ISSN 1561-8331 (Print)
ISSN 2524-2342 (Online)
УДК 577.151; 577.112
https://doi.org/10.29235/1561-8331-2026-62-4-300-308

Поступила в редакцию 11.08.2026
Received 11.08.2026

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ESCHERICHIA COLI EXPRESSION AND PURIFICATION OF HUMAN ANGIOGENESIS REGULATORS VEGF165 AND ANGPT1

Abstract. Human vascular endothelial growth factor 165 (VEGF165) and angiopoietin-1 (ANGPT1) are key regulators of angiogenesis and both play a central role in gene therapeutic strategies for treating critical limb ischemia. The low yield and high production cost of VEGF165 and ANGPT1 limit their application and investigation as potential therapeutic agents. To overcome folding limitations and avoid modifying native sequences with large solubility tags, expression plasmids were constructed using the pET-20b(+) vector with an N-terminal His-tag, and expression parameters (temperature, time) were optimized alongside co-expression with the pGro12 chaperone system. Small-scale optimization revealed that moderate cultivation temperatures (22–26 °C) combined with pGro12 co-expression dramatically reduced inclusion body accumulation and increased the soluble fraction for both proteins. Optimal parameters were established as 48 h at 26 °C for VEGF165 and 72 h at 22 °C for ANGPT1. This study establishes a highly effective expression and purification strategy for yielding soluble VEGF165 and ANGPT1.

Keywords: VEGF165, ANGPT1, angiogenesis, bacterial expression system, protein expression, *E. coli*

For citation. Sachanka A. B., Popicheva E. A., Yantsevich A. V. *Escherichia coli* expression and purification of human angiogenesis regulators VEGF165 and ANGPT1. *Vestsi Natsyyanal'nai akademii navuk Belarusi. Seryya khimichnykh navuk = Proceedings of the National Academy of Sciences of Belarus. Chemical series*, 2026, vol. 62, no. 4, pp. 300–308 (in Russian). https://doi.org/10.29235/1561-8331-2026-62-4-300-308

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ЭКСПРЕССИЯ В КЛЕТКАХ *ESCHERICHIA COLI* И ОЧИСТКА РЕГУЛЯТОРОВ АНГИОГЕНЕЗА ЧЕЛОВЕКА VEGF165 И ANGPT1

Аннотация. Изоформы фактора роста эндотелия сосудов VEGF165 и ангиопоэтина-1 (ANGPT) человека являются ключевыми регуляторами ангиогенеза и играют центральную роль в геннотерапевтических стратегиях лечения критической ишемии конечностей. Низкий выход и высокая стоимость получения VEGF165 и ANGPT1 ограничивают их применение в качестве потенциальных терапевтических средств. Для преодоления проблем, связанных с фолдингом белков, и исключения необходимости модификации нативных последовательностей крупными растворяющими метками были сконструированы экспрессионные плазмиды на основе вектора pET-20b(+) с N-концевой His-меткой, а также были оптимизированы параметры экспрессии (температура, время) и отработана методика совместной экспрессии с системой шаперонов pGro12. Оптимизация процесса в малом масштабе показала, что умеренные температуры культивирования (22–26 °C) в сочетании с совместной экспрессией pGro12 позволяют существенно снизить образование телец включения и увеличить долю растворимой формы обоих белков. Были определены оптимальные условия экспрессии: 48 ч при 26 °C для VEGF165 и 72 ч при 22 °C для ANGPT1. Разработана высокоэффективная стратегия экспрессии и очистки, обеспечивающая получение растворимых форм VEGF165 и ANGPT1.

Ключевые слова: VEGF165, ANGPT1, ангиогенез, бактериальная экспрессионная система, экспрессия белка, *E. coli*

Для цитирования. Саченко, А. Б. Экспрессия в клетках *Escherichia coli* и очистка регуляторов ангиогенеза человека VEGF165 и ANGPT1 / А. Б. Саченко, Е. А. Попичева, А. В. Янцевич // Весці Нацыянальнай акадэміі навук Беларусі. Серыя хімічных навук. – 2026. – Т. 62, № 4. – С. 300–308. https://doi.org/10.29235/1561-8331-2026-62-4-300-308

