Immuno-chemical Study of TSV-PA, a Specific Plasminogen Activator from the Venom of *Trimeresurus stejnegeri*

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Abstract.- Rabbit antibodies were prepared against purified TSV-PA, a specific plasminogen activator from the venom of *Trimeresurus stejnegeri*. They strongly cross-reacted with *Crotalinae* snake venoms like *Trimeresurus stejnegeri*, *Trimeresurus mucrosquamatus*, *Agkistrodon halys* and *Agkistrodon acutus*. In contrast, immunological cross-reactions with *Elapidae* snake venoms, *Ophiophagus hannah*, *Naja naja atra* were relatively lower and no cross-reactions with *Bungarus fasciatus*, *Bungarus multicinctus* venoms. On the other hand, enzymatic assays only revealed the existence of plasminogen activation activity in the venom of *Trimeresurus stejnegeri*. Except trypsin, anti-TSV-PA sera and antibodies did not cross-react with other serine proteases, such as physiological urokinase-type plasminogen activator (u-PA) and tissue-type plasminogen activator (t-PA). Anti-TSV-PA antibodies inhibited both the amidolytic activity and plasminogen activation activity of TSV-PA, but they did not inhibit the plasminogen activation activity of u-PA and t-PA.

Key words.- Snake venoms, antibodies, serine proteases

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Introduction

TSV-PA, a specific plasminogen activator from the venom of *Trimeresurus stejnegeri*, is a serine protease which specifically cuts the Arg560-Val561 bond of plasminogen and forms plasmin (Zhang et al., 1995). Sequence analysis indicates that TSV-PA is a typical snake venom serine protease which show great homology with other snake venom serine proteases, such as thrombin-like enzymes (Itoh et al., 1987; Shieh et al., 1988), protein C activator (McMullen et al., 1989), and factor V activator (Tokunaga et al., 1988). Serine proteases are widely distributed in snake venoms especially in *Crotalinae* snake venoms (Stocker, 1990). TSV-PA is the first specific plasminogen activator found in snake venoms. In this investigation, we reported the immuno-chemical study of TSV-PA.

Material and Methods

Snake Venoms were from the stock of Kunming Institute of Zoology, Chinese Academy of Sciences. TSV-PA from *Trimeresurus stejnegeri* venom is purified as described previously (Zhang et al., 1995). TSV-PA was denatured by adding β-mecaptoethanol to 1% final concentration and boiled at 100 °C for 5 min. Native TSV-PA and denatured TSV-PA were emulsified with 50% Freund adjuvant and were administered s.c. at 2 week intervals. Boosts, which were performed when the serum titers were decreasing, were achieved by injecting s.c. the same components, in the presence of incomplete Freund adjuvant. The immunization protocol was performed by the dose of administered antigen 100 μg per rabbit.

Microtitration plates (96 wells) were coated in phosphate-buffered saline (PBS) by over night incubation of antigen (5 μg/ml) and saturation was carried out with 3% BSA in PBS. Plates were washed with PBS containing 0.1% Tween-20. The solutions to be tested (100 μl/well), diluted in PBS containing 3% BSA, were incubated 1 hour at 37 °C, then washed. Peroxidase-labelled goat antibodies anti-rabbit immunoglobulins (Biosys, Compiegne) were added at a 1:2000 dilutions, incubated 1 hour at 37 °C and washed. Substrate for peroxidase (O-phenylenediamine dihydrochloride in 10 mM sodium phosphate, pH 7.3, 0.01% H_2O_2) were added and the absorbance was recorded at 405 nm with a Dynatech microplate reader.

