Prokaryotic expression, refolding, and purification of functional human vascular endothelial growth factor isoform 165: Purification procedures and refolding conditions revisited

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ABSTRACT

Human vascular endothelial growth factor isoform 165 (VEGF165) is the first known member belonging to the VEGF protein family that plays a critical role in new blood vessel formation *in vivo*. This study presents a new protocol with optimized conditions for rapidly producing untagged recombinant and biological active human VEGF165 (rhVEGF165) using *Escherichia coli* cells. Protein was isolated from inclusion bodies, purified by gel filtration and ion exchange chromatography, and subjected to protein refolding and renaturation. The biological activity of rhVEGF165 is comparable with VEFG from eukaryotic source according to human umbilical vein endothelial cells (HUVEC) proliferation assay. Therefore, the present procedures provide a fast and easy way to produce this therapeutic protein.

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Introduction

Vascular endothelial growth factors and respective receptors promote the growth of blood vessel *in vivo*. Researchers have identified three *VEGFA* homologs, *VEGFB*, *VEGFC,* and *VEGFD* in the human genome. Two major VEGF receptors, *VEGFR-1* and *VEGFR-2* have also been identified [1–3]. Multiple protein isoforms of VEGF-A proteins (VEGF121, VEGF121b, VEGF145, VEGF165, VEGF165b, VEGF189, VEGF206, etc.) through the alternative exon splicing of *VEGFA* gene have been isolated from tissues. VEGF signaling plays a critical physiological role in triggering new blood vessel formation as early as the vasculogenesis of embryonic development. In adults, new blood vessels form by expanding from pre-existing vascular network in response to angiogenic stimuli (angiogenesis). For example, tight regulation of angiogenesis is crucial to wound healing and the regular occurrence of the female menstrual cycle [4,5]. Particularly, the retinal pigment epithelium secretes several isoforms of VEGF. These isoforms differ in molecular size and diffusion ability. The lack of some diffuse forms of VEGF may be responsible for the age-dependent degeneration of retinal pigment epithelium, and results in a pathological condition of visual loss [6]. The discovery of a micro RNA, mir-126, in the

mediation of mechano-sensory of blood flow and VEGF signaling has shed light on the genetic mechanism of new blood vessel formation [7]. These new findings reveal that a distinct and temporary type of VEGF signaling tightly regulates angiogenesis.

Solid tumors require blood supply from new blood vessels to support cell growth and tumor expansion. Inhibiting tumor angiogenesis starves tumors and stops their growth due to nutrient shortage. Several VEGF inhibitors are already in clinical use, including Bevacizumab/Avastin® (Genetech, Inc.), which is a prescription for the treatment of several metastatic tumors. As a humanized monoclonal antibody against endogenous VEGF, Bevacizumab prevents VEGF from binding to receptors. Researchers have developed other inhibitors with anti-angiogenic activity: for instance, the trap for VEGF, antisense oligonucleotide to VEGF mRNA, antagonists of VEGF receptors, and peptide vaccines for VEGF receptors [8–10]. However, the clinical application of anti-VEGF therapy remains problematic due to the side effects of therapeutic intervention [11].

As the first characterized member of VEGF, the VEGF165 isoform is also well known as VEGF or VPF (vascular permeability factor), and is a predominately expressed protein in the human body [12,13]. VEGF165 shows strong mitogenic potency to vascular endothelial cells, and has a strong induction effect on vascular formation *in vitro*. The polypeptide of VEGF165 consists of 191 amino acids. The first 25 residues comprise the signal peptide, which does not appear in mature protein. The part of mature VEGF165 consists of 115 amino acids, including eight cysteine residues. This domain

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folds into a cysteine-knot structure and binds to the VEGF receptor. The C-terminal part of VEGF165 accounts for the last 50 residues, and has several heparin-binding sites. These residues do not directly participate in VEGF receptor binding. Mature VEGF165 is a homo-dimeric protein, and is modified by glycosylation at Asn-101. However, the role of glycosylation in mature VEGF165 remains unclear. Furthermore, proteolytic cleavage of mature VEGF165 between the N-terminal part and C-terminal may be a regulatory mechanism for VEGF165 [14]. Post-translational proteolysis processing may play a role in the binding affinity and diffusion ability of VEGF165. Thus, understanding the biochemical difference among various VEGF isoforms may enable specific target validation in drug development.

