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Yunyun Pei Date

Biomaterial Design: Human Tropoelastin

By

Yunyun Pei

Master of Science
Chemistry

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Biomaterial Design: Human Tropoelastin

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Yunyun Pei

B.S., Jilin University, 2002

Advisor: Vincent P. Conticello, Ph.D.

An Abstract of

A thesis submitted to the Faculty of the Graduate School of Emory University

in partial fulfillment of the requirements for the degree of

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in Chemistry

2010

Abstract

Biomaterial Design: Human Tropoelastin

By Yunyun Pei

Elastin is an important protein that provides connectivity and elasticity in the extracellular matrix. It is an extremely insoluble protein due to the extensive cross-linking at Lys residues. Tropoelastin is the soluble precursor of elastin, and therefore is of great importance for structure-function relationship of elastin study.

The work described herein is focused on design and expression of recombinant human tropoelastin in *E. coli*. In this study, five plasmids pJ246/STEL_1, pJ246/STEL_V2, pJ246/STEL_V3, pJ246/STEL_V4, pJ246/STEL_V5 harboring the gene encoding the tropoelastin 1-5 sequences, respectively were optimized by DNA 2.0 (Menlo Park, CA) and they were employed as sources of the gene. These five sequences encode the same amino acid sequences of tropoelastin while having different GC content and were expected to have different expression levels. These five genes were expressed in different vector systems to determine the optimal conditions for high-level protein expression using regular induction. To enhance the expression yield, auto-induction was used for later experiments in different cell lines. Polyhistidine tagged tropoelastins at N-terminal were constructed and expected to be more stable than the native protein. Future studies with the purified tropoelastin may include cross-linking studies and incorporation of proline analogues to investigate the structure of tropoelastin.

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Introduction

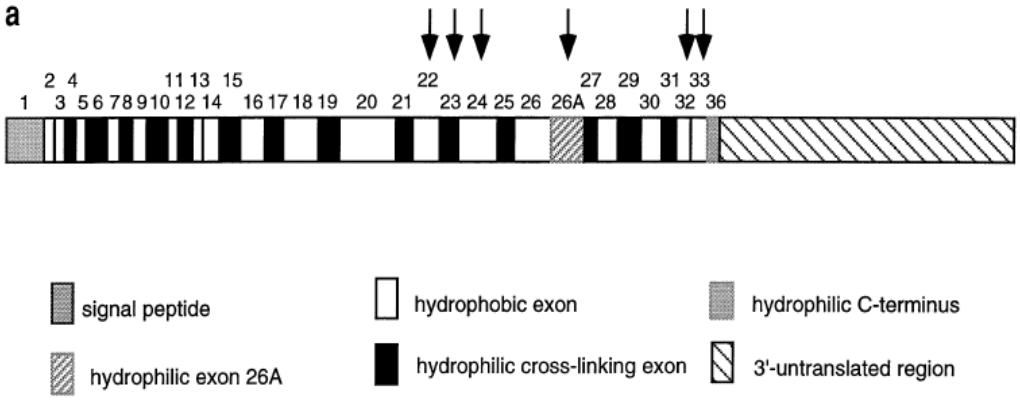
Elastin is an extremely crucial structural protein in a variety of tissues, *in vivo*, as it provides elasticity and resilience to organs and tissues, such as lung, skin and in the arteries of the cardiovascular system. Dysfunction of elastin biosynthesis in the body may cause a series of human diseases (Table 1)[1]. Also, elastin cannot be produced after puberty, so a specific amount of elastin will be consumed in our whole life, which explains the appearance of wrinkles as people age. As a result of the importance of elastin, the study of its structure and function has attracted intensive attention. However, due to the unique physical properties of native elastin, the structural protein is extremely insoluble above the inverse temperature transition[1]. Tropoelastin is the natural, soluble precursor of native elastin. Tropoelastin would be useful in treatments for elastin disorders by injection of recombinant tropoelastin in patients suffering from these disorders, and it may promote wound healing by injection of soluble tropoelastin in damaged tissue. Tropoelastin also has potential for anti-aging applications. In addition, since proline is abundant in native tropoelastin and plays an important role in structural stability, this protein can be used in structural studies involving proline analogue incorporation[2-4].

Disease	Features	Aetiology
Atherosclerosis	Fragmentation of elastin in arteries, increased stiffness of arteries, increased lipid and calcium accumulation in elastin fibers	Uncertain, complex
Buschke-Ollendorff syndrome	Increased thick elastic fibers, decrease in microfibrils, skin lesions, bone dysplasia	Unknown, genetic
Cutis laxa (inherited)	X-linked and autosomally inherited forms, loose sagging skin, increased elastic fiber fragmentation, decreased lysyl oxidase activity	Uncertain, tropoelastin mRNA stability or copper transport defect proposed
Emphysema	Increased compliance of lung, loss of elastin in lung	Unbalanced protease/anti-protease activity suspected
Marfan syndrome	Widespread skeletal, ocular, and cardiovascular defects, loose skin, increased fragmentation of elastin	Mutations in fibrillin gene
Menkes syndrome	X-linked, brittle hair, tortuous blood vessels, elastic fiber fragmentation, neurological defects	Defect in copper-transport ATPase Mc-1 gene
Pseudoxanthoma elasticum	Inelastic skin, cardiovascular defects, fragmentation, clumping, and calcification of elastic fibers, increase in glycosaminoglycans	Unknown, elastin gene defect excluded
Supravalvular aortic stenosis (SVAS)	Narrowing of arteries, reduced elastin content, architecture of aorta disrupted	Mutations in elastin gene
Williams syndrome	SVAS, mental retardation, premature aging of skin, lax joints	Deletion of elastin gene allele and adjacent loci

Table 1. Diseases of elastin fibers[1]

Tropoelastin is composed of two major types of domains: hydrophobic domains rich in non-polar amino acids and hydrophilic domains rich in lysine residues (Figure 1b)[1]. The hydrophobic domains, responsible for the elastic properties of tropoelastin, are characterized by three to six peptide repeats with sequences such as GVGVP. The hydrophilic domains, involved in cross-linking, consist of stretches of lysine residues separated by alanine residues such as in the sequence AAAKAAKAAA. Hydrophilic and hydrophobic domains alternate in native tropoelastin. The hydrophobic domain sequences among species are evolutionarily divergent, while the hydrophilic, cross-linking domain sequences have been highly conserved over time[1].

a



b

Domain	*Sequence*
Hydrophobic Domain (Exon 24)	GLVPGVGVAPGVGVAPGVGVAPGVGLAPGVGVAPGVG VAPGVGVAPGIGPGGVA
Cross-link Domain (Exon 19)	GVVSPEAAAKAAAKAAKY
26A	GADEGVRRSLSPELREGGDPSSSQHLPSTPSSPR
C-terminus	GGACLGKACGRKRK

Figure 1. Structure of human tropoelastin. (a) cDNA structure. (b) Amino acid sequence of selected domains[1].

Tropoelastin subunits construct elastin in the complex process of elastogenesis. At least two major steps are involved: the self-association of multiple tropoelastin monomers and the crosslinking of aligned tropoelastin monomers (Figure 2). The self-association of tropoelastin monomers is proposed as a vital early step in elastic fiber formation, by concentrating the tropoelastin molecules and possibly aligning them into correct form for subsequent cross-linking. So this step is crucial for appropriate crosslinking and is observed as coacervation. Coacervation occurs through an inverse temperature transition. Upon increasing the temperature of a solution of soluble tropoelastin above 20 ºC, the solution becomes turbid due to aggregation of tropoelastin molecules. Aggregation is thermodynamically controlled, and is therefore fully reversible by cooling the solution below the coacervation temperature[5, 6]. Coacervation is believed to be caused by interaction between the hydrophobic domains, and plays an important role in fibrillogenesis[7].

