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Engineering of Harobin for enhanced fibrinolytic activity obtained by random and site-directed mutagenesis

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ABSTRACT

We have previously published a report on the cloning and characterization of Harobin, a fibrinolytic serine protease. However, the broad application of this fibrinolytic enzyme is limited by its low expression level that was achieved in *Pichia pastoris*. To counteract this shortcoming, random and site-directed mutagenesis have been combined in order to improve functional expression and activity of Harobin. By screening 400 clones from random mutant libraries for enhanced fibrinolytic activity, two mutants were obtained: N111R, R230G. By performing site-directed mutagenesis, a Harobin double mutant, N111R/R230G, was constructed and can be functionally expressed at higher level than the wild type enzyme. In addition, it possessed much higher fibrinolytic and amidolytic activity than the wild type enzyme and other single mutants. The N111R/R230G expressed in basal salts medium was purified by a three step purification procedure. At pH of 6.0–9.0, and the temperature range of 40–90 °C, N111R/R230G was more active and more heat resistant. The fibrinolytic activities of Harobin mutants were completely inhibited by PMSF and SBTI, but not by EDTA, EGTA, DTT, indicating that Harobin is a serine protease. N111R/R230G showed much better anti-thrombosis effect than wild type Harobin and single mutants, and could significantly increase bleeding and clotting time. Intravenous injection of N111R/R230G in spontaneous hypertensive rats (SHR) led to a significant reduction in systolic blood pressure (SBP), diastolic blood pressure (DBP) and mean arterial pressure (MAP) ($p < 0.01$), while heart rate (HR) was not affected. The *in vitro* and in vivo results of the present study revealed that Harobin double mutant N111R/R230G is an appropriate candidate for biotechnological applications due to its high expression level and high activity in area of thrombosis and hypertension.

1. Introduction

Hemostasis is a dynamic process of thrombus formation and fibrinolysis [1]. Snake venoms contain a large variety of proteins that affect hemostasis. Many are enzymes such as nucleotidases, phospholipases A2 (PLA2), metalloproteinases and serine proteinases, whereas others, such as disintegrins and C-type lectins, have no enzymatic activity [2]. Various steps in the pathways of the hemostatic system are regulated by specific inhibitors [3]. These inhibitors belong to various protein families, serine proteinase inhibitors (serpins) being the most frequently represented [3,4]. Serine proteinases are very abundant in *Viperidae* and *Crotalidae* venoms in which they may account for 20% of their total protein content, and more than 150 have been identified in several snake venoms [3,4]. They are not lethal by themselves, but they contribute to the toxic effect of the venom when associated with other venom proteins. They affect many steps in the blood coagulation cascade, either nonspecifically, by proteolytic degradation, or selectively, by activating or inhibiting specific blood factors involved in platelet aggregation, coagulation and fibrinolysis [4,5]. Generally, they are classified into several groups as follows: (a) fibrinogen clotting enzymes; (b) fibrino (geno)lytic enzymes; (c) plasminogen activators; (d) prothrombin activators; (e) factor V and factor X activators; (f) hemorrhagins; and (g) platelet aggregation inhibitors [6].

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Fibrin clot formation is a key event in the development of thrombotic disease and is the final step in a multifactor coagulation cascade [7]. Many blood clot-dissolving agents, such as urokinase, streptokinase, and tissue plasminogen activator (t-PA), have been utilized in clinical treatments for thrombotic diseases [8]. However, these agents are often expensive, thermolabile, and can produce undesirable side effects (excessive bleeding and reocclusion) [9,10]. For this reason, a better clot dissolving drug with no hemorrhagic activity must be developed. Fibrinolytic enzymes that cause lysis of blood clots by attacking fibrin directly have been described in various venoms which include Agkistrodon contortrix contortrix, Agkistrodon acutus, Trimeresurus mucrosquamatus, Trimeresurus gramineus, Crotalus atrox, Crotalus basiliscus basiliscus [11–16].

We have previously identified and cloned a serine protease termed Harobin isolated from a sea snake (*Lapemis hardvickii*) venom gland bacteriophage T7 library [17]. The enzyme was a single chain protein with an apparent molecular weight of 30,000 on SDS-PAGE with an isoelectric point of 6.59. Harobin could clot human plasma by cleaving the Aα, Bβ and γ chains of fibrinogen and also exhibited amidolytic activity. The proteolytic activity of Harobin toward N-p-tosyl-L-arginine methylesterase was strongly inhibited by PMSF and moderately affected by DTT, and shared 50–70% similarity to terrestrial snake serine proteases, indicating that it was a serine protease. Meanwhile, Harobin showed stability with wide temperature (35–85 °C) and pH value ranges (pH 4–10).

In the current study, random and site-directed mutagenesis methods were combined in order to improve the functional expression of Harobin in *Pichia pastoris* GS115 [17,18]. This led to two Harobin mutants (N111R, R230G) with a much higher expression level compared to the wild type. The combination of the best mutations led to a double mutant (N111R/R230G) with an even further improved functional expression, a higher activity than the single mutants and wild type. Finally, both *in vitro* and in vivo tests were carried out to verify the activity enhancement of Harobin mutants.