Current protein databank information contains no information on human VEGF165. Moreover, the procedures for producing recombinant human VEGF165 (rhVEGF165) in prokaryotic system remains unclear. Thus, this study revisits the conditions for expressing and purifying VEGF165 using Escherichia coli cells. This study develops a new purification procedure to allow reliable recovery rhVEGF165 from inclusion bodies while maintaining a reasonable overall yield. The coding sequence for human VEGF165 isoform was cloned into a pET vector, and expressed in untagged form. Results show that recombinant protein accumulated in the inclusion bodies of cells. Protein was isolated from the inclusion bodies, then refolded into native state, and finally polished to homogeneity by HPLC. The resulting rhVEGF165 was analyzed by HUVAC cell proliferation assay and gel electrophoresis. These procedures facilitate the investigation of VEGF protein and provide a better understanding of this common angiogenic factor.

Materials and methods

Materials

2-Amino-2-(hydroxymethyl)-1,3-propanediol (Tris base), sodium chloride (NaCl), and magnesium chloride (MgCl2) were purchased from Mallinckrodt Baker, Inc. Phillipsburg, NJ. Guanidine chloride (GuHCl), urea, and L-arginine hydrochloride were purchased from Merck KGaA, Darmstadt, Germany. 2-(Bis(2-hydroxyethyl)amino)acetic acid (Bicine), Lysozyme, deoxyribonuclease I (DNase I), dithiothreitol (DTT) and Triton X-100 were purchased from Sigma Chemical Co., St. Louis, MO. Source 15 Q bulk media, Superdex G200 prep grade, and Sephadex G-25 fine grade were obtained from GE Healthcare, Piscataway, NJ.

Cloning of human VEGF165 protein for protein expression

A cDNA clone of the human VEGF165 gene was purchased from Open Biosystems (Huntsville, AL). The cDNA sequence encoded for A27 to R191 of human VEGF was cloned into pETBlue-1 vector using the AccepTor cloning kit (Novagen, Madison, WI) with the following oligonucleotides:

- 5′Oligo 5′-atggcacccatggcagaaggaggagg-3′

- 3′Oligo 5′-tcaccgcctcggcttgtcacatc-3′

The target DNA sequence was amplified using PCR (95 °C/2 min – one cycle; 95 °C/30 s, 60 °C/30 s, 72 °C/30 s-35cycle; 72 °C/10 min; PfuUltra II Fusion HotStart DNA Polymerase; Stratagene Inc., La Jolla, CA. The resulting DNA fragment was gel purified and ligated with T-vector. ATG start codon and TGA stop codon were added at the 5′ and 3′ ends of the cloned sequence. The expressed rhVEGF165 had an additional methionine residue at the N-terminus. The construction plasmid DNA, pETBlue-1-rhVEGF165, was extracted using QIAGEN plasmid kit (QIAGEN Inc., Alameda, CA) and the cloning sequence was verified by DNA sequencing.

Expression of rhVEGF165

pETBlue-1-rhVEGF165 was chemically transformed into Tuner(DE3)pLacI E. coli competent cells (Novagen, Madison, WI) following the manufacturer's instructions. The transformation cells were plated on a LB–ampicillin–chloramphenicol plate supplied with 2% dextrose and incubated overnight at 37 °C. Doubly resistant colonies were inoculated in 3 mL of LB–ampicillin–chloramphenicol medium containing 2% dextrose and grown 12 h at 37 °C to an OD600 of 0.5–1.0. An equal volume of sterile glycerol solution (60%) was then added, mixed, and aliquoted. The glycerol stock was stored frozen at −80 °C up to one year. For protein production, 50 mL of LB–ampicillin–chloramphencol medium containing 2% dextrose was inoculated with 0.5 mL glycerol stock and incubated at 37 °C. The next day, the 10 mL overnight culture was diluted into 1000 mL of LB–ampicillin–chloramphencol medium, grown to OD of 0.8, and then induced with 1 mM IPTG for 4 h. Cells were collected by 15 min of centrifugation at 5000g and then stored frozen at −80 °C.

