PRODUCTION OPTIMIZATION OF RECOMBINANT HUMAN VASCULAR ENDOTHELIAL GROWTH FACTOR IN TOBACCO (*NICOTIANA BENTHAMIANA*)

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การหาภาวะที่เหมาะสมในการผลิตรีคอมบิแนนท์วาสคูลาร์เอนโดทีเลียลโกรทแฟคเตอร์ของมนุษย์ในต้น *Nicotiana benthamiana*

น.ส.คริสติน จอย ไอ บุลาอน

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Thesis Title	PRODUCTION OPTIMIZATION OF RECOMBINANT HUMAN VASCULAR ENDOTHELIAL GROWTH FACTOR IN TOBACCO (*NICOTIANA BENTHAMIANA*)
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คริสติน จอย ไอ บุลาอน : การหาภาวะที่เหมาะสมในการผลิตรีคอมบิแนนท์วาสคูลาร์เอนโดทีเลียลโกรทแฟคเตอร์ของมนุษย์ในต้น *Nicotiana benthamiana*. (PRODUCTION OPTIMIZATION OF RECOMBINANT HUMAN VASCULAR ENDOTHELIAL GROWTH FACTOR IN TOBACCO (*NICOTIANA BENTHAMIANA*)) อ.ที่ปรึกษาหลัก : รศ. ดร.วรัญญู พูลเจริญ, อ.ที่ปรึกษาร่วม : ผศ. ภญ. ดร.ทักษิณา ชวนอาษา

การผลิตโปรตีนจากพืชเป็นนวัตกรรมทางเลือกในการผลิตรีคอมบิแนนท์โปรตีน วาสคูลาร์เอนโดทีเลียล โกรทแฟคเตอร์ของมนุษย์ (VEGF) เป็นโกรทแฟคเตอร์ชนิดหนึ่งที่มีบทบาทสำคัญในการรักษาบาดแผล การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อพัฒนาสภาวะการแสดงออกที่เหมาะสมและรวดเร็วของการผลิตรีคอมบิแนนท์ VEGF ใน *Nicotiana benthamiana* ในงานวิจัยนี้ เวคเตอร์ที่มียีน VEGF ได้ถูกสร้างขึ้น 4 แบบ ได้แก่ pBYR2e-SP-VEGF-His-SEKDEL (S-V-H-K), pBYR2e-SP-VEGF-His (S-V-H), pBYR2e-SP-His-VEGF-SEKDEL (S-H-V-K) และ pBYR2e-SP-His-VEGF (S-H-V) เวคเตอร์ที่มียีนรูปแบบต่างๆเหล่านี้ถูกนำไปใส่ในเวกเตอร์ geminiviral pBYR2e-K2Md สำหรับการแสดงออกของยีนชั่วคราวใน *N. benthamiana* เพื่อหาปริมาณโปรตีนและระยะเวลาที่เหมาะสมในการเก็บเกี่ยวเพื่อให้ได้ปริมาณโปรตีนที่มากที่สุด หลังจากตรวจหาปริมาณโปรตีน VEGF ที่สะสมในพืช พบว่าทั้งเวคเตอร์ที่มียีน S-V-H-K และ S-V-H มีปริมาณการสะสมที่มากที่สุดในวันที่ 3 หลังจากใส่อะโกรแบคทีเรียในใบยาสูบ รีคอมบิแนนท์โปรตีนถูกทำให้บริสุทธิ์ด้วยเทคนิคโครมาโตกราฟีโดยมีนิกเกิลเป็นตัวจับโปรตีนอย่างจำเพาะ และยืนยันผลด้วยการทดสอบโดยวิธี Western blot ที่ตรวจสอบด้วยแอนติบอดี anti-His และ anti-VEGF ผลการทดลองพบว่าการติด His tag และ SEKDEL ที่ปลายด้านหมู่คาร์บอกซิล นั้นเหมาะสมที่สุดในการผลิตรีคอมบิแนนท์ VEGF ใน *N. benthamiana* จากการศึกษาครั้งนี้สามารถสรุปได้ว่า โปรตีน VEGF ประสบความสำเร็จในการผลิตในพืชและสามารถนำไปใช้ในการศึกษาต่อในด้านการออกฤทธิ์ทางชีวภาพได

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Christine Joy I. Bulaon : PRODUCTION OPTIMIZATION OF RECOMBINANT HUMAN VASCULAR ENDOTHELIAL GROWTH FACTOR IN TOBACCO (*NICOTIANA BENTHAMIANA*). Advisor: ASSOC. PROF. WARANYOO PHOOLCHAROEN, Ph.D. Co-advisor: ASST. PROF. TAKSINA CHUANASA, Ph.D.

Molecular pharming represents the innovative development of plants as alternative production platforms for recombinant proteins. Human vascular endothelial growth factor (VEGF) is one of the growth factors that has displayed eminent roles in wound healing. This study aimed to develop optimized expression conditions and rapid production of recombinant VEGF in *Nicotiana benthamiana*. Herein, four plant expression constructs, particularly pBYR2e-SP-VEGF-His-SEKDEL (S-V-H-K), pBYR2e-SP-VEGF-His (S-V-H), pBYR2e-SP-His-VEGF-SEKDEL (S-H-V-K) and pBYR2e-SP-His-VEGF (S-H-V), were utilized and inserted into geminiviral vector pBYR2e-K2Md for transient expression in *N. benthamiana*. The expression level of all the four constructs was examined and the optimal harvest time for high protein expression was identified. High level of plant-produced VEGF protein expression in both S-V-H-K and S-V-H constructs were apparently detected on day 3 post-infiltration. The recombinant protein was then purified from the crude extracts by using nickel affinity column chromatography and confirmed by Western blot probed with anti-His and anti-VEGF antibodies. Findings revealed that poly-His and SEKDEL labeling at the C-terminal is the most suitable for the optimized production of recombinant VEGF in *N. benthamiana*. Overall, VEGF protein has been successfully produced in plants that could be further characterized for its biological activity

Field of Study:	Pharmaceutical Sciences and Technology	Student's Signature .....
Academic Year:	2019	Advisor's Signature .....
		Co-advisor's Signature .....

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Christine Joy I. Bulaon

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CHAPTER I INTRODUCTION

The development of plant-based biopharmaceuticals such as therapeutic antibodies, growth factors, enzymes and immune complexes have introduced pioneering opportunities for universal production of modern medicines. Molecular pharming is a well-known approach oriented towards the innovative manufacturing of clinical products in plants (1, 2). Currently, a vast expanse of economic prioritization in plant biotechnology has exemplified success for commercial processing of plant-based recombinant proteins. Plants provide amenable advantages complemented with inexpensive costs, scalable capacity, inherent safety and large yields of plant-derived therapeutics (3, 4). Furthermore, plants can synthesize biologically active proteins with proper post-translational modifications (5, 6). Hence, plant expression systems are considered as alternative platforms for high level accumulation of pharmaceutical proteins.

The cumulative progresses in the production technologies of recombinant proteins provide a more promising approach in comparison to direct extraction from the natural material. Most recombinant biologics effectively resolved challenges on product biosafety, source accessibility and purification methods as clinical activity and stability are enhanced (6). Ever since, molecular pharming has had a great deal of importance on the market and biopharmaceutical companies for the production of novel therapeutic targets. A wide range of protein expression hosts, particularly prokaryotic and eukaryotic organisms, has offered multiple favorable amenities for commercial-scale processing of pharmaceutically relevant proteins. In fact, current recombinant biopharmaceuticals are expressed from established cell-based platforms (7, 8). However, demands for strict compliance and adherence to regulatory considerations are highly monitored to guarantee the quality, consistency and security of recombinant products. Thus, only a few of common conventional expression systems such as bacteria and mammalian cells are universally well-characterized for industrial protein production (9). Despite improvements and success of these gold standard expression systems, large-scale product yield, extensive

bioprocessing timeline, contamination risk and high-capital costs remain underlying impugns for these organic cell cultures.

More recently, production techniques of recombinant proteins are devoted to plant-based machineries, with several valuable candidates that have transcended clinical regulations and market demands (10). The plausible commercialization of biomedicines by plant biofactories has drastically expanded due to the success of tobacco-derived ZMapp drug for Ebola outbreak (11). Plant pharming has resolved a number of challenges and limitations presented from other culture systems (4). Thus, substantial growth of molecular pharming using plant hosts has paved a way for the future of affordable, safe and functional biological products (10).

The notable facets and advancement on molecular pharming enlightened the importance of plant and bioengineering technologies to harness protein production efficiency. *Nicotiana benthamiana* is one of the most commonly used plant expression systems that has been explored. This tobacco variety provides a significant cutting edge due to huge biomass, short life cycle, low-risk of contamination and easy scale-up procedures (12). In addition, the pivotal roles of gene synthesis had generated an effective strategy for optimized expression of therapeutic proteins. Indeed, gene expression construct and preferred codon usage provided an array of prospective expression conditions that can be exploited. Specific protein sequences, namely signal peptides and retention signals, have fully demonstrated their evident effects toward successful production of recombinant proteins with protective properties and improved stability (13). Nowadays, various bioengineered growth factors cover remarkable demands for clinical and research applications. The manufacturing of these proteins constantly increases due to cell survival, proliferation and tissue repair activities (14).

In this study, we aim to produce recombinant VEGF protein in plants. The recombinant VEGF gene was codon optimized, cloned in geminiviral vector, and transiently expressed in *Nicotiana benthamiana.* The transient production based on versatile agroinfiltration using geminiviral replicon system allowed convenient expression yields of plant-derived VEGF. The plant-produced VEGF was purified and quantified for its rapid and optimum production in *N. benthamiana.*

1.1. Research Hypotheses

1. The recombinant VEGF can be produced in *Nicotiana benthamiana.*
2. The expression level of VEGF in plants varied depending on the location of poly-His tag and the presence of SEKDEL retention motif on the VEGF gene construct.

CHAPTER II LITERATURE REVIEW

1. Overview of Vascular Endothelial Growth Factor

1.1. Structure and Function of VEGF

Vascular Endothelial Growth Factor (VEGF) is a growth factor that plays a pivotal role in embryogenesis, vascular permeability, wound healing and mostly in angiogenesis (15, 16). The VEGF family comprises 5 members of structurally homologous mammalian glycoproteins, specifically VEGF-A, VEGF-B, VEGF-C, VEGF-D and placental growth factor (PlGF) (17). By custom, VEGF-A gene is represented as VEGF. It was originally discovered from pituitary follicular cells of bovine and is coded by 8 exons spliced at proximal and distal sites forming 4 main subtypes in humans: $VEGF_{121}$, $VEGF_{189}$, $VEGF_{206}$ and the most abundant $VEGF_{165}$ (18). The subscript number identifies the total amino acids present in the mature polypeptide. From a structural standpoint, functional VEGF is generated as a either a homodimeric (40-45 kDa) or heterodimeric (40 kDa) glycoprotein (18, 19). It includes a cysteine-knot motif exemplifying specificity for disulfide bridges in structure and receptor binding in vascular endothelium (20). Moreover, all VEGFs contain 8 conserved cysteine amino acids connected in 3 intramolecular and 2 intermolecular disulfide bonds, respectively. The stable VEGF structure is illustrated as an antiparallel homodimer protein combined by inter-chain disulfide bridges. Numerous relevant pathways associated in angiogenesis, for example tube formation (tubulogenesis), endothelial cell differentiation and production have been characterized thus far and thereby denotes emphasis for an immense analysis.

1.2. VEGF Receptors and Signaling

VEGFs have established signal transduction activities via dimerization of receptors and activation or binding to receptors, also known as tyrosine receptor kinase. There are three primary domains of VEGF receptors (VEGFRs) that differ in activity and reported as follows: extracellular domain initiates binding of VEGF and both transmembrane and intracellular domains activate receptor functions (21). Briefly, the human VEGF is attached firstly to the extracellular domain and

subsequently regulates activation and phosphorylation of tyrosine residues in the cytoplasmic domain, triggering other signaling mechanisms. All 5 members of the VEGF family interact selectively to VEGF receptors: VEGFR-1 (fms-like tyrosine kinase-1), VEGFR-2 (kinase domain region/murine fetal liver) and VEGFR-3 (fms-like tyrosine kinase-4) (Figure 1). They display a distinct VEGF-VEGFR binding pattern that induces migration, hematopoiesis, lymphangiogenesis and other receptor signaling functions. VEGFR-1 receptor has been identified with high binding affinities for VEGF-B, PlGF and VEGF-A but with reduced activity of tyrosine kinase enzyme (22). It serves as a negative regulator of VEGFR-2, restricting the interaction of VEGF ligand and so inhibits VEGR-2 functional potential (20). VEGFR-2 receptor presents the bulk of pro-angiogenic processes as implicated on the VEGF-A binding. Proteolytic signaling of VEGF-C and VEGF-D occurs highly on VEGFR-3, influencing critical roles on morphogenesis of lymph vessels. Likewise, VEGF ligands bind to other proteins or co-receptors including neuropilins and heparan sulfate proteoglycans (22-24). The production of VEGF splice variants is abundant and normally occurs simultaneously. The VEGFR-1 and VEGFR-2 levels are highly prevalent on adult cells of vascular endothelium but also expressed in variety of non-endothelial cells like renal mesangial cells, inflammatory cells, monocytes, hematopoietic stem cells, megakaryocytes, pancreatic cells and even tumor cells. Whereas, VEGFR-3 is predominantly produced on lymphatic cells (25). The VEGFRs share similar signaling mechanism with other related growth factor receptors, hence induce common regulatory outcomes of migration, survival and proliferation. The peculiar roles and bioactivity of VEGFRs have been exclusively defined for angiogenesis, lymphagiogenesis and vascular permeability signals. In exact, VEGFR-1 transduces essential functions dependent to developmental angiogenesis and drives employment and migration of hematopoietic cells, monocytes and macrophages. The VEGFR-3 signaling is significant in enhancement of endothelial cell-lymphatics. Most of VEGF mediated processes are modulated in VEGFR-2 comprising endothelial cell proliferation and development, blood vessel formation or angiogenic activities, vascular permeability and vasculogenesis.

