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Full Length Research Paper

Solubilization and purification of *Escherichia coli* expressed GST-fusion human vascular endothelial growth factors with *N*-Lauroylsarcosine

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Vascular endothelial growth factor (VEGF) is a potent mitogen for tumor angiogenesis. Clinically, VEGF detection in human blood can be expected to be used in the very near future for cancer screening, prognosis, monitoring of therapy and diagnosis. VEGF has been identified as the target for the treatment of cancer. Though prokaryotic expression of VEGF has been done, the solubilization and purification is time consuming and empirical. In this study, $VEGF_{165}$ and $VEGF_{121}$ were cloned into pGEX-4T-1 vector, and GST-VEGF fusion proteins were expressed in *Escherichia coli* at 37°C. The inclusion bodies of GST-VEGF fusion proteins were solubilized with *N*-Lauroylsarcosine (sarkosyl). Briefly, the cell suspension with inclusion body was added with sarkosyl at a final concentration of 1.5%. After the disruption of cells, the clarified supernatant containing sarkosyl was added with Triton X-100 at a final concentration of 3%. The GST-VEGFs were purified by affinity chromatography on glutathione Sepharose 4B. The overall yield was approximately 10 – 12 mg/l cell culture. The binding assay showed that the GST-$VEGF_{165}$ binds to VEGF receptor in a dose dependent manner. The current work provides a novel procedure for solubilization and purification of GST-VEGF fusion proteins, and no laborious procedures for separation of inclusion bodies and renaturation were needed.

Key words: VEGF, sarkosyl, solubilization, purification, GST.

INTRODUCTION

Vascular endothelial growth factor (VEGF), also known as vascular permealility factor, was first described by Senger and Leung (Senger et al., 1983; Leung et al., 1989). VEGF is a disulphide-linked homodimer protein of 34-42 KDa, and a potent and specific endothelial cell mitogen involved in the induction of angiogenesis, that is the growth of new blood vessels. VEGF is synthesized in response to hypoxia and other stimuli. Alternative splicing of a single VEGF mRNA renders four main VEGF isoforms, $VEGF_{121}$, $_{165}$, $_{189}$ and $_{206}$ (Houck et al., 1991; Tischer et al., 1991). $VEGF_{121}$ and $VEGF_{165}$ are soluble isoforms of VEGF, while $VEGF_{189}$ and $VEGF_{206}$ are associated with the extracellular matrix. The predominant form of VEGF is $VEGF_{165}$ (Ferrara and Henzel, 1989). VEGF is expressed and secreted by almost all solid tumors.

VEGF is a potential and unique tumor marker in tumor diagnosis and prognosis. Current data show that: (i) VEGF can be detected in the blood by enzyme-linked immunosorbent assay (ELISA) and in tissues by immunohistochemistry; (ii) VEGF detections could be used for determining the risk of developing cancer, screening for early detection and evaluating the prognosis of cancer, monitoring of cancer therapies especially chemotherapy, monitoring of recurrence of cancer and distinguishing

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benign from malignant diseases (Poon et al., 2001; Pang and Poon, 2006; Kuroi and Toi, 2001). VEGF has also been identified as targets for clinical intervention of cancer patients. Bevacizumab (avastin), a humanized mono-clonal antibody, that binds and inhibits VEGF, has been approved by the Food and Drug Administration (FDA) of the USA in 2004 for the treatment of various cancers. Many other VEGF inhibitors are now been developed for treatment of cancers (Hurwitz et al., 2004a, b).

As VEGF is becoming more and more important in both clinical and basic research, a simple and time-saving procedure for expression and purification of recombinant VEGF is needed. Previously, VEGF had been expressed from bacteria (Siemeister et al., 1996). Although expression as inclusion bodies can often be advantageous for yield, the procedures for separation, washing and solubilization of inclusion bodies and renaturation of the recombinant protein are often time consuming and empirical (Siemeister et al., 1996; Cabrita and Bottomley, 2004). The problems of solubility of some GST-fusion proteins, for example, CMPK, EcR and USP were partially overcome by the use of the alkyl anionic detergent, sarkosyl (Frangioni and Neel, 1993; Elke et al., 1997). However, no literature shows whether $VEGF_{165}$ and $VEGF_{121}$ can be expressed as GST fusion proteins and solubilized with sarkosyl. In the present study, we describe a novel, convenient and effective procedure to solubilize and purify the inclusion bodies of $VEGF_{165}$ and $VEGF_{121}$ from *Escherichia coli* and it could serve as an easily available source of recombinant VEGF for animal immunization and other research purposes.

