Evaluation of arginine esterase from *Agkistrodon halys ussuriensis* venom for gene mutation in Ames test

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ABSTRACT: OBJECTIVE To investigate the gene mutagenesis of arginine esterase from *Agkistrodon halys ussuriensis* venom. **METHOD** The *Salmaonella typhimurium*/ mammalian microsomal test (Ames Test) was conducted using histidine requiring *Salmonella typhimurium* indicator strains TA97, TA98, TA100 and TA102. The arginine esterase was tested by the plate incorporation assay with and without metabolic activation system S_9 mixture at six concentrations, namely, 10.0×10^{-3}, 5.0×10^{-3}, 2.5×10^{-3}, 1.25×10^{-3}, 0.625×10^{-3} and 0.312×10^{-3} U/ mL. **RESULTS** The result demonstrated that either in the presence or absence of metabolic activation system S_9 mixture, arginine esterase was unable to induce gene mutation in these strains at above concen-

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trations and its Ames test result was negative. **CONCLUSION** If its gene mutagenesis is considered as an important factor, arginine esterase is still safe at the dosage of about 1000 times higher than clinical used dosage of the drug "Qingshuanmei" whose main component is arginie esterase.

KEY WORDS: snake venom; arginine esterase; Ames test

白眉蝮蛇毒精氨酸酯酶 Ames 试验

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摘要:目的 研究白眉蝮蛇(*Agkistrodon halys ussuriensis*)毒中精氨酸酯酶的致突变作用。方法 用鼠伤寒沙门细菌营养缺陷型突变株 TA97,TA98,TA100 和 TA102,采用平皿掺入法进行 Ames 试验,将实验分为加和不加代谢激活系统 S_9 2 组平行试验。精氨酸酯酶设 6 个浓度:10.0×10^{-3},5.0×10^{-3},2.5×10^{-3},1.25×10^{-3},0.625×10^{-3}和 0.312×10^{-3} U/mL。结果 加和不加代谢激活系统 S_9 两种条件下,精氨酸酯酶不诱发鼠伤寒沙门细菌营养缺陷型突株的回复突变。Ames 试验结果为阴性。结论 从致突变角度考虑,精氨酸酯酶在高于"蝮蛇清栓酶"(主要成分为精氨酸酯酶)临床治疗剂量约 1000 倍的条件下仍然较安全。

关键词:蛇毒;精氨酸酯酶;Ames 试验

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Introduction

There are many kinds of proteins in snake venom. There is arginine esterase in most of *Agkistrodon* snake venoms. The arginine esterase is a kind of enzyme similar to thrombin in mammal and often is called as snake venom thrombin-like enzyme. The difference between arginine esterase and thrombin is that the former can not activate XIII factor as the later does. When the arginine esterase enters human body and acts with fibrinogen, the hydrolyzed product of fibrinogen can not form stable fibrin clot. The fibrin clot is a kind of gel made up of fibrin soluble monome fibrin, which is dispersed and can be cleaned easily by phagocyte or fibrinolysin in blood. So arginine esterase has the actions of depolymerization of erythrocyte and platelet, reduction of blood viscosity, defibrination and thrombolysis. Some papers [1] reported that arginine esterase in Agkistrodon venoms had the action of blood coagulation *in vitro* and the actions of defibrination and thrombolysis in vivo. Thrombin in mammal can activate a series of blood factors related with blood coagulation and make fibrinogen changed into hinged and insoluble fibrin clot. Mammal thrombin induces blood coagulation not only *in vitro* but also *in vivo*.

