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Expression, purification and characterization of recombinant plasminogen activator from *Gloydius brevicaudus* venom in *Escherichia coli*

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ABSTRACT

The plasminogen activator (PA) in snake venom, a serine protease, can convert plasminogen to active plasmin, indirectly causing the degradation of fibrin. It is difficult to purify sufficient snake venom PA (SV-PA) for clinical applications due to the low SV-PA content in venom. The gene encoding PA was obtained from the venom gland of *Gloydius brevicaudus* using RT-PCR with primers designed according to the N-terminal amino acids of *G. brevicaudus* venom PA (GBV-PA), was cloned into the prokaryotic expression vector pET-42a, and recombinant GBV-PA (rGBV-PA) was expressed via Isopropyl-β-D-1-Thiogalactopyranoside (IPTG) induction. Like human tissue PA, the purified renatured rGBV-PA could significantly reduce the rabbit plasma euglobulin lysis time (ELT) and prevent thrombus formation in the inferior vena cava of rats. Within the dosage range, the dosage and effects were positively correlated.

Introduction

Thrombotic diseases, such as cerebral infarction, myocardial infarction and deep vein thrombosis, represent serious health concerns. Thrombolysis is considered the most effective treatment for thrombotic diseases; however, current thrombolytic agents may induce adverse effects, such as re-embolization and bleeding. It is therefore necessary to develop more efficient thrombolytic drugs. Snake venom, traditionally used in Chinese medicine, has various effects such as anti-tumor, nerve protection, hemostatic and anti-thrombotic properties [1–6]. Gabonase and plasmin represent venom-based anti-thrombotic drugs currently in clinical use. The anti-thrombotic activity of gabonase induces a reduction of fibrin concentration and promotes the release of tissue plasminogen activator ([1]tPA) which activates plasmin, whereas plasmin promotes thrombolysis by the direct degradation of fibrinogen. However, gabonase can form small thromboses during thrombolysis and plasmin can easily cause bleeding during circulating thrombolysis. In 2007, we successfully isolated and purified the plasminogen activator from *Gloydius brevicaudus* venom (GBV-PA) and its N-terminal amino acids were found to be MALIRVLANLLILQLSY [7].

SV-PA can activate plasminogen through a similar mechanism to tPA, by specifically cleaving the peptide bond between Arg561 and Val562 in plasminogen, converting it into active plasmin. Some researchers have expressed rt-PA and its mutants in *Escherichia coli* [8–10].The GBV-PA content of snake venom is only 0.5%, and the clinical application of SV-PA is limited by the high cost of ecological resources, difficulties in product purity and the high cost of separating PA from snake venom. Here, we designed primers according to the N-terminal amino acids of GBV-PA, and employed RT-PCR using total genomic RNA isolated from the venom gland of *G. brevicaudus* as a template to isolate the gene encoding GBV-PA. The gene was cloned into the prokaryotic expression vector pET-42a and expressed by Isopropyl-β-D-1-Thiogalactopyranoside (IPTG) induction in order to purify active recombinant GBV-PA (rGBV-PA).

Materials and methods

Reagents

The restriction enzymes EcoRI and XhoI, *Taq* DNA polymerase and T4 DNA Ligation kit were purchased from Invitrogen (CA, USA). Easy-A High-Fidelity PCR cloning enzyme was purchased from Stratagene (NY, USA). Trizol total RNA isolation kit and PCR kit were purchased from Promega (WI, USA). Primers were ordered from Shanghai Sangon Biotechnology (Shanghai, China). Isopropyl-β-D-1-Thiogalactopyranoside (IPTG) was purchased from Sigma

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[1] *Abbreviations used:* SV-PA, snake venom plasminogen activator; ELT, euglobulin lysis time; tPA, tissue plasminogen activator.