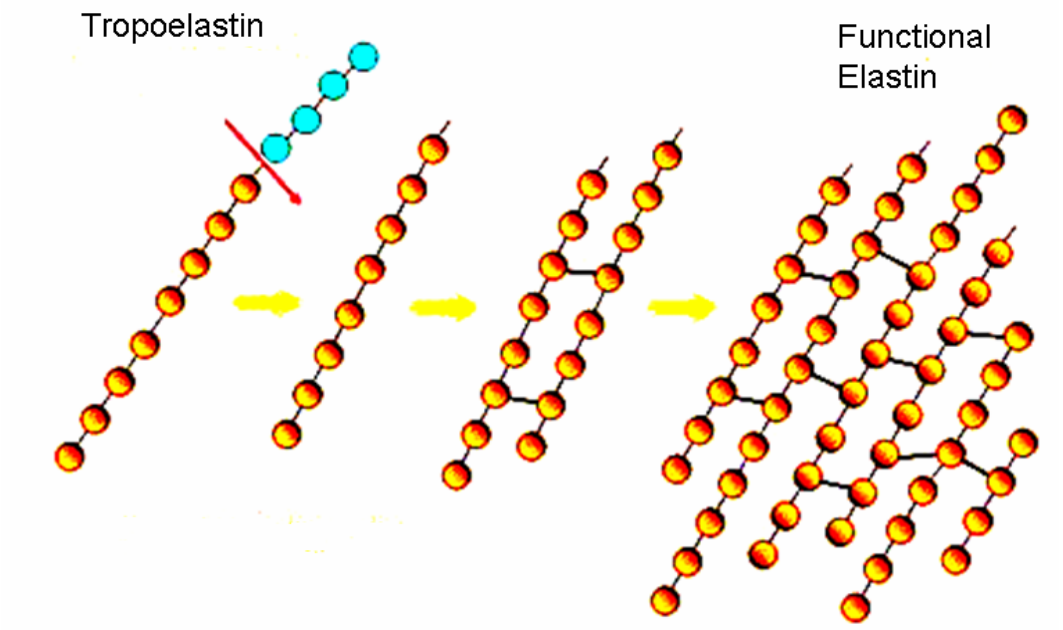
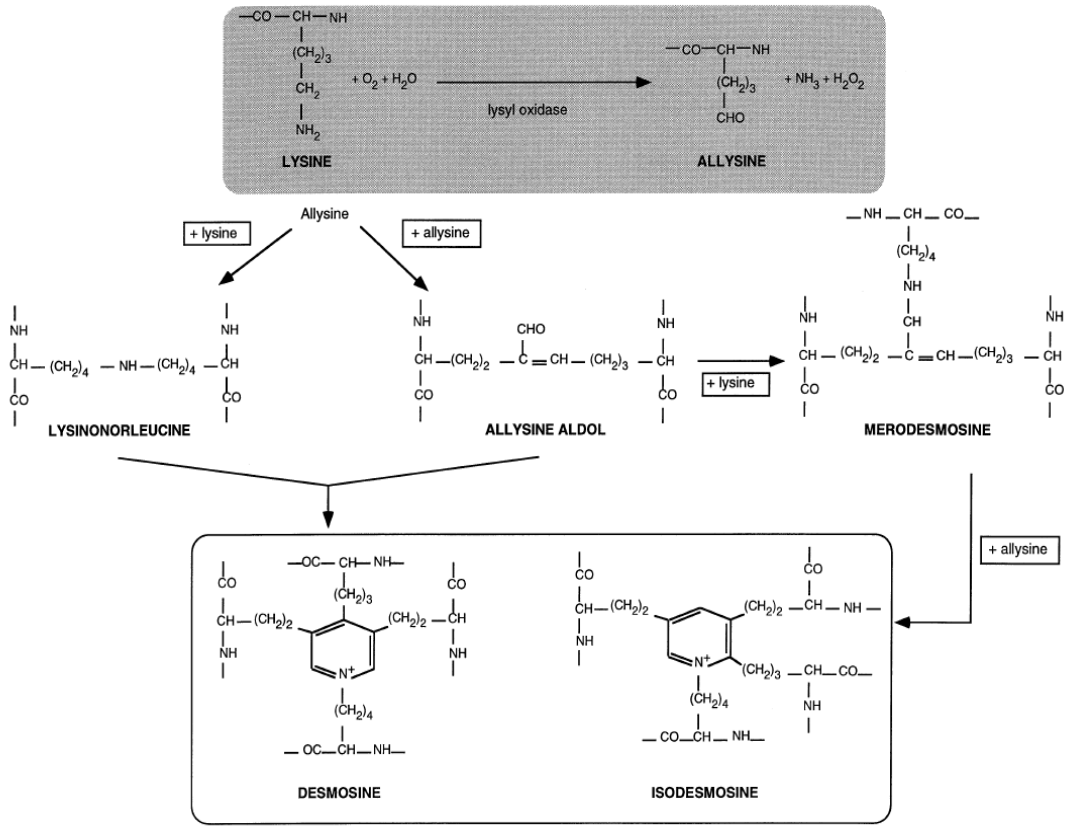


Figure 2. Process of elastogenesis

After coacervation, inter and intra-molecular cross-linking between lysine residues is catalyzed immediately by the copper-dependent enzyme, lysyl oxidase[8, 9]. The initial reaction is an oxidative deamination of ε-amino group on Lys residues by the enzyme lysyl oxidase. The resultant aldehyde can then condense with another aldehyde residue through an aldol condensation reaction or with an unoxidized lysyl amino group though a Schiff base reaction to form the bifunctional crosslink aldol condensation product and dehydrolysinonorleucine, respectively. The two crosslinks can then condense to form the tetrafunctional crosslinks desmosine and isodesmosine (Scheme 1)[1, 10]. Extraction and purification of tropoelastin from animal sources involves deprivation of copper from the diet of the animal, so the inactive lysyl oxidase cannot catalyze the crosslinking and therefore tropoelastin in animal tissues will be accumulated[11, 12]. But this method is time-consuming, and animal-unfriendly. In addition, tropoelastin purified from animal tissues is heterogeneous because different structural isoforms are produced from differential mRNA splicing tissues. Because tropoelastin doesn't undergo posttranslational modification, bacterial expression systems are ideal for preparing relatively large amounts of purified protein. The total synthesis and expression of human tropoelastin has been carried out using an *E. coli* host[13] and in yeast[14] by Anthony Weiss. This group optimized the codon content of the human gene in bacteria as the native gene contains many codons with a low frequency of usage in *E. coli*[13]. They constructed the gene into the pBluescript II SK^+ or pET3d vectors for expression. The ~65 kDa tropoelastin protein was expressed and purified to a level of approximately 30 mg protein/liter culture in the pET3d

vector.

Amino acid analogue incorporation is an interesting area for biomaterials applications of tropoelastin. Effects of amino acid analogue incorporation on the structure of elastin-mimetic[15, 16] and collagen-mimetic polypeptides have been studied. Our initial investigations were focused on high-level expression and purification of recombinant human tropoelastin for biomaterial applications or for use in structural studies involving analogue incorporation or cross-linking of tropoelastin.



Scheme 1. Structure and formation of lysine cross-links in elastin. After conversion of lysine to allysine by the enzyme, lysyl oxidase, spontaneous reaction of allysine with other lysine residues generates the cross-links depicted above[1].

Reliable and reproducible methods for high-throughput production of

proteins are required for protein biochemistry. The auto-induction method has been used for production of recombinant proteins in *E. coli.* Auto-induction takes advantage of the function of *lac* operon regulatory elements under growth conditions. During the initial growth period, glucose is preferentially used as a carbon source. The lac repressor (lacI) binds to the lac operator and prevents transcription from the lac promoter. As glucose is consumed, cells begin to utilize lactose as the carbon source. Lactose binds to the repressor and induces its dissociation from the operator, permitting transcription from the promoter.