MATERIALS AND METHODS

Construction of expression plasmids

$VEGF_{165}$ and $VEGF_{121}$ gene fragments without signal peptide sequences were amplified from human placenta cDNA (Clontech, Mountain View CA, USA) by PCR. The cDNA fragments encoding amino acids 2 - 165 of $VEGF_{165}$ and encoding amino acid 2 - 121 of $VEGF_{121}$ were then cloned into pGEX-4T-1 prokaryotic expression vector with the cloning sites upstream of *EcoRI* and down stream of *Not I*. The recombinant DNA encodes amino acids 2 - 165 of native human $VEGF_{165}$ and amino acids 2 - 121 of native human $VEGF_{121}$ (Semeister et al., 1996; Weindel et al., 1992). The fidelity of the recombinant DNAs were finally confirmed by DNA sequencing analysis. The expression plasmids then were transformed to *E. coli* strain BL21(DE3)pLysS. Recombinant clones were screened with different concentrations of IPTG at different time points. One clone with highest expression of proteins was selected from pGEX-4T-1/$VEGF_{165}$ and pGEX-4T-1/$VEGF_{121}$ respectively.

Expression, solubilization and purification of GST-VEGFs

The expression, solubilization and purification of the GST-VEGF fusion protein were performed essentially as described by Frangioni and Neel (1993) with the following modifications. A single colony of *E. coli* strain BL21(DE3)pLysS transformed with the plasmid of pGEX-4T-1/$VEGF_{165}$ or pGEX-4T-1/$VEGF_{121}$ was grown at 37°C overnight in 50 ml of LB. 1 ml of the overnight culture was added to 50 ml of fresh LB (1:50 dilution) and grown at 37°C to an OD_{600} = 0.6-0.8. 100 µg/ml ampicillin was added to the culture. Bacterial expression was induced by the addition of 1mM IPTG; the culture was further incubated at 37°C for 2 - 3 h. Bacteria were collected by centrifugation, washed with STE buffer (10 mM Tris-HCl, pH 8.0;150 mM sodium chloride; and 1 mM EDTA) and pelleted by centrifugation. The pelleted bacteria were then resuspended in lysis buffer (STE + lysozyme 100 µg/ml) and incubated for 15 min on ice followed by the addition of final concentration of 5 mM DTT and 15 mM PMSF. Sarkosyl (Sigma-Aldrich, St. Louis MO, USA) was added to a final concentration of 1.5% (W/V). After vortexing for 10 seconds, bacteria were disrupted by sonication for 30 s on ice 3 times followed by centrifugation at 13,000 rpm/min for 10 min. The supernatant was transferred to a new tube and treated with Triton X-100 at a final concentration of 3%.

The GST-VEGF fusion proteins were covalently absorbed on glutathione Sepharose 4B. Briefly, the glutathione Sepharose 4B (GE Healthcare, Life Sciences Piscataway, NJ, USA) was equilibrated in PBS and 1 ml of the beads were incubated with cell lysates prepared as previously described overnight at 4°C with shaking. The GST-VEGF fusion proteins were eluted with buffer containing 10 mM reduced gluthathione. Concentrated, the protein samples were stored in glycerol containing Tris-HCl at a final concentration of 10% (v/v) and protein solution was kept at either 4°C for short or -80°C for long storage.

All protein samples were analyzed by 10% SDS-PAGE and visualized by Coomassie Brilliant Blue R-G250 (Zou et al., 1997).

Binding assay

Recombinant extracellular domain of human VEGF receptor (rh-sFlt-1, Sigma Aldrich, St. Louis, MO, USA) was coated onto Nunc 96-well-microplate (0.25 µg/well). After addition of increased concentration of GST-$VEGF_{165}$, wells were incubated with horseradish peroxidase (HRP) labeled VEGF polyclonal antibody. For control, GST-$VEGF_{165}$ was replaced by GST and incubated with HRP-labelled GST polyclonal antibody.