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(CA, USA). The rGBV-PA antibody was prepared in our lab. Goat anti-equine IgG(H + L) was purchased from Southern Biotech (AL, USA). The tPA (Actilyse) was purchased from Boehringer Ingelheim Pharma (Ingelheim, Germany, Lot No.: 082652). Thrombin was purchased from Beijing No.1 Biochemical Pharma (Beijing, China, Lot No.: 101104). Plasmid pET42a, plasmid pGEM-T Easy, JM109 and BL21 (DE3) *E. coli* strains were purchased from Novagen (Merck, Germany) and the BCA kit was purchased from Shanghai Ricky (Shanghai, China). Other reagents were analytical grade.

Animals

G. brevicaudus were purchased from a snake farm in Yiwu, Zhejiang, China, and weighed 80–180 g. The glands weighed 0.04–0.21 g and were separated from the anesthetized snakes and stored in liquid nitrogen. SD rats were obtained from SLAC (Shanghai, China; certificate number SCXK 2007-0005). Rabbits were provided by the Animal Laboratory of Fujian Medical University. Prior to the test, all animals were raised with granulated feed and water. The clean conditions included a room temperature of 23.0 ± 2 °C, humidity of 56%, 12:12 h interrupted lighting, and 24 h regular UV disinfection and ventilation without strong light and sound stimulation.

Plasmid construction

Total RNA was extracted from 100 mg venom glands of *G. brevicaudus* using Trizol, the OD value was measured at 260 nm/280 nm, and the purity and concentration of the total RNA were calculated. For reverse transcription, 2 μg total RNA was used. The target gene was amplified using primers designed according to the N-terminal amino acid sequence of GBV-PA and the restriction sites for XhoI and EcoRI were included at the ends of the primers. The forward and reverse primers were 5′-GGAATTCATGGCGCTGAT-CAGAGTGC-3′ and 5′-CCGCTCGAGTACATGGGGGGCAAGTCACAG-3′, respectively. The recognition sites for XhoI and EcoRI are underlined. His-tag and GST-tag are N-terminals. Appropriate β-actin reference primers were also used. The total PCR reaction volume was 50 μL including 2 μL of the reverse transcription solution as a template. PCR conditions were as follows: initial denaturation at 94 °C for 5 min, then 35 cycles of denaturation at 94 °C for 30 s, primer annealing at 50 °C for 30 s, followed by primer extension at 72 °C for 1 min. A final extension step proceeded at 72 °C for 7 min. PCR products (5 μL) were electrophoresed on a 1.2% agarose gel, treated with ethidium bromide and visualized by UV illumination. The purified *rGBV-PA* gene was cloned into the pGEM-T Easy vector for sequencing. After sequence confirmation, *rGBV-PA* was excised from the cloning plasmid pGEM-rGBV-PA using EcoRI, and then inserted into the expression vector pET-42a, and digested with XhoI and EcoRI to construct the plasmid pET-rGBV-PA. The cloning and expression plasmids were successfully reconstructed by double digestion identification and sequence analysis.

Expression, purification and renaturation of rGBV-PA

The recombinant plasmid pET-rGBV-PA was transformed into 100 μL of *E. coli* BL21 (DE3) cells and incubated in 10 mL LB culture medium containing 30 μg/mL kanamycin with shaking at 250 rpm at 37 °C overnight. Then 500 mL LB containing 30 μg/mL kanamycin was inoculated with 5 mL of the overnight culture and shaken at 250 rpm at 37 °C for 3 h until the OD_{600} measured 0.6. IPTG was added up to a final concentration of 1.0 mM, and the bacterial solution was continuously shaken at 250 rpm at 30 °C for 4 h. The bacterial culture was centrifuged at 5000 rpm for 5 min before the supernatant was removed, suspended in PBS, and sonicated on ice for 30 min using the following conditions: 200 W for 10 s with 10 s intervals, repeated 10 times. The suspension was always kept on ice. The solution was centrifuged at 12,000 rpm at 4 °C for 20 min, and the supernatant and precipitate were collected separately; 50 μL supernatant and precipitant were mixed with 50 μL 12× SDS loading buffer, boiled for 5 min and stored at −20 °C for SDS–PAGE.