Isolation of rhVEGF165 inclusion bodies

All purification procedures were performed at 4–8 °C unless otherwise noted. Cell pellets were resuspended with 4 mL of lysis buffer consisting of 50 mM Tris–HCl, pH 8.0, 1 mM EDTA, 1% Triton X-100 and 10 mM MgCl$_2$ per gram of cells. Lysozyme (1 mg per mL lysis buffer), DNase I (10 µg per mL lysis buffer) and PMSF (1 mM final) were added. The suspension was stirred on ice for 60 min and the cell lysate was centrifuged for 30 min at 13,000g. The pelleted materials were resuspended in 2 mL of 50 mM Tris–HCl, pH 8.0, 1 mM EDTA and 4 M urea per gram of cells, and allowed to mix for 60 min. The insoluble materials were pelleted by centrifugation at 13,000g for 30 min. The pelleted materials were resuspended with 1 mL of 50 mM Tris–HCl, pH 8.0, 1 mM EDTA, 2 M urea, and 10 mM DTT per gram of cells, and allowed to mix for 60 min. The suspension was cleared by centrifugation at 13,000g for 30 min. The extract of solubilized inclusion bodies was then stored frozen at −80 °C.

Denatured purification of rhVEGF165

All chromatographic procedures were performed at room temperature unless otherwise noted. The extract of 5 mL volume was buffer exchanged into 100 mM Tris–HCl, pH 8.0, 5 mM EDTA, 6 M GudHCl and 2 mM DTT using a 25/10 Sephadex G-25 fine column (the gel volume of 50 mL). Fractions with proteins were pooled, concentrated to approximately 10 mg/mL, adjusted to a DTT concentration of 100 mM, and incubated in a 37 °C water bath for 1 h. The denatured protein was then either immediately applied to the pre-equilibrated gel-filtration column and anion-exchange column (below) or temporarily stored at 4 °C overnight. The protein was applied to a 15/70 Superdex 200 prep grade column (15 mm internal diameter, 70 cm column length, and the gel volume of 125 mL) equilibrated with buffer A (50 mM Tris–HCl, pH 8.0, 2 M urea, 0.5 mM EDTA, and 2 mM DTT). Fractions with the purified rhVEGF165 were pooled and immediately applied to a 10/10 Source 15Q column (the resin volume of 8 mL), and washed with one column volumes of Buffer A at a flow rate of 2.62 mL/min. The first linear gradient from 0% to 40% Buffer B (Buffer A + 1 M NaCl) was applied over 8 column volumes followed by the second linear gradient from 40% to 100% Buffer B was applied over 4 column volumes. The elute fractions were analyzed by SDS–PAGE, pooled and concentrated to approximately 10 mg/mL, and then stored at −80 °C.

Refolding and purification of rhVEGF165

The refolding and purification procedures were performed at 4–8 °C unless otherwise noted. Renaturation of rhVEGF165 was carried out using 1:207 dilutions of the purified rhVEGF165 into refolding buffer (50 mM Tris–HCl, pH 8.0, 1.0 M L-arginine, 1 mM EDTA, 0–20 mM glutathione). The denatured protein was thawed and diluted, drop by drop, into the refolding buffer with stir-bar mixing. The protein concentration during renaturation was 2.5 μM (48 μg/mL). After 7 days of renaturation, the protein solution was concentrated and ultrafiltered in Buffer C (20 mM sodium Bicine, pH 8.2) using a Labscale TFF system (Millipore, Billerica, MA). The protein solution was concentrated to approximately 1–2 mg/mL and then stored at 4 °C. Subsequently, 0.1 mg of protein was purified by a MonoS PEEK 4.6/10 column (1.6 mL, GE Healthcare) pre-equilibrated with Buffer C. The unbound materials were washed out with 1 column volume of Buffer C and a linear gradient from 0% to 100% Buffer D (Buffer C + 1 M NaCl) was applied over 10 column volumes. The elution of dimeric rhVEGF165 in fractions was examined by 12.5% SDS–PAGE without reducing agents. The native protein was pooled and concentrated to approximately 1 mg/mL, and then stored at 4 °C or −80 °C indefinitely.

Cell proliferation assay for VEGF

Human umbilical vein endothelial cells from Bioresource Collection and Research Center (BCRC No.: H-UV001; Hsinchu, Taiwan) were maintained in Gibco Medium 199 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum and provided with 30 μg/mL endothelial cell growth supplement (ECGS; Upstate, Lake Placid, NY) and 25U/mL heparin (Sigma Chemical Co.) in a humidified 95% air, 5% CO_2 incubator at 37 °C. Cells were seeded at a density of 5000 cells per well in gelatin-coated 96-well tissue culture plates. Various concentrations of rhVEGF165 or commercial VEGFs were tested at indicated concentrations and incubated with cells for 72 h. ECGS was removed from the culture medium during the assay.