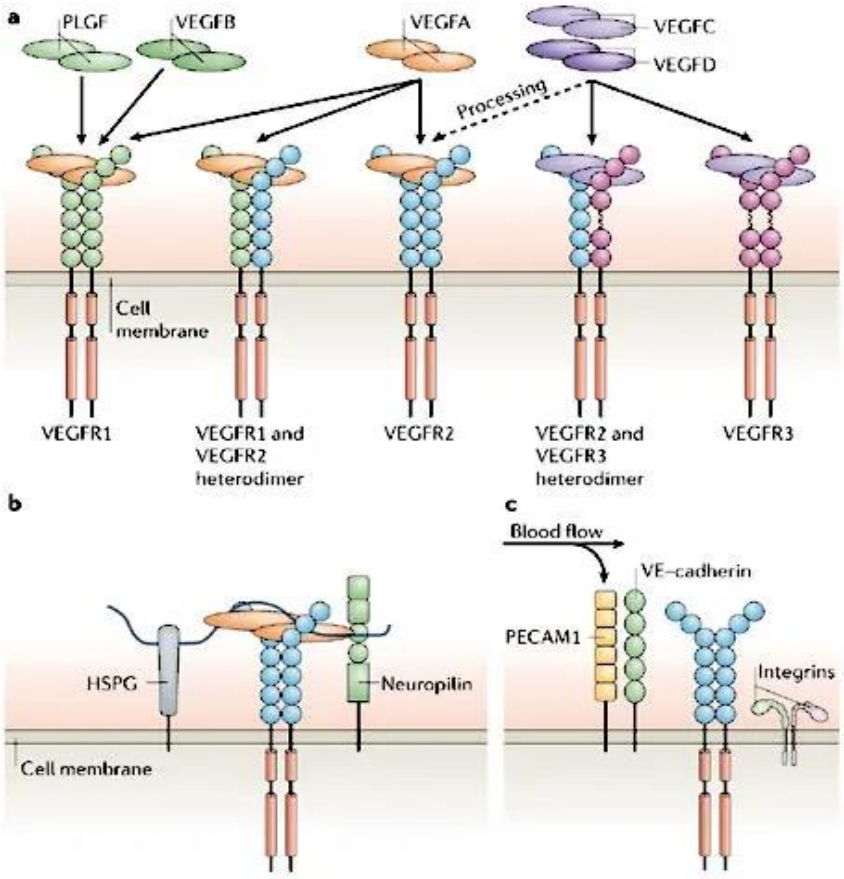


Figure 1 VEGF structure and VEGF receptor binding properties

(20)

1.3. VEGF-VEGFR signaling mechanism

The activity of VEGF receptors is driven by the binding of VEGF (Figure 2). It is regulated by both the availability and affinity properties of the ligand linked by disulfide bonds, thus initiating receptor dimerization (26). VEGFR dimerization is associated with the positive regulation of tyrosine kinase causing autophosphorylation of tyrosine residues and stimulates several signal transduction pathways involving phospholipase-C**γ**–protein kinase C–p38MAPK (27, 28) signaling for endothelial mitogen effect. Moreover, activation of certain cellular mechanisms like P13K/Akt pathway significantly promotes crucial anti-apoptotic role and highly activates soluble co-receptors facilitating cell disruption and migration (29). In line with this, guided by active P13K – p38MAPK pathway, marked aggregation of complexes between adhesion molecules and VEGFRs can decline intracellular

connections and undermine cytoskeletal framework, which induces fenestrae development of endothelial cells and improves cellular migration (30). Finally, it has been considered that acting through Akt mechanism, processing of endothelial nitric oxide synthase (eNOS) is efficiently increased favoring vasodilation and vascular permeability (31). Taken together, important cellular mechanisms implicated in angiogenesis are achieved following VEGF expression and binding to VEGFR, respectively.

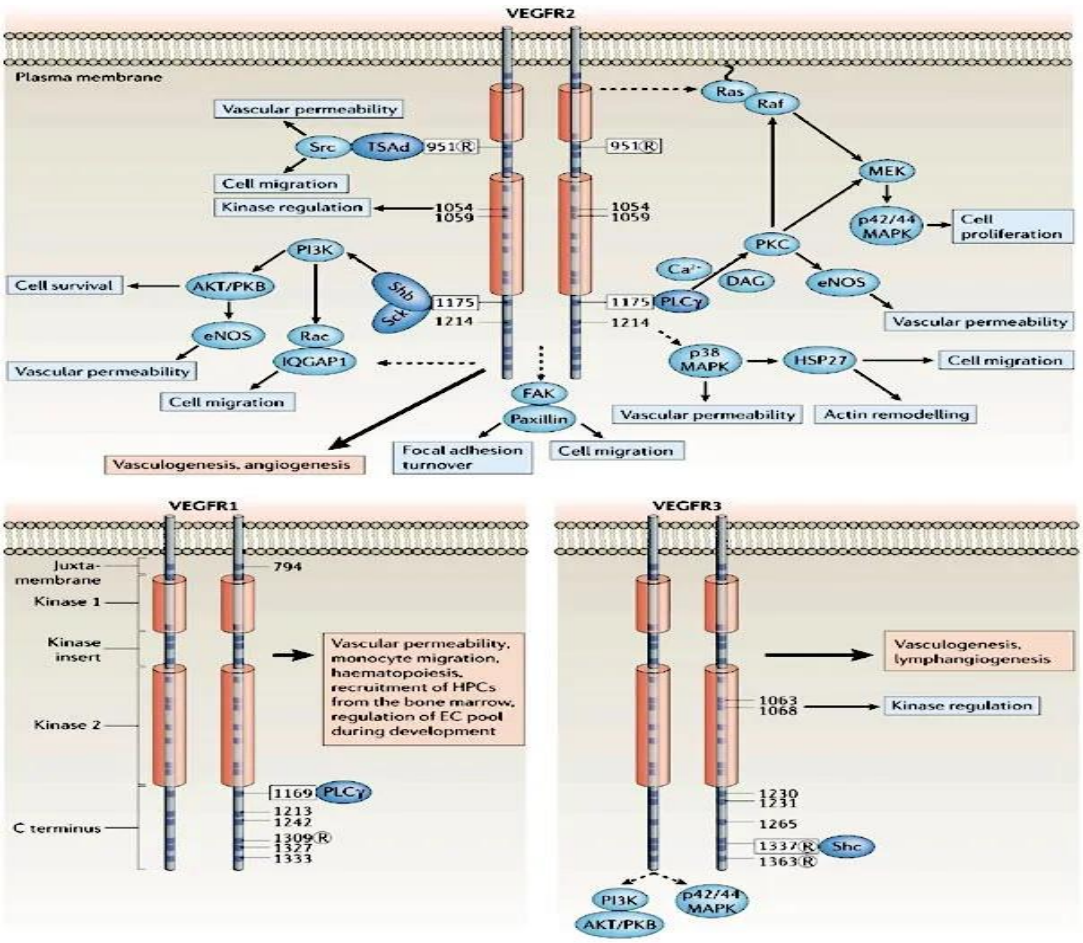


Figure 2 VEGF signaling pathways

(20)

VEGF signaling system plays a central role in a number of physiological processes often implied in hematopoiesis, bone formation and lymphatic development, as described below.

1.3.1. Hematopoiesis

Hematopoietic stem cell development has showed notions by which VEGF differentially controls this cellular process. In fact, it has been discovered that hematopoietic and endothelial cells were originally acquired from a precursor that facilitates VEGFR expression (20). VEGF is the primary regulator of hematopoiesis and promotes survival and differentiation of hematopoietic stem cells (HSCs) via an internal autocrine loop mechanism (32). In the conducted study of Gerber et al., removal of VEGF gene, as well as the presence of intracellular inhibitors of receptor tyrosine kinases, dramatically decreased the rates of survival, colony growth, and in vivo repopulation of HSCs. Although, administration of soluble VEGFR-1, which blocks VEGF and works in the extracellular compartment, only showed minimal effects. The findings concluded the negligible influence of inhibitors that were unable to permeate intracellularly towards VEGF-dependent HSC survival. Further, VEGFR-1 during hematopoiesis revealed the importance of migration of HSCs and monocytes to a VEGF gradient. In this context, repression of inflammatory responses after treatment with VEGFR1 neutralizing antibodies was initially due to immobilization of hematopoietic cells and leukocytes (33). Functional relevance of VEGFR-1 signaling accentuates rescued pro-survival potential of VEGF-expressing hematopoietic progenitors after bone marrow damage (34).

1.3.2. Bone formation

Bone repair and regeneration are physiological processes that attribute both endogenous reformative capacity and construction of new mineralized tissues for bone restoration. Human bones are usually highly vascularized and utilize VEGF for angiogenesis-osteogenesis coupling mechanism (35). VEGF stimulates recruitment and reorganization of blood vessels and bone cells during skeletal remodeling and bone repair. In general, bone disorders can lead to different pathological impaired situations. For instance, around 10% of the bone defective population experience delayed reformation or no reformation and acquire severe unhealed bone fractures (36). In the case of regenerative measures, reconstruction of ample bone injuries and osteogenic distraction occasionally cause unsuccessful results. The prospective

contributors for failed treatment procedures include instable supply of blood, periosteum defects, insufficient amount of osteoprogenitor cells (osteoporosis), impeded movement and infection at the wound site (37). Currently, the underlying mechanisms causing impaired bone repair and regeneration strategies have not been completely elucidated. Functions of VEGF in bone establish cooperative responses between network of blood vessels and bone cells, sustaining physical contact and mutual communication of active cells vital for skeletal development. A hematoma caused by any form of trauma in the bones and the following counters during inflammation instigate bone repair. The expression level of VEGF was reported to be remarkably higher in hematoma with relevance to its adequate concentrations in plasma. Neutrophils demonstrate substantial existence after injury and are recruited to affected sites for removal of bone debris and pathogenic microorganisms (38). VEGF drives neutrophil chemotaxis and enhances bone marrow permeability (39). According to the study of Hu and Olsen (40), during acute inflammation, VEGF induces neutrophil infiltration into the circulation of treated mice. It has been described that VEGF is an effective chemotactic agent for macrophage, which targets macrophage-angiogenic signals in inflammation phase. Interestingly, the roles of VEGF during bone development are recapitulated for skeletal vascularization and normal bone remodeling (41). A well-characterized coupling mechanism associated with vasculogenesis and angiogenesis resulted to the production of bone-forming progenitors and provided nutrients, oxygen and minerals essential for mineralization.

1.3.3. Lymphatic development

Lymphatic development is one of the key processes regulated by VEGFR-3 receptor. Commonly, receptor activation occurs due to binding of VEGF-C and VEGF-D ligands. Endothelial-lymphatics (or lymphatic cells), which produce VEGFR-3, accumulate into lymph sacs and set forth the lymphatic system framework. Studies showed that inadequacy of lymph vessels and deficiency of VEGFR-3 expression in mice yielded serious tissue edema and vascular remodeling defects (42), which later on led to prenatal deaths. The results have implicated the essential impacts of VEGFR-3 in the developmental stage of blood vessels.

1.4. Wound healing mechanism of VEGF

VEGF mediates downstream angiogenic signaling or angiogenesis, which is one of the important processes involved in repair and restoration of injured tissues. Initially, activity of VEGF is regulated upon binding to VEGFR and was specific for endothelial cell functions (43). However, recent studies uncovered additional angiogenic activities of VEGF characterized on wound repair functions (Figure 3). Upstream expression of VEGFRs from non-endothelial cell types such as keratinocytes, mast cells, fibroblasts and macrophages implicate direct response from VEGF for wound healing process (44). The process of wound healing in skin initially occurs with hemostasis and inflammation. Then, it proceeds stages of cell proliferation, extracellular matrix deposition and remodeling and finally scar development (45). In the proliferation stage of effective repair, angiogenesis drives production of blood vessels, which promote adequate supply and delivery of oxygen and nutrients necessary for growth and survival of damaged cell (46). The clinical interpretations of VEGF abundance during the course of tissue repair has demonstrated sufficient wound vascularization. In cases of severe and incurable wounds, low level of VEGF tends to yield reduced activity contributing to inefficient and delayed healing (47). Most of newly formed blood vessels are produced through active angiogenesis after a significant rise in the number of proangiogenic factors, like VEGF, at the lesion site. Studies exploring VEGF in repair aspects implied increased VEGF production in wound fluid for more than 7 days (48) and commonly expressed in the early and late cutaneous repair stages (49). Hypoxia induces upregulated expression of VEGF protein in response to negligible oxygen levels; thus, it drives activated transcription of VEGF gene (50).

The VEGF created due to tissue damage provides a significant role in several facets of wound healing. At the early stage of wound repair, VEGF acts as a robust inducer of vascular permeability (51) affecting expression of intracellular adhesion molecules on endothelium (52) and later on assists in the circulation of inflammatory cells into the tissue. Additionally, VEGF has been extensively studied for its contribution towards overall wound closure and re-epithelialization in animals (53). Majority of the results indicate that increasing VEGF levels from severe and

defective healing models influence rapid repair of inflicted wound, effective angiogenic functions, improved formation of granulation tissue and higher stability of reparative wound (54).