Introduction. Human vascular endothelial growth factor (VEGF) is a key regulator of angiogenesis and promotes the growth of new blood vessels *in vivo*. VEGF consists of two identical polypeptide subunits that form a homodimeric glycoprotein stabilized by disulfide bonds [1]. It is a member of a family of structurally related proteins that act as ligands for the VEGF receptor (VEGFR) family. Ligand-receptor binding initiates an intracellular signaling cascade that drives endothelial cell growth, survival,

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and proliferation, ultimately promoting angiogenesis [2]. Four VEGF homologs have been identified in humans: VEGF-A, VEGF-B, VEGF-C, and VEGF-D [3]. Among these, VEGF-A has been the primary focus of angiogenesis research for more than three decades, making it the best-characterized molecule within the VEGF family [4]. Multiple protein isoforms of VEGF-A differ in their bioavailability. VEGF121 is freely secreted, whereas VEGF189 and VEGF206 are secreted but remain largely bound to heparan sulfate proteoglycans on cell surfaces. VEGF145 and VEGF165 exhibit intermediate properties, existing in both bound and soluble forms [5]. Among these, VEGF165 is the most abundant, the most extensively studied, and the predominantly active VEGF-A isoform, existing primarily as a homodimer with a molecular weight of ~40 kDa [6].

VEGF165 is a homodimeric growth factor characterized by a cysteine-knot motif and a single glycosylation site. Each monomer comprises an N-terminal receptor-binding domain (amino acids 1-110) that participates in disulfide-linked dimer formation, and a C-terminal heparin-binding domain (amino acids 111–165) that mediates interaction with heparan sulfate proteoglycans on cell surfaces. The N-terminal region is primarily responsible for VEGFR engagement, whereas the C-terminal region determines extracellular matrix association and modulates isoform bioavailability [7]. VEGF165 binds to the FMS-like tyrosine kinase 1 (Flt-1) and kinase insert domain receptor (KDR), which are localized on the endothelial cell surface of developing and mature blood vessels [8]. It also interacts with Flt-4, a receptor primarily associated with lymphangiogenesis [9]. Activation of these receptors initiates intracellular signaling cascades, including post-translational modifications, that lead to the formation of new blood vessels.

VEGF165 plays a central role in gene-therapeutic strategies aimed at treating critical limb ischemia [10] and wound healing [11]. Therapy with VEGF-overexpressing mesenchymal stem cell sheets significantly improved cardiac function, reduced fibrosis, and enhanced myocardial neo-angiogenesis and cell proliferation, and may be used in the period after transplantation to shorten recovery time [12]. Moreover, aberrant angiogenesis resulting from overexpression of VEGF165 under stress conditions (hypoxia, chemotherapy, radiotherapy) [13], contributes to diseases such as rheumatoid arthritis, atherosclerotic plaque formation, visual loss in diabetic retinopathy, and tumor angiogenesis [14]. Inhibition of VEGF165 has therefore emerged as a therapeutic strategy for several metastatic tumors [15].

VEGF165 has been reported to be expressed in various systems, such as *Kluyveromyces lactis* GG799 cells at ~5.7 mg/L [16], *Saccharomyces cerevisiae* at ~4.0 mg/L [17], and overproduced in Chinese hamster ovary (CHO) cells at ~20.0 mg/L [18]. Attempts to express VEGF165 in conventional prokaryotic systems typically yield low amounts of soluble protein and are frequently accompanied by misfolding and aggregation into inclusion bodies, which severely compromise recovery of functional protein [19]. Although soluble VEGF165 has been produced in *E. coli* as fusion constructs, process-scale limitations (including low volumetric productivity, proteolytic instability, inefficient tag removal, and challenges in downstream refolding and purification) impede industrial-scale implementation [20]. Optimization of bacterial fermentation media and control parameters to increase production of insoluble VEGF165 and related isoforms has been described, with final purified yields reported in the range of 5–40 mg/L [21] and soluble-fraction yields of ~0.5–1.0 mg/L [19, 22, 23]. Bacterial expression of soluble VEGF165 can shorten fermentation durations and improve scalability, thereby increasing the economic viability and applicability of the protein [24].