The proliferation of cells was measured by the MTT assay. 10 μL of 5 mg/mL 3-(4,5-dimethylthiazol)-2,5-diphenyltetrazolium bromide (MTT; Sigma Chemical Co.) in PBS was added to each well and incubated for 1–4 h. Cell viability was measured by a SpectraMax reader (Molecular Devices, Sunnyvale, CA) using the absorption wavelength of 570 nm with a reference of 650 nm. All tests were performed in triplicate for each sample.

Results and discussion

Prokaryotic expression and denatured purification of untagged human VEGF165

Using pET-1 vector, E. coli expressed the protein sequence of matured human VEGF (GenBank Accession No. NP_001165097) as an untagged protein. After IPTG induction for 4 h, a protein band for a unknown protein at 27.5 kDa was over-expressed (Fig. 1A). Its MW was higher than the expected MW of the rhVEGF165 of 19,297 Da, based on the cloning sequence. In addition, another protein band at approximate 20 kDa, was over-expressed after IPTG induction. To validate these two proteins, the soluble fraction and insoluble fraction were prepared from cells. The 27.5 kDa protein appeared in the soluble fraction, whereas the 20 kDa protein appeared exclusively in the insoluble fraction (Fig 1B). In-gel digestion and LC/MS/MS analysis confirmed that the 20 kDa protein is human VEGF (data not shown). Thus, we purified the 20 kDa protein from the insoluble fraction of E. coli cells. The insoluble fraction was prepared, washed in 4 M urea, and then extracted by
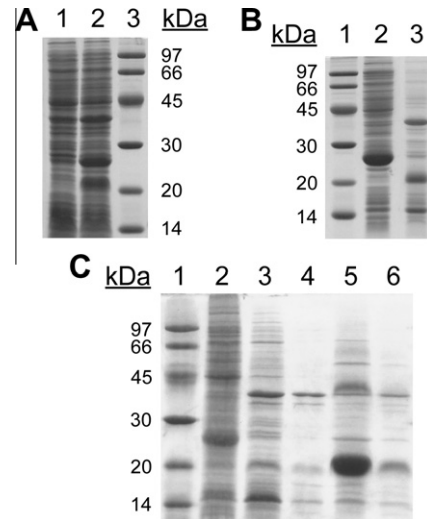

Fig. 1. Recombinant expression and inclusion bodies preparation for rhVEGF165 from E. coli. (A) The total protein of the host bacteria was separated by 12.5% SDS–PAGE and visualized by Coomassie blue stain. Lane 1: the LMW molecular weight standards. Lane 2: cells prior to IPTG induction. Lane 3: cells after 1 mM IPTG induction for 4 h. (B) Preparation of cell lysate. Cells were lysed by lysozyme-Triton X-100 and centrifugal fractionation. Lane 1: the LMW molecular weight standards. Lane 2: the supernatant of cell lysate. Lane 3: the pellet of cell lysate. (C) Extraction of rhVEGF165 from washed inclusion bodies. The inclusion bodies were isolated as (B), washed twice in urea, and then extracted by DTT. Lane 1: the LMW molecular weight standards. Lane 2: the soluble fraction of cell lysate. Lane 3: the soluble proteins from the first washing step. Lane 4: the soluble proteins from the second washing step. Lane 5: the DTT-extraction. It is riched for rhVEGF165. Lane 6: the second DTT-extraction.

reducing agent (Fig. 1C). The crude extract exhibited a heterogeneous composition that included substantial amounts of E. coli proteins (Fig. 1C, lane 5). The crude extract was applied to gel filtration chromatography followed by anion exchange chromatography, producing a high purity preparation of denatured rhVEGF165 (Figs. 2 and 3). The yield of purified rhVEGF165 was approximately 4 mg per liter of starting culture (Table 1) by BCA protein assay (Pierce, Rockford, IL).