Antibodies (IgG) were purified from the sera by double ammonium sulfate precipitations (35% final concentration), and then dialyzed against PBS. Inhibition of enzymatic activity was carried out by preincubating for 30 min at 37 °C a fixed concentration of the enzyme (5 μg/ml) with variable amounts of antibodies

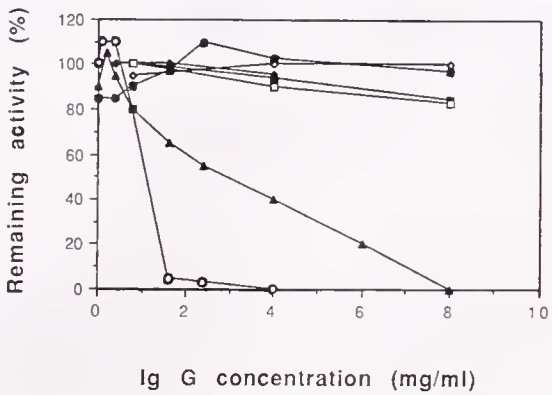

Figure 1. Inhibition of plasminogen activation by IgG against native and denatured TSV-PA. Fixed concentrations of TSV-PA, human two chains lower-molecular weight urokinase (u-PA) or human two chains tissue type plasminogen activator (t-PA) were incubated with various concentrations of antibodies against TSV-PA for 30 min in 37 °C. Then the remaining plasminogen activation activity was determined and expressed as the percentage of the original values. (O) TSV-PA test, (●) TSV-PA control, (Δ) TSV-PA test with anti-denatured TSV-PA antibodies, (◆) u-PA test, (◇) u-PA control, (■) t-PA test, (□) t-PA control.

and by testing the residual enzymatic activity. Plasminogen activation activity and amidolytic activity of the enzymes were assayed as described by Zhang et al. (1995).

Results

We immunized rabbits with purified TSV-PA, both in its native and denatured forms. Table 1 shows that the antiserum directed against native TSV-PA has a much higher ELISA titer than that directed against denatured TSV-PA, showing that native TSV-PA is much more antigenic than denatured TSV-PA. On the other hand, antiserum directed against native TSV-PA has a much lower ELISA titer when coating the plate with denatured TSV-PA, indicating that large parts of the antibodies raised against native TSV-PA are against comformational epitopes of the protein.

In the first series of experiments, we examined the immunological cross-reactions by ELISA. The anti-TSV-PA antibodies (from here, if not specially noted, anti-TSV-PA serum and antibodies means those raised against native TSV-PA) cross-reacted with TSV-PA and *Trimeresurus stejnegeri* venom. In the mean time, they cross-reacted with several other *Crotalinae* snake venoms in which we did not find the activity of plasminogen activation. From the ELISA titers, we can see, first, the immunological cross-reaction with venoms from *Crotalinae* snakes are much higher than with those from *Elapidae* snake venoms. Second,

Table 1. The titers of the antibodies against TSV-PA (a specific plasminogen activator from the venom of *Trimeresurus stejnegeri*)

Antigen	Serum	Immunoglubins
TSV-PA	10^6	200 ng/ml
denatured TSV-PA(I)	6400	2 μg/ml
denatured TSV-PA	800	25 μg/ml
TSV-PA(1)	12800	1 μg/ml
Trimeresurus stejnegeri	10^6	200 ng/ml
Trimeresurus mucrasquamatus	$5X10^4$	2 μg/ml
Agkistrodon acutus	$5X10^3$	2 μg/ml
Agkistrodon halys	$5X10^4$	2 μg/ml
Ophiophagus hannah	10^2	100 μg/ml
Vipera russelli	$5X10^3$	10 μg/ml
Naja naja atra	10^2	200 μg/ml
Bungarus fasciatus	<10	>20 mg/ml
Bungarus mulicitintus	<10	>20 mg/ml
batroxobin	10^4	2 μg/ml
trypsin	640	31 μg/ml
thrombin	<10	>20 mg/ml
u-PA	<10	>20 mg/ml
streptokinase	<10	>20 mg/ml
t-PA	<10	>20 mg/ml
plasmin	<10	>20 mg/ml

ELISA titers were defined as the serum dilution or immunoglubin concentration that produced half of the maximal response. The indicated values are the means of three independent experiments, standard deviations being 10%. (1) means immuno-cross reactions with anti-denatured TSV-PA antibodies.

even though we did not find plasminogen activity in other venoms, but in these venoms such as *Trimeresurus mucrosquamatus*, *Agkistrodon halys* and *Agkistrodon acutus* there are serine proteases which are very similar with TSV-PA in structure. This observation is also coincident with the sequence comparison of TSV-PA with other snake venom serine proteases like thrombin-like enzymes, protein C activator and factor V activator (Zhang et al., 1995). The sequence of TSV-PA shows 64% homology with thrombin-like enzyme from *Trimeresurus flavoviridius*.