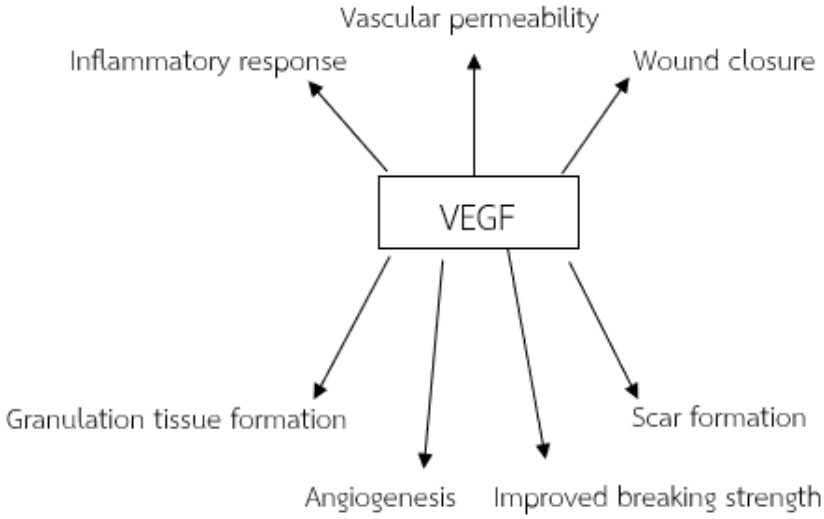


Figure 3 VEGF activities during wound repair

1.5. Source of VEGF

The VEGF has been produced in several cell types including endothelial and non-endothelial cells. In the cardiovascular system, expression of VEGF was evident in cardiac myofibroblasts (55). The co-expression of VEGF-VEGFR regulates autocrine mechanism and VEGF-associated tissue remodeling. In addition, VEGF stimulation in the nervous system may contribute to the cell survival, proliferation and neuronal protection of Schwann cells and hippocampal neurons (56, 57). During bone development, production of VEGF in hypertrophic chondrocytes play key roles in angiogenesis, bone remodeling and formation (58). In most cases, the relevance of VEGF expression in tumor cells has been exploited that impedes apoptotic cell death and promotes improved resistance to cancer therapies (59). Meanwhile, epidermal keratinocytes, fibroblasts, and mast cells produce upregulated levels of VEGF during the course of wound repair (44, 51, 60). The experimental treatment of VEGF to damaged tissues mediates angiogenic, regenerative and healing effects (16,

61). Further exploration towards the potentials of human VEGF protein may provide significant and novel findings for its future applications and thus suggest protein production at sufficient yields.

2. Recombinant Protein Production

2.1. Molecular Pharming via *Nicotiana benthamiana*

Recombinant protein expression using prokaryotic or eukaryotic host systems is the leading method for the production of industrial and research proteins. An increasing interest for recombinant protein production in plants provides an alternative strategy for the production of promising biopharmaceuticals. Plant-based expression systems are advantageous for successful post-translational modifications vital for the biological activity and stability of heterologous proteins (62). The development of plant systems offers remarkable benefits toward product yield with reduced production cost, homogeneity and safety against endotoxins and human pathogens contamination (63, 64). In this study, tobacco, specifically *Nicotiana benthamiana*, was used and characterized as the alternative platform to produce recombinant protein (Figure 4).

Figure 4 Nicotiana benthamiana

Tobacco is the most widely known plant expression system to produce high yields of recombinant proteins. It continues to be the main non-food crop choice for molecular farming of pharmaceuticals. Additionally, *Nicotiana benthamiana* is well established as the plant host with high biomass yield, high purity and cost-effective scalability processes critical for clinical and agricultural applications (65). It is

generally use as the experimental host in plant virology since a number of plant viruses are able to infect it and it is susceptible to several plant-pathogenic agents essential for molecular plant-microbe interaction studies (66, 67). Overall, tobacco has great flexibility and efficient genetic transformation suitable for plant-derived productions of antimicrobial compounds.

Genetic transformation heavily relies on *Agrobacterium tumefaciens* as an effective tool to deliver or introduce the gene of interest into the host plant. There are two kinds of plant genetic transformation namely transient transformation and stable transformation (68). In comparison, transient transformation is an easier, simpler and more rapid method to analyze certain genes than stable transformation. One of its major advantages is that the analysis is accomplished within days or weeks of transformation (69). Among the transient transformation strategies, *Agrobacterium*-mediated or agroinfiltration follows the principle wherein the foreign DNA can transiently remain in the nucleus without integration in the genome of the plant. The two common methods of agroinfiltration are syringe and vacuum infiltration. Both plant-based production strategies accord fast and noteworthy assessment of heterologous protein expression with improved protein stability and activity.

2.2. Recombinant Protein Expression Optimization

Human growth factors have become remarkable candidates for commercial biopharmaceuticals due to several advantageous properties. Advancement on heterologous protein expression from plant systems include strategies related with bioengineering disciplines such as optimized gene expression constructs. A number of recombinant proteins have elucidated the affecting factors that can impact the strength of protein expression, which includes addition of promoter sequences, tag sequences, and retention signals (70-73).

2.2.1. Codon Optimization

An important step used in enhancing protein expression is the optimization of codons where the gene coding regions of the target protein uses codon biases for efficient gene expression and protein translation. The modification of coding

sequence according to the codon usage frequency of the plant expression system improves heterologous gene expression resulting in up-regulated protein and mRNA levels (74). The synonymous substitutions of rare codons to frequent codons based on host genomes demonstrate impacts on rate of translation elongation and therefore result to high protein expression levels (75, 76). In a similar approach, selection of preferred codons is considered to affect mRNA expression through marked-fold increased levels of mRNA indicating regulated translational accuracy and efficiency (77).

2.2.2. Signal Peptide

Proteins tagged with N-terminal secretion sequence or signal peptide are directed to pass through the secretory pathway. The newly synthesized recombinant protein with secretion signal is recognized and translocated in the endoplasmic reticulum (ER)-Golgi pathway to allow protein processing and complete post-translational modifications. Signal peptides are short protein sequences with a length of approximately 16-30 amino acid residues (78). Prior studies have demonstrated that utilizing efficient signal peptides can contribute in the yields of the recombinant proteins (79, 80). Subcellular localization of foreign proteins can be achieved using a signal peptide. Comparatively, high levels of protein expression were observed when secretion is targeted to subcellular compartments (81). The development of effective secretion system in bacterial expression systems have presented high production of alpha-amylase AmyL and AmyS recombinant proteins up to 1352 and 2300 U/mL after optimizing promoter and signal peptide tags (82). In plants, the expression of single-chain antibody fragments (scFv) protein containing signal peptide resulted in higher level of expression with appropriate protein folding and assembly (83). Selection of the most suitable signal peptide has described a method to increase secretion of proteins and improve production levels of recombinant proteins.

2.2.3. Retention Peptide Tag

The course or final destination of nascent recombinant proteins can play as a major determinant for their structure and stability. In concept, expressed proteins go

through to the endoplasmic reticulum (ER) for post-translational modification (PTM) and move to Golgi complex for Golgi-specific modifications (84). Later studies identified a C-terminal peptide (SEKDEL/KDEL) in several proteins that are sorted in the ER (85). It has been indicated that after the addition of the hexapeptide motif in lysozyme, the exogenous protein bypassed secretory pathway and was consequently accumulated in the ER. Further analyses showed that SEKDEL-tagged protein can interact with the KDEL receptor, leave the ER, undergo PTMs in the cis-Golgi and successively retrieved back to the ER by retrograde transport (86).

The essential features of ER include chaperones that assist in the proper folding of nascent proteins (87). ER localization has become promising for protein folding and storage that presumably contributes to increased protein levels. The effect of SEKDEL retention motif labeling in some recombinant proteins indicates enhanced protein expression levels following targeted accumulation at the ER compartment (88). In one context, the SEKDEL peptide boosted the amount of plant-produced anti-cancer monoclonal antibody four-folds higher in *Arabidopsis* plants and were stably expressed without occurrence of any damage to plant cells (89). Nevertheless, improved protein accumulation is not constantly observed in all cases (90). Therefore, the potential of SEKDEL tagging strategy in increasing yields of recombinant proteins suggest further investigations.

2.2.4. Poly-Histidine tag

A well-known affinity ligand such as poly-Histidine (His) tag has been widely used for purification of recombinant proteins via immobilized metal affinity chromatography. It is a small metal chelating tag which consists of 6 to 10 Histidine residues added either at the N- or C-terminus (91). The accessibility of the His tag at the protein surface allows recombinant protein for binding on the affinity resin (92). In accordance to other studies, the poly-His tag length showed greater effects on protein binding property and yield. A strong affinity of Aquaporin Z protein tagged with 10 Histidine residues to the metal-IMAC matrix was observed after elution in high concentration of imidazole (93). The 10x His-tagged recombinant protein eluted later than the 6x His-tagged protein. In addition, higher Aquaporin Z yields was

obtained with recombinant protein bearing 6x His when compared to 10x His. In the present study, the position of poly-His tag at the N- or C-terminus was systematically investigated for its influence on protein expression levels. Previous studies have reported impacts of His-tag location on some proteins resulting to induced overexpression via subcellular uneven distribution, specifically on N-His-tagged recombinant proteins (94). However, some studies have confirmed comparable effects on expression, yield and structure of recombinant proteins associated with shifting His tag locations between N- or C-termini (93).

2.3. Geminiviral Replicon System

Production of recombinant proteins in plants can be improved by the development of transient expression systems with geminiviral vectors. For simple processing of plant pharmaceutical proteins, high expression level of proteins within days to weeks hold important economic advantages. The plant viral vectors in general greatly promote high-yield production of foreign proteins because of efficient replication mechanisms that result to enhanced levels of transgenes and mRNA transcripts (95, 96). Consequently, the use of *Nicotiana* varieties based on geminiviral replicon system are currently exploited. In fact, the geminiviruses derived from the bean yellow dwarf virus (BeYDV) showed useful strategies to achieve high accumulation of recombinant proteins in tobacco (97). In these systems, monopartite geminiviral vectors contain circular DNA genomes that drives replication process in the nucleus via rolling circle mechanism (98). The replication machinery of BeYDV system possess three genes of the Mastrevirus subgroup: virion-sense (V) strands V1 and V2 which directs coat proteins synthesis and complementary strand which encodes virus replication initiator protein (Rep) and RepA (99). In addition, geminiviruses genomes require cis elements such as long intergenic region (LIR) and short intergenic region (SIR) for replication. Specifically, the SIR initiates synthesis of complementary strand and contains termination signals for transcription (100). Meanwhile, the structurally stem-loop LIR contains replication initiators like bidirectional promoter elements and facilitates the rolling circle mechanism of V strand (101). Rep is also vital in the replication machinery as it releases and replicates

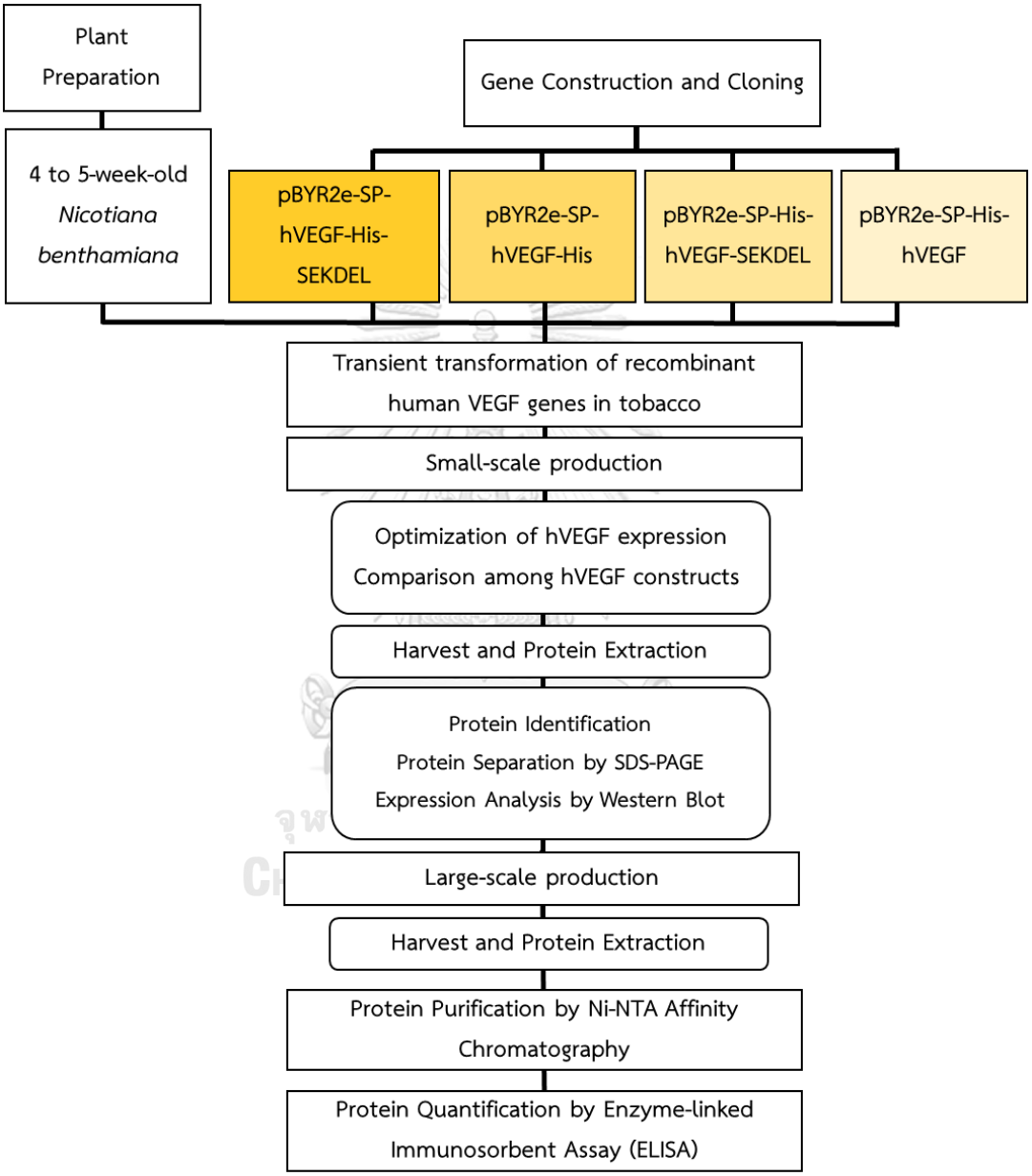
DNA construct replicon that contains the expression cassette for the target gene. It acts as a trans element and thus can be provided from other viral replicon system or transgene (67). It has established peculiar nicking capacity to regulate replicative release of recombinant DNA flanked between LIR tandem repeat or cis acting elements of the virus (98). The DNA transiently introduced into the nucleus replicates and prospectively lead to increased gene expression levels.