Refolding of denatured rhVEGF165

Previous research reports two approaches to the refolding of the cysteine-knot motif in human VEGF protein from the denatured state to the native state. The yield of dimeric, native VEGF is highly dependent on the incorporation of salt additives, such as 0.4 M sodium chloride or 0.5 M guanidinium hydrochloride, in the refolding buffer [15,16]. This study proposes another one-step, dilution-based refolding protocol that dilutes denatured rhVEGF directly into 1.0 M L-arginine and incubates it for 7 days (Fig. 4). The dimeric rhVEGF165 decreased if a disulfide reagent, glutathione, was present in the refolding buffer. However, HUVEC cell proliferation assay reveals that the potency of the refolded protein was higher after incorporating a small amount of glutathione. Accordingly, this study includes glutathione in the refolding buffer. After refolding was complete, the refolding reaction was concentrated by tangential flow filtration followed by diafiltration to exchange buffer with a low ionic strength pH 8.0 buffer. Finally, native rhVEGF165 was polished with Mono S chromatography (Fig. 5). The resulting rhVEGF165 showed more than 95% purity in non-reducing SDS–PAGE analysis and the MW of reduced rhVEGF165 and non-reduced rhVEGF165 were about 19 and 42 kDa (Fig. 5B), respectively. The overall yield was approximately 1 mg protein per liter of starting culture (Table 1).
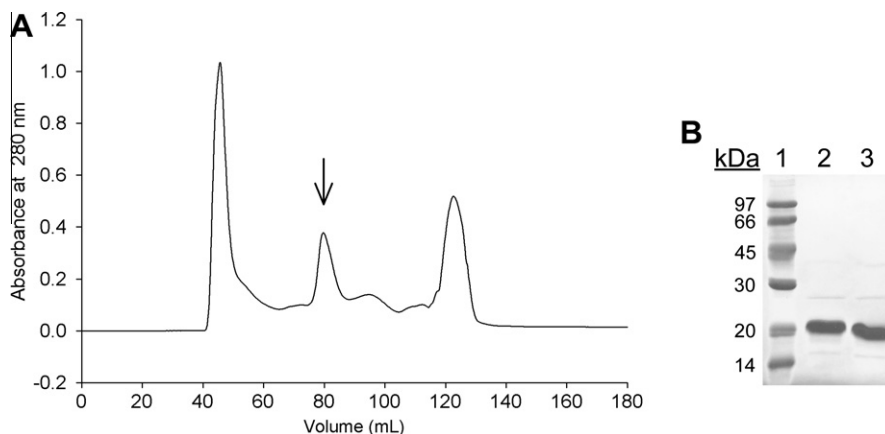
Fig. 2. Purification of untagged rhVEGF165. (A) Superdex 200 chromatography. The arrow indicates the peak containing denatured rhVEGF165. (B) The Coomassie blue stained SDS–PAGE (12.5%) of eluate from (A). Lane 1: the LMW molecular weight standards. Lane 2: DTT-reducing sample. Lane 3: non-reducing sample. No significant difference was found between reduced and non-reduced samples.
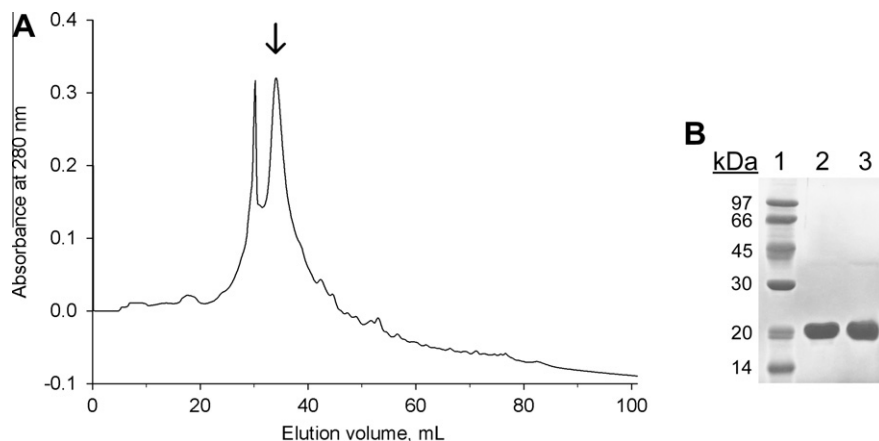

Fig. 3. Purification of partial purified rhVEGF165. (A) Source 15Q chromatography. The arrow indicates the peak around 185 mM of NaCl containing denatured rhVEGF165. (B) The Coomassie blue stained SDS–PAGE (12.5%) of eluate from (A). Lane 1: the LMW molecular weight standards. Lane 2: DTT-reducing sample. Lane 3: non-reducing sample. No significant difference was found between reduced and non-reduced samples.