We further analyzed the immunological cross-reactions of anti-TSV-PA antibodies with other serine proteases. They strongly cross-reacted with batroxobin, a thrombin-like enzyme from *Bothrops atrox* venom. In addition, they only slightly cross-reacted with trypsin (Table 1). Even TSV-PA shares the same biological activity (plasminogen activation) with physiological activators, u-PA and t-PA, there is no immunological cross-reactions among them. These results is in agreement with that the venom serine proteases which have been well studied like thrombin-like enzymes belong to trypsin-kallikrein subfamily (Itoh et al., 1987; 1988, McMullen et al., 1989).

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            1                   20                              40
TSV-PA      VFGGDECNINEHRSLVVLFN----SNG--FLCGGTLINQDWVVTAAHC------
batroxobin  VIGGDECDINEHPFLAFMYY----SPR--YFCGMTLINQEWVLTAAHC------
trypsin I   IVGGYTCPEHSVPYQVSL-------NSGYHFCGGSLINDQWVVSAAHC------
t-PA        IKGGLFADIASHPWQAAIFAKHRRSPGERFLCGGILISSCWILSAAHCFQERFP
u-PA        IIGGEFTTIENQPWFAAIYRRHR-GGSVTYVCGGSLISPCWVISATHCFID-YP

                          60                  80
TSV-PA      DSNNFQLLFGVHSKKILNEDEQTRDPKEKFFCPNRKKDDEV--DKDIMLIKLDS
batroxobin  NRRFMRIHLGKHAGSVANYDEVVRYPKEKFICPNKKKNVIT--DKDIMLIRLDR
trypsin I   YKSRIQVRLGEHNINVLEGDEQF-INAAKIIKHPNYSSWTL--NNDIMLIKLSS
t-PA        P-HHLTVILG-RTYRVVPGEEEQKFEVEKYIVHKEFDDDT--YDNDIALLQLKS
u-PA        KKEDYIVYLG-RSRLNSNTQGEMKFEVENLILHKDYSADTLAHHNDIALLKIRS

               100                         120                 140
TSV-PA      SVSNSEH----IAPLSLPSSP---PSVGSVCRIMGWGKTIPTKEIYPDVPHCAN
batroxobin  PVKNSEH----IAPLSLPSNP---PSVGSVCRIMGWGAITTSEDTYPDVPHCAN
trypsin I   PVKLNAR----VAPVALPSAC---APAGTQCLISGWGNTLSNGVNNPDLLQCVD
t-PA        DSSRCAQESSVVRTVCLPPADLQLPDW-TECELSGYGKHEALSPFYSERLKEAH
u-PA        KEGRCAQPSRTIQTICLPSM-YNDPQFGTSCEITGFGKENSTDYLYPEQLKMTV

                          160                         180
TSV-PA      INILDHAVCRTA-YSWRQVANTTLCAGILQGG------RDTCHFDSGGPLICNG
batroxobin  INLFNNTVCREA-Y--NGLPAKTLCAGVLQGG------IDTCGGDSGGPLICNG
trypsin I   APVLSQADCEAA-YP-GEITSSMICVGFLEGG------KDSCQGDSGGPVVCNG
t-PA        VRLYPSSRCTSQHLLNRTVTDNMLCAGDTRSGGPQANLHDACQGDSGGPLVCLN
u-PA        VKLISHRECQQPHYYGSEVTTKMLCAADPQWKT------DSCQGDSGGPLVCSL

