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Expression, purification, and characterization of recombinant lumbrokinase PI239 in *Escherichia coli*

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ABSTRACT

Lumbrokinase (LK) is an important fibrinolytic enzyme derived from earthworms. It has been found that LK is composed of a group of isoenzymes. To construct and express the mature peptide of LK PI239 in *Escherichia coli*, we amplified and optimized the gene of LK which was then cloned into the prokaryotic expression vector pET-22b(−). The recombinant LK (rLK) protein was expressed as inclusion bodies and we have developed a purification process of rLK from these inclusion bodies. A step-down urea concentration strategy was applied to the rLK renaturation process. The purified and renatured rLK apparently ameliorated the conditions of the model thrombosis rats used, and may be developed into a therapeutic agent for thrombotic-associated diseases.

Atherothrombotic diseases lead to about 26 million deaths every year around the world, and therefore more and more attention is being given to anti-thrombotic drugs. Earthworms, which have long been used as a component in traditional Chinese medicine due to their anti-pyretic and diuretic properties, have been found to have anti-thrombosis effects [1–6]. Further studies showed that lumbrokinases, which are present in the body cavity and digestive organs of earthworms, are a group of fibrinolytic enzymes. Some lumbrokinases have been isolated from earthworms or expressed by recombinant DNA techniques and are being extensively investigated [5,7–10]. It was found that many wild type lumbrokinases are highly stable in common organic solutions *in vitro* over a long period of time, and most importantly, tend to be absorbed through the intestine [2]. Therefore, they have the potential to be developed into therapeutic drugs for thrombotic-associated diseases such as cerebral thrombosis and post-apoplectic sequelae [1,3].

Unfortunately, the life cycle of earthworm is long, and extraction of lumbrokinases is generally a labor intensive and time-consuming activity [11]. What is more, the extract is easily contaminated by multiple components. The enzymes must be purified from the lyophilized powder of the earthworm. As a result, this therapeutically fibrinolytic drug is expensive. So, high yields of recombinant lumbrokinases are needed.

In this study, we cloned and expressed the mature peptide of PI239 in *Escherichia coli*. The expressed insoluble inclusion body was purified and refolded, and the anti-thrombotic and fibrinolytic effects were investigated using a thrombosis rat model. This work may serve as a significant foundation for the bioengineering of lumbrokinases.

Materials and methods

Materials and reagents

Earthworms (*Lumbricus bimastus*), purchased from Beijing Baiao Pharmaceuticals, were the LK source. Restriction enzymes, rTaq polymerase and T4 DNA ligase were purchased from TaKaRa (Dalian, China). Isopropyl-β-D-thiogalactoside (IPTG)[1] was purchased from Sigma (USA). Urea, guanidine hydrochloride, Triton X-100 and Tris were purchased from Serva (Germany). Sephacryl S-300 high resolution resin was purchased from Amersham Pharmacia Biotech (USA). The expression vector pET-22b(−) and its host strain BL21(DE3) were purchased from Novagen. The t-PA (Actilyse) was purchased from Boehringer Ingelheim Pharma (Ingelheim am Rhein, Germany). All other chemicals and reagents were obtained from other commercial sources and were of the highest purity available.

Plasmid construction

Reverse transcription-polymerase chain reaction (RT-PCR) was used to obtain the gene fragment encoding lumbrokinase PI239

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[1] *Abbreviations used:* IPTG, isopropyl-β-D-thiogalactoside; RT-PCR, reverse transcription-polymerase chain reaction; LB, Luria–Bertani; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; MS, mass spectrometry; BSA, bovine serum albumin; PBS, phosphate-buffered saline; ECL, enhanced chemiluminescence.

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in *L. bimastus*. Total RNA was extracted with Trizol reagents (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. The tissue was homogenized manually with single-use pellet pestles (Kontes Glass Company, Vineland, NJ, USA). The concentration and purity of total RNA was estimated by spectrophotometry. The cDNA was prepared using M-MuLV reverse transcriptase (Fermentas Life Science, Canada) and incubated for 5 min at 70 °C, reverse transcribed for 1 h at 44 °C, and inactivated for 5 min at 75 °C. PCR amplification was performed with Pyrobest DNA polymerase (TaKaRa, Dalian, China) and the specific primer sequences used for PI239 mature peptide were 5′-CGG CATATGATTGTCGGAGGAATTGAAGC-3′ (sense) and 5′-GCTCTAGAG CATTAGTTGTTGGTGATGATGTCTG-3′ (antisense) (synthesized by Invitrogen, USA). NdeI and SalI restriction sites are underlined. PCR amplification was programmed as follows on an Eppendorf Mastercycler Gradient PCR System (Eppendorf, Germany): Taq activation for 5 min at 95 °C, 30 cycles of denaturation for 30 s at 95 °C, an annealing process for 30 s at 55 °C, and an extension for 1.5 min at 72 °C. Finally, rTaq was added and the sample was incubated at 72 °C for further 30 min. The amplified gene was recovered using an agarose gel and cloned into pMD20-T vector. The recombinant plasmid was named pMDLK. After DNA sequencing, the lumbrokinase gene was recovered from pMDLK plasmid by digestion with NdeI and SalI and cloned into the pET-22b prokaryotic expression vector. The resulting plasmid was designated pETLK. All constructs were confirmed by DNA sequencing and *E. coli* BL21(DE3) cells were transformed with the plasmid pETLK for protein expression.

