Affinity-Purification of Fibrinogenase with High Proteolytic Activity from *Agkistrodon halys* (Chinese) Venom

Biao Ma¹², Ying Zhang¹, Dan Wu¹, Jianping Jia³, Wentao Xu¹, and Yunbo Luo¹

¹College of Food Science & Nutritional Engineering, China Agricultural University Beijing 100083, China, ²Saisheng Pharmaceutical Company, Beijing 100176, China, and ³Department of Neurology XuanWU Hospital, Capital University of Medical Sciences, Beijing 100069, China

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To purify and characterize the fibrinogenase with high proteolytic activity from *Agkistrodon halys* (Chinese) Venom. Monoclonal antibodies against fibrinogenase were prepared and a novel affinity chromatography equipped with a monoclonal antibody against fibrinogenase was developed and applied for the purification of fibrinogenases. The purified fibrinogenase was identified by fibrinolytic activity assay, and antithrombosis activity assay. HPLC chromatography and SDS-PAGE analysis demonstrated the uniformity and purity of the purified fibrinogenase. In comparison with a conventional A-50 chromatography method, affinity-purified fibrinogenase showed higher activity (3631 U mg⁻¹ vs 501 U mg⁻¹). In addition, the physiological activity of the fibrinogenase both in vitro and ex vivo showed the purified fibrinogenase can specifically degrade β-, γ-fibrinogen and has a high anti-thrombotic activity. In conclusion, the purified fibrinogenase by affinity column were shown to be homogeneous and showed a high and specific proteolytic activity against β-chains of fibrinogen molecules and antithrombosis activity.

Key words: Fibrinogenase, Affinity-Purification, *Agkistrodon halys* (Chinese) venom, β-chains of fibrinogen, Fibrinolytic activity, Antithrombosis activity

INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of mortality worldwide today. Thrombosis, including prosthetic heart valves, atrial fibrillation, and deep vein thrombosis, is a big concern for patients, who have heart disease. Since thrombosis is induced by the activation of coagulation system and platelet system, anticoagulation or anti-platelet agents have been recommended to prevent formation and enlargement of thrombus. The agents activating the fibrinolytic system have been clinically applied for the antithrombosis. The main factor in fibrinolytic system is the plasmin, which is activated from the plasminogen by the plasminogen activator (PA). The plasminogen activator is mainly used for the thrombolytic therapy. There are two types of PA. One is the non fibrin-specific PA such as streptokinase (SK) and two-chain urokinase-type PA (tcu-PA, urokinase), the other is the fibrin-specific PA such as tissue-type PA (t-PA), and single-chain urokinase-type PA (scu-PA) (Ueshima and Matsuo, 2006).

The two types of PA have been used in clinic since 1960s, but they have drawbacks: firstly, they have side effects including hemorrhage; secondly, their *in vivo* half lives are short. Now, scientists are searching for non-hemorrhagic agents with a long half life. One resource of antithrombosis is snake venom, which inhibits blood clotting by several mechanisms, such as proteases that degrade fibrinogen and disintegrins that inhibit platelet aggregation by integrin binding.

Snake venoms are complex mixtures, which contain many enzymes and inhibitors involved in blood coagulation or platelet aggregation (Stocker, 1990). These proteins can be classified into several categories depending on their hemostatic action. Most venoms contain thrombin-like enzymes that clot fibrinogen (Holleman and Weiss, 1976; Itoh et al., 1987), fibrinolytic enzymes that degrade fibrinogen/fibrin (Markland, 1991), coagulation activators, and coagulation inhibitors. The clinical application of the
fibrinolytic factions from snake venom has been studied. As a member of snake venom metalloproteases, it contains Zn$^{2+}$ and lacks arginine esterase activity of fibrinogenases with specificity for the $\alpha$-chain of fibrinogen. They have a molecular weight of ranging 20,000 to 26,000 and disulfide bonds are important for their activity. They preferentially degrade the $\alpha$-chain of fibrinogen. Fibrinogenases have been purified from snake venoms of *Agkistrodon halys* (Ouyang et al., 1983; Fujimura et al., 1995); *Trimeresurus macropsquamatus* (Hung and Chou, 1994); *Bothrops jararacussu* (Lobo de Araujo et al., 1998), and *Lachesis muta rhombeata* (De-Simone et al., 2005). In order to meet the needs of clinical use, we developed a combinational purification method (DEAE-Sephadex plus antibody-affinity chromatography) and obtained a fibrinogenase with high purity and high antithrombosis activity.