3. Objectives of the Study

In the current study, four human vascular endothelial growth factor (VEGF) gene constructs were utilized and transiently expressed using geminiviral vector in *Nicotiana benthamiana*. The gene constructs were modified according to the addition of C-terminal SEKDEL retention motif and poly-Histidine (His) purification tag at either the N-terminal or C-terminal region. Experimentally-optimized parameters between constructs were characterized to fully comprehend their relationship to protein expression level by Western blot assay. The *N. benthamiana* leaves were infiltrated with different constructs and harvested at specified time points to determine best expression condition. The constructs that showed the highest expression of VEGF protein at optimal harvest time were further purified and studied to investigate their effects in protein binding. Optimized expression and purification conditions devised in the study can identify the optimal gene construct to yield VEGF protein in sufficient amounts that will be measured by ELISA.

CHAPTER III EXPERIMENTAL

1. Experimental Design



2. Materials

2.1. Gene sequence

The Human Vascular Endothelial Growth Factor (VEGF; Accession No: NP_001165097.1) has been synthesized (Bioneer company, Korea)

2.2. Enzymes

Restriction enzyme *Xho*I (New England BioLabs, Ipswich, MA, USA)

Restriction enzyme *Xba*I (New England BioLabs, Ipswich, MA, USA)

Restriction enzyme *Sac*I (New England BioLabs, Ipswich, MA, USA)

Taq DNA polymerase (Promega)

Q5 DNA polymerase (Promega)

T4 DNA ligase (Promega)

2.3. Cloning and Expression vectors

pGEMT-Easy Vector (Promega) (Appendix B)

pGEMT-SP Vector

pBYR2e-K2Md Vector (Appendix C)

2.4. Molecular Biology kits

AccuPrep Nano-Plus Plasmid Mini Extraction kit protocol (Bioneer, Korea)

AccuPrep Gel Purification Kit (Bioneer, Korea)

Human VEGF ELISA kit Cat. No: DY293B (R&D system, USA)

2.5. Chemicals

Acrylamide (Himedia, India), Agarose (Vivantis, Malaysia), Ampicillin (ITW Reagents, Darmstadt, Germany), Kanamycin (Bio Basic, Markham, ON, Canada), Rifampicin (Thermo Fischer Scientific, Waltham, MA, USA), Gentamicin (ITW Reagents, Darmstadt, Germany), 2-N-morpholino-ethanesulfonic acid (MES) (ITW Reagents, Darmstadt, Germany), Magnesium Sulphate (MgSO4) (Merck, USA), Tris (Vivantis, Malaysia), Enhanced Chemiluminescence (ECL) plus detection reagent (Abcam, UK), Bovine Serum Albumin (BSA) (HiMedia Laboratories), color reagent

A (stabilized peroxide solution) and color reagent B (stabilized chromogen solution) (R&D Systems), 0.45 μm S-Pak membrane filters (Merck, Massachusetts, USA), Amintra Ni-NTA affinity resin (Expedeon, Cambridge, UK), nitrocellulose membrane (Bio-Rad)

2.6. Bacteria

Escherichia coli (DH10B strain)

Agrobacterium tumefaciens (GV3101 strain)

2.7. Antibodies

HRP-conjugated rabbit polyclonal anti-His antibody (Abcam, UK)

Mouse anti-human VEGF antibody (R&D system)

Biotinylated goat anti-human VEGF detection antibody (R&D system)

HRP-conjugated goat anti-mouse antibody (Abcam, UK)

3. Buffer

3.1. Buffer for VEGF Isolation

Extraction buffer

20 M Tris-HCl pH 7.4, 50 mM NaCl, 5 mM Imidazole

Washing buffer

20 M Tris-HCl pH 7.4, 50 mM NaCl, 20 mM Imidazole

Eluting buffer

20 mM Tris-HCl pH 7.4, 50 mM NaCl, 250 mM Imidazole

3.2. DNA loading 6x dye

38% (w/v) Glycerol, 0.08% (w/v) Bromophenol blue, 0.08% (w/v) Xylencyanol

3.3. Z-buffer non-reducing dye

125 mM Tris HCl, 12% Sodium Dodecyl Sulphate, 10% Glycerol, 0.001% Bromophenol blue pH 6.8

3.4. 1X Tris-buffered saline (TBS)

0.9% Sodium Chloride (NaCl), 50 mM Tris base buffer pH 7.4

3.5. 1X Tris-buffered saline-Tween (TBST)

1X TBS, 0.05% Tween 20

3.6. 1X Phosphate-buffered saline (PBS)

137 mM NaCl, 2.7 mM Potassium Chloride (KCl), 8.1 mM Sodium hydrogen phosphate (Na_2HPO_4), 1.5 mM Potassium dihydrogen phosphate (KH_2PO_4) pH 7.4

3.7. Phosphate-buffered saline-Tween (PBST)

1X PBS, 0.05% Tween 20

3.8. Coomassie® blue stain solution

Coomassie Brilliant Blue R-250, Methanol, Glacial acetic acid, H_2O

3.9. Destaining solution

Glacial acetic acid, Methanol, H_2O

3.10. 1X Running buffer

25 mM Tris, 192 mM Glycine, 1% SDS

3.11. 1X Transfer buffer

25 mM Tris, 192 mM Glycine, 15% Methanol

3.12. 1X Infiltration buffer

10 mM MES, 10mM $MgSO_4$ pH 5.5

4. Media

4.1. Luria Bertani Broth

1% NaCl, 0.5% Yeast, 1% Peptone

4.2. Luria Bertani Agar

1% NaCl, 0.5% Yeast, 1% Peptone, 1.5% Agar

5. Methods

5.1. Codon Optimization of VEGF

The nucleotide sequence coding for human vascular endothelial growth factor was plant codon-optimized and synthesized by Invitrogen GeneArt® Gene Synthesis (Thermo Fischer Scientific). In brief, the optimized gene sequence along with signal peptide (SP), 8x poly-Histidine (His) tag and SEKDEL (Ser-Glu-Lys-Asp-Glu-Leu) retention motif was inserted into the pBHA cloning vector.

To optimize expression of VEGF, three additional recombinant constructs were designed and generated by Polymerase chain reaction (PCR). The list of forward and reverse primers used in the present study is listed in the Table 1. PCR was carried out with forward primers *XbaI*-SP and His-VEGF and reverse primers *SacI*-8xHis and *SacI*-SEKDEL-VEGF; two primers with conserved regions of VEGF coding site. The recombinant VEGF constructs were amplified in a 25 µl reaction volume covering 0.5 µl of pBHA-SP-VEGF-His-SEKDEL plasmid as the template. Subsequently, gene constructs were synthesized using 400 µM of forward and reverse primers. For **SP-VEGF-His** synthesis, *XbaI*-SP forward and *SacI*-8xHis reverse primers were used. For **His-VEGF-SEKDEL** synthesis, *XhoI*-His-VEGF forward and *SacI*-KD-VEGF reverse primers were used. For **His-VEGF** synthesis, *XhoI*-His-VEGF forward and *SacI*-8xHis reverse primers were used. The reaction mix contained 200 µM of dNTP, 1X Q5 reaction buffer and 1 unit of Q5 Taq polymerase. Amplification was done in a MJ Mini 48-well Personal Thermo Cycler (Biorad) with conditions as follows: initial denaturation at 98 ℃ for 2 min, 30 cycles of denaturation at 98 ℃ for 30 sec, annealing at 52 ℃ for 30 sec, and elongation at 72 ℃ for 1 min, and final elongation at 72 ℃ for 10 min. During final elongation step, 0.2 µl of Taq polymerase was added to incorporate poly-A overhang at the 3' ends. The amplified products were investigated by horizontal gel electrophoresis (1% agarose) and visualized under UV illumination.

Table 1 Primer pairs used to generate recombinant VEGFs

Gene	Primer Sequence
SP-VEGF-His	**F (*XbaI*-SP)** 5'TCTAGAACAATGGGCTGG **R (*SacI*-8xHis)** 5'GAGCTCTTAATGATGGTGATGGTGGTGATGATG
His-VEGF-SEKDEL	**F (*XhoI*-His-VEGF)** 5'CCTCGAGCATCATCACCACCATCACCATCATAACTTCCTGCTGTCTTGG **R (*SacI*-KD-VEGF)** 5'CGAGCTCTCAAAGCTCATCCTTTTCAGATCTTCTAGGCTTATCGCACC
His-VEGF	**F (*XhoI*-His-VEGF)** 5'CCTCGAGCATCATCACCACCATCACCATCATAACTTCCTGCTGTCTTGG **R (*SacI*-8xHis)** 5'GAGCTCTTAATGATGGTGATGGTGGTGATGATG

5.2. Gene Cloning

The constructed VEGF sequences were ligated into pGEMT cloning vector by T4 DNA ligase for subsequent cloning steps. The ligated products were artificially transformed into DH10B *Escherichia coli* competent cells by heat shock method for propagation. Selected colonies were screened by Colony PCR and grown in Luria Bertani (LB) broth medium with 100 mg/mL ampicillin overnight at 37 °C. Cloned VEGF fragments in pGEMT vector were subjected to restriction enzyme digestion with *Xho*I and *Sac*I enzymes and VEGF gene constructs were ligated to pGEMT-SP cloning vector to insert the N-terminal SP sequence. The ligated products will undergo artificial transformation into DH10B *E. coli* cells and transformants were screened for successful VEGF gene insertion by Colony PCR. The nucleotide sequences of VEGF fragments in pGEMT-SP vector were confirmed by DNA Sanger sequencing (U2Bio, Thailand). The four different constructs were developed which differ in the location of 8x poly-His tag in N or C-terminal and also with and without C-terminal SEKDEL retention motif.

5.3. Construction of pBYR2e-K2Md expression vector

Subsequently, the nucleotide sequences coding for VEGFs were excised from the pBHA and pGEMT-SP cloning vectors by *XbaI* and *SacI* restriction enzymes, purified by using AccuPrep Gel Purification Kit, and cloned into geminiviral expression vector (pBYR2e-K2Md) by digesting with similar restriction endonucleases. The ligated plasmids (Figure 5) namely pBYR2e-SP-VEGF-His-SEKDEL (S-V-H-K), pBYR2e-SP-VEGF-His (S-V-H), pBYR2e-SP-His-VEGF-SEKDEL (S-H-V-K) and pBYR2e-SP-His-VEGF (S-H-V) were further transformed into DH10B *E. coli* cells by heat shock method. Transformed competent cells were spread on LB agar plate comprising 50 µg/mL kanamycin and incubated overnight at 37 °C. Transformed colonies were analyzed and amplified by Colony PCR using primer pairs specific bearing VEGF variants. PCR positive transformants were inoculated in 5 mL LB broth medium with 50 µg/mL kanamycin and incubated overnight at 37 °C on rotary shaker at 250 rpm. The plasmids were extracted from the bacterial culture according to AccuPrep Nano-Plus Plasmid Mini Extraction kit protocol and stored in -20 °C.