Using the thrombin-like enzyme in clinical as anticoagulant drug began in 1960's at oversea. The earliest drug was named as Arvin and renamed as Ancrod. The application of the arginine esterase of snake venom in our country is later than oversea. But it develops fast. Three kinds of anticoagulants have been developed. The "Qingshuanmei"(Eliminate-embolism enzyme) was developed from the venom of *Agkistrodon halys ussuriensis* in Northeast China[2]. The "Kangshuamei"(Svate) was developed from *Agkistrodon halys Pallas* venom[3,4]. The Defibrinase was developed from *Agkistrodon acutus* venom[5]. The operative component of the three kinds of anticoagulants is arginine esterase. They all have the actions of reducing blood viscosity, increasing blood circulation, thrombolysis and preventing thrombus formation. They are very effective drugs to the diseases such as cerebral thrombosis, thromboangitis, deep phlebitis, coronary heart disease, Raynaud's disease and scleroderma, which are caused by thrombus formation. Although the main component of "Qingshuanmei" is arginine esterase. there are many other proteins which are no use at all. The drugs are not pure[6]. The results and conclusions about the drugs studied before are the comprehensive effects of all the proteins in it and not only the effects of arginine esterase. In order to develop more pure and more effective anticoagulant drugs in the future, we had purified arginine esterase from *Agkistrodon halys ussuriensis* venom. In this study, the Ames test of the pure arginine esterase was conducted using histidine requiring *Salmonella typhimuriu m* indicator strains to investigate its gene mutagenesis and to evaluate its safe properties.

Materials and methods

Bacteria strains The standard histidine-requiring *salmonell typhimurriu m* mutants TA97, TA98, TA100and TA102 were supplied by Shanghai Second Military Medical University and were conformed to posses following genotypes. They are all with histidine mutant and rfa, all with uvrB except TA102, TA97, TA98 andTA100 with plasmid of PKM101, TA102 with plasmid of PKM101 and PAQ1. Sensitivity (positive reaction) to known mutagens is as follows. 9-AA and 2-AF (with S_9) to-

ward stains TA97, TA98 and TA100, NaN_3 and MMS toward strain TA102.

Chemicals 9-AA, 2-AF and Aroclor 1254 were purchased from Pharmacia, Sweden. NaN_3, MMS and other chemicals were analytically pure. 10 mL of S_9 mixture containing 5.0 mL 0.2 M pH 7.4 BP, S_9 2.0 mL, 3.0 mL sterile water.

Preparation of arginine esterase Arginine esterase was prepared according to the method of Wang Xiaofei[7], and the enzyme activity of original solution was 0.25 U/mL. The original solution can be diluted according to test need. The proceses for purification of arginine esterase was that snake venom was purified by chromatography using DEAE-Sepharose CL-6B、Sephacryl S-200、CM-Sepharose CL-6B and hydroxyapatite in sequence. The arginine esterase was punified by SDS-PAGE.

Preparation of S_9 180±20g male Sprague-Dawley rats were injected (ip) with Aroclor 1254 (500 mg/kg dissolved in corn oil at 200 mg/mL) 5 days before sacrifice. Lives were obtained under aseptic condition at 4℃. The S_9 fraction was the 9000g supernatant of liver homogenate (1g wet liver: 30 mL PBS), Protein concentration was determined by the procedure of Lowry *et al*[8]. Sterility of the preparation is determined by showing no increasing of disturbance after adding 0.1 mL S_9 to 20 mL nutrient broth and incubating at 37℃ for 24h. The activating efficiency of S_9 was verified using indirect mutagen 2-AF.

Determination the enzyme activity of arginine esterase α-p-Tosyl-L-arginine methyl ester (TAME) can be hydrolyzed by arginine esterase. TAME can react with hydroxylamine and produce a kind of colourfull substance which may be estimated quantitatively. The activity of arginine esterase was estimated by calculating the decrease of TAME[9].

Bacteria culture 10μL of the test strains which were preserved at -80℃ was taken and incubated in nutrient broth containing 0.5% NaCl in a 37℃ shaking bath for about 10h and finally reached to (1.0~10.0)×10^8 cells per mL.

