大肠杆菌表达的人血管内皮细胞生长因子的复性与纯化研究

Vascular endothelial growth factor (VEGF) is a potent and specific endothelial cell mitogen capable of inducing angiogenesis and growth of new blood vessels. In humans, 5 different VEGF isoforms with 121, 145, 165, 189, and 206 amino acids can be generated as a result of alternative splicing from the single VEGF gene[1]. These isoforms differ in molecular mass and in biological properties such as the ability to bind heparin or heparan-sulfate proteoglycans and different VEGF receptors. In contrast to other forms, $VEGF_{121}$ does not bind heparin or extracellular domain of the VEGF receptor[2]. VEGF-induced angiogenesis results in a therapeutic benefit in several animal models of myocardial or limb ischemia[3],[4]. However, few efforts have been made to obtain biologically active VEGF protein from the inclusion bodies produced by engineered Escherichia coli in high-cell-density culture. High expression levels of recombinant proteins in E.coli often lead to the formation of inclusion bodies[5]. Thus, protein renaturation is usually required.

An efficient downstream process for the production of recombinant VEGF has been developed for industrial application using a novel "direct refolding" method. In this method, $VEGF_{121}$ was expressed as inclusion bodies in E. coli, and the critical step was ultrafiltrated refolding after solubilization of the inclusion body with 81% recovery. After purification by anion chromatography and Sephacry S-100 chromatography, the final purification yield was 31% with purity exceeding 95%, and the yield was twice as high as that with the conventional procedure.

MATERIALS AND METHODS

High-cell-density cultivation

One single colony of E. coli BL21(DE3)/$pET_2$4a- $VEGF_{121}$ on LB plate was inoculated in 5 ml LB and cultured overnight at 37 ℃, then 3 ml of the culture medium was inoculated in 150 ml LB medium and grown for 5 h at 37 ℃ and the culture was used to inoculate a fed-batch fermentation. The fermentation was carried out in a 3.7 L bioreactor (KLF2, Bioengineering, Germany) at 37℃ with the initial working volume of 2.2 L. The culture media (per liter) consisted of 20 ml 50% glycerol, 20 g tryptone, 10 g yeast extract, 15.12 g $Na_2HPO_4 \cdot 12H2O$, 3 g KH_2PO_4, 1 g NH_4Cl, and 0.50 g NaCl. A total of 500 ml feeding medium contained 200 ml 50% glycerol, 20 g tryptone, 40 g yeast extract, and 2.5 g $MgCl_2$.

The pH was controlled at 7 by automatic feeding of 3 mol/L NH_4OH, and dissolved O_2 was maintained at above 30% air saturation by stirrer cascading. IPTG was added to the culture at a final concentration of 0.5 mmol/L for inducing $VEGF_{121}$ expression, and the cells were harvested after another 4-hour cultivation.

Disruption and isolation of the inclusion bodies

The cell pellets were suspended in TE (20 mmol/L Tris-HCl, 5 mmol/L EDTA, pH 8.0) at 4 ℃ and disrupted under a pressure of 120 MPa four times at a flow rate of 130 ml/min at 4 ℃ using a high pressure homogenizer (EmulsiXex-c50, Canada). The pellets of the inclusion body were centrifuged at 10 000 g for 30 min, resuspended in washing solution (2 mol/L urea, 20 mmol/L Tris-HCl, 0.5 mol/L NaCl, 2% Triton X-100, 2 mmol/L mercaptoethanol, pH 8.0) and stirred for 20 min, and centrifuged again at 10 000 g for 15 min at 4 ℃. The above procedures were repeated, and finally the pellets were washed twice in 20 mmol/L Tris-HCl (pH 8.0) and stored at -30 ℃.