In the classical protocol for induction of protein expression in T7 systems, bacteria are grown to mid-log phase, and expression is induced by the addition of IPTG. This requires careful monitoring of the growth of the cultures, so that they can be induced before they reach saturation. Auto-induction requires growth of the bacteria in defined media in which expression of the T7 polymerase is automatically induced in late log-phase growth due to the depletion of carbon sources other than lactose[17]. It is no longer necessary to carefully monitor the bacterial cultures before adding the inducer. Moreover, it has been demonstrated that cultures grown under the modified conditions yield a greater cell mass-and concomitantly greater yield of recombinant protein-since the media is well buffered and the T7 polymerase is induced in late log-phase. Another advantage of auto-induction is that it avoids the high cost of IPTG.

Auto-induction could be developed for any expression system in which the elements driving expression of the target protein are induced by a change in metabolic

state that is brought about by growth of a growth of a culture.

Materials

All chemical reagents were purchased from either Fisher Scientific, Inc. (Pittsburgh, PA) or Sigma Chemical Co. (St. Louis, MO) unless otherwise noted. Isopropyl-β-D-thiogalactopyranoside (IPTG) was purchased from Research Products International Corp. (Prospect, IL). Restriction endonucleases, T4 DNA ligase, and T4 kinase were purchased from New England Biolabs, Inc. (Beverly, MA), shrimp alkaline phosphatase was obtained from Roche Applied Science (Indianapolis, IN), and Platinum *Pfx* DNA polymerase was obtained from Invitrogen Corp. (Carlsbad, CA).

Plasmid pQE-80L was obtained from QIAGEN, Inc. (Valencia, CA). The plasmid, pBAD/HisA, was purchased from Invitrogen Corp. (Carlsbad, CA), and the pET-21a, pET-23d, pET-24d vectors were purchased from Novagen, Inc. (Madison, WI). The plasmid pJ246/STEL_1, pJ246/STEL_V2, pJ246/STEL_V3, pJ246/STEL_V4, pJ246/STEL_V5 harboring the gene encoding the tropoelastin 1-5 sequences were optimized by DNA 2.0 (Menlo Park, CA) and they were employed as sources of the gene. Double digestion of pJ246/STEL_1, pJ246/STEL_V2, pJ246/STEL_V3, pJ246/STEL_V4, pJ246/STEL_V5 with *Nco* I and *Hind* III afforded a duplex DNA cassette of approximately 2200 bp, which were inserted into the *Nco* I/*Hind* III sites of pIL5 (modified plasmid pQE-80) to generate plasmid pYP34-38. Synthetic oligonucleotides were purchased from either Sigma-Genosys, Inc (The Woodlands, TX) and were used as received. TALON® metal affinity resin was purchased from Clontech, Inc. (Mountain View, CA).

Procedures for the manipulation of DNA, the transformation of competent cells, and the growth and induction of bacterial cultures were adapted from the published literature[18] or instructions supplied by manufacturers. Reagents for the manipulation of bacteria and DNA were sterilized by either autoclave or passage through a 0.2 μm filter. Enzymatic reactions were performed in the reagent buffers supplied by the manufacturer. Automated DNA sequencing was performed at the Agencourt DNA sequence center.

Protein electrophoresis was performed using 10-15 % gradient discontinuous pre-cast sodium dodecyl sulfate (SDS) polyacrylamide gels on a PhastSystem from GE Healthcare Bio-Sciences Corp. (Piscataway, NJ). The Perfect Protein Marker was used as a protein standard for SDS-PAGE and western blot analysis and was purchased from Novagen, Inc. (Madison, WI). Silver staining, used for protein visualization, was carried out using a silver staining kit purchased from GE Healthcare Bio-Sciences Corp. (Piscataway, NJ). Western blotting was carried out using the PhastSystem (GE Healthcare). The primary antibody used for binding polyhistidine tagged proteins was the His-tag monoclonal antibody from Clontech, Inc. (Mountain View, CA). The primary antibody for binding elastin or tropoelastin proteins was the mouse anti-elastin monoclonal antibody from Chemicon International, Inc. (Millipore, Temecula, CA). The secondary antibody used was the goat anti-mouse secondary antibody, also from Clontech, Inc. Polyhistidine tagged proteins were visualized by chromogenic detection using the 1-step NBT/BCIP reagent mixture from Pierce Biotechnology (Thermo Scientific) (Rockford, IL).

Proteins were quantified by using the BCA protein assay kit (Pierce Biotechnology).

Experimental Methods

Plasmid Construction and Cloning

1. pQE system

The tropoelastin-containing plasmids (pJ246/STEL_1, pJ246/STEL_V2, pJ246/STEL_V3, pJ246/STEL_V4, pJ246/STEL_V5) and the vector plasmid, pIL5 (modified pQE80L by I-Lin Wu) were double-digested with *Nco* I/*Hind* III, respectively. The tropoelastin genes were then ligated into pIL5 to generate the new plasmids, pYP34-38. The sequences were confirmed by DNA sequencing analysis. The recombinant plasmids were first transformed into Top10F' for expression. LB media 5 mL, supplemented with 50 μg/ mL ampicillin, were inoculated with single colonies, harboring the five recombinant plasmids, and grown overnight at 37 °C. An aliquot (2 mL) of the overnight cultures was transferred to 50 mL LB in a 250 mL flask. The cultures were allowed to grow at 37 °C until OD_{600} reached 0.8 and protein expression was induced with 1mM IPTG. The cultures were incubated for another 4 hours. OD_{600} readings were monitored for each of the cultures. An aliquot (1 mL) of the cell cultures were collected after 4 h. The aliquots were centrifuged for 5 min at 6,300 rpm, the supernatant was discarded, and samples were resuspended in 50 μL ddH_2O and stored at –20 °C. Samples were prepared for SDS-PAGE and western blot analysis by mixing 5 μL sample culture, 12.5 μL 2 x sample buffer, 1 μL DTT (dithiothreitol), and 6.5 μL ddH_2O for a total of 25 μL.

Then the recombinant plasmids were then transformed into BL21 and Origami B, respectively. The protein expression procedure was the same as that in

Top10F'.

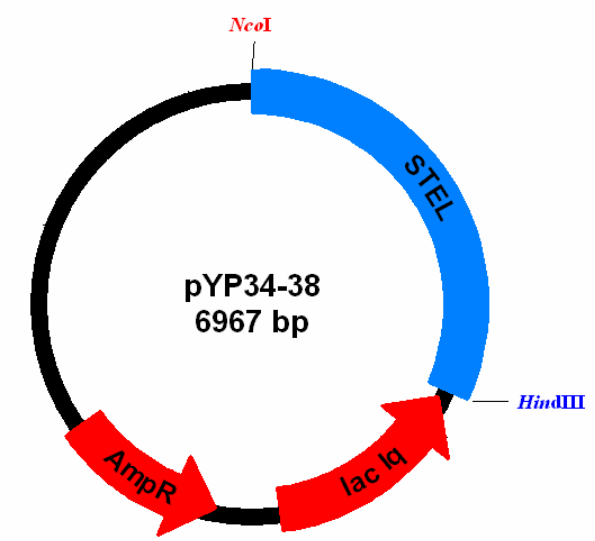


Figure 3. Plasmid map of pYP34-38 based on pQE 80L

2. pBAD/HisA system

The tropoelastin-containing plasmids (pJ246/STEL_1, pJ246/STEL_V2, pJ246/STEL_V3, pJ246/STEL_V4, pJ246/STEL_V5) and the vector plasmid, pBAD/HisA were double-digested with *Nco* I/*Hind* III, respectively. The tropoelastin genes were then ligated into pBAD/HisA. The sequences of new plasmids, pYP44-pYP48, were confirmed by DNA sequencing analysis. The recombinant plasmids were transformed into Top10F' for expression. LB media 5 mL, supplemented with 50 μg/mL ampicillin, were inoculated with single colonies, harboring the five recombinant plasmids and grown overnight at 37 °C. Aliquots (2 mL) of the overnight cultures were transferred to 50 mL of LB in a 250 mL flask. The cultures were allowed to grow at 37 °C until OD_{600} reached 0.8 and protein expression was induced with 0.002 %, 0.02 %, 0.2 % arabinose, respectively. Samples were prepared for SDS-PAGE and western blot analysis by mixing 40 μL sample culture,

20 µL 2 X sample buffer and 1 µL DTT.