Angiopoietin-1 (ANGPT1) promotes the maturation and stabilization of blood vessels during the later stages of vascular development [25]. ANGPT1 is a secreted ligand for the endothelial tyrosine-kinase receptor Tie2, acting as a key regulator of vascular maturation and stability and modulating both physiological angiogenesis and pathological vascular remodeling [26]. Transgenic overexpression of ANGPT1 protects the adult vasculature from plasma leakage, attenuates VEGF-induced adverse effects, and synergistically promotes vessel formation by enhancing endothelial cell survival following injurious stimuli [27]. As demonstrated previously [28], combined administration of VEGF165 and ANGPT1 is a promising strategy for treating conditions such as critical limb ischemia and for enhancing wound regeneration.

ANGPT1 comprises a short N-terminal domain that assembles into ring-like structures to supercluster oligomers into variably sized multimers, a central coiled-coil domain that mediates and stabilizes

oligomerization, and a C-terminal fibrinogen-like domain that primarily mediates binding to the Tie2 receptor [27, 29]. Because of its structural properties, ANGPT1 is prone to aggregation and insolubility, which likely complicates high-level expression. Recombinant expression of ANGPT1 has been reported in mammalian systems including COS cells [27] and HEK293 cells [30], in CHO cells [31] and in insect cells (Sf9) using a baculovirus [32].

In this study, we present an improved strategy for the overexpression and purification of soluble VEGF165 and ANGPT1, achieved through optimized expression conditions and co-expression of molecular chaperones. For the first time, we demonstrate the expression and isolation of ANGPT1 in *E. coli*. Recombinant proteins were purified from the cells by metal-affinity chromatography (via an N-terminal His-tag) followed by size-exclusion chromatography. The isolated proteins are suitable for functional studies.

Materials and methods. Reagents used in this study included: BigDye Terminator v3.1 Cycle Sequencing Kit and BigDye Xterminator Purification Kit (Applied Biosystems, USA); Q5 Hot Start High-Fidelity DNA polymerase; competent *E. coli* DH5α cells; Gel Loading Dye, Purple (6X) with SDS; restriction enzymes NdeI HF and HindIII HF; recombinant shrimp alkaline phosphatase (rSAP); 1 kb Plus DNA Ladder (NEB, UK); T4 DNA ligase; RNase A; dNTPs; PageRuler Plus protein marker, nitrocellulose membrane (Thermo Scientific, USA); silica gel (Serva, Germany); sodium hydroxide and sodium chloride (Five Oceans, Belarus); ethidium bromide, agarose, acetonitrile, and methanol (Sigma, USA); ampicillin (Sintez, Russia); glycerol, ethanol, guanidine thiocyanate, and sodium dodecyl sulfate (SDS) (Acros Organics, Germany); LB and TB media (Conda, Spain); EDTA (PanReac AppliChem, Germany); isopropanol, hydrochloric acid, and acetic acid (Base No.1 chemical reagents, Russia), isopropyl-β-D-1-thiogalactopyranoside (IPTG), phenylmethylsulfonyl fluoride (PMSF), the pET-20b(+) expression vector, N,N,N′,N′-tetramethylethylenediamine (TEMED), ammonium persulfate (USB, USA); imidazole and β-mercaptoethanol (Glentham Life Sciences, UK); Tris (tris(hydroxymethyl)aminomethane) (Melford, USA); peptone and tryptone (Conda, Spain); rabbit polyclonal anti-His IgG, goat anti-rabbit HRP-conjugated secondary antibody, and Excellent Chemiluminescent Substrate for western blot detection (Elabscience, USA); PCR products were purified from agarose gels using the NucleoSpin Gel kit (Macherey-Nagel, USA).

Plasmid construction. The coding sequences of VEGF165 and ANGPT1 were amplified from the pCDNA_VEGF165 and pCDNA_ANGPT1 plasmids (constructed previously) [33] by PCR using Q5 polymerase and primers 1–2 (VEGF165) and 3–4 (ANGPT1; presented in the Table), which generated HindIII and NdeI restriction sites. The PCR products were digested, gel-purified using the NucleoSpin Gel and PCR Clean-up kit, and ligated into the pET20b vector at the HindIII and NdeI sites using T4 DNA ligase, thereby introducing an N-terminal His-tag for affinity purification. Ligation products were transformed into *E. coli* DH5α, colonies with the correct insert were screened by PCR. Sequence identity of all constructs was confirmed by Sanger sequencing.