Refolding and purification of $VEGF_{121}$

One gram of the inclusion body was dissolved in guanidine hydrochloride buffer (7 mol/L, containing 20 mmol/L Tris-HCl, 1 mmol/L EDTA, 100 mmol/L mercaptoethanol, pH 8.0) for unfolding the protein completely, followed by addition of 20 mmol/L Tris-HCl (pH 8.0) into the solution until white precipitate appeared. After centrifugation, the precipitate was dissolved in 8 mol/L urea solution (containing 20 mmol/L Tris-HCl, 1 mmol/L EDTA, 100 mmol/L mercaptoethanol, pH 8.0) and the protein concentration adjusted to 0.2 mg/ml. The dissolved protein (500 ml) was ultrafiltrated in the refolding buffer (2000 ml, containing 20 mmol/L Tris-HCl, 1 mmol/L EDTA, 0.1 mmol/L GSSG, 1 mmol/L GSH, pH 8.0) for 7 h using a Millipore lab scale TFF system (France). The renatured protein was adjusted to pH 8.0 for purification. Firstly, the recombinant protein was purified by DEAE-Sepharose Fast Flow column. Secondly, the major protein fraction was collected and purified by Sephacryl S-100.

SDS-PAGE and immunoblotting

Samples were boiled in sample-buffer with or without DTT reduction and detected in gels with Coomassie brilliant blue staining or Western blotting.

Cell proliferation assay

Biological activity of the recombinant $VEGF_{121}$ was determined by a vascular endothelial proliferation assay using human umbilical vein endothelial cells. Proli- feration of the cells in the presence of $VEGF_{121}$ was determined by MTT assay.

RESULTS

Production of $VEGF_{121}$ in high-cell-density cul- tivation and its isolation as pure inclusion bodies

Fermentations were performed (37 ℃, pH 7.0) with constant substrate feeding. The optimal induction time was 4 h for E.coli to reach high levels of $VEGF_{121}$ expression. When

cultivated for 9 h, the cell quantity reached 46 g dry cell/L, corresponding to $VEGF_{121}$ of about 4.5 g dry cell/L.

After extensive washing, most cellular proteins were separated from the inclusion bodies, and the purity of the inclusion bodies reached 70% (Fig.1).

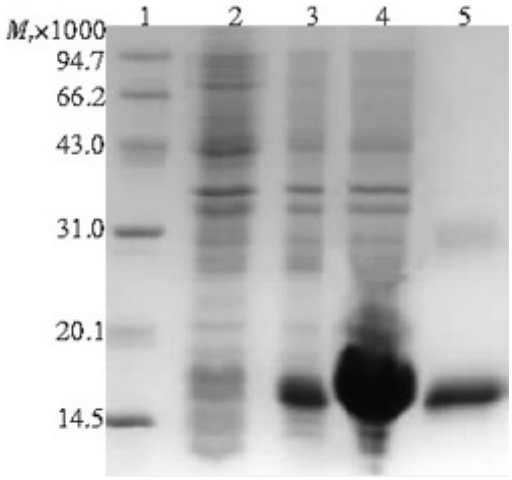

Fig.1 $VEGF_{121}$ inclusion bodies on 15% SDS-PAGE
Lane 1: Protein markers; Lane 2: Supernatant of disruption cell pellet; Lane 3: Raw inclusion bodies; Lane 4: Purified inclusion bodies; Lane 5: Refolded inclusion bodies

Refolding and purification of $VEGF_{121}$

The inclusion body was denatured and ultrafiltrated with the refolding buffer, and subsequently purified on anion exchange chromatography and Sephacryl S-100. The purity of the purified $VEGF_{121}$ was 95% (Fig.2,3) and the overall recovery of the refolded $VEGF_{121}$ from the inclusion bodies of the E.coli was 31% (Tab.1).

