ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 1999 Oct; 20 (10): 944 – 947
http://www.simm.ac.cn; www.chinainfo.gov.cn/periodical

Fibrinogenolytic properties of natrahagin (a proteinase from cobra venom) and its effect on human platelet aggregation

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KEY WORDS cobra venoms; protein-glutamine gamma-glutamyltransferase; platelet aggregation; ristocetin; thrombin; platelet membrane glycoproteins

ABSTRACT

AIM: To study the fibrinogenolytic properties of natrahagin and its effect on platelet aggregation. **METHOD**: SDS-PAGE, fibrinogenolytic activity assay, platelet aggregation. **RESULTS**: Upon incubation of fibrinogen with natrahagin at the ratio of 50 : 1 (w/w), A_α-chains of fibrinogen were almost completely hydrolyzed in 5 min; however, at least 6 h was needed for the complete degradation of γ-chains. Fibrinogenolytic activity of natrahagin was 0.349 ± 0.044 $g \cdot min^{-1} \cdot g^{-1}$ as determined by its ability to reduce the clottable fibrinogen. On the other hand, natrahagin concentration-dependently inhibited platelet aggregation induced by ristocetin in platelet-rich plasma and thrombin (80 $U \cdot L^{-1}$) in washed platelets with IC_{50} (95 % confidence limit) of 56 (40 – 79) and 3.3 (1.4 – 8.0) $mg \cdot L^{-1}$. No inhibitory effect was found on collagen- and ADP-induced platelet aggregation even when the dose of natrahagin reached 200 $mg \cdot L^{-1}$. **CONCLUSION**: Natrahagin is an α, γ-fibrinogenase with an inhibitory effect on platelet membrane glycoprotein Ib (GPIb)-dependent platelet aggregation.

INTRODUCTION

Biological effects of proteinases from cobra venoms are seldom reported, especially those acting on blood coagulation and platelet functions[1]. Although evidence has indicated that Chinese cobra (*Naja naja atra*) venom can effectively inhibit the aggregation of platelets and the formation of thrombosis[2], the components responsible for these remain unknown. Recently, two proteinases from different cobra venoms, mocarhagin and F1, were reported to inhibit platelet aggregation and degrade fibrinogen[3-5]. By using similar purification methods for mocarhagin, a proteinase designated as natrahagin was purified from Chinese cobra venom in our laboratory. The goal of this study was to investigate its fibrinogenolytic properties and its effect on platelet aggregation.

MATERIALS AND METHODS

Reagents and instruments Crude cobra (*N naja atra*) venom was provided by the Institute of Snake Venoms of Guangzhou Medical College. Natrahagin was purified to homogeneity as judged by SDS-PAGE from the crude venom according to the method reported by De Luca M, *et al*[6], except that Sephedex G-150 was used instead in gel filtration. Collagen, ADP, α-thrombin, ristocetin and bovine fibrinogen were purchased from Sigma, USA. Aggregometer (Model SPA-4) was made in Shanghai, China; Mini-Protean Ⅱ electrophoresis system, Bio-Rad, USA; Phenol reagent was a gift from Professor CHANG Hou-Chang of the Pharmacological Department of the First Military Medical University.

Specific cleavage of fibrinogen This was shown on SDS-PAGE of 12.5 % polyacrylamid gels. After incubation at 37 ℃ for 5 min separately, natrahagin 7.5 $g \cdot L^{-1}$ 64 μL and bovine fibrinogen 24 $g \cdot L^{-1}$ 1.0 mL, both in TS (Tris-HCl buffer 0.01 mol $\cdot L^{-1}$, pH 7.4, containing NaCl 0.15 mol $\cdot L^{-1}$), were mixed and incubated at 37 ℃. At 5 min, 30 min, 3 h, 6 h, and 24 h, a sample of 50 μL was withdrawn. The sample was denatured and reduced by

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Received 1998-09-28 Accepted 1999-04-01

immediately adding to the sample buffer (containing 2 % SDS and 5 % mercaptoethanol) and heating in boiling water for 3 min.

