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Optimized production of insulin variant, a recombinant platelet aggregation inhibitor, by high cell-density fermentation of recombinant *Escherichia coli*

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ABSTRACT

Optimal conditions for a high cell-density fermentation of *Escherichia coli* strain harboring a recombinant anti-thrombosis insulin variant (named rAT-INS) were investigated by using fed-batch culture employing pH-stat method. The optimized main medium composition were glycerol 10 g/L, yeast extract 30 g/L, trypton 10 g/L, NaCl 5 g/L. The late-stage induction with 0.05 mM isopropyl-β-D- thiogalactopyranoside showed the highest productivity after 28 h of the fed-batch fermentation. This optimized process yielded about 150 mg of purified rAT-INS from 1 L of wet cell mass with high-homogeneity. The amino acid compositions and mass data of the purified rAT-INS were in good agreement with those as expected. Purified rAT-INS exhibited potent inhibitory activity of platelet aggregation. The in vivo assay showed that rAT-INS had a higher activity in prolonging the bleeding time in mice than native-insulin. The purified rAT-INS had almost no insulin receptor binding activity. Our study demonstrates the promise for mass production of novel recombinant antiplatelet agents.

1. Introduction

Heterologous expression of naturally scarce eukaryotic proteins in *Escherichia coli* has provided abundant source of these proteins for clinical use. Levels of foreign gene expression in *E. coli* are highly system-specific depending on biochemical characteristics of the recombinant proteins, induction mechanism of gene expression, host strains, and cultivation conditions. High cell-density fermentation is a useful process for improving the production yield of recombinant proteins. Understanding the relationship between microbial physiology, host-vector system, and gene expression during fermentation is essential to optimize the production of recombinant proteins [1].

Up to now, fed-batch culture technique has been mainly used for high cell-density fermentation of recombinant *E. coli*. To supply nutrients to the fermentor in fed-batch culture, various feeding systems have been developed, such as predetermined feeding system, DO-stat system, pH-stat system and system using on-line glucose analysis. Under excessive glucose concentration even in the aerobic condition, *E. coli* produces acetate, which inhibits cell growth and product synthesis known as *E. coli* Crabtree effect [2–4]. Among the various factors to be considered for high productivity in a fermentation process, acetate concentration is one important factor. Therefore, for high density culture of recombinant *E. coli* by fed-batch culture technique, it is desirable to maintain DO concentration above 10% of that saturated by air, and glucose concentration at low level to inhibit acetate accumulation [5]. The optimum concentration of nitrogen source in relation to the carbon source is also important to achieve high cell-density and high productivity of targeted recombinant proteins.

Platelet aggregation is a crucial step in thrombotic events and involves the binding of fibrinogen to activated platelets. Since RGD motif mediates the interaction of fibrinogen with cell surface receptors, RGD-containing proteins or peptides have the potency to inhibit platelet aggregation and could be used in the treatment of thrombotic diseases [6]. In our previous study, we introduced RGD motif into an inactive human proinsulin to construct a recombinant anti-thrombosis insulin variant (named rAT-INS). rAT-INS exhibited inhibitory activity of platelet aggregation with an IC50 value of 0.35 μM [6]. However, large scale expression and purification of rAT-INS is challenging in order to maximize the yield and maintain the biological activity of rAT-INS.

In this study, we investigated and optimized the medium composition and induction process of rAT-INS expression for high cell-density culture of recombinant *E. coli*. In addition, we developed an efficient protocol for the high recovery of rAT-INS expressed in *E. coli* as cytoplasmic inclusion bodies.