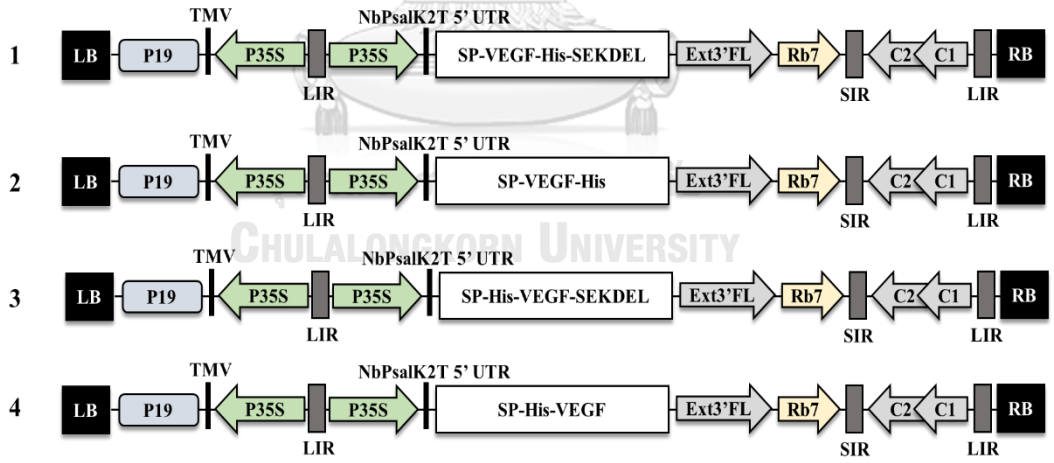


Figure 5 Schematic representation of VEGF gene constructs

Schematic representation of human vascular endothelial growth factor (VEGF) gene constructs in geminiviral vector (pBYR2e-K2Md) that are used in the present study; (1) SP-VEGF-His-SEKDEL: human vascular endothelial growth factor gene with 8X histidine residues and SEKDEL retention tag at the C-terminus and signal peptide at

the N-terminus; (2) SP-VEGF-His: human vascular endothelial growth factor gene with 8X histidine residues at the C-terminus and signal peptide at the N-terminus; (3) SP-His-VEGF-SEKDEL: human vascular endothelial growth factor gene with SEKDEL retention tag at the C-terminus and signal peptide and 8X histidine residues at the N-terminus; (4) SP-His-VEGF: human vascular endothelial growth factor gene with signal peptide and 8X histidine residues at the N-terminus.

5.4. Gene transformation into *Agrobacterium tumefaciens* strain

The ligated pBYR2e-VEGF plasmids were transformed into *Agrobacterium tumefaciens* electrocompetent cells using MicroPulser (Bio-Rad, USA). Transformed *A. tumefaciens* cells were spread on LB agar plate supplemented with 50 µg/mL kanamycin, 50 µg/mL rifampicin and 50 µg/mL gentamicin and grown at 28 °C for 48 h. Selected colonies were screened for successful gene insertions by Colony PCR. Then, positive transformants were cultured in 5 mL LB medium with 50 µg/mL of kanamycin, rifampicin, and gentamicin at 28 °C overnight. The *A. tumefaciens* suspensions containing plant expression vector (pBYR2e-VEGF) were inoculated into 1:100 fresh culture and propagated at 28 °C overnight on rotary shaker at 250 rpm.

5.5. Modified Agroinfiltration of *Nicotiana benthamiana* leaves

Transformation technology on *Nicotiana benthamiana* leaves was achieved transiently by syringe (small-scale) and vacuum (large-scale) infiltration. The overnight grown A. tumefaciens cells were centrifuged at 4, 000 x g for 15 min at 4 °C. The cell pellets were resuspended in 1X infiltration buffer to get final optimal density at 600nm (OD_{600}) of 0.4.

Agrobacterium cells harboring each of the constructs S-V-H-K, S-V-H, S-H-V-K and S-H-V were used for infiltration. Briefly, *N. benthamiana* seedlings were transplanted in sterile soil and grown in pots for 5-6 weeks under controlled temperature of 28 °C and illumination for 16 h daily. In-order to check the expression level, small-scale infiltration was carried out by injection of an *Agrobacterium* solution (OD_{600} = 0.4) containing VEGF onto the underside of *N. benthamiana* leaves

with the use of 1 mL syringe without a needle. Then, infiltrated leaves were collected on day 2, 3, and 4 post-infiltration (d.p.i). Alternatively, large-scale infiltration was followed by submerging *N. benthamiana* leaves at inverted position in a 2 L solution of *Agrobacterium* cells (OD_{600} = 0.4). Vacuum of 600 – 760 mmHg was applied for 1-2 min and then pressure was drawn out gently and returned to atmospheric pressure. Infiltrated tobacco plants will be maintained at an indoor plant room provided with regulated growing condition.

5.6. VEGF Protein Extraction

Small-scale infiltrated leaves were grinded with liquid nitrogen using a mortar and pestle. The fine powdered extract was extracted with 1:2 extraction buffer under cold condition. The crude suspension was mixed thoroughly in a vortex machine for 5 min. The total soluble protein was quantified by Bradford assay (Appendix G). Additionally, large-scale infiltrated leaves were homogenized by blender containing ice-cold extraction buffer and grinded for at least 1 min. Crude extracts were centrifuged at 26, 000 x g for 20 min. The plant extract was filtered by vacuum filtration using sterile 0.45 µm S-Pak membrane filters.

5.7. VEGF Protein Purification

Nickel affinity chromatography was utilized to purify plant-produced VEGF proteins. Initially, the column was packed with Amintra Ni-NTA affinity resin and equilibrated with 3 column volumes of extraction buffer. The clarified plant extract was slowly loaded onto the column with a flow rate of 0.5 mL/min. The resin was washed with 10 bed volumes of washing buffer and 8xHis-tagged VEGFs was eluted with 5 bed volumes of eluting buffer.

5.8. SDS-PAGE and Western Blot

The expression level of VEGF infiltrated with different expression constructs in plants and the purity of protein fractions were analyzed by using SDS-PAGE and Western Blot. The samples were mixed with 10x non-reducing loading buffer,

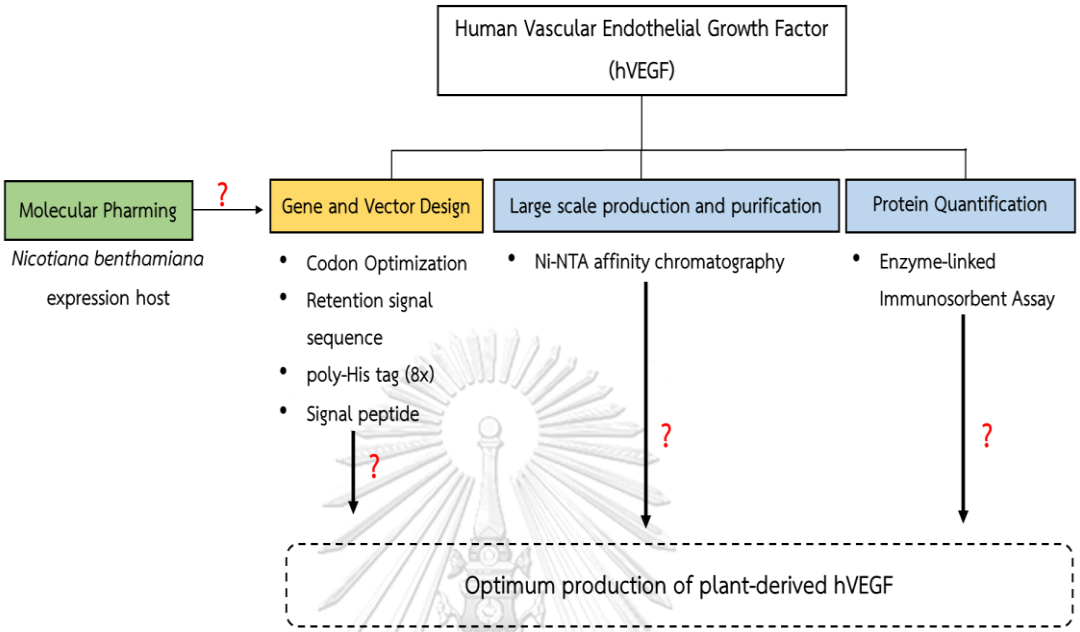
denatured at 95 °C for 5 min and loaded onto 5% stacking gel. The separation by SDS-PAGE (10-12% acrylamide) under non-reducing condition was conducted in 1X running buffer for 2 h. Separated proteins were either subjected to either Coomassie staining or Western blot detection. For Coomassie staining, the gel was stained with Coomassie® blue stain solution and the bands were visualized. For western blot, separated proteins were directly transferred from the gel onto a nitrocellulose membrane in 1X transfer buffer at constant voltage. The membrane will be blocked with 5% skim milk in 1X Tris-buffered saline (TBS) for at least 30 min, washed with TBST buffer and later probed either with HRP-conjugated rabbit polyclonal anti-His antibody or with mouse anti-human VEGF antibody diluted at 1:5000 in 3% skim milk in 1x TBS and HRP-conjugated goat anti-mouse antibody diluted at 1:5000 in 3% skim milk in 1x TBS. The protein band was developed by using enhanced chemiluminescence (ECL) plus detection reagent.

5.9. Enzyme-Linked Immunosorbent Assay (ELISA)

The concentration of plant-purified VEGF was determined by Enzyme-Linked Immunosorbent Assay (ELISA) by following manufacturer's instruction (Human VEGF ELISA kit protocol) (Appendix H). A 96 well microplate was coated with 1 µg/mL of mouse anti-human VEGF capture antibody in PBS buffer and incubated overnight at 4 °C. The microplate was washed with 200 µL of PBST buffer. Further, each well was blocked with 200 µL of 1% bovine serum albumin (BSA) in PBS for 2 h at room temperature (RT). Then, the plate was washed in preparation for sample addition. The VEGF samples and recombinant human VEGF standards was loaded into the wells and incubated for 2 h at RT. Subsequently, the microplate was washed and incubated with 100 ng/mL of biotinylated goat anti-human VEGF detection antibody for 2 h at RT. Then, 50 µL of 40-fold diluted streptavidin-HRP was added in each well and incubated for 20 min in the dark. For color development, 1:1 mixture of color reagent A (stabilized peroxide solution) and color reagent B (stabilized chromogen solution) were added and incubated for 5-10 min. The reaction was stopped with 50 µL of 1M H_sSO_4. Finally, the optical density of each well was measured at 450 nm in

a 96-well plate reader (BMG Labtech, Germany). The experiments were performed in triplicates and data are presented as mean± standard deviation (SD).

6. Research Framework



CHAPTER IV RESULTS AND DISCUSSION

The potentials of growth factors for wound healing applications are widely characterized. Several bacterial expression systems are generally utilized for expression of recombinant proteins due to rapid growth, ease of manipulation, well-defined genetic system and high yields of proteins. In fact, previous studies have illustrated the production of recombinant proteins for wound management i.e., EGF (102), bFGF (103) and VEGF (19, 104) in *E. coli*. However, this system limits the expression of growth factors in soluble form since expressed recombinant proteins are often produced as aggregates or inclusion bodies. Thus, bacterial expression platform requires supplementary methods for solubilization and refolding of the protein inclusion bodies. In this study, the expression optimization and production of human vascular endothelial growth factor (VEGF) in *Nicotiana benthamiana* was investigated. Plants as molecular pharming platform has presented numerous unique benefits including associated low-cost production expenditures, unparalleled scalability, guaranteed safety against adventitious pathogens and increased expression level of recombinant proteins (4, 105). The gene sequence encoding human VEGF protein was synthesized in different expression constructs as shown on Appendix A.

1. Nicotiana benthamiana-produced VEGF

1.1. Gene Cloning

The human vascular endothelial growth factor (VEGF) containing 8x poly-His tag and SEKDEL sequences in the C-terminal was optimized *in silico* according to tobacco expression. To evaluate the expression level of VEGF protein in plants, four gene constructs were synthesized which vary according in the allocation of 8x poly-His tag between N or C-terminus and also with and without C-terminal SEKDEL retention motif. Different constructs were amplified by PCR and determined by horizontal gel electrophoresis. Findings showed positive amplification of VEGF gene constructs at

705 bp for SP-VEGF-His-SEKDEL, at 672 bp for SP-VEGF-His, at 612 bp for His-VEGF-SEKDEL and at 594 bp for His-VEGF as predicted (Figure 6).

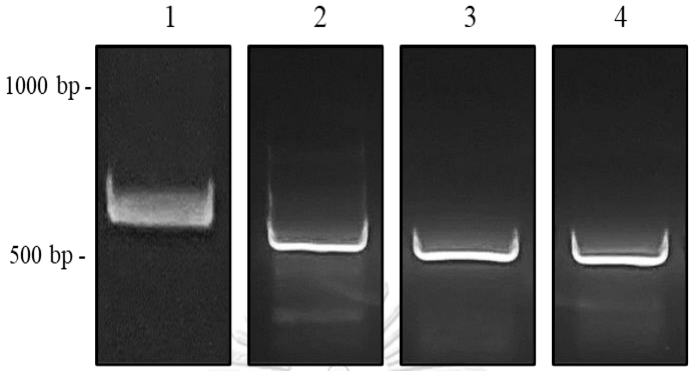


Figure 6 Amplification of VEGF gene constructs

PCR amplification of human vascular endothelial growth factor (VEGF) gene constructs used in the study. Lane 1: SP-VEGF-His-SEKDEL; Lane 2: SP-VEGF-His; Lane 3: His-VEGF-SEKDEL; Lane 4: His-VEGF.

Then, the PCR products obtained were purified, ligated into pGEMT or pGEMT-SP vector and artificially transformed in *Escherichia coli* cells strain DH10B. Clones for each construct were verified by Colony PCR and confirmed by DNA sequencing (Appendices D-F). The constructs developed were identical with the expected nucleotide sequences and subsequently cloned into plant geminiviral vector (pBYR2e-k2Md). The viral vector derived from bean yellow dwarf virus was utilized to produce high copy number of VEGF mRNA transcripts (66). The resulting expression plasmids, pBYR2e-SP-VEGF-His-SEKDEL (S-V-H-K), pBYR2e-SP-VEGF-His (S-V-H), pBYR2e-SP-His-VEGF-SEKDEL (S-H-V-K) and pBYR2e-SP-His-VEGF (S-H-V) were transformed into *Agrobacterium tumefaciens* cells strain GV3101 and infiltrated into tobacco leaves (Figure 6).