Fibrinogenolytic activity This assay was carried out according to the method reported by Huang *et al*[7] with slight modification. Briefly, 0.8 % fibrinogen solution 0.2 mL and natrahagin solution 50 μL, both in TS, were mixed and incubated at 37 ℃ for various intervals. To the above mixture, imidazol saline buffer 3.8 mL containing edetic acid 2.5 mmol·L^{-1} and thrombin 2.6 kU·L^{-1} was added. At least 60 min was allowed to form fibrin clot before it was removed. The clot was washed with sufficient distilled water, then the water was sucked out with filter paper before it was put in a tube containing 1 mL 10 % NaOH and boiled for 30 min. To the tube, 1 mL water and 3 mL 20 % Na_2CO_3 was added, mixed and finally 1 mL of phenol reagent. Twenty minutes later, the absorbance was measured at 540 nm.

Platelet preparation Blood of 7 healthy voluntary male donors, aged from 21 a to 31 a, who had not taken any drugs for at least 2 wk, was withdrawn from venae mediana cubiti (9 mL from each) and collected into sodium citrate (129 mmol·L^{-1}, 9:1 vol/vol). Platelet-rich plasma (PRP) was obtained by centrifuging at 200 × *g* for 10 min. After the removal of PRP, the remnant blood was then centrifuged at 2000 × *g* for the additional 20 min, and the platelet-poor plasma (PPP) was collected and mixed with PRP to give a platelet count about $3 \times 10^{11} \cdot L^{-1}$.

Washed platelet preparation PRP was mixed with TS containing edetic acid 0.02 mol·L^{-1} in a ratio of 4:1 (vol/vol), and centrifuged at 2000 × *g* for 10 min. The platelet pellets were washed twice with TS containing glucose 0.01 mol·L^{-1}, then the platelet count was adjusted to $5 \times 10^{11} \cdot L^{-1}$.

Platelet aggregation PRP or washed platelet suspension 180 μL was placed in a curvette and stirred with natrahagin or control solution 10 μL at 37 ℃ for 6 min, then aggregating agent 10 μL was added (final concentration: ADP 10 μmol·L^{-1}, collagen 200 mg·L^{-1}, ristocetin 1.2 mg·L^{-1}, thrombin 80 U·L^{-1}). The inhibition rate of platelet aggregation was calculated as follows: Inhibition (%) = $(C_{max}-N_{max})/C_{max} \times 100$ % (C_{max} and N_{max} represent the maximal aggregation rates of control and natrahagin group respectively).

Statistical analysis Data were expressed as $\bar{x} \pm s$ and compared with *t* test.

RESULTS

Specific cleavage of fibrinogen At the ratio of 1:50 (w/w), natrahagin almost completely degraded A_α-chains of fibrinogen in 5 min. Slow degradation of γ-chains became more apparent as incubation prolonged, and the band of γ-chains was barely seen at 6 h. At least 7 new bands could be detected by naked eyes at 24 h apart from β-chains (Fig 1).

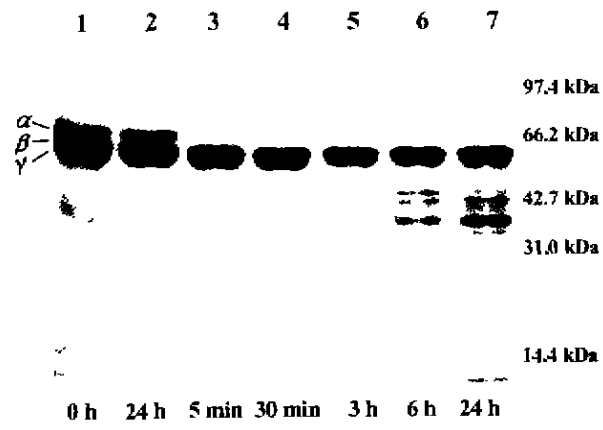

Fig 1. SDS-PAGE analysis of reduced bovine fibrinogen after incubation with natrahagin. Line 1 and 2, control; Line 2 to 7, groups treated with natrahagin.