Materials and Methods

Materials

The dry powder from venoms of *Agkistrodon halys brevicaudus stejneger*, *Agkistrodon ussuriensis emellaov* snake, *Trimeresurus jerdonii* snake, *Agkistrodon acutus* snake (Chinese), *Bothrops jararaca* snake, and vampire bats were obtained from Qingyuan County, Liaoning Province, China. Agarose gel was obtained from Pharmacia (Pharmacia, Uppsala, Sweden). Complete and non-complete Freund’s Agent (CFA/NCFA), polyethylglycerol 8000 (PEG 8000), and FCS (fetal calf serum) was obtained from Sigma (Sigma, St Louis, USA). New Zealand White bats were included in the assay.

Procedures for immunization and screening

Two grams of crude powder of the snake venom were dissolved in 50 mM Tris-HCl buffer (pH 7.6). The suspension was centrifuged at 6,000 rpm for 10 minutes at 4°C. The supernatant was applied to a DEAE-sephadex A 50 column pre-equilibrated with Tris-HCl buffer. After washing with Tris-HCl buffer, the sample was eluted with a linear gradient of 0–0.6 M NaCl in Tris-HCl buffer. The faction with highest fibrinolytic activity was ultra-filtered and lyophilized.

Preparation of monoclonal antibodies against fibrinogenase

Seven-week-old female BALB/c mice were immunized subcutaneously with 50 mg purified fibrinogenase protein emulsified with complete Freund’s adjuvant (Sigma, Saint Louis, USA) in 0.2 mL on day 0 and boosted twice on days 14 and 28 with the same dose of antigens emulsified with incomplete Freund’s adjuvant (Sigma, Saint Louis, USA), respectively. Three days before fusion, mice were injected (i.v.) with 0.2 mL of a fibrinogenase stimulator. Then mouse spleen cells were collected according to the method described previously (Koher and Mdstem, 1975). Briefly, the prepared spleen cells were fused with mouse myeloma cells of the SP2/0 cell line with 50% (w/v) PEG (Chan et al., 2006). Cells were seeded into 96-well cell culture plates and incubated at 37°C under 5% CO$_2$. From the 1st to the 4th day, cells were cultured in HAT medium containing 15% FCS; from 5th to 10th day, cells were cultured in HT medium; finally cells were cultured in RPMI-1640 medium containing 10% FCS. The screening and tittering of the monoclonal antibodies were performed by ELISA method (Xu et al., 2005).

Purification of monoclonal antibody

Ascites fluid was dialysed against 50 mM-Tris/HCl, pH 7.5 at 4°C and subjected to ion-exchange chromatography on Q Sepharose (Pharmacia, Uppsala, Sweden). Bound proteins were eluted with a linear gradient of 0-0.5 M-NaCl in the above buffer. Fractions containing monoclonal antibody were identified by the ELISA, and purity was assessed by SDS-gel electrophoresis (Laemmli, 1970).

Determination of the specificity and reactivity of monoclonal antibodies

To determine their specificity, MAbs were first analyzed by Western blot with the crude fibrinogenase protein and the purified fibrinogenase proteins from venoms (Sambrook and Russell, 2001). The specificity and reactivity of the MAbs against fibrinogenase were also determined by the ELISA using the purified fibrinogenase as antigen. As the fibrinogenase has analogs, the crude fibrinogenase or venom from *Agkistrodon halys* snake (Chinese), *Agkistrodon ussuriensis emellaov* snake, *Trimeresurus jerdonii* snake, *Agkistrodon acutus* snake (Chinese), *Bothrops jararaca* snake, and vampire bats were included in the assay. Briefly, ELISA plates were coated overnight with purified fibrinogenase (4 ug per well) at 4°C and blocked for 1 h with 5% non-fat milk at 37°C, followed by three washes with PBST. The plates were then incubated with hybridoma supernatant at 37°C for 1 h. The bound MAbs were detected with HRP-conjugated rabbit anti-mouse IgG, and developed with o-phenylenediamine dihydrochloride (OPD). Each sample was analyzed in duplicate.

Preparation of monoclonal antibody affinity column

A monoclonal antibody against the fibrinogenase was...