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Auto-induction for high level production of biologically active reteplase in *Escherichia coli*

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ABSTRACT

Reteplase is a third generation tissue plasminogen activator (tPA) with a modified structure and prolonged half-life in comparison to native tPA. As a non-glycosylated protein, reteplase is expressed in *Escherichia coli*. Due to presence of several disulfide bonds, high level production of reteplase is complicated and needs extra steps for conversion to biologically active form. Auto-induction represents a method for high-yield growth of bacterial cells and higher expression of recombinant proteins. Here we have tried to optimize the auto-induction procedure for soluble and active expression of reteplase in *E. coli*. Results showed that using auto-induction strategy at 37 °C, Rosetta-gami (DE3) had the highest level of active and soluble reteplase production in comparison to *E. coli* strains BL21 (DE3), and Shuffel T7. Temperature dominantly affected the level of active reteplase production. Decreasing the temperature to 25 and 18 °C increased the level of active reteplase by 20 and 60%, respectively. The composition of auto-induction medium also dramatically changed the active production of reteplase in cytoplasm. Using higher enriched auto-induction medium, super broth base including trace elements, significantly increased biologically active reteplase by 30%.

It is demonstrated here that auto-induction is a powerful method for expression of biologically active reteplase in oxidative cytoplasm of Rosetta-gami. Optimizing expression condition by decreasing temperature and using an enriched auto-induction medium resulted in at least three times higher level of active reteplase production. Production of correctly folded and active reteplase in spite of its complex structure helps for removal of inefficient and cumbersome step of refolding.

1. Introduction

Human tissue plasminogen activator (tPA) is a member of fibrinolytic system, commonly secreted by blood vessel endothelial cells. It is a 527 amino acids protein with 17 disulfide bonds and five distinct structural domains [1]: Finger domain (F), the epidermal growth factor like domain (E), the two kringle 1 (K1) and kringle 2 (K2) domains, and a serine protease catalytic domain (P) [2,3]. tPA is a serine protease that converts the pro-enzyme plasminogen into the protease plasmin that has the ability of fibrin clot lysis in the case of thrombosis [4].

However, the functional preparation of full-length tPA with multiple disulfide bonds and molecular mass of 69 kDa remains the bottleneck for its production in large scales. Reteplase is a truncated and non-glycosylated variant of tPA consisting of 355 amino acids with 9 disulfide bonds. This deleted variant of tPA contains two (K1 and P) of the five domains of the main protein with total molecular mass of 39 kDa [1,2,5]. Due to reduced hepatic elimination, the half-life of this variant is increased to 13–16 min in comparison to 3–4 min of the full-length alteplase [6].

Prokaryotic systems such as *Escherichia coli* expression system is the preferred choice for the production of recombinant proteins. This is mainly due to low cost, fast growth rate, well-characterized genetics and the availability of large number of host strains [7–9]. Generally, the production of eukaryotic proteins in *E. coli* is strongly controlled by inducible promoter such as *lac* promoter which is induced by isopropyl-β-D-1-ihioglactopyranoside (IPTG) [8]. Although IPTG induction is widely used for the production of heterologous proteins but there are some obstacles. There is always some basal expression of host proteins even in the absence of inducer [10]. In addition, the refolding of inclusion bodies that are insoluble aggregates is a long and cumbersome

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task, particularly for proteins with complex structure and multiple disulfide bonds such as reteplase [10,11].

The main purpose of recombinant protein expression is often to obtain a high degree of accumulation of soluble product in the prokaryotic cells. The auto-induction method that is also based on the function of lac promoter has been used for functional and soluble expression of recombinant protein in *E. coli* [12,13]. In this method target protein is produced automatically without need to monitor cell density and inducer addition at the proper time. Auto-induction media are made from a well-balanced mixture of carbon substrates of glucose, glycerol, and lactose [14,15]. At the early stages of the culture, glucose is the preferred carbon source and catabolic repressor of l*ac* promoter. During the recombinant protein production stage, glycerol could be used as a supporting carbon source while lactose induces the expression. Using auto-induction media results in higher levels of functional target protein compared to conventional and manual induction with IPTG [16].

In this study, we have used three different auto-induction media including auto-induction medium LB broth-base with/without trace elements and super broth-base including trace elements, for soluble and active reteplase production in *E. coli*. Three different *E. coli* strains are used. *E. coli* BL21 (DE3) is the preferred prokaryotic host strain as generally give higher yields for the large majority of proteins and has the advantage of being deficient in the *lon* and *ompT* proteases [1,17]. In Addition two genetically-modified strains including Rosetta-gami and Shuffle T7 were used. We were able to produce high levels of soluble reteplase through optimization of expression condition.

2. Materials and methods

2.1. Bacterial strains and expression vector

For recombinant protein expression, three different *E. coli* strains BL21 (DE3), Rosetta-gami (DE3), and Shuffel T7 were prepared and transformed using standard protocols. During these experiments, pET-ret plasmid is used as described in our previously published report. In pET-ret a codon optimized reteplase gene is cloned in pET21a into *Nde*I and *Hind*III restriction sites therefore the expression of reteplase is controlled by the T7 inducible promoter [1].