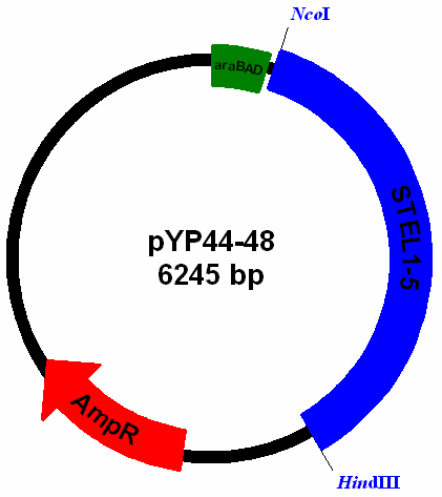


Figure 4. Plasmid of map of pYP44-48 based on pBAD system

To enhance the stability of tropoelastin, polyhistidine tagged proteins at the *N*-terminal were constructed. First, DNA oligonucleotide primers (Histag-F and Histag-R) encoding the sense and anti-sense of polyhistidine tag sequence (Table 2) were chemically synthesized by Sigma-Genosys, and the single-stranded primers were annealed to produce duplex DNA. Annealing was carried out by dissolving the primers in distilled, deionized water (dd H_2O) to a final concentration of 0.5 µg/µL. A 10 µL aliquot of each of the two primers was mixed together with 4 µL of 5 M NaCl, 4 µL of 1 M $MgCl_2$, and 172 of µL dd H_2O. By gradually decreasing the temperature of the reactions from 99 °C to 30 ºC (decreased by 5 °C every 3 min), the DNA strands were annealed together. The annealed DNA was phosphorylated at the 5′ and 3′ positions using a T4 polynucleotide kinase enzyme. The double-stranded DNA fragment was visualized by DNA agarose gel electrophoresis (4 % NuSieve agarose). Annealed DNA duplex was joined enzymatically to the *Nde* I/*Bam*H I-digested pET

21a (Invitrogen) plasmid in the presence of T4 DNA ligase enzyme with incubation at 16 ºC for 12 h. An aliquot (2 μL) of the ligation mixture was used to transform *E. coli* strain, Top10F′. The cells were recovered in 1 mL SOC rich media for 1h. An aliquot (100 μL) of the transformant suspension was spread onto LB media supplemented with ampicillin (50 μg/mL) for antibiotic selection. The plates were incubated at 37 °C for 12-14 h. The inserts in the recombinant plasmids were checked by double digestion by *Nde* I/ *Bam*H I. The recombinant plasmids were double digested with *Nco* I and *Hin*d III and later were ligated with digested STEL1-5 genes. The sequences of new plasmids, pYP64-pYP68, were confirmed by DNA sequencing analysis. The plasmids pYP64-pYP68 were transformed into BLR for expression. The expression procedure was same to that in BL 21.

Name	Sequence (5'-3')
Histag-F	TATGGCTAGCCATCATCATCATCATCATCATCATCACAGCAGCGGCCATA TCGACGACGACGACAAGGCCATG
Histag-R	GATCCATGGCCTTGTCGTCGTCGTCGATATGGCCGCTGCTGTGATGATGATG ATGATGATGATGATGGCTAGCCA

Table 2. DNA sequences for polyhistidine tag oligonucleotide

Auto-induction expression

Recombinant plasmids DNA (containing the tropoelastin gene 1-5) were transformed into BL21 or Origami B strain and plated on solid LB media

supplemented with kanamycin (50 µg/mL) or ampicillin (100 µg/mL). Aliquots of 5 mL non-inducing MDG media, supplemented with appropriate antibiotics were inoculated with single colonies, harboring the appropriate plasmids, and grown overnight at 37 °C on a rotator. For 200 mL MDG media (non-inducing media), these sterile ingredients were added in the following order to 183 mL sterilized water: 400 µL of 1 M $MgSO_4$ and 40µL of 1000X trace metal mix, 2.5 mL of 40 % glucose, 10 mL of 5 % aspartate and 4 ml of 50xM (50xM: 1xM = 50 mM $NaPO_4$, 50 mM NH_4Cl, 5 mM Na_2SO_4). Aliquots of 500 µL of the overnight cultures were transferred to 500 mL ZYM 5052 media (ZY: 10 g N-Z-amine AS, 5 g yeast extract, 1000 ml water; 50x5052: 1 X 5052 = 0.5 % glycerol, 0.05 % glucose, 0.2 % lactose) that has been supplemented with appropriate antibiotics in a 2,800 mL Erlenmeyer flasks.

The cultures were allowed to grow at 37 °C until they became turbid. The cell cultures were incubated at 20 °C until OD_{600} reached a plateau (usually OD_{600} could reach 5). Aliquots (25 µL) of the cell cultures were collected after 4 hours. The aliquots were spun down, the supernatants were discarded and the pellets were resuspended in 100 µL ice-cold ddH_2O. Samples were prepared for SDS-PAGE and western blot analysis by mixing 40 µL sample culture, 20 µL 2 X sample buffer, 1 µL DTT.

Purification of tropoelastin

Expression cultures (500 mL) cultures were spun down at 4,000 x g for 20 min. The cell pellets were resuspended in 50 mL ice-cold ddH_2O with adding 100 µL

protease inhibitor cocktail, respectively and stored at –80 °C. The procedure for purification of tropoelastin was modified from the procedure outlined by Martin *et al*[19]. The frozen cells were lysed by three freeze/thaw cycles. Lysozyme with a final concentration of 1 mg/mL was added to the lysate and incubated with shaking at room temperature for 30 min. Then benzonase (25 units/mL culture) and $MgCl_2$ (1 mM) were added to the lysate and the mixture was incubated with shaking (225 rpm) at 4 °C, overnight. The cell lysate was centrifuged at 10,000 x g for 30 min at 4 °C. The supernatant and pellet were separated and analyzed by SDS-PAGE to determine the location of the target protein. SDS-PAGE analysis indicated that the majority of the target protein was present in the soluble fraction with some residual protein in the pellet.

Purification of tropoelastin was also carried out using propanol-butanol extraction. One and one-half volumes of 1-propanol were slowly added dropwise to aqueous supernatant from cell lysis of tropoleastin. The mixtures were kept stirring at 4°C for 2 hours. Then two and a half volumes of 1-butanol were slowly added dropwise to the mixtures above at 4°C with constant stirring. A white precipitate was observed upon addition of 1-butanol. The suspension was centrifuged for 30 min at 11,000 x g, the alcohol fraction was extracted with a glass pipette. The remaining aqueous fraction, which contained tropoelastin, was added to a pre-massed, 500 mL round bottom flask and the solvent was removed under vacuum using a rotary evaporator. A white precipitate was observed on the inside of the flask. The flask was weighed to determine the mass of the resultant product and the white solid was

resuspended in 20 mL 50 mM HEPES buffer at pH 8.0. SDS-PAGE analysis was conducted to determine the purity of the tropoelastin product.

An alternative method for purification of tropoelastin was also explored, based on thermal cycling methods developed for purification of elastin-mimetic proteins. After the frozen cells were lysed by three freeze/thaw cycles，lysozyme (a final concentration of 1 mg/mL), protease inhibitor cocktail /PIC (430 mg in 2 mL DMSO and 8 mL sterile water; 1 mL PIC was used for 20 g *E. coli* cells), benzonase (25 units/mL culture), and $MgCl_2$ (a final concentration of 1 mM) were added to the lysate and the mixture was incubated with shaking (225 rpm) at 4 °C, overnight. The cell lysate was centrifuged at 10,000 x *g* for 30 min at 4 °C. For the first hot/cold spin, the supernatant from the cell lysate was centrifuged at 10,000 x *g* for 30 min at 4 °C. Aliquots of a 5 M NaCl solution were added to the supernatant containing the protein and the solution was incubated at 37 °C with shaking at 225 rpm for 1 h. A white precipitate appeared. The suspensions were then centrifuged at 10, 000 x *g* for 30 min at 37 °C. The supernatant was collected for SDS-PAGE analysis and the pellet was resuspended in lysis buffer (pH 8.0) with protease inhibitor cocktail and incubated at 4 ºC with shaking for 12-16 h to re-dissolve the tropoelastin protein in the pellet. The hot/cold spins were repeated two more times after the initial spins for a total of three hot/cold spins. Dialysis of the purified tropoelastin was carried out in sterile H_2O to remove residual salts and buffer. 10,000 MW tubing was used for the dialysis. The tropoelastin protein samples from dialysis were concentrated to a volume of 2 mL using Amicon Centriplus centrifugal filter devices (MWCO = 10 kDa).