In this approach, *Agrobacterium*-mediated transformation offers a number of benefits such as its practical usage, cost-effective and well-developed mechanism for DNA integration into host cell (106, 107). It permits transient delivery of plant viral vectors containing the gene of interest into plant cells without the loss of transgene

during transfection and allows accumulation of protein in high yields without the expression of infectious virions (99). These plant viruses are able to release a part of their genome into the host species and subject efficient production of recombinant proteins (67). Hence, virus-based transient expression strategy was devised for versatile and robust expression of VEGF protein in *N. benthamiana*.

1.2. Optimization of Human Vascular Endothelial Growth Factor (VEGF) expression in *Nicotiana benthamiana*

The expression of VEGF according to different gene expression constructs in *N. benthamiana* was evaluated in order to determine the best expression condition. The protein expression levels of each construct were analyzed in a time course fashion to confirm the optimal time to harvest biomass. Here, infiltrated leaves were harvested within 2, 3 and 4 days of post-infiltration (d.p.i) and analyzed by Western Blot using HRP-conjugated anti-His antibody (Figure 7).

Based from the results, signs of leaf necrosis on infiltrated tobacco plants occurred at 3 d.p.i. for all the constructs. The expression of VEGF protein in crude extracts was obtained within 4 d.p.i. A significant level of VEGF expression was exhibited among all constructs from 2 d.p.i. Meanwhile, the optimal level of expression was evidently observed at 3 d.p.i. The day optimization conditions employed for expression of VEGF was restricted due to the rapid occurrence of necrotic symptoms by 4 d.p.i. that may affect the protein expression, protein stability and final leaf biomass (108, 109). Nonetheless, despite limited transfection time, high expression level of VEGF was obtained on 3 d.p.i.

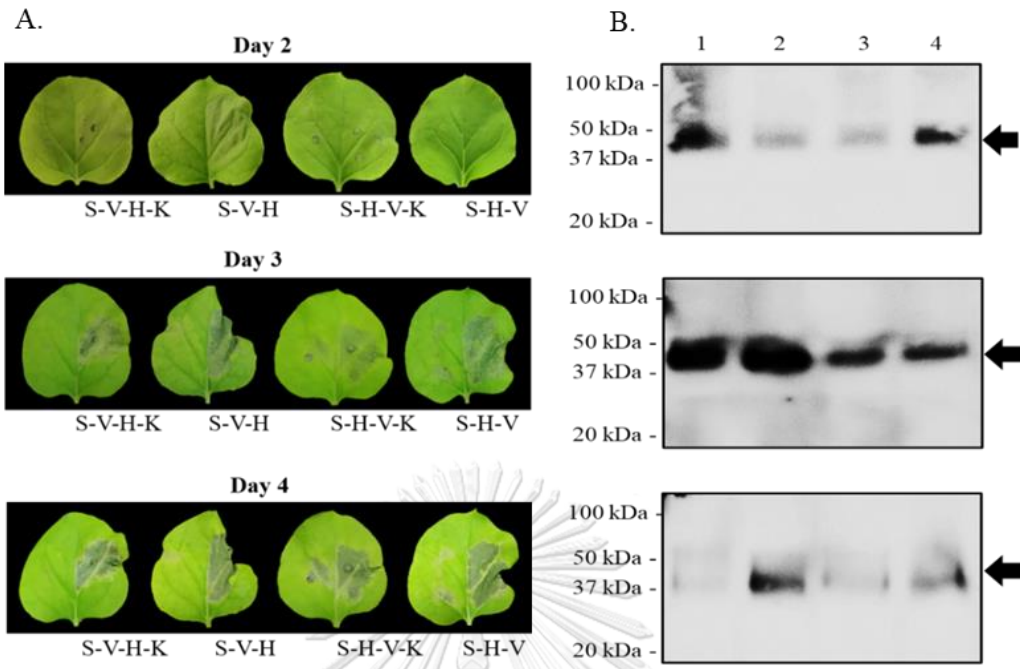


Figure 7 VEGF expression in Nicotiana benthamiana

Expression optimization of VEGF in N. benthamiana leaves. Expression levels of recombinant protein in plants infiltrated with different vector constructs were determined on day 2, 3, and 4 post infiltration. (A) The leaf necrotic symptoms on day 2, 3 and 4 post infiltration with different constructs. (B) Expression of VEGF were shown for all the four constructs used. Western blot probed with anti-His antibody. Lane 1: S-V-H-K; Lane 2: S-V-H; Lane 3: S-H-V-K; Lane 4 S-H-V.

In comparison among the four constructs tested, the expression of VEGF in both S-V-H-K and S-V-H constructs was significantly higher compared with S-H-V-K and S-H-V constructs. An ER retention peptide, SEKDEL, in general has been described to increase recombinant protein production in plants (87). Previous studies demonstrated that SEKDEL directs protein retrieval to ER via retrograde transport and ensures appropriate folding and enhanced protein stability (86, 110). At optimal harvest time, all the constructs expressed VEGF protein and apparently showed no significant difference in the expression when using the constructs with or without C-terminal SEKDEL motif. This phenomenon implicates the possible effects of excessive

protein accumulation triggering ER stress (86). Thereby, as shown from two highest expressing constructs, S-V-H-K and S-V-H, inclusion of SEKDEL sequence did not significantly help in enhancing VEGF protein expression. Furthermore, localization of Histidine residues was analyzed to determine its influence on protein expression efficiency. Initially, the 8x poly-His tag was incorporated to facilitate simple purification of proteins by immobilized metal affinity chromatography. In this study, the DNA sequence of VEGF was engineered to contain His-tag either in N-terminus or in C-terminus and expressed in plants. Although several studies have reported that attachment of His-tag produces negligible or no effect on protein stability and structure (111), few findings confirmed the differential effect of allocation of His tag at the N- and C-termini (18, 112). Here, western blot analysis showed that at day 3 post-infiltration C-terminal His-tagged VEGF constructs have higher protein expression level when compared with N-terminal His-tagged VEGF constructs. Prior studies (19) have utilized C-terminal His-tagged VEGF but with no established comparative data against N-terminal His-tagged protein. In the current study, the obtained results indicate the first evidence that explains the impact of His-tag localization in VEGF expression. However, it should be highlighted that results cannot be implied to other day post-infiltration examined. Consequently, the addition of 8x poly-His tag either at N- or C- terminal has no straightforward effect towards improved VEGF expression. Based in these results, S-V-H-K and S-V-H constructs were used for further experiments.

1.3. Purification of VEGF in *Nicotiana benthamiana*

A one-step purification method was performed by using Ni-NTA affinity chromatography to purify VEGF protein from plant extract. In attempts to examine the possible influence of SEKDEL sequence in the gene expression and purification of His-tagged VEGF, the two constructs with the highest protein expression level *ie*., S-V-H-K and S-V-H were transiently expressed and the crude extracts were purified with Ni-NTA column (Figure 8).

The results showed that VEGF protein in S-V-H-K extract could bind effectively to immobilized nickel ion column and then finally recovered with high concentration of imidazole, denoting accessible binding of plant-derived His-tagged VEGF to the Ni-column. Interestingly, findings proposed potential structure dissimilarity and accessibility of His-tag. However, despite the apparent difference on the binding capacities between S-V-H-K and S-V-H constructs, the study was unable to confirm structure modification attributed to SEKDEL tagging since protein structures were not investigated. Nonetheless, the study presented that SEKDEL peptide can prospectively affect the morphology and can vary the binding of His-tagged VEGF protein. At optimal expression and purification conditions, our results revealed that VEGF protein was best produced in S-V-H-K plant extract. Based on these results, the construct S-V-H-K was further used for large-scale protein expression via., vacuum infiltration and purified by Ni affinity column.

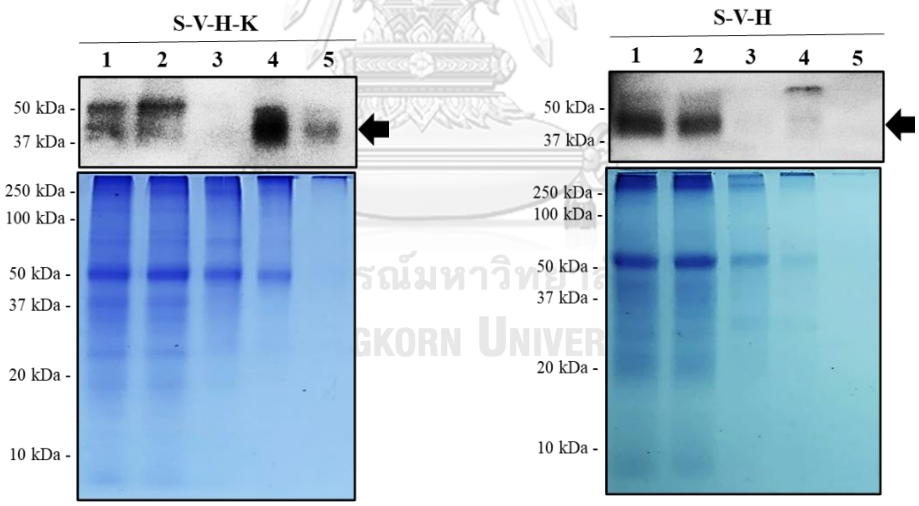


Figure 8 Purification of VEGF protein

The expression and purification of plant-produced VEGF. Purification of VEGF in S-V-H-K and S-V-H plant extracts on day 3 post infiltration was determined by western blot using anti-His antibody and staining of 12% SDS-PAGE gel with Coomassie® blue dye. Lane 1: plant crude extract; Lane 2: flow through; Lane 3: elution fraction at 20 mM Imidazole; Lanes 4: first elution fraction (1 mL) at 250 mM Imidazole; Lane 5: second elution fraction (1 mL) at 250 mM Imidazole.

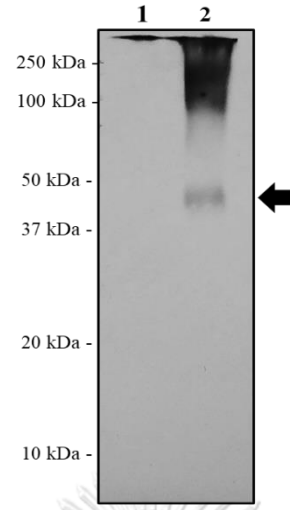


Figure 9 Western blot of plant-purified VEGF

Western blot analysis of purified plant-produced VEGF with S-V-H-K construct analyzed with mouse anti-human VEGF antibody. Lane 1: non-infiltrated plant purified extract; Lane 2: purified plant-produced VEGF.

The integrity and quality of the plant-purified VEGF protein was determined by western blot using mouse anti-human VEGF and HRP-conjugated goat anti-mouse antibodies (Figure 9). The overall molecular weight of the VEGF protein is approximately 23 kDa in monomeric form (19) and 46 kDa after dimerization (18). Under non-reducing condition, the VEGF protein was detected at 45-49 kDa as expected. The recombinant protein expression was measured by ELISA. Results revealed highest expression of VEGF in S-V-H-K construct on day 3 after infiltration at the concentration of 2.45±0.2 ng/g fresh weight.

CHAPTER V CONCLUSION

The optimization of VEGF protein expression and production in *N. bethamiana* have been mainly explored in this study. Here, different gene expression constructs were developed that differ in the location of poly-His purification tag between 3' and 5' terminal ends and with or without SEKDEL retrieval peptide. The outcomes of the study confirmed that VEGF was sufficiently expressed in tobacco plants. The expression level of VEGF was examined within 4 days of post-infiltration (d.p.i). Based on the results, the highest VEGF expression level was obtained on 3 d.p.i. in S-V-H-K and S-V-H constructs. These data confirm that the yield of VEGF in plants did not vary depending on the poly-His and SEKDEL tagging. Further, the study presented potential impacts of C-terminal SEKDEL on the binding capacity of His-tagged protein since recombinant VEGF in S-V-H-K plant crude extract was successfully purified in nickel column. However, structure characterization is needed. Accordingly, the best VEGF expression and purification condition described was with gene construct flanked with poly-His and SEKDEL at the C-terminus. The plant-produced VEGF was optimally expressed on 3 d.p.i. with roughly 2.45 ng/g leaf fresh weight.

In conclusion, the current study demonstrates the feasibility of using plant transient expression system to produce the VEGF protein in *N. benthamiana* leaves. The recombinant VEGF was highly expressed and purified by Ni-affinity chromatography. The production of plant-purified VEGF was quantified by ELISA and confirmed by using anti-His and anti-HVEGF antibodies. Under non-reducing condition, the size of plant-based VEGF was approximately 45-49 kDa as predicted. Taken together, results showed that the plant system has the potential to rapidly produce recombinant VEGF.