PCR primers

ID	Name	Sequence (5′→3′)
1	VEGF_F	gaggtcatatgATGAATTTTCTGCTCTCATGGGTACATTGGAGCTTG
2	VEGF_R	GTACCAAGCTTCCTCCGGGGCTTG
3	ANGPT_F	gaggtcatatgATGACCGTGTTTCTGAGCTTCGCCTTC
4	ANGPT_R	GTACCAAGCTTGAAATCCAGGGGCCGAATC

Optimization of protein expression. For protein expression two-plasmid *E. coli* strain BL21 (DE3) and Terrific Broth (TB) media supplemented with ampicillin and kanamycin were used. For optimization, culture tubes (5 mL TB) were incubated at 37 °C to an optical density of A_{600} = 0.6 and induced with IPTG (1 mM) and arabinose (4 mg/mL) when needed, then incubated at 22, 26, 30, or 37 °C for 24, 48, or 72 h post-induction. Cells were harvested by centrifugation (3000 x g, 4 °C, 20 min) and suspended in R1 buffer (50 mM sodium phosphate, pH 8.0; 0.3 M NaCl; 0.1 mM PMSF). Suspensions were lysed by sonication and clarified by centrifugation (19000 x g, 4°C, 30 min) to separate soluble protein from inclusion bodies. A 100 µL aliquot of the soluble fraction was desalted, precipitated by addition of EtOH

to 90 %, washed with 70 % EtOH, and pelleted by centrifugation (19 000 g, 4 °C, for 10 min). Pellets were resuspended in 50 μL SDS-loading buffer (62.5 mM Tris-HCl, pH 6.8; 10 % glycerol; 2 % SDS; 5 % β-mercaptoethanol; 0.125 % bromophenol blue). Inclusion body pellets were washed with 70 % EtOH, centrifuged (19000 x g, 4°C, for 10 min), and solubilized in 100 μL SDS-loading buffer. Protein expression levels were assessed by western blot (WB) using an anti-His antibody.

For scale-up, cultures were grown in 1 L TB with high aeration at 37 °C to A_{600} = 0.6 and induced with 1 mM IPTG (and arabinose where applicable). Cells were harvested and processed as above, lysed using an Emulsiflex C5 homogenizer, and clarified by centrifugation (30000 x g, 4 °C, for 60 min). The resulting supernatant was used for immobilized metal affinity chromatography (IMAC).

Protein purification. VEGF165 and ANGPT1 were purified from the transparent supernatant using IMAC. The purification utilized a HisTrap HP 5 mL column (Cytiva), which was pre-equilibrated in R2 buffer (pH 8.0; 50 mM sodium phosphate, 0.3 M NaCl). Following equilibration, the column was washed with the same 50–75 mL buffer containing 15–75 mM imidazole and subsequently eluted using a gradient of 50–500 mM imidazole, collecting 2 mL fractions. Protein-containing fractions with > 80 % purity were pooled and concentrated using centrifugal concentrators. The resulting concentrated VEGF165 was then additionally purified by size exclusion chromatography (SEC) on Sephadex G75/100 (Cytiva). This step employed R2 buffer for both equilibration and elution, yielding 0.5 mL fractions. After pooling and concentration of the homogeneous fractions, all proteins were dialyzed against a storage buffer (pH 8.0; 50 mM sodium phosphate, 0.1 M NaCl; 50 % glycerol). Final purity and identity were confirmed by SDS-PAGE, WB, and MALDI-TOF mass spectrometry.

SDS-PAGE and blotting. SDS-PAGE and blotting were performed using the Hoefer SE250 vertical electrophoresis system and the Bio-Rad Trans-Blot Turbo Transfer system. Proteins were separated on a 12 % polyacrylamide gel under reducing conditions and transferred to a nitrocellulose membrane at 25 V and 1.3 A for 45 min. After blocking in 5 % skim milk in phosphate-buffered saline (PBS) and washing with PBS containing 0.05 % Tween-20, the membrane was incubated with an anti-His-tag rabbit polyclonal antibody (diluted 1 : 1 000), followed by a goat anti-rabbit HRP-conjugated secondary antibody (diluted 1 : 2 500). Proteins were visualized using the Excellent Chemiluminescent Substrate (Elabscience) and imaged with an Azure c300 gel imaging system (Azure Biosystems). The amounts of soluble and pellet proteins were quantified using ImageLab software and compared across different conditions.