Polyhistidine tagged proteins were purified by affinity chromatography. The supernatants after lysis were loaded onto the lysis buffer balanced TALON® Co^{2+} metal affinity resin (5 mL) and the nonbinding proteins were washed out with lysis buffer and lysis buffer containing 20 mM imidazole. The target protein was eluted with elution buffer (20 mL, 50 mM sodium phosphate, 300 mM NaCl, 400 mM imidazole, pH 7.0) and dialyzed (MWCO = 10 kDa) against distilled deionized water (5 × 4 L).

Results and Discussion

The natural sequence of human tropoelastin contains significant numbers of rare codons which have low usage in *E. coli* and therefore can drastically affect protein yield. Therefore human tropoelastin genes were designed and synthesized with codon optimization for high-level expression in *E. coli*. The complete tropoelastin genes were constructed by DNA 2.0 (Menlo Park, CA). Restriction endonuclease cleavage sites were incorporated at the 5′ and 3′ termini of the gene by the company. The human tropoelastin gene of interest is 2289 bp in length and was designed based on the known tropoelastin coding sequence from *H. sapiens*. The recombinant tropoelastin gene sequence is characterized by the hydrophilic and hydrophobic block domains characteristic of native tropoelastin. As a result, five tropoelastin genes with identical amino acids sequences were designed. In these five genes, there were no unusual codons that are absent from *E. coli*, but the GC-content of these five genes were different: 69.86% in STEL1; 65.26% in STEL2; 68.61% in STEL3; 66.47% in STEL4; 67.15% in STEL5. Several reports have shown that the high GC content of target genes is biased against E. coli codon usage, which could be the primary reason for decreased protein expression in *E. coli*. The reason might be the decrease in GC-content will decrease stability of stem structures in the coding regions; otherwise stable secondary structures influence the transcription or translation of the protein[20]. So, the expression levels of these five genes were expected: STEL2> STEL4> STEL5> STEL3> STEL1.

Expression of human tropoelastin was carried out in different vector plasmid

systems to determine the optimal conditions for high protein expression yield. Shown below (Figure 5) is 1% agarose gel electrophoresis analysis for recombinant plasmids in pIL5 vector by double digestion.

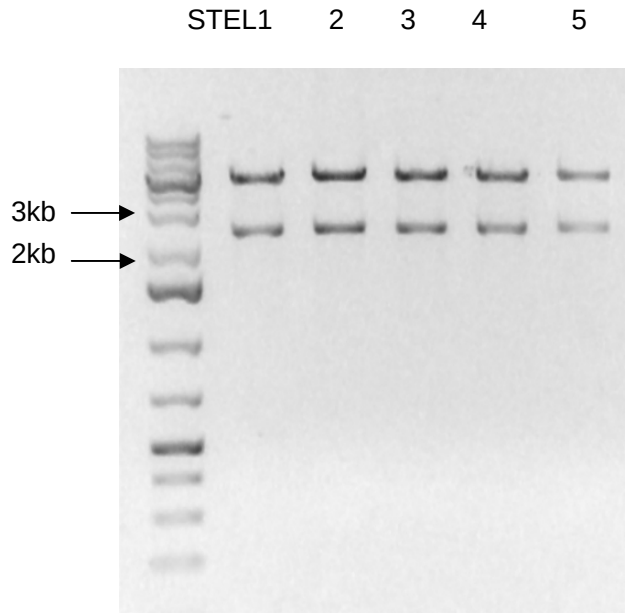


Figure 5. 1% agarose gel electrophoresis analysis for recombinant plasmids by double digestion

Expression of STEL1~5 in the pET and pQE vectors resulted in the detectable protein expression. Better expression was observed in pET 21 plasmid system in BL21(DE3). The higher product yields may have been due to less stringent T7 promoter instead of T7 lac promoter (Figure 6). The Weiss group achieved high levels of tropoelastin expression in a pET vector also under the control of a T7 promoter[19].

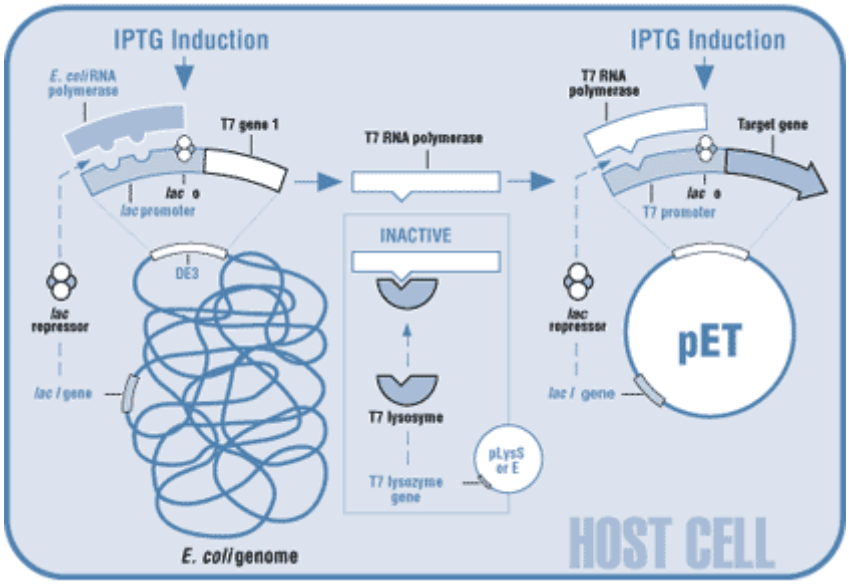
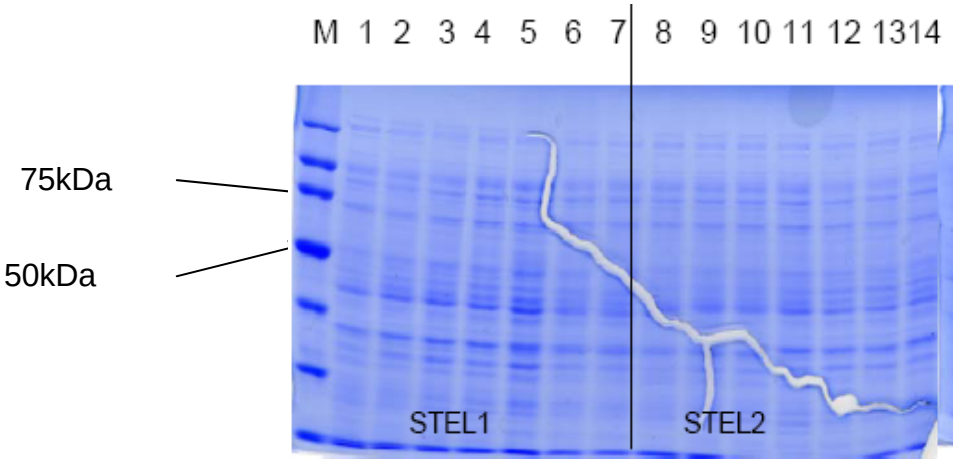


Figure 6. Control elements of the pET system[17].

Since leaky tropoelastin expression may be toxic to the cells, tighter control plasmid pBAD/HisA was used for the expression. Expression of tropoelastin was carried out in the pBAD vector system with arabinose induction in LB media. A series of cultures of sequentially increasing arabinose concentrations were used to induce protein expression to determine if high tropoelastin levels in the cells were leading to toxicity and low protein yields. No tropoelastin was detected by western blot or by SDS-PAGE analysis of expression samples from pBAD expressions (Figure 7).