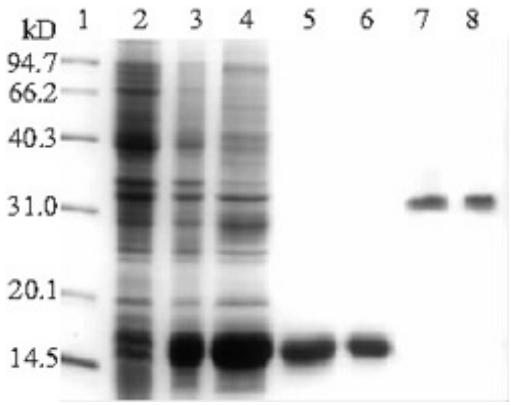

Fig.2 Purity of $rhVEGF_{121}$ inclusion bodies expressed in E. coli by 15% SDS-PAGE
Lane 1: Protein markers; Lane 2: Total proteins of the bacteria without IPTG induction; Lane 3: Total proteins of the bacteria induced by IPTG; Lane 4: Inclusion body of $VEGF_{121}$ before purification; Lane 5: $VEGF_{121}$ purified with DEAE-Sepharose FF chromatography; Lane 6: $VEGF_{121}$ purified with Sephacryl S-100 chromatography; Lanes 7: $VEGF_{121}$ purified with DEAE-Sepharose FF chromatography (non-reduced); 8: $VEGF_{121}$ purified with Sephacryl S-100 chromatography (non-reduced)

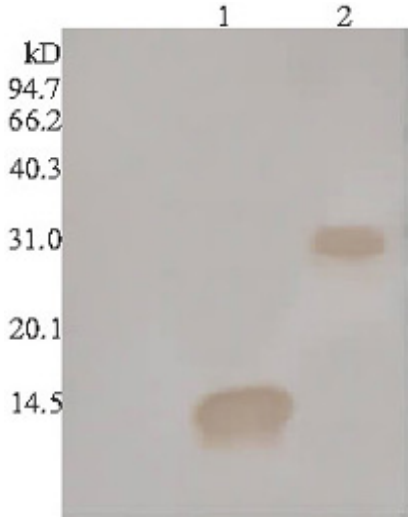

Fig.3 Western blotting of purified recombinant $VEGF_{121}$

The proteins were separated by 15% SDS-PAGE and transferred to a nitrocellulose membrane using a semi-dry electroblotter. The proteins were detected by incubation with rabbit anti-$VEGF_{121}$ polyclonal antibody at 1∶400 dilution followed by goat anti-rabbit IgG conjugated to peroxidase (1∶400 dilution). Lane 1: $VEGF_{121}$ after purification with Sephacryl S-100 chromatography; Lane 2: $VEGF_{121}$ after purification with Sephacryl S-100 chromatography (non-reduced)

Tab.1 Purification of $VEGF_{121}$ from *E.coli*

Purification step	Yield (mg[a])	Purity (%)	Step recovery (%)	Overall protein recovery (%)
Inclusion bodies dissolved	100	70.5[b]	/	/
Refolding from inclusion bodies	81	80.2[b]	81	81
Pooled fractions of $VEGF_{121}$ from anion chromatography	61	90.3[b]	75	61
Pooled fractions of $VEGF_{121}$ from Sephacryl S-100 chromatography	30	95[b]	50	31

[a]Determined using a Lowry assay; [b]Determined using gel densitometry

Cell proliferation assay

The ability of recombinant $VEGF_{121}$ to induce the proliferation of human umbilical vein endothelial cells was studied in vitro. Standard $VEGF_{121}$ was used as the positive control. The growth of human umbilical vein endothelial cells was stimulated by $VEGF_{121}$ in a dose-dependant manner and similar dose-response relationship was found between recombinant $VEGF_{121}$ and native $VEGF_{121}$, which proved that recombinant $VEGF_{121}$ had the same biological activity as native $VEGF_{121}$. The ED50 for this effect is typically 10-20 ng/ml (Fig.4).