Reverting test The concentration of the arginine esterase was indicated by means of enzyme activity (U). The concentrations of arginine esterase in the experiment were 10.0×10^{-3}, 5.0×10^{-3}, 2.5×10^{-3}, 1.25×10^{-3}, 0.625×10^{-3} and 0.312×10^{-3} U/mL. Each concentration of arginine esterase, spontaneous reverting negative control (without arginine esterase) and 2-AF positive indicator were performed with six plates. Six plates were divided into two groups. The first one contained three plates with 0.3 mL S_9 mixture. The second one contained three plates without S_9 mixture. TA97 with 9-AA, TA100 with NaN_3 and TA102 with MMC were positive controls and were performed with three plates without S_9 mixture. All the plates were incubated at 37℃ for 48h and then the reverting colonies in treated groups and control groups were counted and the mean value of three plates was calculated. The estimation standard[10] of reverting test was as follows. If the number of reverting colonies in groups treated with arginine esterase exceeded 2 folds (including 2 folds) of the number of their corresponding spontaneous reverting colonies in any histidine requiring *Salmonella typhimurium* indicator strains, the test results were positive and if the number did not exceed 2 folds, the test results were negative.

Results

The results of Ames test were shown in the following table. The test results revealed that, either in the presence or absence of S_9 metabolic activator, the reverting colonies in the groups treated with arginine esterase did not exceed 2 fold of their corresponding spontaneous reverting colonies in the four test strains and the positive controls exceeded it. From the results, we concluded that arginine esterase is unable to induce gene mutation in these strains at above concentrations, and its Ames test result is negative. If its gene mutagenesis is considered as an important factor, arginine esterase is still safe at the dosage of about 30~1000 times higher than clinical used dosage of the drug "Qingshuanmei" whose main component is arginie esterase.

Tab 1 The results of Ames test on arginine esterase

表 1 精氨酸酯酶的 Ames 试验结果

Treatment	Number of histidine + relevant/plate (mean)								Judgement
	TA97		TA98		TA100		TA102		
	-S_9	+S_9	-S_9	+S_9	-S_9	+S_9	-S_9	+S_9	
Blank control	163.0	167.3	158.0	157.0	156.3	159.0	149.3	158.0	-
Positive control (9-AA 0.3μg/mL)	2018.3[1)]								+
Positive control (2-AF 3.0μg/mL)			87.0[1)]	3089.0[1)]					+
Positive control (NaN_3 0.3μg/mL)					650.0[1)]				+
Positive control (MMS 3.0μg/mL)							4000.1[1)]		+
Arginine esterase (10.00×10^{-3} U/mL)	167.0	168.3	30.3	29.9	195.7	201.0	254.0	245.3	-
Arginine esterase (5.00×10^{-3} U/mL)	158.3	167.0	26.0	34.3	187.0	215.3	235.3	231.7	-

Treatment	Number of histidine + relevant/plate (mean)								Judgement
	TA97		TA98		TA100		TA102		
	- S_9	+ S_9	- S_9	+ S_9	- S_9	+ S_9	- S_9	+ S_9	
Arginine esterase (2.50×10^{-3} U/mL)	168.0	165.0	24.3	30.7	178.0	198.0	251.0	231.1	-
Arginine esterase (1.25×10^{-3} U/mL)	189.3	176.0	30.0	28.0	208.3	206.7	216.7	218.0	-
Arginine esterase (0.63×10^{-3} U/mL)	159.0	160.3	25.7	31.0	187.0	176.0	198.3	200.0	-
Arginine esterase (0.31×10^{-3} U/mL)	169.0	156.3	36.3	30.7	213.0	189.3	189.0	218.3	-

Note: 1) Compared with negative control, $P<0.01$; 9-AA, 9-aminoacridine; 2-AF, 2-aminoflurene; NaN_3, sodium azide; MMS, methyl-methylsulfate.

注: 1) Compared with negative control, $P<0.01$; 9-AA, 9-氨基丫啶; 2-AF, 2-氨基芴; NaN_3, 叠氮化钠; MMS, 甲基磺酸甲酯。

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