Results and discussion. *Optimization of VEGF165 and ANGPT1 expression.* As mentioned before, expression of VEGF165 in *E. coli* is limited by insolubility and aggregation into inclusion bodies [20, 34]. Previous reports describe similar challenges. At the same time, there are no prior reports of ANGPT1 expression in *E. coli*; studies in other expression systems likewise report aggregation and low yields of soluble protein [30, 31]. Low expression levels in *E. coli* frequently result in unfolded or misfolded proteins in the absence of eukaryotic post translational modifications (e.g., glycosylation), difficulties in forming correct disulfide bonds, and accumulation of insoluble protein in inclusion bodies when proteins are overexpressed [35].

To mitigate these limitations, several approaches are employed. Decreasing cultivation temperature and optimizing induction time reduce the rates of transcription, translation, and cell division, thereby providing more time for correct protein folding, decreasing aberrant protein–protein interactions and aggregation, and reducing protease activity [36]. Codon optimization (replacing rare codons with those preferred by the host) is used to improve translational efficiency and yield [35], without such optimization, translation can stall or produce truncated products [36]. Fusion to solubility-enhancing tags (e. g., maltose-binding protein, glutathione S-transferase) is applied to promote folding and increase soluble yield, noting that multiple tags often must be screened to identify the most effective construct [35]. Co-expression with binding partners or molecular chaperones is also employed. Chaperones bind exposed hydrophobic regions of nascent peptides, preventing aggregation and, through iterative ATP-driven conformational cycles, assist substrate folding and refolding [35, 36].

To obtain VEGF165 and ANGPT1 proteins, expression plasmids were constructed using the pET-20b(+) vector encoding each protein with an N-terminal His-tag for affinity purification. All constructs were verified by restriction analysis, PCR, and Sanger sequencing prior to expression testing. At the first stage

of the work, we verified that the expression system (plasmid, promoter, medium, and host strain), which can affect protein expression levels [36], functioned properly by testing expression at 26 °C for 24 h in 5 mL TB medium. Western blot analysis showed that most of the VEGF165 and ANGPT1 proteins were present in inclusion bodies, with only a small fraction soluble, although the total protein yield was high. Based on these results, we focused on optimizing expression conditions and on co-expression with chaperones to preserve the native protein sequences without significant modifications. For optimization, expression was tested at 22, 26, 30, and 37 °C for 24, 48, and 72 h. To increase the amount of soluble protein, we co-expressed the pGro12 (GroEL-GroES) chaperone system, which previously demonstrated promising results for expression of several cytochromes P450 [37, 38].

Under cultivation conditions without co-expression of pGro12 (Fig. 1), *E. coli* produced moderate amounts of soluble protein, with solubility increasing as the cultivation temperature was raised from 22 to 26 °C. Temperatures above 26 °C promoted protein misfolding and aggregation, resulting in pronounced inclusion body formation. Extending incubation from 24 to 72 h increased the soluble fraction only within the 22–26 °C range. At higher temperatures, prolonged incubation exacerbated aggregation and led to near-complete sequestration of the protein into inclusion bodies, as observed at 37 °C.

As shown in Fig. 1, co-expression of VEGF165 with the pGro12 chaperone system substantially increased the proportion of soluble protein and decreased accumulation in inclusion bodies. Raising the cultivation temperature from 22 to 26 °C produced approximately a twofold increase in VEGF165 yield at 24 and 48 h post-induction; however, prolonged expression (72 h) at 26 °C resulted in pronounced protein aggregation despite the high overall yield. By contrast, a 72 h incubation at 22 °C yielded a high proportion of soluble VEGF165, only slightly lower than the peak levels observed at 48 h and 26 °C. Under all tested conditions, co-expression with chaperones at ≥ 30 °C led to substantial insoluble material and poor solubility of the target protein. These results indicate that moderate temperatures (22–26 °C) combined with chaperone co-expression optimize soluble VEGF165 production, whereas higher temperatures and extended incubation promote aggregation and inclusion body formation [22, 23].