2.2. Media and growth conditions

Luria–Bertani medium (LB: 10 g/L bacto-tryptone, 5 g/L yeast extract, 5 g/L NaCl) was used for pre-culture preparation. Three different auto-induction media were purchased from FORMEDIUM. The LB broth base without trace elements (AIMLB0105-M1), LB broth base with trace elements (AIMLB0201-M2) and super broth base with trace elements (AIMSB0201-M3) were prepared by dissolution of 34.85, 34.85 and 74.85 g/L in double distilled water, respectively. Trace elements are a mixture of $CaCl_2$, $MnSO_4$, $ZnSO_4$, $CoCl_2$, $CuSO_4$, $NiCl_2$, $NaMoO_4$, Na_2SeO_3 and $FeCl_3$ salts for a total concentration of 0.03 g/L. Super broth is composed of tryptone 35 g/L, yeast extract 20 g/L, $(NH_4)_2SO_4$ 3.3 g/L, KH_2PO_4 6.8 g/L, Na_2HPO_4 7.1 g/L, Glucose 0.5 g/L, α-Lactose 2.0 g/L and $MgSO_4$ 0.15 g/L.

2.3. Expression of reteplase by auto-induction in different E. coli strains

For pre-culture, 5 ml of LB medium containing ampicillin (100 μg/ml) was inoculated using a single colony from agar plates of three *E. coli* strains and incubated for 12 h at 37 °C and 180 rpm shaking.

For the soluble expression of reteplase, 100 ml auto-induction medium M1, containing ampicillin was inoculated with 1 ml of pre-culture in a 500 ml flask. Expression was carried out in a shaking incubator with 180 rpm at 37°C. After 24 h incubation, cells were harvested by centrifugation (8000 rpm, 10 min). The bacterial pellets were re-suspended in cold lysis buffer (100 mM Tris-HCl, pH 6.8 and 20 mM EDTA) and disrupted by sonication (70% amplitude, 10 cycles of 30 s pulse with 10 s rest intervals). The supernatant was clarified by centrifugation at 13000 rpm for 30 min at 4°C and concentrated with Amicon ultra-15 centrifugal filter units. The concentrated supernatant was analyzed by SDS-PAGE and activity assay.

2.4. SDS-PAGE analysis

A 40 μl portion of supernatant containing soluble reteplase was used for SDS-PAGE. After mixing with 10 μl of 5× electrophoresis sample buffer, it was boiled 5–10 min, and then loaded onto 5% stacking and 12% resolving SDS-PAGE. The gels were stained with Coomassie Blue R-250 dye for 2 h and de-stained overnight.

2.5. Determination of protein concentration

Total soluble reteplase concentration was determined using Bradford assay. This assay is based on the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. A standard curve was constructed by a series of bovine serum albumin (BSA) standards containing 0.78–25 μg BSA (stock solution of 2 mg/ml) in total volume of 1000 μl of deionized water. Total protein concentration of a test sample was calculated using the standard curve.

2.6. Activity assay

The biological activity of the expressed reteplase was evaluated using chromozym t-PA (Roche). Chromozym is a chromogenic substrate cleaved by t-PA and/or reteplase resulting in the formation of 4-nitraniline (pNA), which can be measured at 405 nm. Briefly, a reagent mixture containing chromozym t-PA (4 mM) in Tris buffer (100 mM Tris, pH 8.5, 0.15% Tween 80) was prepared. 5 μl of supernatant was diluted to 5 vol then mixed with 250 μl of reagent mixture and incubated at 37 °C for 30 min. For the blank reagent, 100 mM Tris pH 8.5 was used (25 μl). After stopping the reaction with 125 μl of 10% citric acid, the absorbance was recorded against blank. The activity was calculated by multiplying the corrected absorbance by 1.54. The specific activity was obtained by dividing the measured activity by the protein concentration in the supernatant. All experiments were carried out in triplicates.

2.7. Optimization of temperature and auto-induction media

In order to find the effect of temperature on soluble expression of reteplase, auto-induction was carried out at different temperatures. After inoculation of the M1 auto-induction medium with pre-cultured medium, flasks were transferred to shaking incubators with different temperatures of 18, 25, 30 and 37°C. Auto-induction was continued for 24 h and the optimum temperature was determined for further experiments. All experiments were carried out in triplicates.

In order to improve the soluble production of reteplase by auto-induction, three different auto-induction media (M1, M2 and M3) were used. For medium optimization, the selected *E. coli* strain was cultivated in 100 ml of each auto-induction media containing ampicillin and were incubated in shacking incubator for 24 h.

2.8. Statistical analysis

All experiments were performed at least in triple biologically independent repeats. For statistical analysis, mean and standard deviation were calculated and all data are presented as Mean ± SD. P values ≤ 0.05 were considered as statistically significant.