Fibrinogenolytic activity Natrahagin possessed a fibrinogenolytic activity in a concentration- and time-dependent manner. The specific activity of natrahagin on reducing clottable fibrinogen was estimated to be (349 ± 44) mg·$min^{-1} \cdot g^{-1}$ based on the result of experiments repeated for four times (Fig 2).

Platelet aggregation Natrahagin concentration-dependently inhibited ristocetin- and thrombin-induced platelet aggregation. IC_{50} (95 % confidence limit) was 56 (40 – 79) mg·L^{-1} and 3.3 (1.4 – 8.0) mg·L^{-1} determined by the results of 6 and 5 experiments respectively. The same doses of natrahagin for ristocetin-induced platelet aggregation, *ie* 25 – 125 mg·L^{-1}, were also applied in ADP- and collagen-induced platelet aggregation in PRP, however, natrahagin exhibited no inhibitory effect, even when the

ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 1999 Oct; 20 (10)
E-mail aps@server.shcnc.ac.cn Phn/Fax 86-21-6474-2629

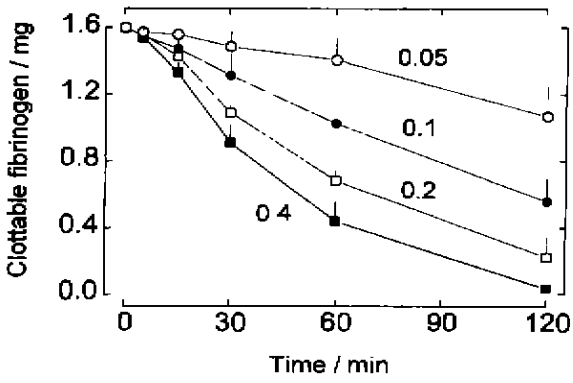

Fig 2. Fibrinogenolytic effect of natrahagin (mg · L^{-1}) on reduction of clottable bovine fibrinogen. $n = 4$ experiments. $\bar{x} \pm s$.

dose was increased to 200 mg · L^{-1} (Fig 3).

DISCUSSION

The results in this study indicated that natrahagin was mainly an α-fibrinogenase. However, the degradation of A_α-chains by natrahagin did not render fibrinogen incoagulable. SDS-PAGE showed that natrahagin almost completely hydrolyzed A_α-chains of fibrinogen in 5 min at the ratio of 1 : 50 (w/w), but the coagulability of remnant fibrinogen was little affected as manifested by fibrinogenolytic activity assay. This observation paralleled that of proteinase F1 from *Naja nigricollis*, which acted on only A_α-chains of fibrinogen[4]. Unlike proteinase F1, natrahagin possessed weak γ-fibrinogenolytic activity. And this might be responsible for the gradual lost of coagulability of fibrinogen as incubation prolonged. What effect natrahagin might exert on the formation of fibrin clot or thrombosis had not yet been explored. Some evidence had indicated that, due to the loss of one of the two complementary polymerization sites required for side-by-side association of fibrin protofibrils, fibrin clot formation was defective after fibrinogen was treated with α-fibrinogenase[8].

GPIb functions as a receptor for von Willebrand Factor (vWF) and thrombin. It has been well established that ristocetin induces platelet aggregation by bridging GPIb with vWF[9]. Natrahagin inhibited platelet aggregation in PRP induced by ristocetin suggested that it blocked either GPIb or vWF. Inhibition by natrahagin of thrombin-induced platelet aggregation in washed platelets, where vWF was eliminated, indicated that natrahagin acted on GPIb. As for ADP- and collagen-induced platelet aggregation, natrahagin did not exhibit any significant inhibitory effect, although natrahagin cleaved A_α-chains readily.