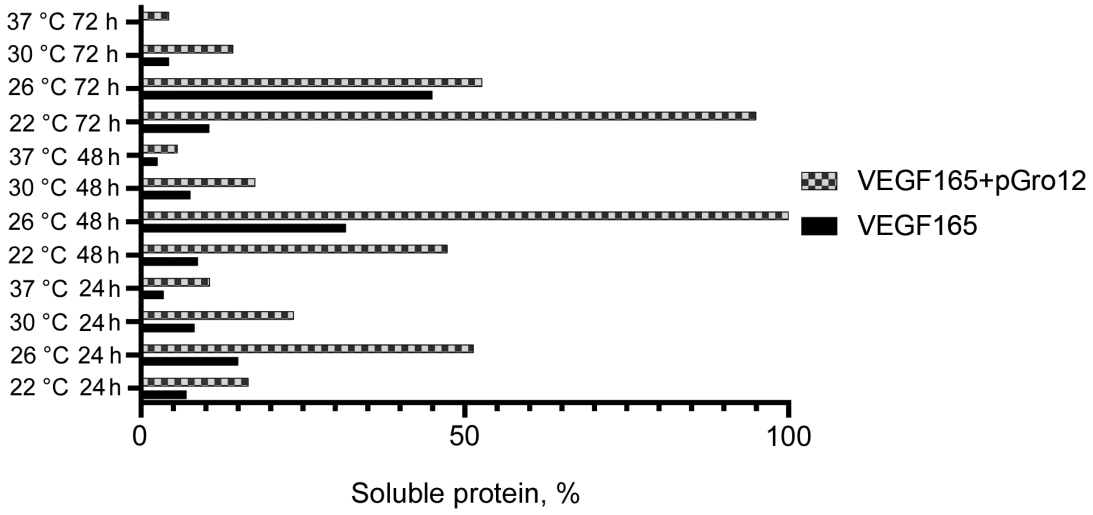


Fig. 1. Western blot analysis showing the percentage of the maximum amount of soluble VEGF165

Expression of ANGPT1 in the absence of chaperone co-expression (Fig. 2) revealed that raising the cultivation temperature from 22 to 30 °C increased the soluble fraction at 24 h, but prolonged incubation at 30 °C and above induced aggregation. Across all tested conditions without chaperones, substantial aggregation and inclusion body formation were observed, severely limiting recoverable soluble protein. Co-expression with the pGro12 chaperone system markedly increased the soluble fraction and reduced inclusion body accumulation. The overall response of soluble ANGPT1 to fermentation parameters paralleled that of VEGF165, with the notable exception that at 30 °C the soluble yield was only ~30 % lower than at 26 °C. The highest soluble yield for ANGPT1 was achieved at 22 °C after 72 h of cultivation.