RESULTS AND DISCUSSION

Expression of VEGFs as GST fusion protein

Expression of GST-VEGF fusion proteins is under the control of the *tac* promoter, which is induced by IPTG (Figure 1a). Prominent protein bands corresponding to a calculated molecular weight of approximately 48 kDa (GST-$VEGF_{165}$), 42 kDa (GST-$VEGF_{121}$) and 26 kDa (GST) were detected. No significant differences were found in the fusion protein amount expressed by incubation for 2 - 3 h. A GST-PC protein was used for positive control of expression, as it is the first time to express GST-$VEGF_{165}$ and GST-$VEGF_{121}$ (Figure 1b).

Solubilization and purification of GST-VEGF with sarkosyl

To solubilize and purify the GST-$VEGF_{165}$ and GST VEG F_{121} fusion proteins, 1.5% sarkosyl, an alkyl anionic detergent, was used for the procedure. The purity of the final products was more than 95% based on evaluation of Coomassie-stained gels and the overall yield was around

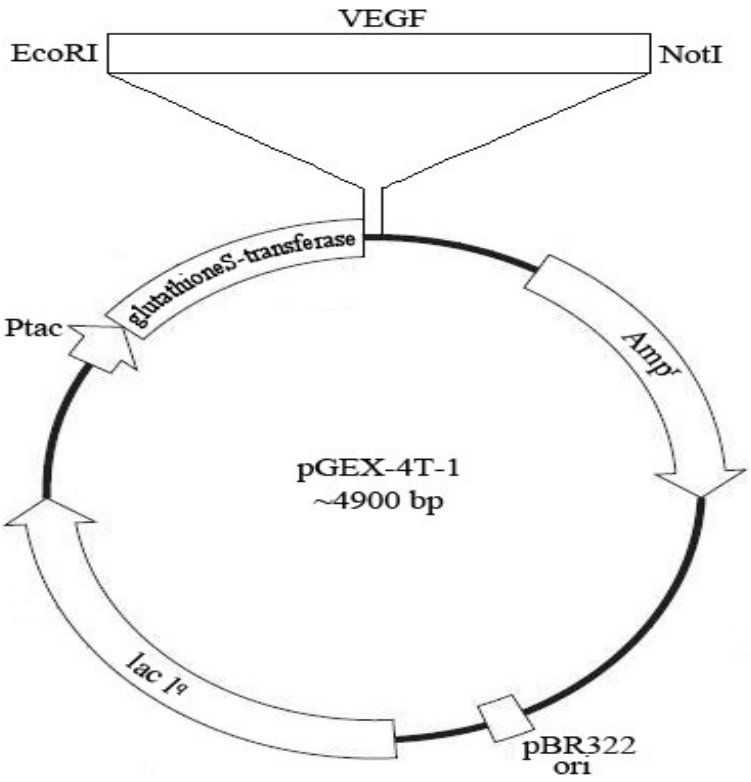

Figure 1a. The schematic diagram of the recombinant plasmid pGEX-4T-1/VEGF expression vector.