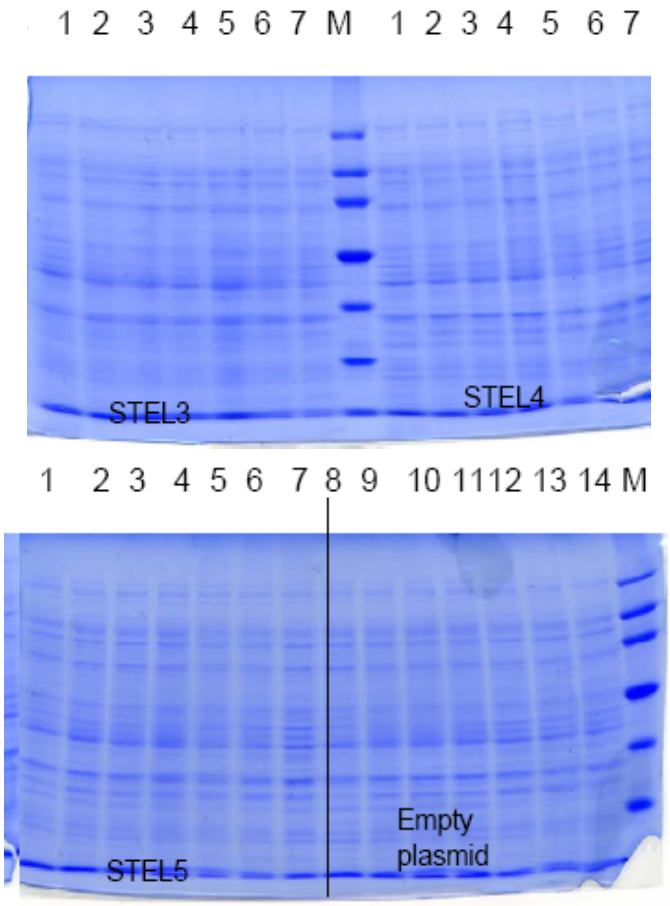


Fig. A		Fig. B		Fig. C	
1: before induction	8: before induction	1: before induction	8: before induction	1: before induction	8: before induction
4hr induction					
2: 0.2% Ara	9: 0.2% Ara	2: 0.2% Ara	9: 0.2% Ara	2: 0.2% Ara	9: 0.2% Ara
3: 0.02% Ara	10: 0.02% Ara	3: 0.02% Ara	10: 0.02% Ara	3: 0.02% Ara	10: 0.02% Ara
4: 0.002% Ara	11: 0.002% Ara	4: 0.002% Ara	11: 0.002% Ara	4: 0.002%	11: 0.002%
Overnight induction					
5: 0.2% Ara	12: 0.2% Ara	5: 0.2% Ara	12: 0.2% Ara	5: 0.2% Ara	12: 0.2% Ara
6: 0.02% Ara	13: 0.02% Ara	6: 0.02% Ara	13: 0.02% Ara	6: 0.02% Ara	13: 0.02% Ara
7: 0.002% Ara	14: 0.002% Ara	7: 0.002% Ara	14: 0.002% Ara	7: 0.002% Ara	14: 0.002% Ara

Figure 7. SDS-PAGE analysis of tropoelastin expression in pBAD/HisA vector (target protein should be around 66kDa)

The hexahistidine tagged tropoelastin gene in plasmid pET 21a was used for western blotting and could be purified using metal-affinity chromatography as an alternative and was expected to be more stable than non-fusion protein. The anti-elastin antibody was fairly useful for western blotting with elastin-mimetic

proteins, although several bands were visible on the blot possibly due to non-specific binding of the primary antibody to other proteins in the cell or to degradation of tropoelastin. Use of the anti-elastin antibody was not effective for blotting tropoelastin samples possibly due to differences in the sequences of elastin-mimetics and tropoelastin proteins although the antibody was used previously for blotting of tropoelastin[21].

In the C-terminal structure of the tropoelastin (Figure 8), beside two hydrogen bonds between A-G, L-S, there is a disulphide bond between two cysteines. So the strain Origami B was chosen for the expression because there is a mutation in the trxb gene, which encodes thioredoxin reductase, and results in enhanced formation of disulfide bond formation in the *E. coli* cytoplasm[22].

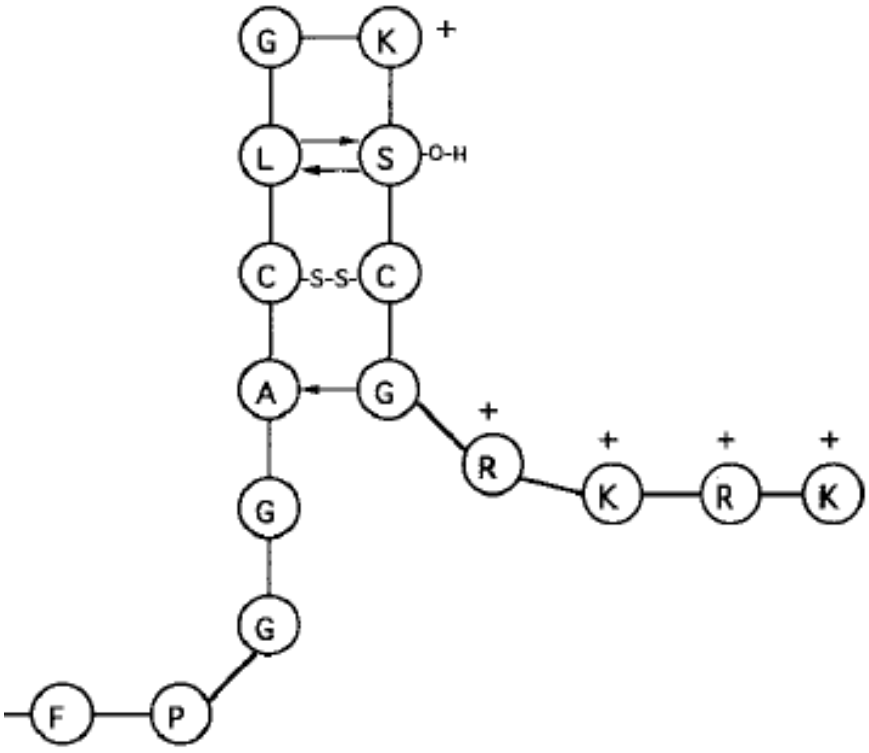
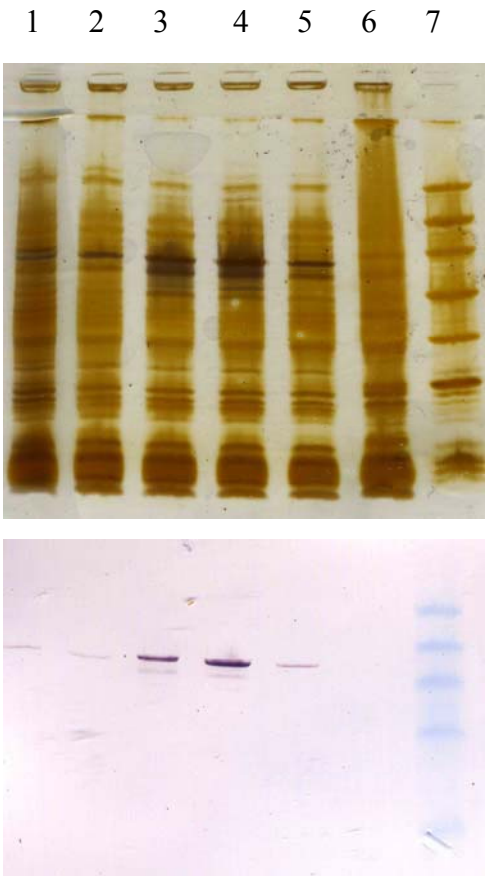