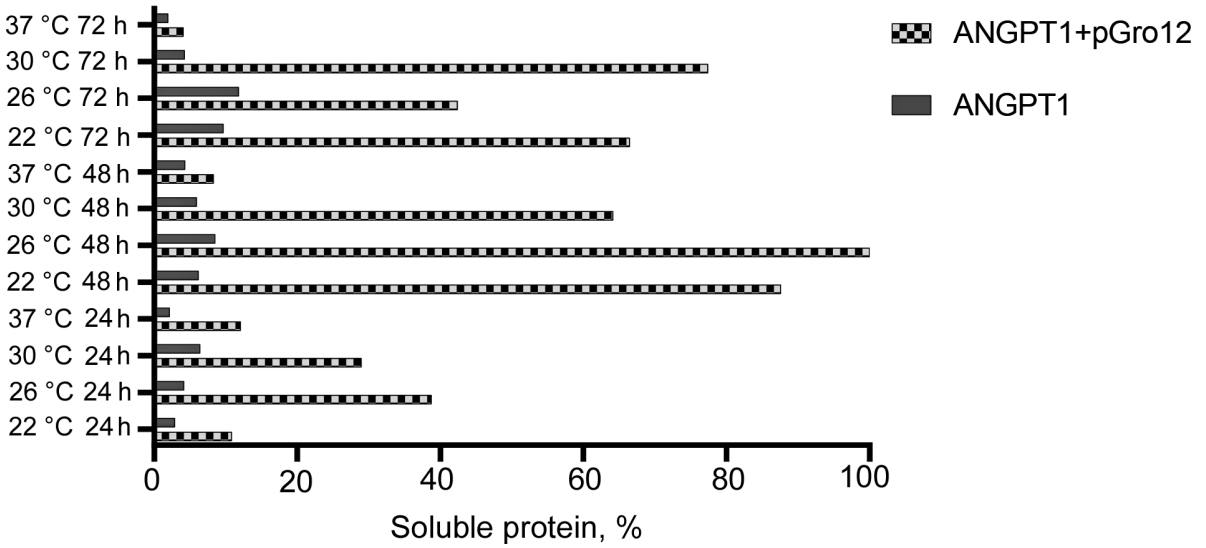


Fig. 2. Western blot analysis showing the percentage of the maximum amount of soluble ANGPT1

Optimal expression parameters were determined as 48 h at 26 °C for VEGF165 and 72 h at 22 °C for ANGPT1. Temperatures above these optima reduced the yield of soluble fraction for both proteins and promoted their partitioning into inclusion bodies, whereas substantially lower temperatures decreased overall expression rates and complicated downstream recovery. Co-expression with the pGro12 chaperone system markedly increased the soluble fraction of both ANGPT1 and VEGF165, consistent with enhanced folding efficiency and reduced aggregation, and therefore represents an effective strategy to improve recoverable yields of correctly folded recombinant protein.

Production of VEGF165 and ANGPT1 in 1 L cultures. Protein expression results obtained at small scale do not always translate directly to larger volumes. Indeed, proteins that are insoluble or poorly expressed on a microscale can sometimes be successfully produced in soluble form when scaled up [35]. To evaluate fermentation under optimized conditions and determine the effect of chaperone co-expression, ANGPT1 and VEGF165 were expressed in 1 L TB cultures. The experiments were conducted at two key conditions: 26 °C for 48 hours (with and without pGro12 co-expression) and 22 °C for 72 hours (with and without pGro12 co-expression). Subsequently, the cells were lysed and prepared for IMAC purification on a 5 mL HisTrap column under identical conditions.

The amounts of VEGF165 recovered after IMAC were 1 mg (26 °C, 48 h, without chaperones), 6 mg (26 °C, 48 h, with pGro12), and 5 mg (22 °C, 72 h with pGro12) from 1 L of culture TB with an estimated purity of ~80% by SDS-PAGE. These results demonstrate a six-fold improvement in the amount of purified VEGF165 achieved by minimizing protein accumulation in inclusion bodies through chaperone co-expression, while maintaining the same level of untargeted proteins. Variation of imidazole concentration (15–75 mM) and wash volume (50–75 mL) had minimal impact on VEGF165 purity or yield. According to the obtained results, the optimal imidazole concentration and wash buffer volume were 50 mM and 50 mL, respectively. Further purification by SEC on Sephadex G75/100 (to remove host cell contaminants of ~70–80 kDa) produced highly purified VEGF165, with a final yield of approximately 4 mg per liter of starting culture and an estimated purity of ~95 % as confirmed by SDS-PAGE and western blot (Fig. 3).

To our knowledge, this is the first report of isolating VEGF165 from *E. coli* with chaperone co-expression, which resulted in a marked increase in soluble protein yield. Previous studies have reported yields of approximately 0.5–1.0 mg of VEGF165 from the soluble fraction [19, 21, 22] and 1–40 mg from inclusion body per 1 L of culture [21, 34, 39]. The refolded protein preparations frequently fail to reproduce the native structure and activity of proteins produced in soluble form, rendering them unsuitable for many downstream applications. Refolding workflows also tend to be low yield: a large fraction of the material can be lost during solubilization, refolding, and subsequent purification steps [40].

