *Bothrops atrox* snake venom, a platelet aggregation inducer, was found to have thrombin-like activity in platelet aggregation, but no prothrombin convertion activity [4]. Botrocetin purified from *Bothrops alteratus* affects aggregation of platelets only in the presence of the von Willebrand factor[5]. Recently, a platelet aggregation inducer from *Cerastes cerastes (Egyptian sand viper)* venom was described[6]. Many coagulation factor activators derived from snake venom have been reported as shown in Fig. 6. Many of these are metal proteases, not serine proteases, so the enzymes are not inhibited by protease inhibitors in the human body[1]. Batroxobin[7] purified from *Brothrops atrox* venom or Ancrod from *Agkistrodon rhodostoma* venom[8] are well known as thrombin-like serine proteases converting prothrombin to thrombin, but these enzymes release only fibrinopeptide A from fibrinogen. The final product reported here most be different from Ancrod because the fraction isolated from *Agkistrodon ralys blomhoffii* by the procedure used here did not have coagulant activator activity. Prothrombinase-like enzymes purified from snake venom have also been reported, for example Ecarin[9], obtained from *Echis carinatus* venom, can convert from prothrombin to thrombin without calcium ions of phospholipids. *Taipan snake (Oxyuranus scutellatus)* venom is also a prothrombinase-like enzyme[10], but it activates prothrombin only in the presence of these cofactors. In addition, many other enzymes activating factors V and X have been described.

Trimeresurus okinavensis venom is well-known to contain a platelet aggregation inducer, a molecular weight 400 Kd mucopolysaccaride[11]. In our study, the partial purified fraction from this snake venom with DEAE column chromatography showed amidolytic activity with S-2251, S-2366, as well as with S-2238, therefore this fraction is considered to contain enzymes like plasmin and activated protein C. The final product is a thrombin-like enzyme converting fibrinogen to fibrin as well as aggregating normal platelets. This has not previously been reported. This enzyme may be used as a hemostatic drug or as a reagent in research of fibrin clotting times.

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