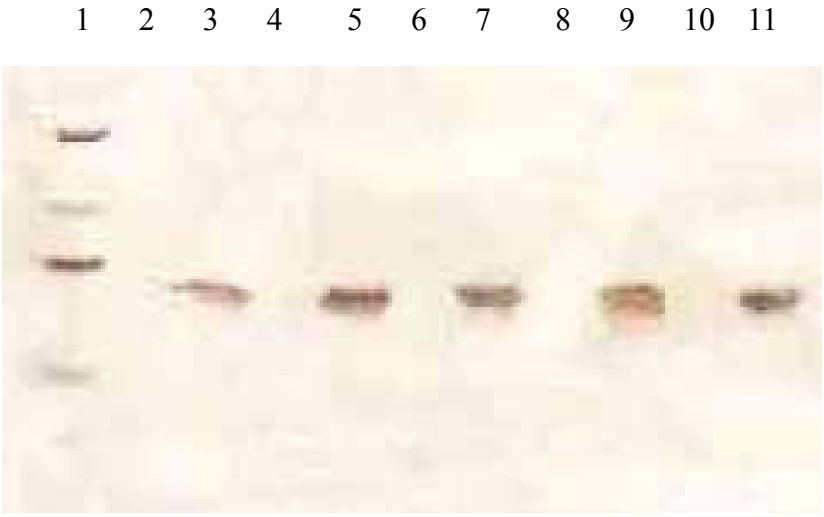
Figure 8. Proposed structure for the C-terminal domain of tropoelastin[22]

Auto induction could result in higher expression level in these vectors because by auto-induction, cell density at 600 nm could reach 5.0, which was much higher that in regular induction. Also, by auto-induction, high cost inducer IPTG was not needed and it was not necessary to monitor the cell density during cell growth. Expression yield could be improved by using auto-induction (Figure 9) or by expression using the pET 21 plasmid for high-level expression using the T7 promoter.



	Corresponding western analysis of expression
Lane 1. whole cell lysate of pYP34	weakly positive
Lane 2. whole cell lysate of pYP35	weakly positive
Lane 3. whole cell lysate of pYP36	positive
Lane 4. whole cell lysate of pYP37	positive
Lane 5. whole cell lysate of pYP38	weakly positive
Lane 6. negative control, pIL5 only	negative
Lane 7. perfect protein marker	

Figure 9. SDS-PAGE & western blot analysis of auto-induction expression results in *E. coli* strain BL21



Lane 1. perfect protein marker

Lane 2. pYP 64 with 0 hr induction

Lane 3. pYP 64 with 4 hr induction

Lane 4. pYP 65 with 0 hr induction

Lane 5. pYP 65 with 4 hr induction

Lane 6. pYP 66 with 0 hr induction

Lane 7. pYP 66 with 4 hr induction

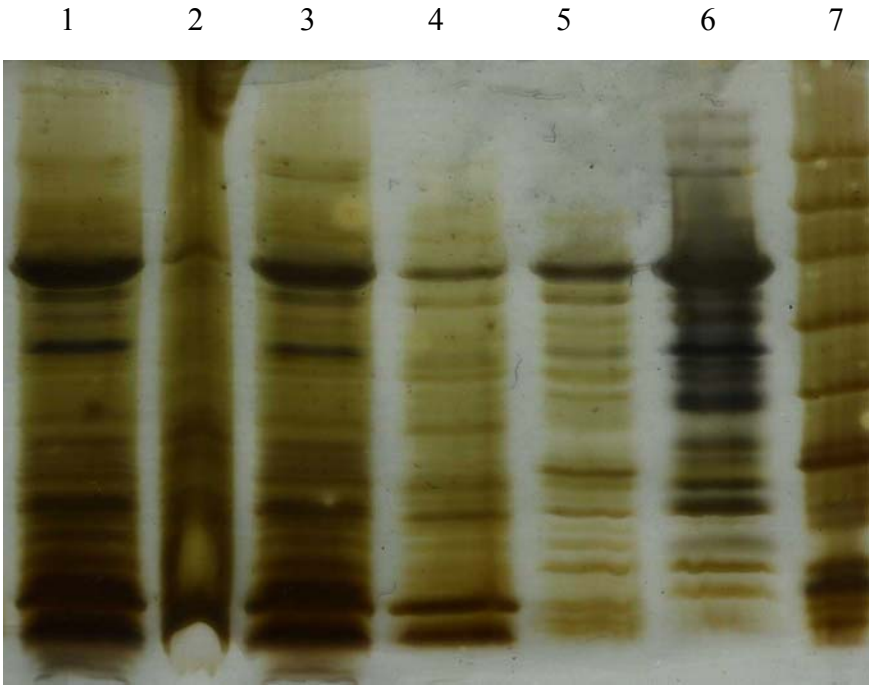
Lane 8. pYP 67 with 0 hr induction

Lane 9. pYP 67 with 4 hr induction

Lane 10. pYP 68 with 0 hr induction

Lane 11. pYP 68 with 4 hr induction

Figure 10. Western blot analysis of whole cell lysate of pYP64-pYP68 in *E. coli* strain BLR (DE3)



Lane 1. supernatant of cell lysate

Lane 2. pellet of cell lysate

Lane 3. flowthrough from Talon Co^{2+} resin

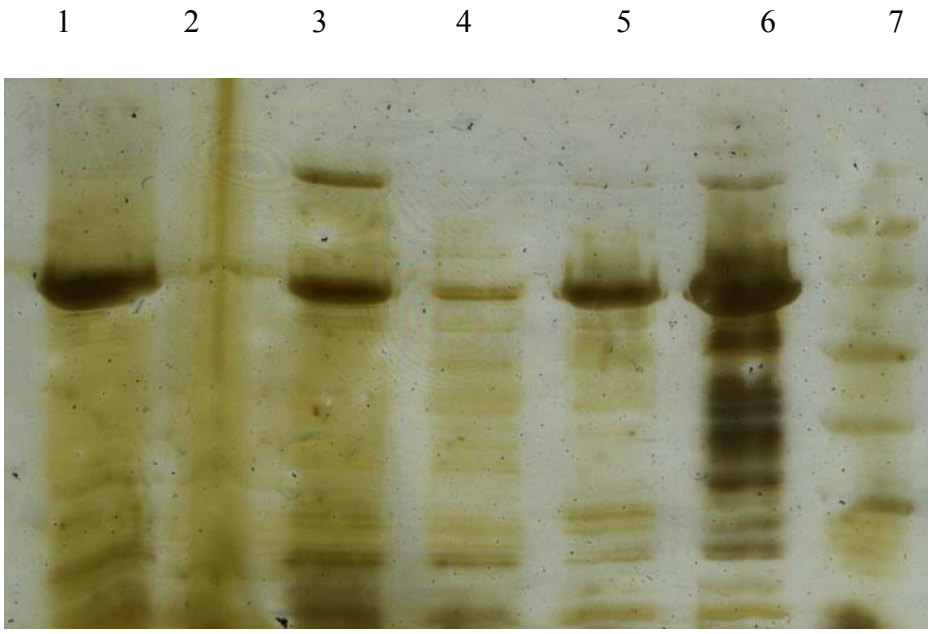
Lane 4. washout with lysis buffer

Lane 5. washout with 20 mM imidazole

Lane 6. eluent with 400 mM imidazole

Lane 7. perfect protein marker

Figure 11. SDS-PAGE analysis with silver stain of pYP64 expression samples from regular expression method in *E. coli* strain BLR (DE3)