2. Materials and methods

2.1. Materials

The *E. coli* host strain used in this study was *E. coli* BL21(DE3) PLysS

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[F⁻, ompT, RB⁻, MB⁻] whose chromosome carries the gene for *T*7 RNA polymerase, *lac*I gene and lac*UV*5 promoter gene. The pET-3a-PICh plasmid harboring rAT-INS gene was constructed by us and described previously [6]. The *E. coli* strain containing the plasmid was designed as BL/pET-PICh. When expressed in recombinant *E. coli* strain, rAT-INS was accumulated as inclusion bodies. Isopropyl-β-D- thiogalactopyranoside (IPTG) was obtained from Sigma, USA. All other chemicals used were of analytical grade obtained from local companies. Trace element stock solution contained following compounds: $FeCl_3{\cdot}6H_2O$ 3.24 g/L, $ZnCl_2$ 0.22 g/L, $CoC1_2{\cdot}6H_2O$ 0.24 g/L, $NaMoO_4{\cdot}2H_2O$ 0.24 g/L, $CaC1_2{\cdot}2H_2O$ 0.12 g/L, $CuSO_4{\cdot}5H_2O$ 0.20 g/L, H_3BO_3 1.0 g/L, and $MgSO_4$ 0.74 g/L.

2.2. High cell-density fermentation of recombinant E. coli

Controlled fermentations were performed in a BIO-FLU-10 L fermentor (New Brunswick Scientific Co.), equipped with an embedded multi-loop controller which regulated agitation, temperature, dissolved oxygen, pH and nutrient. The recombinant *E. coli* BL/pET-PICh was cultured in 100 mL of the seed medium: 3% yeast extract, 1% tryptone, and 0.5% NaCl (w/v) supplemented with 100 μg/mL of ampicillin at 37 °C. This seed culture was transferred with 1% (v/v) inoculums to a 10 L jar fermentor containing 5 L of growth medium (glycerol 10 g/L, yeast extract 30 g/L, tryptone 10 g/L, NaCl 5 g/L, KH_2PO_4 2.0 g/L, K_2HPO_4 0.5 g/L, $MgSO_4{\cdot}7H_2O$ 1.0 g/L, $FeSO_4{\cdot}7H_2O$ 0.20 g/L, $CaCl_2{\cdot}2H_2O$ 0.01 g/L, pH 7.0) and then fermented at 37 °C. Nutrient addition by pH-stat method was controlled with advanced fermentation Software. During fermentation, air and/or pure oxygen were supplied at a rate of 1–1.5 vvm to maintain the dissolved oxygen concentration at 30–60%. The pH was controlled at 7.0 by 30% (v/v) phosphoric acid as the acid and 25% (v/v) ammonium hydroxide as the base. Antifoam-A (Sigma, USA) was used to suppress foam formation. The optical density (OD) of each sample was measured at 600 nm with a UV–visible spectrophotometer (UltraspecIII, Pharmacia). One OD unit was experimentally determined to be 0.45 g dry cell weight/L. After 6 h of growth phase fermentation, the fed-batch process was initiated by adding 800–1200 mL of the feeding medium (glycerol 50 g/L, yeast extract 150 g/L, tryptone 50 g/L, $MgSO_4$ 0.5 g/L, and $(NH_4)_2SO_4$ 1.0 g/L) at the constant rate of 30 m$props_value{literPattern}/L/h. Expression level of the targeted recombinant proteins was examined with sodiumdodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and quantified by densitometry. IPTG was fed for the expression of recombinant rAT-INS at the late stage of fermentation. Plasmid stability was determined by counting colonies on agar-plates (LB medium) with and without 100 mg/L ampicillin.