                       200                 220          234
TSV-PA      I----FQGIVSWGGHPCGQPGEPGVYTKVFDYLDWIKSIIAGNKDATCPP
batroxobin  Q----FQGILSWGSDPCAEPRKPAFYTKVFDYLPWIQSIIAGNKTATCP
trypsin I   Q----LQGIVSWG-YGCALPDNPGVYTKVCNFVGWIQDTIAAN
t-PA        DGRMTLVGIISWG-LGCGQKDVPGVYTKVTNYLDWIRDNMRP
u-PA        QGRMTLTGIVSWG-RGCALKDKPGVYTRVSHFLPWIRSHTKEENGLAL
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Figure 2.Amino acid sequence comparison of TSV-PA with batroxobin, rat trypsin, human t-PA and u-PA. The sequence comparison was performed with a Clustal V software package in a computer. Sequences were from the following sources: TSV-PA, Zhang et al. (1995); batroxobin, Itoh et al. (1987); rat trypsin, MacDonald et al. (1982); human t-PA and u-PA protease domains, Pennica et al. (1983) and Steffens et al. (1982).

In second series of experiments, we tested the inhibitory effect of antibodies directed against TSV-PA on the enzymatic activities of TSV-PA, u-PA, t-PA and other proteases. Figure 1 shows the plasminogen activation inhibition by antibodies against both native and denatured TSV-PA. In agreement with the results above, anti-TSV-PA antibodies did not inhibit the plasminogen activation activity of u-PA and t-PA. On the contrast, both anti-native-TSV-PA and anti-denatured-TSV-PA antibodies inhibited plasminogen activation by TSV-PA. Further experiments expressed that only anti-native-TSV-PA antibodies inhibited the amidolytic activity of TSV-PA, but anti-denatured-TSV-PA antibodies did not. Combining the results of figure 1 and the inhibition of amidolytic activity of TSV-PA, we could find that anti-native-TSV-PA antibodies inhibited both amidolytic and plasminogen activation activities of TSV-PA, on the other hand, anti-denatured-TSV-PA antibodies did not inhibit amidolytic activity of TSV-PA, but inhibited plasminogen activation by TSV-PA. These results indicate that the inhibition of plasminogen activation by anti-denatured-TSV-PA antibodies is not caused by the block of catalytic centre but by the block of the substrate binding site. Probably, this is caused by a piece of peptide in the enzyme which involves in the binding of the substrate, especially for large protein substrate binding, and which appears as a sequential

epitope. Both anti-native and anti-denatured TSV-PA antibodies did not inhibit the thrombin-like activity of *Trimeresurus stejnegeri* and *Agkistrodon acutus* venoms.

Figure 2 shoes the sequence comparison among TSV-PA, batroxobin, rat trypsin, human t-PA and u-PA. TSV-PA shares 63% sequence identity with batroxobin (a thrombin-like enzyme), 42% with rat trypsin, but only 23% with human u-PA and 21% with human t-PA. The sequence comparison results are in agreement with the immuno-chemical studies above.

Discussion

Snake venoms contain numerous different proteases which act on blood cascade (Stocker, 1990). Biochemically, two main classes of these protease are recognized: serine proteases and mealloproteinases. For one group of venom serine proteases, they share a trypsin homologous catalytic domain and the molecular weights are usually around 25 kDa-35 kDa depending on the carbohydrate content of the enzyme. Even the sequence similarity among them are around 65-70%, for example thrombin-like enzymes (Itoh et al., 1987; Shieh et al., 1988), protein C activator (McMullen et al., 1989), factor V activator (Tokunaga et al., 1988), kallikrein-like enzyme (Komori et al., 1988) and newly determined plasminogen activator (Zhang et al., 1995), this group of venom serine protease is characterized by their highly divergent substrate specificity. Unlike their trypsin homologous, they are usually highly specific. The explanation of their highly homology primary sequence verse their highly divergent substrate specificity (for protein substrate) and the determination of their substrate binding site is extremely significant in our understanding of protein structure-function relationship and further the protein reconstruction for medical purpose in future.

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