Lane1. supernatant of cell lysate

Lane 2. pellet of cell lysate

Lane 3. flowthrough from Talon Co^{2+} resin

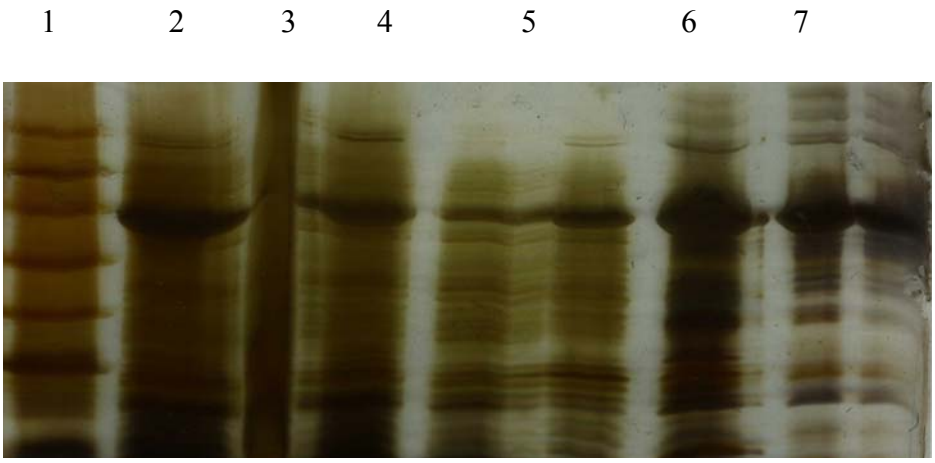
Lane 4. washout with lysis uffer

Lane 5. washout with 20 mM imidazole

Lane 6. eluent with 400 mM imidazole

Lane 7. perfect protein marker

Figure 12. SDS-PAGE analysis with silver stain of pYP65 expression samples from regular expression method in *E. coli* strain BLR (DE3)



Lane1. perfect protein marker

Lane 2. supernatant of cell lysate

Lane 3. pellet of cell lysate

Lane 4. flowthrough from Talon Co^{2+} resin

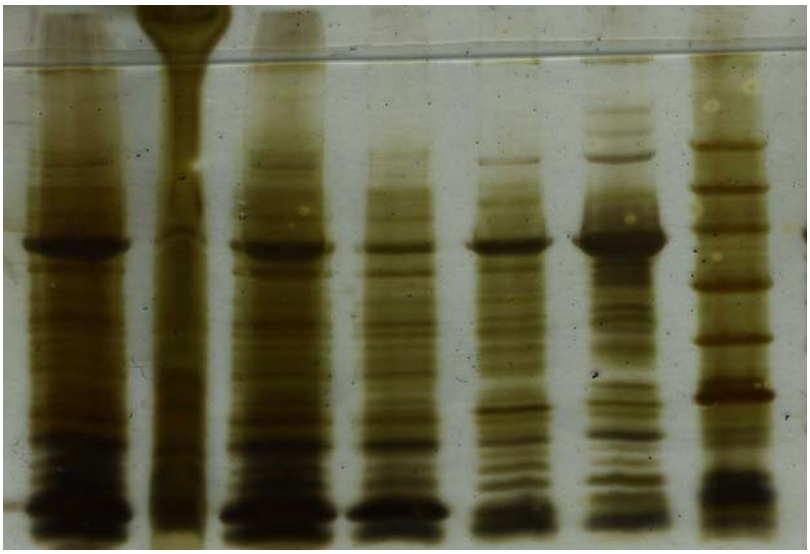
Lane 5. washout with lysis buffer

Lane 6. washout with 20 mM imidazole

Lane 7. eluent with 400 mM imidazole

Figure 13. SDS-PAGE analysis with silver stain of pYP66 expression samples from regular expression method in *E. coli* strain BLR (DE3)

1 2 3 4 5 6 7



Lane 1. supernatant of cell lysate

Lane 2. pellet of cell lysate

Lane 3. flowthrough from Talon Co^{2+} resin

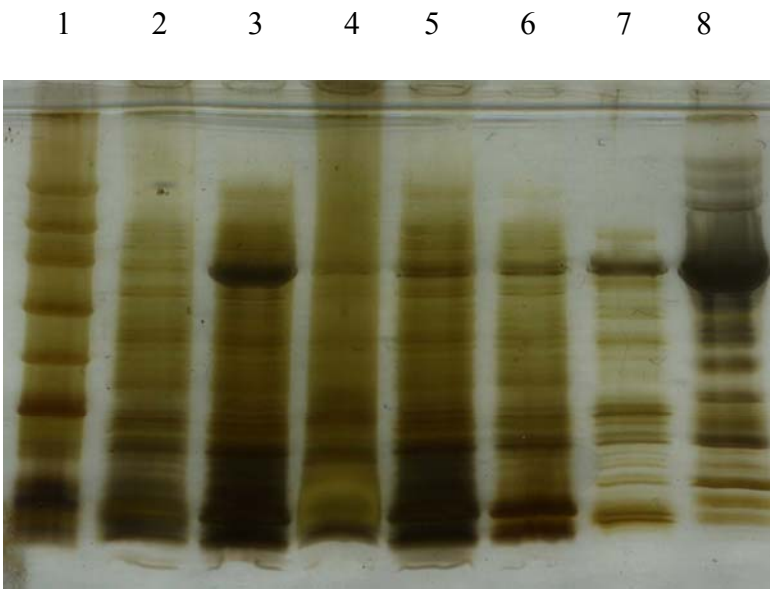
Lane 4. washout with lysis buffer

Lane 5. washout with 20 mM imidazole

Lane 6. eluent with 400 mM imidazole

Lane 7. perfect protein marker

Figure 14. SDS-PAGE analysis with silver stain of pYP67 expression samples from regular expression method in *E. coli* strain BLR (DE3)



Lane 1. perfect protein marker

Lane 2. cell lysate with 0 hr induction

Lane 3. supernatant of cell lysate

Lane 4. pellet of cell lysate

Lane 5. flowthrough from Talon Co^{2+} resin

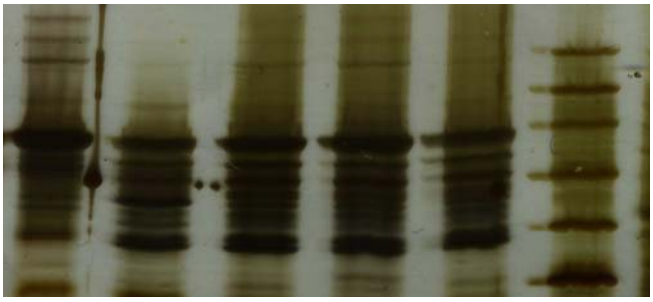
Lane 6. washout with lysis buffer

Lane 7. washout with 20 mM imidazole

Lane 8. eluent with 400 mM imidazole

Figure 15. SDS-PAGE analysis with silver stain of pYP68 expression samples from regular expression method in *E. coli* strain BLR (DE3)

1 2 3 4 5 6



Lane 1. purified pYP 64

Lane 2. purified pYP 65

Lane 3. purified pYP 66

Lane 4. purified pYP 67

Lane5. purified pYP 68

Lane 6. perfect protein marker

Figure 16. SDS-PAGE analysis with silver stain of purified protein samples from regular expression method in *E. coli* strain BLR (DE3) after dialysis

The purification results were not good even the protease inhibitor cocktail was added during purification. Therefore, the degradation might occur during cell growth. The proteins were easily to be degraded possibly because that they were misfolded and therefore not stable. It has been reported that adding 3% (v/v) ethanol will activate chaperon and thus help correct folding of the proteins[23].

Conclusions

Recombinant human tropoelastin was expressed and purified from a bacterial protein expression host. Expressions with tightly controlled promoters were likely the main reasons for low protein expression and purification yields. Expression in the pET 21a vector resulted in the highest protein expression levels due to the less stringent T7 promoter system. Purification using the polyhistidine tagged tropoelastin in pET 21a or hot/cold spin method was useful as future goals for improving tropoelastin yields. Future studies with the purified tropoelastin may include cross-linking studies and incorporation of proline analogues to investigate the structure of tropoelastin. Chemically cross-linked tropoelastin may be useful in structural studies that investigate the *in vivo* folding of native elastin, or in biomaterials applications. Tropoelastin may be useful in a variety of biomaterials applications as elastin-mimetics have shown promise as components of biomedical devices in tissue engineering and in a wide variety of other applications[24].

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