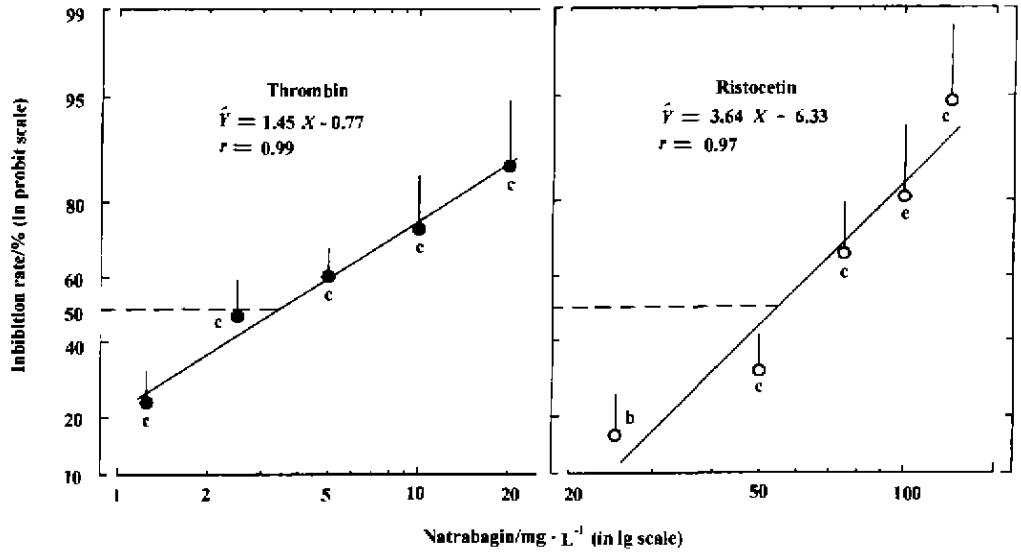

Fig 3. Inhibition by natrahagin for platelet aggregation induced by thrombin in washed platelets (n = 5 experiments) and ristocetin in platelet-rich plasma ($n = 6$ experiments). $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* corresponding controls.

This result agreed with the hypothesis that γ-chains, rather than A_α-chains, were the major sites responsible for the interactions between fibrinogen and platelets[10]. The ability of natrahagin to hydrolyze γ-chains was so weak that, even at high dose, it did not reduce the aggregation rate of platelets.

Another proteinase that acted on GPIb from cobra venoms was mocarhagin, which was purified from *Naja mocambique mocambique* venom, and reported to cleave the binding site for vWF and α-thrombin in GPIb[3] and P-selectin glycoprotein ligand receptor (PSGL-1)[6]. As we used similar purification methods for natrahagin with those for mocarhagin, and as both proteinases inhibit GPIb-mediated platelet aggregation, it is reasonable to assume that natrahagin bear similar proteolytic properties against GPIb and PSGL-1. And these will be determined in the near future.

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944 – 947

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眼镜蛇毒蛋白酶 natrahagin 水解纤维蛋白原的特性及对人血小板聚集的影响

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natrahagin

关键词 眼镜蛇毒类；蛋白质-谷氨酰胺 γ-谷氨酰转移酶；血小板聚集；利托菌素；凝血酶；血小板膜糖蛋白类

目的：研究 natrahagin 水解纤维蛋白原的特性及其对血小板聚集的影响. **方法**：SDS-PAGE. 水解纤维蛋白原活性测定，血小板聚集实验. **结果**：Natrahagin 与纤维蛋白原以 1∶50 (w/w)孵育，5 min 内 A_α-链几乎完全降解，γ-链的完全降解则至少需 6 h；其水解可凝固纤维蛋白原的活性为 0.349 ± 0.044 $g \cdot min^{-1} \cdot g^{-1}$. Natrahagin 浓度依赖性地抑制利托菌素对富血小板血浆和凝血酶(80 $U \cdot L^{-1}$)对洗涤血小板的聚集反应，IC_{50}(95 %可信限)分别为 56 (40 – 79)和 3.3 (1.4 – 8.0) $mg \cdot L^{-1}$. 但即使 natrahagin 达 200 $mg \cdot L^{-1}$，对 ADP 和胶原诱导的血小板聚集仍无抑制作用. **结论**：Natrahagin 是一种 α，γ-纤维蛋白原溶解酶，可选择性抑制血小板膜糖蛋白 Ib 介导的血小板聚集.

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