2. Materials and methods

2.1. Strains, plasmids, culture medium and reagents

Plasmid pPIC9K, *P. pastoris* GS115 strain and Zeocin™ were purchased from Invitrogen (USA). *E. coli* DH5α was used for all plasmid constructions. *P. pastoris* was cultured in shake flask in BMGY (1% yeast extract, 2% peptone, 1% glycerol, 1.34% YNB, 4×10^{-5}% biotin and 100 mM potassium phosphate, pH 6.0) before induction, or BMMY for induction (BMGY with 0.5% methanol instead of 1% glycerol). YPD plates (1% yeast extract, 2% peptone, 2% dextrose, 2% agar) containing 1 mg/ml Zeocin™ or 100 μg/ml Zeocin™ were used for selecting transformants with multiple copies of the expression vector. Other molecular biological reagents were purchased from Takara (Japan) or Invitrogen (USA). Mutagenesis kits were purchased from Stratagene (USA). All other chemicals used were of analytical grade and were procured from Sigma–Aldrich (USA).

2.2. Construction of the Harobin mutant libraries and transformation of P. pastoris

To obtain randomly mutated Harobin derivatives, EP-PCR (error prone-PCR) was performed on the entire Harobin coding sequence from the wild plasmid pPIC9K/Harobin using the GeneMorph II random mutagenesis kit (Stratagene, USA) following the manufacturer's instructions. The conditions for EP-PCR random mutagenesis were as follows: a 100 μl reaction mixture containing 10 mM Tris-Cl (pH 8.3), 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM $MnCl_2$, 1 mM dCTP, 1 mM dTTP, 0.2 mM dATP, 0.2 mM dGTP, 2 μM (each) primer, 20 ng template DNA, and 2.5 units Taq DNA polymerase. PCR was performed in a PCR machine (MyCycler Thermal Cycler; Bio-Rad) for 25 cycles. One cycle consisted of 1 min at 94 °C, 1 min at 54 °C, and 1 min at 72 °C. To increase mutational diversity and reach an appropriate error rate, three consecutive EP-PCRs were conducted [19,20]. PCR products were visualized by agarose gel electrophoresis and purified using the QIAquick gel extraction kit (Qiagen, Germany). Purified PCR products were ligated into the pPIC9K and the plasmid mixture was transformed into *P. pastoris* GS115 by electroporation using a MicroPulse (Bio-Rad, USA) after linearization with Sal I. The transformed cells were cultured on YPD plates containing 100 μg/ml and 1 mg/ml Zeocin™ respectively. After incubation at 30 °C for 3 days, ten of the larger colonies from each Zeocin concentration were selected for protein expression.

Site-mutagenesis of the Harobin mutant gene was carried out, using QuickChange® II Site-Directed Mutagenesis Kit (STRATAGENE®) according to the manufacturer's manual. Mutagenesis primers were designed, according to the instruction of the mutagenesis kit. After digestion with DpnI, the resulting plasmids containing the mutated Harobin gene were transformed into *E. coli* JM109. Mutations were confirmed by DNA sequencing, using an ABI DNA sequencer, according to the manufacturer's manual. The mutated plasmids were transformed into *P. pastoris* GS115 for rational screening.

2.3. Expression of Harobin mutants in P. pastoris

Selected colonies were cultured in 25 ml BMGY in 250 ml shake flask at 28 °C, 250-rpm, for 18 h. Cells were collected by centrifugation at 3000 g for 5 min at 4 °C, and re-suspended in 25 ml BMMY for continued growth under the same conditions. Methanol was added to a final concentration of 0.5% every 24 h to maintain induction. After 72 h with methanol, the culture supernatant was separated from the cells by centrifugation as above and used for protein purification in further experiment. The collected cells were analyzed for determination of gene copy number by real time qPCR [17,21].

2.4. Large-scale expression of Harobin mutants in fermentor

Fermentation of the Harobin mutants transformant of *P. pastoris* was carried out using a 30-L fermenter (Baoxing Bioengineering Equipment, Shanghai, China) equipped with software control of temperature (26 and 30 °C), agitation (1000 rpm), pH, anti-foam addition, supplemental oxygen addition ($dO_2 \geq 30\%$ and $\geq 25\%$), and data logging. A colony containing Harobin mutants from an YPD plate (2% peptone, 1% yeast extract, 2% glucose, and 1.5% agar) with the highest level of secretion was inoculated into 500 ml MGY in a 2.5-L baffled flask. Cultures grew for approximately 36 h until OD_{600} reached 15. The temperature was kept at 29 °C, and aeration and agitation were adjusted to keep the dissolved oxygen well over 25% of the saturation level. Five liters basal salts medium (per liter: 27 ml of 85% phosphoric acid, 0.93 g Ca_2SO_4, 18.2 g K_2SO_4, 14.9 g $MgSO_4 \cdot 7H_2O$, 4.13 g KOH, and 40 g glycerol, adjust pH to 5.0 with concentrated NH_4OH) containing 4.35 ml/L PTM1 trace salts was inoculated with 500 ml inoculum seed. After glycerol exhaustion (23.5 h), glycerol (50% w/v, containing 12 ml/L PTM1) was fed at a rate of 75 ml/h until the wet cells achieved to approximately 200 g/L for about 6 h. Glycerol feed was terminated and methanol (100% containing 12 ml/L PTM1) feed was initiated firstly at a rate of 15 ml/h for about 4 h, followed by a rate of 40 ml/h for about 2.5 h, then changed to 50 ml/h for another 83.5 h until the end of the fermentation [22]. The cells were removed from the culture by centrifugation at 10,000 g for 15 min at 4 °C and the cell-free