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APPENDICES

APPENDIX A

VEGF gene expression construct sequences

1. SP-VEGF-His-SEKDEL (S-V-H-K)

TCTAGAACAATGGGCTGGTCCTGCATCATCCTGTTCCTTGTTGCTACTGCTACCGGCGTTCACT
CTGATGTTCAACTTCTCGAGAACTTCCTGCTGTCTTGGGTGCACTGGTCTTTGGCTCTGCTTCTT
TACCTTCACCACGCCAAGTGGTCACAAGCTGCTCCTATGGCTGAAGGTGGTGGTCAGAATCATC
ATGAGGTGGTGAAGTTCATGGACGTGTACCAGAGGTCTTACTGCCATCCTATTGAGACTCTGGT
GGACATCTTCCAAGAGTACCCTGACGAGATCGAGTACATCTTCAAGCCATCTTGCGTGCCACTT
ATGAGATGTGGTGGTTGCTGCAACGATGAGGGTCTTGAGTGTGTTCCTACCGAAGAGTCCAACA
TCACCATGCAGATCATGCGGATTAAGCCTCATCAGGGTCAGCACATCGGTGAGATGTCTTTCTT
GCAGCACAACAAGTGCGAGTGCAGGCCTAAGAAGGATAGAGCTAGGCAAGAGAATCCTTGCGG
TCCTTGCAGCGAAAGAAGAAAGCACCTTTTCGTTCAGGACCCGCAGACATGTAAGTGCTCATGC
AAGAACACCGACTCCAGATGCAAGGCTAGACAGCTTGAGCTTAACGAGAGGACCTGTAGGTGCG
ATAAGCCTAGAAGACATCATCACCACCATCACCATCATTCTGAAAAGGATGAGCTTTGAGAGCT
C

2. SP-VEGF-His (S-V-H)

TCTAGAACAATGGGCTGGTCCTGCATCATCCTGTTCCTTGTTGCTACTGCTACCGGCGTTCACT
CTGATGTTCAACTTCTCGAGAACTTCCTGCTGTCTTGGGTGCACTGGTCTTTGGCTCTGCTTCTT
TACCTTCACCACGCCAAGTGGTCACAAGCTGCTCCTATGGCTGAAGGTGGTGGTCAGAATCATC
ATGAGGTGGTGAAGTTCATGGACGTGTACCAGAGGTCTTACTGCCATCCTATTGAGACTCTGGT
GGACATCTTCCAAGAGTACCCTGACGAGATCGAGTACATCTTCAAGCCATCTTGCGTGCCACTT
ATGAGATGTGGTGGTTGCTGCAACGATGAGGGTCTTGAGTGTGTTCCTACCGAAGAGTCCAACA
TCACCATGCAGATCATGCGGATTAAGCCTCATCAGGGTCAGCACATCGGTGAGATGTCTTTCTT
GCAGCACAACAAGTGCGAGTGCAGGCCTAAGAAGGATAGAGCTAGGCAAGAGAATCCTTGCGG
TCCTTGCAGCGAAAGAAGAAAGCACCTTTTCGTTCAGGACCCGCAGACATGTAAGTGCTCATGC

AAGAACACCGACTCCAGATGCAAGGCTAGACAGCTTGAGCTTAACGAGAGGACCTGTAGGTGCG
ATAAGCCTAGAAGACATCATCACCACCATCACCATCATTGAGAGCTC

3. SP-His-VEGF-SEKDEL (S-H-V-K)

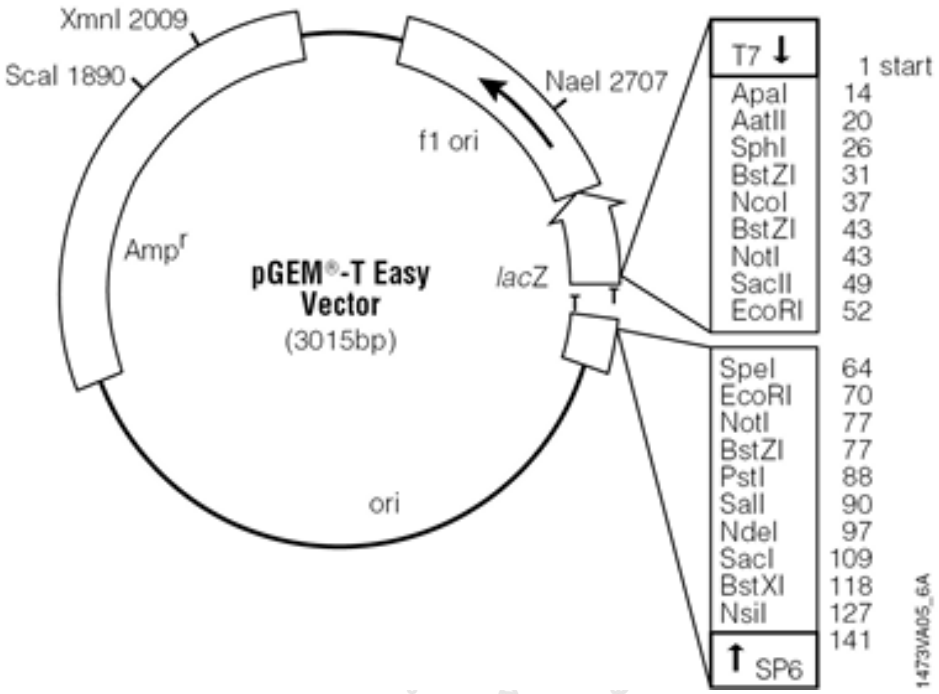
TCTAGAACAATGGGCTGGTCCTGCATCATCCTGTTCCTTGTTGCTACTGCTACCGGCGTTCACT
CTGATGTTCAACTTCTCGAGCATCATCACCACCATCACCATCATAACTTCCTGCTGTCTTGGGTG
CACTGGTCTTTGGCTCTGCTTCTTTACCTTCACCACGCCAAGTGGTCACAAGCTGCTCCTATGG
CTGAAGGTGGTGGTCAGAATCATCATGAGGTGGTGAAGTTCATGGACGTGTACCAGAGGTCTTA
CTGCCATCCTATTGAGACTCTGGTGGACATCTTCCAAGAGTACCCTGACGAGATCGAGTACATC
TTCAAGCCATCTTGCGTGCCACTTATGAGATGTGGTGGTTGCTGCAACGATGAGGGTCTTGAGT
GTGTTCCTACCGAAGAGTCCAACATCACCATGCAGATCATGCGGATTAAGCCTCATCAGGGTCA
GCACATCGGTGAGATGTCTTTCTTGCAGCACAACAAGTGCGAGTGCAGGCCTAAGAAGGATAGA
GCTAGGCAAGAGAATCCTTGCGGTCCTTGCAGCGAAAGAAGAAAGCACCTTTTCGTTCAGGACC
CGCAGACATGTAAGTGCTCATGCAAGAACACCGACTCCAGATGCAAGGCTAGACAGCTTGAGCT
TAACGAGAGGACCTGTAGGTGCGATAAGCCTAGAAGATCTGAAAAGGATGAGCTTTGAGAGCTC

4. SP-His-VEGF (S-H-V)

TCTAGAACAATGGGCTGGTCCTGCATCATCCTGTTCCTTGTTGCTACTGCTACCGGCGTTCACT
CTGATGTTCAACTTCTCGAGCATCATCACCACCATCACCATCATAACTTCCTGCTGTCTTGGGTG
CACTGGTCTTTGGCTCTGCTTCTTTACCTTCACCACGCCAAGTGGTCACAAGCTGCTCCTATGG
CTGAAGGTGGTGGTCAGAATCATCATGAGGTGGTGAAGTTCATGGACGTGTACCAGAGGTCTTA
CTGCCATCCTATTGAGACTCTGGTGGACATCTTCCAAGAGTACCCTGACGAGATCGAGTACATC
TTCAAGCCATCTTGCGTGCCACTTATGAGATGTGGTGGTTGCTGCAACGATGAGGGTCTTGAGT
GTGTTCCTACCGAAGAGTCCAACATCACCATGCAGATCATGCGGATTAAGCCTCATCAGGGTCA
GCACATCGGTGAGATGTCTTTCTTGCAGCACAACAAGTGCGAGTGCAGGCCTAAGAAGGATAGA
GCTAGGCAAGAGAATCCTTGCGGTCCTTGCAGCGAAAGAAGAAAGCACCTTTTCGTTCAGGACC
CGCAGACATGTAAGTGCTCATGCAAGAACACCGACTCCAGATGCAAGGCTAGACAGCTTGAGCT
TAACGAGAGGACCTGTAGGTGCGATAAGCCTAGAAGATGAGAGCTC

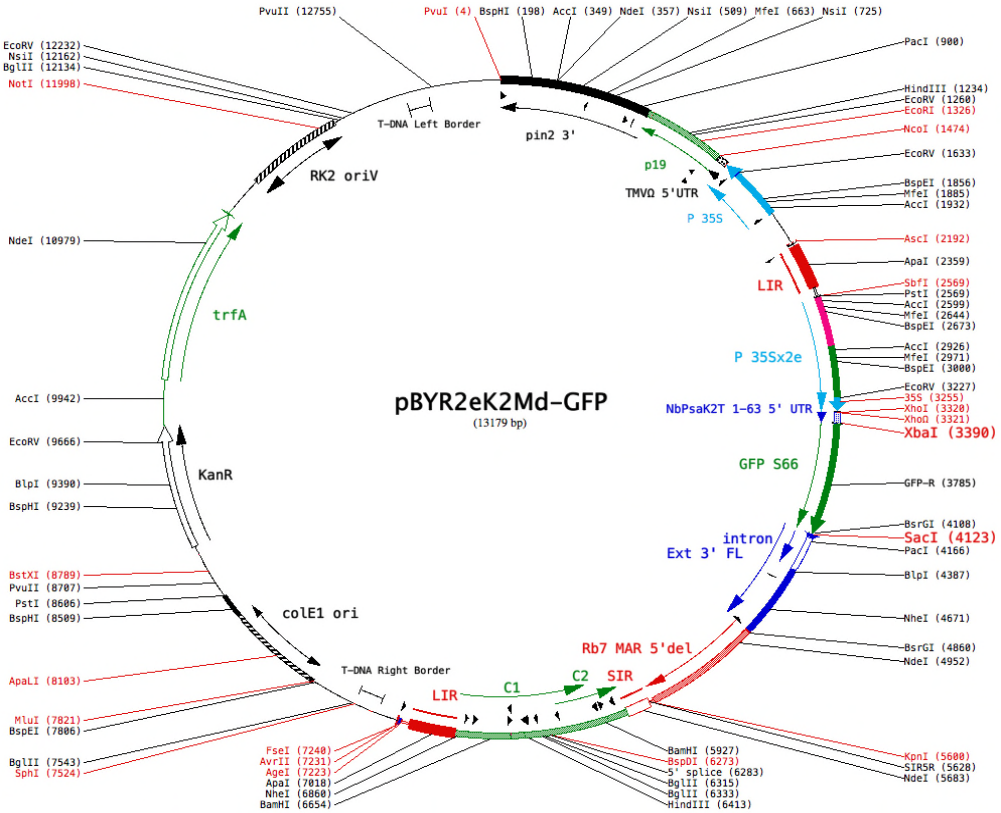
APPENDIX B

pGEM-T Cloning Vector



APPENDIX C

pBYR2e-K2Md Expression Vector



APPENDIX D

The sequencing similarity of experimental SP-VEGF-His with the template SP-VEGF-His

```
Template          1 ------------------------------------------------T      1
                                                                   |
Experimental      1 TTGGCGAATCCATGCTCCGGCCGCCATGGCGGCCGCGGGAATTCGATCCT     50

Template          2 CTAGAACAATGGGCTGGTCCTGCATCATCCTGTTCCTTGTTGCTACTGCT     51
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
Experimental     51 CTAGAACAATGGGCTGGTCCTGCATCATCCTGTTCCTTGTTGCTACTGCT    100

Template         52 ACCGGCGTTCACTCTGATGTTCAACTTCTCGAGAACTTCCTGCTGTCTTG    101
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
Experimental    101 ACCGGCGTTCACTCTGATGTTCAACTTCTCGAGAACTTCCTGCTGTCTTG    150

Template        102 GGTGCACTGGTCTTTGGCTCTGCTTCTTTACCTTCACCACGCCAAGTGGT    151
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
Experimental    151 GGTGCACTGGTCTTTGGCTCTGCTTCTTTACCTTCACCACGCCAAGTGGT    200

Template        152 CACAAGCTGCTCCTATGGCTGAAGGTGGTGGTCAGAATCATCATGAGGTG    201
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
Experimental    201 CACAAGCTGCTCCTATGGCTGAAGGTGGTGGTCAGAATCATCATGAGGTG    250

Template        202 GTGAAGTTCATGGACGTGTACCAGAGGTCTTACTGCCATCCTATTGAGAC    251
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
Experimental    251 GTGAAGTTCATGGACGTGTACCAGAGGTCTTACTGCCATCCTATTGAGAC    300

Template        252 TCTGGTGGACATCTTCCAAGAGTACCCTGACGAGATCGAGTACATCTTCA    301
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
Experimental    301 TCTGGTGGACATCTTCCAAGAGTACCCTGACGAGATCGAGTACATCTTCA    350

Template        302 AGCCATCTTGCGTGCCACTTATGAGATGTGGTGGTTGCTGCAACGATGAG    351
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
Experimental    351 AGCCATCTTGCGTGCCACTTATGAGATGTGGTGGTTGCTGCAACGATGAG    400

Template        352 GGTCTTGAGTGTGTTCCTACCGAAGAGTCCAACATCACCATGCAGATCAT    401
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
Experimental    401 GGTCTTGAGTGTGTTCCTACCGAAGAGTCCAACATCACCATGCAGATCAT    450

Template        402 GCGGATTAAGCCTCATCAGGGTCAGCACATCGGTGAGATGTCTTTCTTGC    451
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
Experimental    451 GCGGATTAAGCCTCATCAGGGTCAGCACATCGGTGAGATGTCTTTCTTGC    500

Template        452 AGCACAACAAGTGCGAGTGCAGGCCTAAGAAGGATAGAGCTAGGCAAGAG    501
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
Experimental    501 AGCACAACAAGTGCGAGTGCAGGCCTAAGAAGGATAGAGCTAGGCAAGAG    550

Template        502 AATCCTTGCGGTCCTTGCAGCGAAAGAAGAAAGCACCTTTTCGTTCAGGA    551
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
Experimental    551 AATCCTTGCGGTCCTTGCAGCGAAAGAAGAAAGCACCTTTTCGTTCAGGA    600

Template        552 CCCGCAGACATGTAAGTGCTCATGCAAGAACACCGACTCCAGATGCAAGG    601
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
Experimental    601 CCCGCAGACATGTAAGTGCTCATGCAAGAACACCGACTCCAGATGCAAGG    650

Template        602 CTAGACAGCTTGAGCTTAACGAGAGGACCTGTAGGTGCGATAAGCCTAGA    651
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
Experimental    651 CTAGACAGCTTGAGCTTAACGAGAGGACCTGTAGGTGCGATAAGCCTAGA    700

Template        652 AGACATCATCACCACCATCACCATCATTGAGAGCTC--------------    687
                    ||||||||||||||||||||||||||.|||||||
Experimental    701 AGACATCATCACCACCATCACCATCATTAAGAGCTCAATCACTAGTGAAT    750
```