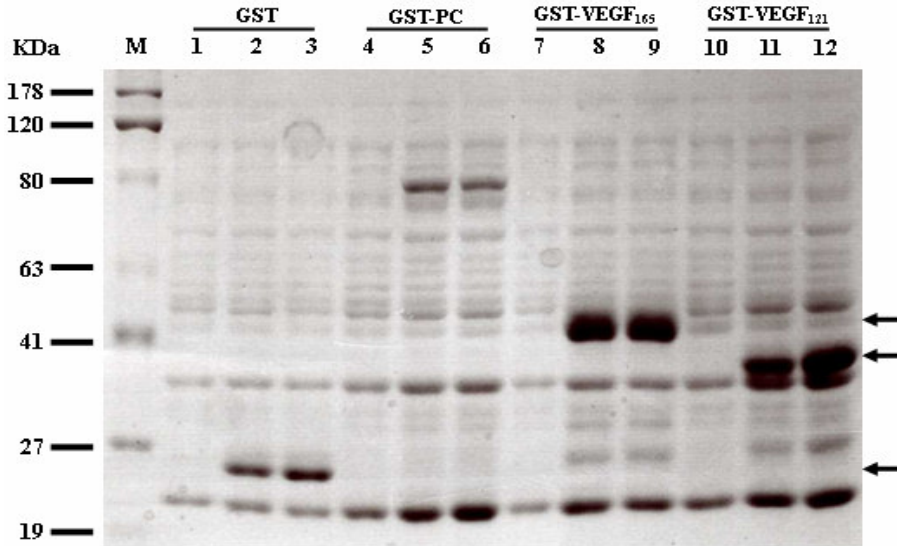

Figure 1b. 10% SDS-PAGE analysis of bacteria expressed GST-VEGF$_{165}$ and GST-VEGF$_{121}$. M: Protein Marker. Lane 1: *E. coli*/GST lysates without IPTG induction for 3 h; Lanes 2 and 3: *E. coli*/GST lysates with IPTG induction for 2 and 3 h respectively; Lane 4: *E. coli*/GST-PC lysates without IPTG induction for 3 h; Lanes 5 and 6: *E. coli*/GST-PC lysates with IPTG induction for 2 and 3 h respectively; Lane 7: *E. coli*/GST-VEGF$_{165}$ lysates without IPTG induction for 3 h; Lanes 8 and 9: *E. coli*/GST-VEGF$_{165}$ lysates with IPTG induction for 2 and 3 hours respectively; Lane 10: *E. coli*/GST-VEGF$_{121}$ lysates without IPTG induction for 3 hours; Lanes 11 and 12: *E. coli*/GST-VEGF$_{121}$ lysates with IPTG induction for 2 - 3 h respectively. Arrows show the GST-VEGF$_{165}$ (up arrow), GST-VEGF$_{121}$ (middle arrow) and GST (low arrow) at 48, 42 and 26 kDa respectively.

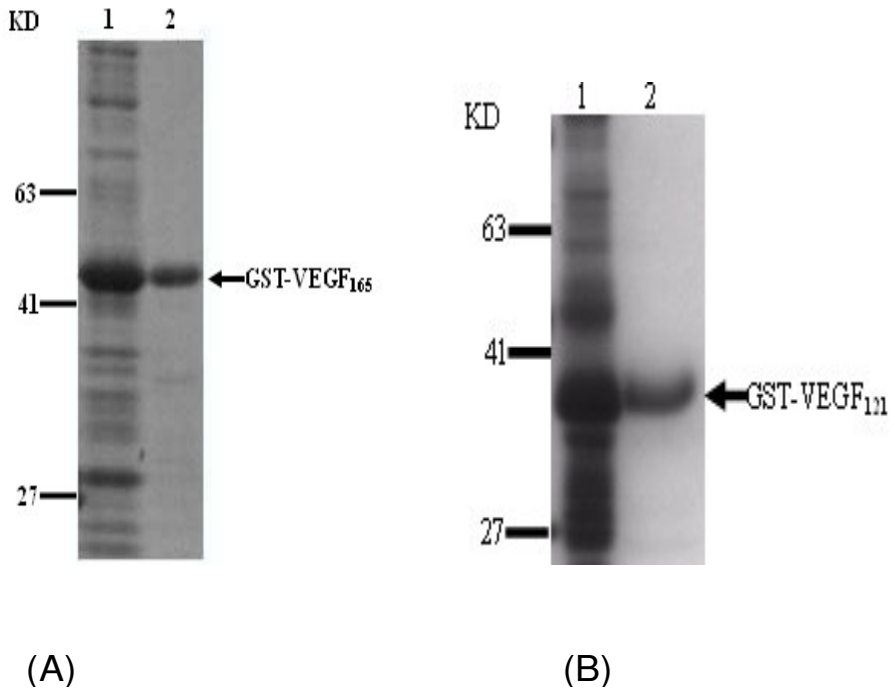

Figure 2. Purification of GST-VEGF fusion proteins analyzed by 10% SDS-PAGE. Lane 1; Bacterial lysates with unpurified GST-$VEGF_{165}$ (A) or GST-$VEGF_{121}$ (B). Lane 2. Purified GST-$VEGF_{165}$ (A) or GST-$VEGF_{121}$ (B). The arrows mark the position of GST-VEGF fusion proteins.