The inclusion bodies were resuspended in 50 mL denaturation lysis buffer and incubated in an ice bath for 3 h so that the proteins were thoroughly resuspended. The insoluble cellular components were removed after centrifugation at 12,000 rpm at 4 °C for 20 min, and the supernatant was filtered using a 0.45 μm membrane filter to obtain the rGBV-PA inclusion body solution. Ni-affinity chromatography was used to purify rGBV-PA. Ni-affinity chromatography resin was loaded onto a 10 × 14 mm column with a column volume (cv) of 8 cm^3, equilibrated using 4 cv of charge buffer (100 mM $NiSO_4$), and re-equilibrated using 3 cv of binding buffer II (0.5 M NaCl, 20 mM Tris–HCl, 8 M urea, pH 8.0). The pretreated inclusion body solution (sample volume, 20 mL) was loaded onto the column at a flow rate of 0.2 mL/min, and was flushed with binding buffer (0.5 M NaCl, 20 mM Tris–HCl, pH 8.0) until a baseline UV reading was reached. Then non-specifically bound proteins were washed with elution buffer (0.5 M NaCl, 20 mM Tris–HCl, 8 M urea, 30 mM imidazole, pH 8.0), and the target proteins were eluted with elution buffer (0.5 M NaCl, 20 mM Tris–HCl, 8 M urea, 1 M imidazole, pH 8.0). The eluted components were collected for SDS–PAGE.

Purified rGBV-PA was renatured by dialysis. The dialysis cassette containing the purified rGBV-PA solution was successively placed into beakers either containing 400 mL dialysis buffer I (0.5 M NaCl, 20 mM Tris–HCl, 8 M urea, pH 8.0), dialysis buffer II (0.5 M NaCl, 20 mM Tris–HCl, 6 M urea, pH 8.0), dialysis buffer III (0.5 M NaCl, 20 mM Tris–HCl, 4 M urea, pH 8.0), dialysis buffer IV (0.5 M NaCl, 20 mM Tris–HCl, 2 M urea, pH 8.0), dialysis buffer V (0.5 M NaCl, 20 mM Tris–HCl), dialysis buffer VI (0.9% NaCl, 20 mM Tris–HCl, 0.1 mM Oxidized Glutathione[GSSG], 0.9 mM Glutathione[GSH], pH 8.0), dialysis buffer VII (0.2% NaCl, 10 mM Tris–HCl, 0.1 mM GSSG, 0.9 mM GSH, pH 8.0) and dialysis buffer VIII (0.045% NaCl, 50 mM Tris–HCl, 0.1 mM GSSG, 0.9 mM GSH, pH 8.0), and stirred at 4 °C for 4 h. The solutions in the dialysis cassettes were combined and filtered using 0.45 μm membranes to ensure the solutions were sterile so that active rGBV-PA was recovered. Active rGBV-PA was purified by chromatography in a Mono-Q anion exchange column as Zhang Y described [14].

Physical and chemical properties of rGBV-PA protein

The protein concentration was determined using a BCA kit. The N-terminal amino acids of rGBV-PA were sequenced by GeneCore (Shanghai). The molecular weight of rGBV-PA was determined by mass spectrometry.

Western blot analysis

After SDS–PAGE analysis, the whole bacterial resuspension and the sonicated precipitate resuspension solutions were transferred onto a nitrocellulose membrane, blocked with 5% nonfat milk for 1 h, followed by incubation with horse anti-*Agkistrodon halys* antibody diluted in 1:500 at 4 °C overnight, the membrane was washed, incubated with horseradish peroxidase-conjugated antibody diluted 1:1000 at room temperature, and developed with diaminobenzidine (DAB).

Determination of rGBV-PA biological activity

In vitro fibrinolytic activity test

Fibrinolytic activity was measured with plasminogen-rich fibrin plates using the Astrup method with tPA as a standard [11].