APPENDIX E

The sequencing similarity of experimental His-VEGF-SEKDEL with the template His-VEGF-SEKDEL

```
Template          1 -CATCATCACCACCATCACCATCATAACTTCCTGCTGTCTTGGGTGCACT     49
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
Experimental    551 GCATCATCACCACCATCACCATCATAACTTCCTGCTGTCTTGGGTGCACT    600

Template         50 GGTCTTTGGCTCTGCTTCTTTACCTTCACCACGCCAAGTGGTCACAAGCT     99
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
Experimental    601 GGTCTTTGGCTCTGCTTCTTTACCTTCACCACGCCAAGTGGTCACAAGCT    650

Template        100 GCTCCTATGGCTGAAGGTGGTGGTCAGAATCATCATGAGGTGGTGAAGTT    149
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
Experimental    651 GCTCCTATGGCTGAAGGTGGTGGTCAGAATCATCATGAGGTGGTGAAGTT    700

Template        150 CATGGACGTGTACCAGAGGTCTTACTGCCATCCTATTGAGACTCTGGTGG    199
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
Experimental    701 CATGGACGTGTACCAGAGGTCTTACTGCCATCCTATTGAGACTCTGGTGG    750

Template        200 ACATCTTCCAAGAGTACCCTGACGAGATCGAGTACATCTTCAAGCCATCT    249
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
Experimental    751 ACATCTTCCAAGAGTACCCTGACGAGATCGAGTACATCTTCAAGCCATCT    800

Template        250 TGCGTGCCACTTATGAGATGTGGTGGTTGCTGCAACGATGAGGGTCTTGA    299
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
Experimental    801 TGCGTGCCACTTATGAGATGTGGTGGTTGCTGCAACGATGAGGGTCTTGA    850

Template        300 GTGTGTTCCTACCGAAGAGTCCAACATCACCATGCAGATCATGCGGATTA    349
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
Experimental    851 GTGTGTTCCTACCGAAGAGTCCAACATCACCATGCAGATCATGCGGATTA    900

Template        350 AGCCTCATCAGGGTCAGCACATCGGTGAGATGTCTTTCTTGCAGCACAAC    399
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
Experimental    901 AGCCTCATCAGGGTCAGCACATCGGTGAGATGTCTTTCTTGCAGCACAAC    950

Template        400 AAGTGCGAGTGCAGGCCTAAGAAGGATAGAGCTAGGCAAGAGAATCCTTG    449
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
Experimental    951 AAGTGCGAGTGCAGGCCTAAGAAGGATAGAGCTAGGCAAGAGAATCCTTG   1000

Template        450 CGGTCCTTGCAGCGAAAGAAGAAAGCACCTTTTCGTTCAGGACCCGCAGA    499
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
Experimental   1001 CGGTCCTTGCAGCGAAAGAAGAAAGCACCTTTTCGTTCAGGACCCGCAGA   1050

Template        500 CATGTAAGTGCTCATGCAAGAACACCGACTCCAGATGCAAGGCTAGACAG    549
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
Experimental   1051 CATGTAAGTGCTCATGCAAGAACACCGACTCCAGATGCAAGGCTAGACAG   1100

Template        550 CTTGAGCTTAACGAGAGGACCTGTAGGTGCGATAAGCCTAGAAGATCTGA    599
                    ||||||||||||||||||||||||||||||||||||||||||.|.||| |
Experimental   1101 CTTGAGCTTAACGAGAGGACCTGTAGGTGCGATAAGCCTAGATGTTCT-A   1149

Template        600 AAAGGATGAGCTTTGAGAGCTC----------------------------    621
                    |||||||||||||||||||||||
Experimental   1150 AAAGGATGAGCTTTGAGAGCTCGAATCGAATTCCCGCGGCCGCCATGGCG   1199
```

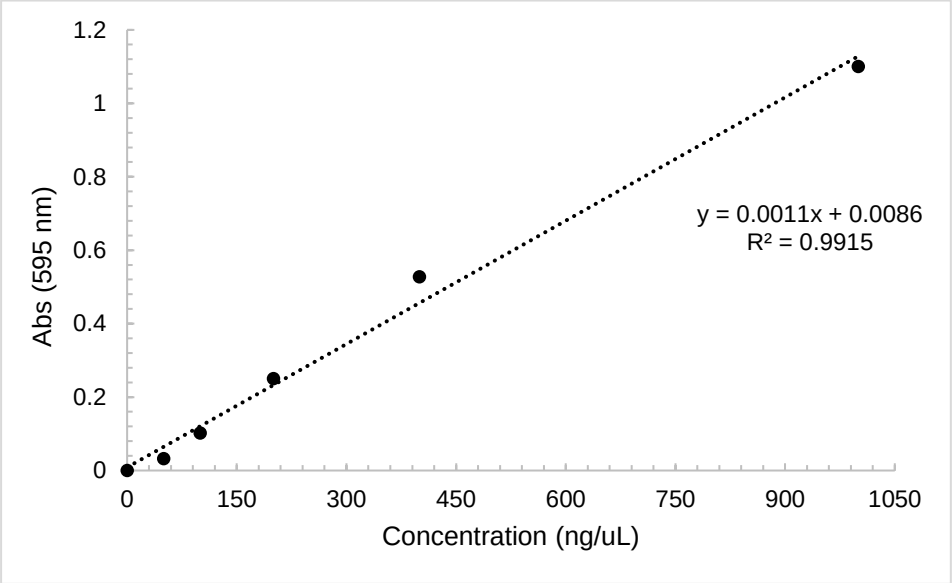
APPENDIX F

The sequencing similarity of experimental SP-His-VEGF with the template SP-His-VEGF

```
Template          1 ---------TCTAGAACAATGGGCTGGTCCTGCATCATCCTGTTCCTTGT     41
                            |||||||||||||||||||||||||||||||||||||||||||
Experimental     51 TTCGATTCCTCTAGAACAATGGGCTGGTCCTGCATCATCCTGTTCCTTGT    100

Template         42 TGCTACTGCTACCGGCGTTCACTCTGATGTTCAACTTCTCGAGCATCATC     91
                    |||||||||||||||||||||||||||||||||||||||||||||||||||
Experimental    101 TGCTACTGCTACCGGCGTTCACTCTGATGTTCAACTTCTCGAGCATCATC    150

Template         92 ACCACCATCACCATCATAACTTCCTGCTGTCTTGGGTGCACTGGTCTTTG    141
                    |||||||||||||||||||||||||||||||||||||||||||||||||||
Experimental    151 ACCACCATCACCATCATAACTTCCTGCTGTCTTGGGTGCACTGGTCTTTG    200

Template        142 GCTCTGCTTCTTTACCTTCACCACGCCAAGTGGTCACAAGCTGCTCCTAT    191
                    |||||||||||||||||||||||||||||||||||||||||||||||||||
Experimental    201 GCTCTGCTTCTTTACCTTCACCACGCCAAGTGGTCACAAGCTGCTCCTAT    250

Template        192 GGCTGAAGGTGGTGGTCAGAATCATCATGAGGTGGTGAAGTTCATGGACG    241
                    |||||||||||||||||||||||||||||||||||||||||||||||||||
Experimental    251 GGCTGAAGGTGGTGGTCAGAATCATCATGAGGTGGTGAAGTTCATGGACG    300

Template        242 TGTACCAGAGGTCTTACTGCCATCCTATTGAGACTCTGGTGGACATCTTC    291
                    |||||||||||||||||||||||||||||||||||||||||||||||||||
Experimental    301 TGTACCAGAGGTCTTACTGCCATCCTATTGAGACTCTGGTGGACATCTTC    350

Template        292 CAAGAGTACCCTGACGAGATCGAGTACATCTTCAAGCCATCTTGCGTGCC    341
                    |||||||||||||||||||||||||||||||||||||||||||||||||||
Experimental    351 CAAGAGTACCCTGACGAGATCGAGTACATCTTCAAGCCATCTTGCGTGCC    400

Template        342 ACTTATGAGATGTGGTGGTTGCTGCAACGATGAGGGTCTTGAGTGTGTTC    391
                    |||||||||||||||||||||||||||||||||||||||||||||||||||
Experimental    401 ACTTATGAGATGTGGTGGTTGCTGCAACGATGAGGGTCTTGAGTGTGTTC    450

Template        392 CTACCGAAGAGTCCAACATCACCATGCAGATCATGCGGATTAAGCCTCAT    441
                    |||||||||||||||||||||||||||||||||||||||||||||||||||
Experimental    451 CTACCGAAGAGTCCAACATCACCATGCAGATCATGCGGATTAAGCCTCAT    500

Template        442 CAGGGTCAGCACATCGGTGAGATGTCTTTCTTGCAGCACAACAAGTGCGA    491
                    |||||||||||||||||||||||||||||||||||||||||||||||||||
Experimental    501 CAGGGTCAGCACATCGGTGAGATGTCTTTCTTGCAGCACAACAAGTGCGA    550

Template        492 GTGCAGGCCTAAGAAGGATAGAGCTAGGCAAGAGAATCCTTGCGGTCCTT    541
                    |||||||||||||||||||||||||||||||||||||||||||||||||||
Experimental    551 GTGCAGGCCTAAGAAGGATAGAGCTAGGCAAGAGAATCCTTGCGGTCCTT    600

Template        542 GCAGCGAAAGAAGAAAGCACCTTTTCGTTCAGGACCCGCAGACATGTAAG    591
                    |||||||||||||||||||||||||||||||||||||||||||||||||||
Experimental    601 GCAGCGAAAGAAGAAAGCACCTTTTCGTTCAGGACCCGCAGACATGTAAG    650

Template        592 TGCTCATGCAAGAACACCGACTCCAGATGCAAGGCTAGACAGCTTGAGCT    641
                    |||||||||||||||||||||||||||||||||||||||||||||||||||
Experimental    651 TGCTCATGCAAGAACACCGACTCCAGATGCAAGGCTAGACAGCTTGAGCT    700

Template        642 TAACGAGAGGACCTGTAGGTGCGATAAGCCTAGAAGATGAGAGCTC----    687
                    |||||||||||||||||||||||||||||||||||||||||||||||||||
Experimental    701 TAACGAGAGGACCTGTAGGTGCGATAAGCCTAGAAGATGAGAGCTCCCAA    750
```

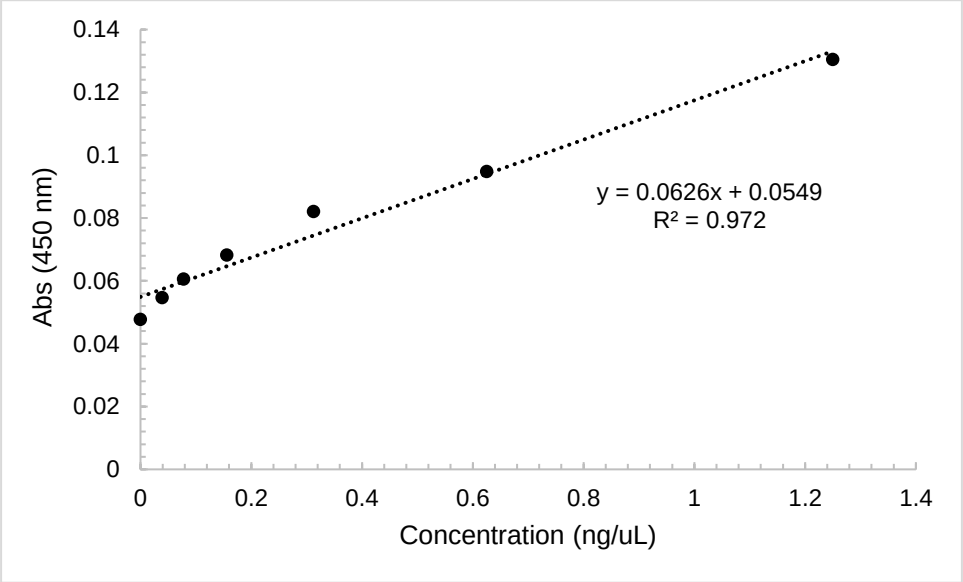
APPENDIX G

The Standard Curve for Total Soluble Protein Determination by Bradford Assay



APPENDIX H

The Standard Curve for VEGF quantification by ELISA Assay



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