10-12 mg/l bacteria culture (Figure 2). All VEGF isoforms are covalently linked homodimers with a glycosylation site at Asn74, which is not required for VEGF biological activity. The molecular weight of $VEGF_{165}$ and $VEGF_{121}$ is 22 and 16 kDa under reduced conditions. The GST is a 26 kDa protein. The sizes of GST-$VEGF_{165}$ and GST-$VEGF_{121}$ are estimated to be 48 and 42 kDa respectively (Siemeister et al., 1996).

The procedure for purification of VEGF expressed from *E. coli* has the following steps generally: (1) separation and washing of inclusion body; (2) protein solubilization and renaturation; and (3) purification. The procedure is complicated, time-consuming and empirical (Siemeister et al., 1996; Lilie et al., 1998). We introduce a novel procedure here to purify GST-VEGF, a method that with only one step, the solubilization with Sarkosyl, was needed, and to skip the complicated procedures of separation, washing and renaturation of inclusion bodies.

Binding activity of GST-$VEGF_{165}$ to rh-sFlt-1

Binding ability of GST-$VEGF_{165}$ expressed in *E. coli* to recombinant human extracellular domain VEGF receptor (rh-sFlt-1) which was expressed from baculovirus, was studied by a sandwich-like assay. The affinity constant was approximately 10^6 mol/l (Figure 3). Frangioni and Neel (1993) reported that the enzymatic activity of tyrosine phosphatase 1B was maintained during the procedure of sarkosyl solubilization and purification. The hygromycin B phosphotransferase with biological activity was solubilized and purified from prokaryotic expression by sarkosyl (Zhuo et al., 2005). Our data show that GST-$VEGF_{165}$ binds to soluble extracellular domain Flt-1 in a dose-dependent manner.

The interaction of VEGF with its signaling tyrosine kinas receptors is a very important biological function. VEGF binds VEGF receptor 1 (VEGFR-1) and receptor 2(VEGFR-2) at different sites. The main cluster of VEGF amino acids that bind VEGFR-1 is located at one end of the VEGF monomer and the main cluster of VEGF amino acids that binds to VEGFR-2 is located at the opposite pole of the VEGF monomer. 3 acidic residues, Asp63, Glu64 and Glu67 in exon 3 are essential for binding to VEGFR-1, and 3 basic residues, Arg82, Lys84 and His86, in exon 4 are essential for binding to VEGFR-2 (Neufeld et al., 1999).

Conclusion

In conclusion, we have established a novel and efficient procedure to express and purify biologically active GST-$VEGF_{165}$ and GST-$VEGF_{121}$ from *E. coli* /pGEX-4T-1 fusion system. Sarkosyl is an alkyl anionic detergent and can be used for solubilization and renaturation of bac-

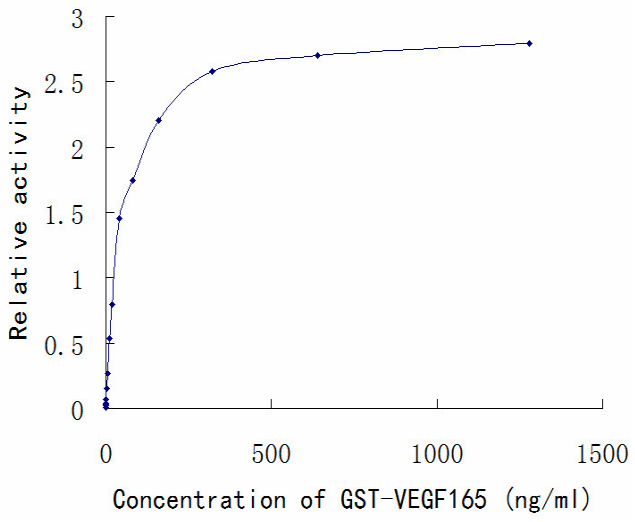

Figure 3. Binding activity of GST-VEGF$_{165}$ to VEGF receptor. Recombinant human fusion VEGF165 binds to VEGF receptor, sFlt-1, in a dose-dependent manner.

terial expressed inclusion bodies of VEGF. With sarkosyl, overall yield of GST-VEGF was up to 12 mg/l cell culture, and two laborious steps; the separation, washing and solubilization of inclusion body and renaturation of fusion proteins were skipped. Therefore, the novel procedure reported here can not only produce high amounts of VEGF fusion protein, which could be used for animal immunization and other research purpose, but also make the procedure much easier, compared to the conventional method.

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