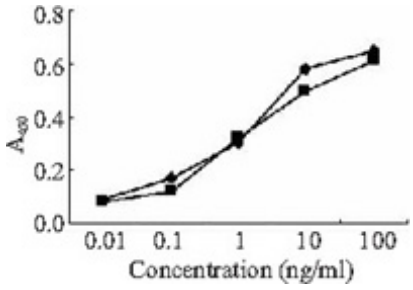

Fig.4 Endothelial cell proliferation assay
■: Recombinant $VEGF_{121}$; ◆: Native $VEGF_{121}$

DISCUSSION

We prepared $VEGF_{121}$ from E.coli with a high yield refolding. The overall yield of the protein using conventional dilution refolding method was less than 10% (data not shown). However, the renaturation procedure in our work obtained an overall yield over 31%. As disulfide bond (S-S bond) breakage abolished the biological activity of a protein, the S-S bond formation is a crucial factor for correct refolding of the protein. Controlling S-S bond formation improved the refolding efficiency of insoluble protein as the inclusion body[6]. $VEGF_{121}$ contains 4 S-S bond, which is difficult to refold[7]. In this study, the mis-pairing disulfide bonds were unfolded sufficiently in 7 mol/L guanidine hydrochloride, and the denaturant was sharply diluted and precipitated, to improve the purity and solubility of the inclusion body. The inclusion body retained its high solubility in 8 mol/L urea. The recombinant protein was allowed with a mild and gradual environment, and refolding was achieved by ultrafiltration with the denaturant replaced slowly. The recovery and specific activity of $VEGF_{121}$ was increased and the refolding efficiency improved.

The purity of the inclusion body was an important factor for refolding. Peptidoglycans, lipids, nucleic acids, lipopolysaccharides, and membrane proteins were the major impurities during inclusion body treatment[8]. In this study, the washing buffer could effectively remove these impurities as shown by SDS-PAGE. The inclusion body was dissolved in guanidine hydro- chloride after washing with the washing buffer, and then diluted and precipitated. The purity of the inclusion body reached 70%, which was helpful for further refolding and purification.

The expressed product was purified by anion exchange chromatography and Sephacryl S-100 chromatography with a purity of above 95%, which is suitable for biological activity evaluation. We provided in this study a practical purification process, which made possible potential large-scale production of biologically active $VEGF_{121}$.

$VEGF_{121}$ was highly expressed in E.coli system, and the study opens a wide prospect for the application of $VEGF_{121}$ in the prevention and treatment of ischemic heart disease and other tissue ischemic diseases such as secondary arterial occlusion in the limbs.

REFERENCES

[1]Tischer E, Mitchell R, Hartman T, et al. The human gene for vascular endothelial growth factor: multiple protein forms are encoded through alternative exon splicing[J]. J Biol Chem, 1991, 266: 11947-54.

[2]Gitay-Goren H, Cohen T, Tessler S, et al. Selective binding of $VEGF_{121}$ to one of the three vascular endothelial growth factor receptors of vascular endothelial cells[J]. J Biol Chem, 1996, 271: 5519-23.

[3]Pufe T, Wildemann B, Petersen W, et al. Quantitative measurement of the splice variants 120 and 164 of the angiogenic peptide vascular endothelial growth factor in the time flow of fracture healing: a study in the rat[J]. Cell Tissue Res, 2002, 309: 387-92.

[4]Miyanishi K, Trindade MC, Ma T, et al. Periprosthetic osteolysis: induction of

vascular endothelial growth factor from human monocyte/macrophages by orthopaedic biomaterial particles[J]. J Bone Miner Res, 2003, 18: 1573-83.

[5]Rudolph R, Lilie H. In vitro folding of inclusion body proteins[J]. FASEB, 1996, 10: 49-56.

[6]Rudolph R. Renaturation of recombinant, disulfide-bonded proteins from inclusion bodies[J].//Tschesche H. Modern methods in protein and nucleic acid research[M]. New York: Walter de Gruyter, 1990: 149-72.

[7]Scrofani SD, Fabri LJ, Xu P, et al. Purification and refolding of vascular endothelial growth factor-B[J]. Protein Sci, 2000, 9: 2018- 25.

[8]Thatcher DR. Recovery of therapeutic proteins from inclusion bodies:problems and process strategies[J]. Biochem Soc Trans, 1990, 18:234-5.

回结果列表