Table 1
Prokaryotic expression, refolding, and purification of recombinant human VEGF 165 isoform.[a]

Purification step	Total protein (mg)	Purity[b]	Overall yield
Solubilized inclusion bodies	61	37%	100%
Superdex 200 and Source 15 Q	4	>95%	6.5%
Refolding and Mono S	1	>95%	1.6%

[a] The results are given for the 6 g of wet weight cells from 1 L of *E. coli* culture.
[b] The purity is determined by SDS–PAGE analysis in which the band intensities of 19 or 45 kDa are used for denatured and renatured rhVEGF165, respectively.
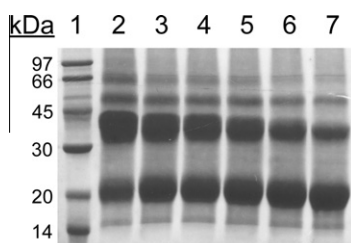

Fig. 4. Refolding of rhVEGF165 analyzed by non-reducing SDS–PAGE (12.5%). Lane 1: the LMW molecular weight standards. Lane 2: refolded rhVEGF165 under disulfide-shuffling reagent free condition. Lane 3–7: refolded rhVEGF165 under 1, 2, 5, 10, 20 mM of disulfide-shuffling reagent (glutathione) condition.

Improved bacterial expression and purification of human native VEGF165

This study describes a new detailed procedure for producing untagged recombinant human VEGF165 by prokaryotic expression and purification. Each step in the presented procedure was optimized and repeatedly validated to ensure a reasonable yield and good reproducibility. Chromatogram and SDS–PAGE data confirmed each step. The proposed protocol is a convenient guide for producing this growth factor or further improving laboratory procedures. Scrofani et al. reported a detailed protocol for producing human VEGF-B isoform 167 (VEGF-B167) from a prokaryotic source, and analyzed the binding affinity of their recombinant protein to VEGF-R1 or VEGF-R2 [15]. Although they provided a feasible protocol for producing VEGF-B, there are two major differences between their protocol and ours. First, we produced rhVEGF165 as untagged protein, whereas Scrofani et al. expressed rhVEGF-B167 as an NH$_2$-terminal His$_6$ tagged protein. Second, the proposed protocol does not use any organic solvents such as acetonitrile. Keyt et al. reported a procedure for preparing unglycosylated VEGF from bacterial expression, and demonstrated that the C-terminal domain of VEGF165 is critical for the endothelial cell mitogenic activity of VEGF165. However, their report contains no data on the protein expression and purification, preventing us from comparing the results of these two protocols [16].
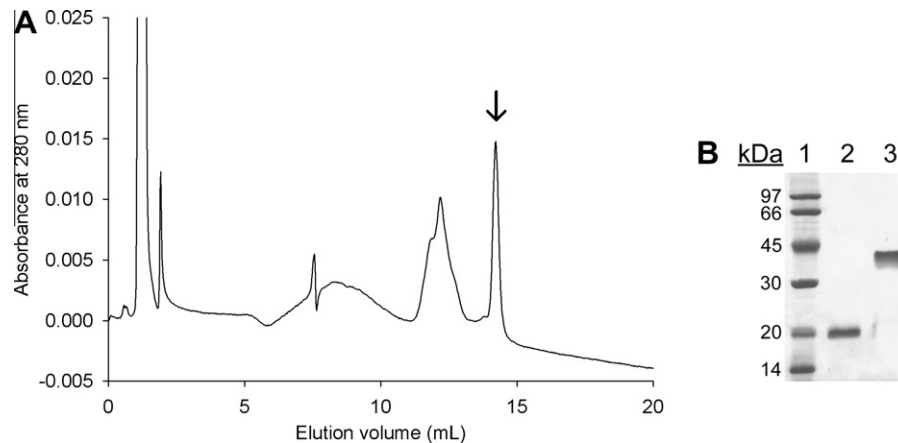
Fig. 5. Purification of native rhVEGF165. (A) Mono S chromatography. The arrow indicates the peak around 650 mM of NaCl contained rhVEGF165 homodimer. (B) The Coomassie blue stained SDS–PAGE (12.5%) of eluate from (A). Lane 1: the LMW molecular weight standards. Lane 2: DTT-reducing sample. Lane 3: non-reducing sample.

In conclusion, this study develops a protocol for semi-preparative preparation of *E. coli*-derived human VEGF. The rhVEGF165 production in bacterial host in this study is equivalent to the mammalian hVEGF165, according to HUVEC proliferation assay. The prokaryotic system is an ideal preparation method because of its high yield, low costs, and bio-safety. Many researches and applications can facilitate the large-scale production of recombinant human protein to satisfy the growing demand for recombinant protein of human VEGF.

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