2.3. Refolding and purification of rAT-INS

The fermented broth was centrifuged at 8000 rpm for 30 min at 4 °C, the pellet was washed twice with 50 mM phosphate buffer (pH 7.4) and lysed in lysis buffer (0.05 M Tris-HC1, 5% Triton, 8% sucrose, 0.05 M EDTA, pH 8.0) by sonication using a vibra cell sonicator (Sonics, USA) with a 19 mm-threaded probe at 40 kHz amplitude of power. The efficiency of cell lysis was optimized by employing different sonication time course. After sonication, the lysate was centrifuged at 4 °C for 30 min to recover the inclusion bodies which was then resuspended in wash buffer (50 mM Tris-HCl, 2.5 g/L TritonX-100, 2 mM EDTA, pH 8.0) and recovered by centrifugation at 25–28 °C for 30 min at 8000 rpm. The lysed pellet was collected by centrifugation at 10,000 g and suspended by stirring overnight at 10 °C with 4 vol of lysis buffer (8 M urea or 6 M guanidine-HC1, 1 mM DTT in 0.1 M Tris/HCl, pH 8.0). After centrifugation, DTT was added to the supernatant to a final concentration of 10 mM and the mixture was incubated at 37 °C for 1 h, then diluted with 4 vol of cold water, with pH adjusted to 4.0–5.0 for precipitation at 4 °C for 2 h. The pellet was collected by centrifugation, and quickly dissolved in 50 mL of cold water with pH adjusted to 10–12. Then the solution was diluted with 2 L of 0.05 M Glycine/NaOH buffer, pH 10.8, and kept at 4 °C overnight for refolding. After ultrafiltration, the refolded protein was loaded onto a Sephacryl S200 column (2 × 100 cm) using Watson Marlow peristaltic pump, and eluted with the formation buffer. The fraction containing rAT-INS was pooled, ultrafiltrated, and lyophilized.

2.4. Platelet aggregation inhibition assay

Blood was collected from a healthy human donor in 3.8% (w/v) sodium citrate (1:9 ratio) and was centrifuged at 150 × g at room temperature for 10 min to obtain platelet-rich plasma. Samples with various concentrations of rAT-INS were incubated with 277 μl of platelet-rich plasma at 37 °C for 2 min before the addition of 3 μl of 300 μM adenosine 5′-diphosphate (ADP). The inhibition of platelet aggregation was determined by light transmission. IC_{50} (molar concentration of peptide required to cause 50% inhibition of platelet aggregation) was calculated for each experiment.

2.5. Assay for bleeding time in mice

BALB/c mice (male, 20 g body weight) were obtained from Institute of Experimental Animal, Chinese Academy of Medicine Science. Bleeding time in mice was measured by the method described by Dejana et al. [7]. Solution of normal saline or various concentrations of rAT-INS was injected intravenously through a tail vein of the mouse. A cut of 4 mm from the tail of mouse was done 8 min after the injection. Bleeding time was recorded from the beginning of bleeding to its end.

2.6. Assay for insulin binding of rAT-INS

Insulin receptor was partially isolated as crude membranes from human placenta [8]. rAT-INS was then diluted into a series of concentrations in KRB buffer (0.119 mol L^{-1} NaCl, 1.2 mmol L^{-1} $MgSO_4$, 0.03 mol L^{-1} HEPES, 0.05 mol L^{-1} KCl, 1.2 mmol L^{-1} KH_2PO_4, 1.3 mmol L^{-1} $CaCl_2$, 0.01 mol L^{-1} $NaHCO_3$, pH 7.4). Equal volume of diluted ^{125}I-insulin and six-fold diluted insulin receptor in KRB buffer containing 2% (w/v) of bovine serum albumin were added and mixed with rAT-INS. The mixtures were incubated overnight at 4 °C and then centrifuged at 10,000 g for 5 min. The radioactivity of the pellet was determined by γ-ray detection assay. Native human insulin with full receptor-binding activity and 0.9% NaCl was set as positive control and negative control, respectively.

2.7. Physicochemical properties of rAT-INS

Matrix-assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) mass spectrometry of rAT-INS was performed at the Chemistry Research Institute Mass Spectrometry Research Centre, Academy of Nature Sciences of China. Purified and concentrated samples were applied to a Voyager-DE STR MALDI-TOF biospectrometry work station (Applied Biosystems) using sinapic acid as the matrix. Whole protein spectra were recorded in a linear mode and bovine serum albumin was used as the calibration marker. The thiol group was determined by Ellman's method [9]. SDS-PAGE and IEF were performed according to the Pharmacia method [10].

3. Results and discussion

3.1. Structural features of rAT-INS

Structural representation of AT-INS was shown in Fig. 1. The space distance between B30 site of B chain and A1 site of A chain is about 5–10 Å. With the similarity of structural characteristics between the C-peptide and the functional RGD motif, it is possible to display an RGD motif on the surface of mutant insulin. The structural simulation of rAT-

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