Expression of rLK in E. coli and fermentation

A single transformed colony was inoculated into 10 mL Luria–Bertani (LB) medium supplemented with ampicillin (100 μg/mL) and grown while shaking overnight at 200 rpm at 37 °C. Three milliliter bacteria culture was transferred to 300 mL fresh LB medium in a 500 mL shake flask. The culture was grown at 37 °C until the OD_{600} reached 0.5 and IPTG was added to 1 mM to induce the expression of recombinant lumbrokinase. The culture was agitated for an additional 4 h, yielding a final OD_{600} of 4. Cells (1 mL) were collected by centrifugation and the pellet was resuspended in 100 μL of ddH_2O, mixed with 20 μL of 6× SDS loading buffer (0.35 M Tris, pH 6.8; 10.28% SDS; 36% glycerol; 5% β-mercaptoethanol and 0.012% bromphenol blue) and heated at 100 °C for 10 min. The sample was centrifuged at 12,000 rpm for 8 min and 10 μL supernatant was analyzed by SDS–PAGE and stained by Coomassie blue R-250.

Fermentation was performed using a fermentor (working volume of 5 L). A single transformed colony was inoculated in 10 mL Luria–Bertani medium supplemented with ampicillin (100 μg/mL) and grown at 220 rpm and 37 °C overnight. Two hundred milliliters of semi-defined medium (16 g/L tryptone, 10 g/L yeast extract, 20 mL/L glycerol, and 5 g/L NaCl) with 100 mg/L of ampicillin was inoculated with an overnight culture and grown at 220 rpm and 37 °C until the culture reached an OD_{600} of 2–3. The culture was used to inoculate the fermentor to a starting OD_{600} of 0.1–0.2. Five liters of sterile medium (5 g/L tryptone, 5 g/L yeast extract, 10 g/L glycerol, 2 g/L KH_2PO_4, 4 g/L K_2HPO_4, and 3 g/L $MgSO_4$) were aseptically added to the fermentor and inoculated with the culture as described above. Fermentation was performed using the following parameters: 37 °C, pH 7.0, airflow 5 L pm (1 vvm), agitation 250–850 rpm, and dissolved oxygen 30%. When the OD_{600} reached 7, cultures were induced by addition of IPTG (final concentration is 1 mM) at 37 °C for 4–6 h. Cells were harvested by centrifugation (Beckman J2-HS) at 15,000 rpm for 20 min at 4 °C.

Cell lysis and inclusion body washing, solubilization, purification, and refolding

About 100 g (wet wight) of the harvested cell paste were resuspended in 500 mL of STE buffer (10 mM Tris–HCl, pH 7.5, 10 mM NaCl, 1 mM EDTA, pH 8.0) and sonicated in ice. The pellet was collected by centrifugation. Inclusion body of rLK was washed with 1000 mL of 4 M urea, 1% Triton X-100, 5 mM EDTA, 5 mM β-mercaptoethanol, 50 mM Tris–HCl, pH 8.0, at room temperature for 30 min twice, followed with 1000 mL of 5 mM EDTA, 50 mM Tris, pH 8.0, at room temperature for 30 min. The final pellets of inclusion bodies (about 250 mg protein) were solubilized by homogenizing in 15 mL of 10 mg/L DTT, 7 M guanidine hydrochloride at 4 °C overnight. The supernatant was collected by centrifugation. A chromatography column (2.4 × 100 cm) was packed with 500 mL of depyrogenated Sephacryl-300 resin for the purification of rLK. The column was equilibrated with 7 M guanidine HCl. The supernatant of solubilized rLK was applied to the column. The chromatography was performed at a flow rate of 1 mL/min and rLK fraction was eluted with equilibration buffer.

The purified protein was diluted with 7 M guanidine HCl to 0.2 mg/mL and refolded by dialyzing against 30 times volume of10 mM Tris–HCl, pH 8.0, supplement with 14 mM β-mercaptoethanol, 5 μM $CuSO_4$ at 20 °C for 8 h and finally dialyzed against PBS at 4 °C for 24 h. The refolded protein was collected by centrifugation at 15,000 rpm for 20–30 min to remove the pellet. The final renatured protein was lyophilized and stored at −80 °C.

Characterization of rLK

The concentration of recombinant protein was measured using the BCA protein assay reagent (Pierce, Rockford, IL, USA). The purity of the protein was evaluated by SEC-HPLC. The sample (5 μg) in PBS was injected onto a 7.5 mm × 300 mm G2000SW column (TOSOH Corporation) at a flow rate of 0.5 mL/min. Peaks were detected by monitoring at a wavelength of 275 nm. The purity of rLK was calculated as a percentage of the total peak area detected. N-Terminal and C-terminal amino acid sequencings were carried out by Shanghai GeneCore Biotechnologies Co., Ltd. (Shanghai, China). The molecular mass of the purified rLK was determined by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS).

Western blot analysis

Proteins were transferred to nitrocellulose membranes (0.22 μm; Invitrogen) after SDS–PAGE using a Bio-Rad Trans-Blot Semi-Dry electrophoretic cell. The unoccupied sites on the membrane were blocked with 1% (w/v) bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 1 h. The membrane was rinsed with PBS and placed in 1:500 diluted rabbit anti-lumbrokinase antibody (Biosynthesis, China) for 1 h with constant shaking. Then, the membrane was washed with washing buffer (50 mM phosphate, pH 7.0, 0.05% Triton X-100) three times (5 min each) and incubated with 1:1000 diluted HRP-labeled goat anti-rabbit IgG (Boster, China). Immune complexes were detected by enhanced chemiluminescence (ECL) according to the manufacturer's specifications (Santa Cruz, CA, USA).

Fibrinolytic activity assay of rLK

Fibrinolytic activity was measured with plasminogen-rich fibrin plates using the method of Nakajima and Zhang et al. [12] with t-PA as a standard. Samples were added to the plate. Total bacterial protein without IPTG induction was used as the negative control and t-PA of several concentrations (2, 4, 6, and 8 IU/ml or 0.2,