ANGPT1 was purified by IMAC using the same protocol as for VEGF165. Yields of ANGPT1 after IMAC were 0.1 mg (26 °C, 48 h, without chaperones), 1.5 mg (26 °C, 48 h, with pGro12 co-expression), and 2 mg (22 °C, 72 h, with pGro12), with an estimated purity of ~95% by SDS-PAGE (Fig. 3). These results confirm that chaperone co-expression is necessary to improve ANGPT1, solubility, and scalable production. Reducing the imidazole concentration in the wash buffer from 50 mM to 35 mM or 25 mM increased co-elution of host proteins and produced only marginal improvements in ANGPT1 recovery, thereby decreasing the overall purity of the preparation. Crucially, increasing the imidazole concentration promoted premature elution of ANGPT1. ANGPT1 has previously been produced in COS, CHO, and Sf9 cells with reported yields ranging from ~0.2 to 200 ng per liter and generally low purity [27, 30–32]. Expression of ANGPT1 in high yield systems such as *E. coli* or yeast has not been described. The single step IMAC procedure reported here is simple, rapid, and bypasses the need for complex denaturation-refolding steps.

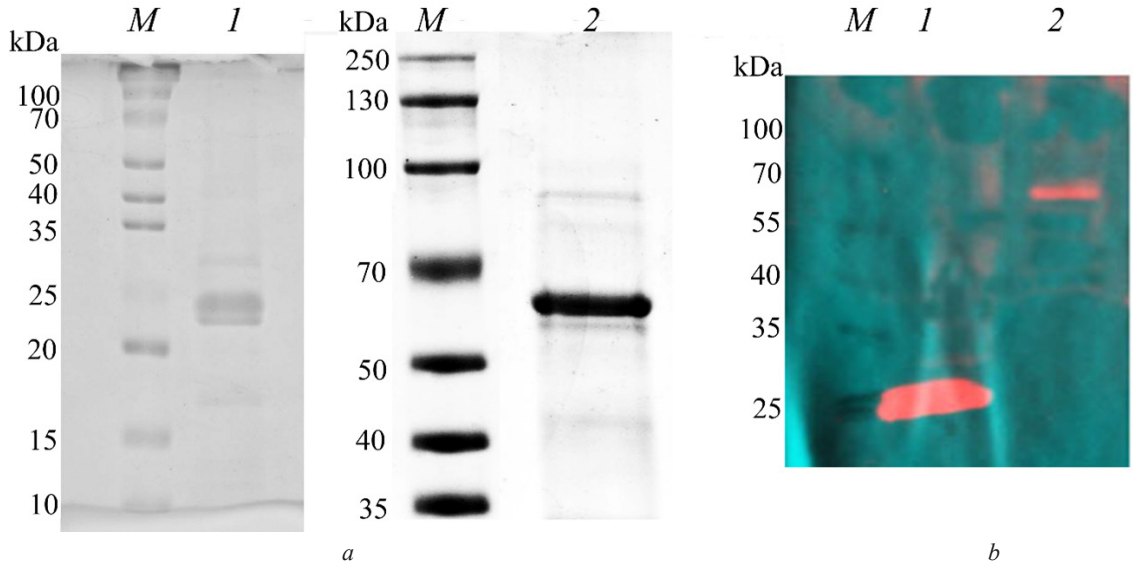


Fig. 3. SDS-PAGE (*a*) and Western blot (*b*) analyses of purified VEGF165 (*1*) and ANGPT1 (*2*). Lanes: M, prestained molecular mass marker

Conclusion. In summary, we developed an advanced expression system for the rapid and scalable production of VEGF165 and ANGPT1 in *E. coli*. This system utilizes co-expression of the pGro12 (GroEL-GroES) chaperone complex alongside a streamlined purification workflow to achieve high-purity protein. By combining lowered induction temperatures with chaperone co-expression, the optimized protocol maximizes soluble product yield and minimizes inclusion body formation, thereby facilitating efficient purification. The recombinant proteins produced using this approach are suitable for research reagents and preclinical applications, including studies of critical limb ischemia, tissue engineering, wound healing, and functional or gene-therapy investigations. However, further process validation (activity assays, endotoxin clearance, and scale-up optimization) is required to confirm suitability for therapeutic use.

Acknowledgements. This work was supported by the Belarusian Republican Foundation for Fundamental Research (grant X2025M-034).

Благодарности. Работа выполнена при финансовой поддержке Белорусского республиканского фонда фундаментальных исследований (грант X2025M-034).

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